VACUOLATION, PROLIFERATION AND NEOPLASIA IN THE LIVER OF BOSTON HARBOR WINTER FLOUNDER (*PSEUDOPLEURONECTES AMERICANUS*)

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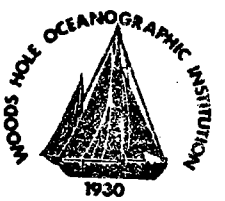

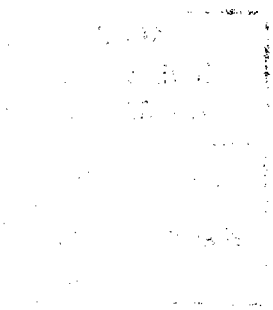

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ABSTRACT

Neoplasia has been found in the livers of bottom-feeding fish taken from heavily contaminated freshwater and marine habitats. This study examined the progressive development and ultimate diversity of liver neoplasia in winter flounder (*Pseudopleuronectes americanus*) from Deer Island Flats, Boston Harbor, MA., U.S.A., and encompassed histopathology, ultrastructural pathology, immunohistochemistry and experimental toxicology. It was found that liver neoplasia was most prevalent adjacent to a major sewage outfall, and that the predominant neoplastic cell type was cholangiocellular. Cholangiocellular neoplasms ranged from non-invasive tubular cholangiomas to invasive anaplastic cholangiocellular carcinomas. The latter were solid, tubular, cystic and scirrhous in form. Hepatocellular adenomas and carcinomas were also present, but only infrequently. Abnormally vacuolated hepatic epithelia were intimately associated with neoplastic lesions of all types. These vacuolated cells were first seen in the center of the hepatic tubule, as vacuolated preductular biliary epithelial cells. Later, cells of the entire hepatic tubule were vacuolated. Foci of vacuolated cells were visible grossly, and often contained or were adjacent to neoplastic lesions. Vacuolation, biliary hyperplasia, aggregation of macrophages and necrosis were first seen in two year old fish. These lesions then appeared to progress, becoming more severe and prevalent as the fish grew. Of the fish for which age data were available, the youngest fish to contain a liver neoplasm was 5 years old. Prevalence of neoplasia did not differ between gender of fish. Liver neoplasia and vacuolation persisted in fish that were maintained in clean water on clean food for five months. However, the prevalence of vacuolation decreased with increasing distance from urban contamination, being absent in fish from Georges Bank.

Ultrastructural examination of winter flounder liver from clean and contaminated sites revealed a loss of hepatic glycogen and lipid stores with increasing environmental contamination, with a concomitant increase of abnormal proliferated endoplasmic reticulum (ER). Fluid accumulation in the cisternal space of the ER, and the perinuclear space and mitochondria led to vesicle formation. These vesicles coalesced, to form large cellular vacuoles that compressed the nucleus and residual cytoplasm to

the margins of the cell. Vacuolation appeared to be a process that affected preductular cells, hepatocytes, cholangiocytes, neoplastic cells, and exocrine pancreatic cells. To assess the role of vacuolated cells in the progression to neoplasia, evidence for replicative nuclear DNA synthesis was sought by assaying for the nuclear incorporation of a nucleotide analog, bromodeoxyuridine (BrdU). Tissue sections from fish labelled with BrdU were stained immunohistochemically using an anti-BrdU monoclonal antibody. Constitutive DNA synthesis was observed in basal gill and intestinal epithelia, and renal hemopoietic cells. Increased levels of DNA synthesis were observed in vacuolated cells, hyperplastic biliary epithelia, and most particularly in neoplastic cells, some of which were vacuolated. These observations were taken to suggest that vacuolated cells were capable of DNA synthesis, and that this, along with their intimate spatial relationship with neoplastic cells implied that they may be involved in the progression to neoplasia. To further investigate these observations, attempts were made to recreate the feral disease in the laboratory. Methods were developed for atraumatic capture, transport and year round maintenance of winter flounder. Long term colonies were established and experiments designed to reproduce the situation in wild-caught fish. The long latency between first exposure of larvae to genotoxic carcinogens in the native fish from Boston, and the actual appearance of neoplasia many years later, leads to the assumption that chronic exposure to epigenetic carcinogens was the rate limiting step in this neoplastic progression. Technical grade chlordane was chosen as representative of the hepatotoxic epigenetic carcinogens present in Boston Harbor sediments. Acute and subacute exposures were conducted, to establish the toxicity of the fish to chlordane, and to examine the resultant histopathology. A chronic feeding study was then conducted for one year, using chlordane and benzo(a)pyrene. Histological alterations induced in treated fish included elevated levels of macrophage aggregations, perisinusoidal edema, necrosis, and a proliferative reaction that involved the formation of structures that were apparently primitive biliary tubules.

These studies have shown that winter flounder exposed to chemical contaminants appear to undergo a set of histopathological changes that precede neoplastic change. Cellular vacuolation is a significant change that may be directly involved in the progression to neoplasia. It is a relatively common lesion and is an excellent marker in winter flounder for the detection of the chronic biological effects of the particular chemical contaminants in the Boston Harbor environment, at a stage long before overt neoplasia is evident.

Thesis supervisor: John J. Stegeman, Senior Scientist, WHOI.

ABSTRACT

Liver neoplasia in winter flounder from Deer Island Flats (Boston Harbor) was examined. Liver lesions were most prevalent in fish captured near a major sewage outfall. The earliest lesions were hydropic vacuolation of biliary preductular cells, and hyperplasia of the biliary system in subadult fish. This was followed by vacuolation of the entire hepatic tubule, and finally by grossly visible foci of vacuolation. Diverse types of cholangiocellular neoplasms were the predominant neoplastic type in older fish. These, and rarer hepatocellular neoplasms, were intimately associated with vacuolated cells. Ultrastructurally, vacuolation was seen to involve progressive dilation, vesiculation and vacuolation of the cisternae of the endoplasmic reticulum, including the perinuclear space. Evidence for replicative DNA synthesis was sought by detecting nuclear bromodeoxyuridine uptake immunohistochemically. Activity suggestive of cell cycling was seen in vacuolated cells, hyperplastic biliary epithelia, and especially neoplastic foci. Foci of epithelial proliferation were observed in winter flounder chronically exposed to chlordane and benzo(a)pyrene. It was concluded that the DNA synthetic activity and close association with liver neoplasms of hydropically vacuolated cells would suggest that monitoring for the prevalence of this lesion is an excellent early warning indicator of the biological effect of hepatotoxic environmental contaminants.

ACKNOWLEDGEMENTS

I feel as if I have been married to both my wife Hannah and the laboratory of John Stegeman for the past half decade. Fortunately, neither has sought to terminate the relationship, for which I owe everybody an enormous debt of thanks. The fact that my time as a graduate student is essentially over is due in large part to the support, patience and tutelage of John Stegeman. Any failure of his attempts to teach me how to write, are mine: his persistence was endless. Patience, support and encouragement was also freely available from Roxanna Smolowitz, Bruce Woodin, Adria Elskus, Mark Hahn, Pam Kloepper-Sams, Jay Gooch and Beth Snowberger-Gray.

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didn't want to party. My immediate peers, Laela Sayigh, Carla Curran and Cindy Van Dover were all enormously friendly and supportive as they buried themselves respectively in dolphins, cunner and wierd and wonderful vent studies. Jake Pierson and Abbie Jackson provided administrative support and direction in spite of my inability to read calendars, or wear golden sneakers. Most recently, Rob Koza read the whole manuscript backwards in a hotel room in Houston.

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My cares and concerns about our planet seem to be percolating through to my children. The following is a caption my three year old son Sam gave to a painting that he recently did at preschool:

"People put dirty stuff in it and the fish won't grow.
It's just plain old dirty water."

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ABBREVIATIONS USED IN TEXT

AHH	Aryl hydrocarbon hydroxylase
AHF	Altered hepatocyte focus
APTS	Aminopropyltriethoxysilane
BrdU	Bromodeoxyuridine
CCC	Cholangiocellular carcinoma
CCF	Cholangiocellular fibrosis
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EROD	Ethoxyresorufin-O-deethylase
HCC	Hepatocellular carcinoma
ODC	Ornithine decarboxylase
MWRA	Massachusetts Water Resources Authority
PAH	Polynuclear aromatic hydrocarbon
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
RNA	Ribonucleic acid
RTLA	Registry of Tumors for Lower Animals, Smithsonian Institute, Washington D.C

MICROGRAPHS

Histological photomicrographs were all made using bright field microscopy, with Kodak T-Max or Tech Pan film, an Olympus OM-2 camera, a Zeiss Axioskop, and a green filter. Figure legends give the magnification of each image as the product of the ocular and objective magnification. Images were all enlarged 5x.

Electron micrograph magnification is given as the final printed value. Scale bar sizes are also given for electron micrographs.

DATABASE AND ARCHIVE

Individual fish morphology and histopathology records, wet specimens, paraffin blocks, histological slides and frozen samples of the winter flounder that gave rise to this study, are archived in the laboratory of John Stegeman, W.H.O.I.

PREFACE

Some species of bottom feeding fish in heavily polluted harbors get liver cancer. Why and how do these cancers arise? What risk do humans face from eating these fish and from recreating in the environment the fish live in? Does the cancer impair the fish or the affected fish populations? What are the early warning signs of the development of cancer, that can be used for the monitoring of less contaminated sites? What can we learn from these fish about the effects of industrial man on our planet? How should we modify our culture to avoid these effects to our globe? What implications do these problems have for the political management of the environment?

Answers to these questions should impact every facet of our industrial society. Obvious concerns include public health, commercial fish and shellfish harvests, sewage outfall management, toxic waste disposal, offshore mining and oil exploration, and marine transportation and recreation. However, these questions are relevant to the whole global community. Each and every domestic and industrial unit that generates a liquid, gaseous, or solid waste stream, however far inland, has an eventual impact on the quality of our aquatic systems, which comprise over 7/10 of the globe.

This thesis examines the natural history of a disease that carries portent far beyond the impact on an individual fish. But until we understand the fundamental biology of what has gone wrong in each individual, we neither understand how best to monitor the effects of chemical contaminants on aquatic systems, nor can we understand how to change the way we live to reduce our impact on the natural world around us.

The disciplines and special fields that this research embraces include waste management, coastal ecology, environmental toxicology, flounder biology, liver anatomy, fish oncology and hepatocarcinogenesis. The introduction strives to present salient observations that tie such diverse subjects into a cohesive whole.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Liver cancer in native fish

Liver cancer in native fish was first reported by Dawe, Stanton and Schwartz (1964). They described the occurrence of bile duct tumors in a population of white suckers (*Catostomus commersoni*) from a lake in a populous part of Maryland. Additional multiple hepatocellular neoplasms were found in one bullhead (*Ictalurus nebulosus*). Important observations made in this study include the following: 1) old fish were the individuals most affected, 2) both suckers and bullheads are bottom feeders, 3) the phenotypes of the tumors were very different in the two species, 4) the environment was impacted by modern man, and 5) gross examination and necropsy findings are insufficient, without histology, to detect neoplasms. These observations established the basis of much of what has been subsequently confirmed by other more extensive studies of liver neoplasia in native fish. Questions that these observations raise are still relevant today. Why only in old fish? Why in bottom feeders? Why do different species react with different cellular responses? What do these observations mean for human health (Dawe 1990, Ahmed 1991)? Other enzootics of liver cancer in bottom feeding fish in contaminated marine and freshwater systems have been studied in the subsequent 26 years. In marine fish these include the English sole and rock sole (McCain et al. 1977, Myers et al. 1987), the white croaker (Malins et al. 1987), the tomcod (Smith et al. 1979, Cormier 1986), the winter flounder (Murchelano and Wolke 1985, Gardner and Pruell 1988) and others, all of which have been reviewed (Mix 1986, Harshbarger and Clark 1990). The common thread behind these

epizootics is that they were found in bottom feeding fish, in sedimentary areas, that were heavily contaminated with industrial and domestic wastes.

Winter flounder have been shown to bear liver tumors at a dump site in central Long Island Sound, in Black Rock Harbor CT., Narragansett Bay R.I., and New Bedford Harbor MA. (Gardner et al. 1989), in Boston Harbor MA. (Murchelano and Wolke 1985, Gardner and Pruell 1988), 2 miles east of Boston Harbor (J. Harshbarger pers. comm.) and in Salem Harbor MA (C. Dawe pers. comm.). All of the above sites are contaminated with various mixtures of chemicals and heavy metals. Boston Harbor is perhaps the most notorious, although in many respects it is outranked by New Bedford and Black Rock Harbors (Gardner et al. 1989, Gardner and Pruell 1988).

Sewage in Boston Harbor

Sewage has been flowing into Boston Harbor since the late 1600's, with a major outfall active at Deer Island since 1894. Primary treatment began in 1969, although up to 1.5 billion gallons of essentially untreated sewage effluent will continue to be discharged daily until 1995. These and many other aspects of the degradation and so-called "clean up" were recently reviewed by Dolin (1990). In the past decade, the publicity over the contamination of Boston Harbor has been both popular (Pickman 1984, James 1987, Deland 1988, Anon 1989), and scientific (Brown 1987, which includes a partial bibliography, Kaltofen and Kessel 1989, Sullivan and Robinson 1990, Robinson et al. 1990). Concerns about failures to adequately treat the sewage of municipal Boston and its surrounding communities have led to the court mandated

"clean up" process by the Massachusetts Water Resources Authority (M.W.R.A.), that includes construction of primary and secondary treatment facilities, landfilling of sludge, redirection of the effluent to an offshore location, a toxics reduction program, and a combined sewer overflow improvement program. Many of the technical documents concerning this project are chronicled in Dolin (1990).

The concern about Boston Harbor has involved conflicting issues that include commercial lobstermen losing markets, recreational fishermen changing their venue, and major sewage rate increases to fund the M.W.R.A. The recreational winter flounder fishing grounds in Boston Harbor and Quincy Bay have traditionally been unsurpassed: however, in the past decade the recreational trade has all but collapsed (Childs 1987). The reasons for this are complex and poorly understood. Contributing factors may include adverse public reaction to media reports detailing neoplasia in fishes and harbor pollution, a local change in winter flounder distribution and a steady decline of the winter flounder stocks in all Massachusetts coastal waters from 1981 to the present (Witherell et al. 1991).

The favored location for catching winter flounder in Boston is just north-west of the major Deer Island outfall on the south-eastern corner of Deer Island Flats (Lightsey 1987, and pers. obs.). This suggests that the winter flounder can survive and thrive in the face of massive sewage contamination. The sediments of the flats are densely populated with polychaete worms. Flounder caught in the spring on Deer Island Flats contained up to 100 *Nereis diversicolor* in their stomachs (pers. obs.). These polychaetes feed on the organic and bacteria rich sediment from the sewage. Sewage

outfalls are highly nutritious, because of the high loading of organic carbon and nitrogen rich suspended solids. The M.W.R.A. estimates its annual nitrogen discharge into Boston Harbor to be 12,937 metric tonnes (M.W.R.A. 1990). However the sewage effluent is not only highly nutritious, it also contains a number of potential toxicants.

Toxic chemicals and heavy metals in Boston Harbor

Sewage, road runoff, industrial outfalls, and storm drains, are all sources of lipophilic chemical contaminants such as the halogenated and polynuclear aromatic hydrocarbons and heavy metals such as lead. The lipophilic compounds partition out of water, into suspended solids (Gearing et al. 1980, Readman et al 1984), which add to the organic rich bottom sediments. The levels of contamination in Deer Island sediment, and at other sites show a decreasing gradient with increasing distance from urban centers (Boehm 1984, Shiaris and Jambard-Sweet 1986, NOAA 1988a, Duston et al. 1990 and Table 1-1). In a national ranking of levels of contamination of bivalve molluscs, Boston Harbor ranked in the top 10% of sites for chlorinated pesticides, polynuclear aromatic hydrocarbons, polychlorinated biphenyls, and some forms of lead, mercury and tin (NOAA 1987b). Only one other site (Raritan Bay/ Hudson River, NY) had more rankings in the top 10 percentile.

Thus, there seem to be nutritional benefits and toxic risks for animals living near an outfall. So what are the chronic sublethal effects to a winter flounder that is breeding and feeding adjacent to a major municipal sewage outfall? To address this issue there are a number of indicators that can be examined.

TABLE 1-1

Concentrations of a subset of chemical contaminants in sediment, winter flounder liver and mussel tissue.

Station	Sediment				Flounder Liver			Mussel tissue			
	tPAH	tPCB	tChlP	tDDT	tPCB	tChlP	tDDT	tPAH	tPCB	tChlP	tDDT
BHDI[1]	6.2	.36	.048	.14				4.3	1.4	.14	.13
BOS[1]	43	.923	.016		10.5	1.1	0.83				
BH 2[1]	880	0.14									
CC2	1.4	0.03									
GB	0-0.3										

tPAH = total polynuclear aromatic hydrocarbons tDDT = DDT,DDE,DDD, tChlP = total chlorinated pesticides less tDDT. BHDI and BOS: Deer Island stations (NOAA 1987a and 1988a). BH2 and CC2: Deer Island and Southern Cape Cod Bay respectively (Boehm 1984). GB: Georges Bank (Boehm and Farrington 1984). Values given as µg/g dry weight. NOAA sediment data is normalized to percent fines, 78% BHDI, 63% BOS.

[1]These stations are all within 1/4 mile of each other suggesting a highly heterogeneous bottom, or inconsistencies between laboratory method (Farrington et al. 1988)

Indicators of environmental degradation

Indicators include analysis of specific chemicals within the sediment, analysis of contaminants within organisms that accumulate toxic chemicals such as shellfish, and analysis of the biological effect of these compounds on organisms, such as fish, that have the metabolic capacity to bio-activate these compounds. These indicators are most useful when used in parallel.

Chemical analyses of selected sets of individual compounds and metals have been conducted extensively in sediments, shellfish, and fish (Farrington et al. 1983, Boehm 1984, Boehm and Farrington 1984, NOAA 1987b, NOAA 1988a, NOAA 1988b, NOAA 1989, Duston et al. 1990). Earlier data for Massachusetts have been recently collated for fish and shellfish (McDowell Capuzzo et al. 1987).

There are a number of variables that need consideration in interpretation of data on chemical contaminants in sediment. Sediment grain size and carbon content are important considerations. Physical parameters such as deposition rates, small scale patchiness of the sampling area, with depressions being the major sediment sinks, and periodic storm erosion are also important. Biological variables include the physical and chemical reworking of the sediment by infaunal organisms. These animals can mix the sediment to the depths of their burrowing. In addition to these sources of variation, there is an artefactual problem in the comparability of chemical data from different analysts (Farrington et al. 1988).

Bivalve molluscs have a very limited capacity to metabolize most synthetic chemical compounds (Moore et al. 1980, Stegeman and Kaplan 1981, Livingstone and Farrar 1984), and their filter feeding habit encourages maximal accumulation of

contaminants from the water and suspended solids. For these reasons, bivalves are widely regarded as good indicators of biological accumulation of toxic chemicals, and have been the subject of the U.S. Mussel Watch program (Farrington et al. 1983), started in 1976, which evolved into the National Status and Trends Program (N.O.A.A. 1987b, NOAA 1988a and NOAA 1989).

In contrast to bivalves, interpretation of chemical contaminant data in fish is complicated by their ability to metabolize and excrete some compounds. Their livers contain mixed function oxidase systems (cytochrome P450s) capable of metabolizing a range of polynuclear aromatic and halogenated hydrocarbons (Stegeman and Kloepper-Sams 1987, Sijm and Opperhuizen 1989). Major experimental inducers of cytochrome P450 in fish include beta-naphthoflavone, benzo(a)pyrene, dimethylbenzanthracene, 3-methylcholanthrene and specific coplanar polychlorinated biphenyl congeners. These inducers are commonly referred to as inducers of aryl hydrocarbon hydroxylase (AHH) activity (Stegeman 1981). This hydroxylation is catalyzed by a cytochrome P450 isozyme, which in fish has been referred to as P450E (Klotz et al. 1983), though more appropriately is termed P4501A (Stegeman 1989). P4501A is inhibited by 7,8 benzoflavone in fish (Klotz et al. 1983). Winter flounder have been shown to have a range of AHH activity (Foureman and Bend 1982) which is inhibited by 7,8-benzoflavone. Catalytic activity of P4501A can be also be assayed by ethoxyresorufin-O-deethylase activity (EROD). AHH and EROD activities were measured in winter flounder from a series of coastal Massachusetts sites (Stegeman et al. 1987): significant activities were recorded from all sites, although correlation with

PAH levels was unpredictable, reflecting recent observations of inhibition of catalytic efficiency by contaminants such as PCBs at high concentrations (Gooch et al. 1989, Elskus et al. 1989). Gray (1988) has shown that P4501A activity is higher in mature male than female winter flounder.

The capacity to metabolize organic compounds aids in the excretion of xenobiotics, as the metabolites are less lipophilic, and more hydrophilic than the parent compound, and thus more readily secreted in the bile. This increase in hydrophilicity allows clearance of metabolizable substrates from the fish, resulting in low body burdens of chemical contaminants, in spite of severe chemical exposure. Analyses of bile metabolites has been suggested (Varanasi and Stein 1991) as an appropriate indicator for compounds that fish can excrete, but this approach only indicates the very recent exposure history of the individual. In contrast, fish are good indicators of biological accumulation for poorly metabolized compounds such as some of the heavily chlorinated polychlorinated biphenyl congeners (Gooch et al. 1989).

When fish metabolize hydrocarbons, the first steps often include oxidation with subsequent hydroxylation. In this pathway an epoxide is formed that can covalently bond with nucleotide residues. The resultant bulky adduct can lead to somatic mutations in genes that include those that normally maintain a dynamic balance between cell division, cell death and differentiation. Loss of this balance can lead to neoplastic change. Different cytochrome P450 isozymes of rodents can affect the balance between bioactivation vs. detoxication and excretion (Guengerich and Leibler 1985), and the same may be true for fishes.

Carcinogenesis

Mutation and other changes in genetic structure form the basis of the first stage of carcinogenesis. Farber (1984) defined the stages of carcinogenesis as initiation, promotion and progression. Initiation involves a change in the DNA of a cell that makes that cell a potential tumor progenitor. Cell proliferation is necessary at this stage to stabilize the change, before DNA repair reverses it (Columbano et al. 1981). Agents that induce such a change include genotoxic chemicals, such as benzo(a)pyrene, radiation, and viruses. Farber suggests that promotion, the second stage, is the rate limiting step in carcinogenesis: it involves the proliferation of initiated cells into nodules of altered phenotype. Thus, initiation is necessary but often insufficient to cause a tumor to grow. Most nodules regress, making the term "pre-neoplastic" a misnomer for most altered foci. Progression reflects the continued expansion of a minority of these nodules to tumors. In mammals the cytochrome P450 content of these nodules is reduced by 75-80%, whilst the detoxifying enzymes such as epoxide hydrolase and glutathione transferase are frequently elevated (Buchmann et al. 1985). Thus, the nodules may be resistant to ongoing xenobiotic insult. Promotors, those compounds that cause initiated cells to proliferate (Schulte-Herman 1974, Ochs et al. 1986), include hyperplastic and hypertrophic agents such as estradiol, and agents that induce atrophy and cell damage, allowing pockets of resistant cells to proliferate.

The initiation - promotion - progression paradigm in chemical carcinogenesis is useful to formulate hypotheses, and design experimental protocols. Therefore, even

though the focus of this thesis is on descriptive studies of the pathogenesis of liver tumors in winter flounder, I will first review what is known about the genetic basis for carcinogenesis in the liver, so that field and laboratory histopathological studies can be interpreted in the context of current concepts of molecular carcinogenesis. Any consideration of current issues in carcinogenesis should also acknowledge the belief of some that the initiation-promotion paradigm confuses mechanistic issues (Ames and Gold 1991). Indeed, the relevance of such concepts and resultant protocols for assessing the risk of exposure to synthetic compounds has been seriously questioned by these authors (Ames and Gold 1990). Nonetheless much current research centers on the activity of so-called genotoxic initiators and epigenetic promotors.

Changes in nuclear DNA associated with initiation may involve the pathological activation of genes that have physiological functions in the control of cell differentiation. Such genes have been termed proto-oncogenes in their normal state, and when deregulated, oncogenes. These genes are activated by changes in the nature or quantity of their expression, with a resultant effect at various stages of the pathways and networks that control cell proliferation. Oncogenes are typically assayed for tumorigenicity *in vitro*, by transfecting the gene into a fibroblast monolayer and thus transforming the phenotype to one of contact inhibition independence (Fasano et al. 1984). Such genes can be further shown to be oncogenic by implantation of foci of these transformed cells into the subcutis of nude mice. The mechanism by which a gene becomes oncogenic is variable, but can involve single base mutation (Barbacid 1987). Oncogenes have various sites of action and are widespread and highly

conserved in eukaryotes, including fish (McMahon 1988; Nemoto 1986; Van Beneden 1988). Oncogenes, such as *myc* and *ras*, can synergise in their tumorigenicity (Sinn 1987). The *myc* gene affects gene transcription directly, whereas others such as the *ras* family (Barbacid 1987) affect signal transduction through second messenger networks at the plasma membrane, the mutant form losing GTPase activity (Sweet et al. 1984). A hint of the physiological importance of the H-*ras* gene has been suggested by the inhibition of cleavage in axolotl eggs by an H-*ras* monoclonal antibody (Baltus et al. 1988).

One of the most common molecular abnormalities associated with human cancers is seen in the *ras* oncogene family. There are three c-*ras* genes, H-*ras*, N-*ras* and K-*ras*. Benzo(a)pyrene diol-epoxide has been shown to activate H-*ras* in rodents by mutation (Marshall et al 1984). It has been shown that the K-*ras* oncogene has a specific 12th codon mutation in hepatocellular carcinomas from rats exposed to aflatoxin B1 (McMahon et al. 1986).

Winter flounder from Deer Island Flats have mutant transforming K-*ras* oncogene alleles (McMahon et al. 1988 and 1990). Grossly visible lesions were taken from the livers of winter flounder from Deer Island Flats (Boston Harbor). DNA was extracted from these nodules and sequenced directly, and transfected into NIH 3T3 fibroblasts in culture. Resultant foci of transformed fibroblasts were then injected subcutaneously into nude athymic mice, and resultant tumors harvested for DNA extraction. Portions of the K-*ras* first exon DNA were amplified by polymerase chain reaction and sequenced from the flounder nodules, and the nude mice tumors. For each flounder

nodule, and resultant mouse tumor, identically altered codons were found (McMahon et al. 1990). The mutations comprised GC → AT or GC → TA base changes which are suggestive of a chemical exposure history (Eisenstadt 1981).

These molecular tools have great potential for the study of liver neoplasia in general, and for winter flounder in particular. However, as presented below, necessary morphological information had to be obtained prior to employing more mechanistic approaches. Support for this altered approach is also found in the review by Farber and Sarma (1987):

> "*Basic for any analysis of the genesis of disease is an appreciation that the manifestation of altered structure and function that constitute the phenotype of any disease should be delineated as far as possible before the extensive use of exciting new tools in modern biochemical, molecular, and genetic technology*".

Heeding this admonition, the focus of this thesis is to provide a detailed description of the morphogenesis and cellular relationships of liver disease in winter flounder from contaminated habitats. Necessary background information include the general biology of winter flounder, the histology and ultrastructure of the liver of teleosts, a review of current issues of mammalian hepatocarcinogenesis, and a review of hepatic neoplasia in wild and experimental fish populations.

Winter flounder biology

The literature on the biology of winter flounder is extensive, and has been reviewed by Klein-MacPhee (1978). A shallow-water, coastal flatfish, this teleost feeds on small invertebrates, and prefers sand and mud substrates. Found from the Straits of Belle Isle to the Chesapeake Bay, a major factor affecting its movements is water temperature; 15°C being the upper optimum limit. South of Cape Cod this results in a summer migration to cooler deeper water. North of the Cape, with cooler water from the Labrador Current, the summer offshore migration is limited to 1-2 miles (Howe and Coates 1975). It is thus a better indicator of the effects of a particular habitat at the latitude of Boston Harbor, than it is south of Cape Cod.

Winter flounder in Boston Harbor grow to a total length of about 100 mm in the first year, to about 200 mm in the second year, and thereafter grow at variable rates. In the first two years males grow slower than females (Jay Barnett unpubl. data). In the spring of the third year the fish mature gonadally. Spawning occurs progressively later with increasing latitude, with the season being February to April in the Mystic River, CT. (Pearcy 1962), March in Boston Harbor (Adam Smith pers. comm., and pers. obs.) and April to June in Newfoundland (Kennedy and Steel 1971). Females ripen and spawn completely, whereas males remain in milt for an extended period through the spring (pers. obs.). Gravid females lay sticky demersal eggs on sand and mud. The eggs hatch after about three weeks, and remain as plankto-benthonic larvae until settling out as metamorphosed flatfish by two months of age (Sullivan 1915, Scott 1929, Williams 1975, Rogers 1976).

The effects of chemical contaminants on young fish spawned in contaminated

habitats are important to study: to this end, the influence of contaminant burdens in the progeny of winter flounder from Deer Island Flats were recently examined (NOAA 1990). Gravid fish from Deer Island Flats and a reference site in Long Island Sound were spawned in the laboratory. Resultant progeny from Deer Island showed reduced egg and larval size, reduced yolk sac volume, a mild increase in embryo mortality, and an increase in skeletal abnormalities. It was also shown that the concentrations of lipophilic contaminants, such as polychlorinated biphenyls, were high in larvae from Deer Island Fish, and presumably passed from adults to their progeny. In an experimental exposure of winter flounder eggs to DDT and dieldrin, Smith and Cole (1973) induced a high level of abnormal gastrulation and vertebral deformities. These and other chemical toxicities could have an incremental impact on growing flounder feeding in contaminated habitats. As discussed above, the primary organ for the detoxification and excretion of metabolizable xenobiotics is the liver. An understanding of the normal structure and function of this organ in winter flounder is necessary before a study of hepatic malfunction can be made.

The histology and ultrastructure of teleost liver

A major factor in diseases of mammalian liver systems is the organization of the liver parenchyma into a lobular pattern, with zonation of hepatocytes within the lobule. In contrast, teleosts lack this level of organization. The teleost liver was first described as tubular (Shore and Jones 1889) referring to the tubules of hepatocytes. Later it was classified as tubulosinusoidal (Elias and Scherrick 1969). These latter authors used the

word tubule to describe the vascular sinusoids as being tubular, or of small diameter and saccular, widened into sac-like spaces. However, the liver of teleosts is best described as tubulosinusoidal, referring to the tubules of hepatocytes, partially surrounded by sinusoids. The tubule of hepatocytes is the basic unit of fish liver (Weiss 1972, Welsch and Storch 1973, Hinton and Pool 1976, Hacking et al. 1977, Hinton et al. 1984, Hampton et al. 1985).

The tubule of hepatocytes probably has a blind ending. The central lumen of each tubule is formed by a biliary passageway, the canaliculus. Ultrastructurally, the canaliculus is seen to be lined by the microvillous hepatocyte apices, which are joined together by many cellular junctions (Hampton et al. 1988). Canaliculi in some species, cyprinids especially, can be intracellular, extending as a finger-like depression lined by plasma membrane to penetrate to a point near the nucleus (Yamamoto 1965). The canaliculus drains into the biliary preductule, which is formed both by hepatocyte apices, and biliary epithelia. The preductule is marked by its centrotubular location and, at the light microscopic level, by small ovoid darkly staining nuclei, and ultrastructurally by an electron-dense preductular nucleus, with scant fingers of preductular cell cytoplasm interdigitating and in union with the hepatocyte apices (Hampton et al. 1988). The preductule then drains into biliary ductules, which are passageways still surrounded by hepatocytes, but mural elements of which are completely made up of biliary epithelial cells. Biliary ductules then drain into biliary ducts, which are formed by columnar epithelia, and surrounded by a sheath of fibroblasts. A basal lamina is absent from ductules, but present between duct epithelia

and periductal cells. Partially surrounding the hepatocyte tubules is an anastomotic network of vascular sinusoids (Fujita et al 1980, Tanuma and Ito 1980). Adjacent tubules may be separated, at some sites, by the space of Disse, and not sinusoids; these inter-tubular spaces are rich in fat storing Ito cells (Wake 1980, McCuskey et al. 1986).

Hepatocytes normally contain varying amounts of lipid and glycogen stores. These stores change in composition and extent during the spawning season in some flatfish (Timashova 1982). Ultrastructural responses of teleost liver to hepatotoxins include changes in lipid content, and dilation and vesiculation of the endoplasmic reticulum and the perinuclear space (Weis 1974, Scarpelli et al. 1974, McCain et al. 1978, Klaunig et al. 1979, Hawkes 1980). These changes are species and chemical specific, but are not seen uniformly through the liver. This heterogeneity of response gives the potential for chronic selection of resistant liver epithelia that may undergo neoplastic transformation. However, before the current understanding of hepatocarcinogenesis in fish is reviewed, I will first review some pertinent aspects of liver cancer in mammals.

Hepatocarcinogenesis in mammals

A central question in the study of liver cancer today is the relevance of the axiom that hepatocellular carcinomas arise from adenomatous nodules, which arise from tinctorially and histochemically altered foci of hepatocytes (Farber and Sarma 1987, Pitot et al. 1989, Bannasch 1990). However, a growing minority of researchers have examined the role in chemical hepatocarcinogenesis of a family of stem cells of

putative cholangiolar origin, called oval cells (Farber 1956, Grisham and Porta 1964, Sell and Leffert 1982, Sell and Salman 1984, Hayner et al. 1984, Yaswen 1985, Marceau 1990, Sell 1990). The relevance of oval cells to hepatocellular carcinogenesis is debated. In a recent review, Fausto (1990) concluded that oval cells were a facultative liver stem cell compartment, that was activated by certain hepatocarcinogens. In contrast, Bannasch (1990) held that oval cells have little relevance to the development of hepatocellular carcinoma, favoring instead the hepatocyte as the cell of origin. Experimentally however, transformed oval cells can generate hepatocellular and cholangiocellular neoplasms (Tsao and Grisham 1984). The oncogenetic changes associated with oval cells have been shown to be diverse (Fausto and Shank 1983, Braun et al. 1987, De Feijter et al. 1990). Oval cells can be maintained in culture (Grisham 1980, Sirica 1990), and it has been shown that lineage switching can result from changes in single transforming oncogenes (Garfield et al. 1988). The role of oncogenes in changing the level of gap junctional communication in oval cells has also been studied (Feijter et al. 1990). Gap junctional communication has been shown to be inhibited under the influence of epigenetic carcinogens (Yotti et al. 1979).

Hepatocarcinogenesis in fish

The above studies of hepatocarcinogenesis in mammals involved diverse disciplines: molecular genetics, biochemistry, histopathology, histochemistry, immunohistochemistry, and cell culture. Many of these tools have also been applied to the study of liver cancer in fish. Following pioneering studies on

hepatocarcinogenesis in the danio (*Brachydanio rerio*) with diethylnitrosamine (Stanton 1965) and the rainbow trout (*Oncorhynchus mykiss*) with aflatoxin (Halver 1967), the rainbow trout, guppy (*Poecilia reticulata*), *Poeciliopsis monacha* and medaka (*Oryzias latipes*) have been utilized in chronic carcinogenesis bioassays. All of these studies are described in an important monograph edited by Hoover (1984). There is also a growing literature concerning oncogenes and anti-oncogenes in fish (Nemoto et al. 1986, Nemoto et al. 1987, McMahon et al. 1988, Smith et al. 1988, Van Beneden et al. 1988, Mangold et al. 1989, Wirgin et al. 1989, McMahon et al. 1990, Read-Connole 1990). Essentially, there is evidence that, not surprisingly, proto-oncogenes exist in fish, and presumably they can be activated by similar changes to those found in mammals. In contrast, the study of promoters in fish (Bailey et al. 1987a, 1987b, 1988) is limited to a demonstration of the promotional effects of the PCB formulation Arochlor 1254 (Hendricks et al. 1990), and estradiol (Nunez et al. 1989) on initiated rainbow trout. Promoters are thought to act by the direct or indirect stimulation of initiated cells to proliferate. The study of cell proliferation in fish has been limited to short term responses to chemical exposures (Droy et al. 1988, Schultz et al. 1989, and Nunez 1990). However, the role of cell proliferation in chronic exposures to cytotoxins and hyperplastic agents has not been examined in fish, particularly in species showing environmentally associated disease.

The availability of outbred native species, which when taken from contaminated areas, exhibit enzootic neoplasia, offers a significant complement to the study of hepatocarcinogenesis in traditional domesticated fish species, which are presumably of

a healthy, but more limited genetic diversity. The native populations more closely model hepatocarcinogenesis as it pertains to humans, than do the generally less genetically-diverse, more easily transformed laboratory strains, whether they be mouse or minnow (Dawe and Couch 1984). However, one group of experimental studies in fish that has direct relevance to modelling the process in feral fish is that group of studies that utilized polynuclear aromatic hydrocarbons, in as much as polynuclear aromatic hydrocarbons are a major component of the genotoxic carcinogens present in many contamianted sediments (Table 1-2). It is remarkable how in those studies the proportion of hepatocellular versus cholangiocellular neoplastic and other non-neoplastic cell types seems not to vary between species; the data seem to indicate a strong involvement of hepatocytes in each case. This is in distinct contrast to observations made on some native fish. The studies listed in Table 1-3 demonstrate how hepatocellular carcinoma is the dominant neoplasm in english sole (*Parophrys vetulus*), and mummichogs (*Fundulus heteroclitus*), whereas cholangiocellular carcinoma is the dominant neoplasm in winter flounder (*P. americanus*), suckers (*Catostomus commersoni*), and bullheads (*Ictalurus nebulosus*). These differences presumably reflect the enormous diversity of species-specific responses and chemical contaminants found in the habitats of these native fish. A factor not compared in Tables 1-2 and 1-3 is the age of sampling in relation to the life span of the fish. The experimental studies, for reasons of economy of time and effort, are young adults, whereas the feral samples are of all ages, but probably predominantly fish nearing the end of their life span. To gain a deeper understanding of how each feral enzootic develops, it is essential to

know what changes precede overt neoplasia. Only a few studies have attempted to trace back the development of neoplasia in younger fish from natural populations (Rhodes et al. 1987).

In reviewing all of the above literature a number of questions emerged as significant and addressable in regard to enzootic hepatic neoplasia in winter flounder:

1) What are the characteristic end-stage lesions in neoplasm-bearing winter flounder from Boston Harbor? (Chapter 2)

2) How does liver disease progress through the life cycle of the flounder? Is the early exposure of larvae to contaminants sufficient to initiate a neoplastic response, or is a cumulative repeated or prolonged exposure necessary to promote frank neoplasia? (Chapter 3)

3) Can the phenotype of each abnormal cell be determined by ultrastructural studies? (Chapter 4)

4) Which abnormal cell lineages persist and proliferate through the progression of the disease process? (Chapter 5)

5) Can the progression to neoplasia be modelled experimentally? (Chapter 6)

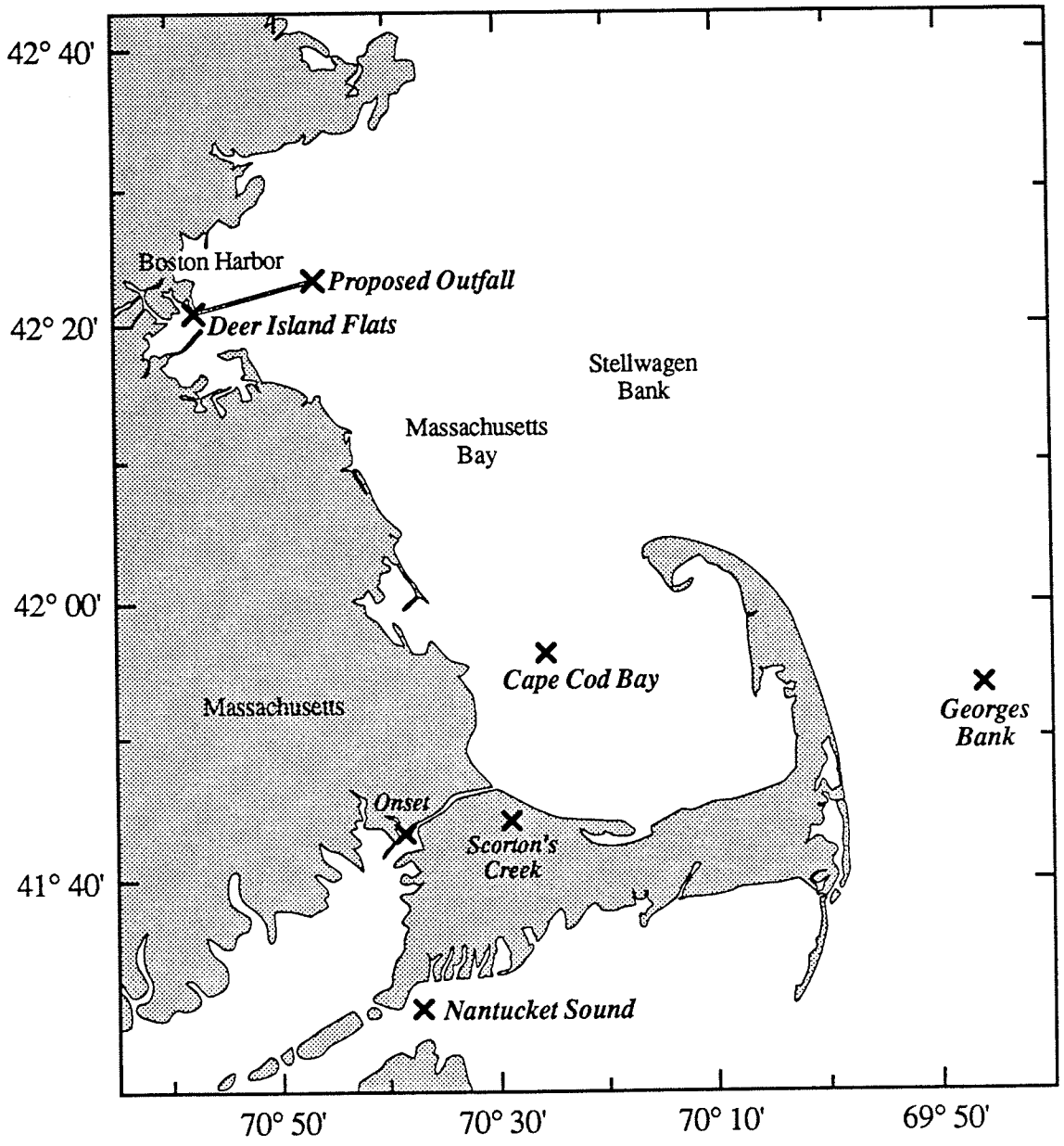


Figure 1-1

A chart of the coast of Massachusetts and Georges Bank illustrating the field stations (X) occupied for the sampling of winter flounder during this study.

TABLE 1-2

Experimental studies exposing fish to contaminated sediment or PAHs.

Species	Treatment	Route	N	%HCC	%CCC	Reference
Oncorhynchus mykiss[1]	Sediment extract	Egg	148	8	0	Metcalfe et al. 1988
Oncorhynchus mykiss	Benzo(a)pyrene	Egg	23	9	0	Black et al. 1985
Oncorhynchus mykiss	Benzo(a)pyrene	i/p	28	46	0	Hendricks 1985
Poeciliopsis monacha	Dimethylbenzanthracene	Bath	21	33	0	Schultz & Schultz 1984
Oryzias latipes[2]	Benzo(a)pyrene	Bath	73	36	0	Hawkins et al. 1990
Poecilia reticulata[3]	Benzo(a)pyrene	Bath	70	19	0	Hawkins et al. 1990
Poecilia reticulata	Dimethylbenzanthracene	Bath	50	46	0	Hawkins et al. 1990

HCC = Hepatocellular carcinoma, CCC = Cholangiocellular carcinoma, HCC/CCC = Normalized ratio of HCC vs CCC

[1]Rainbow trout. This species was, until recently, called *Salmo gairdneri*. [2]Medaka [3]Guppy

Quantitation of altered hepatocyte foci prevalence was not available in these studies.

TABLE 1-3

Prevalence of neoplastic and non-neoplastic lesions in fish from contaminated sites, where normal and grossly abnormal livers were examined histologically.

Species	N	%AHF[6]	%CCF	%HCC	%CCC	HCC/CCC	Reference
Fundulus heteroclitus[1]	60	73	12	33	0	1	Vogelbein et al., 1990
Parophrys vetulus[2]	151	31	5	15	5	.75	Myers et al 1987
Ictalurus nebulosus[3]	456	17	8	17	27	.39	Baumann et al., 1990
P. americanus[4]	200	4	-	3	7	.30	Murchelano and Wolke 1985
Catostomus commersoni[5]	456	-	68-77	2[7]	11[7]	.15	Hayes et al., 1990
P. americanus	236	2	25	1	9	.10	This study
Ictalurus nebulosus	170	-	36	1[7]	10[7]	.09	Hayes et al., 1990

AHF = Altered Hepatic Foci, HCC = Hepatocellular carcinoma, CCF = Cholangiocellular fibrosis, CCC = Cholangiocellular carcinoma, HCC/CCC = Index of neoplastic cell types (1 = all neoplasms were hepatocytic, 0 = all neoplasms were cholangiocytic). [1]Mummichog [2]English sole [3]Brown bullhead [4]Winter flounder [5]White sucker

[6]Where prevalences for AHF were given independently for each type, the value for the most prevalent type was used.

[7]Estimated from published figure.

CHAPTER 2

HEPATIC NEOPLASIA IN WINTER FLOUNDER, *PSEUDOPLEURONECTES AMERICANUS*, FROM BOSTON HARBOR, MA., U.S.A.

INTRODUCTION

Epizootics or enzootics of hepatic neoplasia have been described in a number of bottom-feeding fish from freshwater, estuarine, and marine habitats. The first cases described were in the white sucker and brown bullhead (Dawe et al. 1964). Other important examples include English sole (Myers et al 1987), tomcod (Cormier 1986) and mummichog (Vogelbein et al 1990). Hepatic carcinomas have also been described in winter flounder from Deer Island Flats in Boston Harbor (Murchelano and Wolke 1985). These and other epizootics of hepatic neoplasia in fish have been recently reviewed (Harshbarger and Clark 1990).

In most cases thus far described, epizootics of hepatic neoplasia in fish have been associated with the presence of environmental contaminants in the sediments of urbanized harbors and rivers. Sewage has been discharged into Boston Harbor via open drains and pipes since before 1700; a major sewage outfall has been active off Deer Island since 1894. Primary treatment was initiated in 1969 (Dolin 1990), although largely untreated sewage will continue to be discharged until 1995. Domestic and industrial sewage discharges have resulted in diverse and extreme chemical contamination of the sediments of the harbor. Sediments in Boston Harbor contain high levels of polynuclear aromatic hydrocarbons, halogenated hydrocarbons, and heavy metals (NOAA 1988a, Boehm 1984, Shiaris and & Jambard-Sweet 1986). Many candidates exist, yet the precise causes of hepatic neoplasia in native fish are not known.

Winter flounder from relatively uncontaminated sites have grossly normal livers, and the histology of uncontaminated fish has been described elsewhere (Bodammer and Murchelano 1990, Moore et al. 1989). Essentially flounder from relatively pristine sites show a homogeneous hepatic parenchyma consisting of vascular sinusoids which partially surround tubules of lipid and glycogen rich hepatocytes; the hepatocyte apices in the center of the tubule form the biliary canaliculi. The canaliculi drain into biliary preductules, and thence into the larger biliary system. Large bile ducts, lined with columnar epithelial cells, and surrounded by a sheath of fibroblasts, appear only rarely in histological sections of fish liver. In contrast, livers of winter flounder from Boston exhibit a wide range of neoplastic and non-neoplastic gross and histological changes. Hepatic neoplasia in winter flounder in Boston Harbor has been described (Murchelano and Wolke 1985, Gardner and Pruell 1988), but an illustrated description of the full histological diversity of neoplasms present in these fish has not been published.

In this chapter the diversity of hepatic neoplasia in the winter flounder from Deer Island Flats, in Boston Harbor, is described and illustrated. Neoplastic hepatic epithelial phenotypes ranged from poorly differentiated biliary duct carcinomas, to differentiated tubular cholangiomas, to trabecular carcinomas and adenomas of hepatocellular morphology. These descriptions have stimulated studies of the pathogenesis of the disease, of experimental protocols to model the disease in the laboratory, and of the interrelationships between histological and molecular lesions found in these fish.

METHODS

Fish

Winter flounder were caught either by otter trawl or hook and line in a variety of sampling efforts in the years 1985 to 1990. Fish were either necropsied at once, or returned to the laboratory alive. To transport 100 adult flounder to the laboratory, fish were taken from the net and placed in 35 gallon barrels, 3/4 filled with seawater. The water was changed every 30 minutes. Fish were transferred to a circular glass fiber tank in a 3/4 ton truck. The tank was 1.2 m in dia. and height, and contained 300 gallons of aerated seawater. In the laboratory, fish were maintained in running filtered sea water at ambient temperature and then examined, usually within 48 hours, although some fish were held for longer periods.

Fish were killed by cervical section, and then opened by a ventral incision around the margin of the abdominal cavity. The liver was dissected free of peritoneal attachments and examined for surface abnormalities. The liver was then cut into slices at 4 mm intervals. Each cut surface was examined for grossly visible lesions. Alternate slices were preserved in neutral buffered formalin or liquid nitrogen. All fixed tissue was processed if lesions were visible. If no visible lesions were evident, then one slice was processed for histology. Frozen samples were archived for other studies. Protocols for the maintenance, examination and euthanasia of fish used in this study were approved by the Institutional Animal Care and Use Committee of the Woods Hole Oceanographic Institution.

Processing

Samples for histology were routinely dehydrated and embedded in paraffin, and stained with hematoxylin and eosin. Adjacent sections from selected fish were stained with special stains (Luna 1968) for glycogen (periodic acid Schiff), collagen (Masson trichrome), nucleic acids (Menzies) or iron.

Histologic evaluation

Slides were evaluated for the presence of non-neoplastic abnormalities, and neoplastic lesions. Criteria used in assigning lesions to specific diagnostic categories are listed in Table 2-1. Non-neoplastic cell types are described more fully in Chapter 3. Neoplastic lesions were defined largely by criteria that have been used to classify lesions in rats (Institute of Laboratory Animal Resources 1980) and in the English sole (Myers 1987). Neoplastic hepatocytes were recognized as focal, non-tubular, masses of cells having obvious basophilic cytoplasm, large spherical nuclei with prominent nucleoli; these cells formed trabecular arrays. In contrast, neoplastic cholangiocytes were recognized as cells with less cytoplasm, a lesser degree of basophilia, and smaller nuclei with less distinct nucleoli than hepatocytes. These cells had a tendency, to varying degrees, to form tubules. Cholangiocellular lesions were classed as bearing one or more of the following characteristics: tubular, solid, cystic, papillary and scirrhous. Neoplastic lesions were staged as benign if the surrounding parenchyma was compressed but not invaded, the neoplastic cells were well differentiated, and few mitotic figures were present. In contrast, carcinomas were invasive structures with

TABLE 2-1

A summary of criteria used in assigning lesions to specific diagnostic categories.

Non-neoplastic lesions

Abnormal arrangements and/or appearances of differentiated cell(s).

Cholangial hyperplasia - increased number of duct and ductular profiles, but with normal biliary architecture, with, or without surrounding fibroplasia.

Hydropic vacuolation - a cell with a large non-staining cytoplasm, and a small basophilic nucleus. Seen singly in the center of the hepatic tubule, in tubular arrays, and in grossly visible foci.

Macrophage aggregation - aggregations of large, golden-brown macrophages often present adjacent to larger blood vessels and biliary structures.

Necrosis - Nuclear pyknosis and fragmentation, with cytoplasmic eosinophilia and swelling.

Neoplastic lesions

General features

Failure to fully differentiate a normal architecture.
Multiple repetition of an abnormal structure, resulting in a lesion at least 1 mm in diameter; often intimately associated with hydropically vacuolated cells.
Abnormal nuclear, nucleolar, and cytoplasmic morphology.
Exclusion of macrophage aggregates.

Hepatocellular adenoma and carcinoma

Abnormal trabeculae of hepatocytes with the above general features. Hepatocytes typified by basophilic cells with round nuclei and rhomboid cell outlines.

Cholangioma and cholangiocellular carcinoma

A diverse array of forms which more or less resemble immature cholangiocytes, with the above general features, seen in various blends of tubular, solid, cystic, papillary, and schirrous arrangements. Cholangiocytes typified by more or less tubular arrays of cells with smaller, less circular nuclei, and a more eosinophilic cytoplasm.

Staging of neoplasms

Non-invasive, compressive, differentiated lesions classed as benign.
Large lesions with increased mitotic index, invasive borders, undifferentiated pleomorphic phenotype, classed as carcinomas.

poorly differentiated cells. Carcinomatous cells in these structures had nuclei of diverse size and shape and, at times, a high mitotic index. Extra-hepatic organs were examined visually, but not histologically in this study, so the presence or absence of micro-metastases could not be determined.

Statistics

The significance of observed differences between lesion prevalence in males and females was tested by χ^2. Differences between mean length of fish of each gender that did and did not contain neoplasms were tested for significance by the Student's t test.

RESULTS

Fish

A total of 293 winter flounder (198 females, 87 males and 8 gender unrecorded) from Deer Island Flats (Boston Harbor) were examined grossly and histologically, in the period 1985 -1990. One or more hepatic neoplasms were found in 29/293[1] (9.9%) of the fish examined (data were available on the gender of all fish with neoplasia). The difference between the prevalence of neoplasia in males compared to females was insignificant (males 11.5%, females 9.6%, χ^2 $p = 0.84$). Analysis of mean total length showed that female fish with neoplasms were significantly longer than those without

[1]This prevalence is artefactually high, because a minority of these fish came from archival material where only grossly abnormal fish were examined histologically. In a random sample for the years 1987-1990 the mean prevalence of neoplasia was 6.5% (Table 3-4).

neoplasms (396 ± 32 mm, N = 19 vs. 370 ± 39 mm, N = 179; t test p = 0.0055). The same trend was present for males, but it was not statistically significant (357 ± 16 mm, N = 10 vs. 338 ± 48 mm, N = 77; t test p = 0.21).

Gross hepatic lesions

Present in 59/293 (20.1%) of all fish examined, gross lesions were absent in only 1/29 (3.5%) of those fish containing histological evidence of neoplasia. The diversity of the lesions is illustrated in Figure 2-1. The commonest appearance was as shown in Figure 2-1a, where single or multiple raised creamy white nodules were found, both on the surface and deep. These nodules varied in size from 2 to 20 mm.

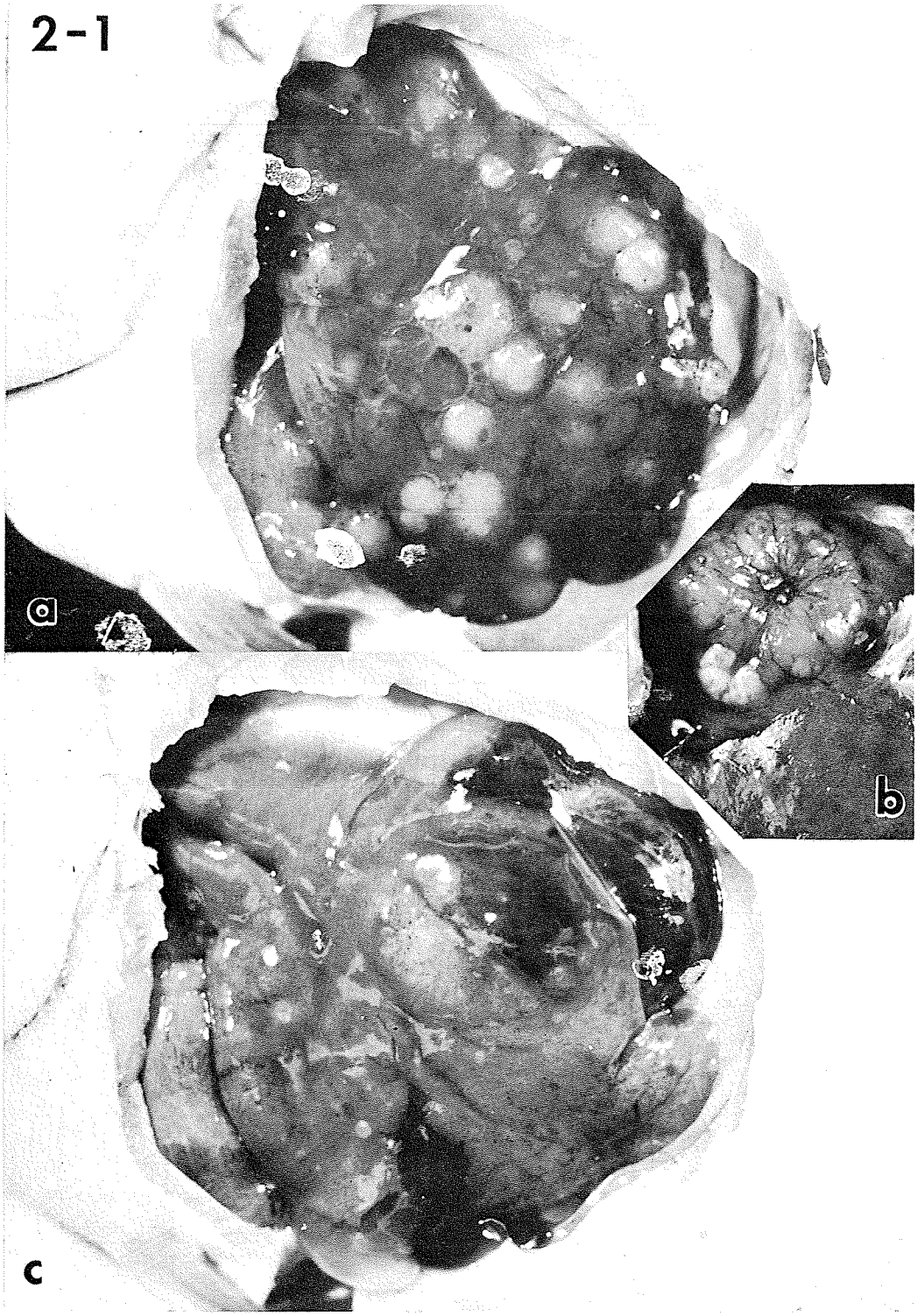
Figure 2-1

Ventral views of exposed surfaces of winter flounder livers caught on Deer Island Flats, Boston Harbor. The liver lies below the ventral body wall that has been cut away. The opercular flap is visible to the left.

a) RTLA # 5233. Multiple creamy white nodules pervade the liver. Additional non-nodular clear patches are also evident. Histologically this liver had at least 50 distinct neoplastic lesions and foci of vacuolation. Neoplastic cell types involved a spectrum of cholangiocellular phenotypes. 1.5x

b) RTLA # 5229. A large fleshy umbilicated hepatic mass is seen overlying the stomach. A small creamy lesion is adjacent. Histologically both of these lesions were cholangiomas. 1.1x

c) RTLA # 5231. The major portion of the liver in this fish consisted of distended cysts, filled with serosanguineous fluid. Histologically these lesions were cystic and papillary cholangiocarcinomas. 1.1x


2-1
a
b
c

Additional gray, translucent 2 to 8 mm lesions were seen flush with the surface and were less nodular. Histologically the creamy white and gray translucent lesions consisted of focal aggregations of vacuolated cells (described in Chapter 3), with or without neoplastic lesions within any given focus. Other less common lesions included 5 to 30 mm fleshy masses, often the same color as the surrounding tissue, as shown in Figure 2-1b. A final gross lesion type consisted of multi-loculated, thin-walled, as opposed to fleshy, cysts filled with sero-sanguineous fluid (Figure 2-1c). The cystic lesions proved to be exudative papillary cholangiocarcinomas (Figure 2-7).

Non-neoplastic cells associated with neoplastic lesions

Winter flounder from Boston Harbor commonly contained a set of non-neoplastic changes that included, in order of decreasing prevalence, 1) macrophage aggregations, 2) hydropically vacuolated cells, 3) biliary duct hyperplasia, 4) undifferentiated spindle-shaped cells within a focus of vacuolated cells, and 5) tinctorially altered foci of hepatocytes (these were seen extremely rarely). Of the above lesions only the vacuolated cells were found in close spatial association with neoplastic lesions.

Vacuolated cells have been described elsewhere (Bodammer and Murchelano 1990, Moore et al 1989, and Chapter 3). They are hydropically swollen epithelia, found adjacent to cholangiocytes, hepatocytes and exocrine pancreatic cells. These cells are first seen in 2-3 year old fish as abnormal preductular biliary epithelia, in the center of the hepatic tubule (Moore et al 1989). In adults the lesions often involve the

entire hepatic tubule. The end stage involves grossly visible (Figure 2-1) creamy white or gray focal aggregations of vacuolated cells as illustrated in Figure 2-1a.

Vacuolated cells were associated with hepatocellular and cholangiocellular neoplastic lesions in one or more of the following three ways: 1) As large foci of vacuolated cells containing smaller areas of neoplastic cells, 2) As single or multiple vacuolated neoplastic cells within a neoplastic focus, or 3) As vacuolated cells in the edge of a neoplastic focus invading surrounding parenchyma. The interrelationships between vacuolated cells and neoplastic lesions are summarized in Table 2-2.

Histochemistry

Iron: Non - neoplastic hepatocytes showed variable, but at times intense deposits of cytoplasmic iron. Macrophage aggregations also stained intensely for iron. In contrast, all hepatocellular and cholangiocellular neoplastic lesions present in 6/6 different fish were negative. Intra-vacuolar fluid in vacuolated cells did not stain.

Collagen: Positive staining was extensive immediately surrounding proliferated bile ducts, and inter-woven among many of the larger neoplastic lesions. Apparent desmoplastic fibroplasia filled voids that seemed to be associated with degeneration of neoplastic tissue. Intra-vacuolar fluid in vacuolated cells did not stain.

Glycogen: Glycogen, present in the cytoplasm of some hepatocytes of Boston Harbor fish, was found in far greater abundance in the hepatocytes of fish from uncontaminated areas. Brush borders of some neoplastic cholangiocytes also stained positively with this technique. Intra-vacuolar fluid in vacuolated cells did not stain.

TABLE 2-2

Proportions of neoplastic lesions intermingled by, in foci of, and fringed by vacuolated cells (vc).

Neoplasm type	Number of neoplasms	% of neoplasms containing vc	% of neoplasms in foci of vc	% of neoplasms fringed with vc
Hepatocellular	6	17	17	100
Cholangiocellular	54	37	65	91

23 fish contained one or more cholangiocellular neoplasms, 5 contained one or more hepatocellular neoplasms, and 1 contained both hepatocellular and cholangiocellular lesions. Neoplasms and their relationship to vacuolated cells are described in the text. Neoplasms were classed as distinct if there was obvious spatial and phenotypic discontinuity between adjacent lesions.

Nucleic acids: Menzies stain revealed DNA to be in the nuclei, and RNA to be in the nucleolus and non-vacuolated cytoplasm of all cells. The fluid within hydropically vacuolated cells did not stain for either DNA or RNA.

Diversity of neoplastic phenotypes

The overwhelming impression in reviewing this collection of hepatic neoplasms was that each and every lesion had a unique nature. Nevertheless, many lesions were of varying degrees of similarity, both within and between fish. Cholangiocellular carcinomas proved to be most common (87% of all histologically distinct neoplastic lesions) yet extremely heterogeneous. The lesions have been classified descriptively to illustrate their diversity. The histo-morphological characteristics of each neoplasm type are given in Table 2-3, the prevalence of each lesion type is listed in Table 2-4, and Table 2-5 lists selected individual cases.

Hepatocellular adenoma

Differentiated hepatocellular neoplasms were rare; only three cases were observed. Figure 2-2a shows one of these lesions, that was walled off with a fibrous capsule and that compressed the adjacent parenchyma. This lesion appeared to have overgrown and displaced a focus of vacuolated cells, with sheets of monomorphic hepatocytes whose nuclei were round with prominent nucleoli, and whose cellular outlines were indistinct, but generally rhomboid. Vascular sinusoids permeated the lesion, although the number of rows of hepatocytes between individual sinusoids was 4 to 6, in contrast to two in longitudinal sections of normal liver tubules. Vacuolated cells were irregularly interspersed through the body and edges of the focus.

TABLE 2-3

Histo-morphological characteristics of neoplastic lesions in winter flounder from Deer Island Flats, Boston Harbor, MA.

Lesion type	Tissue architecture	Invasion of surrounding parenchyma	Nucleus	Nucleolus	Cytoplasm staining	Nucleus: cytoplasm ratio	Cell size
Hepatocellular adenoma	Trabecular	–	Round; monomorphic	Variable	Basophilic	Normal	Large
Hepatocellular carcinoma	Trabecular	+	Round; pleomorphic	Prominent	Basophilic	High	Large
Cholangioma	Tubular	–	Circular to ellipsoid	Variable	Normal to eosinophilic	Low/ normal	Large
Cholangiocellular carcinoma	Tubular	+	Ovoid	Small	Eosinophilic	Normal	Normal
	Solid	+	Ellipsoid	None	Eosinophilic	High	Small
	Scirrhous	+	As tubular or solid, but interwoven with fibrotic sheets				
	Cystic	+	Ellipsoid Variably sized cysts, lined with flattened cholangiocytes	None	Eosinophilic	High	Small

Sizes are as compared to untransformed hepatocytes or cholangiocytes

TABLE 2-4

Prevalence of neoplasm type in 29 flounder collected from Deer Island Flats between 1985 and 1990†. A total of 60 histologically distinct neoplastic lesions were observed.

	N	%
Hepatocellular adenoma	3	5.0
Hepatocellular carcinoma	3	5.0
Cholangioma	2	3.3
Cholangiocellular carcinoma		
Tubular	15	25.0
Solid	18	30.0
Tubular/solid	1	1.7
Tubular/scirrhous	4	6.6
Tubular/cystic	7	11.7
Cystic/scirrhous	5	8.3
Cystic/papillary	2	3.3

† Lesions are described in the text, and in Table 2-3. Lesions were classed as distinct if there was obvious spatial and phenotypic discontinuity.

TABLE 2-5

Summary of neoplastic lesions in winter flounder liver from Deer Island Flats, Boston Harbor 1985-1990, described in this paper and archived at the Registry of Tumors for Lower Animals, Washington D.C. Multiple lesions from the same, or different tissue block of one animal have the same RTLA #.

	R.T.L.A. #	Length (mm)	Sex	Lesion size (mm)
Hepatocellular adenoma				
Trabecular	5227	410	F	20
Hepatocellular carcinoma				
Trabecular	5235	415	F	1.8
	5236	360	F	1.2
Cholangioma	5229	450	F	30.0
	5226	360	M	10.0
Cholangiocellular carcinoma				
Tubular	5230	395	F	1.0
	5232	389	F	8.0-16.0
	5233	395	F	1.4-4.8
	5226	360	M	2.0
Solid	5228	420	F	2.0-3.6
	5230	395	F	2.0
	5233	375	F	4.0
	5234	398	F	8.0
	5237	360	M	2.1
Tubular/solid	5228	420	F	2.2
Tubular/scirrhous	5233	375	F	4.0
Tubular/cystic	5225	390	M	2.1
	5228	420	F	4.0
Cystic/scirrhous	5225	390	M	2.0
Cystic/papillary	5228	420	F	3.3
	5231	436	F	27.0

Hepatocellular carcinoma

Pleomorphic hepatocellular lesions were uncommon (3 fish). Small 1 to 2 mm invasive lesions, more basophilic than the surrounding parenchyma, were evident (Figure 2-2b). Nuclear profiles were round and had prominent nucleoli, but their size was variable, with some nuclei being twice the size of adjacent non-neoplastic parenchymal hepatocytes. Mitotic figures were evident throughout the lesions. The lesion was at times heavily permeated by dilated vascular sinusoids. Invasion occurred by the radial intercalation of normal tubules by fingers of neoplastic trabeculae.

Cholangioma

Non-invasive, differentiated, monomorphic cholangiocellular lesions were also rare. Only two cases were observed in this collection. In one fish (RTLA # 5229) a 30 mm diameter raised, umbilicated lesion (Figure 2-1b) had a subsidiary, creamy white nodule on one surface. Histologically (Figure 2-3) the entire lesion was a mass of densely packed biliary tubules. Nuclei varied from circular, to ovoid, to ellipsoid. Nucleoli varied from prominent to absent. Many of the tubules lacked lumens. In the center of the lesion, nests of dilated tubules were seen, lined with cuboidal epithelia and filled with eosinophilic cellular debris. Portions of the center of the lesions were necrotic,

Figure 2-2

Hepatocellular neoplasms in winter flounder liver from Deer Island Flats, Boston Harbor. Paraffin embedded sections stained with hematoxylin and eosin.

(a) RTLA # 5227. Hepatocellular adenoma: A large fleshy hepatic mass was evident grossly and histologically. Normal tubulosinusoidal structure was replaced by masses of monomorphic neoplastic hepatocytes (N). No invasion was evident. The lesion was walled off by a thick fibrous capsule (F). Many of the cells in the surrounding parenchyma were vacuolated (V). 100 x.

(b) RTLA # 5236. Hepatocellular adenocarcinoma: multiple 1-2 mm yellow lesions were observed grossly in this fish. An invasive trabecular focus is shown (arrowheads delineate border). 100 x.

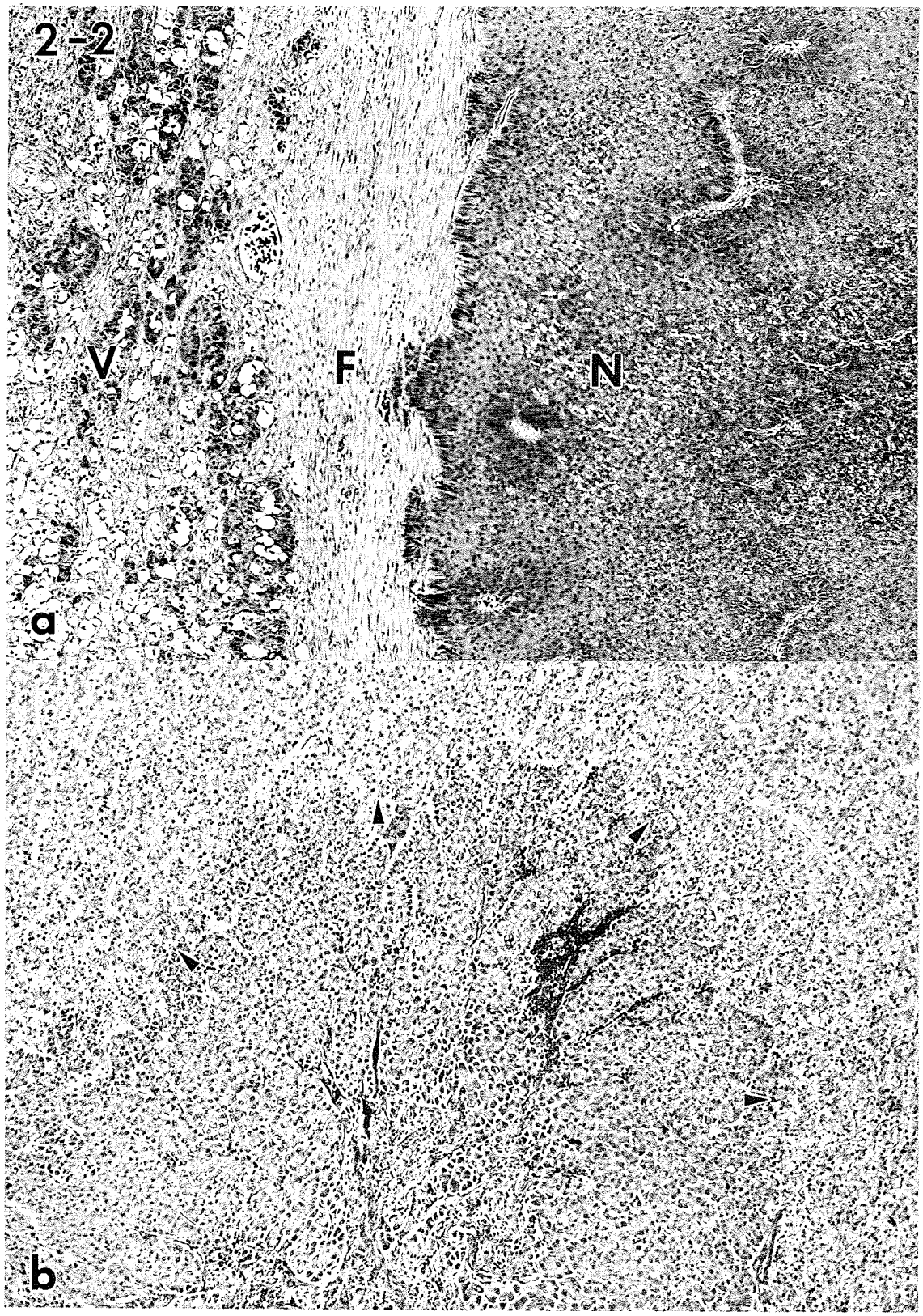
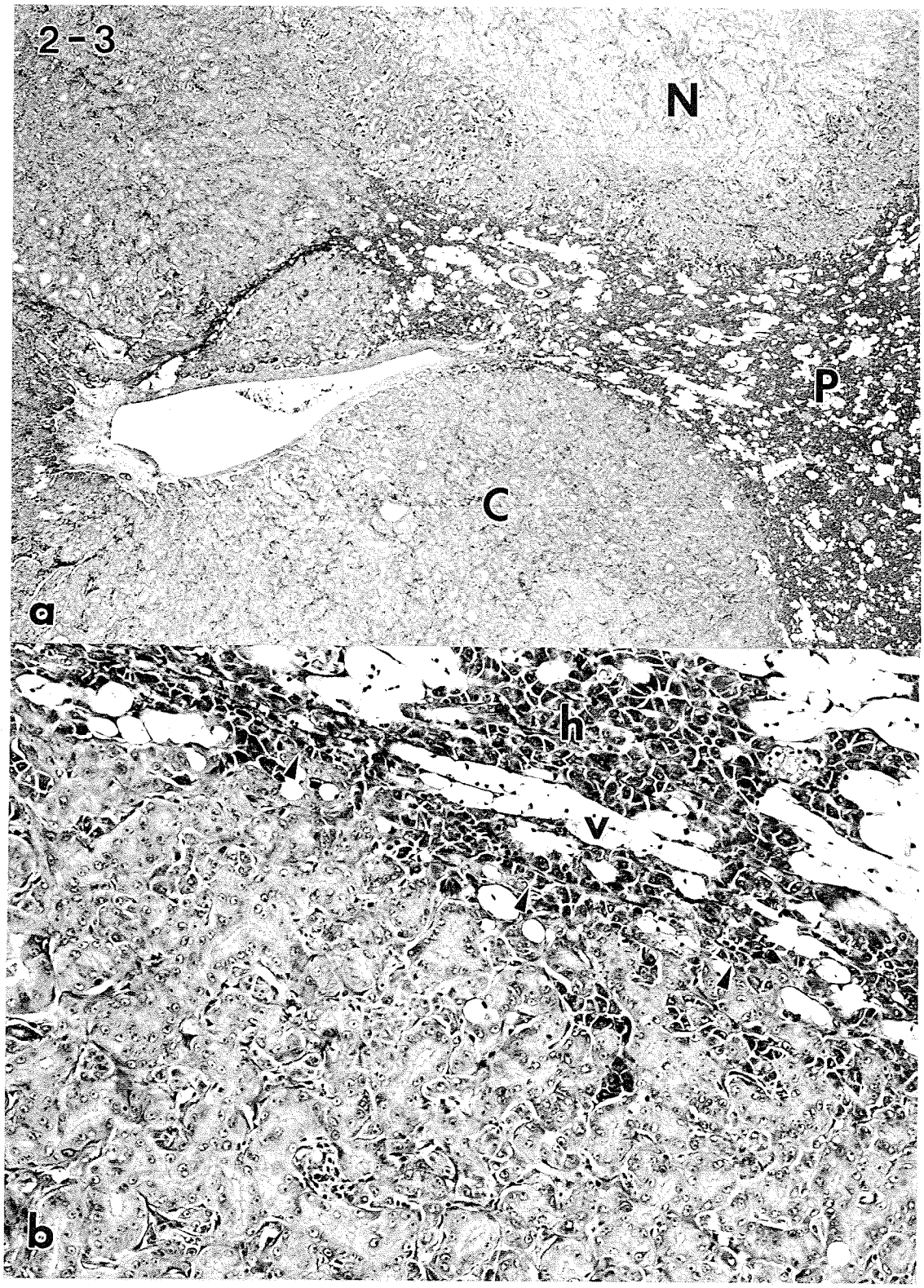

2-2
V
F
N
a
b

Figure 2-3

A large cholangioma in a winter flounder liver from Deer Island Flats, Boston Harbor. Paraffin embedded sections stained with hematoxylin and eosin. The gross appearance of this lesion is illustrated in Figure 2-1b. RTLA # 5229.

a) Cholangioma. Section through the border of the neoplasm. A solid mass of neoplastic tubules is present (C), with a wedge of heavily vacuolated parenchyma (P). Above this, a further lobe of the neoplasm is evident, with a necrotic center (N). 25x

b) Detail of the area between "C" and "P" in 2-3a above. The tubular nature of the neoplasm is evident, with compression of adjacent parenchyma (arrowheads). The tubular stage of vacuolation is evident in the parencyhma, which consists of vacuolated cells (v) and basophilic hepatocytes (h). 200 x.


2-3
N
P
C
a
h
v
b

with extensive scirrhous cords of eosinophilic fibrous tissue running through the mass of neoplastic tissue. Histologically, the grossly visible but smaller adjacent creamy protuberance consisted of a solid, non-tubular mass of neoplastic cholangiocytes. These cells were continuous with the adjacent larger mass of tubules, and compressed but did not invade the overlying band of normal parenchyma.

Tubular cholangiocellular carcinoma

This subtype (Figure 2-4), seen in 15 lesions, had many similarities to the cholangiomas described above, with obvious tubules. However, the cell margins were

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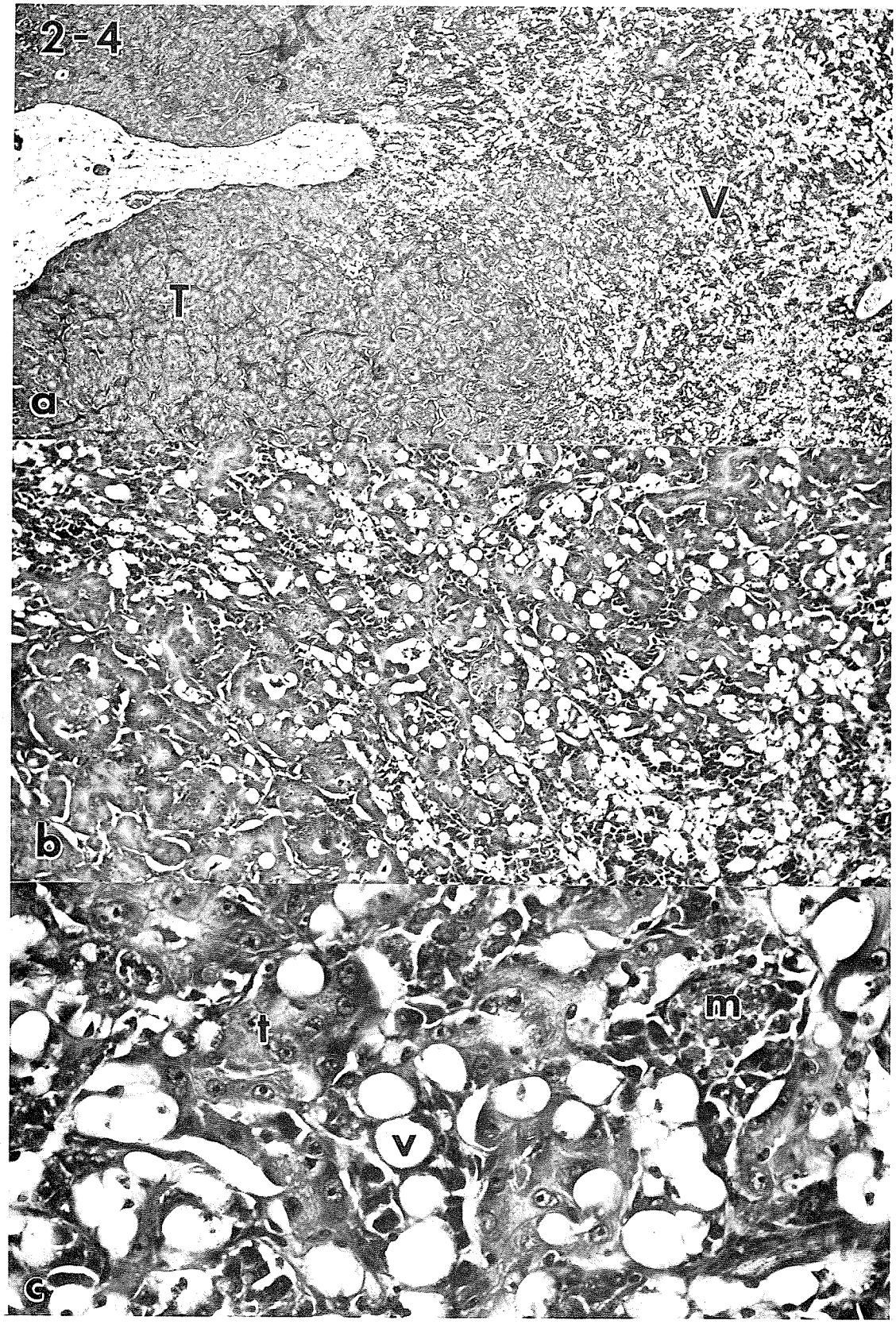
Figure 2-4

An invasive tubular cholangiocellular carcinoma in a winter flounder liver from Deer Island Flats, Boston Harbor. Paraffin embedded sections stained with hematoxylin and eosin. The gross appearance of this fish is illustrated in Figure 1a. RTLA # 5233.

a) A mass of biliary tubules (T). The neoplasm interdigitates an adjacent focus of vacuolated cells (V). 25 x.

b) A higher magnification of the left edge of the neoplasm in (a). Note the close proximity of vacuolated and neoplastic cells. 100 x.

c) A higher magnification of (b). Vacuolated cells (v), neoplastic cholangiocytes (t), and a macrophage aggregate (m) are evident. 400 x.


2-4
V
T
a
b
t
m
v
G

less distinct, the nuclear outlines were more variable, and the tumors were seen at times to invade rather than compress the adjacent parenchyma. Invasion occured by sinuous interweaving of neoplastic biliary tubules around normal tubules of hepatocytes. Vacuolated cells were common at the invasive edge of the tumor lesions.

Solid cholangiocellular carcinoma

A number of cholangiocellular neoplasms (18) lacked an obvious tubular phenotype, with poorly differentiated cholangiocytes arranged in solid masses and whorls (Figure 2-5). Nuclei were smaller than those in the cells of the tubular subtype. The nuclei were elongated, pleomorphic, and irregular in outline and the nucleoli were indistinct. The eosinophilic cytoplasm had a low volume, except where hydropic vacuolation of neoplastic cells occured. Many of these lesions were invasive, with masses of cells diffusely invading adjacent parenchyma.

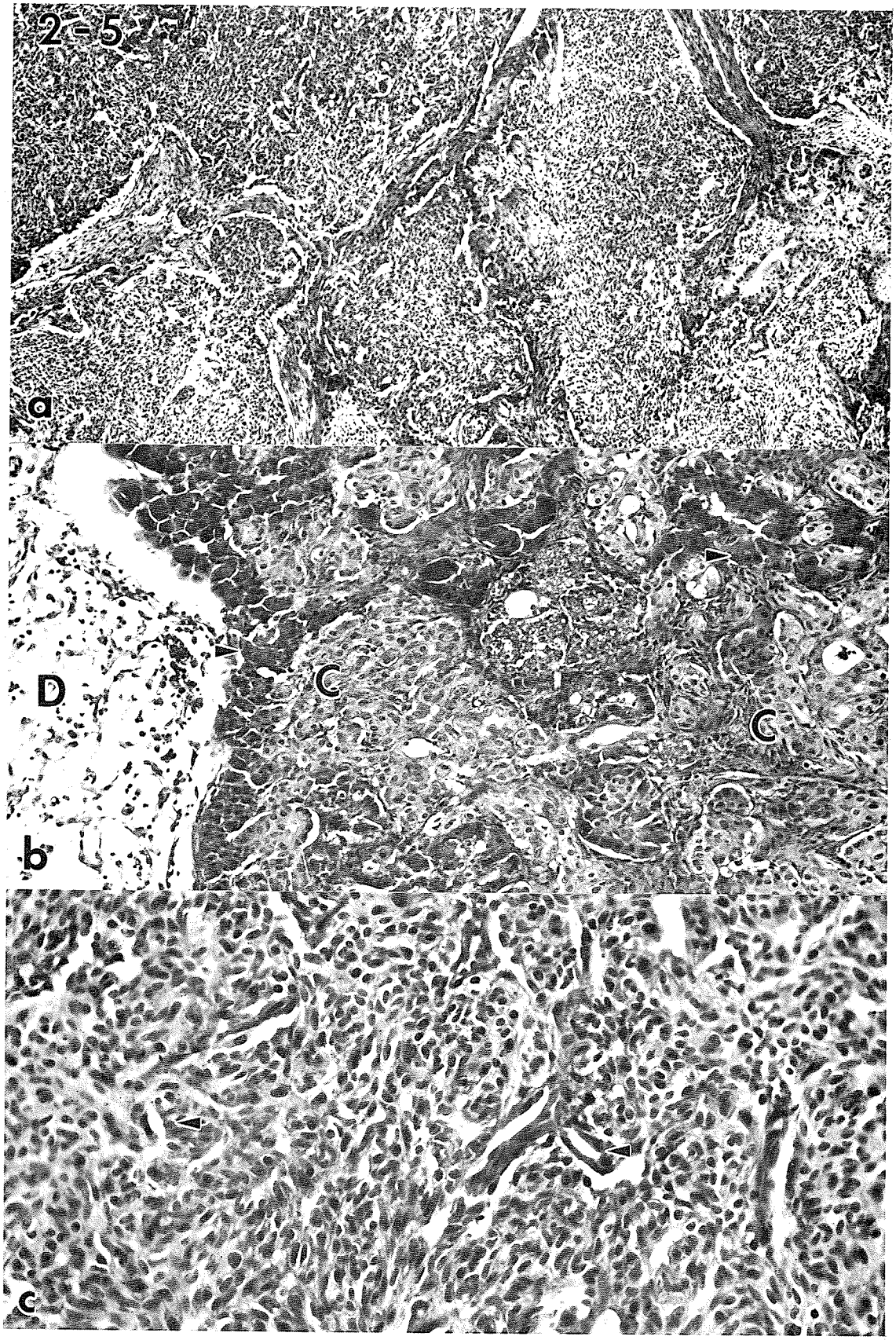
Figure 2-5

An invasive solid cholangiocellular carcinoma in a winter flounder liver from Deer Island Flats, Boston Harbor. Paraffin embedded sections stained with hematoxylin and eosin. The gross appearance of this fish is illustrated in Figure 1a. RTLA # 5233.

a) Masses and whorls of neoplastic cholangiocytic epithelia. 25 x.

b) A higher magnification of a different area of the same lesion as in (a). Masses of neoplastic cholangiocytes (C) are interspersed with trabeculae of more basophilic non-neoplastic hepatocytes (arrowheads). A degenerative area (D) on the left margin has been filled in with fibroplastic tissue. 100 x.

c) Detail of part of figure 5(a). Occasional primitive tubular structures are evident (arrowheads). 400 x.


a
D
C
C
b
c

Cystic cholangiocarcinoma

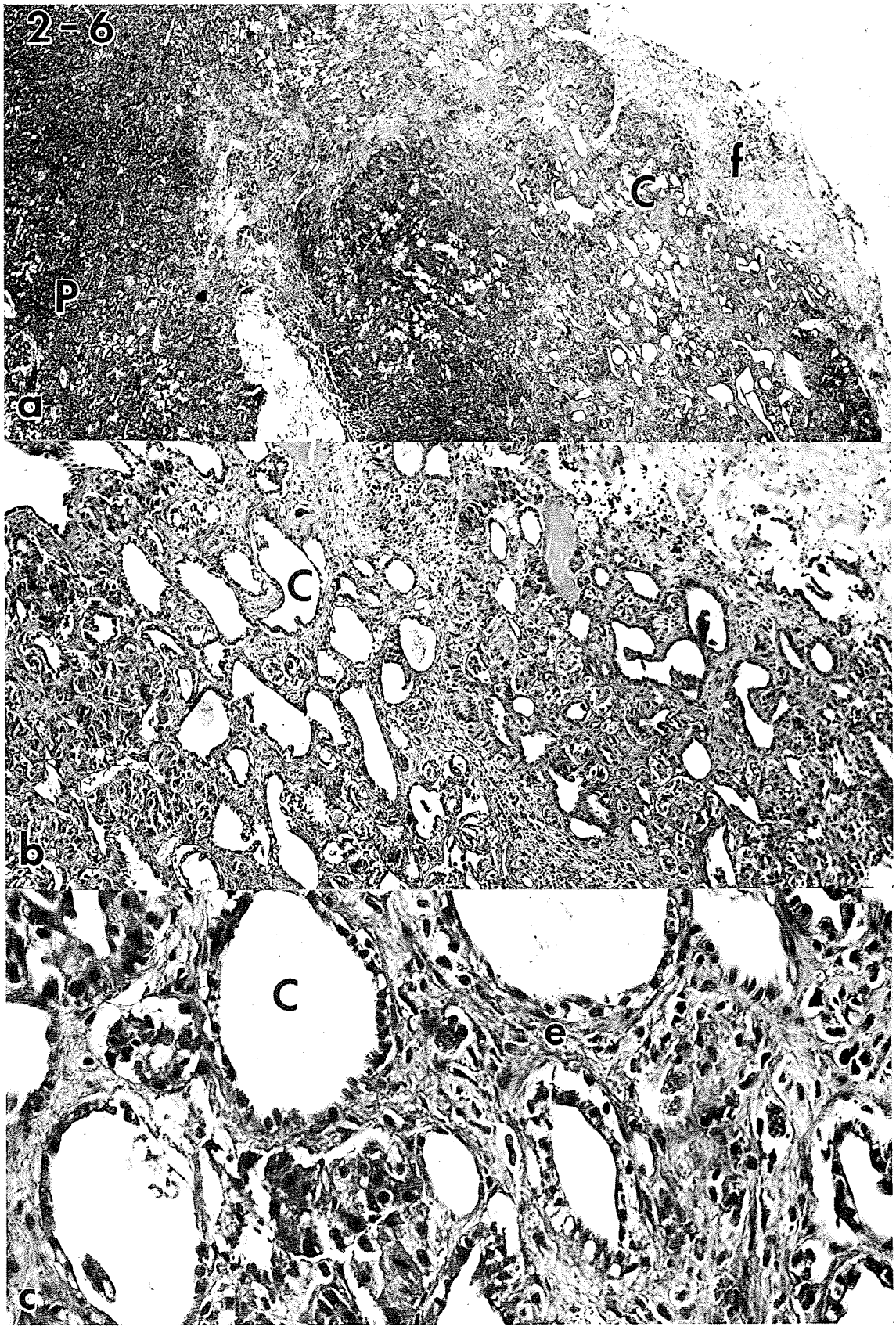
Some tubular cholangiocellular carcinomas had areas dominated by more or less circular cysts and dilated tubules (Figure 2-6), lined by small, flattened, cuboidal epithelia, with ellipsoid nuclei and sparse cytoplasm. In others, the entire lesion was cystic. Cysts were interspersed by more pleomorphic cholangiocytes typical of those described in the solid tumor subtype above.

Figure 2-6

An invasive cystic cholangiocellular carcinoma in a winter flounder liver from Deer Island Flats, Boston Harbor. Paraffin embedded sections stained with hematoxylin and eosin. RTLA # 5228.

a) Cystic cholangiocellular neoplastic tissue (C) is bordered by a fibrotic necrotic capsule (f): the parenchyma contains many vacuolated cells (P). 25 x.

b) 100 x and (c) 400 x of the lesion in (a). Multiple cysts (C) lined with small flattened cholangiocytes are surrounded by a stroma of similar cells.


2-6
f
C
P
a
C
b
C
e
c

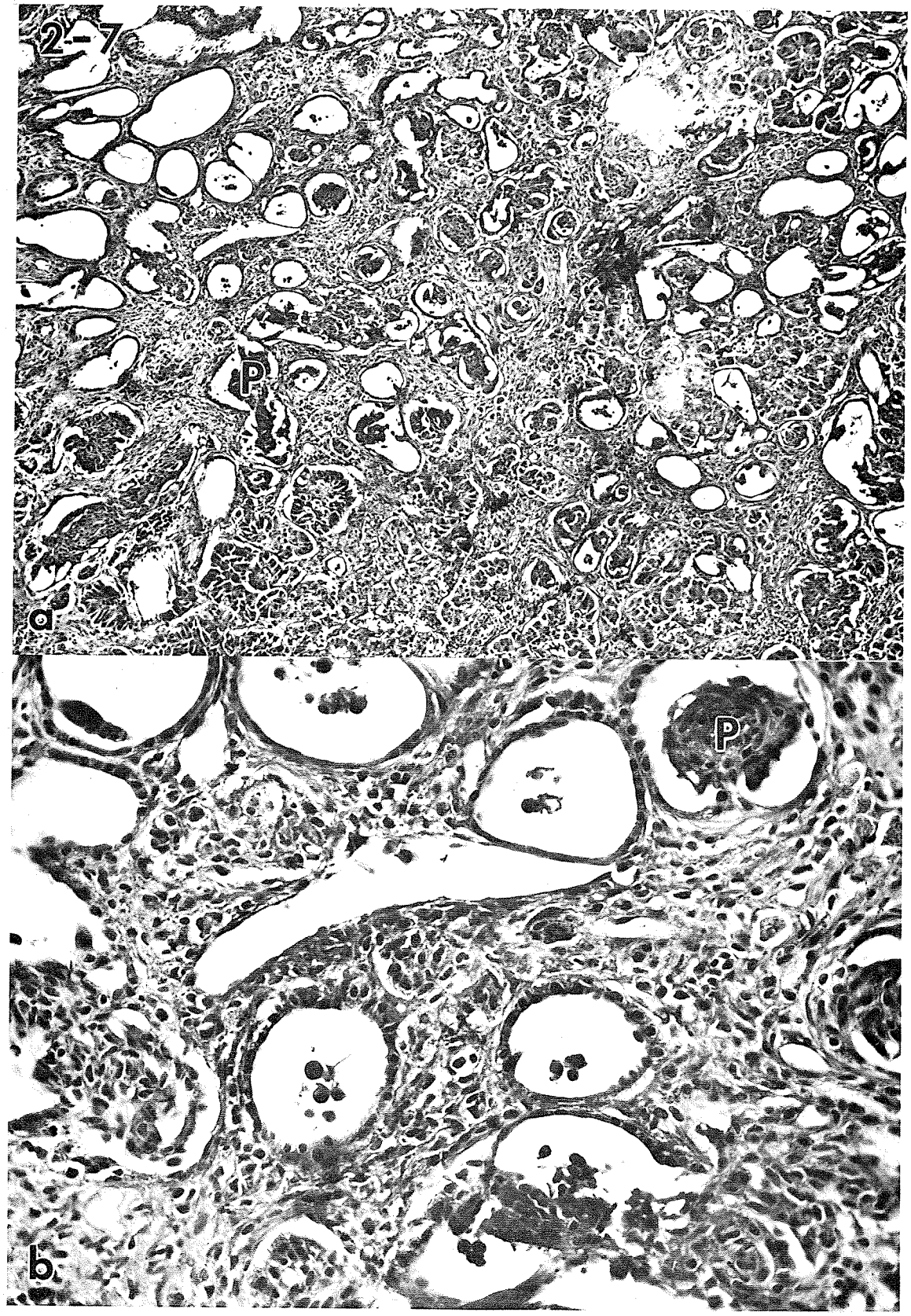
Papillary cholangiocellular carcinoma

A minority of the cystic cholangiocellular carcinomas had papillary tufts growing into the cyst lumen. These were comprised of cells with a similar morphology to those lining the surrounding cysts, namely small poorly differentiated cells with scant cytoplasm (Figure 2-7). Free macrophages were also present in the lumina of these cysts that contained papillary tufts. Some of these cysts had enlarged enormously to form grossly visible structures filled with sero-sanguineous fluid (Figure 2-1c).

Figure 2-7

An invasive papillary cholangiocellular carcinoma in a winter flounder liver from Deer Island Flats, Boston Harbor. Paraffin embedded sections stained with hematoxylin and eosin. RTLA 5228.

a) 100 x and (b) 400 x Papillary tufts (P) are evident in the lumina of many of the cysts.


2-7
P
a
P
b

Degenerative changes in cholangiocellular carcinomas

Fibrotic strands infiltrated many of the lesions. However, a minority showed an extensive fibrosis, giving a scirrhous appearance (Figure 2-8a). These lesions appeared senescent, with whorls and tubules of neoplastic cholangiocytes intercalated by a swirling network of fibroblasts. These tracts were often continuous with spaces in the tumor focus, which were wholly or partially filled with desmoplastic fibrosis (Figure 2-8c). In a minority of lesions, these spaces were filled with many smaller empty structures lined by effete neoplastic cells, interspersed with single macrophages. This arrangement had the appearance of spongiosis hepatis (Bannasch et al. 1981), and seemed to be the end stage of degeneration of the neoplastic focus (Figure 2-8b).

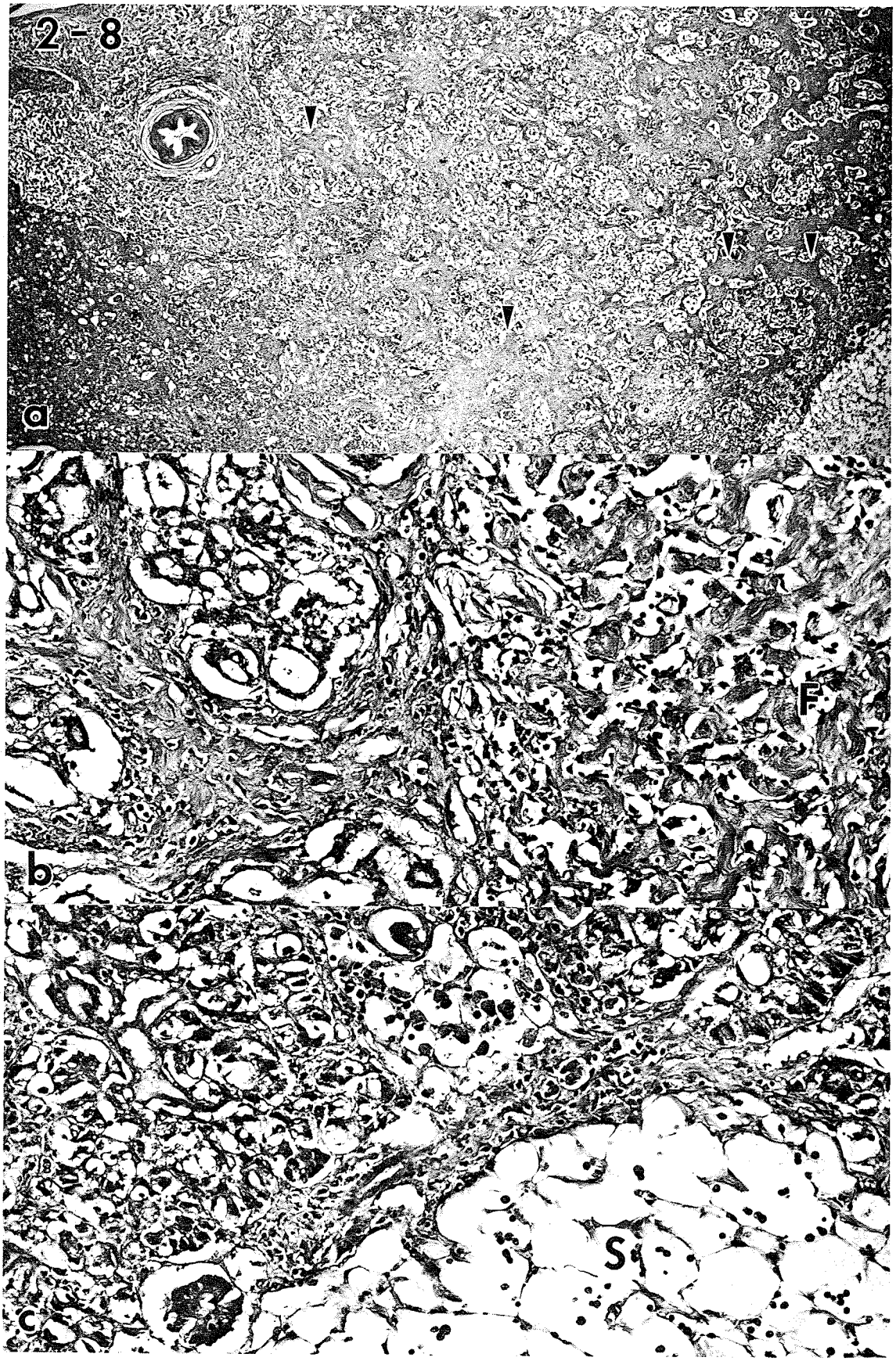
Figure 2-8

An invasive schirrous cholangiocellular carcinoma in a winter flounder liver from Deer Island Flats, Boston Harbor. Paraffin embedded sections stained with hematoxylin and eosin. This tissue section was not deposited with the RTLA.

(a) Extensive fibrotic tracts ensheath degenerate neoplastic cells (arrowheads). Collagen specific stains, not shown, confirmed the presence of collagen. 25 x.

(b) An area of (a) where fibroplasia is extensive (F). 200 x.

(c) A higher power view of the bottom right corner of (a). Spongiosis hepatis is evident (S). Note the differences between spongiosis hepatis as illustrated here and the vacuolated cells evident in many of the other figures. 200 x.


2-8
a
b
c
F
S

DISCUSSION

Important conclusions arising from these observations are: 1) In collections of 1985 to 1990 an array of cholangiocellular carcinomas was the dominant neoplastic cell type in winter flounder from Deer Island Flats, Boston Harbor, MA. 2) Vacuolated cells were closely associated with most of the neoplasms observed. 3) All of the neoplastic lesions that were stained for iron showed complete exclusion of iron, in contrast to surrounding hepatocytes. 4) The response of winter flounder liver to a broad range of sediment contaminants, albeit unique to each site, was very different to that of some other coastal flatfish, such as the English sole.

The cholangiocellular nature of the majority of liver neoplasms in winter flounder can be compared with reports of hepatic neoplasia in bottom feeding fish from contaminated sites (Table 1-3). Thus, cholangiocellular lesions predominated in the brown bullhead, *Ictalurus nebulosus* (Baumann et al. 1987, Hayes et al. 1990), the white sucker, *Catostomus commersoni* (Hayes et al. 1990), and the winter flounder (Murchelano and Wolke 1985 and this study). In contrast, hepatocellular neoplasms were much more common than cholangiocellular neoplasms in the English sole, *Parophrys vetulus* (Myers et al. 1990). In the mummichog, *Fundulus heteroclitus* (Vogelbein et al. 1990) hepatocellular carcinomas were also most common. In fact cholangiocellular carcinomas were not reported.

Hepatocellular carcinoma has been the predominant diagnosis in the experimental fish tumor model species exposed to chemical carcinogens. The group of experimental studies most relevant to feral epizootics in contaminated habitats is that where

polynuclear aromatic hydrocarbons were used, as these compounds are commonly found in contaminated sediments. Aquarium species exposed to polynuclear aromatic hydrocarbons include the rainbow trout, *Oncorhynchus mykiss* (Hendricks et al. 1985, Black et al. 1985), *Poeciliopsis monacha* (Schultz and Schultz 1984), guppy, *Poecilia reticulata* (Hawkins et al. 1990) and medaka, *Oryzias latipes* (Hawkins et al. 1990). The resultant neoplasms were all hepatocellular in the above studies, although in the *Poeciliopsis* study an illustration of one lesion suggests that cholangial components were also present.

The relatively homogeneous histology of the hepatocellular carcinomas of the feral epizootic described in *Fundulus heteroclitus* (Vogelbein et al. 1990) is very comparable to the results obtained in the above experimental studies. *Fundulus* were exposed to very high doses of a mixture of the genotoxic polynuclear aromatic hydrocarbons in creosote from a nearby wood treatment plant, and presumably other contaminants. This exposure is somewhat comparable in result to the common experimental protocol of early exposure to a relatively high dose of a single genotoxic carcinogen. In contrast, the other feral epizootics of neoplasia have usually involved chronic exposure to a broader suite of genotoxic and epigenetic carcinogens. Those compounds include polynuclear aromatic hydrocarbons, halogenated hydrocarbons, and heavy metals. This greater diversity of exposure may have given rise to the wider diversity of phenotypes, with multiple subtypes in these other feral species including the winter flounder.

The similarities and differences between species that develop mainly

cholangiocellular carcinomas, such as the winter flounder, and those where hepatocellular carcinomas dominate, such as the English sole, should be closely compared.

These differences in neoplastic cell type resulting from each field and experimental exposure may very well reflect a number of factors: 1) The nature of the chemical(s) involved, the dose(s) and the duration of exposure are unique to each site. 2) The spawning behavior, feeding habits and seasonal migration are unique to each species and, to a degree, to each site. 3) The cellular and molecular responses of each species to the chemical contaminants depends on the unique genetic, biochemical and morphological constitution of each species. 4) The relative stage in the life span at which the fish were examined varies in each experiment and field study. 5) The resultant histopathology is interpreted uniquely by each histopathologist. These factors are considered below.

With regard to the age at sampling (4), D. Hinton (pers. comm.) has found that cholangiocellular neoplasms appear later than hepatocellular lesions in an experimental medaka system. The differences between experimental and feral studies may have arisen from the fact that most experimental fish are killed at one year or younger, whereas many feral fish are significantly older at the time of sampling.

With regard to the idiosyncracies of histopathological diagnosis (5), Lee, Hendricks and Bailey (1989) have recently acknowledged that the usage of "hepatocellular" in the diagnosis of fish tumors may have in the past included cholangiocellular structures. The relevance of non-hepatocellular tumor data to

carcinogenicity studies had, according to these authors, not been established. In fact, Hendricks now regards the majority of carcinomas in rainbow trout liver exposed to aflatoxin B1 to be mixed hepatocellular and cholangiocellular (Hendricks, pers. comm.), rather than primarily hepatocellular as described in previous studies (Sinnhuber et al. 1976).

Fish pathologists are not alone in having difficulty standardizing diagnostic criteria. Edmonson and Craig (1987) state that in the classification of human hepatic neoplasms sampled at autopsy, the terms cholangiocarcinoma, mixed cholangiocellular carcinoma, adenocarcinoma, and hepatocellular carcinoma with ductal transformation have been used for the same lesion type by different institutions.

A question, therefore, is whether there is any fundamental difference between a "poorly differentiated hepatocellular carcinoma" and a "poorly differentiated cholangiocellular carcinoma" beyond subjective diagnostic interpretation? Are we looking for two distinct oncogenetic mechanisms or just one? Is there a single epithelial stem cell population in fish liver, with a common origin of all hepatic epithelial neoplasms? These questions are still being debated in the field of mammalian hepatocarcinogenesis, and perhaps an analysis of the pathogenesis of these lesions in winter flounder will add to our understanding of liver cancer in the broader context of comparative oncology.

Recent studies using cytokeratin immunohistochemistry support the view that some mammalian species have a single hepatic epithelial progenitor cell type (Marceau 1990). Whether alterations that lead to carcinogenesis are in a progenitor cell or in

hepatocytes that have already differentiated is a central issue. The hepatocyte / altered hepatocytic focus / hepatocellular carcinoma sequence with concomitant focal changes in morphology and histochemistry is regarded by many to be the central paradigm of hepatocarcinogenesis (Bannasch 1990). However, increasing numbers of researchers are now focussing on the so-called oval cell and its pluripotent abilities. The issue of oval cells as stem cells was reviewed recently (Sell 1990). Oval cells transformed in culture, when injected into rats gave a broad spectrum of hepatocellular and cholangiocellular neoplasms (Tsao and Grisham 1987). Oval cells are believed to arise from the terminal bile ducts (Sell and Salman 1984). Are there any non-neoplastic cell types that may have a role in winter flounder comparable to oval cells in rodents?

In the winter flounder the cell type most intimately associated with the majority of the neoplastic lesions described in this study is the vacuolated cell (Table 2-1). This cell appears to be an abnormal hepatic epithelial cell that contains a large vacuole, apparently filled with water and electrolytes, that develops in response to chronic exposure to cytotoxins. In other studies (Chapters 3 and 4) we have shown the first cell that undergoes vacuolation to be the biliary preductular cell. It is possible that there is some form of functional homology between biliary preductular epithelia in winter flounder and the oval cell in mammals.

Investigations prompted by these questions are described in subsequent chapters. The morphogenesis of vacuolated and other abnormal cells is described histologically and ultrastructurally in Chapters 3 and 4, the potential for cell proliferation is evaluated in Chapter 5, and protocols designed to model the disease are described in chapter 6.

CHAPTER 3

EPITHELIAL VACUOLATION AND BILIARY EPITHELIAL PROLIFERATION PRECEDE NEOPLASIA IN THE LIVER OF WINTER FLOUNDER, *PSEUDOPLEURONECTES AMERICANUS*.

INTRODUCTION

The presence of vacuolation and hepatic neoplasia in winter flounder from Boston Harbor was first reported by Murchelano and Wolke (1985); this report focused on some of the salient features of the end stage of the disease syndrome. More recently Gardner and Pruell (1989) presented findings suggestive of an association between neoplasm prevalence and contamination by polynuclear aromatic hydrocarbons. Additionally, Moore et al. (1989) described the earliest stage of vacuolation to be centrotubular, and Bodammer and Murchelano (1990) described the ultrastructure of vacuolated and aberrant hepatocytes from two Boston Harbor winter flounder. The detailed morphology of neoplastic lesions were described in chapter 2, but to date there has been no description of the stepwise morphological changes leading up to frank neoplasia, nor any quantitative analysis of prevalence of lesions between different age and length classes of fish, all of which could be extremely important in understanding the disease.

This chapter examines the following questions concerning the pathogenesis of this disease: 1) What are the most obvious cellular changes that precede and accompany hepatic neoplasia in these fish? These changes were examined in the context of the histology of winter flounder from Georges Bank; the least contaminated area within which winter flounder can be caught. 2) How does the prevalence of neoplastic and associated non-neoplastic lesions change as fish grow in length and age? 3) How are the different lesions related to each other structurally? 4) How prevalent are these lesions in less contaminated habitats? 4) How stable over time are these

lesions after removal and transfer of hosts to a cleaner environment? 5) How has the prevalence of lesions in fish from Boston changed during the course of the past seven years?

Knowledge of the lesions and how their prevalences change with age and length will serve to better study the molecular and biochemical rate-limiting steps in the progression from normal liver to frank neoplasia. An understanding of how the different lesions relate to each other will allow better testing of hypotheses about the cells of origin for hepatocellular and cholangiocellular lesions.

In comparative terms, a more complete understanding of this disease in winter flounder will allow a meaningful comparison between the winter flounder and other species such as the English sole, that show markedly different prevalences of hepatocellular vs. cholangiocellular neoplastic disease (Myers et al. 1990).

MATERIAL AND METHODS

Collections of Fish

Winter flounder were collected from several sites within Boston Harbor, as well as from other coastal and offshore sites. These sites ranged widely in levels of chemical contamination of the sediments, see Table 3-1.

Boston Harbor: Sexually mature winter flounder greater than 200 mm in length were collected from Deer Island Flats in Boston Harbor by otter trawl; 52 were collected on March 18th 1988, and 102 on April 30th 1990. An additional 73 adult fish were also obtained in a May 13th, 1989 survey of Boston Harbor (30 from Deer Island, and 43 from other sites in Boston Harbor). These fish were collected within

a four hour period from eight areas by hook and line (Sullivan and Robinson, 1990). All of the above Boston Harbor collections were a subset of the fish examined in Chapter 2. Fish for the current study were only used from collections in which every fish caught was examined histologically, irrespective of its size or gross liver appearance.

In addition to the fish killed soon after capture, 52 adults collected by otter trawl in April 1987 were held in 90% recirculated Woods Hole seawater, at a temperature of 15°C, or ambient, whichever was lower, at a bottom density of 10 fish m^{-2}. The fish were fed chopped surf clams from Georges Bank for five months before sacrifice. Analysis, by gas chromatograph, mass spectroscopy, of a subsample of these clams from this site showed total Arochlor 1254 to be 25 ng/gm dry weight (Emily Monosson pers.comm.).

Additional collections, were made at 4-6 week intervals between July-November 1987 using a 70 cm wide scallop drag with a 4 mm mesh liner. These collections revealed a cohort of young-of-the-year fish on Deer Island Flats. These fish ranged in size from a minimum of 27 mm in early July, up to 100 mm by November. This cohort remained resident on Deer Island Flats until the end of December, when they were found on the rise from the deeper water to the south of the Flats. The stomach contents of all these fish were examined visually. Subadult fish of the size range of 100-200 mm[1], were rare on Deer Island Flats.

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[1]These were probably two-year-old fish, on the basis of unpublished growth curve data for winter flounder from Boston Harbor (J. Burnett, National Marine Fisheries Service, Woods Hole, MA).

Coastal and Offshore Fish: Winter flounder were also collected by otter trawl in Cape Cod Bay on May 15, 1988, in Nantucket Sound April 28, 1988, and at Onset, Buzzards Bay on June 12, 1989. Young of the year were caught by beach seine in Scorton Creek, Sandwich MA during September, 1987. Additional histological slide collections of winter flounder livers from Massachusetts Bay and Georges Bank were obtained from the New England Aquarium histological section depository. The various coastal and offshore site locations are given in Figure 1-1 and Table 3-2.

Age determination

For the April 30th 1990 collection, a sample of scales was scraped off the dorsum of the caudal peduncle of each animal, and submitted for age ring counts to Jay Burnett, National Marine Fisheries Service, Woods Hole, MA.

Dissection of fish

Fish were killed by cervical section. Young of the year were killed at capture. Adult fish were either killed at the time of collection, or were held for 1-3 days in ambient temperature, aerated seawater before dissection in the laboratory. Fish were placed blind side up and measured for total and standard length. An oval incision was made in the ventral body wall overlying the liver and anterior ventral gonad. Gonads were either white and triangular in males, pink and elongated in females, or small and blue-gray in immature fish. All tissue samples were fixed in 10% neutral buffered formalin. Fish shorter than 50 mm in total length were fixed whole and then

decalcified in saturated ethylenediaminetetraacetic acid for 48 hours (Luna 1968) before being embedded in paraffin. Livers from larger fish were removed by severance of the peritoneal attachments. Livers from 50-100 mm long fish were fixed whole. For fish longer than 100 mm in length the liver was cut into 4 mm thick slices. Each slice was examined for grossly visible abnormalities. A routine sample was taken from the central but non-hilar portion of the liver. Area(s) of visibly abnormal liver were also sampled. Other viscera, gonads, heart and gills were inspected for gross lesions. Fixed specimens were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. Selected fish were also stained for glycogen by the periodic acid Schiff (PAS) method of McManus, and for iron: these methods are described by Luna (1968).

Histological analysis

After an initial survey of the material, the prevalences of the following lesions were recorded:

1) Vacuolation, seen in three stages: a) Centrotubular vacuolation - isolated groups of 1-2 vacuolated cells in the center of the hepatic tubule). b) Tubular vacuolation - linear arrays of vacuolated cells, filling the hepatic tubule, often extending into biliary duct structures. c) Focal vacuolation - foci of thirty to several hundred contiguous vacuolated cells. 2) Macrophage aggregation. 3) Biliary duct proliferation. 4) Neoplasia. 5) Necrosis.

Prevalences of macrophage aggregation and biliary duct proliferation were scored

by examining five randomly selected views of one slide, using a Zeiss axioskop. Macrophage aggregations were scored as present if there was a mean of more than 1 per 100x view. Biliary duct proliferation was scored as present if there were more than 4 bile duct profiles, whose fibrotic sheathes were not touching each other, per 25x view. Vacuolation, necrosis, and neoplasia were scored as present if evident in any part of one slide, examined completely. Neoplasms were defined as focal accumulations of atypical hepatocytes, or cholangiocytes, that were larger than 1 mm diameter and which showed various degrees of failure to differentiate, and invasion or compression of the surrounding parenchyma. Full descriptions of these and other neoplasms can be found in Chapter 2.

Analysis of data

The prevalence of lesions was compared between classes of fish defined by differences in sex, length, and site of collection. Many lesions co-occurred: they were thus not statistically independent. Gross hepatic lesion prevalence was regarded as the best indicator of severe pathology, and was thus tested for significant differences between groups of fish by chi-square analysis. Significance of differences between mean age and length of groups of fish that did, and did not, contain each lesion were analyzed by the Student's t test. One potential bias in the data stemmed from multiple slices being examined from gross lesion-bearing fish, in contrast to single slices for visibly normal fish. This may have underestimated the neoplasm prevalence for visibly normal fish, however lesions larger than 1 mm in diameter were visible grossly, if

exposed by the 4 mm slicing technique described above. Inevitably a proportion of the very small lesions, were overlooked. However, only one neoplasm was found in 207 fish that lacked grossly visible hepatic lesions. Furthermore, where the prevalence of non-neoplastic lesions was recorded independently for different slices from the same liver, there was excellent agreement between blocks. These observations strongly suggest that any bias introduced in the sampling process was minimal.

RESULTS

Offshore and Coastal Fish

Georges Bank fish were remarkable in their lack of histological abnormalities (Figure 3-1 and Table 3-2). Hepatocyte cytoplasm stained poorly with hematoxylin, but PAS stains showed abundant glycogen. Ultrastructural studies (Chapter 4) showed an abundance of lipid in the cytoplasm of hepatocytes. The most remarkable feature of the liver histology of these fish was their homogeneity. Larger biliary structures were very rare. Essentially sections of these livers were comprised of large expanses of lipid and glycogen filled hepatocytes in random tubular arrays. Only in the hilar region were larger vessels and ducts at all noticeable. Macrophages, whether single or aggregated were extremely rare in fish from Georges Bank.

Young of the year from Scorton Creek showed a homogeneous liver structure, with basophilic hepatocyte cytoplasm, and minimal PAS staining. Of the fish from Massachusetts Bay and Cape Cod Bay, a minority (20% or less) had mild but distinct changes reminiscent of some of the non-neoplastic changes seen in winter flounder from Boston. In the Massachusetts Bay sample mild, diffuse vacuolation was the most prevalent lesion. Macrophage aggregation was also present at a low level in both of these coastal sites. This was also seen in flounder from Onset, Buzzards Bay, and south of Falmouth in Nantucket Sound. The sample size of these latter two sites (5, and 6 fish respectively) precluded meaningful numerical analysis. These changes were far less severe than similar changes in Boston fish, which are described below. In a few fish, more commonly from the coastal sites, than from Georges Bank, the juxtasinusoidal portions of hepatocyte cytoplasm stained more densely with hematoxylin. This was regarded as a normal variation.

TABLE 3-2

Summary of lesion prevalence in winter flounder livers from Georges Bank, Massachusetts Bay, Cape Cod Bay, and Boston Harbor.

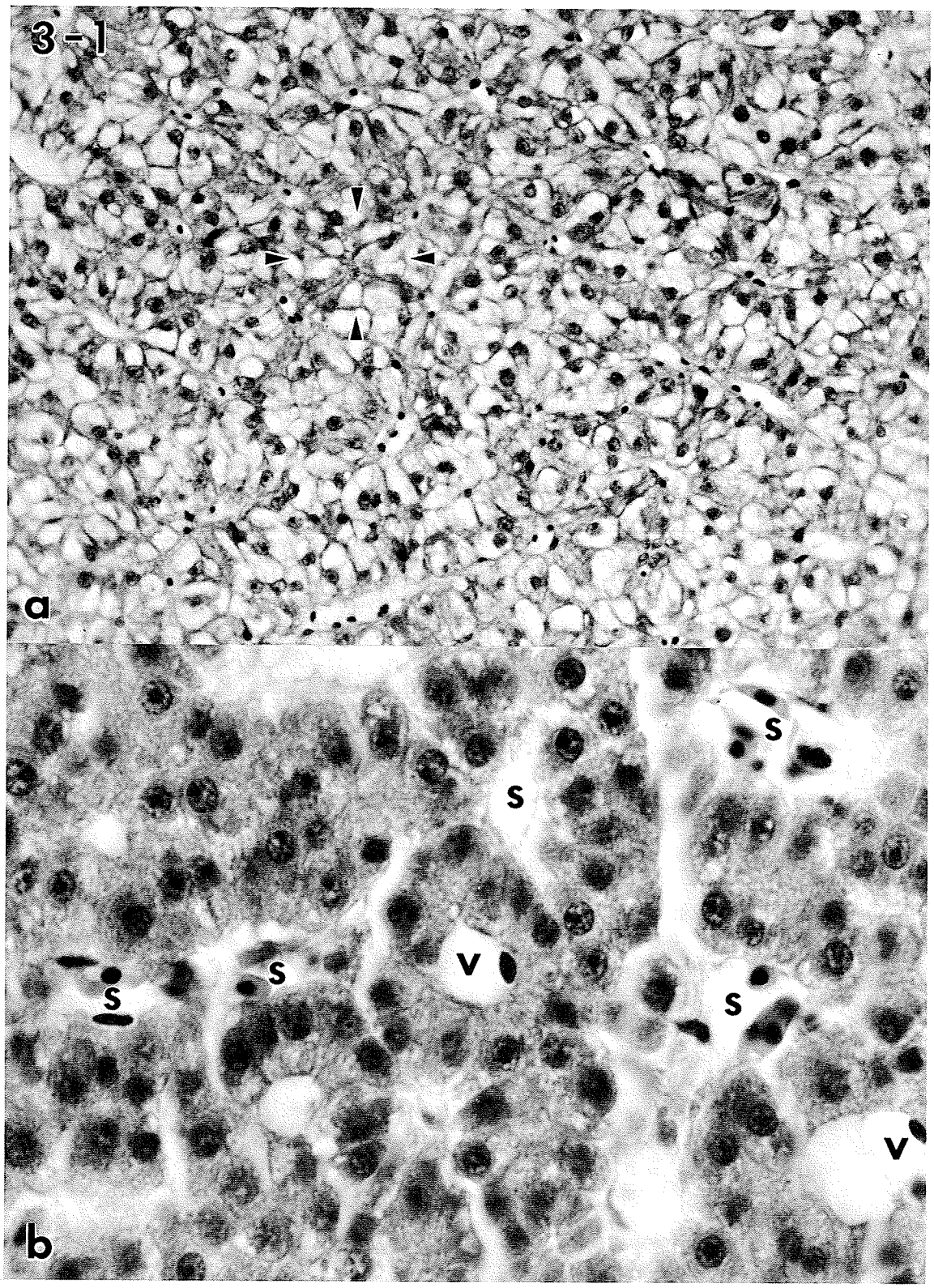
	Georges Bank[1]	Massachusetts Bay[2]	Cape Cod Bay[3]	Boston Harbor (Deer Island)[4]
Total sample size	36	34	43	82
Mean Length±S.D.(mm)	372 ± 47	243 ± 39	232 ± 80	370 ± 39
Macrophage aggregation	0 (0)	1 (2.9)	1 (2.3)	32 (39.0)
Vacuolation	0 (0)	4 (11.8)	1 (2.3)	46 (56.1)
Gross lesions	0 (0)	0 (0)	0 (0)	17 (20.7)
Biliary proliferation	0 (0)	0 (0)	1 (2.3)	21 (25.6)
Liver neoplasia	0 (0)	0 (0)	0 (0)	7 (8.5)

Lesion prevalences given as the number of cases per station, with the percentage prevalence in parentheses. Lesions are defined in the text. Vacuolation includes any or all of the three stages described. Sampling stations are illustrated in Figure 1-1. [1]Pooled samples from 7 sites within the area bounded by 40° 52'N and 41° 41'N, and 71° 41'W and 67° 29'W. Samples were obtained from New England Aquarium depository. [2] Pooled samples from 3 sites 3.5-7.3 nautical miles east and northeast of Deer Island, caught 7-8 July 1987. Samples obtained from New England Aquarium depository. [3] 3.5 miles south east of Manomet Point, Cape Cod Bay, caught 10 May 1988. [4]Deer Island Flats, caught March 1988 and May 1989.

Figure 3-1 - Histology of winter flounder from Georges Bank and Boston Harbor. Five micron section stained with hematoxylin and eosin. Note the magnification of the 1b is higher than 1a.

a) Normal winter flounder liver. Random tubular (arrowheads) arrangements of hepatocytes surround a centro-tubular canaliculus. Lobules typical of mammalian liver are not seen. Hepatocytes are rich in normal vacuoles which stain lightly with hematoxylin. These vacuoles contain glycogen and lipid. Biliary ductule and duct structures, and macrophage aggregations are all absent from this image and rare from animals from this site in general. 200x.

b) Centrotubular vacuolation (v) in the liver of a winter flounder of 180 mm total length. Vascular sinusoids (s) surround tubules of hepatocytes. At the center of a number of tubules one or more vacuolated preductular cells are present. 1000 x.


3-1
a
b
S
S
S
S
S
S
V
V

General features of Boston Harbor Fish

Erosion and deformation of the rays of the fin and tail were commonly seen in adult winter flounder from Boston Harbor. Young of the year fish caught between July and November 1987 on Deer Island Flats showed no such changes.

Post-spawning adults and immature fish were found to have stomachs filled with polychaete worms. Polychaetes were also seen in great number in the fine meshed drag.

The liver histology of the young of the year from Boston Harbor was comparable to the samples of the same age class from the Scorton Creek fish described above. In contrast, the liver histology in adult fish from Boston Harbor was markedly different from that in adults from cleaner sites (Table 3-2 and Figures 3-1b and 3-2). Most hepatocytes lacked the lipid and glycogen-filled cytoplasm seen in normal fish. Hepatocytes were smaller than those of reference fish, and sinusoids were less prominent. The hepatocyte cytoplasm stained with increased basophilia (Table 3-3). Degree of hepatocyte basophilia did not differ significantly between males and females.

Figure 3-2
Histology of tubular and focal vacuolation in the liver of a Boston Harbor winter flounder.

a) Focal and tubular (t) vacuolation in the liver of a winter flounder from Boston Harbor of 350 mm total length. The large focus (F) of vacuolation contains islands of non-vacuolated basophilic cells (b). Extensive biliary hyperplasia and fibroplasia (bf) is evident. 100 x.

(b) A higher magnification of tubular vacuolation (tv), biliary hyperplasia, fibroplasia and vacuolation of bile ducts (vc). 200 x.

(c) A series of vacuolated tubules with an adjacent aggregation of macrophages (ma).

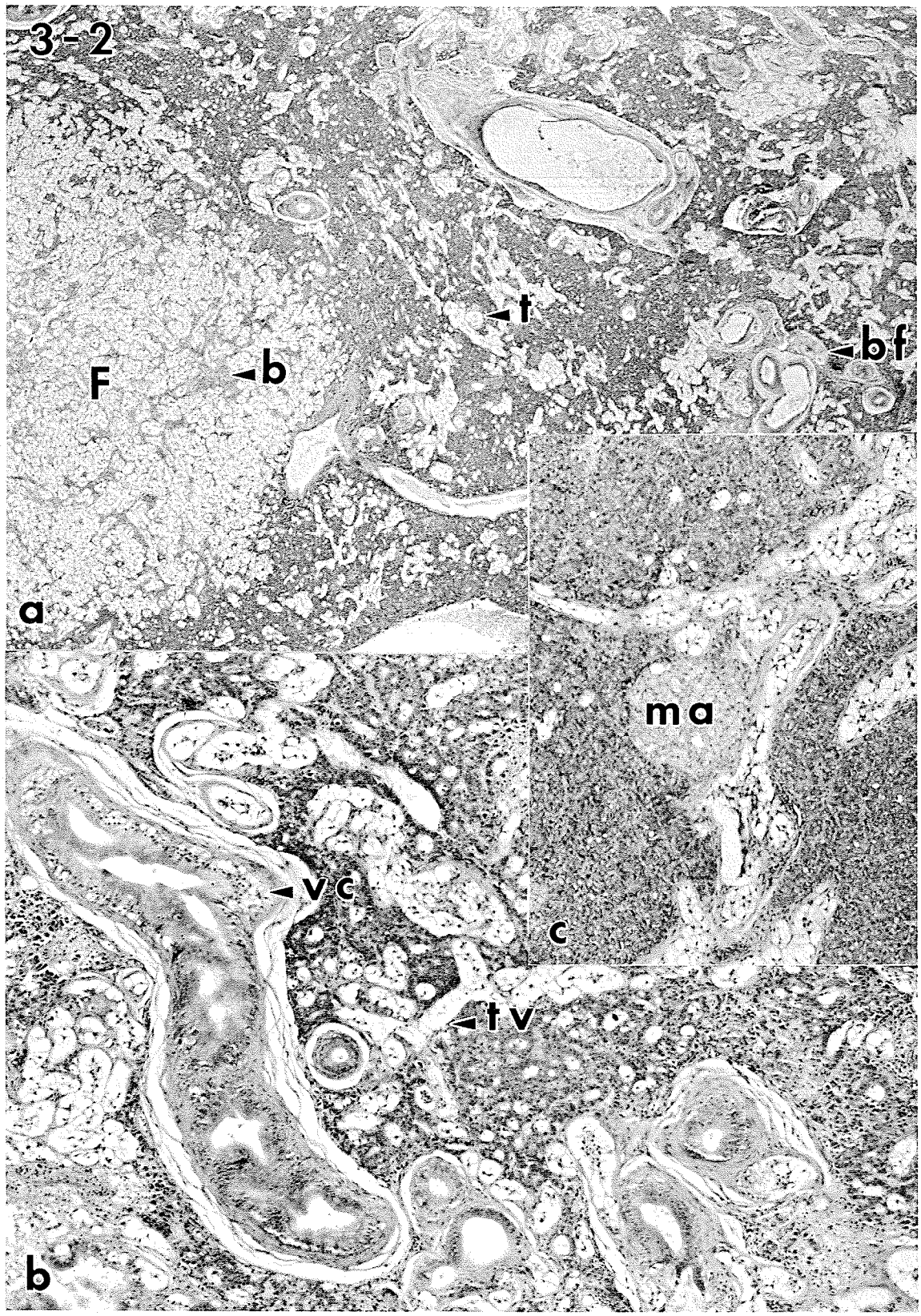

3-2
F
b
t
bf
a
ma
c
vc
tv
b

TABLE 3-3

Comparison of levels of hepatocyte basophilia[1] for adult flounder from offshore, coastal and urban sites.

	Georges Bank[2]	Massachusetts Bay[3]	Cape Cod Bay[4]	Boston Harbor (Deer Island)[5]
Sample size	36	34	43	82
Basophilic Index	0.1 ± 0.4	1.8 ± 1.1	2.0 ± 1.3	3.3 ± 0.7

Stations are shown in Figure 1-1.
[1]Hepatocyte basophilia was scored on an arbitrary scale, 0-4, from very little cytoplasmic staining (0) to intense purple (4). No gender differences were observed from any site.
[2]Pooled samples from 7 sites within the area bounded by 40°52'N and 41°41'N, and 71°41'W and 67°29'W. Samples were obtained from New England Aquarium depository.
[3]Pooled samples from 3 sites 3.5-7.3 nautical miles east and northeast of Deer Island, caught 7-8 July 1987. Samples obtained from New England Aquarium depository.
[4]3.5 miles southeast of Manomet Point, Cape Cod Bay, caught 10 May 1988.
[5]Caught March 1988 and May 1989.

The whole biliary system appeared to have undergone a marked proliferation, and/or metaplasia from hepatocytes, and an increase in wall thickness. Bile duct lumina were often dilated and contained densely staining material suggestive of cholestasis. Biliary and vascular structures commonly had adjacent macrophage aggregates. Vascular sinusoids were noticeably less prominent, and essentially absent from focal aggregations of vacuolated cells. Vacuolar swelling, distinct from the lipid-rich, poorly-stained hepatocytes of normal fish, was seen in hepatocytes, cholangiocytes and intrahepatic exocrine pancreatic cells. Grossly visible foci of vacuolated cells and or neoplastic cells were often present. Macrophage aggregations were usually absent from foci of vacuolated cells. Foci of basophilic cellular alteration described in this

species (Murchelano and Wolke 1985), were seen very rarely, and eosinophilic and clear cell foci comparable to those described in the English sole (Myers et al. 1987) were not seen. Further description of each of these lesions is provided below, and followed by an analysis of lesion prevalence, and how they vary within the population.

Biliary duct proliferation

The earliest change in the biliary system in Boston Harbor fish was characterized by an increased number of bile ductule and duct profiles, with many branches evident. These were often associated with macrophage aggregations. This condition progressed in larger fish to form a tortuous, proliferated network of biliary channels, often with thick fibrotic sheaths, and vacuolated cholangiocytes, described below (Figure 3-1b and 3-2).

Vacuolation

Centrotubular vacuolation - Single and paired cells each containing a large non-staining vacuole were first seen at all commonly in fish of 200 to 300 mm total length. These cells were usually within a tubule of hepatocytes and were rarely seen adjacent to a vascular sinusoid (Figure 3-1b). Previously, we have shown (Moore et al. 1989) the following ultrastructural evidence for these cells to be abnormal preductular epithelia: 1) Numerous cellular junctions with the apices of surrounding hepatocytes - a hallmark of the center of the teleost hepatic tubule, and 2) Small electron-dense nuclei characteristic of preductular cells; these contrasted with the hepatocyte nuclei that

were larger, circular and had a prominent nucleolus. The vacuoles were devoid of lipid. Seen with light microscopy in this study, these centrotubular vacuolated cells were swollen, and they filled the pre-ductular lumen. Their cytoplasm did not stain with hematoxylin, eosin or with PAS for glycogen, iron, or nucleic acids (chapter 2). They had peripheral, small, densely staining nuclei, and they were significantly enlarged in their cytoplasmic volume as compared to normal flounder preductular cells.

Tubular vacuolation - In the livers of more severely affected fish the abnormally vacuolated cells were seen in curvilinear arrays or cords, many cells long, though only 1 to 4 cells wide (Figure 3-2b and c). These cords of vacuolated hepatocytes seemed to be confluent with partially vacuolated bile ducts in some areas. The dividing border between vacuolated bile ducts, and the vacuolated tubules of hepatocytes with fibrotic sheaths, was hard to define, suggesting that the vacuolated state may be a single epithelial type arising within the teleost tubular liver, from hepatocytes and from cholangiocytes at this stage, and from biliary epithelia at the centrotubular stage.

Focal vacuolation - In fish with moderate to advanced lesions, vacuolated cells also appeared in aggregations of several hundred cells, sometimes forming nodules that were grossly visible (Figure 3-2a). Within the large nodules of vacuolated cells there were occasional islands of small, apparently undifferentiated, non-vacuolated basophilic cells.

Necrosis

Many fish from Boston Harbor showed diffusely distributed patches of hepatic

necrosis, with loss of cellular outline and nuclear condensation; this change was often accompanied by fibrosis.

Gross lesions

Grossly visible lesions have been described in Chapter 2. One to three millimeter creamy cysts were also seen in the visceral peritoneum, usually of the intestine, but occasionally involving other viscera including the liver capsule. These were microsporidian cysts of *Glugea stephani* (Stunkard and Lux, 1975).

Neoplasia

Fifteen fish that bore one or more hepatic neoplasms were observed. Detailed descriptions of these lesions, and others from additional non-randomly sampled fish, were given in Chapter 2.

Macrophage aggregation

Aggregations of 10 to 20 contiguous macrophages in section were not uncommon in flounder from Boston Harbor (Figure 3-2c). Single macrophages were seen in the liver parenchyma and in the walls of larger bile ducts in fish from all sites, including Georges Bank, but aggregations were only rarely seen in fish from cleaner sites. The large aggregations seen in Boston Harbor flounder were most often adjacent to areas of aggregated vacuolar cells, biliary proliferation, and necrosis.

Variations in lesion prevalence with:

Length

A low frequency of non-neoplastic lesions was seen in the 100 to 200 mm length class. Marked increases in lesion frequency were seen after the onset of sexual maturity (Figure 3-3). Lesions of any type were very rare in young of the year fish (100 mm).

Age

Age data were available only for the 1990 collection. Lesion prevalence for 3 age classes of that collection is illustrated in Figure 3-4, which shows that prevalences increased as the fish grew older. Fish with neoplasms, gross lesions, and macrophage aggregates were significantly older than fish lacking any of those lesions (Table 3-4).

Figure 3-3

Relative frequencies of each winter flounder liver lesion type in each 100 mm length class. Flounder shorter than 200mm were collected through the summer and fall of 1987, and larger fish were collected on March 18, 1988. All fish came from Deer Island Flats, Boston Harbor. 121 fish were evaluated for the presence of each of the six lesion types shown, as described above. Successive 100 mm length class sample sizes were: 0-99:38, 100-199:17, 200-299:16, 300-399:34, 400-499:16.

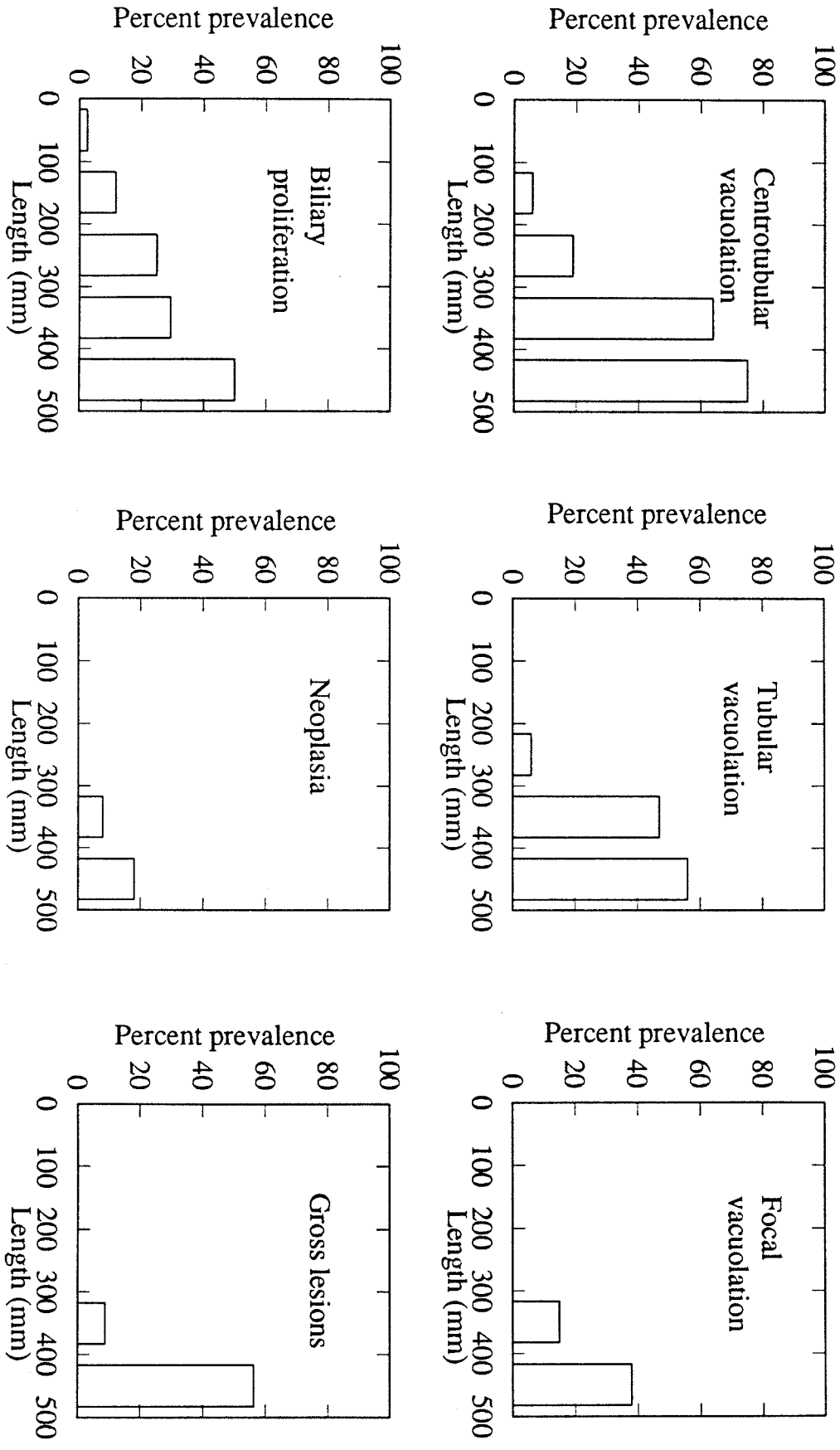

Percent prevalence
Centrotubular vacuolation
Length (mm)
Percent prevalence
Biliary proliferation
Length (mm)
Percent prevalence
Tubular vacuolation
Length (mm)
Percent prevalence
Neoplasia
Length (mm)
Percent prevalence
Focal vacuolation
Length (mm)
Percent prevalence
Gross lesions
Length (mm)

TABLE 3-4

A comparison between age and length for 1990 fish that did and did not contain various abnormalities.

		n	Age (years)	Length (mm)
Liver neoplasia	Absent	93	5.09 ± 1.23	369 ± 34
	Present	5	6.50 ± 1.35 *	386 ± 26
Gross visible lesions	Absent	88	5.01 ± 1.14	369 ± 35
	Present	10	6.30 ± 1.83 *	386 ± 32
Centrotubular vacuolation	Absent	60	5.12 ± 1.40	369 ± 39
	Present	38	5.18 ± 1.08	373 ± 28
Diffuse vacuolation	Absent	70	5.07 ± 1.33	368 ± 37
	Present	28	5.32 ± 1.16	376 ± 29
Aggregated macrophages	Absent	56	4.82 ± 0.97	363 ± 35
	Present	42	5.57 ± 1.49 **	380 ± 32 **

Data given as a mean ± S.D of length or age. Age data were available for 98 fish. For this subset significance of observed differences of mean age and length between fish that did and did not contain each lesion lesions were tested using Student's t test. Significant p values are indicated as follows: * = $p < 0.025$, ** = $p < 0.005$. No significant differences were observed between body weight and lesion presence

Sex

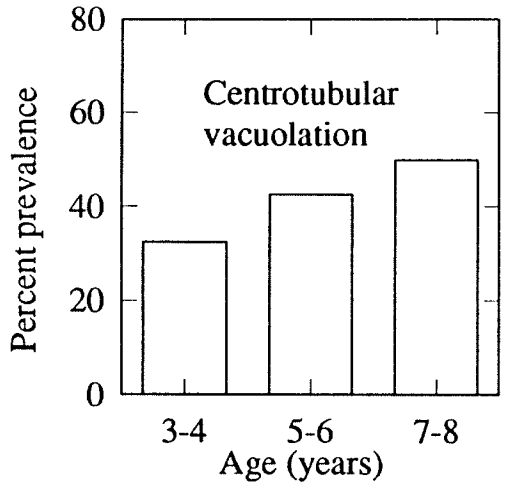
No obvious differences were observed in neoplastic or non-neoplastic lesion prevalence between males and females.

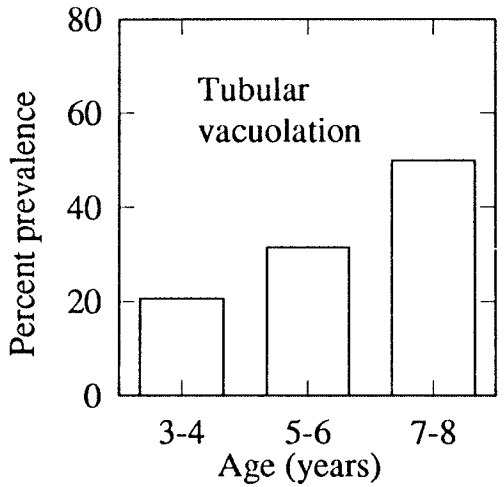
Location in Boston Harbor

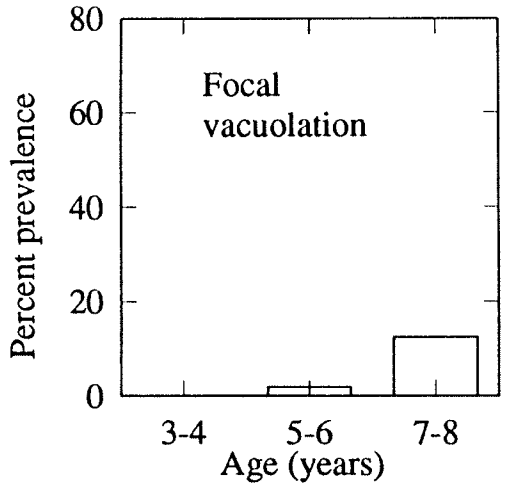
Figure 3-5 compares fish from Deer Island with a pooled sample of fish collected at the same time from other parts of Boston Harbor, but not including Nut Island, the other major sewage outfall. Many of the lesions characteristic of Deer Island fish were seen in the fish from other sites in Boston Harbor, but at lower prevalences. However, in this collection, there were some lesions, namely aggregations of vaculated cells, gross lesions, and neoplasia, that were only seen in flounder from Deer Island. Gross lesion frequencies differed significantly between Deer Island and other sites in Boston Harbor. Furthermore, in comparing the Deer Island fish in this 1989 collection with previous Deer Island collections in 1987 and 1988, overall lesion prevalence was markedly lower.

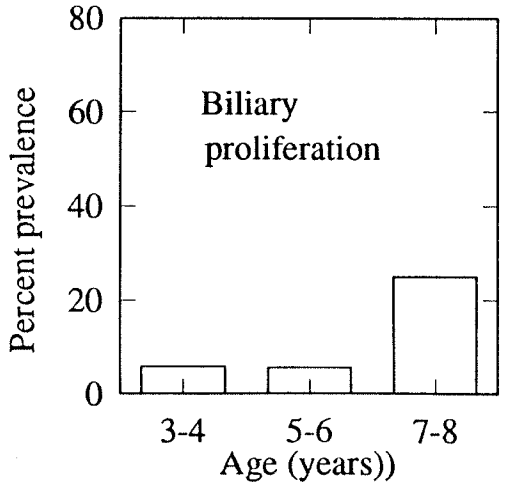
Figure 3-4

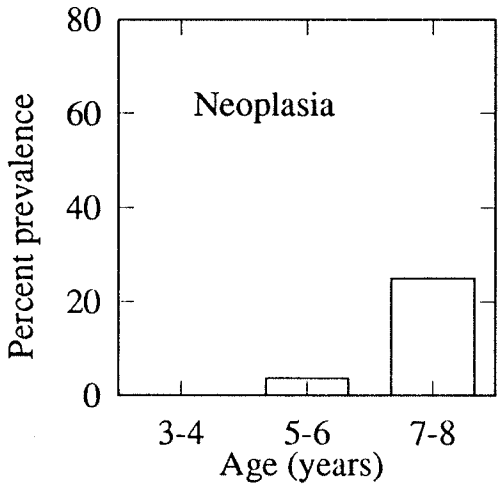
Relative frequencies of each winter flounder liver lesion type in three age classes. Flounder were collected April 30, 1990, Deer Island Flats, Boston Harbor. Age classes were for two years each (3-4, 5-6, 7-8) to achieve appropriate sample sizes, which were 3-4 = 34, 5-6 = 54, and 7-8 = 8.

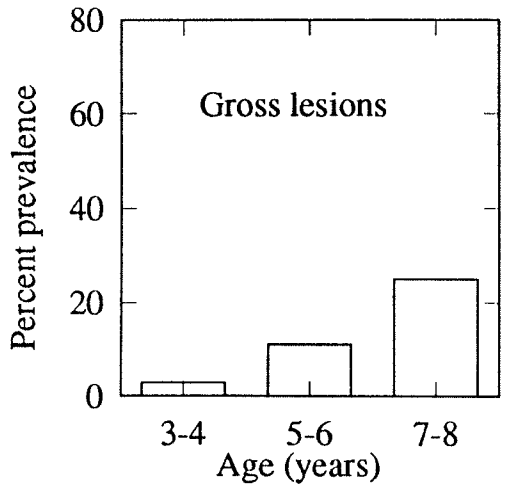

Centrotubular vacuolation
Percent prevalence
80
60
40
20
0
3-4
5-6
7-8
Age (years)


Tubular vacuolation
Percent prevalence
80
60
40
20
0
3-4
5-6
7-8
Age (years)


Focal vacuolation
Percent prevalence
80
60
40
20
0
3-4
5-6
7-8
Age (years)


Biliary proliferation
Percent prevalence
80
60
40
20
0
3-4
5-6
7-8
Age (years))


Neoplasia
Percent prevalence
80
60
40
20
0
3-4
5-6
7-8
Age (years)


Gross lesions
Percent prevalence
80
60
40
20
0
3-4
5-6
7-8
Age (years)

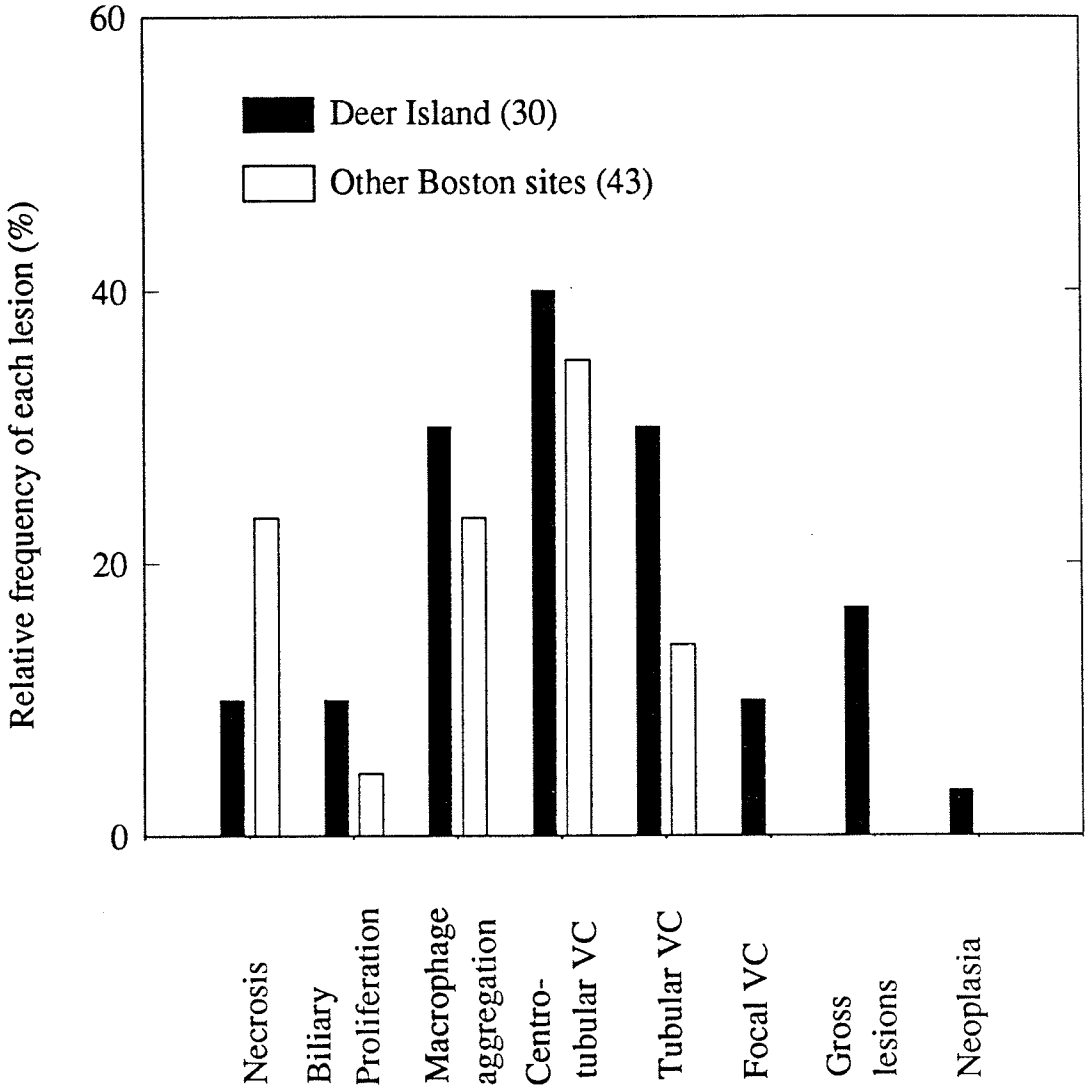


Figure 3-5

Relative frequencies of liver lesions in winter flounder from Deer Island Flats compared with a pooled sampling of sites elsewhere in Boston Harbor. These sites were pooled, as individual site sample sizes were inadequate for all sites except Deer Island. Gross lesions were significantly more prevalent in Deer Island fish ($p<0.05$).
VC = vacuolated cells. All fish were caught on May 13, 1989.

Depuration

Figure 3-6 compares lesion frequency for two groups of flounder caught on Deer Island Flats. One group was killed within a week of capture, the other was maintained in clean water for five months as described in the methods section. There was little difference in the frequency of any of the lesions.

Year of collection

Table 3-5 and Figures 3-7, 3-8, and 3-9 compare the lesion prevalences between years. In this comparison the 1987 depurated fish have been included, given the seeming irreversibility of these lesions. Data were analyzed to discern possible trends. Figure 3-7 suggests a diminution of single cell vacuolation in recent years. Figure 3-8 shows a less clear cut trend for neoplasms, and Figure 3-9 illustrates a potential confounding variable. Only one collection was made in any one year, but it appears, from this data at least, that collections made later in the year have lower prevalences of neoplastic lesions.

To evaluate this trend further, three samplings were completed in 1991, in March, April, and May. To date, the histology has not been examined, however gross lesion prevalence decreased through the season, March 12: 21%, April 8: 8%, and May 22: 3%, suggesting that lesion prevalence does indeed decreaase with samplings made later in the year.

TABLE 3-5

A comparison of winter flounder data from Deer Island Flats 1984 to 1990

	1984[1]	1987	1988	1989	1990
Date sampled	April 2 & June 6	April 21	March 18	May 13	April 30
Sample size	200	51	52	29	100
% Female		57	92	66	71
Body length ± S.D. (mm)	353	364 ± 32	378 ± 38	367 ± 31	370 ±31
Gross lesion prevalence (%)	ND	17.7	28	13.3	10
Neoplasm prevalence (%)[2]	7.5	5.9	11.5	3.3	5
Gross lesion size ± S.D. (mm)	ND	4.9 ± 3.0	12.5 ± 11.0	3	8.1 ± 5.0
Neoplasm size ± S.D. (mm)	ND	6.3 ± 5.2	11.5 ± 10.0	1	1.5 ± 0.6
Centrotubular vacuolation %	)	77	65	40	39
Tubular vacuolation %	) 68	71	48	30	29
Focal vacuolation %	)	14	22	10	2
Macrophage aggregation (%)	68	75	63	30	43

[1] Data from Murchelano and Wolke 1985. Fish from Deer Island and elsewhere (ND = Not described).

[2]Mean neoplasm prevalence for 1987-1990 is 6.5%. The prevalence given in chapter 2 (9.5%) is higher because some fish sampled for that analysis were excluded from this data set because they were sampled non-randomly.

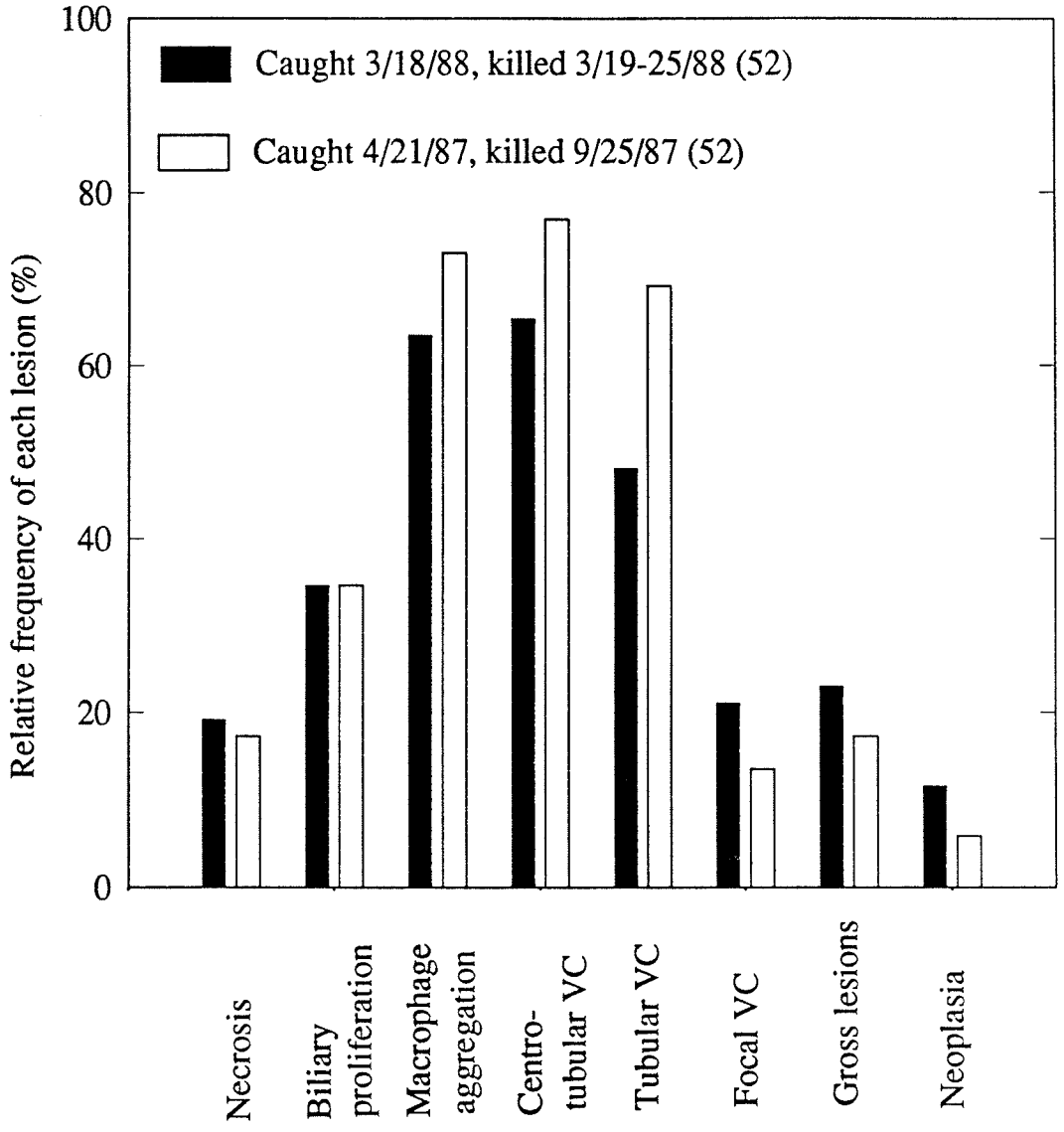


Figure 3-6

The effect of five months maintenance, with relatively clean food and water, on flounder from Deer Island Flats. Open bars represent fish depurated for five months before examination. Solid bars represent fish examined soon after capture. Relative frequencies of each lesion type are shown. Note that any one fish often had multiple lesion types. VC = vacuolated cells.

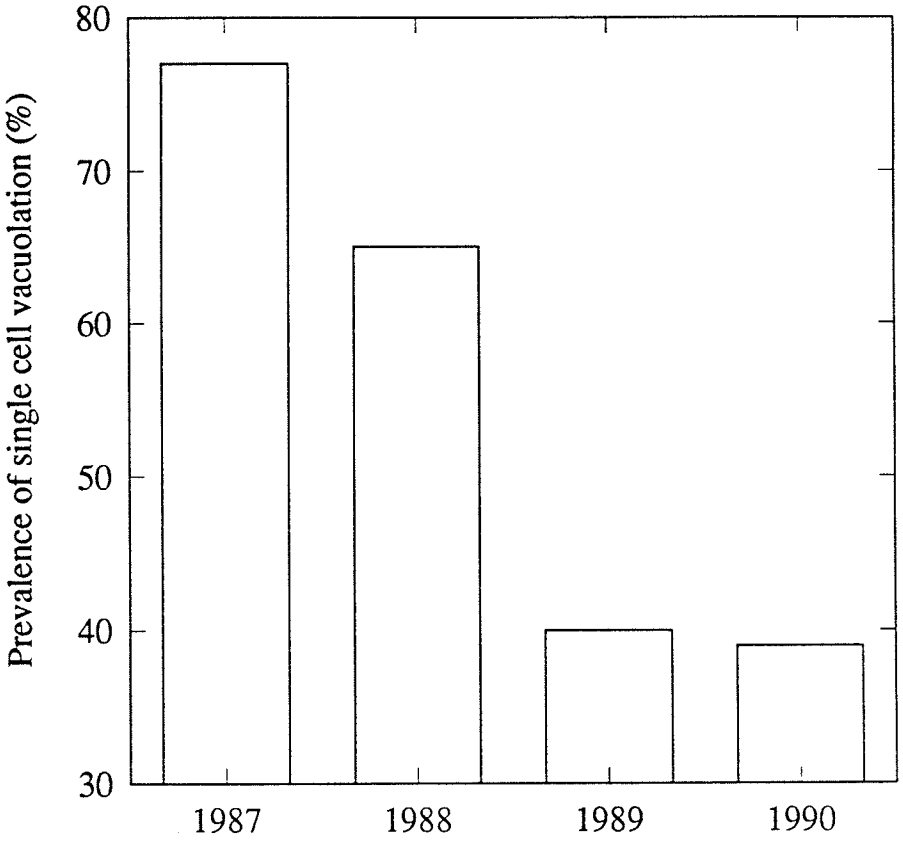


Figure 3-7

Prevalence of centrotubular cell vacuolation in sexually mature winter flounder from Deer Island Flats, Boston Harbor, 1987 to 1990. The 1987 collection was kept in clean water for 5 months before sacrifice.

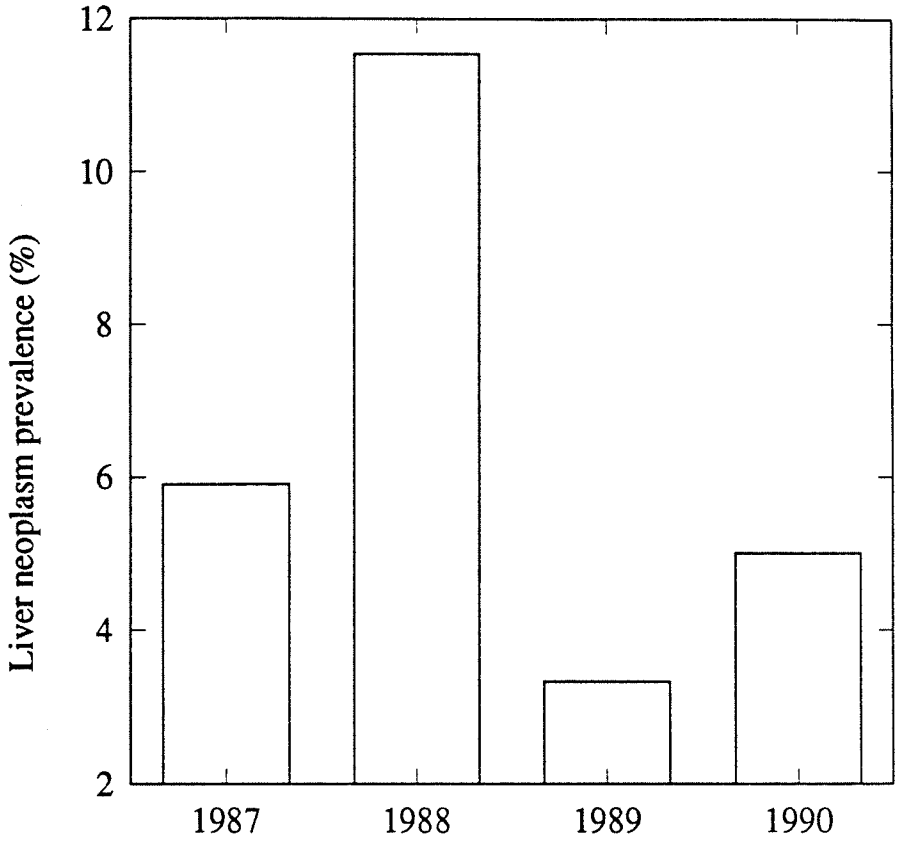


Figure 3-8

Liver neoplasm prevalence in sexually mature winter flounder from Deer Island Flats, Boston Harbor, 1987 to 1990, plotted by year in which collection was made. The 1987 collection was kept in clean water for 5 months before sacrifice.

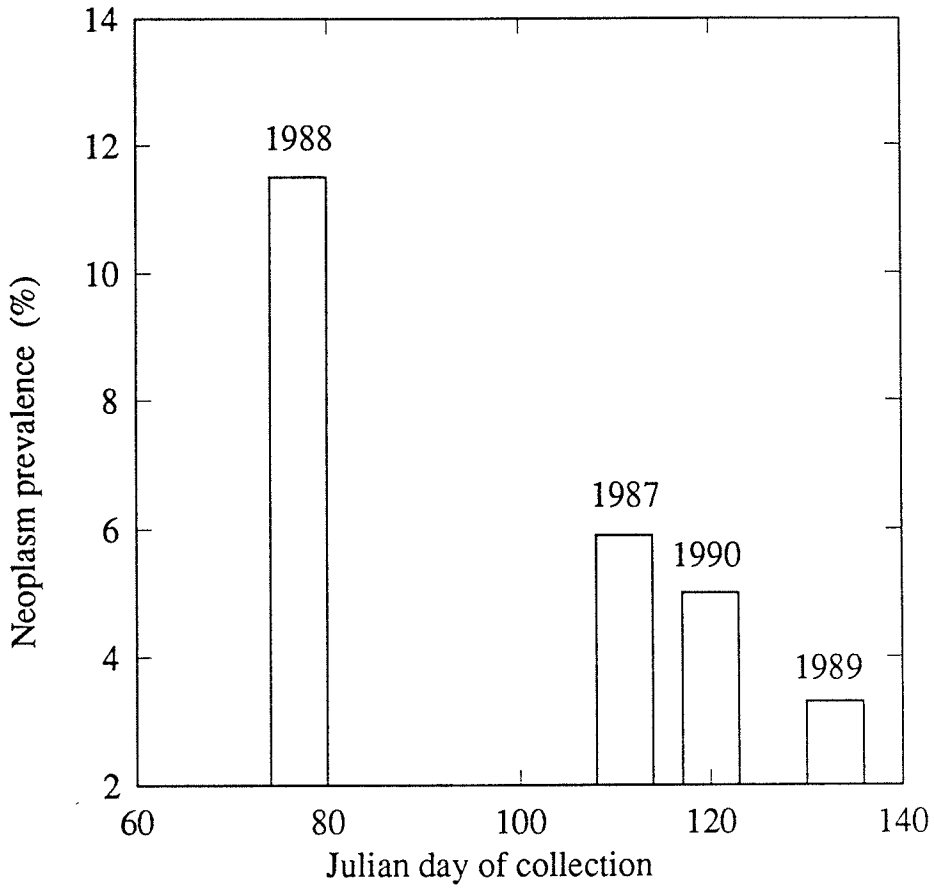


Figure 3-9

Liver neoplasm prevalence in sexually mature winter flounder from Deer Island Flats, Boston Harbor, 1987 to 1990, plotted by Julian day that collection was made. The 1987 collection was kept in clean water for 5 months before sacrifice.

DISCUSSION

This study has shown that winter flounder from Boston Harbor contain a set of hepatic lesions that precedes liver neoplasia. These lesions first appeared in young fish and became more prevalent with increasing length and age. Similar, less severe changes were seen occasionally in coastal fish outside of Boston Harbor, whereas hepatic morphological change was absent in offshore (Georges Bank) winter flounder (Table 3-2 and Figures 3-1a, 3-3 and 3-4). Differences in lesion frequency between males and females from Boston Harbor were insignificant, and lesion frequency did not decrease after five months maintenance of Boston Harbor winter flounder in clean water with clean food (Figure 3-6).

Vacuolation

Vacuolated cells were first seen in Boston Harbor winter flounder in the center of the hepatic tubules of sub-adult fish. In longer fish the lesion expanded to fill the entire hepatic tubule. In 10-20% of fish, vacuolation was also found in large focal aggregates that were often visible grossly. Vacuolation was also observed in bile ductules and ducts and in exocrine pancreatic cells.

A central question is whether vacuolation, common to all the above hepatic epithelia, represents a chronic injury resulting from exposure to hepatotoxins that always progresses to cell death, or whether vacuolation is some form of cytotoxin resistant epithelial phenotype, arising from each of the three differentiated hepatic

epithelia, or from their common stem cell? If the latter is the case, do vacuolated cells have the potential to proliferate and transform into a variety of neoplastic phenotypes? We have previously suggested that vacuolated cells are an active part of the proliferative process (Moore et al., 1989). Murchelano and Wolke (1985) suggested that the vacuolation process is irreversible, with some vacuolated cells progressing to necrosis, and that surviving cells may be committed to neoplasia. Our findings in this study support a role for vacuolated cells beyond that of impending cell death. If these cells were susceptible to cytotoxins, cell death would likely be the fate of the majority of vacuolated cells and one could expect to see a loss of vacuolated cells through either necrotic, or apoptotic cell death. If so, some highly proliferative liver compartment would have to serve as the ongoing source for the increasing numbers of vacuolated cells seen in the livers of larger and older flounder (Figures 3-3 and 3-4). Such a compartment has not been described. Necrotic hepatocytes have been described in this study, and by Bodammer and Murchelano (1990). An atypical form of apoptosis was described by the latter authors, in the non-vacuolated "dark" hepatocytes[2] but not in the majority of vacuolated cells. Figure 3-6 in this study suggests that the majority of vacuolated cells persist, or are replaced by new vacuolated cells, in the absence of ongoing exposure to a contaminated environment and food, at least for five months. This persistence makes it unlikely that apoptosis is occuring in hydropically vacuolated cells to a degree sufficient to explain the widespread persistent vacuoles observed. The vacuolated phenotype appears therefore to have some inherent stability,

[2]The nature of dark hepatocytes is discussed in chapter 4, page 154.

independent of continued exposure to cytotoxic insults, unless removal to clean water and food allowed an ongoing release of hydrophobic cytotoxins from lipid stores in the animal. Bodammer and Murchelano (1990) concluded that they were "*not .. successful in describing a population(s) of hepatotoxin resistant cells*". On the contrary, we believe that the vacuolated cells that they and we have described may be such a population, and that the depuration data suggest these cells to be persistent in the absence of ongoing exposure to hepatotoxins.

Understanding the relationship between neoplastic and vacuolated cells in winter flounder liver may be crucial to unravelling the pathogenesis of neoplasia in this species. Vacuolated cells are found within and around foci of all types of neoplasia, certainly implying a close relationship. Which came first? Many foci of vacuolated cells contain small islands of relatively normal basophilic cells (Figure 3-2a), others contain foci of neoplasia. Murchelano (1990) has suggested the basophilic cells may have neoplastic potential. This would imply that foci of vacuolation develop, and that resistant (non-vacuolated) cells within the foci then transform to a neoplastic phenotype. However, some neoplastic foci examined in this study had only a small peripheral ring of vacuolated cells which itself was surrounded by more normal parenchyma. These may have been large foci of vacuolated cells, almost entirely overgrown by neoplastic cells. On the other hand, one might speculate that the vacuolated cells were in some way part of the advancing edge of the neoplasm, and these cells with time acquired the transformed, atypical neoplastic phenotype.

The vacuolated cells not immediately associated with obvious neoplastic change

could be in a state of cellular diapause[3], surviving in a hepatotoxic, possibly hypoxic cellular milieu. Such a condition has not to our knowledge been described before, but these cells seem to be unique to some species of flatfish living in contaminated waters (Stehr 1990). These diseased liver tissues are likely to be somewhat hypoxic, given the marked reduction in the prominence of vascular sinusoids, and the almost total absence of sinusoids from many of the foci of vacuolated and neoplastic cells. It is notable that the first cell type to undergo vacuolation is the cell farthest from the capillary bed, namely the biliary preductular cell. This cell may also be closest to a hepatic epithelial stem cell, if other investigators (Hampton et al. 1988, Nunez et al. 1990) were correct in presuming these cells to be oval cell equivalents. Further understanding of the dynamics and consequences of vacuolation must await the study of cell proliferation indices for each cell type in Boston Harbor winter flounder liver, and the development of an experimental model to generate these changes in the laboratory.

Promotion

The complexity of these cellular changes suggests that there are multiple etiological factors involving stages of initiation, promotion and progression of the neoplastic process. Deer Island Flats are heavily contaminated with polynuclear aromatic hydrocarbons, halogenated hydrocarbons, and heavy metals (Boehm 1984,

[3]Diapause is a term native to reproductive endocrinology, used when gestation is arrested, to allow parturition to be scheduled to occur at the optimal season. The term is used loosely here to connote a sense of suspended animation.

Shiaris and Jambard-Sweet 1986, N.O.A.A. 1988a). It is reasonable to assume that all post-metamorphic winter flounder, that are spawned on Deer Island Flats, are heavily exposed to genotoxins through the diet and via the peribranchial respiratory epithelium. Whilst direct evidence for this is lacking, experimental data supports the assumption, for when polychaete worms were exposed to benzo(a)pyrene and fed to winter flounder, benzo(a)pyrene accumulated in the flounder (McElroy and Sisson 1989). Polychaete worms are a very common diet item in juvenile and adult flounder collected on Deer Island Flats. Does this exposure lead to a sufficient genotoxic insult to initiate a neoplasm?

A 6 to 12 month latency has been reported between first exposure to genotoxic carcinogens and the development of neoplasia in aquarium fish species (Hoover 1984). Additionally, young fish are more sensitive than adults to genotoxins (Halver 1967, Hendricks 1981). If genotoxin exposure alone were necessary and sufficient to elicit a neoplastic response in winter flounder from Deer Island Flats, one might expect to see frank neoplasia in 1 year old winter flounder. The data presented in Figures 3-3 and 3-4 relate lesion frequency to body length and age. Whilst age data are only available for a fraction of the fish examined, none of the neoplasm-bearing flounder for which age is available were less than five years old, and the body length of the unaged neoplasm bearers would suggest four years to be a likely minimum age for neoplasm occurrence. This long latency from first exposure to genotoxins to the appearance of frank neoplasia suggests that some additional factors are acting. Halogenated hydrocarbons, other promoters, a series of multiple genetic hits, and or

endogenous agents favoring cell proliferation could be rate-limiting in this disease process. However, the absence of any gender bias in lesion prevalence, in contrast to a previous report (N.O.A.A. 1987a), would suggest that the influence of pollutants was more significant than that of potential endogenous promoters of neoplasia, such as estradiol.

The higher prevalence of lesions in fish from Deer Island Flats, as compared with other areas of Boston Harbor, and other less contaminated areas, adds further circumstantial evidence to the tenet that neoplasia in bottom feeding fish is caused by pollutants in municipal and industrial discharges (Mix 1986, Harshbarger and Clark 1990). It also suggests that the amount of movement by flounder between different areas of the harbor is limited, as previously indicated (Howe and Coates 1975).

The lack of any gender bias in neoplastic and non-neoplastic lesion prevalence was a surprise. Estradiol is a well-recognised promoter, and is found at much higher levels in mature female winter flounder than in males (Gray 1988). The lack of any excess of lesions in females compared to males could well reflect the enormity of the exposure to exogenous promotors, many of which are estrogenic (Nelson et al. 1978), thus obscuring any endogenous differences between males and females.

In summary the progression of vacuolation and other changes in Boston Harbor winter flounder have been described. It seems unlikely that most vacuolated cells are about to die. They, or their identical progeny, have been shown to persist in a poorly vascularized, highly abnormal liver. Perhaps the concept of cellular diapause, in a hypoxic, toxic liver environment is an appropriate one. The ultrastructural basis for

the vacuolation observed, is described in Chapter 4, and the issue of whether vacuolated cells are actively involved in the neoplastic process of cellular proliferation in these flounder is considered in Chapter 5.

CHAPTER 4

ULTRASTRUCTURE OF NORMAL, VACUOLATED AND NEOPLASTIC CELLS IN THE LIVER OF WINTER FLOUNDER

INTRODUCTION

The mammalian liver is characterized histologically by the presence of discrete lobules. The appropriate description of teleost liver structures has been discussed extensively (Elias and Bengelsdorf 1954, Ito et al. 1962, Elias and Scherrick 1969, Hampton et al. 1985). Essentially the lobular level of organization is less apparent or absent in the liver of fish, which can be described as having simple tubules of hepatocytes as their putative basic unit. Irregularly oriented, these tubules have a central drainage to the biliary system and are surrounded by tubular vascular sinusoids. Hepatocyte apices in the center of the tubule form the microvillar walls of the biliary canaliculus. The canaliculi drain into biliary preductules. The preductule is a transitional pasageway formed by the interdigitation of hepatocyte apices and plasma membrane from bile preductular cells (Hampton et al. 1988).

Studies of the ultrastructural pathology of fish exposed to hepatotoxins have shown the commonest hepatic response to be proliferation, dilation and vesiculation of the endoplasmic reticulum (Scarpelli et al. 1963, Klaunig et al. 1974, Scarpelli et al. 1974, McCain et al. 1978, Hacking et al. 1976, Hawkes 1980). Examination of the ultrastructure of fish liver from contaminated sites has included studies of the English sole (Stehr et al., 1988 and 1990) where the ultrastructure of hepatocellular and cholangiocellular carcinomas were described, the tomcod (Cormier 1986 and Cormier et al. 1989), where an elevation of lipid content was seen in hepatocytes of fish from a contaminated site, and the European flounder (Kohler 1989), where fish removed from a contaminated area were monitored for their capacity to regenerate healthy livers.

In this last study regenerative changes included loss of lipid inclusions, resumption of glycogen synthesis, and loss of macrotubules.

Chapters 2 and 3 in this study described the salient features of the histological progression and end stages of contaminant associated liver disease in winter flounder. As environmental quality decreased the following trends were apparent: 1) Hepatocytes became more basophilic; 2) Vacuolated cells became common, first in the center of the tubule, then filling the tubule, and then in grossly visible foci; 3) A diverse array of mainly cholangiocellular neoplasms became increasingly prevalent in older fish from the most contaminated site, which was Deer Island Flats (Boston Harbor). These histological observations raise a set of questions concerning their ultrastructural basis.

These questions include: 1) What is the ultrastructural basis for the different degrees of hepatocyte basophilia that was seen light microscopically in winter flounder from habitats of different levels of contamination? 2) What is the ultrastructure of the vacuolated cell at each stage of its development in winter flounder liver? 3) What ultrastructural changes are evident in neoplastic cells in winter flounder liver? 4) How does knowledge of these ultrastructural changes contribute to our understanding of the pathogenesis of hepatic neoplasia in winter flounder from Boston Harbor, and in other poikilothermic and homeothermic vertebrates? Answers to these questions will be valuable in interpretation of histological observations made in the use of winter flounder as bioindicators of the chronic biological effect of contaminants. In particular, understanding the significance of vacuolated cells is important in assessing the value of examining winter flounder, and other similarly affected flatfish species, for the

presence of hydropically vacuolated cells as indicators of cellular changes that precede hepatic neoplasia.

MATERIALS AND METHODS

Sample collection and preparation

Male and female winter flounder were collected by otter trawl from four locations: 1) Boston Harbor, near Deer Island, 2) Nantucket Sound, one mile south of Falmouth Heights, 3) Onset, 1/4 mile northwest of the state pier, and 4) Georges Bank. Within 48 h of capture the fish were measured, killed by cervical section, and the livers removed as described previously (Chapters 2 and 3). Slices of liver 4 mm thick from various regions of each liver were made and 1 to 4 were randomly selected and trimmed to 4 x 4 mm cubes. Using a clean, new razor blade, cubes were diced into 1 mm cubes in an ice cold drop of modified Karnovsky's fixative (2% formaldehyde, 2.5% glutaraldehyde, 0.1M cacodylate, pH 7.2; Ito and Karnovsky 1968) and then held in the fixative on ice for 12 to 24 h. Chemicals and materials for electron microscopy were obtained from Electron Microscopy Sciences (Fort Washington, PA.) Samples of grossly abnormal liver were processed in addition to the randomly selected sections. After fixation, samples were washed twice in ice-cold 0.1M cacodylate buffer. Samples collected offshore were held on ice in buffer, after the second wash, for return to the laboratory. Specimens were then post-fixed in 1% osmium tetroxide for 1 h, dehydrated for two periods of 5 min in each of 30, 50, 70,

80, and 95 % ethanol and then placed in four changes of 100% alcohol for 15 min each. This was followed by immersion in two changes, for 5 min each, of propylene oxide. Then the specimens were placed for 1 h in 50% Spurrs epoxy (Polysciences, Warrington PA. - standard mix) in propylene oxide before infiltration for 1 h in each of two changes of 100% Spurrs. Tissues were then embedded in Beem capsules, and incubated overnight at 60°C. Epoxy embedded sections were cut semi-thin to be stained with toluidine blue for light microscopy, and thin to be stained with 3% uranyl acetate for 15 min, and 2.5% lead citrate for 3 min, for transmission electron microscopy. The remainder of each liver slice was fixed in neutral buffered formalin, embedded routinely in paraffin, sectioned at 5μm and stained with hematoxylin and eosin.

Transmission electron microscopy

A Zeiss 10 transmission electron microscope was used to examine stained grids. Images were evaluated in the context of structures evident in the corresponding light microscopic material. Survey and detail micrographs were exposed randomly, and of structures of interest. Negatives were processed by standard methods, and images printed on Ilford multigrade resin-coated paper.

Analysis of ultrastructure

Micrographs were examined in the light of the questions listed above. The differences and relationships between normal and pathological cells were examined.

Particular attention was paid to the appearance of the nuclear margin, chromatin, nucleolus, lipid and glycogen stores, mitochondria, golgi, cell junctions, endoplasmic reticulum, and lysosomes, and to the presence of apoptotic bodies (Wyllie et al. 1980).

RESULTS

Fish sampled

A subset of the fish described in Chapter 3 were sampled for ultrastructural studies. This chapter describes studies with transmission electron microscopy on the following fish: 17 from Deer Island Flats (Boston Harbor), 1 from Onset, 4 from Nantucket Sound, and 8 from Georges Bank.

Hepatic ultrastructure compared between sites

Georges Bank

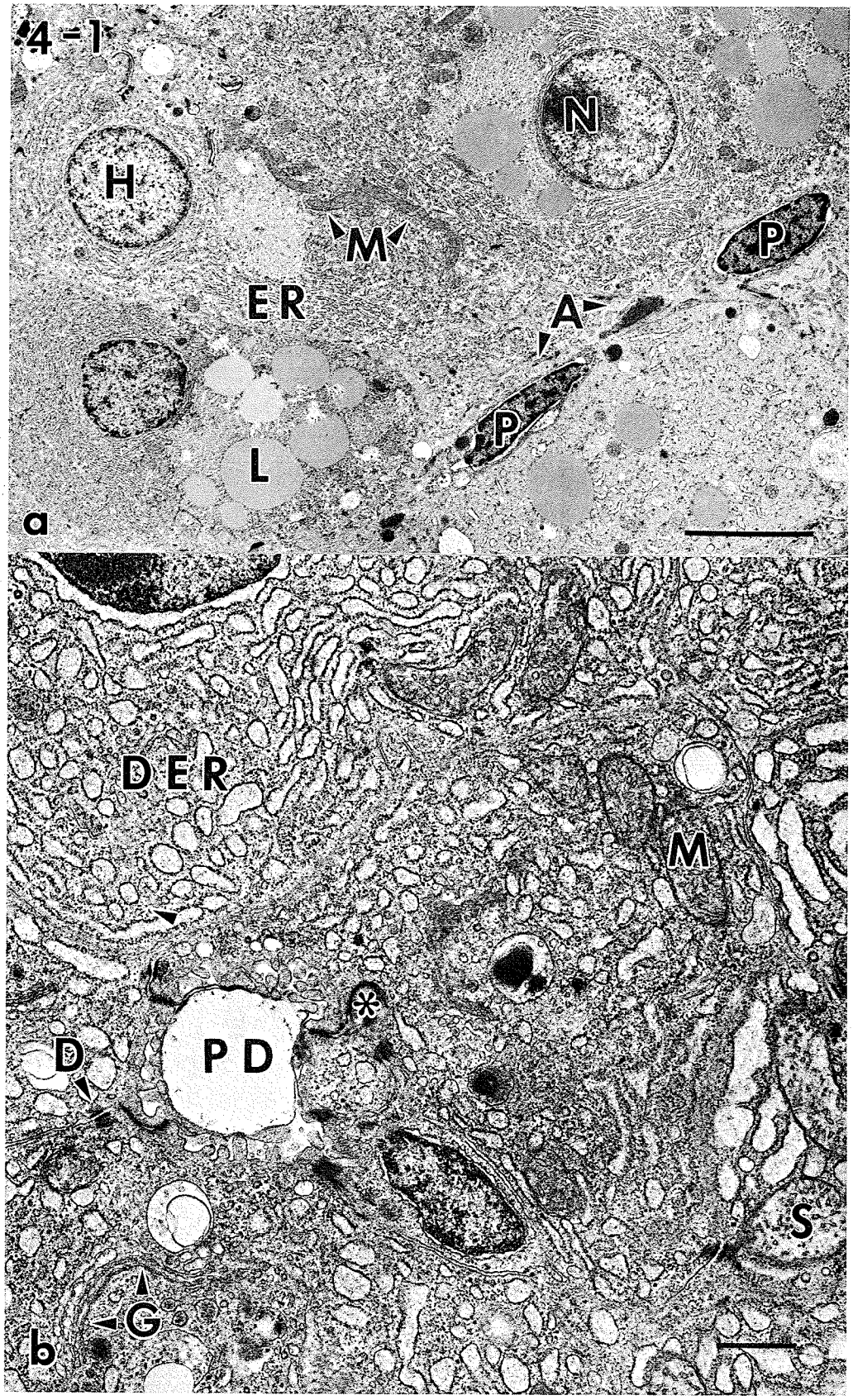
Hepatocytes in winter flounder from Georges Bank had an ultrastructure (Figure 4-1a) that was very comparable to that published for other species (Welsch & Storch 1973, Hinton & Pool 1976, Hacking et al. 1977, Hampton et al. 1985 and 1988 and 1989). Histologically the hepatocyte cytoplasm stained poorly with hematoxylin. Their ultrastructure reflected this lack of basophilia, with large portions of the cytoplasm of each hepatocyte being filled with lipid droplets, and clusters of glycogen particles and rosettes. The remainder of the cytoplasmic matrix was primarily occupied by crescentic stacks of rough endoplasmic reticulum. There was no dilation of the cisternal space, and dilation of the perinuclear membrane, which is an extension of the cisternal space, was seen only rarely. The hepatocyte apices with their microvillar borders formed the boundary of the lumen to the biliary canaliculus. Adjacent to the microvilli,

Figure 4-1

Ultrastructure of winter flounder hepatocytes from offshore and coastal sites. Note 1b is at a higher magnification than 1a.

a) Transmission electron micrograph of winter flounder liver from Georges Bank. Section through hepatocytes, and a biliary preductule. Hepatocyte nuclei (H) are circular in section, with a prominent nucleolus (N). Hepatocyte cytoplasm contains lipid droplets (L), normal rough endoplasmic reticulum (ER), and normal mitochondria (M). The biliary preductule is formed by microvillous apices of hepatocytes (A), and by fingers of preductular cell cytoplasm. The preductular cell nucleus (P) is small, ovoid, and electron dense in comparison to the hepatocyte nucleus. Uranyl acetate and lead citrate. x 4305. Bar = 5 μm

b) Transmission electron micrograph of winter flounder liver from Nantucket Sound, MA. The image shows mild ultrastructural abnormalities. Detail of the center of an hepatic tubule. Significant changes from the above figure are evident. The lumen of the biliary preductule (PD) is enlarged. The cisternal space of the rough endoplasmic reticulum is dilated (DER); this dilation continues into the perinuclear space. Mitochondria show mild (M) to severe (S) swelling. Centrotubular cellular junctions (*) and desmosomes (D) are evident between hepatocyte apices, and between hepatocyte apices and preductular cells. Golgi apparatus is present (G). Uranyl acetate and lead citrate. x 6744. Bar = 2 μm


4-1
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b

epithelial junctional complexes and desmosomes were evident between the apices of adjacent hepatocytes, and between hepatocyte apices and biliary preductular cells. Hepatocyte nuclei were in general smooth edged, and round in section. The hepatocyte nucleolus was distinct and located in the center or periphery of the nucleus. Figure 4-1a is representative of the fish from Georges Bank.

Preductular cells contained scant cytoplasm, with rudimentary leaflets of endoplasmic reticulum, a few small mitochondria and many junctional complexes with adjacent hepatocyte apices (Figures 4-1a). These findings are comparable to those in the rainbow trout described by Hampton et al. (1985, 1988 and 1989). The preductular nuclei were characteristically small, elongated ovals, and significantly more electron dense than hepatocyte nuclei. They were always situated in the center of the hepatic tubule.

Onset and Nantucket Sound.

Winter flounder from these coastal habitats had a histology comparable to that of fish from Georges Bank. However, macrophage aggregations were present at times, and some of the hepatocytes were somewhat basophilic. This histological basophilia was seen, at the ultrastructural level, to involve a mild decrease in the amount of glycogen and lipid stores, and a concomitant increase in the amount of rough endoplasmic reticulum in the hepatocytes in these fish (Figure 4-1b). In some cases the endoplasmic reticulum was slightly dilated, as was the perinuclear space and some of the mitochondria. The canalicular and preductular structures were as described

above, although the diameter of the preductular lumen was at times increased, and the length of the microvilli somewhat reduced (Figure 4-1b).

Deer Island Flats (Boston Harbor) winter flounder lacking vacuolated cells

Histologically, the non-vacuolated hepatocytes of winter flounder from Deer Island Flats, as compared to those from Georges Bank, were significantly more basophilic. Ultrastructurally this was found to reflect an almost total loss of lipid and glycogen stores in many of the fish. A minority of the fish, especially the subadult fish, retained significant lipid stores. The major cytoplasmic constituent was rough endoplasmic reticulum. Endoplasmic reticulum comparable to that from Georges Bank fish was occasionally present. However, the majority of the fish contained endoplasmic reticulum that was either dilated (figure 4-2) or vesiculated (Figure 4-4a), by an accumulation of an electron-lucent fluid. A few apoptotic bodies (Wyllie 1980) were present (Figure 4-4a). These contained fragmented chromatin, endoplasmic reticulum, and mitochondria, but autolysosomes were more common. Mitochondria were usually distended by fluid accumulation (Figure 4-4 and Figure 4-6b). Apical microvilli varied from normal to absent. There was a great variation in the electron density of hepatocytes (Figure 4-2). "Light" hepatocytes predominated, although "dark" hepatocytes were at times common. Nucleoli varied in form from indistinct to obvious, and in shape from circular to elongate. In fish where vacuolation was absent, the preductular and endothelial cells were unremarkable.

Ultrastructure of vacuolated cells of winter flounder liver from Deer Island Flats

Hydropic vacuolation in the liver of winter flounder was first found in the center of hepatic tubules. This then progressed to involve the entire tubule. The most advanced stage involved grossly visible foci of vacuolated cells. Vacuolation has been described in hepatocytes, biliary ductular cells, and in exocrine pancreatic cells (chapter 3). Ultrastructural material was available for the study of the centrotubular (Figure 4-2 and Figure 4-3), tubular (Figure 4-5) and focal (Figure 4-6) stages. The process of vacuolation involved progressive ballooning of the dilated endoplasmic reticulum, of the perinuclear space and of swollen mitochondria. As these ballooned structures increased in size they coalesced, and formed one pancytoplasmic vacuole that filled the majority of the cell volume, pushing the nucleus and residual cytoplasmic matrix to the margin of the cell. The vacuoles were filled with electron-lucent fluid which contained a diffuse, floccular, moderately electron-dense material. Histochemical studies of intra-vacuolar fluid gave negative results for glycogen, iron, nucleic acids, and protein (see

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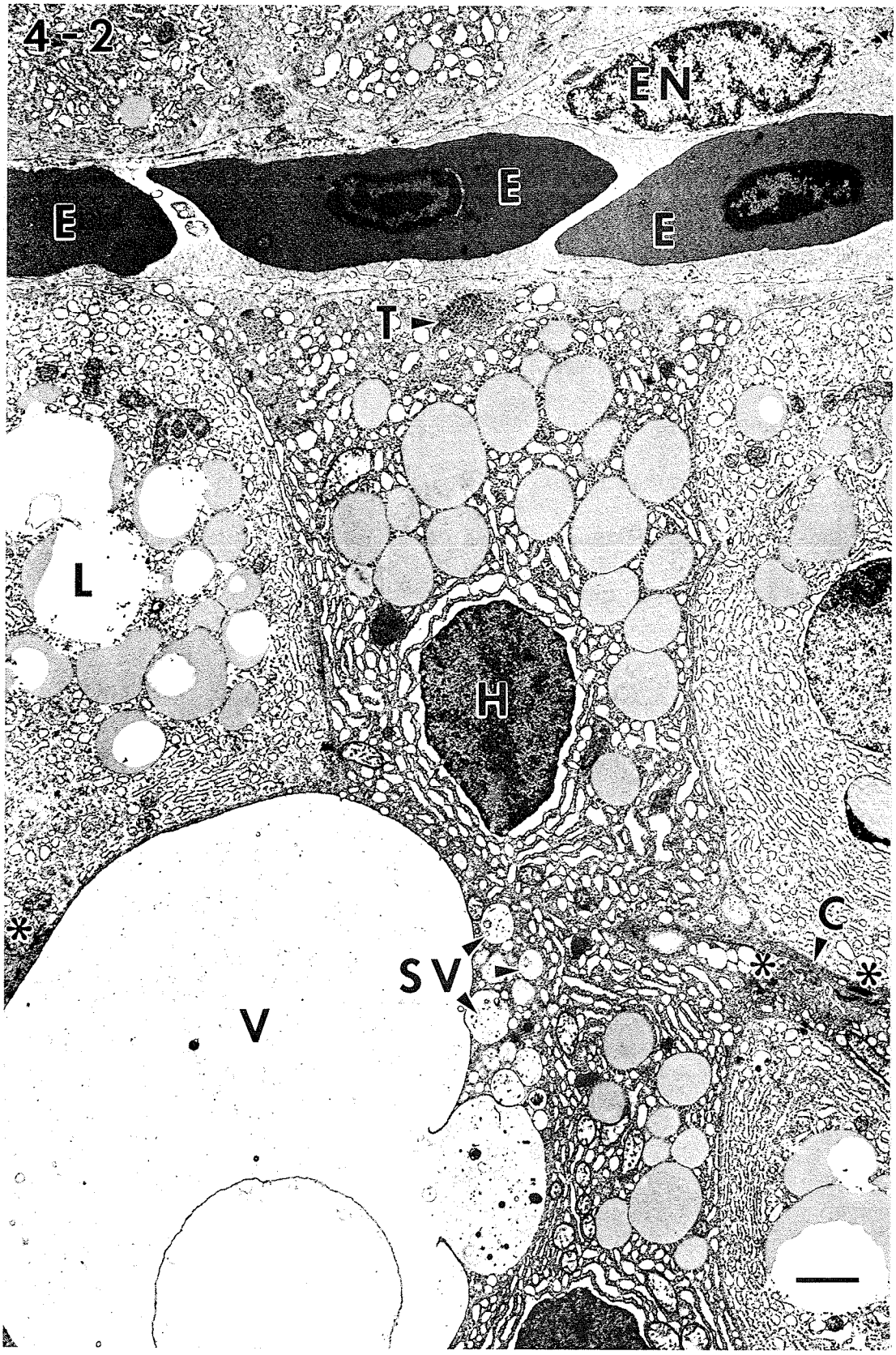
Figure 4-2

Transmission electron micrograph of winter flounder liver from Deer Island Flats, Boston Harbor. This subadult female fish was 280mm in length. The section through an hepatic tubule illustrates early vacuolar change. A centrotubular cell is undergoing extreme hydropic vacuolation. Whether the vacuolated cell is an hepatocyte or preductular cell is not evident from this image. The centrotubular location of the cell is shown by its contiguity with cellular junctions (*). A large vacuole (V) fills the majority of the cell, with which smaller vacuoles (SV) are at times confluent. This suggests, although is not certain from this static picture, that the smaller vacuoles are coalescing to add to the major vacuole. An adjacent hepatocyte (H) shows dilation of the rough endoplasmic reticulum. A biliary canaliculus (C) is also present. The periphery of the hepatic tubule is shown at the top of the image. Erythrocytes (E), which are nucleated in flounder, are seen within a vascular sinusoid, formed by endothelia (EN). Tonofilaments are evident in the space of Disse (T). The voids (L) within the larger lipid droplets are an artefact commonly seen due to loss of lipid during processing. Uranyl acetate and lead citrate. x5238. Bar = 2 μm


4-2
EN
E
E
E
T
L
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V
SV
C
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*
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*

Chapter 2). The images presented here rule out the presence of lipid, and thus it seems likely that the primary constituent is water and electrolytes, with flocculent cell debris.

Centrotubular vacuolation: Ultrastructurally, the single centrotubular vacuolated cells were never found adjacent to sinusoidal structures (Figure 4-2 and 4-3). They were always surrounded by a circle of hepatocytes in transverse section, or a row of hepatocytes in longitudinal section. The nucleus of the vacuolated cell had an oval shape, often flattened on one side, and was electron dense. The nuclear chromatin, when present in the plane of section, was comparable to that of normal non-vacuolated preductular cells. Mitochondria in these centrotubular vacuolated cells were often swollen. In these sections the microvillar borders of the biliary canaliculi appeared normal. There were many cellular junctions between centrotubular vacuolated cells and adjacent hepatocyte apices. Vacuolated centrotubular cells were surrounded by circlets of hepatocytes undergoing micro-vesiculation of the endoplasmic reticulum perhaps as a prelude to tubular vacuolation (Figure 4-3 and Figure 4-4). These observations led to the conclusion that vacuolated centrotubular cells were primarily abnormal preductular cells. One specimen contained a stage intermediate between centrotubular and tubular vacuolation (Figure 4-4). The preductule was grossly swollen by the ballooning vacuole, and an adjacent hepatocyte showed clear evidence (Figure 4-4d) that the fluid accumulation in the endoplasmic reticulum was in the cisternal space.

Tubular vacuolation: The second stage of vacuolation described as tubular vacuolation was seen ultrastructurally to involve entire tubules filled with vacuolated

cells with large ballooned vacuoles that marginated the nucleus and residual cytoplasm (Figure 4-5). Vacuolated tubules were often ensheathed in a sleeve of fibrotic tissue. Histologically these vacuolated tubules were often seen to be continuous with vacuolated bile ductules and ducts. The precise nature of the cell of origin of each vacuolated cell was thus hard to determine on morphological criteria.

Foci of vacuolation: The third stage of vacuolation involved grossly visible foci of vacuolated cells (Figure 4-6). The foci were disorganized aggregates of vacuolated cells, and other less vacuolated cells, that were basophilic histologically. Vascular structures were rare. Ultrastructurally the vacuolated cells were of the same description as those above. In one case vacuoles were also apparent in the nucleus of a vacuolated cell.

A summation of the above observations in conjunction with those observations on hydropic vacuolation in chapter 3 is given in Figure 4-7.

Figure 4-3

Ultrastructure of hydropic vacuolation in preductular cells of winter flounder from Deer Island Flats, Boston Harbor.

a) A series of large vacuoles is present in preductular cells. The electron dense ovoid nuclei typical of preductular cells (P) have enormously expanded perinuclear spaces. Many small contiguous vacuoles appear to be in apposition where they might coalesce (SV). This early stage of vacuolation is limited to the center of each hepatic tubule as shown by the proximity of the vacuoles (V) to cellular junctions (*) and lack of contiguity with any vascular structures. The vacuole in the lower right hand corner of the image appears to be in a small centrotubular portion of an hepatocyte. The endoplasmic reticulum in surrounding hepatocytes is mildly dilated, but the mitochondria appear normal. Uranyl acetate and lead citrate. x 4305 Bar = 2 μm

b) Transmission electron micrograph of winter flounder liver from Deer Island Flats, Boston Harbor. Detailed view of centrotubular vacuolation. The centrotubular location of the vacuolated cell is shown by proximity to canalicular microvilli (C), and cellular junctions (*). The vacuole (V) is an extreme dilation of the perinuclear space. The nucleus (P) of the vacuolated cell is typical of a preductular cell. It is unclear from this image whether the small vacuoles are part of the vacuolated cell or in an adjacent cell. Uranyl acetate and lead citrate. x 6744 Bar = 2 μm

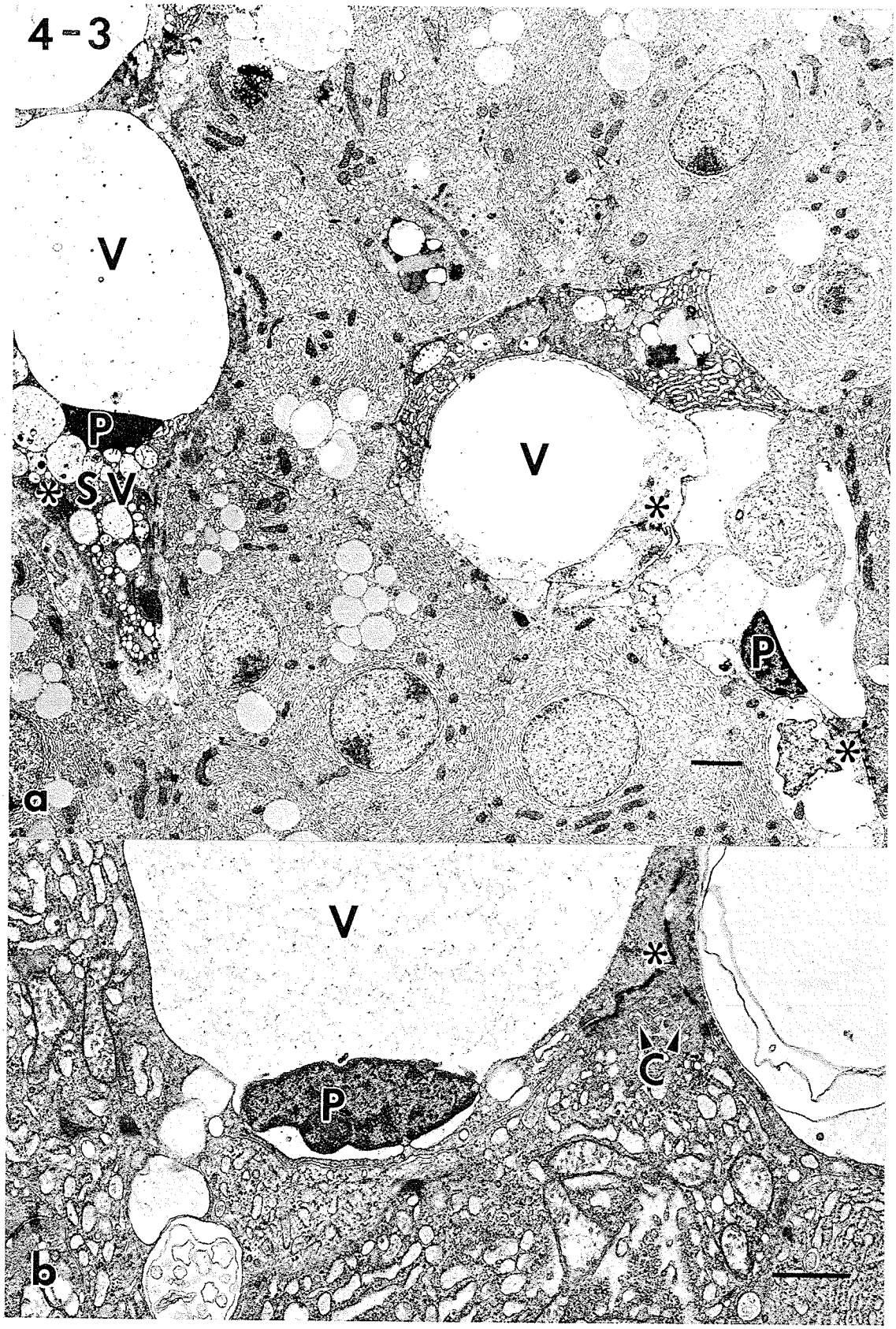

4-3
V
P
SV
V
P
a
V
P
C
b

Figure 4-4

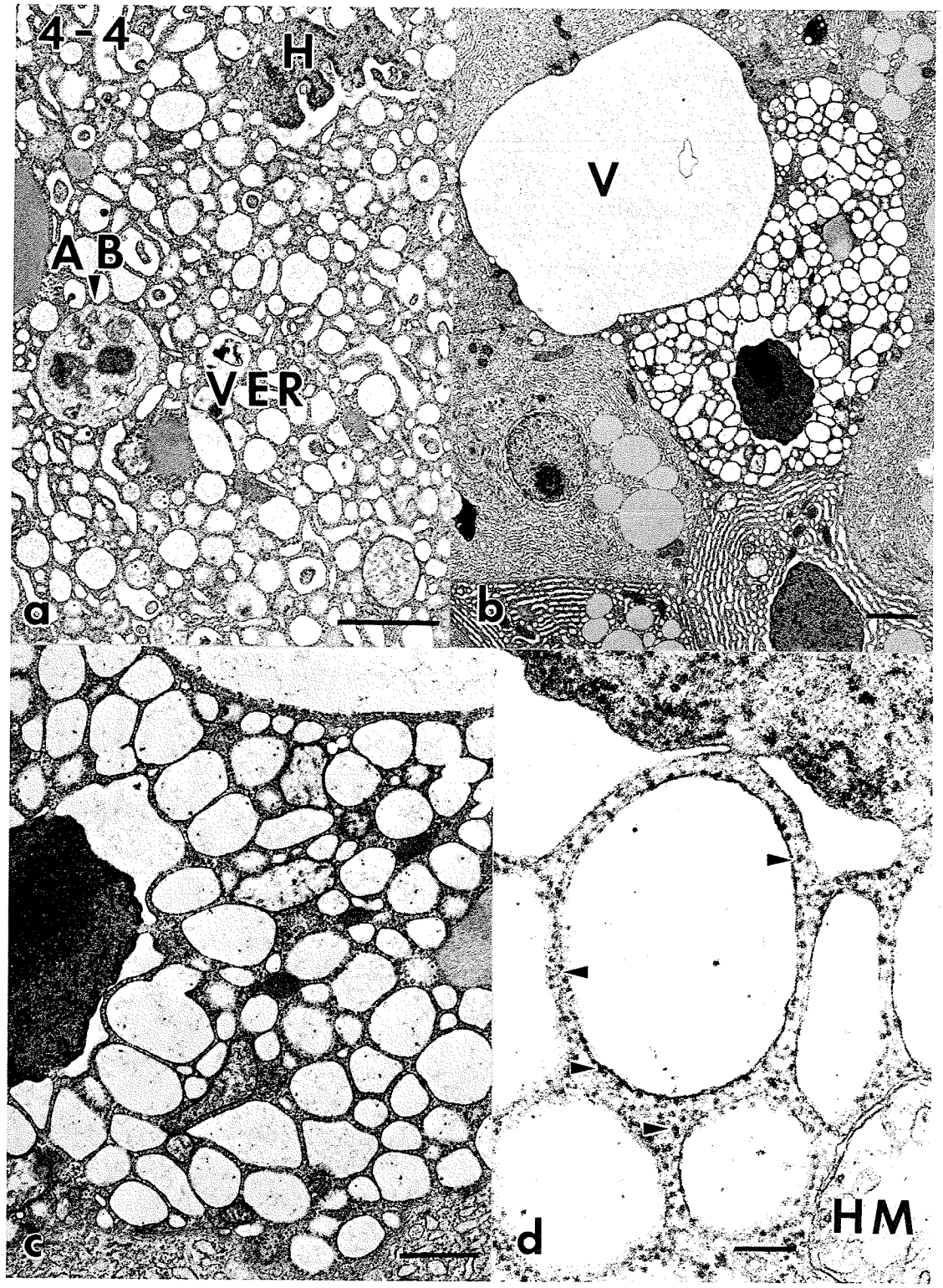
Ultrastructural changes in winter flounder hepatocytes from Deer Island Flats, Boston Harbor.

a) Detail of vesiculated rough endoplasmic reticulum (VER) of an hepatocyte (H). This vesicular appearance is essentially the "norm" for the majority of non-vacuolated hepatocytes in winter flounder from Deer Island Flats, Boston Harbor. An apoptotic body (AB) is also present. Uranyl acetate and lead citrate. x 8466. Bar = 2 μm

b) Transmission electron micrograph of winter flounder liver from Deer Island Flats, Boston Harbor. View of a centrotubular vacuolated (V) cell and an adjacent hepatocyte apparently undergoing vacuolation by extreme dilation and vesiculation of the cisternal space of the endoplasmic reticulum. Uranyl acetate and lead citrate. x 4305. Bar = 2 μm

c) Higher power of Figure 4.4B. x 13108. Bar = 1 μm

d) Higher power of Figure 4.4C. The location of the fluid accumulation is shown to be within the cisternae of the endoplasmic reticulum, by the presence of ribosomes on the outside of the membranes bounding the vesicles (arrows). A swollen mitochondrion (HM) is also present. x 53632. Bar = 0.2 μm


4-4
H
V
A
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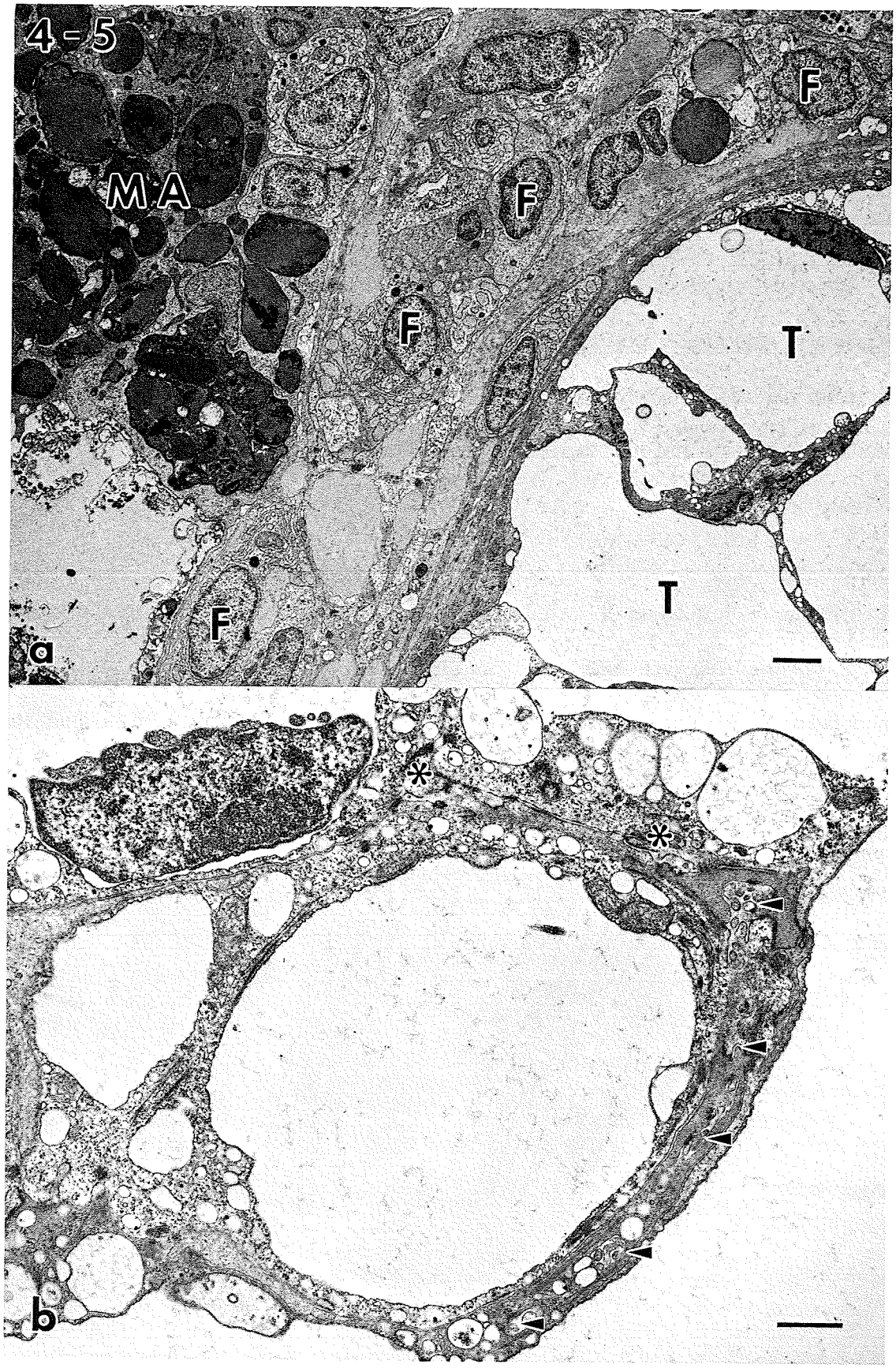
VER
a
b
c
d
HM

Figure 4-5

Ultrastructure of tubular hydropic vacuolation in winter flounder liver from Deer Island Flats, Boston Harbor

a) Tubular stage of vacuolation. The entire tubule (T) is vacuolated, and ensheathed in fibroblasts (F). An aggregation of macrophages is evident in the upper left corner of the image (MA). Uranyl acetate and lead citrate. x 4305. Bar = 2 μm

b) Detail of tubular stage of vacuolation. Section through contiguous vacuolated cells. Arrows indicate the course of the center of the tubule, marked by the microvilli of the vacuolated hepatocytes around it. Cell junctions (*) are common. Uranyl acetate and lead citrate. x 11552. Bar = 1 μm


4-5
MA
F
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F
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b

Figure 4-6

Ultrastructure of focal vacuolation in the liver of winter flounder from Deer Island Flats, Boston Harbor.

a) The focal stage of vacuolation. Prominent features of this advanced stage of vacuolation are evident in this image: Large vacuoles (F), pleomorphic nuclei, numerous cellular junctions (*), and multiple smaller vacuoles and vesicles in various stages of formation and disintegration. Uranyl acetate and lead citrate. x 4305. Bar = 2 μm

b) A higher magnification of vacuolated cells within a focus of vacuolation, illustrating the association of vacuolation and swollen mitochondria (SM), and vesicles of endoplasmic reticulum (ER). These structures can be distinguished, even after the mitochondrial cristae have disappeared by the presence of a double membrane in the vesicles originating from mitochondria. 11552 x. Bar = 1 μm

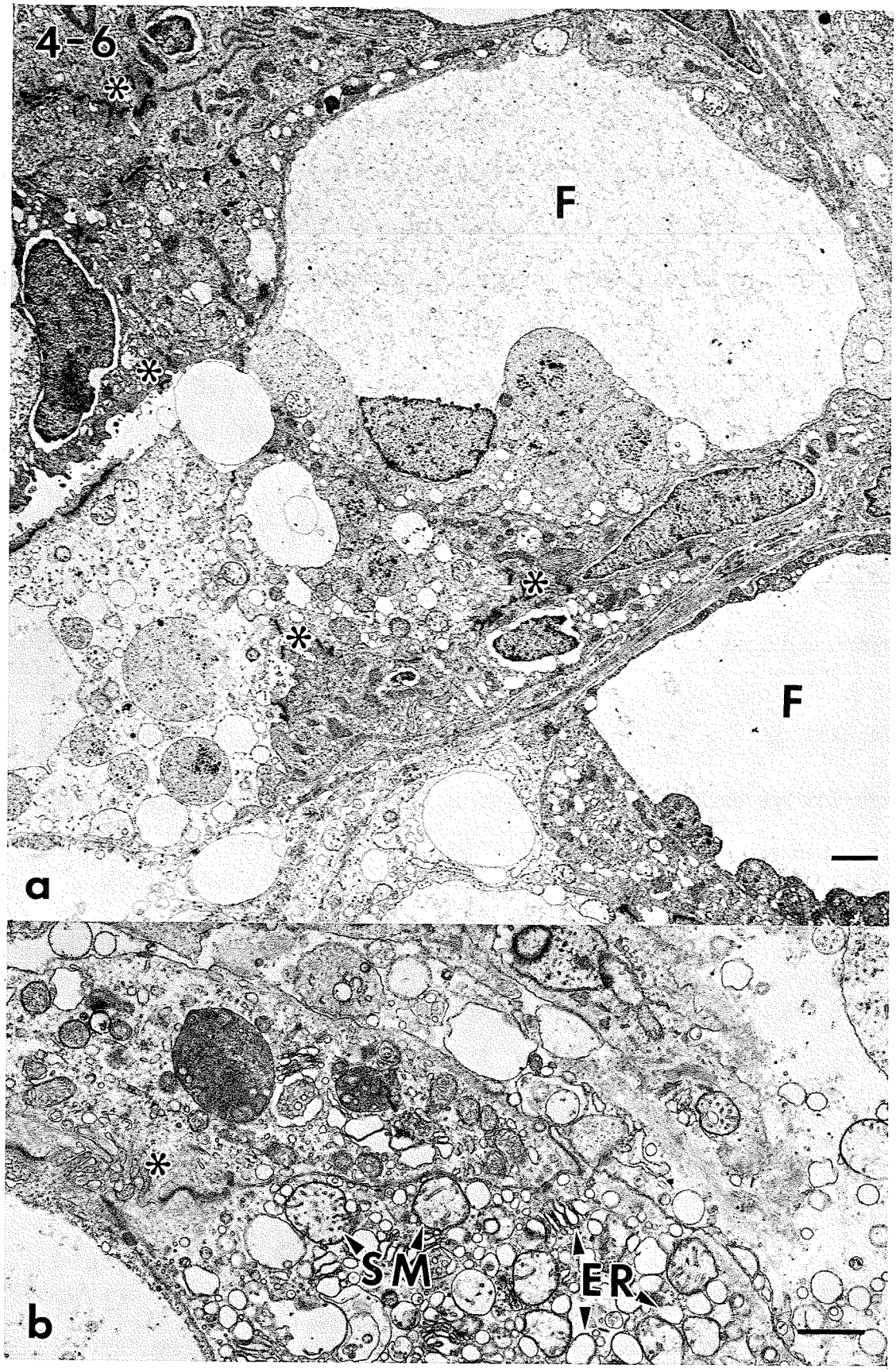
4-6
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Figure 4-7

Schematic drawings of the progressive nature of hydropic vacuolation, with ultrastructural images of each stage. These drawings were developed using histological information from Chapters 2 and 3, and ultrastructural information from this Chapter. The drawings are intended to convey the structural relationships between each cell type involved. Vascular and perivascular structures have been omitted. Each micrograph is presented as a reduced (magnification 2583x) portion of figures described and annotated above as follows: a) Figure 4-1a, b) Figure 4-3a, c) Figure 4-5a, d) Figure 4-6a.

a) A normal tubule of hepatocytes. The central biliary canaliculus drains into the biliary preductule that is partially lined by small preductular epithelial cells.

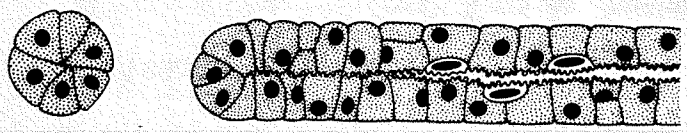
b) Centrotubular vacuolation. The first stage involves hydropic vacuolation of individual preductular cells in the center of the tubule. This then proceeds to involve a series of preductular cells.

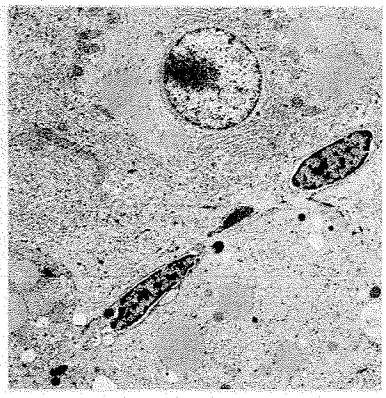
c) Tubular vacuolation. The process of hydropic vacuolation has now spread to involve the entire tubule. Fibroplasia surrounding the tubule is common.

d) Focal vacuolation. These grossly visible foci of hydropically vacuolated cells are presumably formed by the total vacuolation of an area of hepatocytes and biliary epithelia.

4 - 7

a





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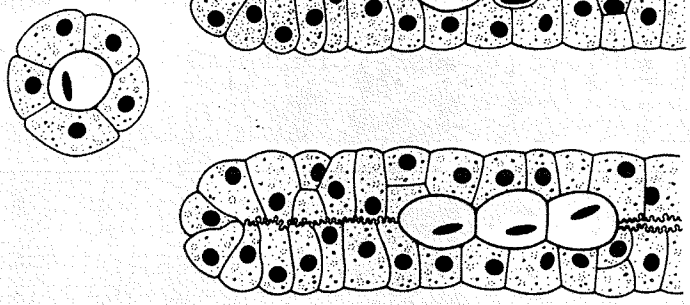

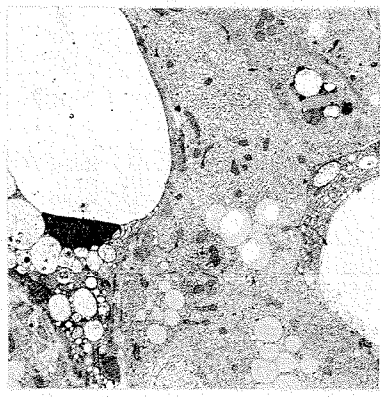

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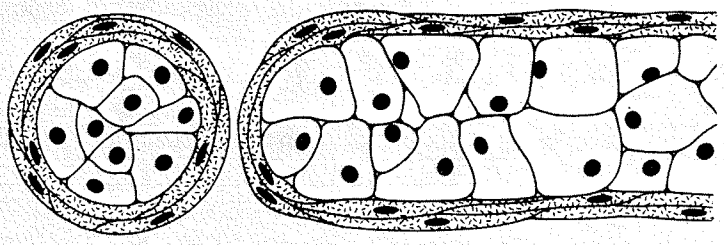

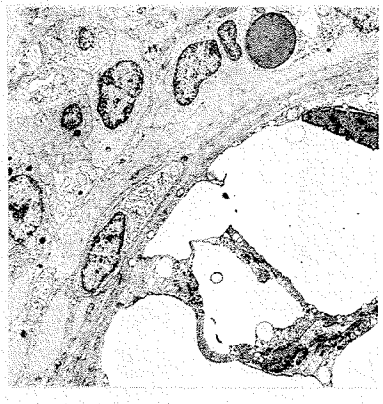

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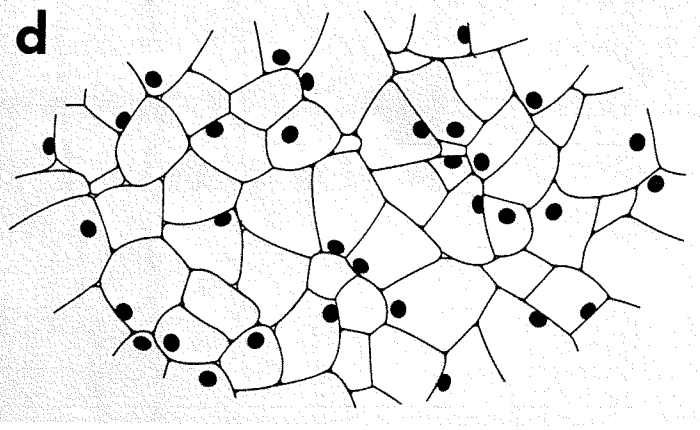
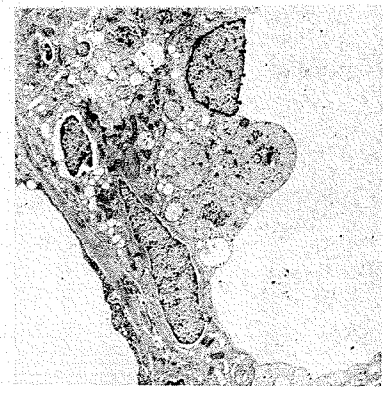




Ultrastructure of cholangiocellular carcinomas

1) Tubular cholangiocellular carcinoma - RTLA # 5226

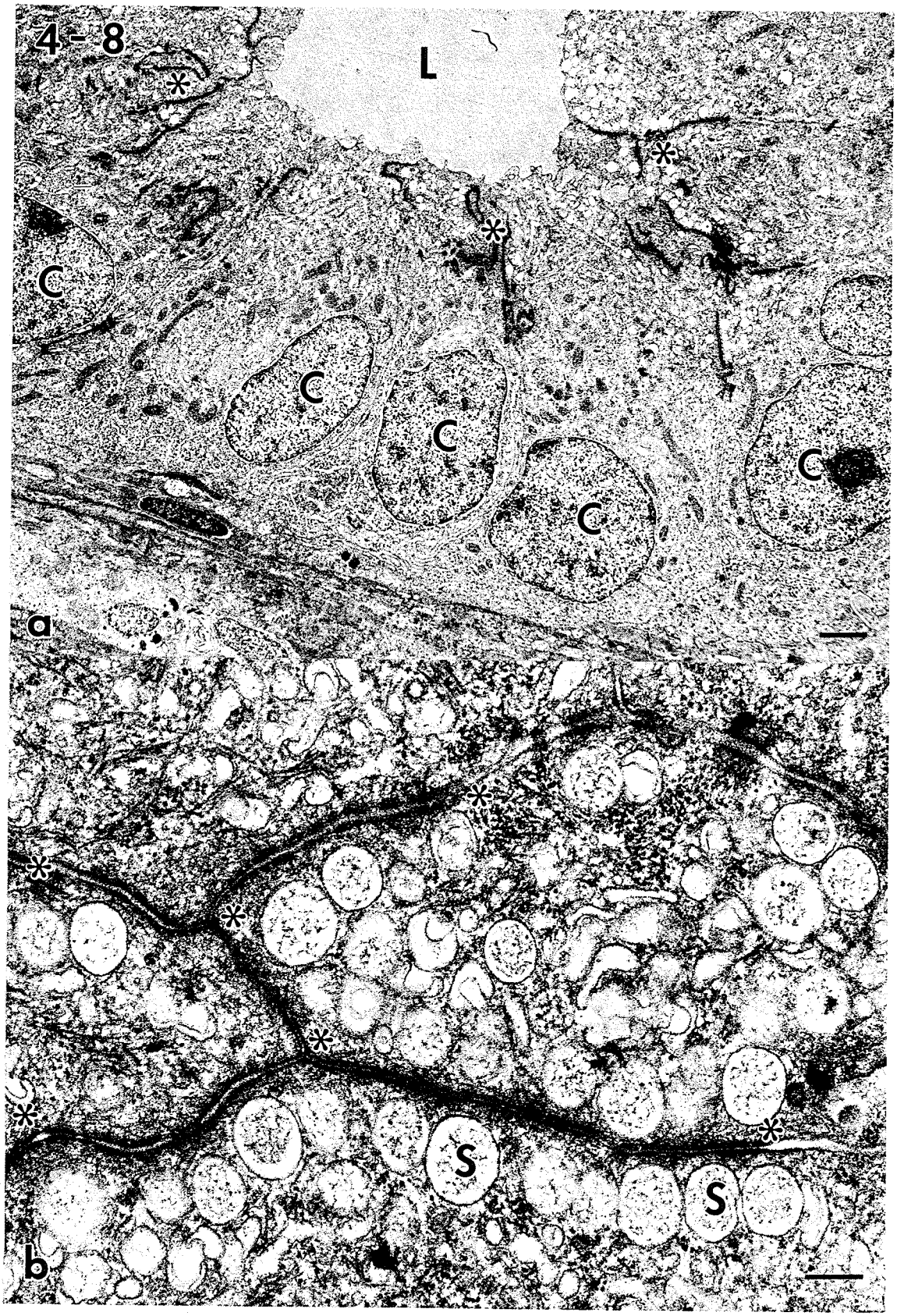
The tubular nature of this neoplasm was readily apparent ultrastructurally (Figure 4-8). Each tubule had 8 - 12 columnar cholangiocytes in cross section. The lumenal apex of each cell contained many vesicular structures, possibly of a secretory nature. Apices of adjacent cells were strongly bonded by extensive cellular junctions, and desmosomes (Figure 4-7b). The nuclei were somewhat pleomorphic, with a mildly irregular outline. Nucleoli were occasionally evident against the nuclear margin. Mitochondria were electron dense, elongated and appeared functional.

Figure 4-8

Ultrastructure of a tubular cholangiocellular carcinoma in a winter flounder from Deer Island Flats, Boston Harbor.

a) RTLA # 5226. Section through a tubular cholangiocarcinoma. The tubular lumen (L) is encircled by numerous columnar cholangiocytes (C), whose nuclei are somewhat pleomorphic. The apices of the cells contain numerous vesicular structures, and extensive cellular junctions (*). Uranyl acetate and lead citrate. x 4305. Bar = 2 μm

b) Higher magnification of the apical portion of a tubule in another part of the same tubular cholangiocarcinoma illustrated in 4-7A. The extensive cellular junctions (* to *), and vesicular structures (S) are illustrated. Uranyl acetate and lead citrate. x 53632 x. Bar = 0.2 μm


4- 8
L
C
C
C
C
C
a
S
S
b

2) Solid, anaplastic cholangiocellular carcinoma

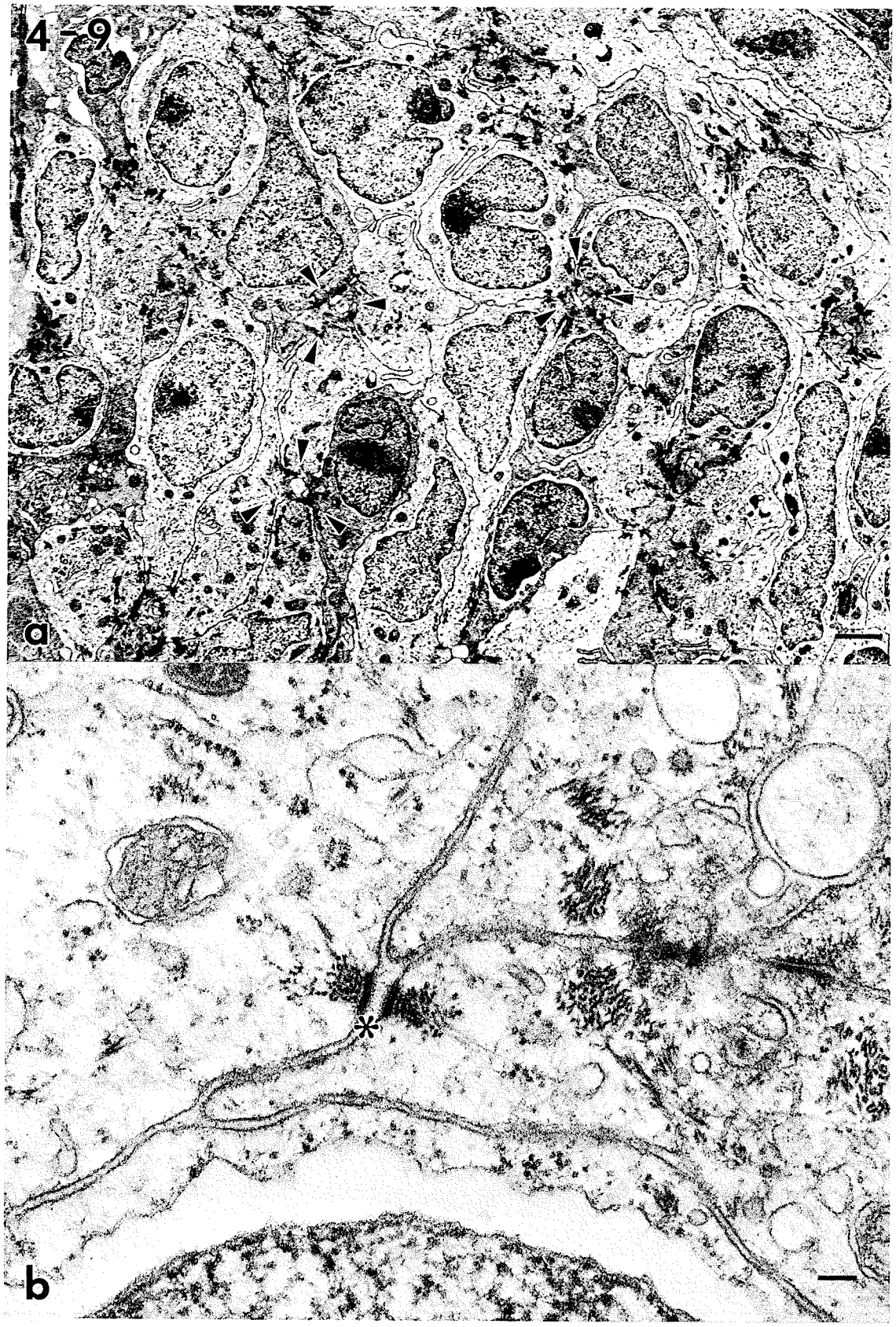
In contrast to the lesion described above, this neoplasm had a marked increase in the nucleus to cytoplasm ratio (Figure 4-9a). Nuclear size and shape was highly variable, with many deep indentations into the nuclear margin. Nucleoli were variable and irregular in their size, shape, position and appearance. The perinuclear space was mildly enlarged. The cytoplasmic matrix was disorganized, with some glycogen, a few disorganized leaflets of mildly dilated endoplasmic reticulum and a few small mitochondria. In contrast to the neoplasm described above, cellular junctions were much less extensive. A few short desmosomes with obvious filaments were present in clusters around small but patent tubular lumens (Figure 4-9). The presence of these lumens, which were indistinct with the light microscope, confirmed the diagnosis of a cholangiocellular lesion in parallel with the more obviously tubular lesion described above. Surrounding hepatocytes were typical of those described above for Deer Island Flats, Boston Harbor fish. They contained micro-vesiculated endoplasmic reticulum throughout the cytoplasm.

Figure 4-9

Ultrastructure of an anaplastic cholangiocellular carcinoma from a winter flounder from Deer Island Flats, Boston Harbor.

a) Section through an anaplastic solid cholangiocarcinoma. The tubular basis of this structure is still evident, as shown by the numerous clusters of cellular junctions around very small lumens (arrowheads). The nuclei of the neoplastic cells are extremely pleomorphic, and the nucleus to cytoplasm ratio is high. Uranyl acetate and lead citrate. x 4305. Bar = 2 μm

b) Higher magnification of the specimen in Figure 4.8a. The disorganized nature of the cytoplasmic matrix and extremely truncated cellular junctions are evident (*). 34066 x. Bar = 0.2 μm


4-9
a
b

DISCUSSION

The findings of these ultrastructural studies include: 1) The demonstration that the histological basophilia observed in the hepatocytes of winter flounder from Deer Island Flats, Boston Harbor, as compared to cleaner sites is due to a loss of hepatocyte lipid and glycogen stores, and a proliferation of the rough endoplasmic reticulum. 2) Fluid accumulated in the cisternae of the endoplasmic reticulum, the perinuclear space, and in ballooned mitochondria. It seems likely that these membrane-bounded balloons of fluid coalesced repeatedly, to eventually form one very large perinuclear cytoplasmic vacuole that compressed the nucleus and remaining cytoplasm to the margins of the cell. 3) The first cells to undergo the vacuolation process were in the center of the hepatic tubule. The majority of these cells were preductular biliary epithelial cells. 4) The second, tubular stage of vacuolation included both preductular cells, and hepatocytes. 5) Neoplastic cells showed plasticity of phenotype comparable to those described for mammalian lesions.

The association of a basophilic hepatocyte cytoplasm with an increase in rough endoplasmic reticulum has been described in rainbow trout (Scarpelli et al. 1963) and in mammals (Ghadially 1988). The histological basophilia results from hematoxylin staining nuclear and ribosomal nucleic acids. In contrast, the fish from Georges Bank had a poorly staining cytoplasm reflecting a high lipid and glycogen content. It appears that various species of fish respond differently when exposed to hepatotoxins. Some species increase their lipid content (Scarpelli 1974, Kohler 1989, Cormier 1989),

whilst in others it is reduced (Weiss 1974, and this study) when exposed to hepatotoxins. Differences are also evident in the same species, depending on toxicant, dose, and duration of exposure.

Dilation of the endoplasmic reticulum has been described following many insults to the liver (reviewed by David 1964, and Smuckler and Arcasoy 1969), including hypotonic solutions, starvation, dietary deficiency, anoxia, carbon tetrachloride exposure, hepatitis, yellow fever, liver neoplasia, exposure to dimethylnitrosamine, bile obstruction, and intra-hepatic cholestasis. Smuckler and Arcasoy (1969) published electron micrographs of dilated and vesiculated endoplasmic reticulum resulting from exposure to carbon tetrachloride that are very reminiscent of that illustrated here (especially Figure 4-4a) for winter flounder from Deer Island (Boston Harbor). The fundamental lesion in all cases of dilation of the endoplasmic reticulum involves an ingress of water (Ghadially 1988). These changes were unlikely to be an artefact in this study, as the fixative used was hypertonic. Starvation was not an issue, as all of the animals were killed within 48 h of capture, and many had food in their intestines. From the remainder of the above list, the likely factors active in this situation may include exposure to hepatotoxins, anoxia and cholestasis.

Exposure to hepatotoxins is a highly likely occurrence in fish inhabiting the severely contaminated sediments of Boston Harbor. The presence of tissue anoxia is harder to evaluate. Recent monitoring of water quality in Boston Harbor (Robinson et al. 1990), has failed to show prolonged anoxic events in the water column.

However, reduced tissue oxygenation could still occur if oxygen transport were impaired by a functional or absolute loss of hemoglobin.

The role of cholestasis in the development of these ultrastructural changes is unclear. Histologically, dilated and hyperplastic bile ducts and ductules were commonly encountered. These tubes contained plugs and lakes of bile fluid within them. However, classical ultrastructural signs of canalicular cholestasis were lacking. In mammals the cardinal ultrastructural features of cholestasis are the presence of granular and vesicular secretions in dilated canaliculi that have lost their microvillar border (Phillips et al. 1987). In winter flounder from Deer Island Flats, Boston Harbor, the canaliculi were not dilated and canalicular secretions did not accumulate to any great extent. In some fish the microvilli were somewhat reduced. Thus, the reason(s) for the development of vacuolated cells remain unclear.

The identity of the cell of origin of the vacuolated cell at each stage was important to establish. In a recent paper, Bodammer and Murchelano (1990) report ultrastructural findings upon analysis of two Deer Island winter flounder, both of which appeared to correspond to the tubular vacuolation stage as defined in this study. These authors describe in detail the differences between light and dark hepatocytes[1] and the possible role of apoptosis (Wyllie 1980). These authors discuss the question of whether vacuolated cells are hepatocytes or preductular cells. Bodammer and

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[1] Light and dark hepatocytes were also seen in this study, although their significance is unclear. Ghadially (1987) discusses the issue of light and dark hepatocytes at length. The dark hepatocyte is dehydrated: this can result from impending cell death, differences in metabolism or deficiencies in fixation and/or processing.

Murchelano referred to a previous publication (Moore et al. 1989), which they appear to have interpreted as suggesting that vacuolated cells are all preductular. Our report did focus on the centrotubular stage, but it was also stated that hepatocytes and other cells probably also underwent vacuolation. This current study reinforces the opinion that the first cell type to vacuolate was centrotubular, and preductular in origin, but that hepatocytes also probably vacuolate in the second stage of the process. Centrotubular vacuolation was seen more frequently in younger fish than tubular vacuolation (Chapter 3). Evidence for the early vacuolated cells being preductular included location within the tubule, nuclear morphology, and the presence of many cellular junctions.

Hydropically vacuolated cells have also been recently described as "apparent apoptotic parenchymal cells" (Carr et al. 1991). The usage is not justified by these authors, nor is any appropriate publication cited. It is unlikely that apoptosis is an appropriate descriptor for hydropically vacuolated cells for two reasons. Firstly, it was shown in chapter 3 that hydropically vacuolated cells persist for a period of months. This is in distinct contrast to apoptotic cells that have a half-life of a few hours (Wyllie 1980). Secondly, the ultrastructural studies described here fail to show any structures associated with vacuolated cells that in any way correspond to previously described apoptotic bodies in mammals (Wyllie 1980).

The next, tubular stage of vacuolation involved the entire hepatic tubule, occupying the space of both biliary and hepatocytic components. A potential way in which the centrotubular to tubular progression could occur is as follows: Assume that hepatotoxins render the parenchymal hepatic milieu physiologically marginal, whether

due to pH imbalance, ionic imbalance, or lack of tissue oxygen. The portion of the parenchyma that is likely to succumb first is that furthest away from the vascular supply, namely the preductular cells, thus initiating the vacuolation process at the center of the tubule. The presence of dysfunctional, ballooned preductular cells might then reduce the flow of bile secretions from the hepatocytes. This would in turn result in spreading of the vacuolation process throughout the tubule (Figure 4-4 and Figure 4-5). The reduced flow can only be mild, otherwise there would have been evidence of canalicular stasis. However, it has been suggested in the human condition of congenital dilation of the bile ducts, that chronic bile stagnation, without acute obstruction, allows survival of the individual, whilst causing chronic carcinogenic irritation of the biliary tubules by stagnant bile (Gallagher et al. 1972). There is an elevated risk of cholangiocarcinoma in this condition (Kagawa et al. 1978). A further possibility to account for the progression from centrotubular to tubular vacuolation could be that the vacuolated centrotubular cell has the capacity to proliferate. This capacity could reflect a resistance to the stagnant bile fluid, which in fish from a contaminated site such as Boston, will have many partially metabolized carcinogens and procarcinogens. Additionally bile salts can be metabolized to compounds such as methylcholanthrene (Burns et al. 1990). As hepatocytes undergo necrosis and apoptosis in the face of these toxins, the resistant vacuolated cells in the center of the tubule might proliferate to fill the void, with concomitant peritubular fibroplasia. This fibroplasia involved layers of flattened cells, with scant cytoplasm, and small oval nuclei, illustrated in Figure 4-5a, may reflect a proliferation of the fat storing cells of

Ito (Wake 1980, and McCuskey et al. 1986). These cells are presumed to be the teleost equivalent of the mammalian stellate, fat-storing, putative vitamin A storing cells of the space of Disse, which have the capacity to assume a fibroblastic phenotype. A fundamental question that is currently unanswered is whether the process of vacuolation spreads to fill the tubule by recruiting hepatocytes into the vacuolation process, or by the proliferation of centrotubular vacuolated cells to fill the entire tubule, replacing effete hepatocytes, or by a combination of these two processes? The identity of vacuolated cells and their peripheral neighbors of the same tubule is essential in an understanding of the generation of tubular vacuolation and, likely, focal alteration as well.

The subsequent progression from tubular vacuolation to grossly visible foci of vacuolated cells is open to study at this time. How this progression occurs depends on whether vacuolation is a step in the path to cell death, or a state that does not preclude multiplication? The latter would seem plausible if it were not for the fact that fully vacuolated cells have very few functional organelles remaining. However, what little cytoplasm they do retain appears relatively normal, and their nuclear morphology is essentially unchanged.

To determine whether the vacuolated cells filling the hepatic tubules were vacuolated hepatocytes, or proliferated preductular cells, one could compare cell specific markers such as the cytokeratins (Marceau 1990) using histochemistry, if reagent antibodies were available that reacted specifically with teleost cytokeratins. A second test of these alternatives would be to look for evidence of cell proliferation

within these vacuolated cells. The next chapter describes a technique for the study of cell proliferation in winter flounder.

CHAPTER 5

CELL PROLIFERATION IN THE LIVER OF WINTER FLOUNDER, *PSEUDOPLEURONECTES AMERICANUS*, FROM BOSTON HARBOR

INTRODUCTION

Winter flounder living on the contaminated sediments of Boston Harbor undergo a progressive liver disease (Chapter 3). This disease is first seen in 200 to 300 mm long sexually immature fish, as bile duct and ductule proliferation and an abnormal hydropic vacuolation of the cells in the center of the hepatic tubules, primarily the biliary preductule cells (Chapter 4). In older fish vacuolation is widespread, occurring throughout the hepatic tubule, in grossly visible foci, and in cholangiocytes of the bile preductules, ductules and ducts. Vacuolated cells are commonly found in winter flounder from Boston Harbor, yet they are still somewhat enigmatic, in spite of descriptive studies (Moore et al. 1989, Bodammer and Murchelano 1990, Chapter 4). Vacuolation appears to be a stable change, persisting in the absence of ongoing exposure to cytotoxins (see chapter 3). In severely affected fish vacuolated cells are focally aggregated, and often intimately associated with diverse foci of cholangiocellular and hepatocellular neoplasia. Their persistence, increasing prevalence with age of fish, and their association with neoplasia raises the following question. Do vacuolated cells have the ability to proliferate? The identity of proliferating cell type(s) is central to the understanding of the development of liver neoplasia in winter flounder from Boston Harbor.

Cell proliferation has been evaluated immunohistochemically in mammals (Gratzner 1982), and in fish (Droy et al. 1988) by using the non-radioactive thymidine analog bromodeoxyuridine (BrdU). In this study endoscopy was used to select groups

of fish with visibly normal and visibly abnormal livers from Boston Harbor. The selected fish from Boston, and other fish from Georges Bank, were then labelled with BrdU. Immunohistochemical methods for detection of BrdU were optimized for use in formalin-fixed, paraffin-embedded flounder liver samples.

DNA synthetic activity as assayed by BrdU incorporation was found in normal, vacuolated, and neoplastic liver cells. This is, to our knowledge, the first immunohistochemical investigation of replicative DNA synthesis in a neoplastic disease of wild-caught fish exposed to chemical carcinogens in their natural habitats. These findings further our understanding of the pathogenesis of pollutant related liver disease in winter flounder. The methods employed will also have broad application to the study of cell proliferation in the pathogenesis of diverse infectious and non-infectious fish diseases.

METHODS

Materials

Bromodeoxyuridine, fluorodeoxyuridine, protease Type XIV, aminopropyl-triethoxysilane (APTS) and ethyl-m-amino benzoate methane sulfonic acid (MS222) were obtained from Sigma (St Louis, MO). A PAP pen was obtained from Newcomer Supply (Oak Park, IL). Cell proliferation assay kits (RPN 20) consisting of bromodeoxyuridine/ fluorodeoxyuridine labelling reagent, anti-bromodeoxyuridine primary antibody (with nuclease), peroxidase anti-mouse secondary antibody and diaminobenzidine substrate with nickel chloride color intensification were obtained from

Amersham Corporation (Arlington Heights, IL).

Fish

Flounder were obtained by commercial otter trawl from Georges Bank in November 1989 and from Deer Island Flats, Boston Harbor, in April 1990. Fish were returned to the laboratory alive, maintained in running filtered sea water at 15 °C. Protocols for the maintenance, examination and euthanasia of fish used in this study were approved by the Institutional Animal Care and Use Committee of the Woods Hole Oceanographic Institution.

Screening

Within 24 hours of capture, 111 fish from Boston Harbor were examined endoscopically. They were anaesthetized with MS222. Satisfactory anaesthesia involved the minimum exposure necessary to cause a loss of reflex flexion upon being held by the head out of water. Adequately anaesthetised fish lose this reflex, and their bodies are limp and flaccid when gently shaken. At this stage, body equilibrium in the water is also lost. Opercular movements were retained at all times, although fish whose opercular movements are imperceptible will often recover in time. Each fish was then positioned blind side uppermost, and a 5 mm incision made through the skin and body wall, parallel to the myomeres over the center of the abdominal cavity. A 4 mm diameter 70° sidescan rigid endoscope (EDER Instr.Co., Chicago, IL) was then inserted parallel to the ventral surface of the liver. The endoscope was gently lifted

against the body wall to gain perspective and avoid obstruction of the viewing window with peritoneal fluid. A systematic examination of the ventral surface of the liver was made by gradually introducing the endoscope through the incision whilst rotating the instrument to scan cranially and caudally. To complete the survey, the endoscope was removed and reinserted in the opposite direction to the first entry, and the remainder of the ventral surface was scanned in the same way. The wound was repaired with a cruciform suture. No mortalities resulted from the endoscopy. Four fish were identified as exhibiting gross lesions on the ventral surface of the liver. These fish, and 7 visibly normal fish were labelled with bromodeoxyuridine. The fish that were not selected for labelling were necropsied, at which time a further four fish were found to have gross visible lesions of the liver. These lesions were all on the dorsal surface of the liver, and were thus obscured from the endoscope.

Labelling and sampling

Both *in vivo* and *in vitro* labelling techniques were employed within 48 hours of capture. Flounder from Boston Harbor were labelled *in vivo*, by intraperitoneal[1] injection of an aqueous solution of 30 mg/ml bromodeoxyuridine, and 3 mg/ml fluorodeoxyuridine, at 10ml/kg. Three hours later the fish were anaesthetized with MS222 and sacrificed by cervical section. An oval incision was made in the ventral body wall, over the anterior edge of the ventral gonad, along the ventral margin of the

[1]Preliminary trials revealed that suturing the endoscopy wound, as described above, rendered a leak-proof seal, when manual pressure was applied across the body wall overlying the visceral space.

kidney, through the ventral pectoral girdle and the underlying pericardial sac, and then through the caudal third of the ventral operculum, over the vent and then back to the start of the incision. This exposed the liver, kidney, heart, and anterior ventral gill bar. The liver was dissected free of the gall bladder and underlying viscera and cut into 4 mm slices. One piece was selected from the central, but non-hilar portion of the liver. Additional slices of any visible abnormality were also taken. Four millimeter slices of kidney, heart, and upper small intestine and the anterior ventral gill bar were also removed.

Tissues from Georges Bank fish were labelled with BrdU *in vitro*. Heart, liver and kidney were dissected as described above. Liver pieces that were 1x2x2 mm were incubated for one hour *in vitro*, in the bromodeoxyuridine/fluorodeoxyuridine labelling reagent. The reagent was diluted 1/1000 in Eagles minimal essential medium, and 10% fetal bovine serum. Tissue fragments were then washed in phosphate buffered saline (PBS) pH 7.6 for 15 minutes before fixation.

Processing

All tissues were fixed in 10% neutral buffered formalin for 24 hours and then processed immediately to avoid excess cross-linkage by formalin. Tissue samples were routinely dehydrated and embedded in paraffin (Luna 1968). Microscope slides were dipped in 95% ethanol, five dips in each of three changes. Slides were then air dried, and dipped five times in 2% aminopropyltriethoxysilane (APTS) in acetone, and then dipped five times in each of three changes of distilled water, and air dried

(Goldsworthy et al. 1989). Paraffin blocks were sectioned at 5 μm, and sections floated onto the APTS coated slides, and left to dry at room temperature overnight. Routine hematoxylin and eosin stained sections were prepared.

Immunohistochemistry

Sections on APTS coated slides were processed in batches of 20 slides. Slides were immersed for 3 min in each of the following: 1) clearant 3 times, 2) 100% ethanol twice, 3) 70% ethanol once, and 4) PBS 3 times. Slides were then shaken dry, and blotted around each section. Each section on a slide was ringed with a hydrophobic circle drawn by a PAP pen. Sections were incubated in a humidified chamber[2] as follows: 1) 50 μl 0.05% protease in PBS, prewarmed to 37° C, but with the slide at room temperature, for 60 min; 2) 50 μl anti-bromodeoxyuridine (containing nuclease for DNA denaturation) for 16 h; 3) 50 μl Amersham peroxidase labelled anti-mouse IgG for 1 h. After each of these steps reagent drops were shaken off, and slides immersed in PBS 3 times for 3 min each. Slides were then immersed as follows: 1) Amersham diaminobenzidine and color intensifier for 10 min; 2) Distilled water for 3 min 3 times; 3) Eosin for 5 min; 4) 95% ethanol for 2 min; 5) 100% ethanol for 2 min twice; and 6) Clearant for 2 min twice. Slides were then mounted and coverslipped. Tissues not labelled with BrdU and tissues labelled with BrdU but with PBS replacing the primary antibody, were used as method controls. Each slide

[2]Plastic petri dishes, 150mm in diameter, were used as humidified chambers. Slides, 4 to a dish were lain on two 2 mm diameter wooden sticks that rested on a disc of PBS soaked filter paper that was lain in the petri dish.

carried two serial sections; the first was stained with the full protocol, and the second with PBS instead of the primary. Slides were stored in the dark.

Measurement of proliferation index

Gill, kidney and intestinal sections were examined to ascertain that the bromodeoxyuridine had been administered properly. Criteria included positive reactions in normally proliferative tissues, i.e. gill and intestinal basal epithelia, and renal hemopoietic cells, and negative reactions in non-proliferative tissues, such as the majority of renal epithelia, and gill cartilage. Liver cell proliferation indices were then quantified from single blocks from four fish from Georges Bank and a total of 17 blocks from 11 fish from Boston Harbor. The blocks for quantification from the Boston fish were selected for representation of different histological stages of the disease, after examination of slides stained with hematoxylin and eosin. Each slide was examined randomly at 1000x using an eyepiece graticule with a 10x10 grid as a frame of reference. The proportion of nuclei that were positive for BrdU was counted in hepatocytes, vacuolated cells, and neoplastic cells. One thousand nuclei of each cell type were evaluated, when present. In some slides, counterstaining was too faint to adequately recognise BrdU negative nuclei. In these cases, a serial, hematoxylin and eosin stained section was used to define the area of the section that contained 1000 nuclei of any one cell type.

RESULTS

Immunohistochemical protocol development

The standard protocol provided with the BrdU kit failed to generate positive results in any organ in the winter flounder. The standard protocol describes a one hour primary antibody incubation with no pre-treatment with a protease. The primary antibody contains a nuclease that aids in penetration of the antibody to the epitope. Extension of the incubation time with the primary antibody (with nuclease) from 1 to 16 h generated positive signals in the gill, intestine and kidney, but not the liver. This result was interpreted to mean that the nuclease needed a longer time to expose the epitope in fish than in mammal tissue, and that shielding of the epitope was most marked in the liver. Positive hepatic cells were only observed once the primary antibody incubation was preceded by an incubation with 0.05% protease for one hour. These modifications are described in the methods above.

The results of the *in vivo* and *in vitro* labelled fish were compared. Serial sectioning of *in vitro* labelled kidney samples from Georges Bank showed that the label penetrated 150 μm, into the tissue block; in contrast, the *in vivo* labelled Boston Harbor kidney samples were uniformly labelled throughout the tissue block. The *in vitro* method relied on local percolation of the label into the tissue block, whereas the *in vivo* intraperitioneal injection gave a vascular perfusion throughout every organ of the body, giving rise to uniform staining.

Extrahepatic controls

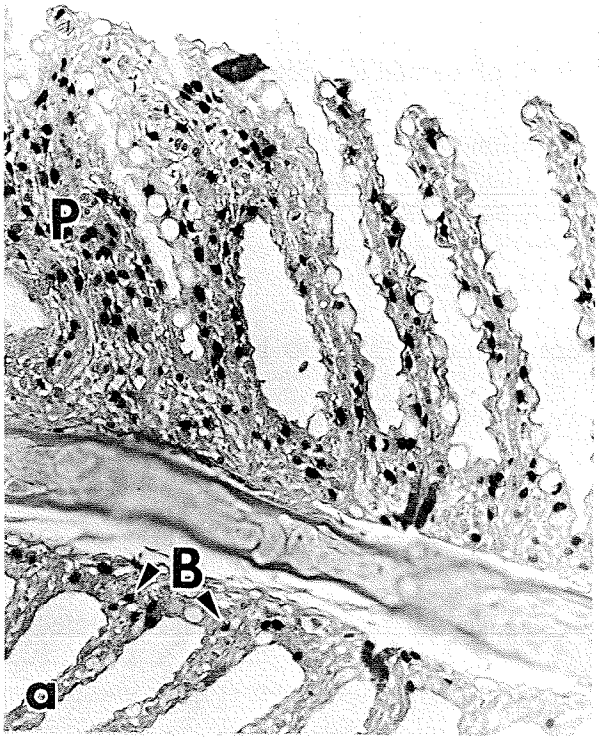
Nuclei of renal hemopoietic cells, intestinal crypt epithelia, normal gill basal epithelia, and proliferative gill lamellar epithelia stained strongly (Figure 5.1a), consistent with the normal proliferation expected in these cells. By contrast, the nuclei of cardiomyocytes, normal gill tip epithelia, and renal tubular nuclei did not stain. No staining was seen in extrahepatic organs of unlabelled fish, or of labelled fish incubated with PBS, in place of the anti-bromodeoxyuridine monoclonal antibody. These patterns were similar in the Boston Harbor and Georges Bank fish.

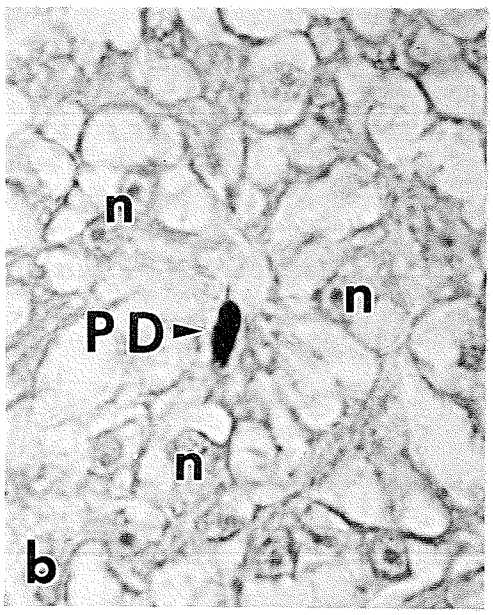
Figure 5-1
Anti-BrdU staining in winter flounder tissues

a) Boston Harbor winter flounder gill lamellae. The lower lamellae are normal, with DNA synthesis restricted to the base of the lamellae (B): in contrast, extensive fusion and proliferation (P), both epithelial and mucous cell, is seen above the gill bar. The abnormal upper portion reflects a concordance between morphological and histochemical evidence for cell proliferation. BrdU monoclonal antibody, eosin counterstain. 200 x

b) Normal tubulosinusoidal hepatic histology of Georges Bank winter flounder. DNA synthesis in a biliary preductular epithelial cell (PD). Hepatocyte nuclei are inactive (n). BrdU monoclonal antibody, eosin counterstain. 400 x

c) An aggregation of macrophages in a Boston Harbor winter flounder liver. Some macrophages are synthesizing DNA (A). 70% were inactive (I). The majority of aggregations were totally inactive. BrdU monoclonal antibody, eosin counterstain. 1000 x





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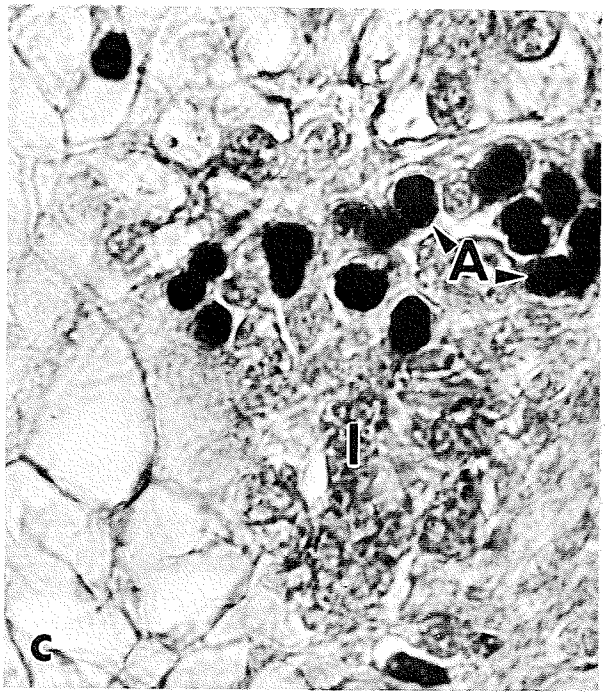


DNA synthesis in cells of the liver

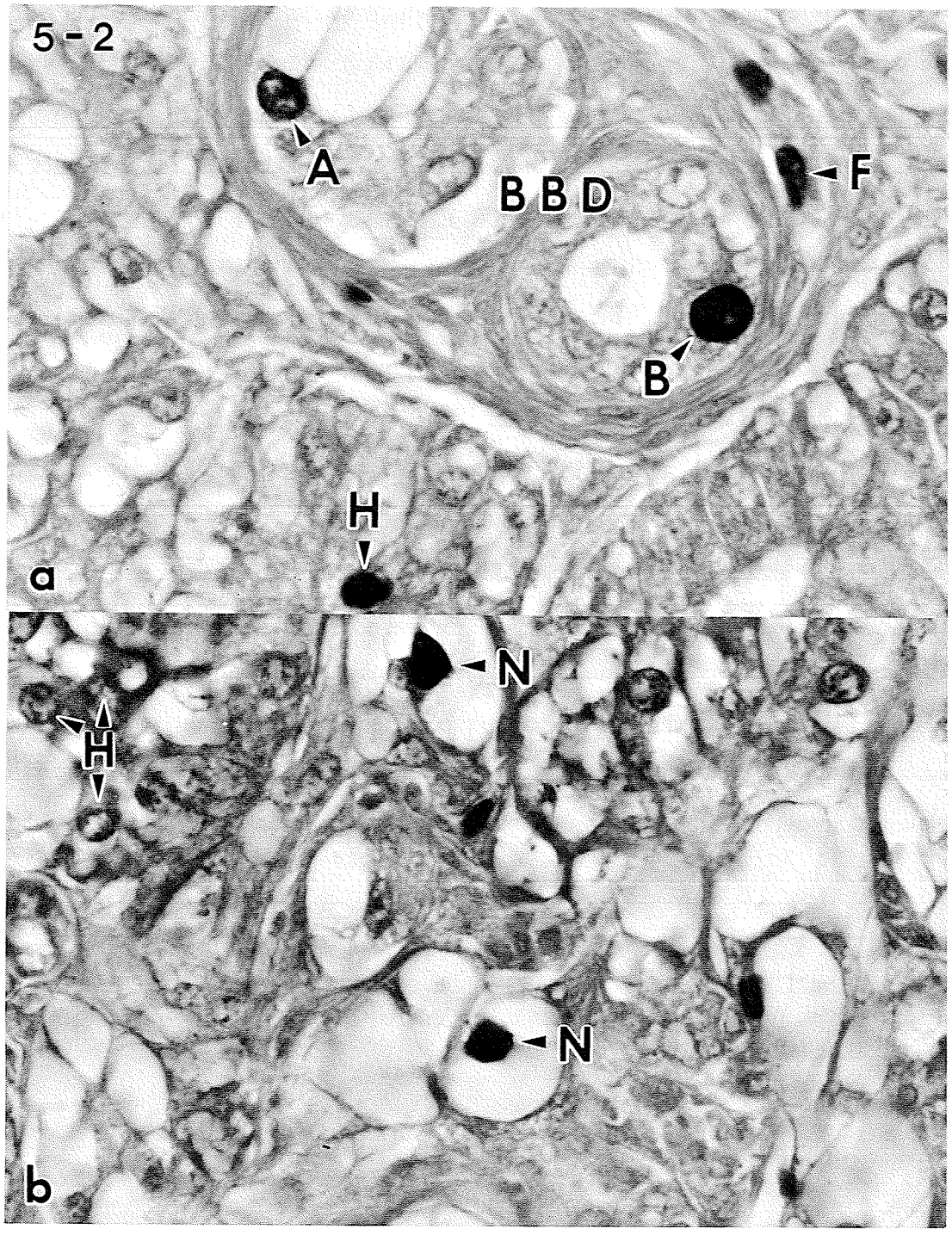
Non-vacuolated hepatocytes in the liver of winter flounder from Boston Harbor were observed to stain for BrdU only occasionally. Hepatocytes undergoing DNA synthesis were not obviously associated with pathological structures, and in particular they were not associated with neoplastic or vacuolar change. Small, ovoid nuclei located in the center of tubules were more frequently seen to be positive (Figure 5-1b). These appeared to be nuclei of normal preductular cells, which have been shown to be the first cell type to undergo vacuolation in winter flounder from Boston Harbor (Moore et al. 1989 and Chapter 4). Bile ductule and duct epithelia and the fibroblasts that ensheathed them stained occasionally, especially in bile ducts and ductules that branched frequently and that were wholly or partially vacuolated (Figure 5-2a).

Figure 5-2

Anti-BrdU staining in Boston Harbor winter flounder liver lesions

a) Branched small bile duct (BBD), with fibrotic sheath. DNA synthesis is occurring in vacuolated (A) and normal (B) cholangiocytes, ensheathing fibroblasts (F), and an hepatocyte (H). BrdU monoclonal antibody, eosin counterstain. 1000 x

b) DNA synthesis is seen in a number of vacuolated cell nuclei (N) in a Boston Harbor winter flounder liver. Non-vacuolated hepatocyte nuclei (H) and aggregated macrophages (MA) are inactive in this image. 1000 x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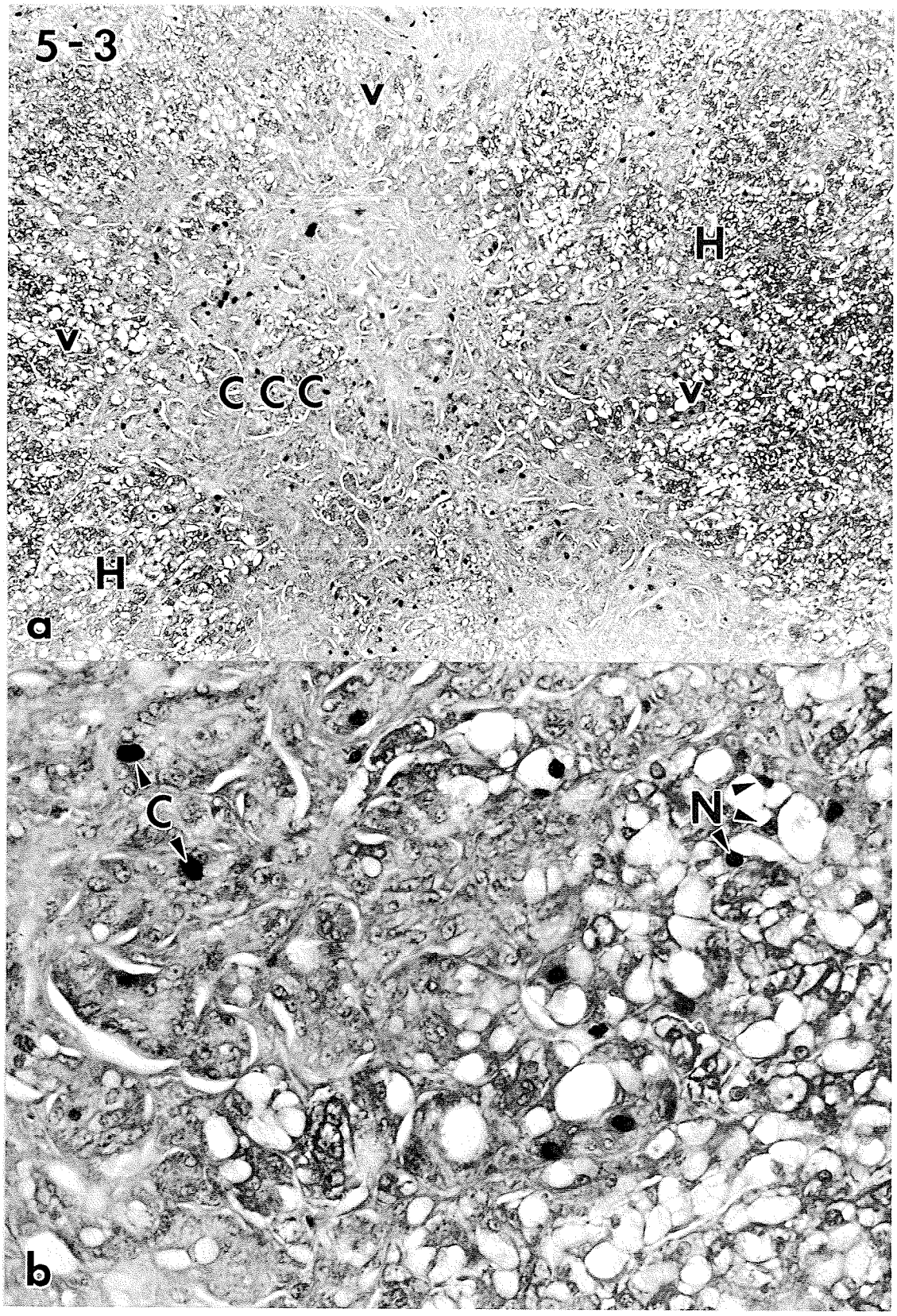
Vacuolated cells that filled the hepatic tubule not uncommonly stained for BrdU (Figure 5-2b). These cells had a small nucleus which was often flattened on one edge. Additionally, nuclei of fibroblasts in the fibrotic sheath around the vacuolated tubules were also seen to stain at times. Large aggregations of vacuolation are the commonest histological component of grossly visible lesions in Boston Harbor fish (Chapter 3). Anti-BrdU staining was clearly present in the vacuolated cell nuclei in these aggregates, but was usually infrequent. In some aggregates there appeared to be more staining in the periphery of the focus, than in the center. Aggregates of vacuolated cells frequently contain islands of cells that stain well with hematoxylin. These cells were usually negative for incorporation of BrdU.

Macrophage aggregates are also a common feature of winter flounder liver from Boston Harbor. Most such aggregates did not stain with anti-BrdU; however, a few showed intense BrdU staining (Figure 5-1c).

Only two of the fish from Boston that were labelled with BrdU proved to contain neoplasms histologically. One animal, fish 15 (numbers refer to individual fish listed in Table 5-1), contained three small, focal, cholangiocellular carcinomas, and the other contained a cholangiocellular carcinoma and an hepatocellular adenoma. Comparable neoplasms in winter flounder from Boston Harbor have been described elsewhere (Chapter 2).

Figure 5-3
Anti-BrdU staining in a focal cholangiocellular carcinoma.
a) An atypical, pleomorphic, tubular cholangiocellular carcinoma (CCC) infiltrate the hepatocytic parenchyma (H). The neoplasm is fringed with fingers of vacuolated cells (v). 100 x

b) Higher magnification of the box in Figure 3a. DNA synthesis is evident in neoplastic biliary epithelial cells (C), and vacuolated cell nuclei (N). 400 x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b

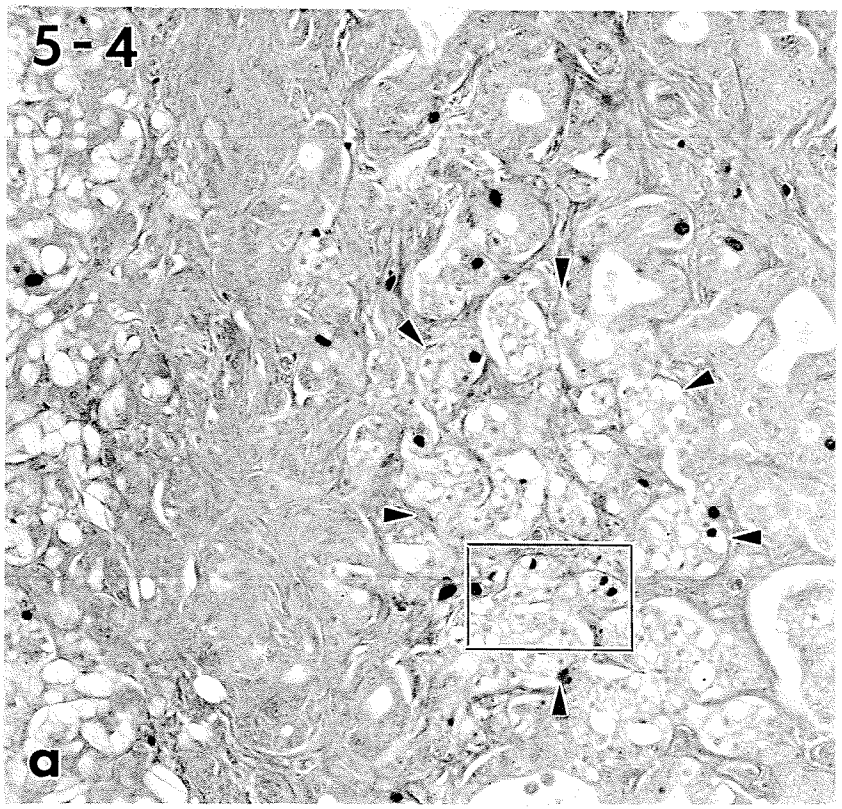
The three cholangiocarcinomas in fish 15 consisted of multiple tubules of biliary epithelial cells with large oval nuclei and indistinct nucleoli. In the first cholangiocarcinoma analyzed, the BrdU staining was observed along the invading perimeter of the lesion, in fingers of neoplastic cells. In the second lesion examined staining was seen diffusely in cholangiocytes throughout the neoplasm (Figure 5-3). In the third neoplasm, BrdU staining was seen primarily in a cluster of vacuolated neoplastic tubular cells within the neoplasm (Figure 5-4). Additionally, vacuolated cells immediately surrounding each of the cholangiocarcinomas were more often positive (Figure 5.3A), than those vacuolated cells at a greater distance from the neoplasm, that were not associated with a neoplastic focus.

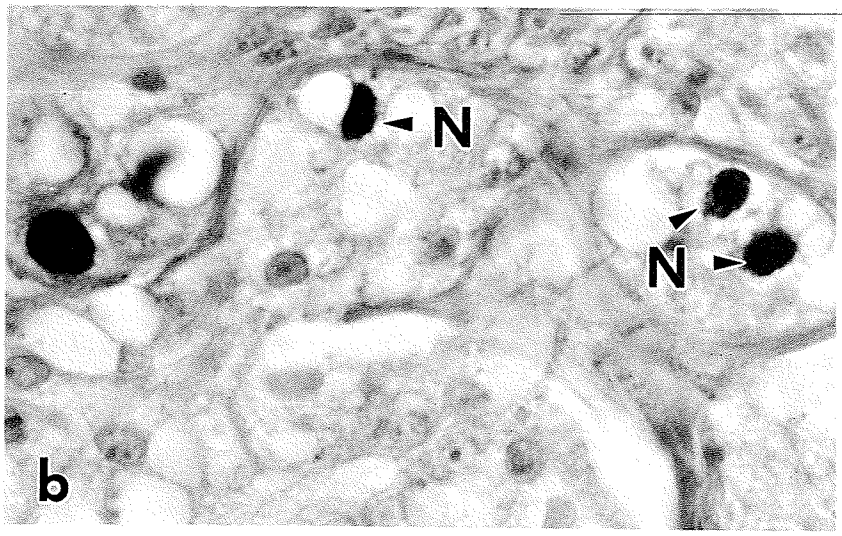
The cholangiocarcinoma in fish 14 differed morphologically from those described in fish 15, in that it consisted of clusters of dilated tubules with extremely flattened epithelia, interspersed with less condensed biliary epithelia and surrounded by sheets of desmoplastic fibroplasia. BrdU staining was not seen in the flattened epithelium, but was observed in the neoplastic cholangiocytes between the tubules and in surrounding fibroblasts. In contrast to the above neoplasms, the single trabecular adenoma in fish 14 that was BrdU labelled, was entirely negative for BrdU staining.

Figure 5-4

Anti-BrdU staining in a partially vacuolated cholangiocellular carcinoma
a) Part of a cholangiocellular carcinoma in which a portion of the neoplastic tubules were partially vacuolated (arrowheads). 200 x

b) A higher magnification of the boxed area of Fig 7a. DNA synthesis was predominantly in this vacuolated portion of the neoplastic cells (N). BrdU monoclonal antibody, eosin counterstain. 1000 x


5 - 4
a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The proliferation indices for hepatocytes of Georges Bank fish, and for hepatocytes, vacuolated cells, and neoplastic cells from Boston fish are given in Table 5-1. Livers from the Georges Bank fish were unremarkable histologically (see Chapter 3) with a homogeneous array of hepatic tubules and sinusoids. Anti-BrdU staining of nuclei was limited to occasional positive hepatocytes in two of the four fish examined.

TABLE 5-1

Site of collection, histology, fish #, and proliferation indices (P.I.) for hepatocytes, vacuolated cells, and neoplasms from Boston Harbor and Georges Bank winter flounder.

Site	Histology	Fish #	Hepatocyte P.I.	Vacuolated cell P.I.	Neoplastic cell P.I.
Georges Bank					
	Normal	1	0	-	-
		2	3	-	-
		3	1	-	-
		4	0	-	-
Boston Harbor					
	Normal	5	1	-	-
		6	0	-	-
		7	1	-	-
		8	0	-	-
		9	1	-	-
		10	0	-	-
	Tubular Vacuolation				
		11	0	0	-
		12	0	15	-
		13	3	4	-
	Focal vacuolation				
		14.1	0	10	-
		14.2	0	2	-
		14.3	0	3	-
	Neoplasia				
Cholangiocarcinoma		14.4[1]	2	1	60[2]
	Adenoma	14.5	1	3	0
Cholangiocarcinoma		15.1	5	3	68
Cholangiocarcinoma		15.2	3	3	24
Cholangiocarcinoma		15.3	4	2	42

Bromodeoxyuridine was used to label normal winter flounder livers from Georges Bank, and livers from Boston Harbor winter flounder, that represented four stages of disease development. Nuclei synthesizing DNA replicatively were stained immunohistochemically. Proliferation index is given as a percentage of BrdU positive nuclei in a random sample of 1000 cells of each cell type present.

[1]Multiple blocks were stained with BrDU for fish #'s 14 and 15.

[2]This lesion had multiple dilated neoplastic tubules, surrounded by extensive desmoplastic fibroplasia.

DISCUSSION

The primary conclusion to be drawn from this study is that vacuolated cells in the liver of winter flounder appear to have, at times, the capacity to synthesize DNA, and that they may have the capacity to proliferate. To make these observations, winter flounder that contained visibly normal and abnormal livers were selected endoscopically and cell proliferation indices calculated. Endoscopy was necessary, as the overall prevalence of grossly visible lesions in fish available in 1990 was very low. The selection of fish with gross lesions, and hence advanced cases of the disease avoided the need to label a large number of fish with bromodeoxyuridine in order to obtain a full range of cases. A protocol was then developed to assay the levels of nuclear DNA synthesis in these fish. This protocol utilized the immunohistochemical detection of bromodeoxyuridine incorporation. Specific modifications of the standard protocol included predigestion with protease, and prolonged incubation with the primary monoclonal antibody together with nuclease. Extrahepatic tissues were used as positive and negative controls. Constitutive proliferation was present in gill and intestinal epithelia, and renal hemopoietic tissue, whereas cardiomyocytes were inactive.

DNA synthesis was evident occasionally in hepatocytes, and cholangiocytes from both Boston Harbor, where fish show a diversity of hepatic lesions, and from Georges Bank where they do not. Uncontrolled variables in the comparison between the two sites included season of capture, and BrdU labelling protocol, however the indices for non-vacuolated hepatocytes from both sites were comparable and very low. Surprisingly, in the livers of Boston Harbor fish, increased levels of DNA synthesis

were found in all stages of vacuolated cells, and most particularly in neoplastic lesions. In one case the cells within the neoplasm that stained most frequently were vacuolated neoplastic cells. This observation strongly suggested a potential linkage between vacuolated epithelia and the progression of the neoplastic phenotype.

Vacuolated cells contain a minimum of normal cytoplasmic components (see Chapter 4). It was thus unexpected that vacuolated cells would show DNA synthesis typical of proliferating cells, as there is presumably a need, in proliferating cells, for a certain amount of cytoplasmic productivity to generate adequate cellular constituents for two daughter cells. The possibility that we have observed DNA repair synthesis should be considered. It has, however, been shown that BrdU histochemistry fails to detect reparative DNA synthesis in rainbow trout liver (Droy et al. 1987). Additionally, a recent study by the same authors (Miller et al. 1989) showed the failure of BrdU to detect DNA repair synthesis in isolated rainbow trout liver cells. DNA is repaired by infilling of short nicks in the nucleotide chain. The short stretches of nucleotides being inserted do not generate a signal that is above the detection threshold for immunohistochemical methods, whereas autoradiographic methods have an adequate sensitivity. In contrast, replicative synthesis involves the widespread incorporation of label and generates a potentially very strong signal, if the detection protocol is optimal. It thus seems reasonable that this technique highlighted cells undergoing replicative DNA synthesis. Was this replicative DNA synthesis in preparation for mitosis or for the development of polyploidy? Polyploid fish liver cells, as in mammals, increase their nuclear size as the number of chromosomes increases (Brasch 1980). Vacuolated

cell nuclear diameter did not increase as compared to adjacent non-vacuolated cells. It thus seems reasonable that increased DNA synthesis detected by this protocol indeed does reflect proliferation of vacuolated cells, and not the onset of polyploidy. However, it should be noted that preparation for mitosis and polyploidy are not necessarily mutually exclusive.

The observations of increased DNA synthesis in the various types of vacuolated cells within parenchymal and neoplastic flounder liver tissue raises several questions. 1) Do the vacuolated cells that are found in the position of hepatocytes, cholangiocytes and exocrine pancreatic cells arise from each of those cells types, or are they arising from a single cell line that replaces the above cell types? 2) What relationships are there between these various stages of vacuolation? 3) Are the vacuolated cells precursors to neoplasia, or are they end stage cells that are dying? An understanding of the relative rates of cell proliferation in the different cell types found in the liver of Boston Harbor flounder is necessary to address these questions.

Proliferative cell types other than vacuolated cells included biliary epithelial cells. The biliary system starts as canaliculi which are formed solely by hepatocyte apical plasma membrane. The canaliculi drain into biliary preductules, lined by hepatocytes and single biliary epithelial cells, and thence into ductules and ducts, completely lined by epithelial cells (Hampton et al. 1988). Many of these biliary epithelial cells in ductules, and ducts, and the fibroblasts surrounding the ducts stained positive for BrdU, suggesting that these cells were contributing to the biliary hyperplasia and fibroplasia that is such a marked part of the disease in winter flounder from Boston Harbor

(Chapter 3).

Other studies addressing DNA synthesis in normal or diseased fish liver have utilized mitotic indices, and tritiated-thymidine incorporation in experimental studies. Kyono-Hamaguchi (1984) exposed medaka *Oryzias latipes,* to diethylnitrosamine, and showed a mitotic index in the liver parenchyma of 1-2%, and a peak labelling index, using ^{3}H-thymidine autoradiography, 3 days after exposure. Similar results were observed following partial hepatectomy. Aoki and Matsudaira (1981 and 1984) exposed medaka to methylazoxymethanol acetate and ^{3}H-thymidine and showed, by scintillation counting, an elevated incorporation of radioactivity in the liver for 1 to 60 days post-exposure. Schultz et al. (1989) exposed *Poeciolopsis lucida* to dimethylbenzanthracene and demonstrated elevated mitotic indices at 2-14 days post-exposure, the precise timing depending on duration of exposure. Nunez et al. (1990) demonstrated tritiated thymidine incorporation in biliary epithelial cells of rainbow trout 14 days after exposure to aflatoxin B_1; 10 to 30% of the biliary epithelia were positive autoradiographically after being labelled with tritiated thymidine.

BrdU, and tritiated thymidine labels have been shown to be very comparable (Lanier et al. 1989, Eldridge et al. 1990); however, a direct comparison between proliferation indices of hepatic cell compartments in the above studies and this study is not possible because of the disparity between the duration of labelling protocols. A further complexity may be that each abnormal cell type may have a unique cell cycle length, and a unique proportion of the cycle that is devoted to S-phase. These differences, if significant, will further complicate any comparison of labelling indices

between different cell types and species. Nonetheless, if the data shown here is compared with the above studies, the labelling indices in Table 5-1 are somewhat lower than other studies. However all of the other studies are the peak response following experimental exposures to carcinogens. In contrast, the field studies here are of steady-state fish that have been exposed to mitogens steadily for many years, thus a lower level of activity is in no way surprising. In the study of rodent hepatic cell proliferation some researchers use a 7 day continuous exposure protocol, with BrdU in implanted mini-osmotic pumps (Eldridge et al. 1990). This maximises the chances of labelling cells with protracted cell cycle times, such as hepatocytes. In contrast, pulse labelling, as used in this study is likely to underestimate the number of cells in S-phase.

It is possible to speculate that the absence of BrdU staining in the adenoma described above reflects a longer cell cycle in the hepatocellular neoplastic cells, than in the cholangiocellular neoplasms. Future studies in flounder should include the use of continuous labelling protocols. However, this study has shown evidence of DNA synthetic activity in biliary epithelia, vacuolated hepatic epithelia and neoplastic cholangiocytes. What is the significance of these observations, given the observation in Chapter 4 that the first cells to undergo vacuolation are biliary preductular cells, and in this chapter that they seem to have the capacity to synthesize DNA?

Subsequent to my conclusions presented in Chapter 4 concerning the identity of centrotubular vacuolated cells as preductular epithelia, and my speculation as to the possibility of them being analogous to oval cells in rodents, I encountered other

publications suggesting teleost biliary preductular cells to be to be presumptive oval cells (Hampton et al. 1988, Nunez et al. 1990). Oval cells, first described in mammals by Farber (1956), are believed to be liver stem cells with the potential to form diverse hepatocellular and cholangiocellular neoplasms (Tsao and Grisham 1987). The possibility of there being an oval cell equivalent in fish is interesting, given the early involvement of preductular biliary epithelia in vacuolar change in winter flounder, (Moore et al. 1989, Chapter 4), and the suggestion by Sell (1984) that oval cells originate from bile ductules in mammals. Thus, chemical carcinogenesis in fish liver may have significant parallels to the situation in rodents. Furthermore, the significantly larger contribution that biliary epithelia make to parenchymal volume in fish, as compared to mammals (Hampton et al. 1989), may mean that study of systems such as that described here in flounder may contribute fundamental understanding to mammalian hepatocarcinogenesis.

In the later stages of vacuolation, cells that appeared to be vacuolated hepatocytes were also common. The role of hepatocytes in the proliferation of hepatic lesions in these fish will remain unclear until the vacuolated phenotype has been better defined in terms of the differentiation of normal and abnormal flounder liver epithelia. However, it should be noted that three recent studies of teleost liver proliferation indices in the face of chemical insult have all shown low levels of non-vacuolated hepatocyte proliferation (Droy et al. 1987, Nunez et al. 1990, and this study). This suggests that parenchymal hepatocytes may not be the most active proliferative compartment in fish liver.

Seventy to ninety per cent of the neoplasms seen in winter flounder from Deer Island Flats in Boston Harbor were cholangiocellular (Murchelano and Wolke 1985, Chapter 2, and Myers, pers. comm.). Biliary hyperplasia, hepatic epithelial vacuolation, and cholangiocellular neoplasms are central to the disease process in winter flounder from Boston Harbor. Previously, we (McMahon et al. 1990) have shown activated K-*ras* oncogenic sequences in hepatic neoplasms from Boston Harbor winter flounder, and from fish that contained vacuolated cell aggregations, but no apparent neoplasm. The frequent contiguity of vacuolated cells to transformed cell types, the possibility that vacuolated cells contain active oncogenes, and their apparent ability to proliferate, would suggest that the vacuolated cell may be closely tied to the development of the neoplastic phenotype.

With regard to the presence of vacuolated cells in species other than winter flounder, vacuolated cells have been described in starry flounder (*Platichthys stellatus*) and rock sole (*Lepidosetta bilineata*) (Myers et al. in press, Stehr et al. 1990), and probably in the European flounder (*Platichthys flesus*) (Simpson, ICI, UK - pers. comm.). How closely associated the vacuolated cells in these species are with neoplastic cells is unclear at this time.

POSTSCRIPT

1. Recently, archival frozen winter flounder liver, collected in this study, was analyzed for ornithine decarboxylase (ODC) activity. High levels of specific activity were observed in samples of parenchyma and of focal neoplasms. ODC activity in fish from Boston was in general higher than in fish from Georges Bank. Analysis of focal aggregations of vacuolated cells revealed a very low total protein content, but the ODC activity, normalized to protein content was as high, or higher than that of the surrounding parenchyma (Koza, Moore and Stegeman 1991). Elevated ODC activity is an excellent indicator of a tissue undergoing active proliferation. These observations serve to corroborate the observation in this chapter that vacuolated cells have the potential to proliferate.

2. Basophilic and eosinophilic foci of altered hepatocytes were not seen at all commonly in our studies of winter flounder. This contrasted to observations on a similar epizootic of neoplasia in English sole (Myers 1987), where tinctorially altered foci were common, and the major cytotoxic lesion was the appearance of megalocytic hepatocytes. It seems that the English sole has a primarily hepatocellular disorder, with tinctorially altered hepatocyte foci, megalocytic hepatocytes and predominantly hepatocytic neoplasms. To investigate these differences, a small sample of English sole from the Duwamish Waterway in Puget Sound was recently labelled, by Mark Myers, with the BrdU protocol described in this chapter. Tissue blocks from these fish were submitted to this author for staining. Control tissues that were comparable to the winter flounder were observed with strong anti-BrdU staining evident in basal gill and gut epithelia, and renal hemopoietic tissue. Parenchymal hepatocytes stained much more frequently than did those in winter flounder. An hepatocellular carcinoma stained with a level comparable to the cholangiocellular neoplasms described above. Megalocytic hepatocytes stained strongly and frequently. This would suggest that these

cells are undergoing replicative DNA synthesis as they become polyploid and aneuploid.

CHAPTER 6

DEVELOPMENT OF THE WINTER FLOUNDER AS AN EXPERIMENTAL MODEL FOR HEPATOCARCINOGENESIS IN BENTHIC FISH FROM CONTAMINATED ENVIRONMENTS

INTRODUCTION

Bottom feeding and other fish acquire liver neoplasia when they live on severely chemically contaminated sediments (Harshbarger and Clark 1990). There are two lines of evidence, which when taken together, suggest there may be a causal link between the chemicals in the environment and the liver cancers: 1) There are statistical correlations between lesion prevalence and degree of sediment contamination (Malins et al. 1987, Landahl et al. 1990); and 2) Liver neoplasia has been induced experimentally in domesticated fish species by exposure to polynuclear aromatic hydrocarbons (Schultz and Schultz 1984, Black et al. 1985, Hendricks et al. 1985, Hawkins et al. 1990). Liver neoplasia has also been induced in rainbow trout with extracts of contaminated sediments (Metcalfe et al. 1988 and 1990). However, there has yet to be a published report of experimental induction of liver cancer in a species known to exhibit liver neoplasia in a natural population exposed to contaminated sediments. To confirm a causal link between specific chemical carcinogens and liver cancer in a natural population of fish, it is necessary to experimentally recreate the disease in the same species, in a manner comparable to that seen in the wild (Dawe 1977). The aim of this study was to develop methods for the experimental replication of the non-neoplastic and neoplastic lesions in winter flounder from contaminated environments (see Table 2-4).

Numerous fish species have been successfully developed as aquarium models for hepatocarcinogenesis (Hoover 1984). These species include the danio (Stanton 1965), rainbow trout (Halver 1967, Sinnhuber et al. 1976, Hendricks et al. 1980, 1981 &

1985), the medaka (Ishikawa et al. 1975, and 1984), *Poeciliopsis* (Schultz and Schultz 1984) and the guppy (Hawkins et al. 1990). A characteristic of all these species is that they have been subjected to chronic selective breeding programs, and therefore are, by definition, to some ill-defined degree, of limited genetic diversity. Experimentally induced liver lesions in fish from natural populations have been reported less commonly. Winter flounder were exposed to contaminated sediment, and vacuolated cells were observed but no hepatic neoplasia was evident (Gardner et al. 1987). Juvenile English sole were exposed to contaminated sediments, and altered hepatocyte foci were found, but again no neoplasms were induced (Myers pers. comm.). Although the reasons for this limited success are probably multiple, an important consideration in the distinction between results with wild and domesticated aquarium fish is that, through the process of domestication, the aquarium species have presumably undergone a reduction, albeit non-lethal, of genetic diversity. This potential inbreeding, even in the face of periodic out-breeding, a practice pursued by at least one laboratory using rainbow trout (G. Bailey pers. comm.) may well have created genetic loci already predisposed to oncogenic progression, thus reducing the number of carcinogenic steps necessary for overt neoplasia. The influence of captive breeding is illustrated by the observation that one particular strain of rainbow trout (shasta) is apparently more susceptible to aflatoxin carcinogenesis (Sinnhuber et al. 1976), than other strains. This situation, whilst analogous to rodents, is speculative, and would bear evaluation by comparing appropriate genetic loci in domesticated and wild fish species.

To compensate for the probable lack of a genetic predisposition towards liver

neoplasia in out-bred fish, any attempt to model neoplasia in feral stocks should include chronic exposure to both genotoxic and epigenetic carcinogens. Chronic sublethal cytotoxicity and chronic hyperplastic stimuli have been largely unexplored as experimental protocols in aquarium model systems of teleost carcinogenesis, and in studies with wild stocks. With these observations in mind, the winter flounder was evaluated for its potential to meet the goal of the study.

Culturing winter flounder is currently not a practical proposition until they are established, feeding juveniles, as rearing flounder from eggs or even pre-metamorphic larvae is extremely difficult (Klein-MacPhee 1978). For this reason this study started with 60 - 100 mm juveniles collected from the field. This study developed the necessary protocols for the year-round maintenance of juvenile and adult winter flounder. It also demonstrated the acute, subacute, and chronic effects of specific chemicals, present in Boston Harbor sediments, on the liver of winter flounder. It was shown that at sublethal concentrations, the compounds in technical grade chlordane were hepatotoxic. Chronic exposure to benzo(a)pyrene and chlordane gave rise to focal epithelial proliferation or metaplasia with early duct formation, and perisinusoidal edema and a resultant spongiosis hepatis, which are all conditions seen in fish from Boston Harbor (only the first of these lesions is seen at all commonly in the field).

GENERAL METHODS

Collection of fish

Collection sites were sought that had an abundance of fish that were histologically normal. Adults collected inshore had low but significant levels of histological change. Inshore juveniles were histologically normal, although they had presumably been exposed to whatever contaminants caused the lesions observed in older fish. Histologically lesion-free adults were best collected from offshore sites such as Georges Bank. Collection from Georges Bank dictated the use of a heavy commercial otter trawl, whereas the inshore fish could be collected by a skiff trawl of short duration, or by a beach seine for juveniles. The heavy commercial otter trawls significantly traumatized the fish, resulting in poor survival due to catching and transport stress. Juveniles were used from inshore, and adults from both areas.

The skiff trawl had a one inch mesh at the mouth, with a half inch mesh liner at the cod end. Ten minute trawls, at 1 knot of speed, were made in 1 - 2 m of water. A trip line with a surface float was employed to allow for retrieval of the net in the event of serious hang-ups. The fish were gently transferred from the net to 120 l plastic barrels, lined with a domestic garbage liner, and half filled with sea water. During collection 70% of the water was changed every 30 minutes. For transport to the laboratory, a 12 volt air pump with bubbling air stones in the water was used, with the air line to the stone sleeved with a short piece of plastic hose, with an inside diameter of 10 mm. The liner was tied off around this hose, excluding air

above the water, but allowing escape of bubbled air. This arrangement controlled water surge and the plastic liner minimized abrasion of the fish against the barrel sides.

Housing

Chronic exposures demand good control of water quality and temperature. For this study filtered sea water from Nantucket Sound, at a supply rate of 30 gallons per minute, heated or chilled to a range of 12-17°C, was available year-round. Water and air supplies were remotely sensed to allow immediate response to system failure. Square tanks with sharp corners were avoided. Cylindrical tanks, 1 to 2 m in diameter, were constructed of moulded fiberglass, plexiglass, or prefabricated flexible fiberglass sheeting (Kalwall brand). The Kalwall tanks had a plywood base. Housing density was calculated to allow each fish to have twice its surface area of bottom, at its final projected size. Water depth ranged from 50 to 100 cm. Water depth was maximized to give an adequate reserve of oxygen in the event of power loss to the water and or air supply. The tanks were illuminated for 12 h each day. Daylight intensity was low. Where water quality was marginal, a recirculating filter was used. This consisted of a 30 cm disk of 5 cm thick styrofoam floating on the tank surface. Through the center of the disk ran a 3 cm diameter PVC pipe, protruding 3 cm above the disk, and extending to within 2 cm of the bottom of the tank. On top of the disk, a buffing pad from a commercial floor polisher was laid, with the pipe protruding. An air stone was lowered to the bottom of the pipe. The air stone induced a rising column of water and debris off the tank floor that then percolated back to the tank through the pad.

The pad was removed as necessary to hose off organic debris trapped in the fibers of the pad.

Diet

Freshly dredged, shucked and refrigerated surf clams (*Spisula solidissima*) were obtained, via commercial vessel, from Nantucket Shoals and Georges Bank. The concentration of total Arochlor 1254 in a pooled subsample of one set of these clams was 25 ng/g dry weight (Emily Monosson pers. comm.). Another subset was tested for saxitoxin concentration, which was at a low non-toxic level (Donald Anderson pers. comm.). Within 24 h of collection, the refrigerated clams were rinsed in sea water, broken pieces of shell removed and the clams frozen at -20°C. For feeding, the clams were semi-thawed to a solid, but workable state, in a microwave oven and diced to approximately 2x2x5 mm pieces. Flounder were fed a ration that was 5% body weight, 2-3 times per week. Juveniles adapted to the diet within two weeks of capture, whereas adults took 2 - 4 months to acclimatize. Initially, food was ignored and consumed under cover of darkness. Later the fish learned to snatch the food before it landed on the floor of the tank. Acclimation to the clam diet was accelerated if fish were fed 100% chopped *Nereis diversicolor* immediately after capture, and then weaned onto clams over a two week period. Flounder were kept on a control diet for at least two months before onset of experimentation.

Chemicals

Data for sediment and shellfish contaminant loadings in Boston Harbor were examined (NOAA 1987b and 1988a) to select appropriate genotoxic and epigenetic chemicals. Contaminant levels in winter flounder were not considered because of the high capacity of teleosts to biotransform and excrete many xenobiotic compounds. This capacity in part lies with their inducible system of hepatic mixed function oxygenases (Stegeman et al. 1987). A notable feature of Boston Harbor data is the high ranking, compared to other sites, of polynuclear aromatic hydrocarbons, non-DDT chlorinated pesticides and heavy metals such as tin, lead and mercury. A major fraction of the pesticides consisted of the various constituents of technical grade chlordane. Chlordane is a non-genotoxic, cytotoxic promoter (Tsushimoto et al. 1983, Ashby and Tennant 1988, Moser and Smart 1989, Ruch et al. 1990). Technical grade chlordane has multiple chlorinated hydrocarbon constituents. It was used rather than an analytical grade product, to provide a general exposure to a semi-controlled range of one class of chlorinated pesticide. Chlordane was obtained from Velsicol Corporation (Marshall, IL), lot number L-5171041: constituents as determined by gas chromatography (using the labels assigned by the Velsicol laboratory that conducted the analysis), were listed on the certificate of analysis that accompanied the shipment. These were C-58: 1.4%, "C": 7.5%, Heptachlor: 8.7%, B,G-"237": 14.3%, F,G,G': 10.3%, G,A-Chlordane: 40.1%, Nonachlor:9.2%. Benzo(a)pyrene (98%), obtained from Aldrich (Milwaukee, WI), was selected as a representative polynuclear aromatic hydrocarbon. Two carriers were used: dimethylsulfoxide (DMSO) and trioctanoin, an extract of goat

lipid, both from Sigma (St Loius MO).

Chemical Treatments

Semi-thawed clams were sliced as above, and refrigerated, whilst chemicals were prepared. Diet contamination was carried out in a fume hood with the operator wearing full protective clothing and a respirator. To prepare each diet 100 g of final mix contained 60 g clams, 35.5 ml water, 4 g gelatin powder (Knox brand), and 0.5g dimethylsulfoxide (DMSO). Each chemical was added to DMSO in a sealed disposable 50 ml centrifuge tube. Chlordane dissolved with vigorous vortexing, whereas benzo(a)pyrene remained as a suspension, even after vigorous vortexing and warming to 60°C. Benzo(a)pyrene preparation was conducted under gold fluorescent safe light. Each mix of chemicals in DMSO was added to a solution of gelatin, which had been dissolved, with vigorous stirring, in boiling water and cooled to 60°C. This mix of chemical, DMSO and gelatin was poured over the clams which were in a double lined plastic bag within a bucket. The diet was then thoroughly mixed by massaging the outside of the bags for two minutes, and then "poured" onto a disposable foil baking dish to a depth of 8 - 10 mm and allowed to gel at 4°C. It was important to allow the clams to equilibrate with room temperature before the mixture was added, otherwise the gelatin gelled before the mix could be poured into the foil trays. The gelled slab was then packaged in ziplock bags, and frozen in the dark till needed. Daily rations were carved off, weighed and crumbled into the tanks with gloved hands. The preparation sank once thawed. Doses used are given in experiment 4 (below). Effluent from tanks containing contaminated fish, or receiving contaminated food was

passed over a bed of activated charcoal before discharge. All contaminated materials and carcasses were disposed of by the institutional safety officer.

GENERAL RESULTS

Collection and culture

These studies, in part, were carried out to establish the most appropriate conditons for capture and maintenance, to maximize survival and health, and minimize suffering of captive winter flounder.

Mortality factors

1. Trauma. Fish caught by skiff trawl had minimal mortality. However fish caught by a standard commercial otter trawl, with 20 - 30 minute tows had a 40-60% mortality, 2 - 8 weeks after capture. This mortality resulted from bacterial infections secondary to net trauma. The first clinical sign was a white mucoid exudate that resulted from bruising of the dorsum of the caudal peduncle. This was followed by acute erosion of the fin rays, with frank hemorrhage being evident, at times, in the water above the fish. Animals that survived more than a week often died 2-8 weeks later with chronic hemorrhagic peritonitis. Bacterial isolates of peritoneal lesions were primarily *Vibrio* spp. (Louis Leibovitz pers. comm.).

2. Plumbing. Fish were regarded as being ready for experimentation once they were feeding aggressively. After initial trauma related deaths had ceased, the only significant cause of mortality, other than dose related effects, was mechanical crisis, such as failure of water or air supplies. To maximize the reservoir of oxygen and

clean water, the tanks were as large as possible. In one instance a dormant water line was reactivated which resulted in an acute mortality in a number of tanks. This mortality was probably due to a toxic build up of sulfide. Recurrence of this problem was avoided by removing the water lines from each tank for at least six hours after a dormant line had been reactivated. During this time good aeration was especially important.

3. Reproductive activity. Water at ambient temperature was supplied to one tank of adults through the winter of 1987-88. In December the water temperature was 6-7 °C and the females began to show ovarian swelling. However, spawning did not occur, and a steady mortality resulted from apparent egg binding. In contrast, males were in steady milt and seemed healthy at temperatures between 0 and 17 °C. Surviving females were moved from winter ambient temperature to the "constant" temperature (12-17 °C) water supply. These females rapidly lost their ovarian swelling, and thrived. This phenomenon is discussed by Klein Macphee (1978). Therefore, it is only practical to maintain female sexually mature winter flounder year-round if the females are kept at a temperature higher than 10°C. The maximum sustainable temperature for adult winter flounder is about 17°C. Juveniles can withstand higher temperatures. For all of the experimental studies water temperature was maintained at 12-17°C on a year round basis, for all fish.

4. Feeding. On four separate occasions isolated deaths occurred 6 to 12 h after feeding. Autopsies revealed a full stomach with large pieces of inadequately sliced clam obstructing the esophagus and stomach. These mortalities were unique in that in contrast to most other causes of mortality, the carcasses floated, as a result of the rapid

decomposition of the clams. The remedy was to ensure that all clam pieces were adequately chopped.

5. Gas bubble disease. One isolated case of mass mortality occurred when a water line was changed from heating to ambient, at the end of the winter. The degassing column was turned off, and 24 to 48 h later a number of isolated deaths occurred. Bubbles were evident behind the orbit of some fish, and in the gill vasculature seen in a wet mount. The diagnosis was gas bubble emboli, due to supersaturation of the water supply. The remedy was to reactivate the degasser. An air leak was found at the shorefront lift pump.

Water Quality

For the period January 1988 to December 1990 the ranges of water quality parameters in tank effluent were as follows: pH 7.8 - 8.0, salinity 29.0 - 31.0 ppt, temperature 12.5 - 15.3 °C, ammonia 0.4 - 2.3 mg/l, nitrite 0.004 - 0.01 mg/l, oxygen 8.98 - 11.58 ppm.

Conclusions concerning general results

There are three critical aspects to successful long term maintenance of winter flounder in captivity. 1) Fish must be collected with as little trauma as possible. 2) There must be adequate holding tanks, with filtered, degassed, flow-through water at a temperature between 10 and 15°C. 3) A diet of appropriate food is essential.

CHEMICAL TREATMENTS

Having established optimal conditions for collection and maintenance of winter flounder, a number of experimental treatments were undertaken. The methods, results and conclusions for each experiment are described here. Four major areas were investigated experimentally:

1. Characterization of the acute and subacute effects of chlordane on juvenile and adult winter flounder (Experiments 1 & 2).
2. Characterization of the effects of extracts of Boston Harbor sediment on winter flounder (Experiment 3).
3. Evaluation of the effects of chronic feeding of benzo(a)pyrene and chlordane on winter flounder liver (Experiment 4).
4. Grow out of "young of the year" winter flounder from Deer Island Flats in clean water with clean food (Experiment 5).

Acute and subacute effects of intraperitoneal chlordane

Experiment 1

Before long-term exposures could be attempted, the acute toxicity of chosen compounds had to be established.

Methods: Juvenile winter flounder caught by beach seine at Great Pond, Falmouth, were used for a pilot study. Treatment groups, of six fish each, were given 20000, 2000, 200, 20, and 0 mg/kg of technical grade chlordane, by intraperitoneal injections in 0.02 ml trioctanoin per gram body weight, on day 0 and day 25, with survivors being sacrificed at day 34.

Results: Mortality results are given in Figure 6-1. The 24 hour LD_{50} calculated from these data, using the probit method described by Rand and Petrocelli (1984), was 11,000 mg/kg. Mortality accelerated after the repeat dosing on day 25. Dead fish that were not autolyzed, and survivors to the end of the experiment, were evaluated histologically. In the treated and control fish that lasted 20 days or more, the liver capsule and the intestinal mesenteries had multiple granulomas, with ostensibly empty, spherical central cavities (Figure 6-2 a and b). These lesions appeared to be lipid granulomas. Although frozen samples suitable for lipid specific stains were not prepared, the granulomatous tissue seemed to have walled off the droplets of trioctanoin, which probably sequestered the chlordane, giving a chronic slow release. Another lesion common to the treated and control fish was the appearance of non-staining intranuclear vacuoles, with margination of the chromatin (Figure 6-2c).

Histological changes seen only in groups treated with chlordane, in a dose dependent manner, included pyknosis and karyorrhexis of hepatocyte nuclei and eosionphilia of the cytoplasm suggestive of necrosis. Pyknosis and necrosis was seen most extensively in those fish that died. In the treated fish that survived for 34 days, there was a dramatic increase in the number and size of macrophage aggregations in the higher dose groups, especially in the two survivors in the 200 mg/kg group (Figure 6-2c). A macrophage index was calculated as a percentage of the liver section occupied by aggregated macrophages (Table 6-1).

Conclusions: 1) The administration of chlordane in trioctanoin appeared to be a possible way of giving a chronic exposure, with infrequent dosing. 2) The injection volume was high, to allow for a practical injectable volume in 5-20 g fish. At the

volume used, trioctanoin granulomas and margination of the nuclear chromatin were an undesirable side effect, and so larger fish and smaller volumes of carrier should be used. 3) The LD_{50} for chlordane given in trioctanoin in winter flounder was extremely high, (11,000 mg/kg). This contrasted with a much lower oral 24 h LD_{50} for rats (390 mg/kg, EPA 1985). This difference suggests that in the trioctanoin treated flounder, chlordane may have been sequestered in the lipid droplets in the granulomas.
4) Chlordane at high doses induced widespread hepatic necrosis and pyknosis. 5) At non-lethal doses, the necrogenic effect persisted, and resulted in aggregation of macrophages.

This experiment led to a plan to repeat the protocol with lower doses, larger group sizes, and with a lower injection volume of carrier. Given the appearance of granulomas in control fish exposed solely to trioctanoin in addition to the treated fish, the effect of using dimethylsulfoxide (DMSO) rather than trioctanoin as a carrier was also evaluated.

TABLE 6-1

Macrophage indices in the livers of winter flounder after a range of doses of technical grade chlordane in trioctanoin were given by intraperitoneal injection. Fish were examined after 21-30 days.

Dose mg/kg i/p	0	20	200	2000
Number of fish	3	3	2	1
Macrophage index	0.0083 $\pm$ 0.003	0.11 $\pm$0.036	2.8 - 4.2	0.51

Macrophage index was calculated as a percentage of the liver section occupied by aggregated macrophages. Values given as a range for sample sizes less than 3, and mean $\pm$ S.D. for groups of 3 fish.

Figure 6-1

Mortality resulting from the exposure of juvenile winter flounder to technical grade chlordane in trioctanoin by intra-peritoneal injection on days 0 and 21. Data expressed as a per cent of each group surviving. Each group began with six fish.

△ = 20000 mg/kg technical grade chlordane in trioctanoin
□ = 2000
▽ = 200
○ = 20
◇ = Trioctanoin control

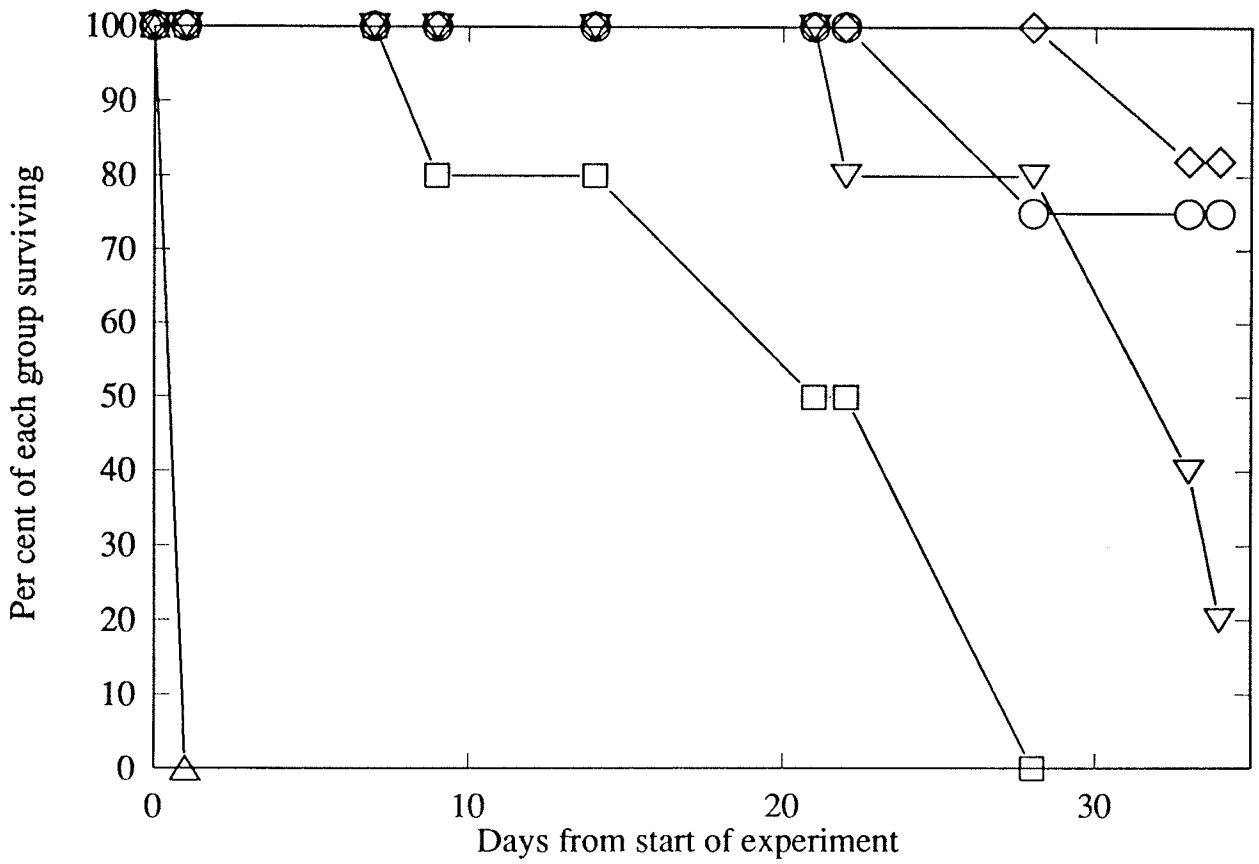


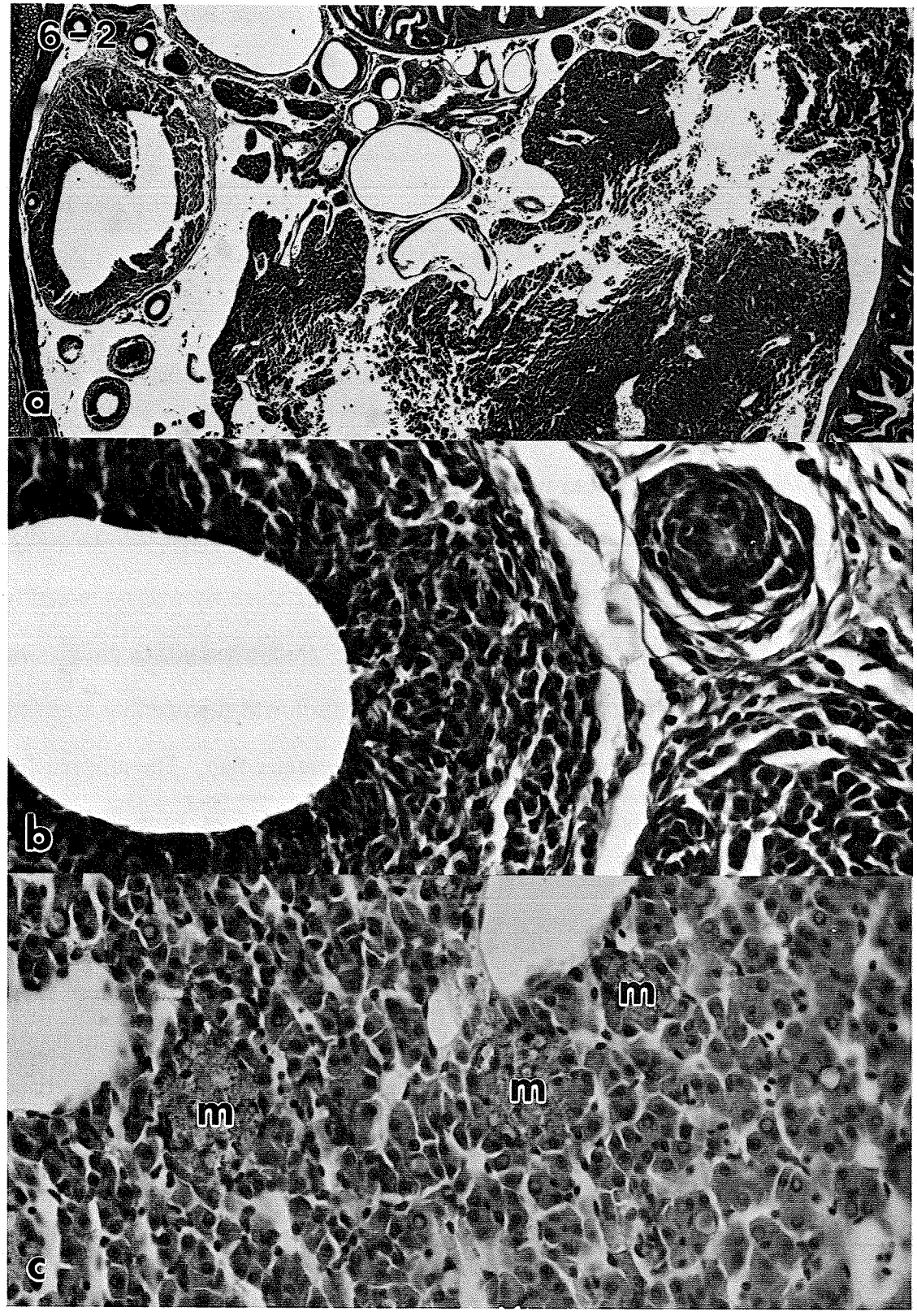
Figure 6-2

Experimental lesions in winter flounder liver after 30 days exposure to technical grade chlordane in trioctanoin, by intra-peritoneal injection.

a) Section through the liver capsule and adjacent viscera. The large spherical spaces are presumably lipid droplets from the trioctanoin injection that have been walled off by a granulomatous reaction. 25 x

b) Detail of a granuloma from a different section from the same fish as in a). 400 x

c) Macrophage aggregations (m) in the liver of winter flounder exposed to 100 mg chlordane in trioctanoin by intraperitoneal injection. Hepatocyte nuclear swelling with margination of the chromatin is also present. 400 x.


6-2
a
b
m
m
m
c

Experiment 2

Methods: Chlordane was administered to 4 groups of 10 adult winter flounder, from Onset, Buzzards Bay, at 0, 25, 50 and 100 mg/kg in trioctanoin and to 4 groups of 10 fish at the same doses of chlordane in dimethylsulfoxide. Dosing was by one intra-peritoneal injection at 1 ml/kg. Mortalities were recorded, and livers removed from survivors for histopathology at day 26.

Results: An acute dose related mortality occurred when flounder were exposed to chlordane when DMSO was used as a carrier, but not when trioctanoin was used as a carrier. Figure 6-3 illustrates the mortality resulting from intraperitoneal exposure of adult winter flounder to technical grade chlordane using either trioctanoin or dimethylsulfoxide as carrier. Fish exposed to either carrier alone showed no mortality.

DMSO as a carrier: The mortality seen in the chlordane/DMSO groups was acute and severe. In the first 36 h of this experiment death was preceded by a marked elevation of the rate and extent of movement of the opercular flap. The affected fish showed oral "gasping" with each opercular movement, at a rate of 30 to 40 per min. Other clinical signs included circling and mouthing at the surface, hyperexcitability and apparent disorientation. Control fish showed barely perceptible movements of the opercular flap at a rate of 10 to 20 per min. Starting on day 3 the fish were killed

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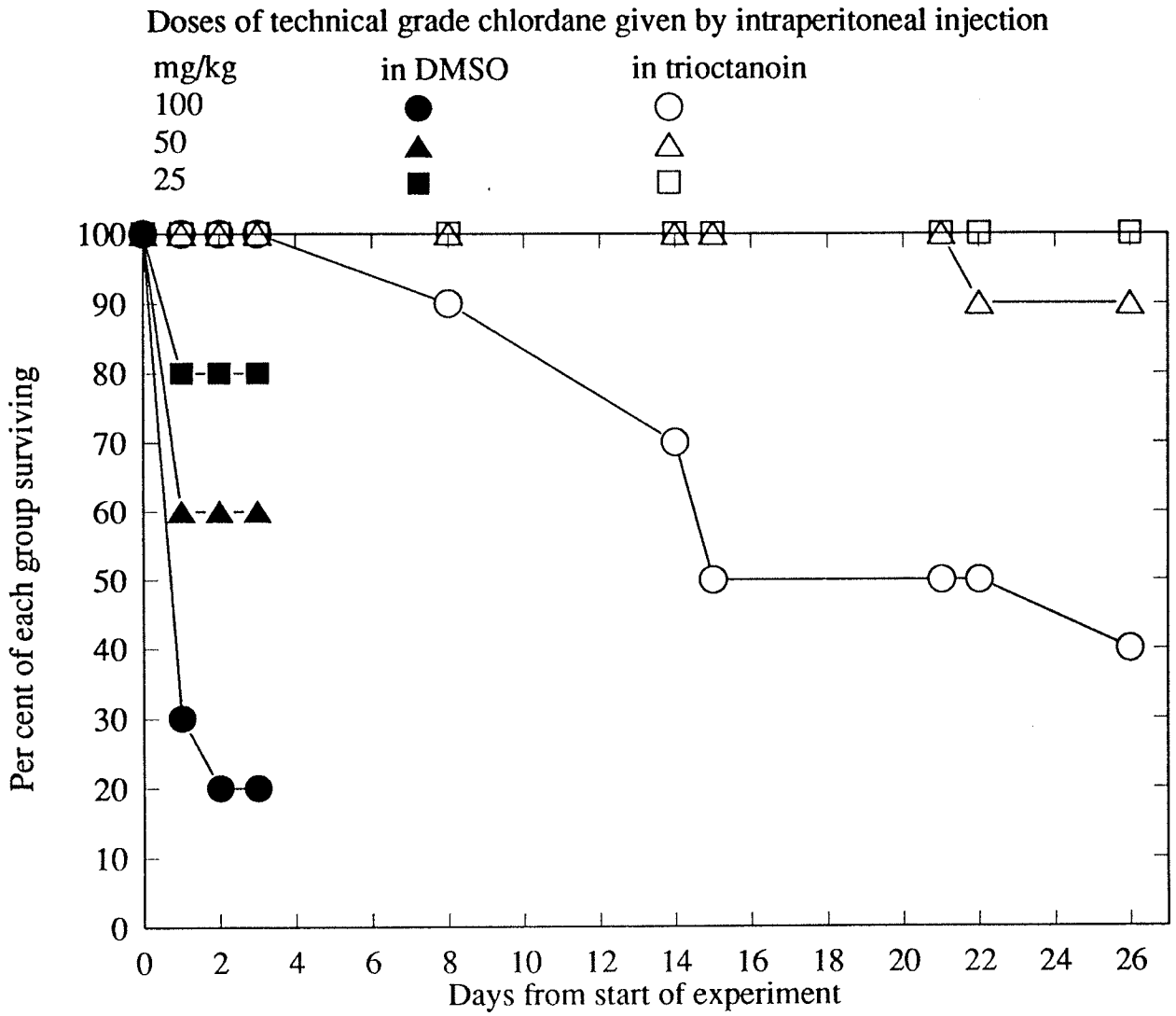
Figure 6-3

Mortality of adult winter flounder exposed to technical grade chlordane: fish were exposed by intraperitoneal injection on day zero, with either trioctanoin as carrier (open symbols), or with dimethylsulfoxide (DMSO) as carrier.


Doses of technical grade chlordane given by intraperitoneal injection
mg/kg
in DMSO
in trioctanoin
100
50
25
Per cent of each group surviving
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
Days from start of experiment

at 3 day intervals, and frozen liver samples archived. In the fish that were not killed, no further mortality was observed after 48 h. In fish remaining alive, behavioral signs persisted intermittently for the next 5 to 7 days. Fish that died acutely showed histological evidence in the liver of perivascular edema, and pyknosis.

Trioctanoin as a carrier: In contrast to DMSO as a carrier, the same dose range administered in trioctanoin showed a far less acute effect. Chlordane in trioctanoin at 50 mg/kg i/p appeared to be the maximum dose tolerated over four weeks. Clinical signs as described above were occasionally present, although far less marked. Hepatic histology of fish that died during the experiment showed areas of the parenchyma where the majority of hepatocytes appeared necrotic. Nuclei were small, densely staining and pyknotic, and the cytoplasm was eosinophilic and cloudy. Survivors of the exposure to chlordane in trioctanoin were killed at day 26. In those survivors 2 to 4 mm shallow "craters" were evident on the liver surface. Histologically the craters were erosive necrotic ulcerated lesions, probably resulting from local diffusion of chlordane from residual droplets of trioctanoin on the surface of the liver. Granulomas were also present on the liver surface of treated and control fish, but less marked than in experiment 1. The primary histological change in the parenchyma of the liver was an increase in the number of macrophage aggregates present. Macrophage aggregate content was scored on a scale of 0-3. The data are presented in Table 6-2. Macrophage content was significantly elevated in groups treated with 25 and 50 mg/kg chlordane. Macrophage aggregation in the 100 mg/kg group was very extensive, but the sample size was inadequate for statistical comparison. Other lesions included

perivascular edema, where there was a diffuse accumulation of non-staining fluid, widening the space between the endothelia and the tubules of hepatocytes (Figure 6-3c).

Chlordane/trioctanoin treated fish from experiment 2 were analyzed for induction of cytochrome P-4501A, the polynuclear aromatic hydrocarbon and planar polychlorinated biphenyl inducible isozyme, and for ornithine decarboxylase activity. Neither the catalytic activity of P-450, nor the amount of the isozyme in any of the treatment groups were significantly different from the control group (Bruce Woodin pers. comm.) In contrast, the activity of hepatic ornithine decarboxylase was significantly decreased in the chlordane-treated groups (Robert Koza pers. comm.).

TABLE 6-2

Hepatic macrophage indices for winter flounder exposed to technical grade chlordane in trioctanoin by intraperitoneal injection.

Treatment (mg/kg chlordane)	Sample size	Hepatic macrophage index of survivors (Mean $\pm$ S.D.)
100	4	1.3 $\pm$ 0.6
50	9	1.4 $\pm$ 1.1 **
25	10	1.0 $\pm$ 0.7 *
0	10	0.5 $\pm$ 0.5

Macrophage index was scored on a visual range of 0-3. Significance of differences between mean macrophage indices were compared, by Students t-test, between the control (0 mg/kg) and each treatment group. ** $p < 0.025$, * $p < 0.05$.

The level of macrophage aggregation in the control fish from experiment 2 (Table 6-2), and in other untreated fish from the same site (data not shown) was not as low as had been seen in fish from Georges Bank (Chapter 3, Table 3-1). The collection site for this experiment was Onset, one third of a mile northeast of the Buzzards Bay entrance to the Cape Cod Canal. This site is heavily travelled by recreational vessels, and adjacent to a major ship canal, and may be significantly contaminated. Therefore, the experiment was repeated with a small number of fish from a relatively pristine area, Georges Bank, to further examine the development of macrophage aggregates. Four fish per group were exposed to 50, 25, 12.5 and 0 mg/kg chlordane in trioctanoin. Interestingly, chlordane in trioctanoin was much more acutely toxic to Georges Bank fish than to Onset fish. In both the 50 and 25 mg/kg groups all the fish died within 24 h. Focal necrosis was evident in fish from both groups. None of the fish in the 12.5 mg/kg group died, but when killed at 7 days after injection they showed perivascular edema.

Conclusions: 1) If the results of experiments 1 and 2 are compared, it appears that chlordane, which is highly lipid soluble, was approximately 180 times less acutely toxic when administered in trioctanoin, than in DMSO. It seems likely that chlordane was sequestered when administered intraperitoneally in trioctanoin. In contrast it was rapidly absorbed when given by the same route in DMSO, allowing the more acute toxicity. 2) Histological lesions resulting from exposure to chlordane when injected in trioctanoin included perisinusoidal edema, pyknosis and necrosis of hepatocyte nuclei, and aggregation of macrophages. No such changes were seen in the control group.

3) Biochemical analyses of samples from the same fish suggested that chlordane is not an inducer of cytochrome P-4501A (Bruce Woodin pers. comm.); however it severely reduces the activity of ornithine decarboxylase in a dose-dependent manner (Robert Koza pers. comm.). This latter observation reinforces the concept that the constituents of technical grade chlordane caused chronic hepatotoxicity reflected by perisinusoidal edema, pyknosis and macrophage aggregation. A compensatory proliferative response was absent in these studies. 4) The winter flounder from Georges Bank had an increased sensitivity to chlordane in trioctanoin, as compared to flounder exposed to the same doses and carrier but collected from Onset. The reason for this is unclear, but may involve differences in levels of lipid stores between the two groups of fish as suggested by the increase of hepatocyte basophilia in coastal fish (Table 3-3), or perhaps genetic differences in their capacity to handle xenobiotic compounds, resulting from a coastal selection pressure to withstand chemical pollution since the onset of the industrial revolution.

The toxicity of Boston Harbor sediments

Experiment 3

A major aim of this experiment was to yield flounder livers exposed to Boston Harbor sediment extract, for DNA adduct analysis. These samples were taken, and have been archived in the laboratory of Dr. Tannenbaum at Massachusetts Institute of Technology (Cambridge, MA). Limited sampling for histology was also conducted.

Methods: One litre glass jars were rinsed sequentially in methanol, acetone,

hexane, acetone, and then methanol. They were then air dried and baked in a muffle furnace for 18 hours at 510 °C, cooled and then closed with caps lined with solvent rinsed foil. Grab samples of bottom sediment were made using a $0.1m^2$ Van Veen grab, on Deer Island Flats, and at Onset. The top two centimeters of sediment were scraped with a solvent rinsed plastic spatula into the glass jars. Samples were placed on ice, and frozen on return to the laboratory. Sub-samples for chemical analysis were archived. Sediment extraction was performed by Jay Gooch (Chesapeake Biological Laboratory, MD.). The extraction method largely followed that of Metcalfe et al. (1988): briefly, 500g sediment, mixed with 200 g sodium sulfate, was extracted 3 times in a 1:1 mix of acetone and hexane using sonication. Extracts were pooled, filtered and reduced by rotary evaporation. One hundred milliliters of acetone was added and reduced by rotary evaporation twice. Particulates were allowed to settle, and supernatants drawn off and reduced to 5ml in a stream of nitrogen. Forty milliliters of DMSO was added and the acetone removed by a stream of nitrogen. Extracts were stored at 4°C in the dark.

Juvenile flounder from Onset were injected intraperitoneally with sediment extract from Deer Island Flats (Boston Harbor) and from Onset. The fish were injected with the equivalent of 0.24, 0.12, 0.06, and 0.0 grams sediment per gram of fish, in 0.26 ml DMSO per 100 gm of fish. Each group consisted of three fish. Survivors were killed 10 days later and their livers examined histologically.

Results: The Boston sediment extract had the visual consistency of flocculent, used motor oil. In contrast the Onset extract was homogeneous and green. Boston

sediment extract equivalent to 0.24 g sediment/g of fish was acutely toxic with all the fish dying in 3 days. The mortality is illustrated in Figure 6-4. No fish died when injected with Onset sediment extract at the same doses, nor with DMSO alone. Histologically, the survivors at 10 days after injection with lower amounts of Boston sediment extract showed perivascular edema very similar to that described in the chlordane/trioctanoin exposed fish (Figure 6-5c). The histology of control animals injected with Onset sediment extract, or DMSO was unremarkable.

Conclusions: 1) The Boston Harbor sediment was acutely toxic at the highest dose; in contrast the Onset sediment was not acutely toxic. 2) Perivascular edema was seen in the survivors of the Boston Harbor extract when examined at 10 days.

In review of the above studies, it was felt that whilst exposure to chlordane had not induced the beginnings of any of the vacuolated, proliferative or neoplastic histological changes characteristic of flounder from Deer Island Flats, it was nonetheless a hepatotoxin in winter flounder. Chlordane induced cytotoxic changes comparable to those induced by Boston Harbor sediment extract, and should, at a sublethal chronic dose provide an appropriate cytotoxic selection of resistant initiated cells, that might prove appropriate for a carcinogenesis model in winter flounder. It was concluded that repeated monthly injections of chlordane in trioctanoin was a potential chronic exposure protocol, but that the artefactual complications of granulomas and nuclear inclusions rendered that method less attractive than chronic exposure in the diet.

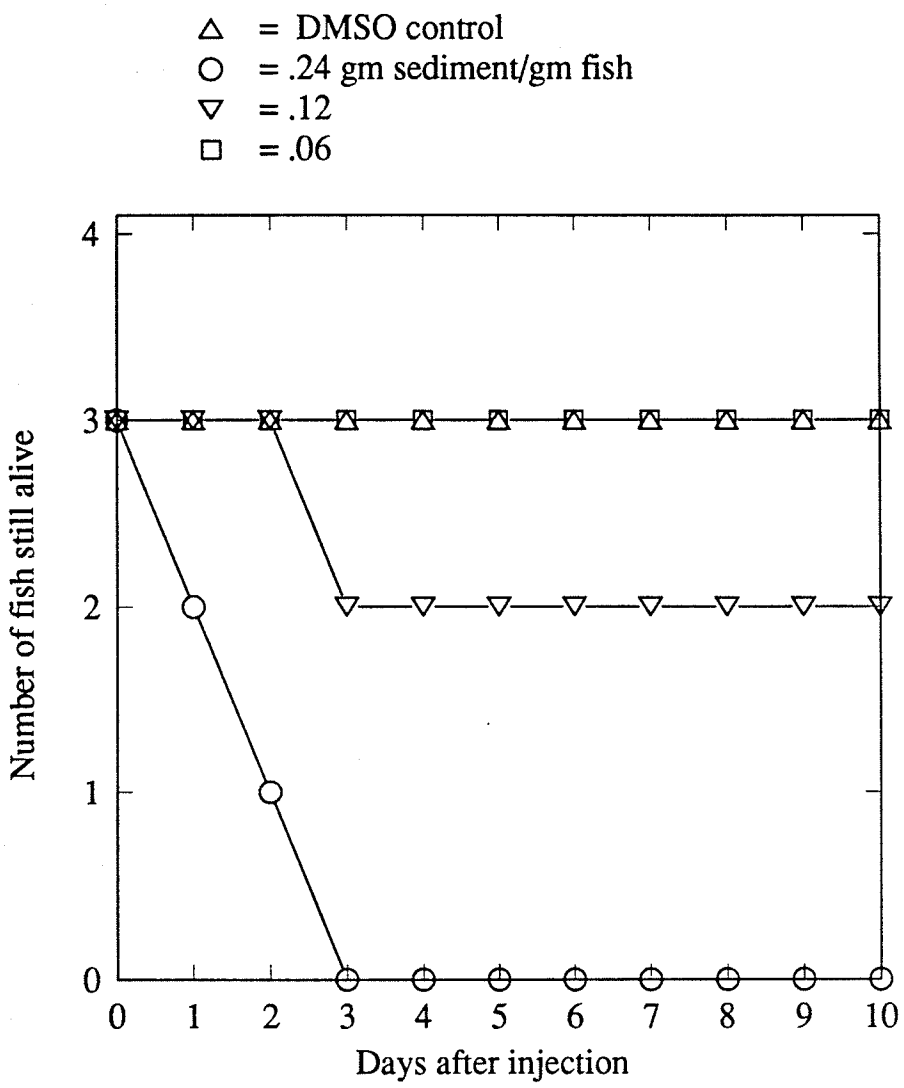


Figure 6-4

Juvenile winter flounder from Onset were exposed, by intra-peritoneal injection, to three dose levels of an extract of Boston Harbor sediment in DMSO, or to DMSO alone. The resultant mortality over 10 days is shown.

Chronic dietary exposure of winter flounder to an initiator, and a cytotoxic promoter

Experiment 4

Methods: Four clam-based diets were prepared as described in the general methods above, respectively containing 900 ppm benzo(a)pyrene, 900 ppm benzo(a)pyrene and 10 ppm chlordane, 10 ppm chlordane, or carrier (control diet). The benzo(a)pyrene dose was chosen in the light of a chronic feeding study in rainbow trout (Hendricks et al. 1985). The chlordane dose was selected on the basis of an unpublished study by Eller (1971), and by approximate calculations of the daily available dose in experiment 2, assuming all of the chlordane was absorbed in 30 days from the trioctanoin depot, and 10% of the chlordane was absorbed from the diet.

Food was given three times per week: At each feeding, each tank received a mass of the clam diet that equalled 5% of the biomass of fish in the tank. Every three months the biomass was recalculated by weighing the fish in a tared container of sea water.

At the termination of the experiment each fish was labelled for 3 h with an intraperitoneal injection of bromodeoxyuridine and fluorodeoxyuridine (Chapter 5). Frozen liver and bile samples were archived, and specimens preserved for histology.

Histology samples were processed routinely, and stained with hematoxylin and eosin. Liver sections that contained histological lesions evident in the hematoxylin and eosin stained slides were also stained immunohistochemically for the uptake of bromodeoxyuridine in the nuclear DNA, and for the presence of cytochrome P-4501A protein. The immunohistochemical protocol was as described in Chapter 5. Three

sections were placed on each slide, one was incubated with anti-BrdU, one with anti-P-4501A (Smolowitz et al. 1991), and one as a control with PBS. The proteolysis that preceded the primary monoclonal was omitted for the P-450 stained section.

Results

Mortalities

At the start of the experiment contaminated food was given three times per week. By the end of three months significant mortality was occurring in the benzo(a)pyrene with chlordane, and the chlordane only groups. Two of the three weekly feedings were therefore replaced with uncontaminated food. The mortalities ceased after three weeks of this regime. Other mortalities in this experiment were caused by mechanical system failure. The entire DMSO control group died six months into the experiment due to failure of the water supply to those tanks. However, a parallel group of juveniles from Boston had been reared on uncontaminated clams, and was used as a reference group. At the outset, each group had 18 fish; by the end of one year 10-13 fish survived in each group, at which time the experiment was terminated. Body weight and hepatosomatic index did not differ significantly between the four groups.

Histopathology

In 3/10 fish exposed to benzo(a)pyrene there was a lesion (Figure 6-5) very comparable to that described in fish exposed to Boston Harbor sediment (above). The

perisinusoidal space was significantly enlarged by the accumulation of non-staining fluid, that appeared to represent interstitial edema fluid. In one case this lesion was confluent with areas of a condition indistinguishable from spongiosis hepatis in adult fish from Boston Harbor, as described in Chapter 2.

In 7/9 of the survivors in the chlordane treated group, a lesion was observed that was adjacent to small blood vessels and bile ducts (Figure 6-6). Numerous small, intensely basophilic cells were present in this lesion, intermingled with aggregates of macrophages. Many of these basophilic cells were lymphocytes, with circular nuclei and scant cytoplasm, but a proportion of the cells had larger more pleomorphic nuclei, characteristic of epithelial cells and reminiscent of the cells present in many of the bile duct tumors described from Deer Island winter flounder (chapter 2). These cells at times appeared to form simple ductular structures. These lesions ranged in size from a few cells in width to 1.0 x 0.4 mm. The same lesion type was also seen in 6/16 of the group treated with chlordane and benzo(a)pyrene, and 2/10 of the benzo(a)pyrene treated group. This lesion will be referred to as focal epithelial hyperplasia. In the benzo(a)pyrene treated group 3/10 fish showed areas of perisinusoidal edema, which in one fish appeared to progress into zones of spongiosis hepatis (Figure 6-4a and 6-4b). The spongiosis had a superficial resemblance to the tubular stage of vacuolation seen in flounder from Deer Island, but on closer examination was a different entity. Mitotic figures were absent from the control group, but were found at significant prevalences in all of the treatment groups. These figures were present within the focal lesions, and in the surrounding parenchyma.

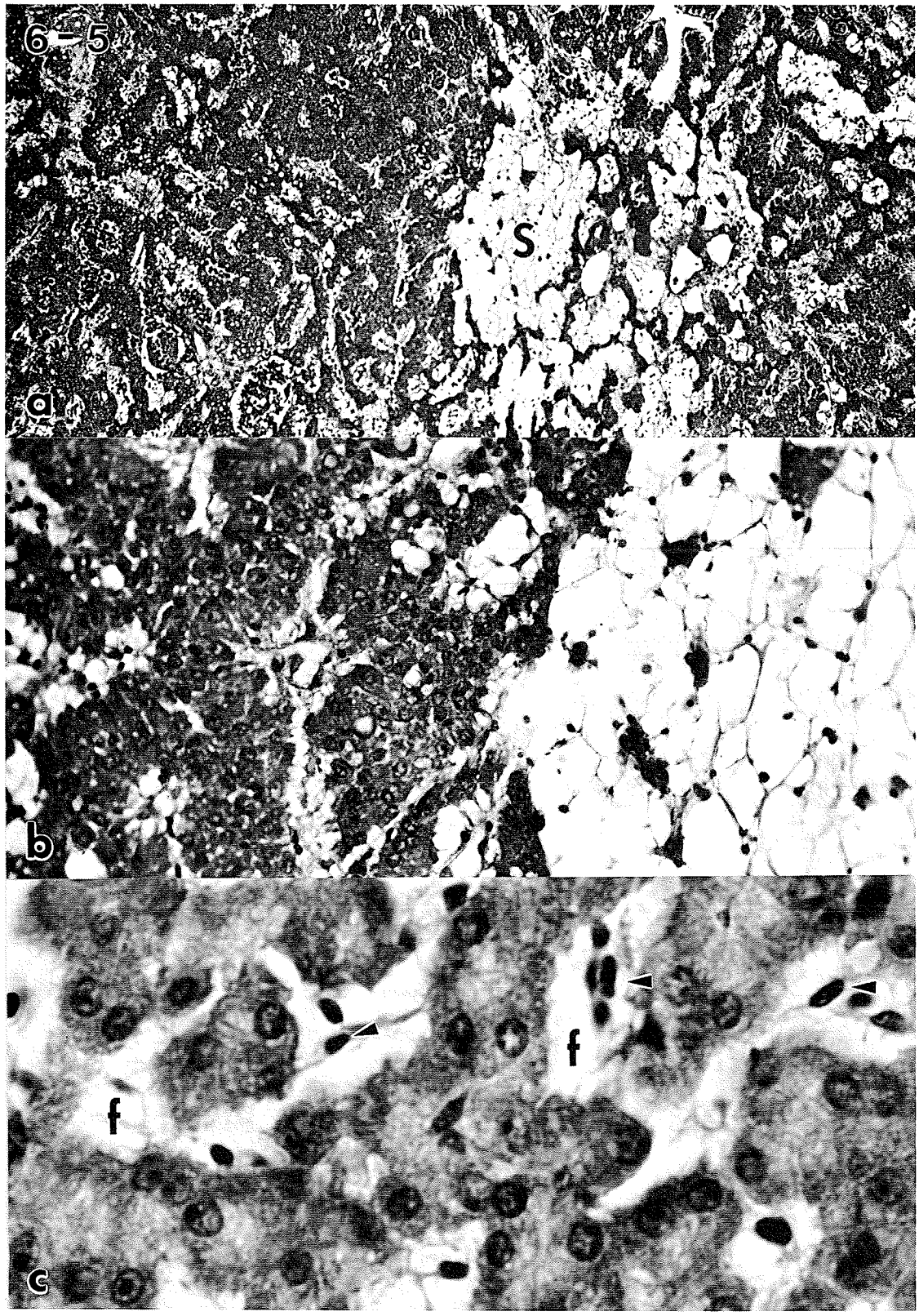
Figure 6-5

Experimental liver lesions in winter flounder liver after twelve months exposure to a weekly dietary exposure to benzo(a)pyrene.

a) Section showing perisinusoidal edema and an area of spongiosis (S). 100 x

b) Detail of 6-2a. Spongiosis and perisinusoidal edema. 400 x

c) Perisinusoidal edema in a fish chronically exposed to benzo(a)pyrene. The erythrocytes are compressed into the center of the vascular space (arrowheads), by interstitial fluid (f) that has accumulated in the perisinusoidal space. The hepatocytes are relatively intact. This lesion should be carefully distinguished from hydropic vacuolation which was never seen in any experimentally treated fish. 1000 x


6–5
S
a
b
f
f
c

Immunohistochemical results are summarized in Table 6-3 and illustrated in Figure 6-6b and 6-6c. The parenchymal hepatocytes of lesion-bearing fish showed a low level of cell proliferation, and variable levels of cytochrome P-4501A expression. The foci of epithelial hyperplasia were remarkable for their intense staining with anti-BrdU and their complete absence of staining with anti-P-4501A (Figure 6-6b and 6-6c). The proportion of nuclei staining for BrdU was comparable to the cholangiocellular carcinomas described in Chapter 5. The perisinusoidal edema and resultant spongiosis hepatis lesions stained for neither BrdU nor P-4501A.

Figure 6-6

Focal epithelial hyperplasia in the liver of a winter flounder exposed to dietary technical grade chlordane weekly for one year.

a) Small ovoid basophilic epithelia (e) intermingled with inflammatory cells are evident adjacent to a biliary ductule. Hematoxylin and eosin. 200 x

b) A similar lesion stained with anti-BrdU, counterstained with eosin. DNA synthesis is evident in the periductular epithelia, but absent from the surrounding parenchyma. 200 x

c) The same lesion as (b) stained with monoclonal antibody 1-12-3, anti-P4501A. The proliferative lesion did not stain, whereas the surrounding parenchyma did. 200x

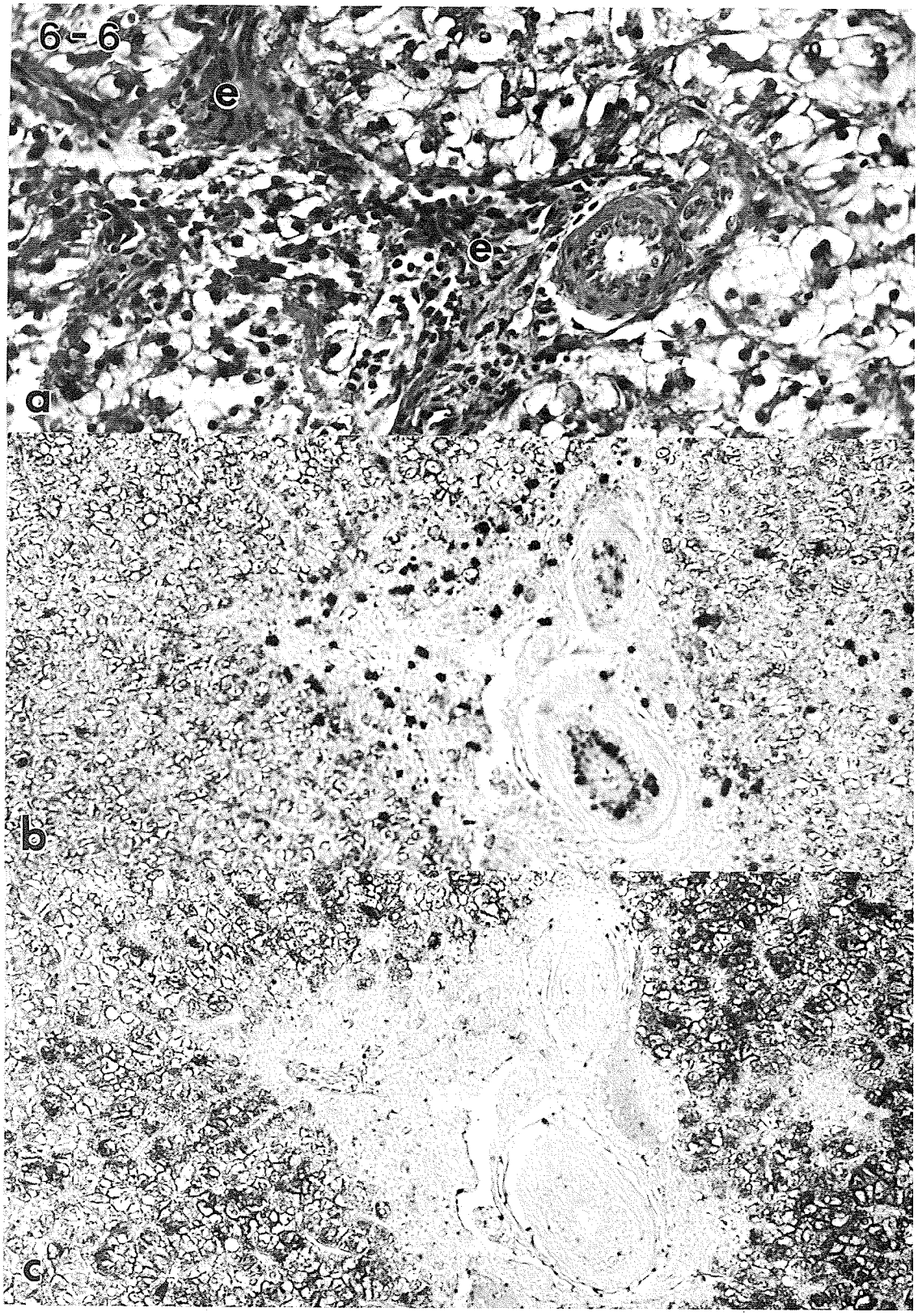

6-6'
e
e
a
b
c

TABLE 6-3

DNA synthetic activity and cytochrome P-450E expression in parenchymal hepatocytes, focal epithelial hyperplasia and perisinusoidal edema in the liver of winter flounder. The fish were fed 10 ppm chlordane (CD) and or 900 ppm benzo(a)pyrene (BP) weekly at 5% body weight for 12 months.

Treatment	Parenchyma		Focal epithelial hyperplasia			Perisinusoidal edema		
	BrdU	P450E	Prevalence	BrdU	P450E	Prevalence	BrdU	P450E
CD	+	+	7/9	+++	-	0/9		
CD & BP	+	++	6/16	+++	-	0/16		
BP	+	++	2/10	+++	-	3/10	-	-
Control	-	-	0/8			0/8		

Liver sections bearing histological lesions, as seen in slides stained with hematoxylin and eosin, were stained immunohistochemically for the incorporation of bromodeoxyuridine (BrdU) into nuclei. BrdU was injected into the fish 3 hours before sacrifice: nuclear detection of incorporation of BrdU reflects replicative S-phase DNA synthesis suggestive of cellular proliferation. Serial sections were also stained immunohistochemically for the presence of cytochrome P-450-1A1 (P450E). Immunohistochemical stain density was ranked -, +, ++, +++.

Conclusions

1. Juvenile winter flounder were successfully maintained on a contaminated diet for a year.

2. Frank neoplasia was not found in any of these fish, but significant evidence of proliferation was present. In particular, the chlordane and benzo(a)pyrene treatments induced a proliferative lesion that included nascent ductular structures with BrdU incorporation that equalled cholangiocellular carcinomas in winter flounder taken from Deer Island Flats, Boston Harbor. The largest lesion was by no means a neoplasm, but the possibility of it becoming one, had the experiment run for longer, should be evaluated by repeat of the experiment with a two year time course. The occurrence of a proliferative lesion involving cells similar to biliary epithelial cells supports the field observations in previous chapters that suggest that biliary proliferation is a significant event in the response of winter flounder liver to chronic exposure to hepatotoxins.

The cytochrome P-4501A content of these lesions was markedly reduced as compared to the surrounding parenchyma. The loss of P-450 expression is commonly seen in undifferentiated cells that at times may progress to neoplasia (Buchman et al. 1985).

3. Perisinusoidal edema was observed in benzo(a)pyrene treated fish, which in one fish appeared to have progressed to spongiosis hepatis. This lesion showed no staining for either BrdU incorporation, or cytochrome P-4501A.

Grow out of "young of the year" winter flounder from Deer Island Flats in clean water with clean food.

Experiment 5

A supposition arising out of the field and experimental studies described so far is that chronic cytotoxicity is a necessary sequel to genetic changes before overt neoplasia can arise in winter flounder from Deer Island Flats. A more general supposition is that there are no inherited or infectious conditions limited to contaminated areas that could lead to the observed epizootics of neoplasia. To further discriminate between these possibilities, juveniles from Deer Island Flats were held to grow out to adult[1] size in clean water and on clean food. Our field collections made through the summer and fall of 1987 had shown a continued residence of the "young of the year" from spawning in March, through till December, when they reached the 80 - 100 mm juvenile stage (Chapter 3). The assumption was that rearing of these fish on clean food and water would result in histologically normal adults. This assumed that although the fish would have been exposed to genotoxic carcinogens, and probably bear initiating genetic lesions by the time of capture, they would be deprived of the necessary chronic exposure to epigenetic carcinogens that they would have received had they remained in the Boston Harbor system over the next three years. If they did show histological changes as adults after maintenance on clean food, it would not be possible to discern, without further study, between sufficient initiation before capture, or inherited or infectious causes. Absence of histological change after a "clean grow

[1]This experiment was planned to run for at least 3 years. The conclusion was not expected within the time frame of this thesis.

out" would at least suggest that continued exposure to one or more factors peculiar to the Boston Harbor environment was a necessary condition for the progression to neoplasia.

Methods

Sixty "young of the year" winter flounder were caught by skiff trawl on Deer Island Flats in November 1988. They ranged in length from 60-90 mm. Since then they have been maintained in 12-15 °C seawater, and fed uncontaminated clams, at 5 % of the body weight 3 times weekly. Six of these fish were killed in March 1990. Apart from mild evidence of macrophage aggregation, there were no histopathological abnormalities. The remainder of these fish (currently 30 in number) will be grown out to 350 mm total length. At this length, had they remained on Deer Island Flats, the majority would have distinct pathological changes. As of March 1991 they ranged in size from 150-300 mm in total length.

Conclusion: From these limited observations there is as yet no reason to suspect that there is any inherent capacity of winter flounder from Deer Island Flats to develop their characteristic adult hepatic histopathology, without recurrent exposure to the contaminated sediments of that area over a number of years, although no firm conclusions can be made until termination of the experiment.

DISCUSSION

This study has demonstrated a practical system for the long term maintenance of winter flounder in the laboratory, and it has made significant advances towards the goal of eliciting liver neoplasia in winter flounder with chemicals found in the sediments of Deer Island Flats in Boston Harbor.

The feeding study has shown that the chronic exposure of winter flounder to dietary chlordane and benzo(a)pyrene induced a cytotoxic and proliferative response that appeared to involve epithelial cells with biliary characteristics. This finding is potentially highly significant. It is very possible that this result is an experimental illustration of the speculation in previous chapters, that preductular biliary epithelia in winter flounder may behave, when exposed to hepatotoxins, in a manner comparable to rodents. In particular the poorly differentiated biliary epithelia described in the chronic feeding study may indeed be teleost oval cells.

Benzo(a)pyrene also induced perivascular edema, which in one fish was associated with spongiosis hepatis. The duration of the experiment was insufficient to elicit any frankly neoplastic lesions, but given the presence of proliferative lesions in the treated groups, it seemed imperative to repeat the study with a longer duration, with an added regenerative stimulus. A repeat of experiment 4 was begun in Jan. 1990. Additions to the protocol included a weekly feeding of clams, dosed with 10 ppm estradiol, to half of each of the four original treatment groups, and an extension of the experiment to two years duration. Estradiol has been shown to be a promoter in rainbow trout

(Nunez et al. 1989) and may actively select the cells resistant to chlordane cytotoxicity.

The acute experimental studies have achieved a number of necessary advances towards the ultimate goal of carcinogenic chronic exposures in winter flounder. These studies have highlighted the significance of choice of carrier in the injection of chemicals. The high lipid solubility of trioctanoin permitted a high loading dose that gave a continuous exposure over a number of weeks. In contrast, the use of DMSO as a carrier resulted in a rapid release of the compound soon after dosing.

The subacute experimental exposures of winter flounder to chlordane have shown it to be an acutely toxic compound at high doses. Sublethal doses were hepatotoxic, with pyknosis, perisinusoidal edema and aggregation of macrophages being the primary histological changes found. Perivascular edema was also induced when extracts of Boston Harbor sediment were injected. High doses of this extract were acutely toxic.

The Boston fish being grown out on clean food have been maintained in the laboratory since Nov. 1988, and as of March 1991 were growing well. Prior to this, the long term maintenance of winter flounder was regarded to be difficult, and many unsubstantiated opinions as to the necessary conditions were proposed. The debates between investigators concerned optimum substrate, tank shape, light regime, and other physical parameters. However, this study found that these considerations paled into insignificance in comparison with two overriding factors: 1) The quality of the animal on arrival at the laboratory - excellent post-catching husbandry could not help a traumatized fish; it seemed that once the mucous integrity of the epidermis had been disturbed, the fish was open to opportunistic pathogens, and 2) for females, the water

temperature had to be maintained between 10 and 17 °C.

Each of these experiments has furthered our understanding of how to manipulate winter flounder to achieve the ultimate experimental goal. That goal is still being sought, with two experiments currently underway.

Alternative strategies for future studies could include: 1) Selection of a more potent initiator, such as dimethylbenzanthracene; 2) Initiation followed by partial hepatectomy; 3) Exposure of embryonic and yolk sac stage winter flounder to carcinogens, with subsequent grow out. Whilst technically impractical at present the technology is currently developing and this last strategy may well be a reality soon.

CHAPTER 7

SUMMARY AND CONCLUSIONS

INTRODUCTION

This study examined the development of hepatic neoplasia in winter flounder from Boston Harbor. This harbor is heavily polluted with a wide range of chemical contaminants, many of which may cause chronic hepatotoxic changes in bottom-feeding fish that result in cancerous lesions. The results of this study include:

1) A characterization of the advanced pathology of neoplasm-bearing winter flounder (Chapter 2).

2) A description of the progression and epizootiology of contaminant-associated liver disease in juvenile and adult winter flounder (Chapter 3).

3) An ultrastructural characterization of hepatotoxic and neoplastic lesions in winter flounder (Chapter 4).

4) An evaluation of DNA synthesis, as an indicator of cell proliferation in winter flounder liver (Chapter 5).

5) The development of the winter flounder as an experimental model to study the etiology and pathogenesis of this disease (Chapter 6).

METHODS

A diversity of field sampling techniques were employed to collect juvenile and adult winter flounder from a number of sites. These techniques included otter trawling, rod and line, and beach seining. The fish were examined by routine histopathological methods, transmission electron microscopy, and immunohistochemical methods.

Additionally, winter flounder were maintained in the laboratory for periods of one month to two years, and treated with various chemicals found within the sediments of Boston Harbor. The details of each methodology have been given in previous chapters.

RESULTS

Oncology

Liver neoplasia in winter flounder from Deer Island Flats (Boston Harbor, MA) has been shown by these studies to be a complex end-stage of a progressive disease. The mean prevalence of neoplasia in sexually mature fish collected in the years 1987 to 1990 was 6.5%. Ninety percent of the neoplastic lesions were cholangiocytic, the remainder were hepatocytic. Some contained well-differentiated, but abnormal structures, whilst others were poorly differentiated, invasive lesions. The phenotypic diversity of the cholangiocellular lesions was large: tubular, solid, cystic, and papillary lesions were observed in varying stages of differentiation, invasion and degeneration. Many neoplasms occurred within large aggregations of vacuolated cells; in some cases there was only a small fringe of vacuolated cells. Many vacuolated cells were also found singly within neoplastic lesions.

Epizootiology and pathogenesis

The significance of vacuolated, and other abnormal cells to the development of neoplasia was evaluated by studies of site-, sex-, length-, and age-specific differences in lesion prevalence in winter flounder from Boston Harbor and other less contaminated

areas. Hepatic lesions including neoplasms, were most prevalent in old fish caught close to a major sewage outfall. In younger fish, the first lesions to be seen included a hydropic swelling of cells in the center of the hepatic tubules, and a proliferation of biliary ductules and ducts. These changes were accompanied by necrosis and aggregations of macrophages. In older fish, vacuolated cells were seen to fill entire hepatic tubules, and to form large aggregations, which were often grossly visible. These aggregations were a common site for the development of neoplastic lesions as indicated above. Vacuolated cells were shown to involve larger parts of the liver with increasing age, to be stable in the absence of ongoing exposure to contaminated sediments and food, and to precede the appearance of neoplastic change. The latter parts of this study were aimed at understanding the structure and development of vacuolated cells, and their potential role in the progression to neoplasia.

Ultrastructure

Vacuolation of hepatic epithelia was shown to involve an accumulation of electron-lucent fluid in the cisternal and perinuclear space of the endoplasmic reticulum, and in mitochondria. The dilated endoplasmic reticulum then rounded up into many small vesicles. These vesicles, and the swollen mitochondria then appeared to coalesce to form a single large vacuole that filled the majority of each affected cell, compressing the remaining organelles and nucleus to the margin of the cell. The nuclear morphology appeared normal, and a few apparently functional mitochondria remained. The first cells to vacuolate were centrotubular biliary preductular cells, as

shown by their location, nuclear morphology, and cellular junctions with the apices of non-vacuolated hepatocytes. The second, tubular stage of vacuolation was shown to involve biliary and hepatocytic components, and was often ensheathed in fibroblasts. The final focal stage involved a diverse aggregation of vacuolated hepatic epithelia with little vascularization.

DNA synthesis

Vacuolated cells had a number of interesting attributes: they were closely associated with neoplastic lesions; they seemed to persist after the fish was removed from the contaminated environment; and their prevalence increased in older fish, which also contained diverse hepatic neoplasms. For these reasons the capacity of vacuolated cells to synthesize DNA was examined. A protocol for the immunohistochemical detection of bromodeoxyuridine (BrdU) incorporation in paraffin embedded fish tissues was optimized. Histological sections were pre-digested with a protease, and then incubated with anti-BrdU and a nuclease overnight. Nuclear BrdU incorporation was then detected with standard peroxidase chromogenesis. Constitutive staining was observed in the gill, small intestine, and renal hemopoietic tissue. The technique was then applied to a range of pathological cases from Boston Harbor, and reference fish from Georges Bank. DNA synthesis, i.e. BrdU incorporation, was rarely seen in parenchymal hepatocytes in liver of fish from either site. An increase in DNA synthesis was seen in vacuolated cells of all stages, and in hyperplastic biliary epithelia. A major increase in DNA synthesis was observed in neoplastic lesions, including

vacuolated neoplastic cells.

Experimental modelling

Experimental studies to model the disease as seen in winter flounder liver from Boston Harbor requires the ability to obtain and maintain animals without undue stress, and to condition them to a maintenance diet. Such methods were not available at the start of these studies. Therefore, appropriate methods were developed for atraumatic capture and transport of winter flounder. A dietary regime was then established to allow long term healthy survival in the laboratory. Acute, subacute and chronic exposures were made with a model initiator, benzo(a)pyrene, and a model group of promoters, technical grade chlordane. Chlordane was shown at high doses to be acutely toxic, at lower doses to induce hepatotoxicity, with pyknosis and necrosis, and at low chronic dietary doses to induce multifocal proliferation of an epithelial cell with biliary characteristics. Chronic exposure to benzo(a)pyrene was also shown to induce the same biliary proliferation, and at times a perisinusoidal edema. In one case this latter lesion was contiguous with, and appeared to blend into areas of spongiosis hepatis. The chronic protocol has been repeated with further groups being exposed to chlordane, benzo(a)pyrene and estradiol. This study will be terminated after two years in Dec 1991.

DISCUSSION

Oncology

This study found a broad diversity of cholangiocellular neoplasms to be the predominant neoplastic lesion in winter flounder from Boston Harbor. In comparing the lesions described here with those in other bottom-feeding fish, such as English sole (Myers et al. 1987) and mummichog (Vogelbein et al. 1989), there is an apparent difference in that the latter two species had a predominance of hepatocellular neoplasms with a concomitant high prevalence of tinctorially altered foci of hepatocytes. These differences are clouded by interpretational and site-specific contaminant differences, but nonetheless there appears to be significant species-specific differences between how these fish respond to chronic exposure to sediments heavily contaminated with chemicals and heavy metals. These observations make an in-depth study of the comparative oncology of different species using archival material an important task. In this way, along with carefully controlled experiments using multiple species in parallel, inconsistencies of nomenclature, and real pathogenetic differences should become apparent. Definition of inter-species differences and similarities is essential if we are to use any one species to its maximum potential in the field and laboratory.

Role of vacuolated cells

In collaboration with other investigators we have shown that vacuolated cells probably contain activated K-*ras* alleles (McMahon et al. 1990). We have also, in collaboration, shown that nodules of vacuolated cells in these fish have ornithine

decarboxylase activity equalling or exceeding that of surrounding non-vacuolated parenchyma (Koza, Moore and Stegeman 1991). These observations taken with the findings presented in this thesis, of intimate association of vacuolated cells with neoplastic lesions, the apparent stability of vacuolated cells in the absence of ongoing toxicity, and their capacity to synthesize DNA replicatively, strongly suggest that the vacuolated cells may play an important role in the progression to neoplasia.

Given the apparent multiple origin of vacuolated cells, their varied fate, and their persistence, the best descriptive term for this process is "hydropic vacuolation". This terms avoids any commitment to a particular fate, such as degeneration, or proliferation. The hydropically vacuolated cell appeared to arise from cholangiocytes at first, but also hepatocytes at the tubular stage. This duality could be a reflection of the common stem cell origins of these two differentiated hepatic epithelia. On the evidence discussed here, the cells can probably proliferate, and are intimately associated with neoplastic cells. Vacuolated cells are much more prevalent than neoplastic cells, and are found in fish 2 to 3 years younger than the youngest fish to develop neoplasia, and are found, at lower prevalences, in less contaminated sites where neoplastic lesions are absent. However, the question as to whether the vacuolated cells are "incomplete neoplasms" (Foulds 1969) has not been answered by these studies.

In further consideration of tumor progression in winter flounder, two observations in this research are relevant. The finding of vacuolated cells to first arise from biliary preductular cells lead to the consideration of parrallels between mammalian oval cells and telost preductular cells. Additionally the chronic experimental exposure of winter

flounder to chlordane and benzo(a)pyrene lead to peribiliary epithelial proliferation. Taken together it is not unreasonable to focus on the location of teleost preductular epithelia as a potential stem cell site for the abnormal development of hepatic epithelia in the face of chronic chemical hepatotoxicity.

Environmental monitoring

The above observations suggest that the prevalence of vacuolated cells is a useful indicator of the exposure of winter flounder in Boston Harbor to a complex mixture of contaminants. Therefore, efforts to monitor the biological effects of contaminants on winter flounder should place significant emphasis on the prevalence of the vacuolated cell lesions, at each of the three stages described. With respect to the relevance of using the prevalence of vacuolated cells for monitoring exposure to contaminants in other flatfish species, discussion of this issue with other investigators has lead to the discovery of similarly vacuolated cells in the livers of starry flounder (*Platichthys stellatus*) and rock sole (*Lepidosetta bilineata*) (Myers et al. in press), and probably in the European flounder (*Platichthys flesus*) (Simpson, ICI, UK - pers. comm.). These lesions are apparently less prevalent than in winter flounder, but are of comparable morphology. It therefore appears that the prevalence of vacuolated cells in certain species of flatfish in the Pacific and Atlantic Oceans may be an excellent indicator of the chronic biological effects of certain chemical contaminations on coastal bottom-feeding fish.

A number of biological changes resulting from chronic exposure to chemical

contaminants in coastal sediments are currently applied to benthic finfish. These "biomarkers" include histopathological lesions shown to be associated with exposure to chemicals, immunohistochemical and biochemical assays for the induction of mixed function oxygenases and chemical assays for the presence of biliary metabolites and bulky nuclear adducts of various contaminants. Histopathology essentially records the phenotype resulting from a summation of all contaminant effects and other pathogenic influences. The mixed function oxygenase studies use cytochrome P-450 induction as an indicator for the exposure to procarcinogens that may be bio-activated, and as a surrogate indicator for the other less well understood effects of xenobiotics binding the cytosolic Ah receptor (Goldstein and Safe 1989). The bile metbolism assay indicates exposure to hydrocarbons including genotoxic compounds, and the adduct assay indicates the potential for genetic damage by genotoxic compounds. Within the current initiation/promotion/progression paradigm of chemical carcinogenesis, there is no assay that focusses on the effects of non-genotoxic compounds. This is reasonable if it is assumed that genotoxic initiators are involved in the rate-limiting step of the progression to neoplasia. This assumption is valid in the light of most experimental carcinogenesis studies in aquarium species of fish. A single dose of a genotoxic carcinogen is often adequate to elicit overt neoplasia in a matter of months (Hoover 1984). However, the much longer latency and gradual increase in prevalence of neoplasia with age, as shown in this thesis, would suggest that in the feral "natural" situation other factors in addition to early genotoxicity may be important. Epigenetic promoters, exerting continuous chronic proliferative effects, may be the rate-limiting

step, and therefore should be subject to an appropriate monitoring technique. The activity of chronic repeated hits from genotoxic carcinogens should also not be overlooked.

Until recently the only histological assay for cell proliferation involved autoradiography, and was not practical for use on multiple field specimens. The optimization, in this study, of the use of immunohistochemistry to assay the uptake of bromodeoxyuridine (BrdU), to highlight nuclei undergoing replicative DNA synthesis, allows for a potentially useful and robust field assay for monitoring the effects of chemical promoters on bottom-feeding fish. The technique is currently being field-tested by the author, at a series of stations, in a study of winter flounder pathology for the Massachusetts Water Resources Authority.

Cell proliferation

With respect to the study of cell proliferation, further development of the BrdU assay is warranted. Liver cells have an unusually long cell cycle, even when actively proliferating (Eldridge et al. 1990); it is therefore important to examine the effect of prolonged (3-7 day) continuous labelling with BrdU. This could be achieved with an intraperitoneal pump exposure, or with repeated injections.

Other ways of examining the role of cell proliferation in the pathogenesis of this disease would be to asses the usefulness of other current markers in mammalian hepatocarcinogenesis. There is a potentially useful endogenous marker of cell proliferation, namely Ki67 (Gerdes et al. 1983), or the so-called proliferating cell

nuclear antigen. Preliminary studies of this antigen in winter flounder were equivocal (data not shown), and parallel problems with this antigen in mammalian studies make it of uncertain value. Other markers include hepatocyte growth factor (Zarnegar and Michalopoulos in press) and transforming growth factor alpha (Hsia et al. 1991).

Any consideration of cell proliferation in a feral species has to be made in the context of seasonal migration and reproductive patterns. The annual hepatomegaly in pre-spawning gravid females has to be considered in the interpretation of cell proliferation data. Additionally, winter flounder migrate into Boston Harbor in the early spring prior to spawning. At the same time that they are preparing to spawn, they are also revisiting an area heavily contaminated with chemicals and metals that may induce a compounding effect on pre-spawning hepatic cell proliferation. Thus, reproductively active fish that move in and out of contaminated areas on a seasonal basis will be undergoing a series of complex endogenous and exogenous influences that impact the rate of hepatic cell proliferation. This cyclical exposure to agents inducing cell proliferation may have parallels in benzene carcinogenesis, where it has been shown, that cyclical exposure has a greater carcinogenic effect than continuous exposure.

Future experimental studies

Related to the use of vacuolated cells as markers of biological effect is the question of what specific chemicals are causing the lesion to develop? Experimental studies within this thesis have shown that benzo(a)pyrene, and technical grade chlordane

elicit a proliferation of biliary epithelia. Vacuolated cells were not induced.

With regard to laboratory experiments with the winter flounder, in addition to on-going experiments that directly extend the work of Chapter 6, I plan to expose winter flounder to a number of different agents present in the sediments of Boston Harbor. These studies will be advanced enormously if the technological barriers to the survival of larval winter flounder through metamorphosis can be overcome. Such an advance would allow genotoxic damage to be induced in embryonic fish, with subsequent chronic exposure of juveniles to promoting agents.

Molecular pathology

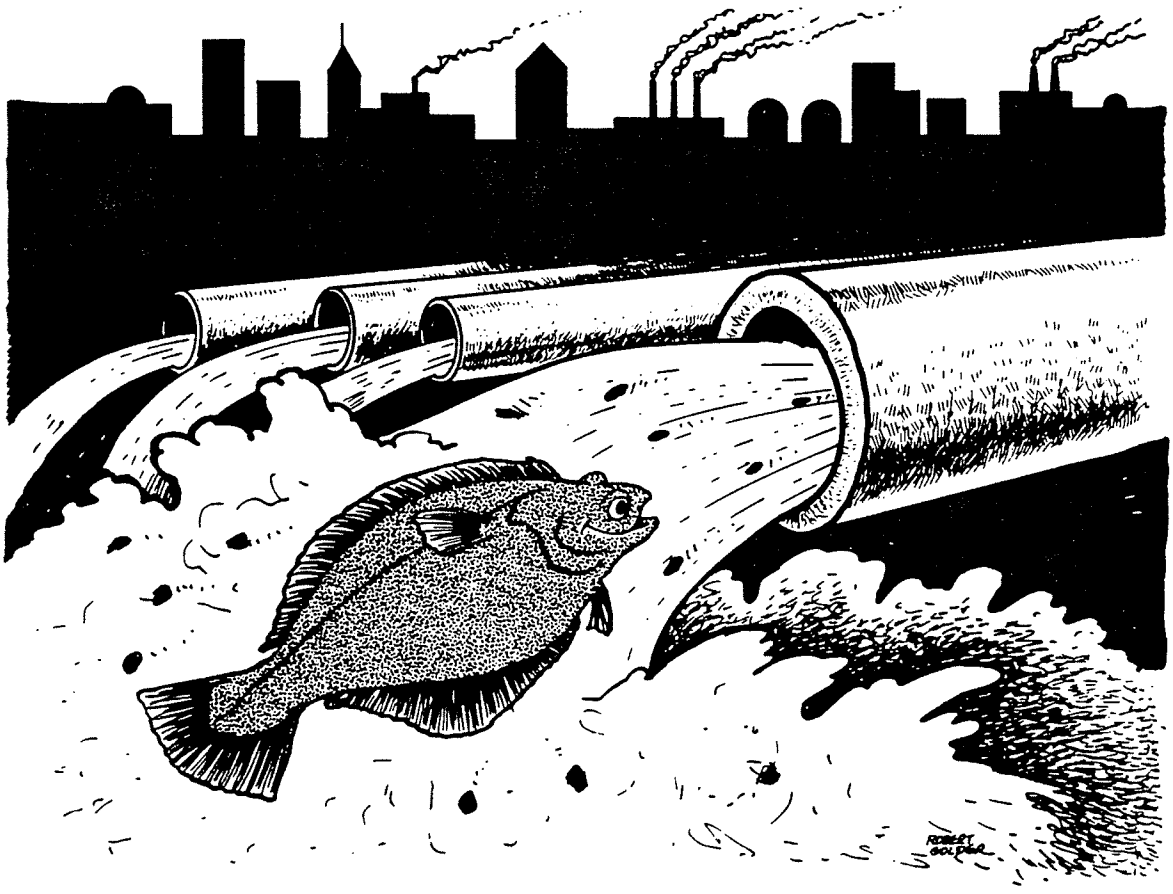
The original aim of this thesis was to localize oncogenic changes, within neoplasm-bearers, to specific abnormal cell types. Four years ago our primary interest was in the *ras* oncogene family. Reagents were unavailable at that time to successfully achieve that aim. Our knowledge of the natural history and morphology of the disease was also too limited to address that aim. Today, two developments have changed this. First this thesis has overcome the limitations in knowledge of the cellular aspects of the disease itself. Second, there is a growing interest in a genetic change associated with neoplasia, and appropriate reagents are, or may soon be available. In particular, in the past three years the presence of mutations in the p53 tumor suppressor gene have been shown to be highly prevalent in neoplasms from a diversity of organ systems. In the liver there is a particular mutation hot-spot at codon 249 (Bennett et al. 1991). The wild type gene is undetectable immunohistochemically, and is usually

heavily overexpressed if mutant, unless the mutation involves a stop codon. Thus, analysis of the flounder p53 genetic sequence, and screening of available antisera for appropriate specificity in the flounder may well yield important information about the role of p53 mutations in this species.

CONCLUSION

To conclude, these studies have, as usual, raised more questions than provided answers, but it seems to be that the hydropically vacuolated cell is central to progressive liver disease in contaminant-exposed winter flounder, and an excellent indicator of the early biological effect of potential hepatocarcinogens in contaminated sediments. Furthermore, these studies have lead me to believe that far greater emphasis should be put on the role of non-genotoxic carcinogens in studies of the pathogenesis of hepatic neoplasia in feral bottom-feeding fish.

The costs and benefits of being an "old-age mutant ninja flounder" are illustrated below.



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