



The effects of pentachlorophenol on the liver and gills of cutthroat trout (*Salmo clarki*)
by John Kevin Morrison

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in
Veterinary Science
Montana State University
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Abstract:

The effects of progressive concentrations of pentachlorophenol (PCP) on the liver and gills of cutthroat trout were examined both histologically and ultrastructurally. Tissues were collected from fish immediately after they became moribund and at the termination of a 96-hour acute toxicity test.

Cellular alterations in the liver at the light microscopic level included: focal cytoplasmic vacuolation and degeneration, dilation of sinusoids with apparent degeneration of sinusoidal endothelium, and mild cellular necrosis. Alterations at the ultrastructural level included: decreased glycogen stores, occasional swelling of mitochondria with an increase in the granularity of the intramitochondrial matrix, and degeneration of mitochondrial cristae. The granular endoplasmic reticulum and the perinuclear cisterna were dilated. There was an apparent increase in the number of nuclear pores in hepatocytes from exposed fish. Large autophagic vacuoles were abundant in some hepatocytes from fish dying from PCP exposure. Hepatocyte projections into the space of Disse were hypertrophic, and membrane bound fragments similar to these projections appeared to slough into the sinusoidal lumen. No consistent or significant cellular alterations were observed in the gills of test or control fish.

This study suggests that both light and electron microscopy can be of value in identifying the pathologic insults caused by exposure of cutthroat trout to PCP.

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A thesis submitted in partial fulfillment
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MONTANA STATE UNIVERSITY
Bozeman, Montana

May, 1983

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ABSTRACT

The effects of progressive concentrations of pentachlorophenol (PCP) on the liver and gills of cutthroat trout were examined both histologically and ultrastructurally. Tissues were collected from fish immediately after they became moribund and at the termination of a 96-hour acute toxicity test.

Cellular alterations in the liver at the light microscopic level included: focal cytoplasmic vacuolation and degeneration, dilation of sinusoids with apparent degeneration of sinusoidal endothelium, and mild cellular necrosis. Alterations at the ultrastructural level included: decreased glycogen stores, occasional swelling of mitochondria with an increase in the granularity of the intramitochondrial matrix, and degeneration of mitochondrial cristae. The granular endoplasmic reticulum and the perinuclear cisterna were dilated. There was an apparent increase in the number of nuclear pores in hepatocytes from exposed fish. Large autophagic vacuoles were abundant in some hepatocytes from fish dying from PCP exposure. Hepatocyte projections into the space of Disse were hypertrophic, and membrane bound fragments similar to these projections appeared to slough into the sinusoidal lumen. No consistent or significant cellular alterations were observed in the gills of test or control fish.

This study suggests that both light and electron microscopy can be of value in identifying the pathologic insults caused by exposure of cutthroat trout to PCP.

CHAPTER 1

INTRODUCTION

Pentachlorophenol (PCP) is reported to be the most toxic member of the chlorophenol series. Fish kills, losses to wildlife, toxic effects in farm and domestic animals, and human fatalities have resulted from exposure to PCP (4, 7, 8, 19, 25, 33, 37, 47). Low but detectable levels of PCP have been found in river water, municipal water supplies, human food stuffs, as well as in blood, urine, and fat of nonoccupationally exposed humans (25). Toxicity studies using PCP have shown that it is extremely toxic to aquatic animals (9, 13, 36, 38). It has also been shown that fishes can concentrate PCP to levels 400 times that of an initial, nonlethal, water concentration (18, 23, 24, 26). This bioaccumulation of PCP represents a potential hazard in the food chain (14, 40).

Chlorophenols, including PCP, are environmentally and economically important. They are used as wood preservatives, fungicides, herbicides, insecticides, and bactericides. Some are degradation products of chemically similar herbicides such as 2,4-D (2,4 dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5 trichlorophenoxyacetic acid, popularly referred to as Agent Orange).

The sensitivity to lethal concentrations of PCP varies according to different species of fishes. Representative 96-hour LC 50 (median

lethal concentration) values are (Montana State University Fisheries Bioassay Laboratory [MSU/FBL], unpublished data):

Rainbow Trout ----- (<u>Salmo gairdneri</u>)	0.115 mg/l
Fathead Minnow ----- (<u>Pimephales promelas</u>)	0.266 mg/l
Channel Catfish ----- (<u>Ictalurus punctatus</u>)	0.132 mg/l
Bluegill ----- (<u>Lepomis macrochirus</u>)	0.202 mg/l

Reported lethal values for some mammals under experimental conditions are (6, 46):

Rats (adults) -----	125-200 mg/kg body wt.
Calves -----	140 mg/kg body wt.
Sheep -----	120 mg/kg body wt.

There are few reports in the literature of studies directly relating, or attempting to correlate, histopathologic changes with PCP toxicity in aquatic animals (14, 32). Histopathological descriptions of mammalian toxicity studies involving PCP vary from rather vague descriptions such as fatty changes in the liver or cloudy swelling of hepatocytes (19, 43), to describing statistically significant alterations of nuclei and organelles using detailed morphometric techniques at the light and electron microscopic levels (16).

The primary functions of the fish gill include: respiration, absorption, excretion, and osmoregulation. Anatomically the gill lamellae, because of their vast surface area and direct exposure to the environment, can be considered primary target tissues in aquatic pollution assessment. The liver, the primary organ of detoxification,

should also receive special attention because of its key role in the partial metabolism of many xenobiotics (5, 17). Figure 1 shows the major detoxification pathways for PCP in fishes (21).

This study was designed to characterize the alterations induced by PCP in the liver and gill of cutthroat trout (Salmo clarki) under experimental conditions. A secondary objective of the study was to determine the feasibility of using histopathologic and ultrastructural evaluations in conjunction with morbidity and mortality to evaluate insults of toxic substances to aquatic animals.

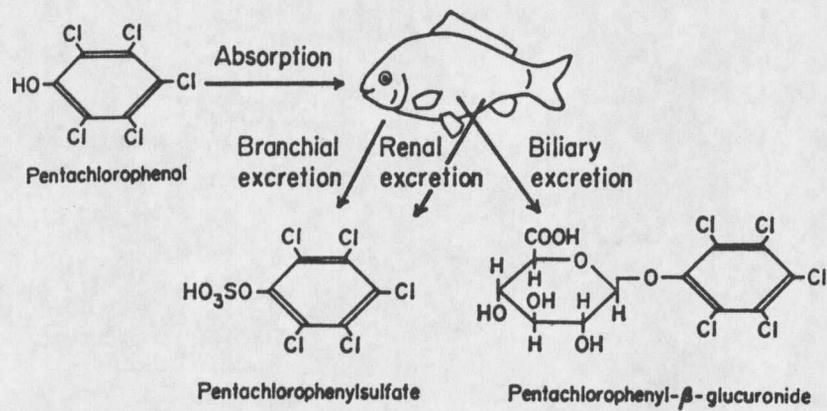


Figure 1. The major detoxification pathways for PCP in fishes. (From Kobayashi and Nakamura, 1979).

CHAPTER 2

METHODS

Two flow through toxicity tests were conducted on cutthroat trout to determine possible pathologic effects in the liver or gills induced by PCP. The tests were conducted at the MSU/FBL. The PCP used in this study was obtained from a stock supply at the MSU/FBL. It was reported to be comparable to that of a 98% PCP, Lot #6-192 obtained from Chem Service, West Chester, PA 19380.

The water source for the tests was a natural groundwater spring located at the U.S. Fish and Wildlife Service, Fish Cultural Development Center (FCDC), Bozeman, Montana. Chemical characteristics of this spring water are shown in Table 1 and a summary of water chemistry analyses are presented in Table 2.

Temperature, dissolved oxygen, and pH were determined for water in the test tanks as follows: dissolved oxygen was measured using a Yellow Springs Instrument (YSI) Model 54 A Oxygen meter, temperature with a calibrated mercury thermometer, and pH with a Beckman Model 3500 Digital pH meter. Total alkalinity, total hardness, and specific conductance were determined in accordance with the American Public Health Association (APHA), Standard Methods for the Examination of Water and Wastewater (3). Chemical analyses from a previous PCP study, 1 week prior to this study, were used to report total alkalinity, total hardness, and specific conductance. These three variables

Table 1. Chemical characteristics of the dilution water.

Al < 0.02 mg/l	Mg 9.8 mg/l
B < 10.0 mg/l	Mn 6.0 mg/l
Ba 31.0 µg/l	Mo < 0.05 mg/l
Be < 2.0 µg/l	Na 0.6 mg/l
Ca 45.9 mg/l	Ni < 0.05 mg/l
Cd < 0.5 mg/l	P < 1.0 mg/l
Cr < 0.1 mg/l	Pb < 0.3 mg/l
Cu < 2.0 µg/l	Si 0.3 mg/l
K 0.48 mg/l	Sr < 0.05 mg/l

Table 2. Summary of water chemistry analysis
Test No. 2

DATE	4-16-82	4-19-82	4-19-82	4-9-82	4-9-82	4-8-82
TANK	D.O. (mg/l)	pH	Temp. °C	Alk. (mg/l)*	Hardness* mg/l	S.E.C.* μ mhos
1	8.3	7.64	11.4	173	206	391
2	8.2	7.69	10.9	172	206	392
3	8.0	7.73	10.9	172	205	394
4	7.9	7.70	10.9	173	205	394
5	7.3	7.79	11.3	173	205	399
6	7.4	7.89	10.9	--	--	--

*Measurements from a similar PCP bioassay

are relatively constant in the water supply used, and would be expected to be similar in both tests.

The PCP toxicant solution was made by placing 2 grams (g) of PCP in each of two 20-liter naphthalene containers. These were then filled with 20 liters (l) of spring water and allowed to stand, with occasional agitation, for 24 hours prior to starting the bioassays. 2 g in 20 l exceeds by 25% the aquatic solubility (80 mg/l) of PCP (41).

Stock toxicant was added to each of five test tanks (#2-#6) by means of a proportional diluter (dilution factor 0.75) of the basic design of Mount and Brungs (30); a sixth tank served as the control (#1). The water flow rate from the diluter into each tank was 500 ml every 2 to 3 minutes. Water replacement time was approximately 2 hours, and full test concentration was effectively reached within 8 hours.

Water was analyzed for PCP twice during the tests (on day 2 and day 4), using gas chromatography by the following method: extraction of PCP from water samples was accomplished at room temperature by acidifying a 125 milliliter (ml) water sample to a pH of less than 2 with 200 microliters (ml) of concentrated H_2SO_4 . Fifty ml of this acidified sample were then placed in a 100 ml volumetric flask and 50 ml of toluene were added. This mixture was stirred using a magnetic stirring device for 1 hour and then allowed to settle for 1 hour. A 1 ml aliquot of sample extract was then derivitized using a solution of 2 ml ether, 2 ml hexane, and 100 ml of diazomethane. The sample was allowed to equilibrate for 1 hour and then analyzed using

gas chromatograph electron capture detection (2). Specific gas chromatograph settings are listed in Table 3.

Prior to testing, fish were reared at 10 C under hatchery conditions at the FCDC.

Fish were exposed to toxicant in glass tanks (50 x 25 x 30 cm) containing a test water volume of 21.8 l. Figures 2 and 3 show the operational system employed in this study. Fish were acclimated to the test tanks for 24 hours prior to introduction of toxicant and were not fed during the acclimatization period nor during the test.

This study differed from a typical 96-hour acute toxicity test in that its primary objective was to obtain numerous fish samples for histologic examination. To accomplish this the maximum carrying capacity for each tank needed to be determined. The "Flow Index" formula (34) used by fish culturists to determine carrying capacity of fish rearing units was used to determine the permissible number of fish that could be held in each test tank, without inducing unwanted metabolites, which could influence the toxicity test results (Table 4).

From a rearing tank, holding approximately 5,000 cutthroat trout, 40 fish were randomly sampled and individually weighed and measured. Average values were used to determine the permissible number of fish to be placed in each test tank. A total of 75 fish were placed in each tank (Table 5).

During the toxicity tests, observations were made at 4-hour intervals. Moribund fish were removed at the observation times and prepared for histologic examination. Dead fish, as evidenced by

Table 3. Gas chromatograph settings for PCP standard curve

Compound: PCP

Concentration Range: 0.02 - 0.5 mg/l

Column: 2 ft. x 1/4" o.d. x 2 mm i.d. glass column packed with 1.5%
OV-17, 1.95% QF-1 on 80/100 mesh (Applied Science, State
College, PA)

Oven Profile: Gas Chrom Q

Oven Temperature: 140°C

Injector Temperature: Isothermal

Detector: Electron capture

Detector Temperature: 300°C

Attenuation:

Sampling Method: Hewlett Packard 7672A Autosampler, 1 µl injections
Hewlett Packard 5880A Gas Chromatograph
(Hewlett Packard, Avondale, PA)

Comments:

Standards were 0.025, 0.05, 0.1, 0.5 mg/l. 50 ml samples were
extracted for 1 hour with 50 ml toluene using 100 ml volumetric
flasks and a magnetic stirring apparatus.

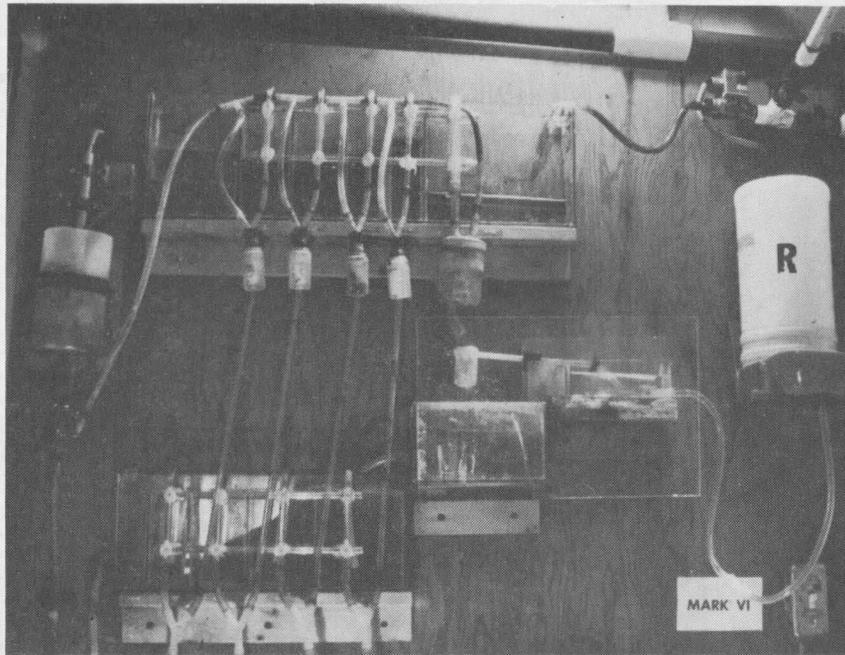


Figure 2. Proportional diluter. (R) toxicant reservoir.

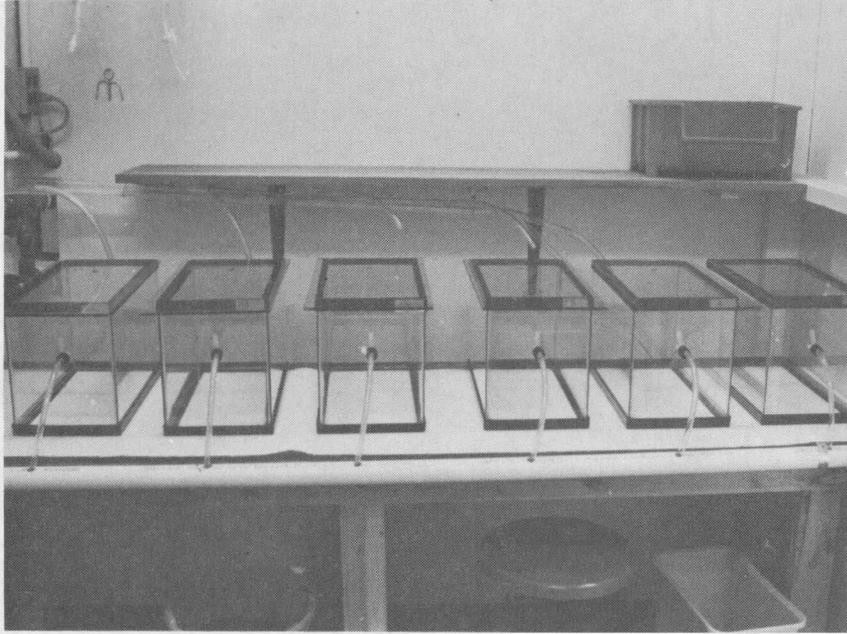


Figure 3. Test tanks.

Table 4. Flow Index formula

$$W = F L I$$

W = total fish weight (pounds)

F = loading factor (1.5)

L = fish length (inches)

I = water inflow (gallons per minute)

Table 5. Carrying capacity of test tanks

Flow into test tanks = 12 liters/hour (0.053 gpm)

Fish length = 1.85 inches

Fish weight = 0.89 grams/fish

Loading factor (F) = 1.5 (established empirically for
this water supply)

$W = F L I = (1.5) (1.85) (0.053) = 0.147$ pounds

0.147 pounds = 66.74 grams

$\frac{66.74 \text{ grams}}{0.89 \text{ grams/fish}} = 75$ fish per tank

complete immobilization and lack of operculation were not used for histologic examination because of possible post-mortem autolysis (29).

Fish taken for light microscopic examination were incised ventrally to maximize exposure of internal organs, and fixed in Bouin's solution for 12 to 24 hours. Samples were then transferred to 65% ethanol until processing. Whole fish were embedded in paraffin, sectioned at 5 microns (m) and stained with hematoxylin and eosin. Gill and liver tissues were examined histologically in an attempt to identify any cellular alterations induced by PCP. These two organs were specifically chosen because the gill is considered a major absorptive, and excretory organ and the liver is a major detoxifying center for xenobiotics.

Fish collected for electron microscopy were placed directly into a solution of 4% formaldehyde and 1% glutaraldehyde, buffered to a pH of 7.2 in phosphate buffer (45). After 5 to 10 minutes, the left operculum was removed from each fish to expose the gills, the fish was incised ventrally, and visceral organs were pulled from the body cavity to maximize exposure to fixative. After 2 to 3 days in fixative, a number of filaments from the outermost left gill arch and a small portion of liver from each sample fish were post-fixed in 1% aqueous osmium tetroxide, dehydrated in graded ethanol, and embedded in Spurr's embedding medium (41). Silver-gold (50 to 70 nanometers) sections were placed on copper grids and stained with uranyl acetate and Reynold's lead citrate (27). They were examined at 80 to 100 Kv with a JEOL - 100 CX transmission electron microscope.

Liver and gill tissues from the same fish collected for electron microscopy were also processed for light microscopy. These samples were embedded in glycol methacrylate, sectioned at 3 μ m and stained with hematoxylin and eosin.

In addition to collecting moribund fish for examination, fish were also collected at the termination of the 96-hour test. At this time, 10 fish for light microscopy (LM) and 5 for electron microscopy (EM) were preserved from each of tanks 1 thru 4. At termination only 13 fish remained in tank 5; therefore, only 4 fish were collected for each LM and EM. All fish in tank 6 were dead by the end of 96 hours.

CHAPTER 3

RESULTS

A decrease in dissolved oxygen as toxicant concentration increased was observed. This is probably due to the increased respiration of test fish induced by exposure to PCP. However, even the lowest reported dissolved oxygen concentration (7.3 mg/l) is more than adequate for the cutthroat trout, under the experimental conditions of this study (35).

The concentrations of PCP to which fish were exposed are presented in Table 6. Test #1 exposed fish to acutely lethal concentrations of PCP in all test tanks. At the highest exposure concentration (tank #6, 0.630 mg/l PCP) death occurred rapidly, with all fish succumbing after only 2½ hours exposure. At the lowest exposure concentration (tank #2, 0.192 mg/l PCP) all fish had succumbed after 8 hours. In test #2, fish were exposed to both acutely lethal and sublethal concentrations of PCP, with the highest exposure concentration (tank #6) averaging 0.165 mg/l (range 0.125 - 0.200) PCP. Mortality at this concentration began to occur after 20 hours (Figure 4). After 40 hours, 19 of the original 75 fish remained alive, with no more mortality occurring for the next 28 hours (68 hours total). After 64 hours had elapsed in the test more PCP stock solution was added to the reservoir of the proportional diluter. The stock PCP had apparently become more saturated upon standing because mortality

Table 6. PCP water analyses (mg/l)

Test No. 1							
Date	Time	Tank 1	Tank 2	Tank 3	Tank 4	Tank 5	Tank 6
4-11	0900	0.000	0.192	0.272	0.362	0.473	0.630
				*		*	

* calculated using dilution factor 0.75

Test No. 2							
Date	Time	Tank 1	Tank 2	Tank 3	Tank 4	Tank 5	Tank 6
4-16	0900	0.000	0.040	0.050	0.069	0.084	0.125
4-19	0900	0.000	0.058	0.077	0.102	0.128	0.200
	Mean	0.000	0.049	0.064	0.086	0.106	0.165

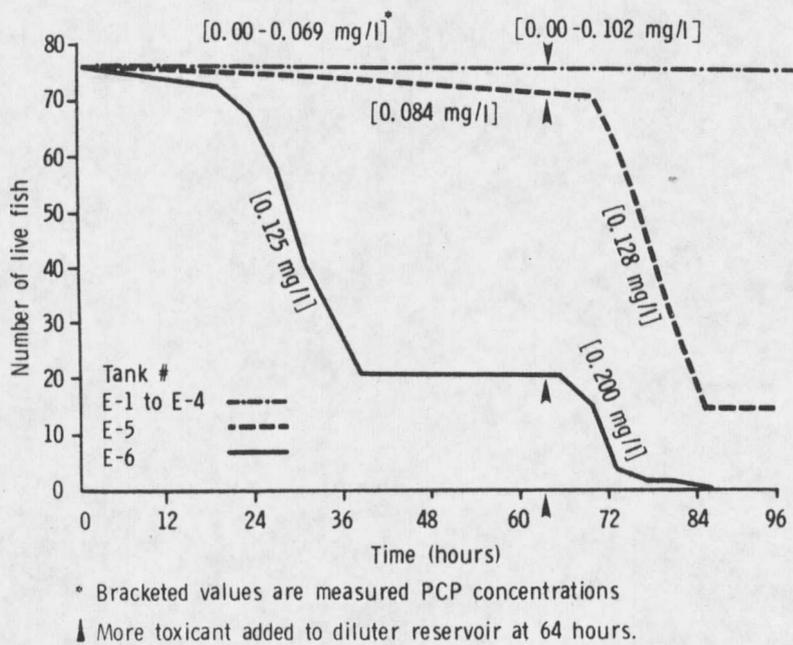


Figure 4. Mortality versus exposure time and PCP concentration.

began occurring once again at the highest exposure concentration (tank #6) and also began occurring at the second highest concentration (tank #5) (Figure 4).

Although these tests weren't intended to establish an LC 50 value for PCP to cutthroat trout, the value would be between 0.084 mg/l and 0.125 mg/l according to these results.

Clinical Signs

Clinical signs in cutthroat trout exposed to lethal concentrations of PCP included: 1) increased and labored operculation (respiration), 2) lethargy and swimming at water surface, 3) loss of equilibrium (twirling), 4) inability to maintain position in the water column (laying on the tank bottom) and 5) death.

Clinical signs in fish exposed to sublethal concentrations of PCP were limited to increased operculation (respiration).

Normal Histology

Gills: The trout gill consists of gill arches, gill filaments and gill lamellae. There are 4 gill arches under each operculum. Two rows of gill filaments are present on each arch and the lamellae branch from the filaments. The gill lamellae are the primary sites of respiration and also function in excretion, absorption, and osmoregulation. They are supplied with blood from the afferent filament artery via the afferent lamellar arterioles. Blood leaves the lamellae via efferent lamellar arterioles. The lamellae appear as leaflike projections that arise alternately from the upper and lower

surfaces of the gill filaments. The epithelium of the gill lamellae consists of a layer of overlapping and/or interdigitating squamous epithelium that is supported by a basement membrane and a thin layer of connective tissue. Contractile pillar cells support the lamellae and have flanges which surround the blood sinusoids. The lamellae are open, except for the pillar cells which separate one side of a lamellae from the other. Chloride cells, the primary osmoregulatory cell in fishes, are generally located at the base of and between the gill lamellae (28) (Figure 5).

Liver: Liver parenchyma in cutthroat trout is not divided into distinct lobules. It is composed of branching and anastomosing, two cell thick laminae of hepatocytes that are separated by distinct endothelial lined sinusoids. In cross section these may appear as rosettes of several cells radially arranged about sinusoids (Figures 6 and 7). Each hepatocyte has an irregular polygonal shape and most contain a single spherical nucleus with a distinct nucleolus. A normal complement of organelles, including mitochondria, endoplasmic reticulum, lysosomes, and golgi are easily recognizable (10) (Figures 8 and 9).

Hatchery reared salmonids typically have large amounts of glycogen within the hepatocytes that appear histologically as extensive

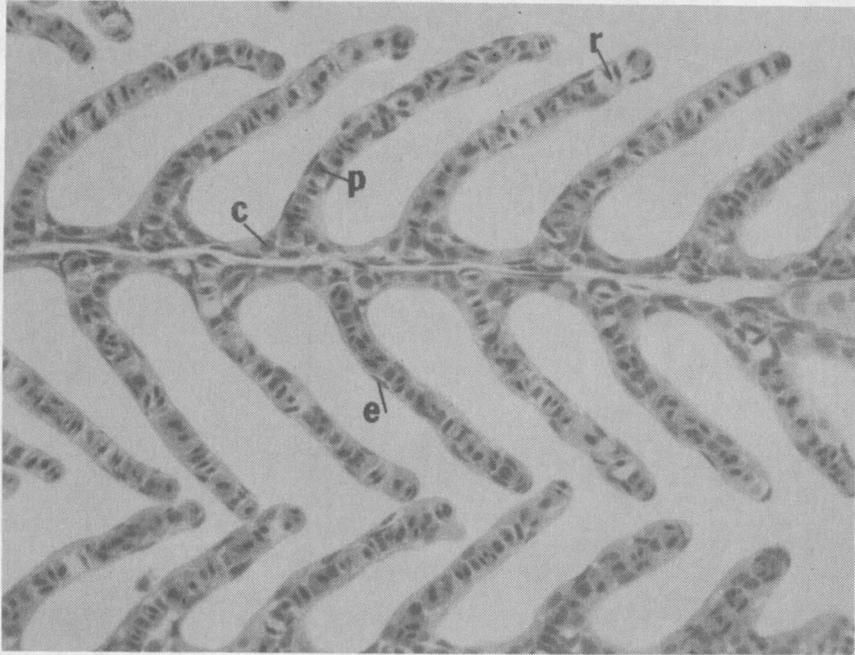


Figure 5. Light Micrograph (LM). Control gill from cutthroat trout. Gill lamellae projecting from gill filaments. (c) chloride cell, (e) squamous epithelium, (p) pillar cell, (r) nucleated red blood cell (rbc) in blood sinusoid. x 175, H & E.

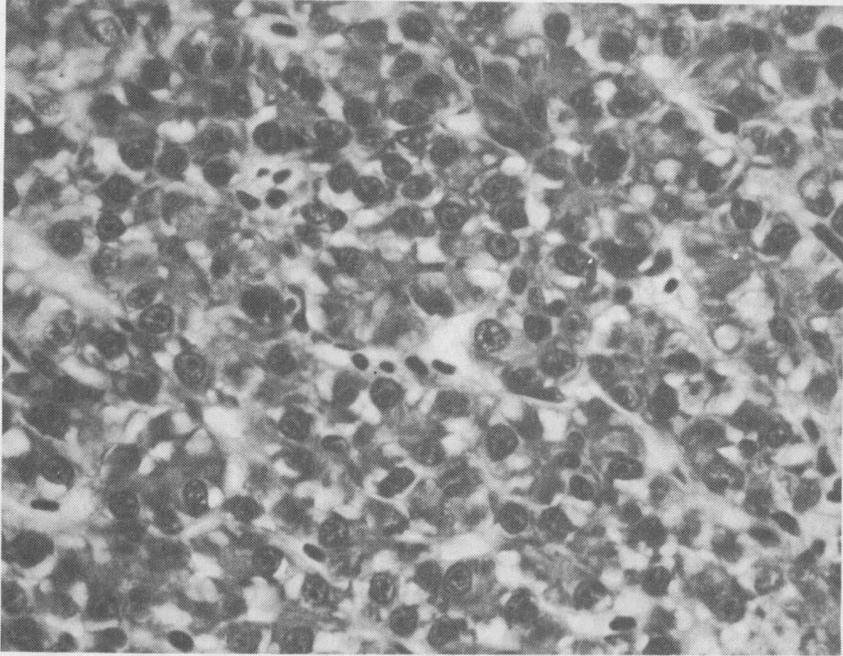


Figure 6. LM. Control (normal) liver. Cutthroat trout. Note: glycogen vacuolation and anastomosing laminae of hepatocytes. x 475. H & E.

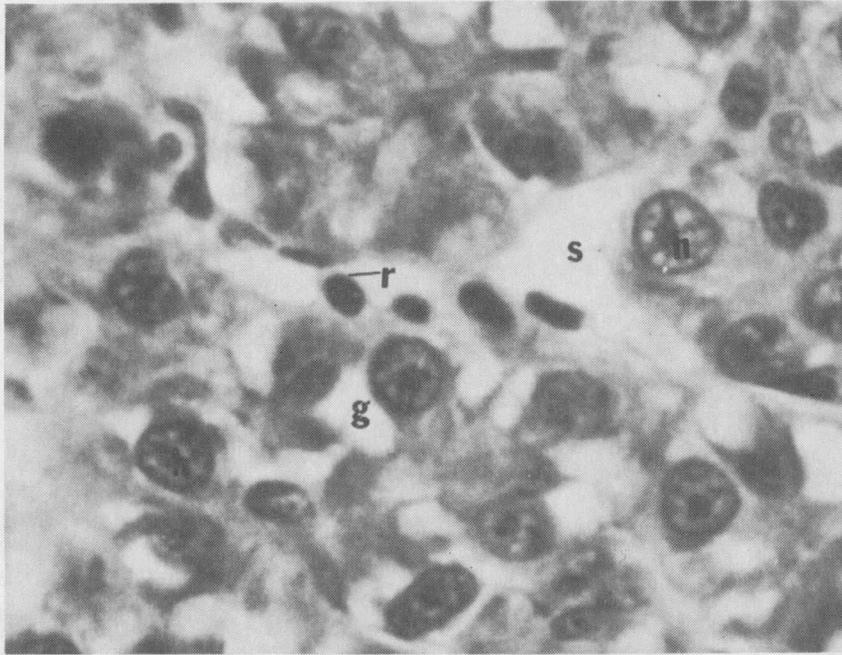


Figure 7. LM. Control (normal) liver. Note: glycogen vacuolation (g), hepatocyte nuclei (h), red blood cell (r) in sinusoid (s). x 1190, H & E.

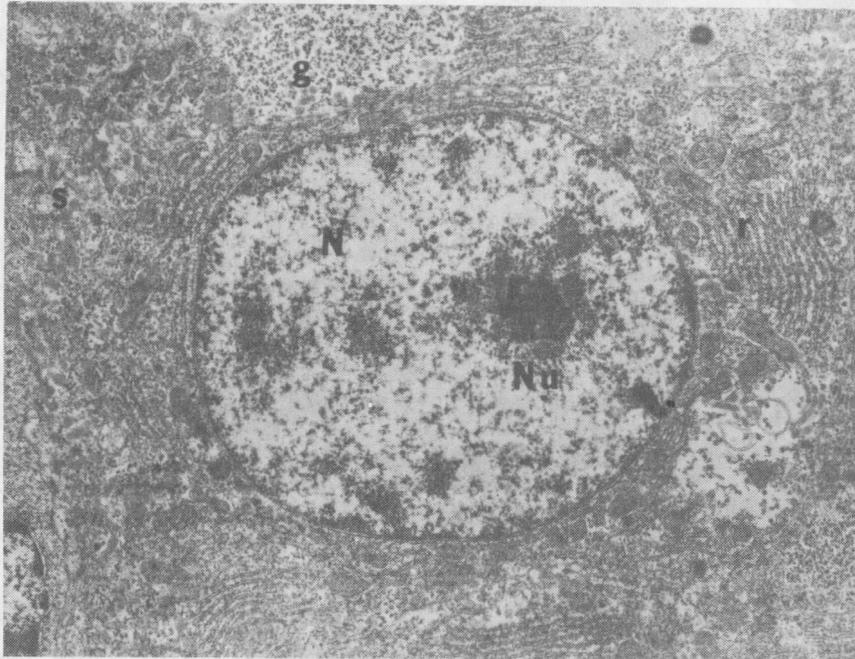


Figure 8. Transmission Electron Micrograph (TEM). Hepatocyte from a control cutthroat trout. (N) nucleus, (Nu) nucleolus, (g) glycogen particles, (r) granular endoplasmic reticulum (GER), (s) agranular endoplasmic reticulum. Many mitochondria are also evident. x 7,700.

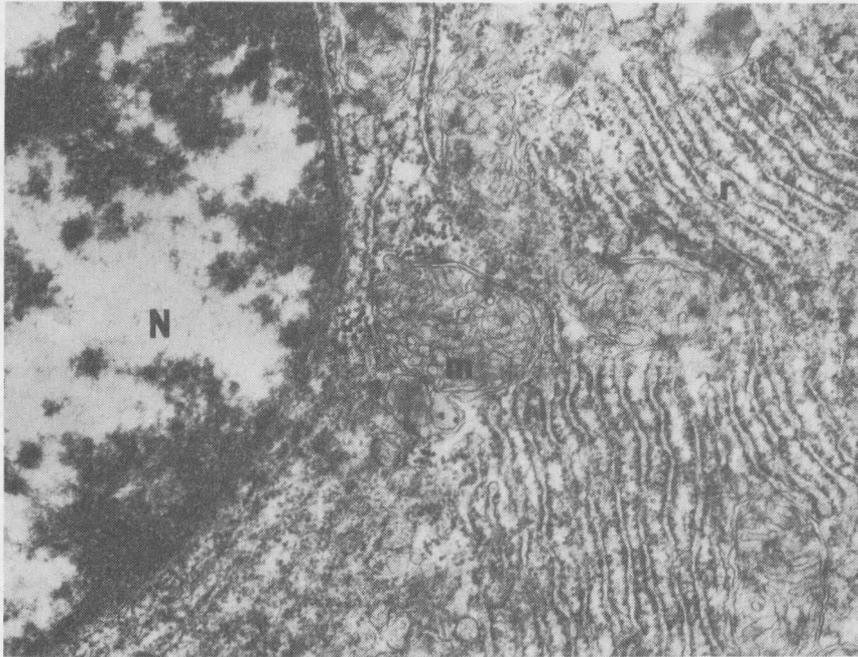


Figure 9. TEM. Control hepatocyte. (N) nucleus, (m) mitochondrion, (r) GER. Note: lack of nuclear pores, and attached ribosomes on GER. x 30,500.

irregular vacuolations in light microscopy and as electron dense bodies in electron microscopy (Figures 6 through 9).

Blood flows from branches of the hepatic portal vein and hepatic artery, through the sinusoids to central veins which empty into the hepatic vein. Hepatic triads are represented by distinct bile ducts, portal veins, and arteries. Bile canaliculi drain bile from hepatocytes. They are located within the laminae of hepatocytes and are formed by the close apposition of two to four hepatocytes. There are numerous microvilli extending into the lumen of each bile canaliculus. The hepatocyte surface facing the sinusoids has numerous microvilli projecting into the space of Disse. The space of Disse is separated from the sinusoidal lumen by fenestrated endothelium (10, 20).

Histology of PCP Exposed Fish

Gills: Gills from fish exposed to PCP (Figure 10) showed no consistent cellular alterations which could be considered a significant variation from control animals.

Liver: The livers contained cellular alterations which were interpreted to be PCP-induced. Changes included focal cytoplasmic vacuolation and degeneration, and dilation of sinusoids with apparent degeneration of sinusoidal endothelium (Figures 11 through 14). The liver of 1 fish showed extensive sinusoidal dilation (Figure 15).

Fish succumbing after exposure to PCP for longer time periods (2 to 4 days) had occasional necrotic cells as evidenced by karyorrhexis (Figure 16).

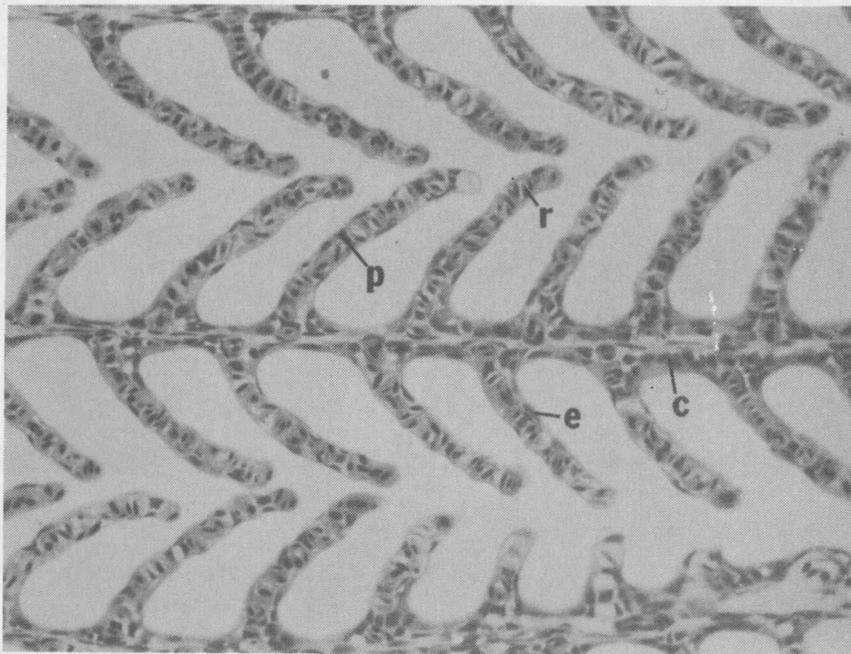


Figure 10. LM. Gill from a PCP-exposed fish. Gill lamellae projecting from gill filaments. (96 hours, 0.128 mg/l PCP [avg.]). (c) chloride cell, (e) squamous epithelium, (p) pillar cell, (r) rbc in lamellar sinusoid. x 175, H & E.

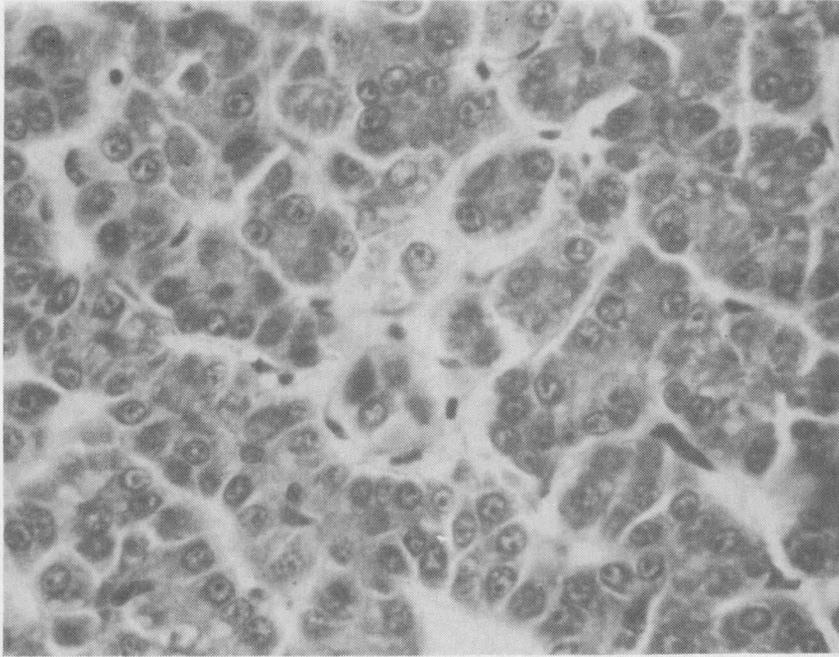


Figure 11. LM. Cutthroat trout liver from a PCP-exposed fish. (76 hours, 0.165 mg/l PCP [avg.]). Note: focal degeneration with slight sinusoidal dilation. x 475, H & E.



Figure 12. LM. Higher magnification of Figure 11. Note: cytoplasmic degeneration and apparent degeneration of sinusoidal endothelium. x 1190, H & E.

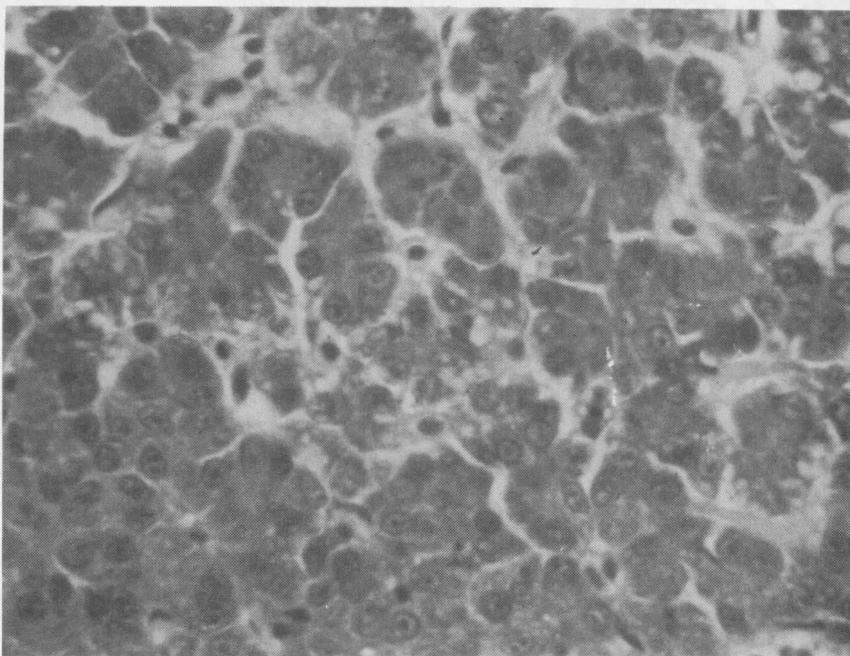


Figure 13. LM. Cutthroat trout liver from a PCB-exposed fish. (76 hours, 0.165 mg/l PCB [avg.]). Note: cytoplasmic vacuolation at center. x 475, H & E.

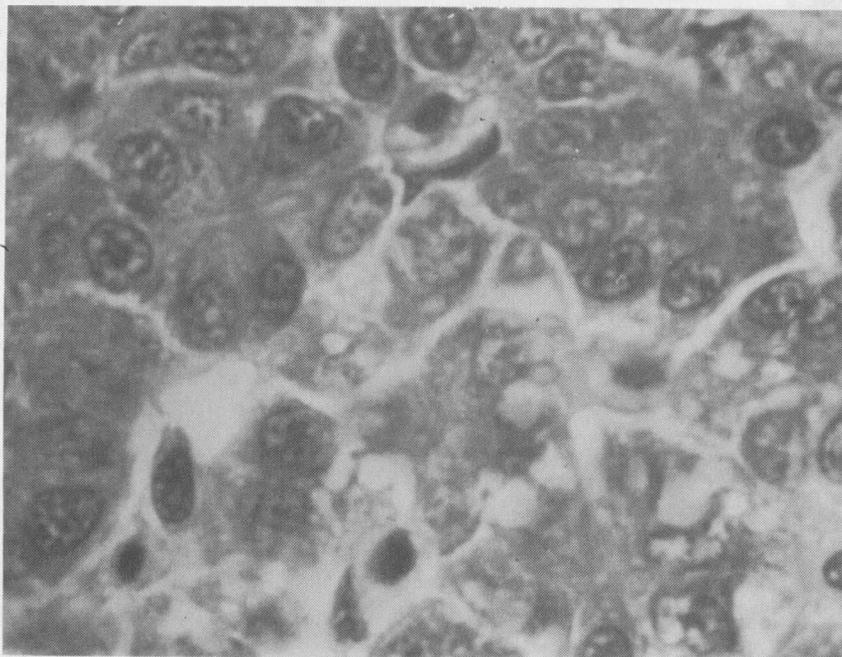


Figure 14. LM. Higher magnification of Figure 13. Note: cytoplasmic vacuolation and degeneration in lower portion of photo. x 1190, H & E.

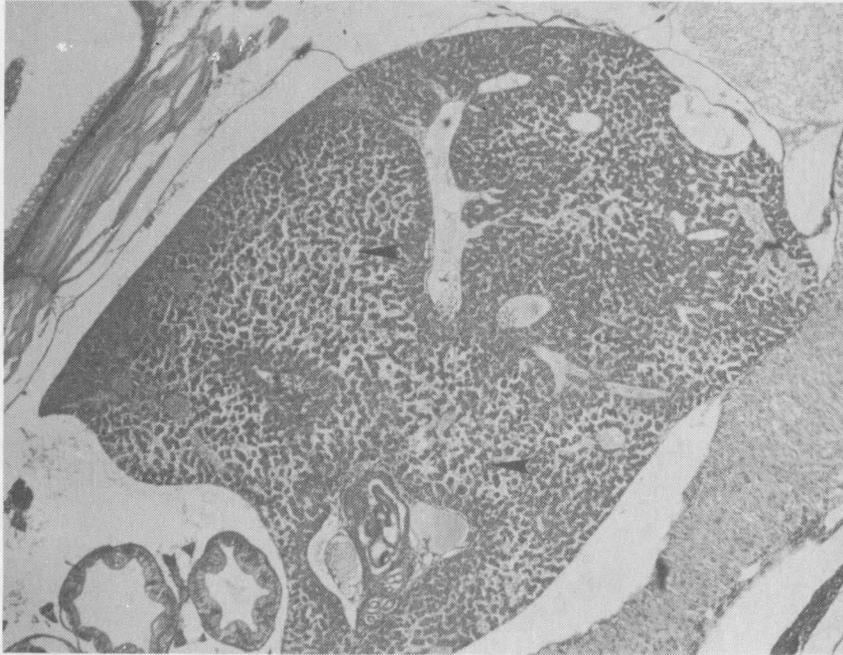


Figure 15. LM. Cutthroat trout liver from a PCP-exposed fish. (76 hours, 0.165 mg/l PCP [avg.]). Note: extensive sinusoidal dilation. (arrows) x 54, H & E.

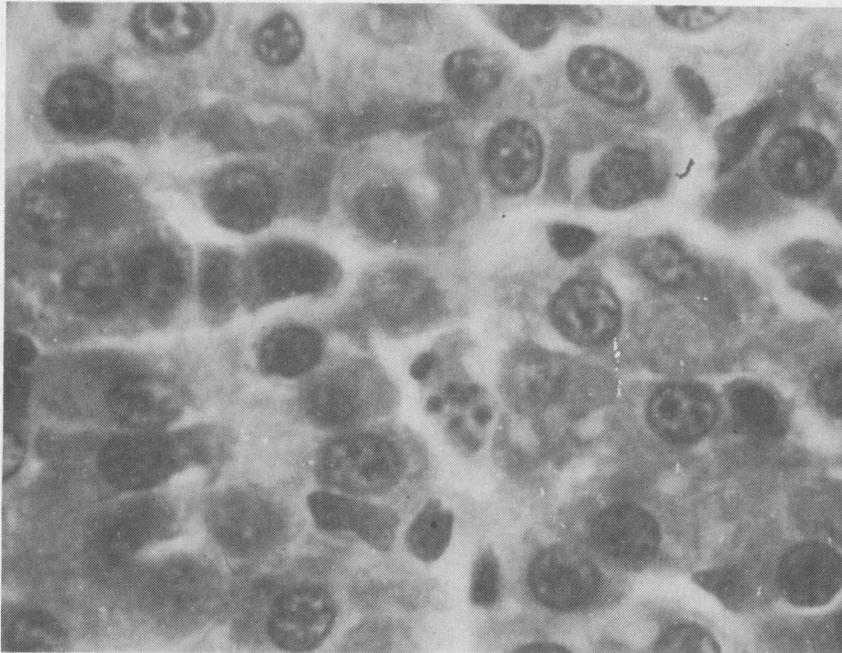


Figure 16. LM. Cutthroat trout liver from a PCP-exposed fish. (52 hours, 0.084 mg/l PCP). Note: necrotic cell at center. x 1190, H & E.

Ultrastructural observations in livers from PCP-exposed fish included: decreased glycogen stores, occasional swelling of mitochondria with mild to moderate increase in the granularity of the intramitochondrial matrix, and degeneration of mitochondrial cristae (Figure 17). The granular endoplasmic reticulum (GER) was consistently dilated and often the same was true for the perinuclear cisterna (Figure 18). Degranulation of the GER was also evident (Figure 19). In some cases electron-dense material was observed within the dilated GER and the perinuclear cisterna (Figure 20). Fish succumbing after the longer exposure periods, more than 24 hours, showed a consistent increase in the number of nuclear pores (Figure 21). Large autophagosomes were abundant in fish dying from PCP exposure (Figure 22). Hypertrophic microvilli or pleomorphic cell surface projections from hepatocytes were evident in the space of Disse. Membrane bound fragments similar in appearance and density to these projections were observed in the lumen of sinusoids (Figures 23 and 24).

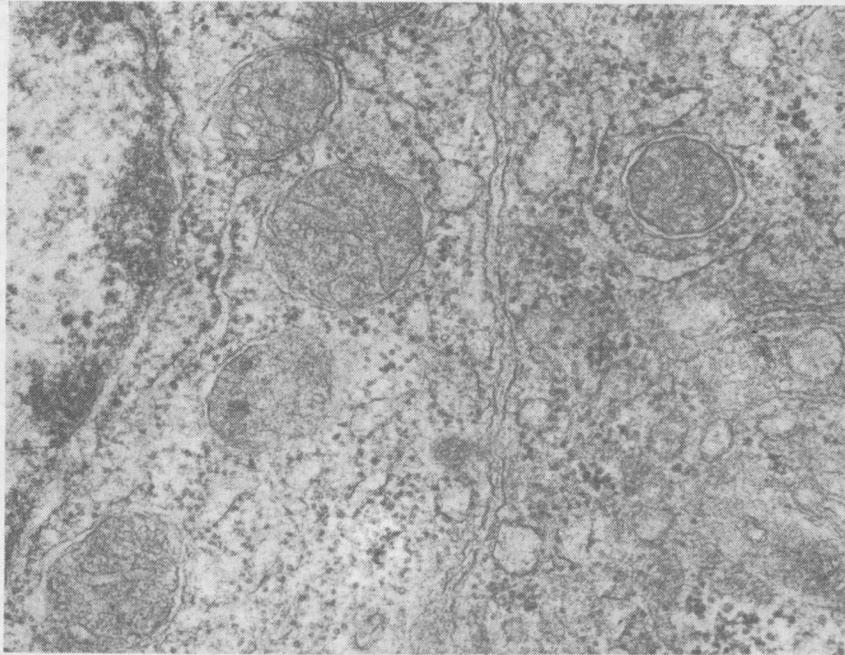


Figure 17. TEM. Hepatocyte from a PCP-exposed fish. (2.5 hours, 0.630 mg/l PCP). Note: increase in granularity of intramitochondrial matrix and apparent degeneration of cristae. x 42,000.

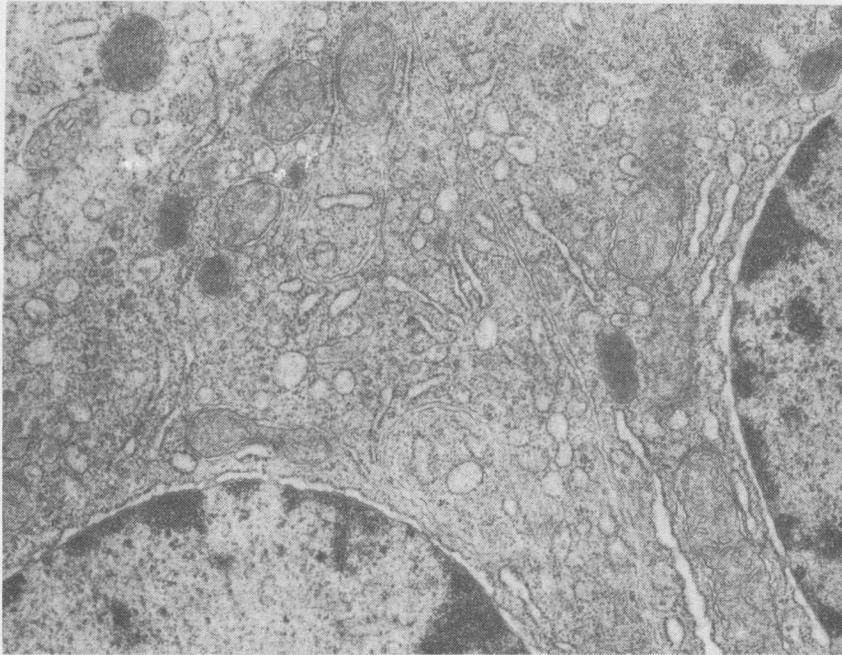


Figure 18. TEM. Hepatocyte from a PCP-exposed fish. (80 hours, 0.106 mg/l PCP [avg.]). Note: dilation of granular endoplasmic reticulum and perinuclear cisterna. x 19,600.

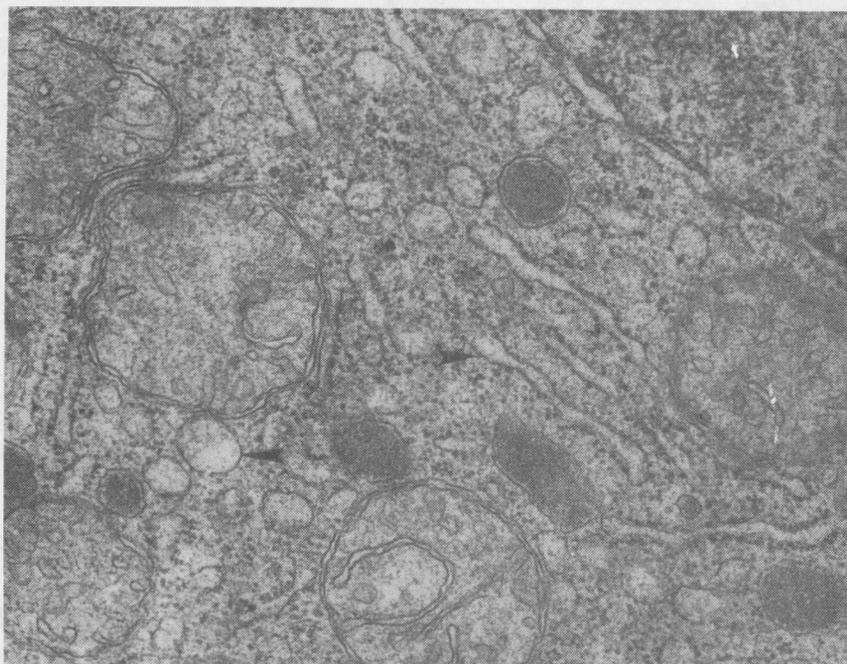


Figure 19. TEM. Hepatocyte from a PCP-exposed fish. (24 hours, 0.125 mg/l PCP). Note: degranulation of endoplasmic reticulum (arrows). x 37,700.

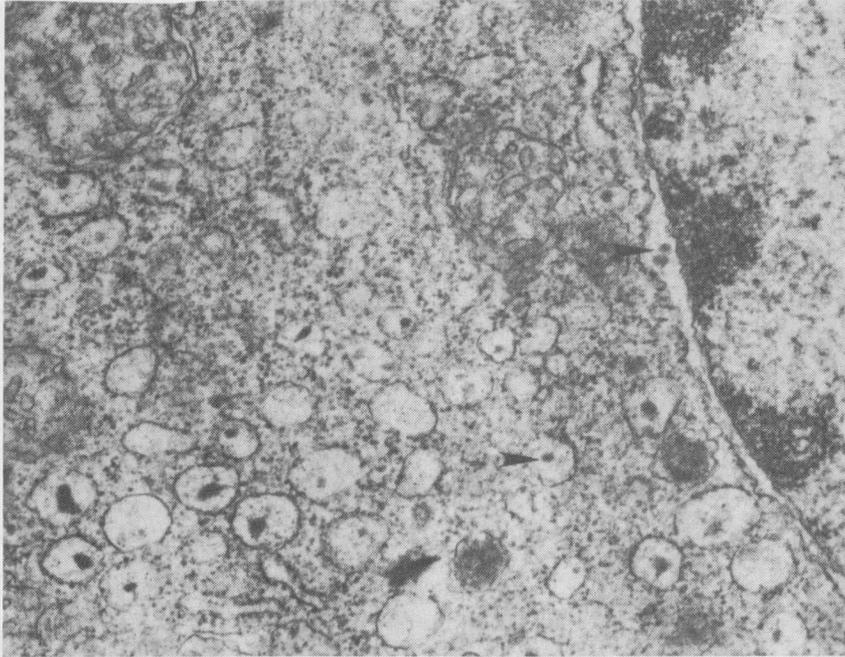


Figure 20. TEM. Hepatocyte from a PCP-exposed fish. (85 hours, 0.106 mg/l PCP [avg.]). Note: electron densities within dilated granular endoplasmic reticulum and perinuclear cisterna (arrows). x 43,300.

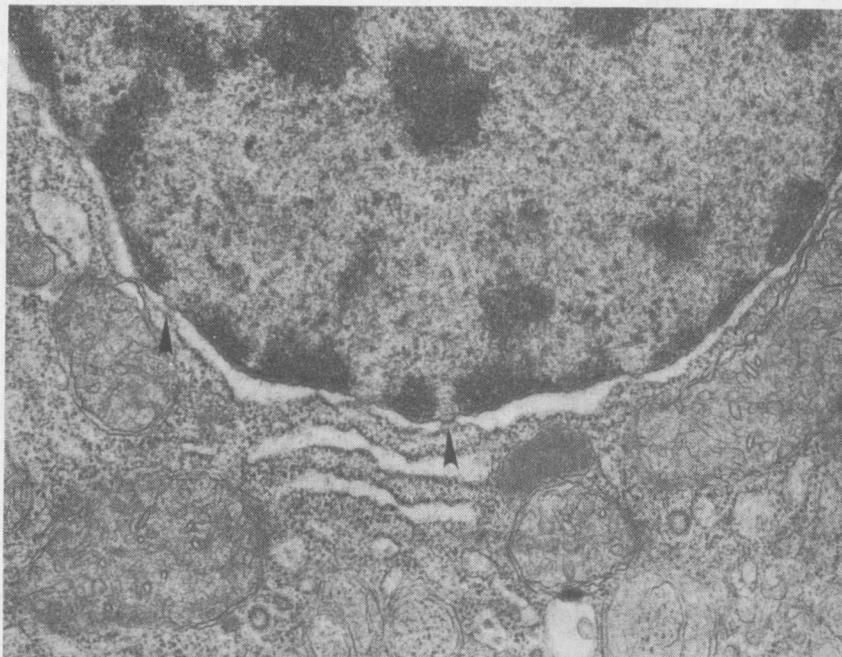


Figure 21. TEM. Hepatocyte from a PCP-exposed fish. (80 hours, 0.106 mg/l PCP [avg.]). Note: nuclear pores (arrows). x 23,800.

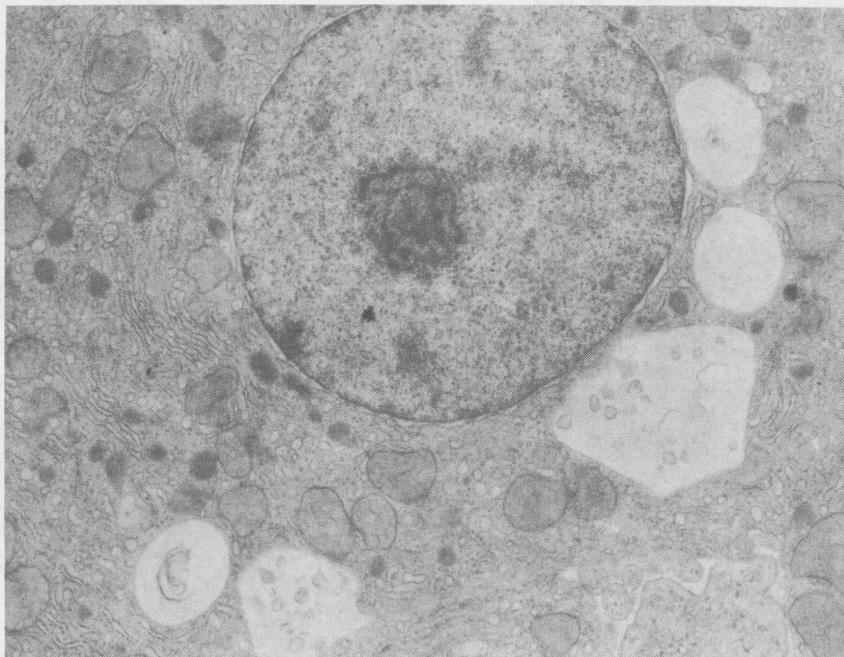


Figure 22. TEM. Hepatocyte from a PCP-exposed fish. (24 hours, 0.125 mg/l PCP). Note: autophagic vacuoles. x 7,700.

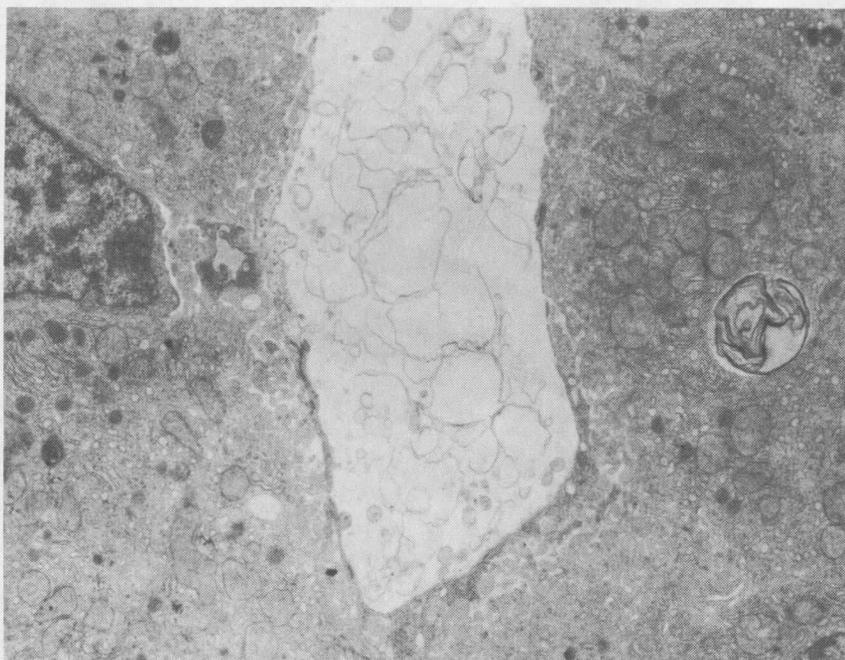


Figure 23. TEM. Hepatocyte sinusoid from a PCP-exposed fish. (24 hours, 0.125 mg/l PCP). Note: debris and membrane bound fragments within sinusoid. x 5,100.

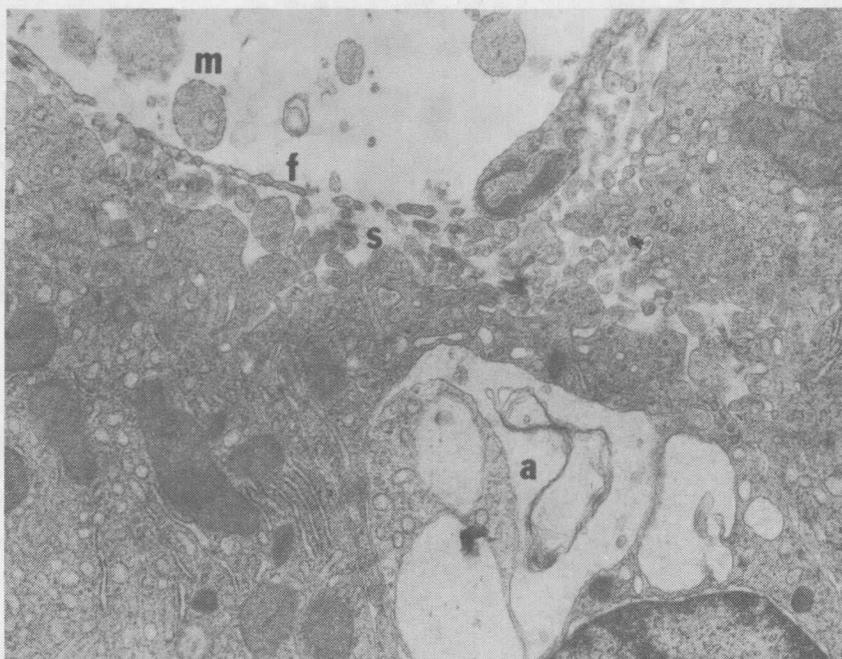


Figure 24. TEM. Hepatocyte and adjacent sinusoid from a PCP-exposed fish. (24 hours, 0.125 mg/l PCP). Note: autophagic vacuole (a), membrane bound fragments (m), fenestrated endothelium (f) and pleomorphic projections in the space of Disse (s). x 9,900.

CHAPTER 4

DISCUSSION

Pentachlorophenol is a powerful uncoupler of oxidative phosphorylation. It also binds with actomyosin and causes muscle fibers to shorten. In vitro studies with mitochondria have shown that PCP uncouples oxidative phosphorylation, inhibits mitochondrial and myosin ATP, inhibits glycolytic phosphorylation, inactivates respiratory enzymes, and damages mitochondrial structure (1, 12, 46). Any of the cellular alterations observed in this study could have been induced by an uncoupling of oxidative phosphorylation.

In the present study only the liver demonstrated cellular alterations which were considered induced because of exposure to PCP. Alterations such as hypertrophy of gill epithelium, and the presence in the liver of mitotic figures and megalohepatocytes were noted, but proved indistinguishable from controls.

PCP-induced alterations observed with light microscopy, including focal cytoplasmic vacuolation and degeneration, dilation of sinusoids with apparent degeneration of sinusoidal endothelium, and occasional necrotic cells, have been described in many studies involving the histologic effects of toxic substances (1, 19, 20, 21, 31, 32, 44). Few studies, however, have correlated changes at the light microscopic level with those observed ultrastructurally. The ultrastructural changes observed in the present study are as follows:

Glycogen: Glycogen is the polysaccharide that constitutes the storage form of carbohydrate in animal cells. It is broken down to yield glucose, and the enzymatic degradation of glucose in turn provides energy and also supplies short-chain carbon skeletons that are reused in the synthesis of various basic components of cytoplasm (15).

Glycogen stores were depleted or decreased in hepatocytes from PCP-exposed fish. This observation is probably directly related to the increased respiration observed in PCP-exposed fish.

Mitochondria: Evaluation of the ultrastructural morphology of mitochondria can be an important diagnostic tool in predicting mitochondrial function (11, 15, 39, 44). Altered function can at times be characterized by features such as: the loss of mitochondrial granules, a condensation of the mitochondrial matrix, swelling of mitochondria, and some dissolution of cristae. While these alterations are considered reversible they can be implicated in causing some functional loss of respiratory control and at least partial uncoupling of oxidative phosphorylation.

Alterations observed in hepatocyte mitochondria from PCP exposed fish included mitochondrial swelling, loss of mitochondrial granules, increased granularity of intramitochondrial matrix, and degeneration of mitochondrial cristae. Since PCP is considered to act as an uncoupler of oxidative phosphorylation, and mitochondria are the primary site of oxidative phosphorylation, some evidence of mitochondrial dysfunction was expected. It is of interest to note that altered mitochondrial structure appeared to be asynchronous. Both

normal and abnormal appearing mitochondria were observed within different regions of the same cell. In as much as fish may be affected differently by exposure to a toxicant, it could be anticipated that subcellular organelles may also be affected differently.

Endoplasmic Reticulum: Many types of cell injury involve changes in the ribosome-endoplasmic reticulum relationship. Detachment of ribosomes from the GER surface is an early, yet reversible, change induced by many chemical toxins. Interference with the ribosome membrane interactions also effects protein synthesis (44).

Some of the more obvious alterations seen in hepatocytes from PCP-exposed animals occurred in the granular endoplasmic reticulum (GER). These included dilation of the cisterna, and some degranulation. Dilation of the GER did not occur in hepatocytes from fish dying less than 8 hours after exposure to PCP. It is possible that a certain time period is necessary before PCP-induced altered GER morphology occurs.

At least three possibilities can be suggested to explain the acute PCP-induced dilation of the GER: 1) production of a protein entity necessary for conjugation, or for the transport of a biotransformed substance; 2) the increased demand for digestive enzymes associated with the increased number of autophagic vacuoles observed in hepatocytes; and 3) the production of replacement membrane proteins necessary to maintain homeostasis.

The endoplasmic reticulum is a continuous membrane-limited system of tubules and cisternae. In continuity with the GER is the agranular endoplasmic reticulum (AER). It is this latter region which is

credited with detoxifying or biotransforming xenobiotics within hepatocytes. It has been demonstrated that some xenobiotics (e.g., barbituates, halogenated hydrocarbons, and pesticides) which are metabolized within the liver are powerful inducers of drug metabolizing enzymes and thereby cause extensive hypertrophy and hyperplasia of the AER (11, 15, 16, 31, 44). This was not a morphologic feature in hepatocytes from PCP-exposed fish, possibly because of the limited duration of the tests.

Hypertrophy and hyperplasia of AER is considered to be a basis for drug tolerance (11, 16, 21). After mortality had subsided at a given concentration of PCP, an increase in the PCP concentration caused additional mortality (Figure 5). This, along with the lack of hyperplastic AER, suggests that development of PCP tolerance is not a critical factor in fish.

Nucleus- Nuclear Pores: The interaction of the nucleus and the cytoplasm is a two-way path that involves informational transfer of macromolecules. Messenger RNA molecules move from the nucleus to the cytoplasm and specific proteins move from the cytoplasm into the nucleus. Nuclear pores are demonstrated and identified by small areas of lower density that interrupt an otherwise continuous layer of heterochromatin. These are considered to be pathways of exchange between the nucleoplasm and cytoplasm (15).

The increased number of nuclear pores evident in hepatocytes from PCP-exposed fish suggests that increased communication between the nucleus and the cytoplasm may be important in toxicity. The apparent

increased informational exchange may be an important aspect of detoxification or the metabolizing process.

Perinuclear cisterna: As was the case for the granular endoplasmic reticulum, the perinuclear cisterna was dilated in hepatocytes from fish dying from exposure to PCP. Electron dense granular accumulations were observed in the GER and in the perinuclear space. Studies have shown that these accumulations first appear in the perinuclear cisterna and subsequently in the lumen of the GER. This suggests that the nuclear envelope participates early in the biosynthesis of new products (15).

Autophagosomes: Autolysosomes are a specific type of lysosome which function in the controlled autolytic elimination of organelles and/or inclusions, in the course of normal renewal or in response to an altered state of physiological activity (15). The rate of autophagocytosis increases markedly in many cases of sublethal injury. The gradual accumulation of autophagic vacuoles could lead to cell dysfunction and death.

The autophagic vacuoles observed in hepatocytes from PCP-exposed fish are most likely the intracytoplasmic vacuolations seen with the light microscope. These autophagic vacuoles may also be associated with the cytoplasmic degeneration observed. The autophagic vacuoles were only observed if fish had been exposed to PCP for a relatively prolonged period of time. Therefore, one could speculate that they might increase in number with longer exposure to PCP.

Microvilli or hepatocyte membrane: Membrane alterations occurring at the hepatocyte surface facing the hepatic sinusoids were unexpected. These alterations appeared to be due either to the hypertrophy and sloughing of the hepatocyte microvilli which project into the space of Disse or to the exocytosis (in an apocrine-like fashion) of some hepatocyte contents, as evidenced by the presence of membrane bound vesicles within the hepatic sinusoidal space. It is not known whether this is an exocytotic adaptation by which the cell is attempting to rid itself of PCP or a PCP product, or whether it is induced externally from the blood sinusoid.

These ultrastructural changes correlate well with the apparent degeneration of the sinusoidal endothelium observed at the light microscope level.

CHAPTER 5

SUMMARY

Cutthroat trout were exposed to progressive concentrations of pentachlorophenol (PCP) in two 96-hour acute toxicity tests. In the first test the PCP concentrations ranged from 0.630 mg/l to 0.192 mg/l and in the second test from 0.200 mg/l to 0.040 mg/l.

Light microscopic alterations in the livers included: focal cytoplasmic vacuolation and degeneration, dilation of sinusoids with apparent degeneration of sinusoidal endothelium, and some cell necrosis.

Alterations at the ultrastructural level included decreased glycogen stores, occasional swelling of mitochondria with some apparent increase in the granularity of the intramitochondrial matrix, and degeneration of mitochondrial cristae. The granular endoplasmic reticulum was consistently dilated, and often the same was true for the perinuclear cisterna. There was an increase in the number of nuclear pores in hepatocytes from exposed fish as compared to controls. Large autophagic vacuoles were abundant in hepatocytes from fish dying from PCP exposure. Either hypertrophic microvilli or pleomorphic cell surface projections from hepatocytes were evident in the space of Disse. Membrane bound fragments, similar in appearance and density to these projections were observed in the lumen of sinusoids, suggesting a sloughing of cellular material.

Most of the cellular alterations observed in this study are considered reversible, however, they may also be important indicators of toxicity. Histopathologic examination can be an extremely important adjunct of toxicity tests. The findings not only reveal pathologic alterations induced by specific toxicants but also secondary or unsuspected conditions which may otherwise go undetected. These aspects alone make routine histologic examination a valuable addition to toxicity studies.

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