COPPER AND ZINC POISONING IN BROOK TROUT (*Salvelinus fontinalis* Mitchell)

By

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INTRODUCTION

The realization that various base metals, including copper and zinc, could readily eliminate fish and other aquatic life has been known for at least a century (Penny and Adams, 1863). In that time, extensive efforts have been made by many investigators to determine maximum tolerated limits. The resulting literature has assumed massive proportions and diversity. Thorough reviews are readily available, however, which catalogue the literature on the toxicity of individual metals to individual fish (Ellis, 1937; Cole, 1941; Doudoroff and Katz, 1953; Jones, 1957, 1959; and Hynes, 1960); consequently, only accounts of investigations pertinent to the current study will be considered.

Two metals, copper and zinc, selected for this study because they are common pollutants. These metals occur not only in waters draining mine wastes but also commonly in the drainage from galvanizing plants, brass works, electroplating industries and certain synthetic plants (Klein, 1957).

The difficulty in determining safe maximum limits of heavy metals soon became apparent because of the great number of variables modifying the toxicity of each metal. It was readily realized, for example, that temperature, dissolved oxygen, pH, and numerous other factors comprising water quality criteria profoundly influenced the toxicity of metal ions. Further investigation revealed that two or more metals would often interact in such a way as to produce a toxicity greater than the sum of the two applied individually (synergism) or could tend to neutralize the toxic effect of each other (antagonism).

Doudoroff (1952) found copper and zinc acted synergistically on minnows (Pimephales spp.). Ellis (1937) noted that calcium and sodium acted antagonistically towards copper on goldfish (Carassius auratus L.). Jones (1938) observed calcium to act antagonistically toward lead and zinc salts on three-spined sticklebacks (Gasterosteus aculeatus L.).

Defferential sensitivity among species of fish was noted by Carpenter (1927) and confirmed by Ellis (1937), but in spite of attendance to these variables, almost no attention was paid to the age or size of the fish tested.

Shelford (1917) pointed out that the injurious effects of a specific fish toxicant should be studied at the weakest stage in the life cycle. Cole (1941) reiterated the same attitude stating:

> "Ecologically it is immaterial how long adults can live in a given concentration of a pollutant is such a concentration prevents the eggs from hatching or stops development of fry or fingerlings."

Certain age or size-based variations in reaction to non-metallic toxicants have been reported, however, only two investigations are known to have made any reference to age variation in response to heavy metals. One of the investigations (Grandell and Goodnight, 1962) makes only passing reference to the problem as an observed, but unexplored, factor. The other report (Jones, 1938) specifically concerns itself with the problem but suffers from inability to maintain a control group and the adoption of uncertain analytical technique.

Aqueous tar extract (phenol, naphthalene, cresol, pyridine, etc.) tolerance in brown trout decreased with age from alevin to yearling, and spermatozoa, ova and zygotes appeared especially resistant (Gardiner, 1927). Carbon dioxide and sulfite waste tolerance had been similarly observed to decrease with age (Wells, 1913; and Nightingale, 1931; respectively, cited by Cole, 1941), however, gas liquors and certain drugs (nicotine and digitaline among them) were tolerated better by adult than juvenile fish (Shelford, 1917; and Sollman, 1905; respectively, cited by Cole, 1941).

Jones' (1938) results indicated that the survival times of young three-spined sticklebacks (18-20 mm) were appreciably longer than for adult sticklebacks when subjected to identical lethal lead solutions. However, as a result of rapid mortality in the adult control fish and his unusual technique for comparing age-based tolerances, his conclusions were to the contrary. In addition, he (Jones, 1939) later confuses matters with an unexplained statement that, "Very small fish proved somewhat less resistant" (than adults), perhaps referring to results from his earlier publication. Crandell and Goodnight (1962) also observed that for two mg. per l. lead sulphate, 5.0 mg. per l. zinc sulphate, or 0.2 mg. per l. sodium pentachlorophenate a higher mortality appeared in immature guppies (Lebistes reticulatus L.) than adults.

However, both the discrepancies and limited range of these investigations indicated clearly that further experimental study was required

to resolve the relation between age and tolerance to heavy metals. Brook trout (Salvelinus fontinalis Mitchell) were used in this study of age-based tolerance to copper and zinc solutions.

In addition, certain discrepancies surrounded the cause of lethal poisoning by heavy metals. The publications of Carpenter (1925, 1927, 1930), Behrens (1928), Dilling, Healey and Smith (1926), Jones (1935, 1938, 1939, 1947), Ellis (1937) and Westfall (1945) all attribute mortality to asphyxia produced by muco-metallic filming of the branchial epithelium. Ellis (1937) tempered his discussion of the cause of mortality by suggesting that in addition to mucous filming, intracellular protein precipitations of the branchial cells might occur. Schweiger (1957) and Parry (1960) found, from histological studies, that heavy metal salts elicited swelling and necrosis of the branchial epithelium, frequently, with desquamation. Neither observed any indication of an increase in the activity of mucous cells. With this disparity in the type of branchial damage, revised histological examinations of the gill platelets were made in this study using yearling brook trout.

Until recently (Saiki et al, 1958; Hibiya and Oguri, 1961; Joyner, 1961) contention surrounded the possibility that heavy metal ions penetrated the body tissues in significant quantities. Carpenter, (1927) could find no trace of lead within the bodies of lead nitrate poisoned minnows after washing them in dilute acetic acid, ashing and then analysing the remains colorimetrically. Behrens (1928), however,

using radio-isotopic lead noted some penetration into internal tissues but considered it insignificant relative to branchial damage. White and Thomas (1912) reported the absorption of copper, and Thomas (1924) the absorption of nickel from sea water by the killifish, Fundulus heteroclites. Dawson (1935) reported tissue damage after extended periods of chronic lead poisoning. His observations included secondary anemia followed by large numbers of immature erythrocytes (erythroblasts) in the general circulation. Associated with the anemia were excess deposits of pigment in the liver, spleen and kidneys (opisthonephros) presumed to be from phagocytosed erythrocytes. Predominant in leukocyte changes were large increases in numbers of monocytes and eosinophiles and the appearance of large numbers of atypical "spindle cells" which Dawson believed may have been of monocytic origin. Other tissue changes reported were a marked proliferation of erythropoietic sites in the cardiac endothelium and formation of large clusters of spindle cells on the surface of the ventricular trabeculae.

Increases in the iron and hemoglobin content of rats subsequent to trace copper ingestion is well known, however, no comparable reports are known for fish immersed in copper or any other metal. Elvehjem and Sherman (1932) found that young rats could be made anemic by feeding only cow's milk, following early weaning (21 days). Iron reserves in the spleen and liver were severely depleted by the rapid animal development and resulted in reduced hemoglobin. Administration of inorganic iron for two weeks quadrupled the levels of hepatic iron

and it doubled the splenic iron but effected less than a five-percent increase in hemoglobin. Administration of 0.05 mg. of copper per day, however, for two weeks increased the hemoglobin concentration over 60 percent and reduced both hepatic and splenic iron concentrations below the anemic level. In addition, in the absence of copper, the hepatic iron accumulation and storage were proportional to iron intake while hemoglobin remained unchanged. When copper was added, however, the hemoglobin formation was roughly proportional to iron intake while hepatic iron concentration remained unchanged until 0.3 mg. was fed daily after which it increased in proportion to the iron intake. Elvehjem and Sherman conclude that copper catalyses the conversion of inorganic iron into hemoglobin. Total iron determinations were performed on the blood of chronic copper and zinc-poisoned yearlings in order to observe whether or not these ions, if absorbed, produced similar effects to the above.

The concentration and accumulation of zinc in tissue has been the subject of several investigations. Feaster et al (1955) determined the major areas of distribution of the zinc radio-isotope (Zn^{65}) in adult rats which indicated the greatest accumulations occurred in the kidney followed in turn by the liver and then the pancreas. Wakely et al (1960) made similar observations with male rats but found the dominant area of deposition to be the prostate, which contained up to 10 times the Zn^{65} concentration of the other tissues. Rapid elimination of peritoneally injected zinc in the fecal

matter led Wakely et al to agree with the conclusions of Sheline et al (1943) and Montgomery et al (1943) that it was excreted into the digestive tract. Very little zinc was found excreted in the urine. Saiki et al (1958) observed active excretion of Zn^{65} in the intestine of the medaka, Oryzias latipes. Similar results were reported from air-bladder injected goldfish, Carassius auratus L., except that high intestinal Zn^{65} concentrations were observed (Hibiya and Oguri, 1961). Appreciable accumulations occurred in the liver, pancreas, air-bladder and pronephros; slightly smaller deposits occurred in the opisthonephros, spleen, gills and vertebrae; still smaller quantities occurred in the muscle and gonads. No apparent variations were observed between male and female gonads. Observations made on the brown bullhead immersed in zinc solutions, containing Zn^{65} as a tracer, (Joyner, 1961) confirmed the results of Hibiya and Oguri that the greatest zinc accumulations are in the gastro-intestinal tract. He (Joyner) found, however, that kidney and gill accumulations contained only slightly less of the isotope after 96 hours immersion. Accumulations in the liver and spleen were extensive but only about half as active as the gut, kidneys or gills. Muscle and bone Zn^{65} activity was less than one-tenth and one-eightieth respectively of the gut. In order to determine whether zinc absorption occurred predominantly in the gills or the gut, Joyner compared tissue Zn^{65} accumulation in normal bullheads and those whose esophagus had been plugged with paraffin. Zinc accumulation in the tissue of fish with plugged esophagus was practically identical to accumulation in corres-

ponding control animals, however, gill Zn^{65} concentration was noticeably lower in animals with the plugged esophagus. Overall, therefore, these results indicate zinc absorption occurs almost entirely in the gills. Another important observation (Joyner, 1961) was the initially rapid gross uptake of zinc for a period of less than twelve hours, followed by a very reduced absorption thereafter. This phenomenon suggests one of two causes: a) absorption continues until reaching a saturation level in the tissues beyond which an equilibrium is established; b) about twelve hours is required to produce a physical change, such as cloudy swelling of the branchial epithelium which reduces permeability to heavy metals.

The absence of other investigations concerned with the actual pathology of chronic zinc and copper poisoning in fish, rather than simply heavy metal accumulations, led to the preparation of certain tissues for histological examination in this investigation. Liver, kidney (opisthonephros), spleen, heart and stomach from yearlings in lethal and sublethal solutions of copper and zinc were examined. Total erythrocyte counts were taken from fingerlings subjected to sublethal solutions of copper and zinc for 11 days and from yearlings in sublethal solutions for 21 days.

1. Survival In Zinc and Copper

(i) Experimental Fish

With the exception of a very small number of yearlings*, all trout were reared from the eyed-egg and maintained, until required for acclimation, in the Whiteshell trout hatchery, operated by the Fisheries Branch, Dept. of Mines and Natural Resources, Province of Manitoba. The eyed-eggs were obtained by the Hatchery from the Ontario government's Dorion hatchery (Port Arthur) or the privately operated hatchery of Mr. K.G. Drew (Spokane, Washington). The thermal history of the eggs until received by the Whiteshell hatchery is not known.

During the holding period, all groups of fish were fed a dry biscuit preparation of an appropiate size every other day and liver sausage once weekly.

The ages of the three groups tested, early fry, young fingerlings, and yearlings are accurately known because of their hatchery confinement:

Early Fry - young fish, two to six weeks past egg-sac absorption, average weight 0.12-0.15 grams.

Young Fingerlings - fingerlings in July and August that were hatched in February and March, held at 5°C. (or less) until late May, thereafter, in water rapidly increasing in temperature to 15°C. about mid-July. Average weight 2.15-2.29 grams.

Yearlings - trout, twelve to eighteen months beyond hatching. Average weight 6.8-8.8 grams.

(*) A small number of yearlings were seined from Stoney Creek, three miles east of Bethany, Manitoba. All specimens caught were either hatchery reared or descendents of hatchery stock.

(ii) Acclimation

Acclimation of the fish to temperature and to the test water were both performed prior to commencement of the tests.

Temperature acclimation was carried out first, with maximum temperature changes of one-half degree Centigrade per day from hatchery temperature (6 - 10°C.) to 10°C., the test temperature. This was followed by a holding period of one week at 10°C. for fry and three weeks or longer for fingerlings and yearlings.

The fish were maintained in ten-gallon aquaria immersed in refrigerated constant-level water baths thermostatically controlled to maintain the temperature at 10°C $\pm$ 0.5°C. Tap water cooled by passage through a long pyrex coil, immersed in the water bath, continuously entered the aquarium. The aquarium overflow syphoned into the water bath.

The fish were acclimated to the soft water (72 p.p.m. T.D.S.) used in test solutions. Trout to be utilized were transferred, 24 hours prior to an experiment, to a mixture of three parts tap water to one part deionized water, then they were transferred to the test water, a mixture of one part tap water to one part deionized water, for a period of at least three days.

(iii) Survivorship

Tests were carried out in static aquaria, the solutions of which were changed once daily. Aquaria of three types were utilized, one for each age group. Fry were tested in 600 ml. pyrex beakers filled to the five hundred milliliter level. Fingerlings were tested in polyethylene

aquaria containing four liters of test solution. Yearlings were tested in eight-liter polyethylene aquaria maintained at six liters.

Constant aeration supplied each aquarium with compressed air that had been bubbled through water. No filtration mechanism was used because of the danger of its constituents absorbing metallic ions from solution.

Temperature was controlled at 10°C. ± 0.5°C. either by immersion of aquaria in the refrigerated water coolers described previously or in a constant-temperature room at 10°C.

Preliminary establishment of upper limits for concentrations of metallic ions was derived from data in Jones (1938) and in Doudoroff and Katz (1953). Maximum concentrations were chosen from those which Jones observed to cause complete mortality in sticklebacks after one day. A series of test concentrations was chosen between this level and the concentration estimated to leave only 70 percent survival at the termination time of 264 hours. Testing was continued until four or more trials were within these limits and at least one more showed a survival greater than 70 percent at the end of testing. Each age group in each metal, therefore, consists of six or more trial groups. A control group for each battery of tests (metal) was also maintained.

(iv) Solutions and Solution Preparation

Stock solutions were prepared by weighing specified amounts of reagent grade copper sulphate ($CuSO_4.5H_2O$) and zinc sulphate ($ZnSO_4.7H_2O$).

Test solutions were prepared by diluting aliquots of stock solution to final test volumes. Water used for dilutions was maintained at 10°C. in

100-gallon reservoirs. Tap water was examined daily for chlorine by the orthotoluidine Flash Test Method (Standard Methods, APHA pp. 91-92) but at no time was it detectable during acclimation or test periods.

Table I

Mean Weight of Trout per liter of Test Solution

Age Group	Metal Series	Number of Fish/Test	Number of Fish/Liter	Mean Weight/Liter
Fry	Copper*	10	20	0.12 grams
Fingerlings		10	2.5	2.3
Yearlings		6	1	6.8
Fry	Zinc*	10	20	0.15
Fingerlings		10	2.5	2.2
Yearling		6	1	8.8

(*) includes the weight of fish in the control solution

Diluent (one part tap to one part deionized water) was accumulated in adequate quantity at one time to supply all aquaria for a complete series of tests. Since diluent was of uniform quality, conductance, alkalinity (methyl-orange test), hardness (EDTA for calcium and magnesium, and also for

calcium alone) determinations were made only once for each series (Table II). Hydrogen ion activity was determined on a Beckman Zeromatic pH meter once for each test concentration immediately after its preparation.

Table II

Experimental diluent quality criteria utilized in copper and zinc survivorship tests. Diluent properties determined were alkalinity (methyl orange test), total dissolved solids (electrical conductivity), EDTA hardness (EDTA and Eriochrome Black T indicator), calcium hardness (EDTA and Murexide indicator); pH determinations were made on final test solutions.

Series	Alkalinity (ppm $CaCO_3$)	Conductivity (mhos per cc)	EDTA Hardness (ppm Ca CO_3)	Calcium Hardness (ppm Ca CO_3)	pH
Zinc	41.1	83.3	56.8	29.8	7.6 ± 0.2
Copper	42.1	83.3	57.5	30.6	7.6 ± 0.2

(v) Handling and Observing Fish

Following introduction of experimental fish into the appropriate aquaria the fish were examined every twelve hours for mortality. Cessation of opercular movement and failure to respond to prodding were the criteria employed to establish mortality. Dead animals were immediately removed, dried on filter paper, weighed and the time of death recorded as the inspection time.

On alternate days immediately prior to daily renewal of test solution, the fish were fed dry pellets as quietly as possible. About thirty minutes later the fish were rapidly transferred to fresh solutions.

(vi) Analysis of Observations

In all tests the measure utilized for comparison of the tolerance of the different age groups was the 70 percent survival time, or S_{70}; that is the time required to reduce survival in a particular test population to 70 percent of its original. The S_{70} for any particular concentration in each age group was determined by calculated interpolation in a line derived by the method of least squares of individual survival times.

S_{70} values were used rather than S_{50} (the usually recorded statistical value) because calculation of confidence intervals was considered imperative and was not possible from the data recorded. Individual survival times for the last 40 percent of the population were almost invariably greatly in excess of the extropolated values indicated by the first 60 percent, consequently, these last results were discarded. Without data for the last 40 percent of the animals in each test, no confidence intervals could be determined below the S_{50} mean.

II. Histological Studies

(i) Acute Copper and Zinc Poisoning

(a) Branchial and Visceral Preparation

Specimens observed in this part of the study were yearling speckled trout only. It was assumed that, although the sensitivity of the different age groups might vary appreciably, the anatomical site, or sites, of the heavy metal poisoning would remain the same.

A rudimentary continuous flow apparatus (Fig. 1) was used in this portion of the study. The diluent was supplied directly from a deionizing unit at a constant rate and an aspirator bottle dripped a constant volume of heavy metal and calcium chloride standard solutions into this water. The resulting control solutions indicated an average electrical conductivity equal to 5 ppm as calcium chloride. Three ppm of this was calcium chloride, deliberately added in the aspirator solution.

Six groups, of two trout per group, were studied. Two of these groups were exposed to zinc solutions at concentrations of 1 mg per l. and 50 mg. per l., solutions which caused total mortality within 48 and 8 hours respectively. Two groups were exposed to copper solutions at concentrations of 0.1 mg. per l. and 10 mg. per l., solutions resulting in total mortality within 54 and 6 hours respectively.

Gill, liver, spleen, kidney (opisthonephros), and heart tissues were removed from these animals usually an hour or less after death and fixed in Zenker's solution.

Figure 1.

Constant Flow Apparatus (Acute Lethal Toxicity)

A) Tap Water Line - Inflow

B) Penfield Deionizing Unit

C) Constant Level Diluent Reservoir - Float Controlled

D) Constant Temperature Cooling Bath - Contains Diluent Cooling Coil

E) Diluent Flow Control Valves

F) Aquaria

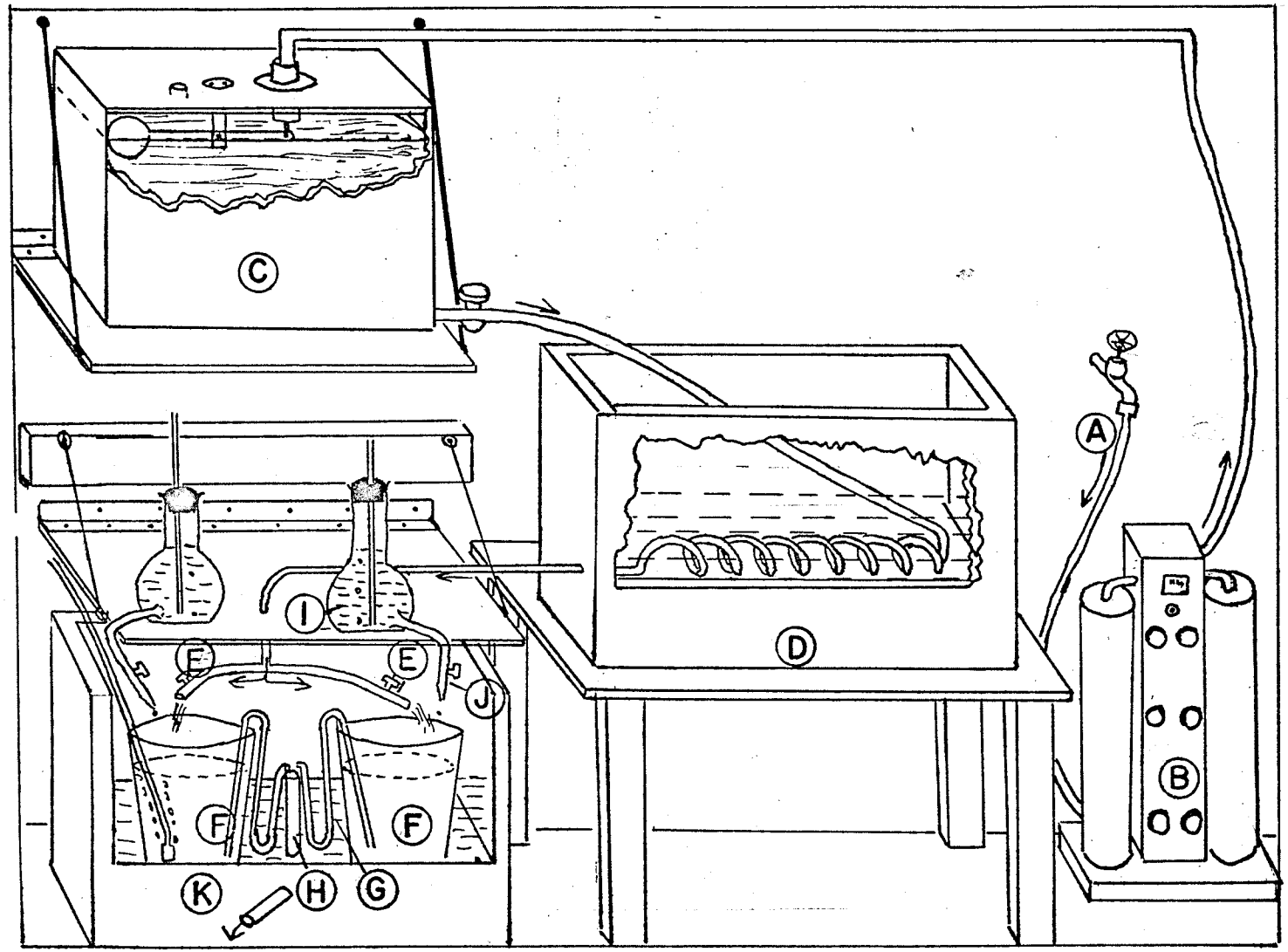
G) Constant Level Aquarium Effluent Syphon

H) Constant Temperature Bath Overflow Standpipe

I) Aspirator Constant Flow Bottles - Contains Standard Solutions of Toxicant

J) Toxicant Flow Control Valves

K) Constant Temperature Bath - For Aquaria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Branchial cell sections were subsequently cut at 6μ and stained by periodic acid Schiff (PAS) reaction for demonstration of mucous cells and mucoid secretions (Sawyer, 1959), and with Harris' hemotoxylin for general histological detail.

The other tissues were cut at 8μ, stained with Harris' hemotoxylin and counterstained with eosin.

Trout from the remaining two groups were used as control animals. Experimental conditions for these fish varied from those of the test fish in only one respect; the aspirator toxicant solution contained no heavy metal ions. Gill, liver, spleen, kidney, and heart tissues were removed from the controls and prepared histologically as outlined above.

(ii) Chronic Copper and Zinc Poisoning

(a) Acclimation

The fish had previously been held in hatchery water at approximately 11°C. with maximum fluctuations of $\pm$ 2°C. Test temperature was, therefore, set at 11° $\pm$ 0.5°C. so that no temperature adjustments were required. All fish were held at this temperature for 10 days prior to testing.

Acclimation to test water was considered unnecessary since the dechlorinated tap water used in this portion of the experiment has approximately the same conductivity as the hatchery water in which they were reared.

(b) Maintenance Technique

Yearling trout were maintained in sublethal solutions of zinc and copper and a control solution of diluent (dechlorinated tap water), in order to investigate some aspects of sublethal pathology. Tap water diluent quality is recorded in Table III.

Figure 2.

Constant Flow Apparatus - (Chronic Poisoning)

A) Activated Charcoal Filter

B) Constant Head Reservoir - Diluent

C) Diluent Flow Regulating Mechanism

D) Standard Toxicant Reservoirs - Equipped With Air Lift Solution Pumps and Constant Head Overflow Chamber

E) Toxicant Flow Regulating Mechanism

F) Diluent, Toxicant Mixing Tanks - Equipped with Electric Stirrers

G) Test Aquaria

H) Control Aquaria

I) Drain

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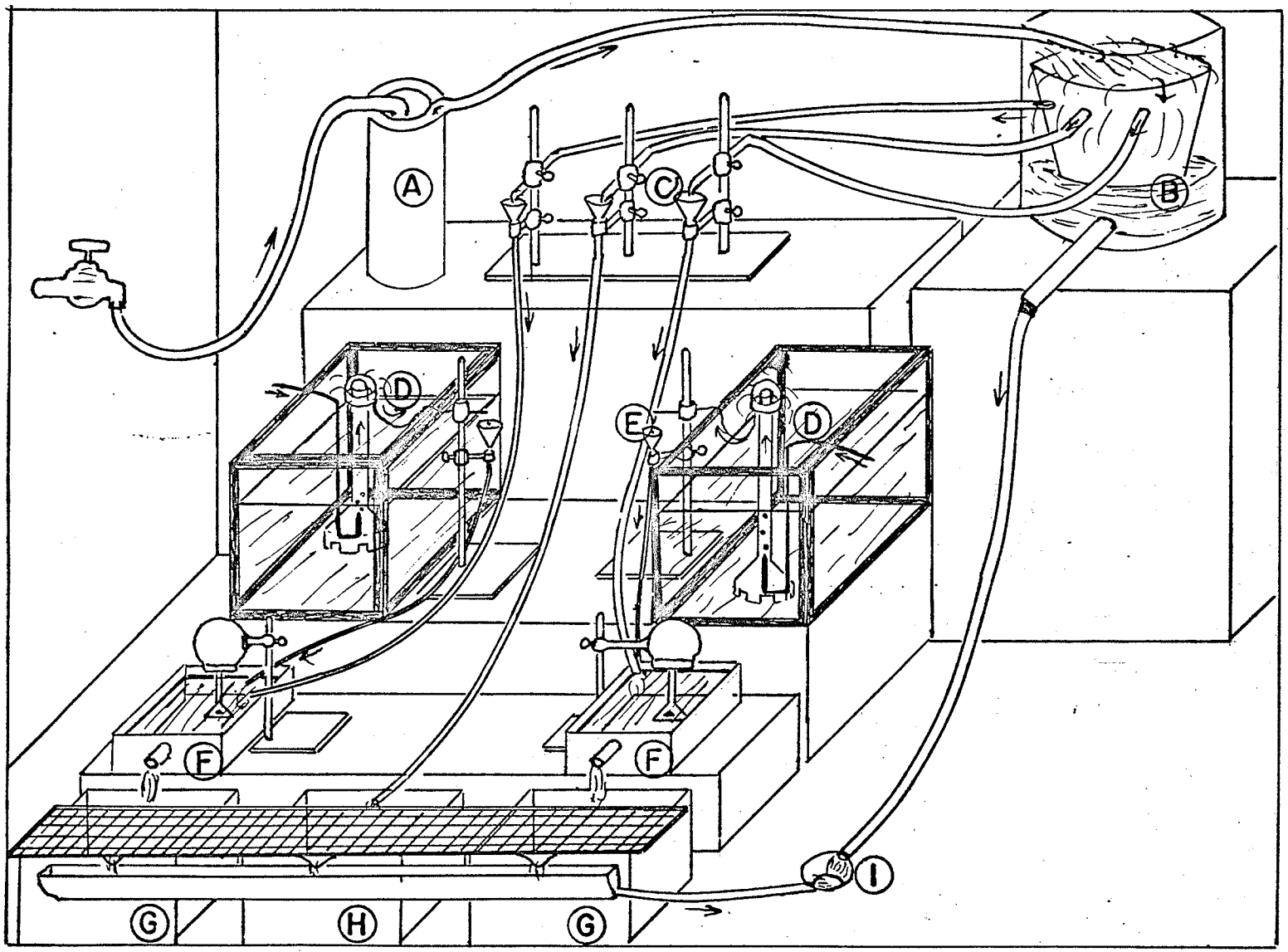


diluent flow-regulating mechanisms (Fig. 2C) of the individual aquaria. Each flow-regulator consists of:

1) a pyrex pipe held by a clamp on a laboratory stand. The height of the pipe being adjustable along the length of the stand. Raising and lowering the pipe raises and lowers the hydrostatic pressure within the pipe.

2) a funnel and tygon hose assembly, similarly mounted on the stand, to catch the diluent and carry it to the respective mixing tank.

A sixty liter reservoir (Fig. 2D), set above the level of each aquarium, provided concentrated copper or zinc sulphate solutions at a rate of about 2 ml. per minute. Solution from these reservoirs was piped to the mixing tanks (Fig. 2F) by the metal salt solution flow-regulators (Fig. 2E). The salt solution regulators consisted of:

1) an air-lift pump continuously lifting an excess of metal solution to a constant level overflow dish. The base of the dish connects on the inner wall with a catheter tube. The peripheral end of the catheter tube passes out of the reservoir to the second part of the apparatus, the capillary tube.

2) the capillary tube is mounted in a clamp in a laboratory stand, which can be raised or lowered to control the drip rate. A small funnel mounted below the capillary tube catches the metal solution and connects to a tube carrying the solution to the mixing tank.

Salt and diluent solutions were stirred with electric mixers in the mixing tanks. Overflow from the mixing tanks was carried to the aquaria (Fig. 2G) at about 100 ml. per minute. Each aquarium was provided with an overflow pipe which drained from the bottom.

Preliminary flow of diluent and concentrated salt solution was established by adjusting each to about 100 ml. per minute and 2 ml. per

minute, respectively, with 500 ml. and 10 ml. volumetric flasks and a stopwatch. Final adjustments were made by regulating the flow of stock solution of the salt until a satisfactory concentration, determined by standard quantitative analysis, was determined.

Zinc in water was determined by the dithizone method according to "Standard Methods for the Examination of Water and Waste Water", A. P. H. A., 11 ed. pp. 265 - 268.

Copper in water was determined by means of Sodium Diethyl-dithiocarbonate modified from Thresh, Beale and Suckling; "The Examination of Waters and Water Supplies",7 ed. p. 229 (Appendix).

When test aquaria were filled, and at suitable concentrations, about a dozen yearlings were introduced into each. Thereafter, zinc and copper analyses of the water were performed every other day. On the fifteenth day, salt reservoir levels were replentished to about their original height by addition of 175 mg. per l. zinc and 12.5 mg. per l. copper.

All groups were moved once each week to a separate aquarium, allowed to rest about one and one half hours after the transfer, and then fed a dry piscine pellet preparation mixed with equal amounts of liver sausage. The fish were allowed about a half hour for feeding before they were returned to the test aquarium.

After the first two days of test, aquaria were illuminated 24 hours per day. For the first two days the lights were turned off automatically at 8:00 p.m. and on at 8:00 a.m., however, at some period between 9:00 p.m. and 9:00 a.m., more fish jumped from the aquaria than throughout the rest of the day.

At the conclusion of the 21-day test period, the yearlings were anaesthetized individually in 2 percent aqueous solution of amyl alcohol.

(c) Branchial and Visceral Studies

The same organs previously examined from fish in the acute poisoning namely, gills, liver, kidney, spleen and heart, plus the stomach (not previously examined) were removed from three animals in each test solution and fixed in Zenker's fixative. Paraffin blocks were prepared in the conventional series of water, graded alcohols, xylene and paraffin. Sections were subsequently cut at six (gills only), seven, or eight μ. Harris' hemotoxlin and eosin were the stains utilized in the examination of all sections for histological variation.

(d) Hematological Study

(1) Fingerlings

In this section of the study all surviving fingerlings from the survivorship tests were used for individual erythrocyte counts. The trout were weighed, anaesthetized with Sandoz M.S.$_{222}$, and the peduncle cleanly removed with a sharp scalpel. Blood from the dorsal aorta was collected in a pocket of a procelain spotplate. To prevent coagulation, several crystals of the disodium salt of ethylene diamino-tetra-acetic acid (EDTA) were placed in each pocket. From the spotplate the blood was sucked to the 0.5 mark (0.5 mm^3) on a Thoma blood diluting pipet for erythrocytes and diluted to one part in 200 with Hendrick's diluting fluid.(Appendix). Routine erythrocyte counts were made on bright line improved Nebaeur hemocytometers without the necessity of a stain. The usual group of eighty squares counted in routine clinical R.B.C. (red blood cell) determinations (Bull. A. O. Spencer 'Bright Line Hemocytometer' p. 12) was

followed in each of the two chambers. A mean erythrocyte count, for each test solution, was calculated from the individual constituent counts.

(2) Yearlings

The fish of this study were the yearlings maintained for 21 days in 2.9 mg. per l. zinc and 0.3 mg. per l. copper and with tap water as diluent. Total iron, erythrocyte count and hematocrit determinations were required from each individual. Blood was collected as above by sectioning the caudal peduncle and dipping the trunk into a polyethylene cup containing crystals of EDTA. Hematocrit samples were taken first by allowing micro-hematocrit tubes (75 mm x 1.5 mm dia.) to fill to about three-quarters capacity and sealing them with wax. Centrifugation was performed in an International Micro-Capillary centrifuge at 11,500 R.P.M. for five minutes. The packed red cell lengths and cell and serum length were each measured on a millimeter rule to compute hematocrit.

During centrifugation, 0.5 mm^3 of blood was collected in a Thoma blood pipette and diluted to one part in 200 with Hendrick's diluting fluid. After a thorough shaking the first 15 drops in the pipette were discarded. Small drops were placed in the wells of the Nebauer hemocytometers and the chambers allowed to fill by capillary action. Allowing the blood cells a few minutes to settle, provided an opportunity to add 0.02 ml. (20.λ) of blood to a test-tube containing 0.2 ml. of water. The blood was picked up in an ultra-microsyringe continuously calibrated from 1-100 λ. Before injecting the blood into the test-tube, the tip was thoroughly wiped around its outside surface. The blood was expelled directly under the surface of the water to avoid any loss on the sides of the tube. These samples were set aside until three to four accumulated at which time they

were examined photometrically with a blank and a standard. The same syringe was used for all iron determinations. After each sample was expelled, the syringe was flushed eight to 10 times with fresh Hendrick's diluting fluid, blown dry with compressed air, flushed twice again and dried.

When the iron sample had been taken, the hematocrit was ready for measurement and the erythrocytes ready for counting. As in the case of the fingerlings, hemoblasts (Catton, 1951) were included as erythrocytes in blood counts. The routine clinical technique of counting 80 squares was again followed. Both sides of the counting chamber were counted for each fish. Erythrocyte counts per cubic millimeter were calculated as follows:

$$\text{No.} = \frac{\text{No. R.B.C.'s counted} \times \text{dilution factor} \times 4000}{\text{No. of small squares counted}}$$

for 1:200 dilution and 80 small squares counted, the formula reduces to:

$$\text{No.} = \frac{\text{No. counted} \times 200 \times 4000}{80} = 10{,}000 \times \text{No. R.B.C.'s counted}$$

Total iron was determined by the clinical micromethod of Naltelson (1957), with only minor modifications.(Appendix).

(e) Histochemical Studies

Spleen, liver, and kidneys from all specimens except the nine used for histological material were discarded before the need to determine their iron and copper content was realized. Consequently, the potassium

ferrocyanide test for ferric iron and the rubeanic acid (dithio-oxamide) test for copper had to be modified from qualitative to rough quantitative histochemical assays; each is described below.

(1) Potassium Ferrocyanide Determination of Tissue Iron

Liver, spleen and kidney sections of at least two different animals per copper or zinc solution were compared with sections from at least two controls. The sections, prepared for histological examination as above, were brought to water through xylene and alcohols and then immersed in a freshly prepared one percent acidified potassium ferrocyanide solution at room temperature for thirty minutes. The potassium ferrocyanide solution was prepared as follows: 100 ml. distilled water, one ml. concentrated hydrochloric acid and one gram potassium ferrocyanide. Sections were timed carefully in the ferrocyanide solution so maximum immersion time variation was $\pm$ 10 seconds between any control and test group. Following staining, sections were rinsed in distilled water, dehydrated in alcohols and cleared in xylene, all as a battery. Permount mounting medium was added and number one cover glasses were placed over the sections.

The Prussian blue density of several representative fields of view at 400X were recorded of each tissue section measured with a Science and Mechanics cadmium-sulphide, photo-electric meter. The wave-length was adjusted to about 500 mμ by means of a dark-green filter placed immediately over the sub-stage illuminator of a Wild M-20 microscope. The light intensity, falling on each section, was maintained at 8 volts input to the tungsten lamp and was not observed to fluctuate. To record this light intensity, the cadmium-sulphide probe of the meter was flatly secured over the microscope eye-piece and the room light extinguished to

eliminate any extraneous light being recorded. On this basis, according to Beer's law, the iron concentration is proportional to the reciprocal of the galvanometer deflection. The fields of view recorded were areas in which very little, if any space, was unoccupied by tissue. The tissue was observed to be fairly homogeneously stained although at 400X some cellular sites appeared to be more densely stained than other areas.

(2) Rubeanic Acid Determination of Tissue Copper

The same Zenker fixed paraffin sections were prepared for copper examination, prior to any staining. Sections of spleen, kidney, and liver from copper, zinc and control solutions were taken down to distilled water with xylene and alcohols and then immersed in a fresh aqueous solution of one percent rubeanic acid at 36°C. (oven) for 24 hours. Sections were all removed at the same time $\pm$ 20 seconds, dehydrated, cleared and mounted in permount with number one coverslips. The sections were mounted on the stage of a microscope containing a Whipple eye-piece at 400X magnification.

When Whipple cells contained a definitely discernable green-black rubeanate particle, they were considered occupied. Three representative areas on each slide from each of two specimens held in zinc, copper or control solutions were counted and averaged to provide a rough estimate of tissue copper.

RESULTS

I. Survival in Zinc and Copper Poisoning

(i) Tolerance and Resistance

Age-based variations in susceptibility to fatal zinc or to fatal copper poisoning were found among early fry, small fingerlings and yearlings. Data relating seventy percent survival time, "S_{70}", to the corresponding causative metal ion concentrations form the basis of the comparison. Seventy percent survival time is used as a measure of resistance.

Resistance, or resistance time, as described by Fry (1947) and as employed here, describes the time interval ('Effective Time', or survival time) that an organism endures a lethal environment. Although it need not be a straight-line relationship, resistance time is related to the intensity of the modifying factor, in this case, heavy metals. Tolerance, as suggested by Fry (1947) and used here, represents the range (lower incipient lethal to upper incipient lethal level) of a factor within which an organism can exist without shortening of its lifespan. In the current tests, survival of the test population to the 264-hour test termination time constitutes tolerance.

(a) Age-Based Variation to Zinc

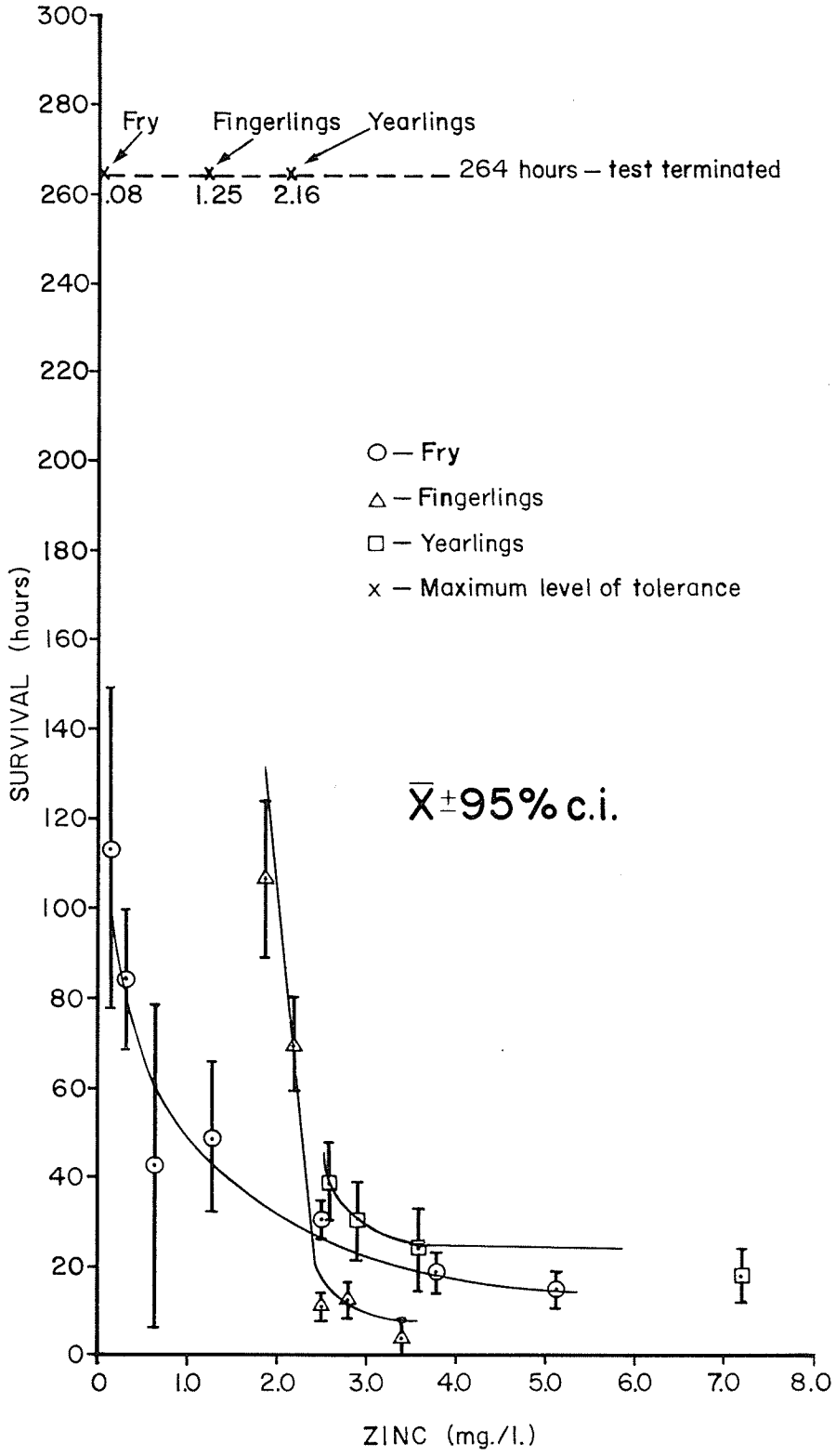
Figure 3, Table IV, shows that young fry are considerably less tolerant to zinc solutions than are fingerlings and yearlings. A concentration of 1.25 mg. per l. produced an S_{70} of 49 hours in fry, but was tolerated by fingerlings. Similarly, a concentration of 2.16 mg. per l.

Table IV

Influence of age on survival time of brook trout in experimental zinc solutions. Experimental error calculated at the level of the 95 percent confidence interval (c.i.)

Group	No. Fish	Concentration (mg/l Zn)	S_{70} a/ (hours)	95% c.i.
Fry	6	Control	264 b/	
	6	0.16	113.71	77.8 - 149.6
	6	0.32	84.42	68.8 - 100.0
	6	0.64	42.28	6.4 - 78.1
	6	1.28	49.39	32.7 - 66.1
	6	2.50	30.63	26.4 - 34.9
	6	3.84	18.63	14.4 - 22.9
	6	5.12	14.63	10.4 - 18.9
Fingerling	6	Control	264 b/	
	6	1.25	264 b/	
	6	1.88	106.63	89.2 - 124.1
	6	2.20	69.86	59.5 - 80.2
	6	2.50	14	8 - 14
	6	2.80	12.45	8.6 - 16.3
	6	3.40	8.00	0 - 8
Yearling	4	Control	264 b/	
	4	2.16	264 b/	
	4	2.52	39.12	30.4 - 47.9
	4	2.88	30.48	21.8 - 39.2
	4	3.60	24.48	15.8 - 13.2
	4	7.20	24.00	12 - 24

a/ Seventy percent survival time
b/ Most or all animals surviving at test termination (264 hours)


300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
SURVIVAL (hours)
Fry
Fingerlings
Yearlings
264 hours – test terminated
.08
1.25
2.16
○ – Fry
△ – Fingerlings
□ – Yearlings
x – Maximum level of tolerance
X̄ ± 95% c.i.
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
ZINC (mg./l.)

did not produce mortality in yearlings but caused an S_{70} in fingerlings in approximately 80 hours and in approximately 35 hours in fry.

Although tolerance to zinc is lowest in fry, resistance is lowest in fingerlings at concentrations greater than 2.5 mg. per l. The survival curve for fingerlings is almost linear and nearly vertical between 1.9 and 2.8 mg. per l. and within this range resistance to zinc increases extremely rapidly with minute decreases in concentration. Above 2.8 mg. per l., there is an inflection in the curve indicating that changes in concentration produce lesser changes in survival time or resistance. Fry and yearlings roughly parallel one another in S_{70} at concentrations greater than 2.5 mg. per l. Furthermore, the 95 percent confidence interval indicates the two could be identical. Above 2.5 mg. per l., the slope is gentle for yearling and fry indicating that resistance increases slowly with decreasing concentration. This slope is maintained by the fingerling curve to about 1.3 mg. per l. Below 1.3 mg. per l., extreme increases in resistance appear with minor decreases in concentration. The yearling curve turns upward sharply from 2.5 mg. per l. as is indicated by tolerance to zinc at 2.2 mg. per l.

(b) Age-Based Variation to Copper

Differences in tolerance and in resistance with age are recorded in Figure 4, Table V. Fry were considerably less tolerant of the various test solutions of copper ions than were fingerlings and yearlings. Yearlings appear to be slightly less tolerant of copper than fingerlings. Fingerlings tolerated 0.15 mg. per l. copper ion. Yearlings were not tested at this concentration but their resistance time at the mutually

Table V

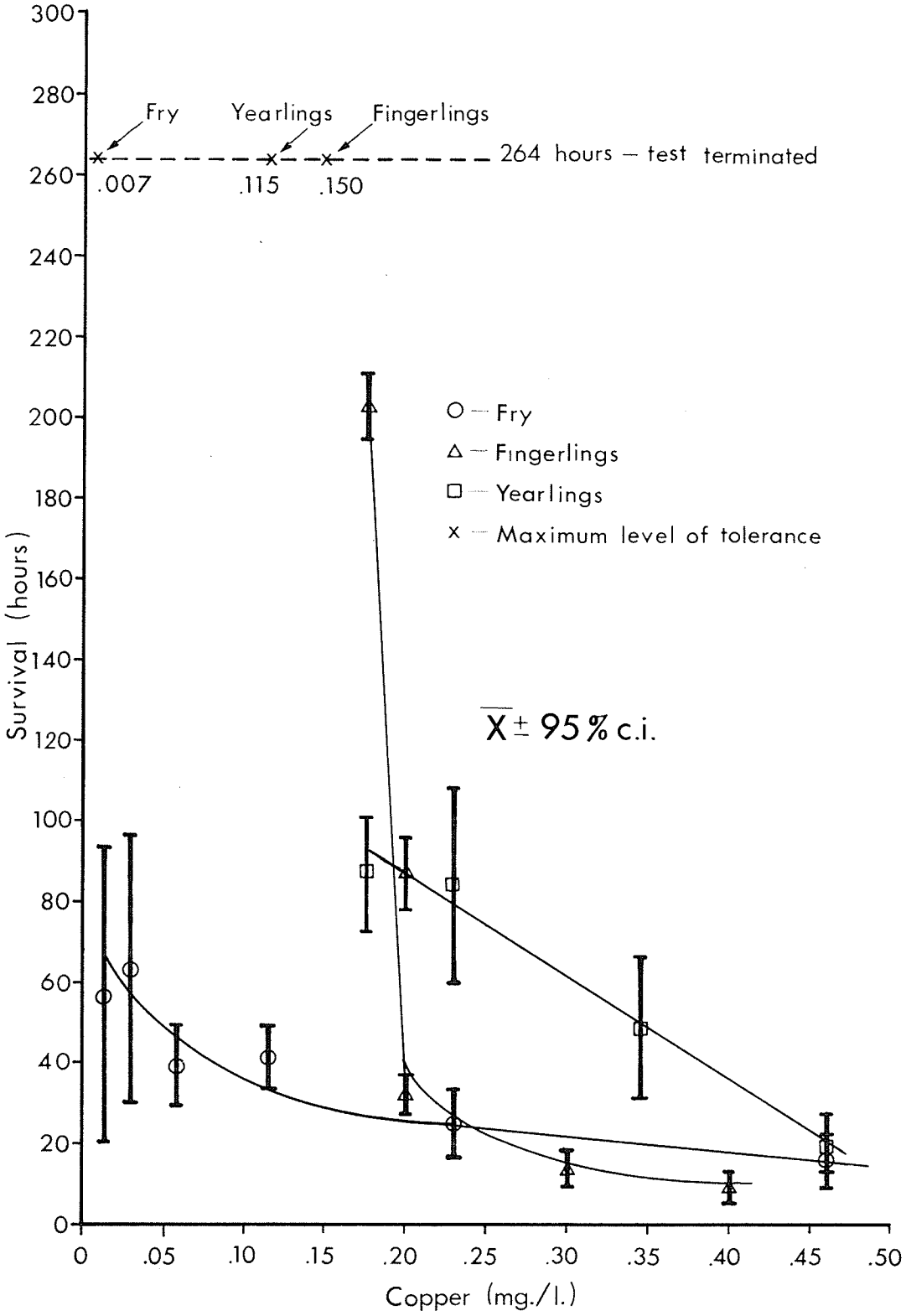
Influence of age on survival time of brook trout in experimental copper solutions. Experimental error calculated at the level of the 95 percent confidence interval (95% c.i.)

Group	No. Fish	Concentration (mg/l Cu)	S_{70} a/ (hours)	95% c. i.
Fry	6	Control	264 b/	
	6	0.007	264 b/	
	6	0.014	57.03	20.6 - 93.4
	6	0.028	63.47	30.6 - 96.4
	6	0.057	39.54	29.8 - 49.3
	6	0.115	41.49	33.8 - 39.2
	6	0.230	25.25	16.8 - 33.8
	6	0.460	15.77	8.9 - 22.6
Fingerling	6	Control	264 b/	
	6	0.150	264 b/	
	6	0.175	202.85	194.6 - 211.1
	6	0.200	32.77	27.7 - 37.2
	6	0.300	14.14	9.6 - 18.7
	6	0.400	9.45	5.6 - 13.3
Yearling	4	Control	264 b/	
	4	0.115	264 b/	
	4	0.175	86.81	72.6 - 101.1
	4	0.202	87.12	78.4 - 95.9
	4	0.230	84.32	60.2 - 108.5
	4	0.345	48.96	31.5 - 66.4
	4	0.460	20.48	13.5 - 27.5

a/ Seventy percent survival time
b/ Most or all surviving at test termination (264 hours)

Figure 4

Age-based variation in the resistance and tolerance of brook trout to solutions of copper. The variations represent differences among fry, fingerlings, and yearlings. The mean survival time for each solution represents the 70 percent survival time (S_{70}). Significance limits shown are the 95 percent confidence interval (95% c.i.).


300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
Survival (hours)
Fry
Yearlings
Fingerlings
264 hours — test terminated
.007
.115
.150
O — Fry
Δ — Fingerlings
□ — Yearlings
x — Maximum level of tolerance
X̄ ± 95% c.i.
0
.05
.10
.15
.20
.25
.30
.35
.40
.45
.50
Copper (mg./l.)

lethal level of 0.175 mg. per l. was about half the level for fingerlings. The maximum experimentally tolerated concentration for yearlings was 0.115 mg. per l. Fry appeared to tolerate a concentration of the order of 0.007 mg. per l. but were unable to tolerate 0.014 mg. per l.

Ninety-five percent confidence intervals of the S_{70} indicate resistance times for fry and fingerlings within the range of concentration of 0.2 to 0.46 mg. per l. to be of nearly identical magnitude. Considering the 70 percent survival time, fingerlings are less resistant within most of this range but become rapidly more resistant at concentrations less than 0.23 mg. per l. Within this range (of similarity), the slopes of curves for both fry and fingerlings are gentle, that is, S_{70} values increase slowly with reductions in concentration. Below 0.2 mg. per l., the fry resistance remains relatively constant until approximately 0.057 mg. per l. below this concentration, the slope becomes steeper. Between 0.014 and 0.007 mg. per l., the slope rises almost vertically indicating that, in this range, tolerance occurs.

Yearling resistance appears linear between 0.2 to 0.46 mg. per l. The resistance is roughly equal to that of fry and fingerlings at 0.46 mg. per l., however, on any further decrease in concentration, the resistance time of yearlings rapidly exceeds resistance in the younger groups because of its steeper slope. At approximately 0.175 mg. per l., fingerling resistance time rises nearly vertically, the maximum tolerated concentration appears to occur at about 0.150 mg. per l. Yearling resistance time shows an abrupt rise in slope below 0.175 mg. per l. The maximum experimentally determined concentration tolerated by yearlings was 0.115 mg. per l. as previously noted.

II. Histological Studies

(i) Acute Copper and Zinc Poisoning

(a) Branchial Degeneration

The normal gill filaments of brook trout (Fig. 5A) are identical in histological organization to those of the closely related rainbow trout (Herbert and Merkins, 1961). The platelets are composed of simple squamous epithelium closely linked laterally to form a continuous layer of fairly uniform thickness. These cells overlay a thin basement membrane. Pilaster cells link the epithelial cells of a single platelet. The cells of the interplatelet region are rounded or cuboidal in type forming compact masses between adjacent platelets. Mucous cells (Figs. 5C and 5D) are sparsely distributed among the nonsecretory cells of both the platelet and interplatelet areas but are prevalent at the filamentary tips.

The epithelial cells of fish exposed to zinc, at a concentration of 1.0 mg. per l. (Fig. 5C) and of copper, 0.1 mg. per l. (Fig. 5D) appeared cloudy and swollen. This condition was very evident in both the platelet and interplatelet regions of the filaments. Evidence of desquamation was apparent in the uneven appearance of the platelet epithelium, and in the occurrence of a few loose necrotic cells. Karyorrhexis has appeared in some of the cells of the platelet and interplatelet regions. Several mucous cells and mucous cell secretions were observed but there was no evidence of either increased mucous cell activity or a generalized mucoprecipitation reaction.

Similar, but more pronounced changes, accompanied death of trout in 50 mg. per l. zinc (Fig. 5E) and 10 mg. per l. copper (Fig. 5F). The epithelial cells of the platelet and interplatelet areas appear to have sloughed off completely, leaving little more than the pilaster cells, and

Figure 5

A histological examination of gill filaments (all 400 X) exposed to copper or zinc poisoning.

5A Transverse section of a filament removed from control fish illustrating the normal straight parallel arrangement of gill platelets.
1 - normal platelet squamous epithelium
2 - pilaster support cells
3 - normal cuboidal interplatelet epithelium

5B Transverse section from trout subjected to sublethal zinc for 21 days.
1 - moderate cloudy swelling of platelet epithelium
2 - moderate cloudy swelling of the interplatelet epithelium
3 - initial state of karyorrhexis
4 - epithelium and pilaster cells closely adherent

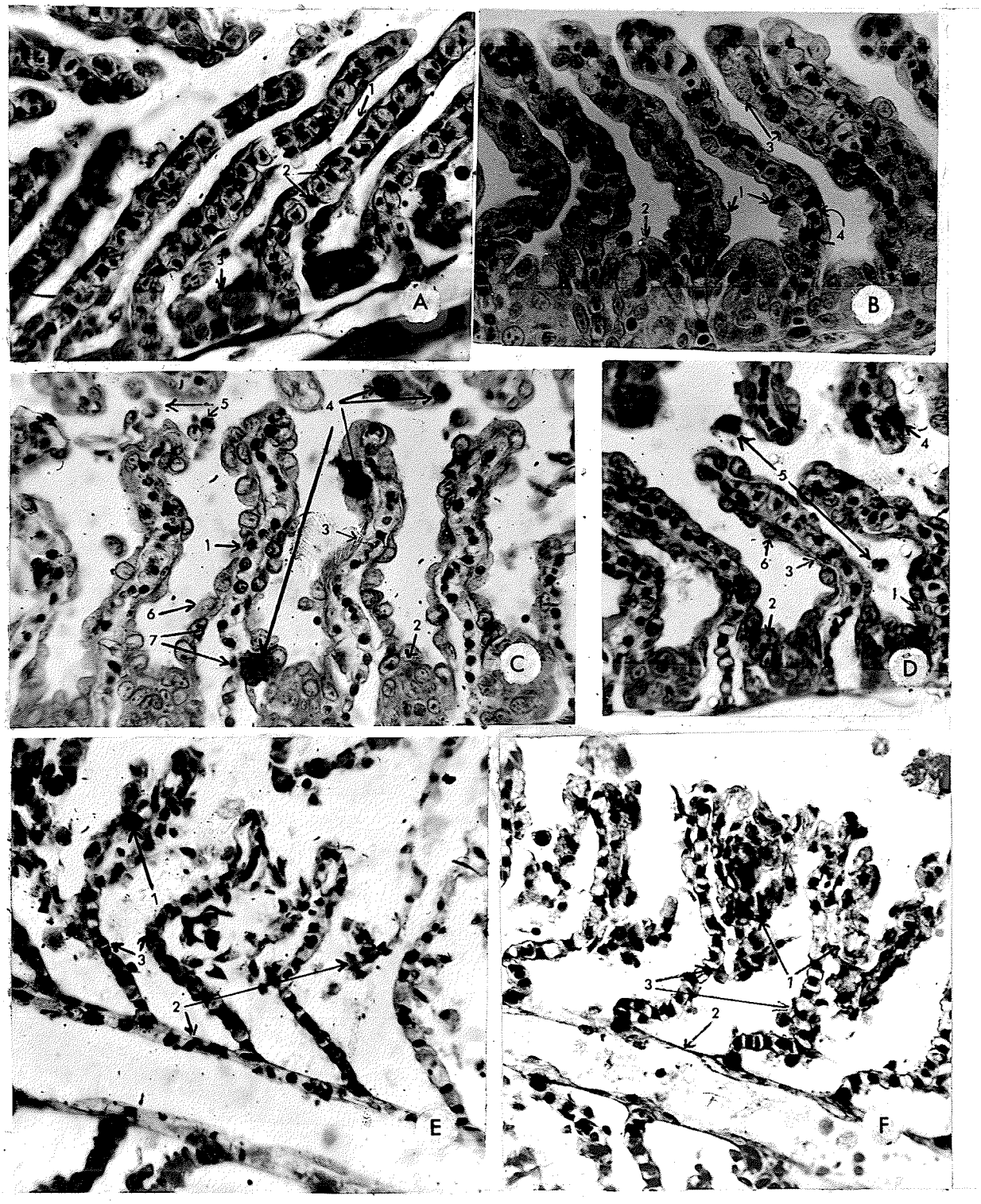
5C Transverse section from trout killed by 1.0 mg. per l. zinc
1 - severe cloudy swelling in the platelet epithelium
2 - karyorrhexis and necrosis of epithelial cells
3 - karyolysis of the epithelium producing an exposed platelet
4 - mucous cells and secretions stained with P.A.S.
5 - desquamated necrotic epithelial cells
6 - karyorrhexis of epithelial cell
7 - separation of epithelium from the pilaster column

5D Transverse section from trout killed by 0.1 mg. per l. copper.
1 - severe cloudy swelling in the platelet epithelium
2 - interplatelet cloudy swelling
3 - karyolysis of epithelial cells producing exposed platelet
4 - mucous cell secretion of mucous (P.A.S.)
5 - desquamated necrotic epithelial cells
6 - karyorrhexis of epithelial cell

5E Transverse section from trout killed by 50 mg. per l. zinc.
1 - desquamated sheets of necrotic platelet epithelium
2 - desquamated interplatelet epithelium and barren interplatelet area
3 - totally stripped platelets consisting only of pilaster cells

5F Transverse section from trout killed by 10 mg. per l. copper.
1 - loose necrotic sheets of branchial epithelium
2 - totally stripped interplatelet area
3 - totally stripped platelets

5



probably the basement membrane. The loose masses of degenerate cells lying between adjacent platelets presumably represent the remnants of sloughed off epithelial cells. No mucous cells could be distinguished in any of the sections examined, and again, there was little to suggest the occurrence of an extensive mucoprecipitation reaction.

(b) Visceral Pathology

Histological sections of heart, spleen, liver, gall-bladder and kidney were prepared from acute zinc poisoned and control yearling trout. The specimens killed in both 1.0 mg. per l. and 50 mg. per l. solutions showed no definite histological changes in any of the above structures, except the gall-bladder. Microscopic cross-sections of this organ showed it to be appreciably distended in trout held in 1.0 mg. per l. zinc. Furthermore, gross examinations of trout poisoned by 2.52 mg. per l. zinc[*] and by 0.175 and 0.345 mg. per l. copper[*], indicated a high incidence of severe gall-bladder distention. The contained bile was observed to be an atypical brownish color in these animals. No such peculiarities were detected in control animals.

A few glomeruli in the kidney of two of the yearlings killed by 1.0 mg. per l. zinc appeared swollen and completely occupied the enclosing Bowman's capsule. Small localized groups of erythrocytes were observed interstitially in several cases, particularly pericapsularly.

([*]) Lethal in 48 hours or longer

(ii) Chronic Copper and Zinc Poisoning - No mortality in 21 days of testing, with the exception of fish leaping from aquaria.

(a) Branchial Degeneration

Both macroscopic and microscopic examinations of gill filaments of yearlings subjected to a sublethal level of copper appeared indistinguishable from the filaments of control specimens, however, filaments removed from trout maintained in sublethal zinc (Fig. 5B) displayed signs of exposure to an inflammatory agent. The platelet and interplatelet epithelium appeared considerably swollen and somewhat cloudy. The nuclei of this epithelium appear to be in an initial state of karyorrhexis, or obscurity. The condition of the epithelium in this case is distinctly different from the epithelium of fish in lethal solutions of 1.0 mg. per l. zinc or 0.1 mg. per l. copper in several respects. In filaments from trout in sublethal zinc solutions, the platelet epithelium is still closely linked laterally; there are no breaks in its surface due to desquamation. Furthermore, the epithelium closely adheres to the lateral surface of the Pilaster cells whereas in the observed lethal cases (Figs. 5C and 5D), the epithelium tended to pull away from the Pilaster cells. Finally, there does not appear to be the extensive picnosis in the individual epithelial cells of the sublethal zinc specimens that was observed in the filaments of yearlings in lethal levels of zinc or copper.

(b) Visceral Pathology

(1) Liver and Gall-Bladder

Histological section of liver and gall-bladder from yearlings subjected to sublethal zinc and copper solutions displayed no distinguish-

able changes when compared to sections taken from liver and gall-bladder of yearlings in control solutions. Examination for changes in pigments, noted in the spleen and kidney of animals treated with zinc, was thorough but revealed no such changes in the liver.

(2) Spleen

Histological sections of spleen from fish maintained in sublethal solutions of zinc and of copper as well as control specimens, all displayed pigment derived from phagocytosed erythrocytes. The quantity of these pigment deposits was, however, appreciably higher in the case of chronic zinc poisoning (Fig. 6B). No perceptible difference appeared between spleen of control fish (Fig. 6A) or those chronically poisoned with copper. The sites of the deposits appeared similar to those observed by Dawson in the brown bullhead (Ictalurus nebulosus) during chronic lead poisoning. Normally, pigmentation is scanty and quite evenly distributed (Fig 6A), but in zinc poisoning granules increase in number particularly around the venous sinuses.

(3) Kidney (opisthonephros)

Degenerate hemoglobin pigments from phagocytosed erythrocytes form a normal constituent of the fish kidney (Fig. 5C). These pigments are gathered in the hemopoietic intertubular connective tissue. In histological sections of kidney from trout poisoned in sublethal zinc, the site of pigment accumulation is the same but, far greater numbers of granules are found (Fig. 6C). No increase in pigmentation was detected when sections from trout in sublethal copper were compared with control animals.

Figure 6

Comparison of the phagocytosed pigment accumulations in the spleen and kidney of control and sublethal zinc-poisoned brook trout subsequent to 21 days immersion.

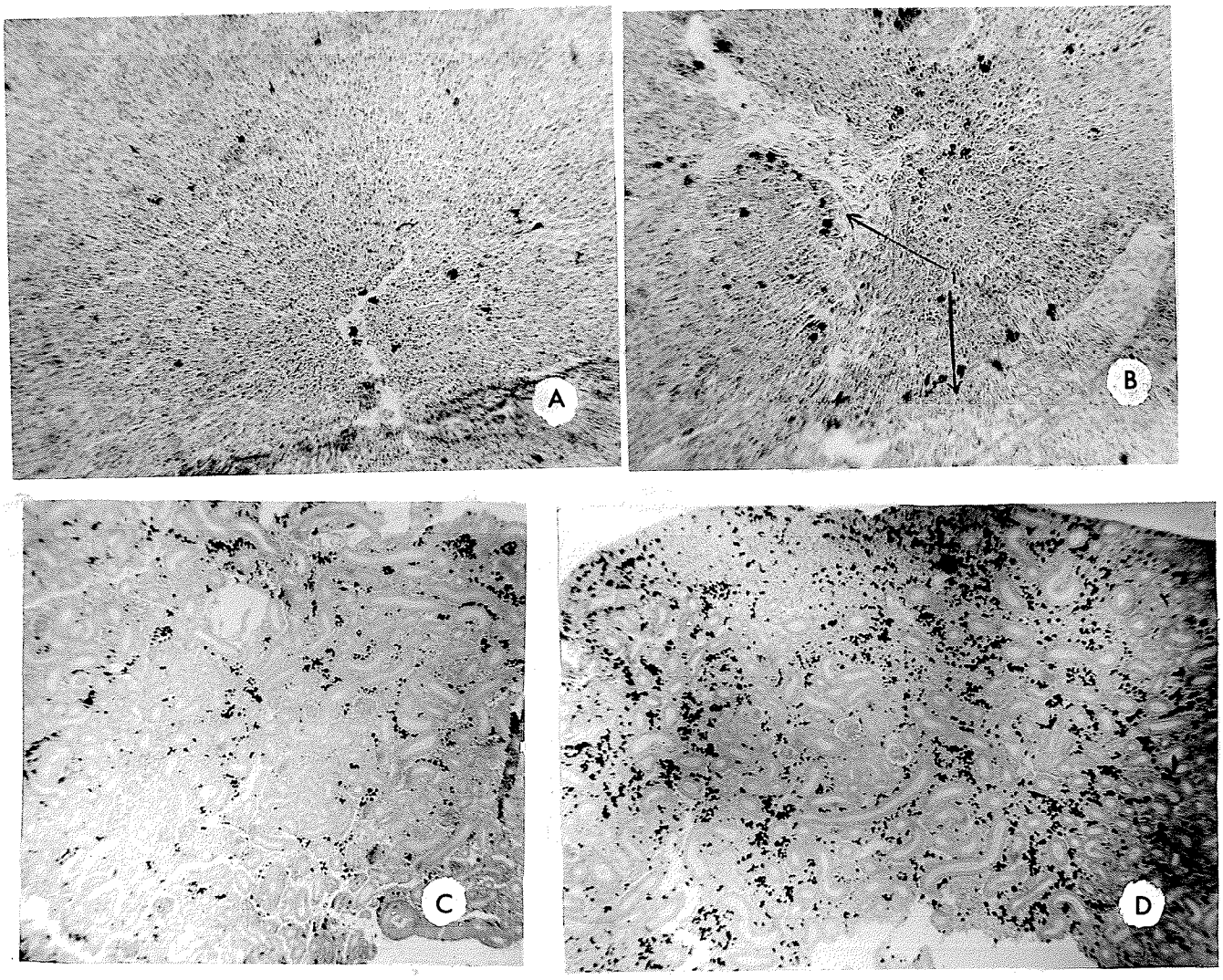
6A Cross-section of spleen from control trout (250 X) illustrating the small amount of phagocytosed hemoglobin in healthy trout spleen.

6B Cross-section of spleen from trout subjected to sublethal zinc illustrating the greatly increased amount of hemoglobin accumulation, indicative of hemolysis.

6C Dorsal longtitudinal section of kidney (frontal plane) from control trout showing the normal distribution and intensity of phagocytosed hemoglobin.

6D Dorsal longtitudinal section of kidney (frontal plane) from trout subjected to sublethal zinc. Section shows much greater density of accumulated hemoglobin pigment than in the control section, another indication of increased phagocytosis.

6



(4) Stomach

Cross-sections of the cardiac region of the stomach were examined to assess mucous cell activity in the mucosa and to determine hemopoietic activity in the submucosa. The mucous cells of specimens from both sublethal copper and zinc solutions appear to be no more or less active than those of the controls. Furthermore, hemopoietic activity in the stomach appears to be minimal; no pigment macrophages were observed and no proliferation of blood cells was detected.

(5) Tissue Hemoblast Activity

Histological examination of the ventricle of the heart of specimens from the control solution and those subjected to chronic copper or zinc poisoning indicated only limited hemopoietic activity. There was a slight proliferative response of the endothelium producing small lymphoid hemoblasts[1] (s.l. hemoblasts). Active proliferation of s.l. hemoblasts was observed on the trabecular muscles of the atrium (Fig. 7A). These cells which are small, spherical, darkly staining bodies, are found in aggregates arising within the trabeculae as well as on the surface (Fig. 7A). Presumably, they are produced by endothelial capillary channels and carried to the lumen of the atrium for release into the circulation. S.l.hemoblast aggregates were also visible in the atrial lumen (Fig. 7B) and in large hepatic sinuses. The presence of this activity is apparently normal as it was observed in all control specimens. The number of extensive s. l. hemoblast aggregates per atrial section, was used as a measure

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(1) Name used by Catton (1951) designating erythrocyte stem cell.

Figure 7

Histological longtitudinal sections (400 X) of portions of the atrium from normal trout indicating the proposed erythropoietic activity of the trout endothelium.

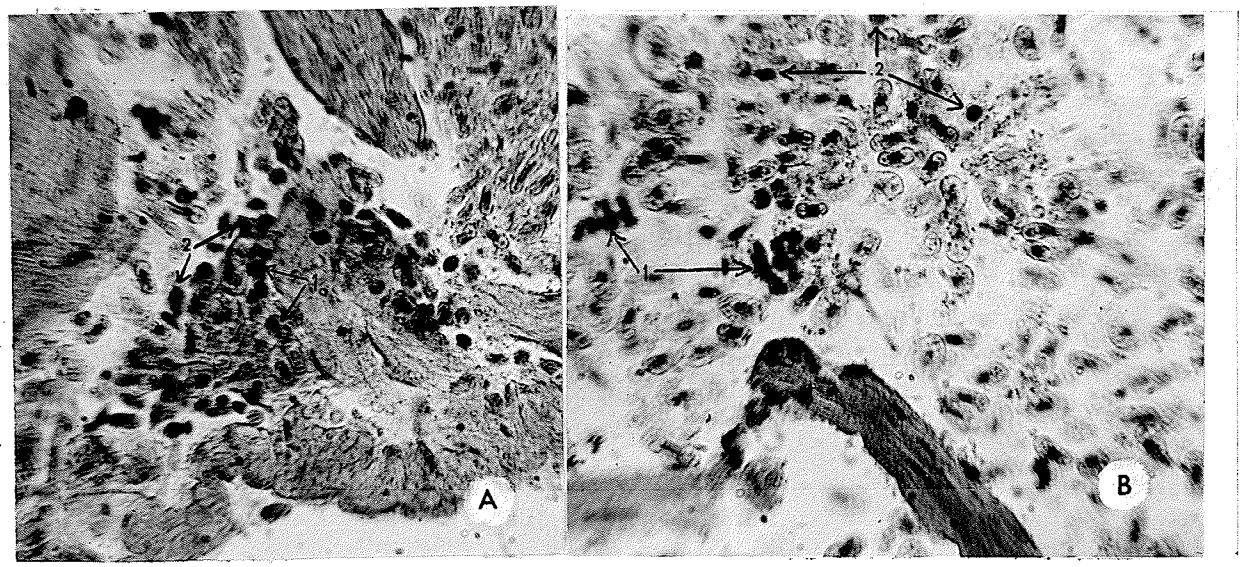
7A Section illustrating probable origin of the erythrocyte stem cell (Catton, 1951), the small lymphoid hemoblast (s.l. hemoblast), in capillary channels and on the surface of the trabecular muscle of the atrium.
1 - illustrates s.l. hemoblasts presumably nearly fully formed within a capillary channel or invagination of the trabeculae.

2 - illustrates s. l. hemoblasts released into the general circulation.

7B Section indicating the presence of s.l. hemoblasts in the circulation (lumen of the atrium).
1 - aggregates presumably resulting from multiplication within the circulation.

2 - individual s.l. hemoblasts dispersed with mature erythrocytes.

7



of the relative proliferative activity in control, sublethal copper and sublethal zinc-poisoned fish (Fig. 7A). The histological sections compared were of uniform diameter, thickness and atrial position. S.l. hemoblast aggregates in the circulation were not counted. More extensive proliferation was observed in specimens exposed to sublethal copper than in controls. Very reduced s.l. hemoblast proliferative activity was observed in animals exposed to chronic zinc poisoning. Zinc-poisoned specimens showed an average of only two areas of proliferation per slide. Sections from control fish averaged five to seven proliferative sites and chronic copper-poisoned specimens averaged seven to nine sites. The number of circulating s.l. hemoblasts was greatly reduced in keeping with the reduced trabecular hemoblast production in individuals subjected to chronic zinc poisoning. The abundant distribution of individual and aggregated s.l. hemoblasts in control specimens is shown in Fig. 7B.

(c) Hemotological Studies

(1) Fingerlings

Erythrocyte counts taken from the blood of fingerlings immersed for 11 days in marginally sublethal copper or zinc displayed very marked differences to counts from controls. Table VI (Fig. 8) records the hemocytometer counts.

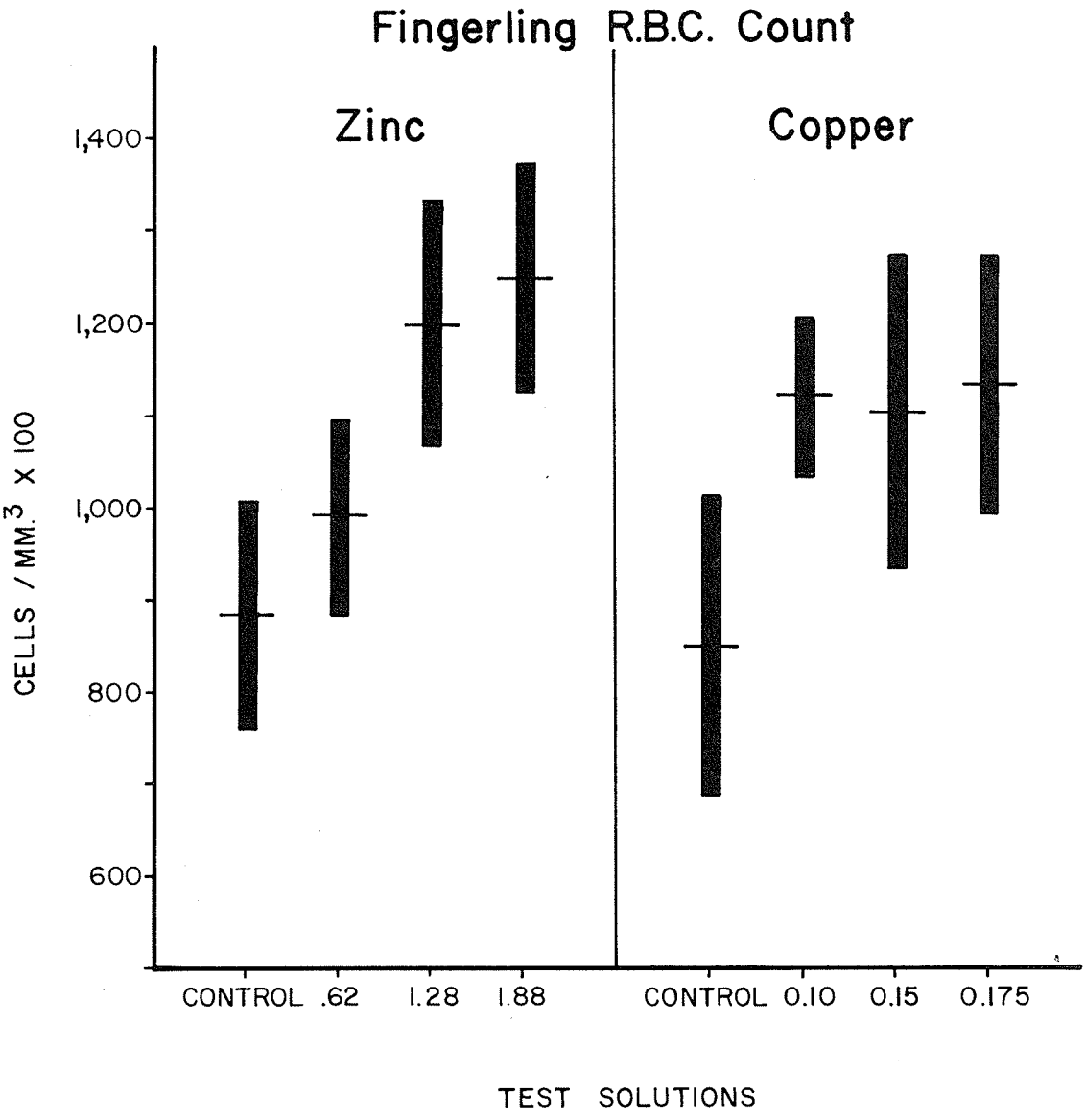
There was a large proportion of s.l. hemoblasts among the greatly-increased number of circulating erythrocytes in both copper and zinc solutions. S.l. hemoblasts were found in the blood of control fish but in apparently less numbers. They were regarded as erythrocytes and counted as such.

Table VI

Number of erythrocytes in thousands per cu. mm. in the blood of fingerling brook trout exposed to sublethal solutions of zinc and copper for 11 days.

	(1) Zinc Solutions			
	Control	0.625 mg/l	1.28 mg/l	1.88 mg/l
No. R.B.C's. (Mean)	884	991	1,200	1,250
No. Animals	7	7	5	5
S. D.	132	114	139	130
No. Counts (n)	14	14	10	10
S. E.	41	36	44	41
$\bar{X} \pm 3S_{\bar{X}}$	761-1007	883-1097	1068-1332	1127-1373

	(2) Copper Solutions			
	Control	0.10 mg/l	0.15 mg/l	0.175 mg/l
No. R.B.C's. (Mean)	851	1,121	1,105	1,135
No. Animals	8	8	8	6
S. D.	214	114	225	130
No. Counts (n)	16	16	16	12
S. E.	54	29	56	38
$\bar{X} \pm 3S_{\bar{X}}$	687-1013	1034-1208	937-1273	1028-1242


Fingerling R.B.C. Count
Zinc
Copper
1,400
1,200
1,000
800
600
CELLS / MM.3 X 100
CONTROL .62 1.28 1.88
CONTROL 0.10 0.15 0.175
TEST SOLUTIONS

(2) Yearlings

(a) Erythrocyte Number

Results of individual erythrocyte counts in each of the three test solutions are shown in Table VII, Fig. 9.

These results would tend to indicate that the number of erythrocytes in the blood of yearling trout maintained in sublethal levels of copper increases. On the other hand, the R. B. C's of specimens in sublethal zinc remains the same or undergoes a decrease.

(b) Hematocrit

The percentage volume that erythrocytes occupied in the blood of yearling trout was determined by centrifugation in microhematocrit tubes. The results are shown in Table VIII, Fig. 10.

The results of the hematocrit indicate that there is very little difference in the percentage of the blood volume occupied by erythrocytes in yearlings regardless of the test solution. Those subjected to zinc poisoning might, perhaps, have a slightly smaller hematocrit.

(c) Total Blood Iron

Results of the micro-analyses of iron in the blood of yearling trout by the Natelson thiocyanate technique are shown in Table IX, Fig. 11.

The results of total blood iron show a tendency to divide into three groups (Fig. 12). In trout immersed in sublethal copper, the blood iron concentration appears to be slightly greater than in the corresponding control animals. Trout subjected to sublethal zinc show a similar, or slightly reduced, blood iron concentration to the controls.

Table VII

Number of erythrocytes (in thousands) per cu. mm. in the blood of yearling brook trout exposed to sublethal solutions of copper and zinc for 21 days.

Group	No. R.B.C's (Mean)	S. D.	S. E.	$\bar{X} \pm 3S_{\bar{x}}$
Control	1,243	77	27.2	1161-1325
Copper	1,573	49	15.5	1558-1589
Zinc	1,158	152	12.7	1114-1202

Table VIII

Percentage of total blood volume occupied by erythrocytes in the blood of yearling brook trout exposed to sublethal solutions of copper and zinc for 21 days. (PCV - packed cell volume)

Group	PCV (Mean)	S. D.	S. E.	$\bar{X} \pm 3S_{\bar{x}}$
Control	32.9%	2.65	0.94	31.0-34.8
Copper	32.5	1.71	0.54	31.4-33.6
Zinc	31.3	2.99	0.86	29.6-32.0

Figure 9

Erythrocyte counts among groups of yearling brook trout in control or sublethal solutions of copper or zinc for 21 days. The results reported are significant to three standard errors.

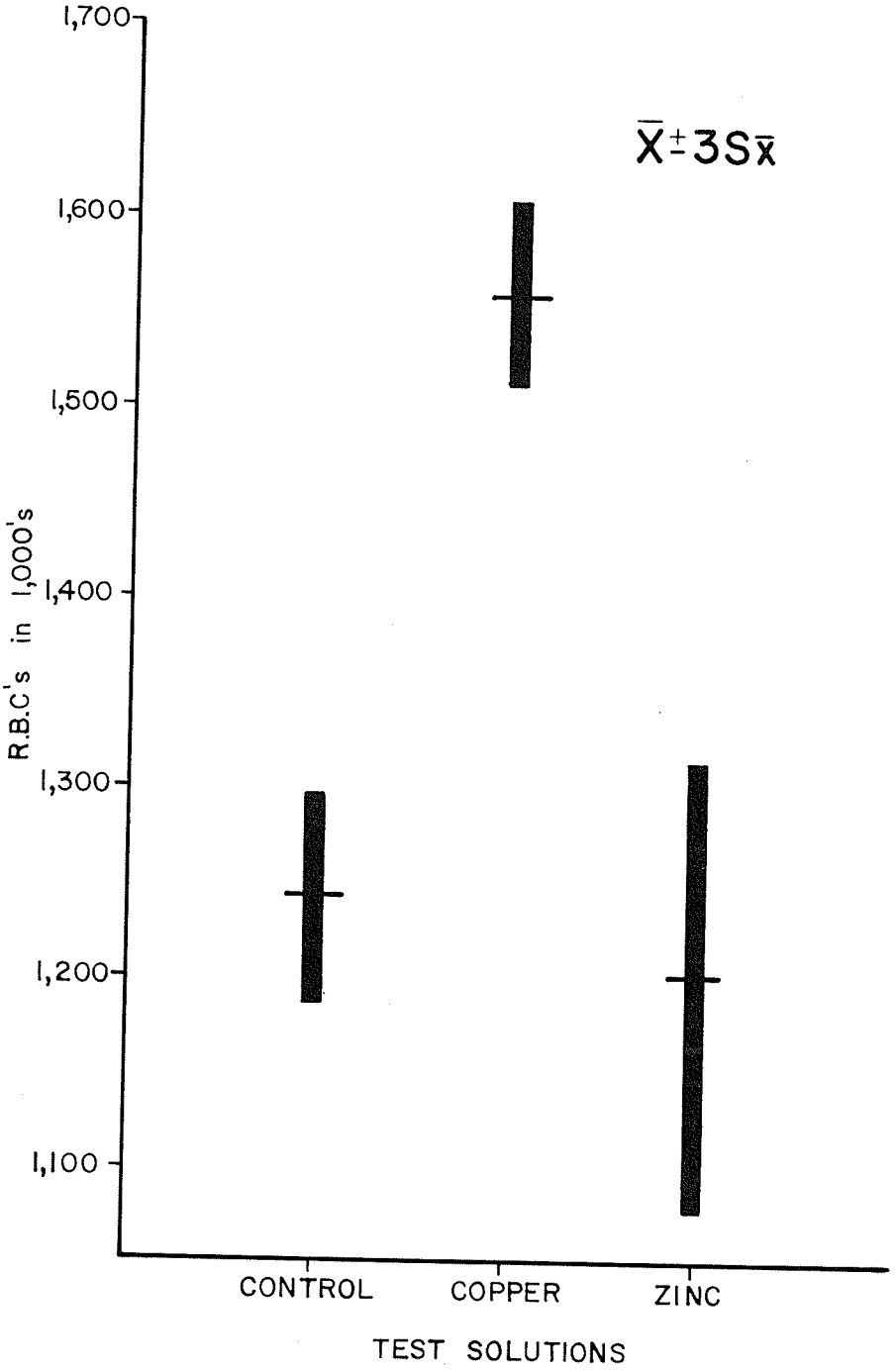

1,700
1,600
1,500
1,400
1,300
1,200
1,100
R.B.C's in 1,000's
$\bar{X} \pm 3S_{\bar{x}}$
CONTROL
COPPER
ZINC
TEST SOLUTIONS

Figure 10

Hematocrit or packed corpuscular volume (PCV) or erythrocytes in the blood of yearling brook trout subjected to sublethal copper or zinc solutions for 21 days.

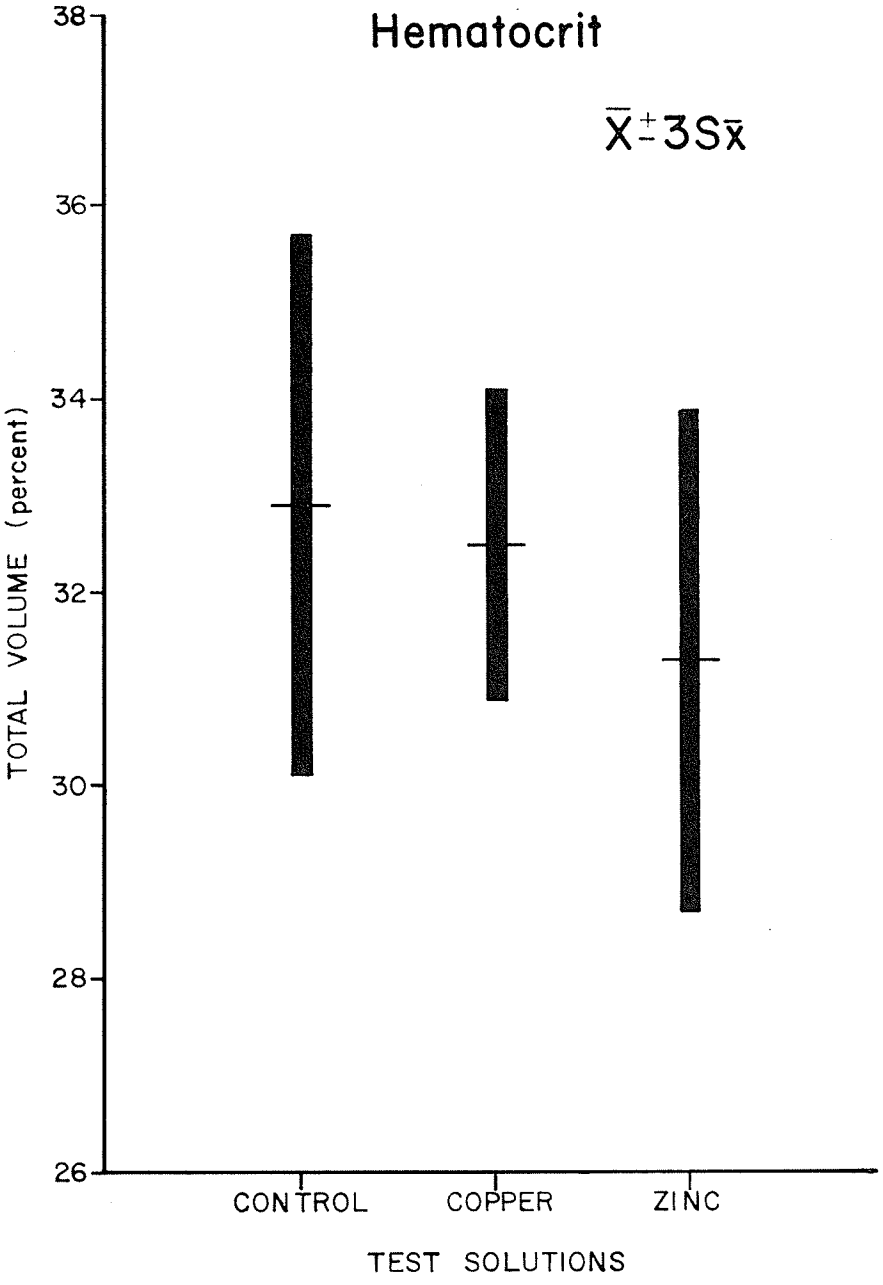

Hematocrit
$\bar{X} \pm 3S\bar{x}$
38
36
34
32
30
28
26
TOTAL VOLUME (percent)
CONTROL
COPPER
ZINC
TEST SOLUTIONS

Table IX

Total iron concentration (mg. per 100 ml. blood) in the blood of yearling brook trout exposed to sublethal concentrations of copper and zinc for 21 days.

Group	Iron Concentration	S. D.	S. E.	$\bar{X} \pm 3S_{\bar{X}}$
Control	35.5	1.78	0.63	33.6-37.4
Copper	41.3	4.36	1.40	39.9-42.7
Zinc	33.2	1.30	0.49	31.7-33.7

Table X

Theoretical hemoglobin concentration in the blood of yearling brook trout exposed to sublethal copper or zinc solution. Values based on factor used for converting from human blood iron concentration to blood hemoglobin.

	Test Solutions		
Mean Conc.	Control	Copper	Zinc
Hemoglobin grams per 100 cc.	10.4	12.1	9.7

Figure 11

Total iron determinations in the blood of yearling brook trout subjected to sublethal copper or zinc solutions for 21 days. Hemoglobin values obtained by using the human conversion factor:

mg. iron per 100 ml. x 0.294 = gm. hemoglobin per 100 ml.

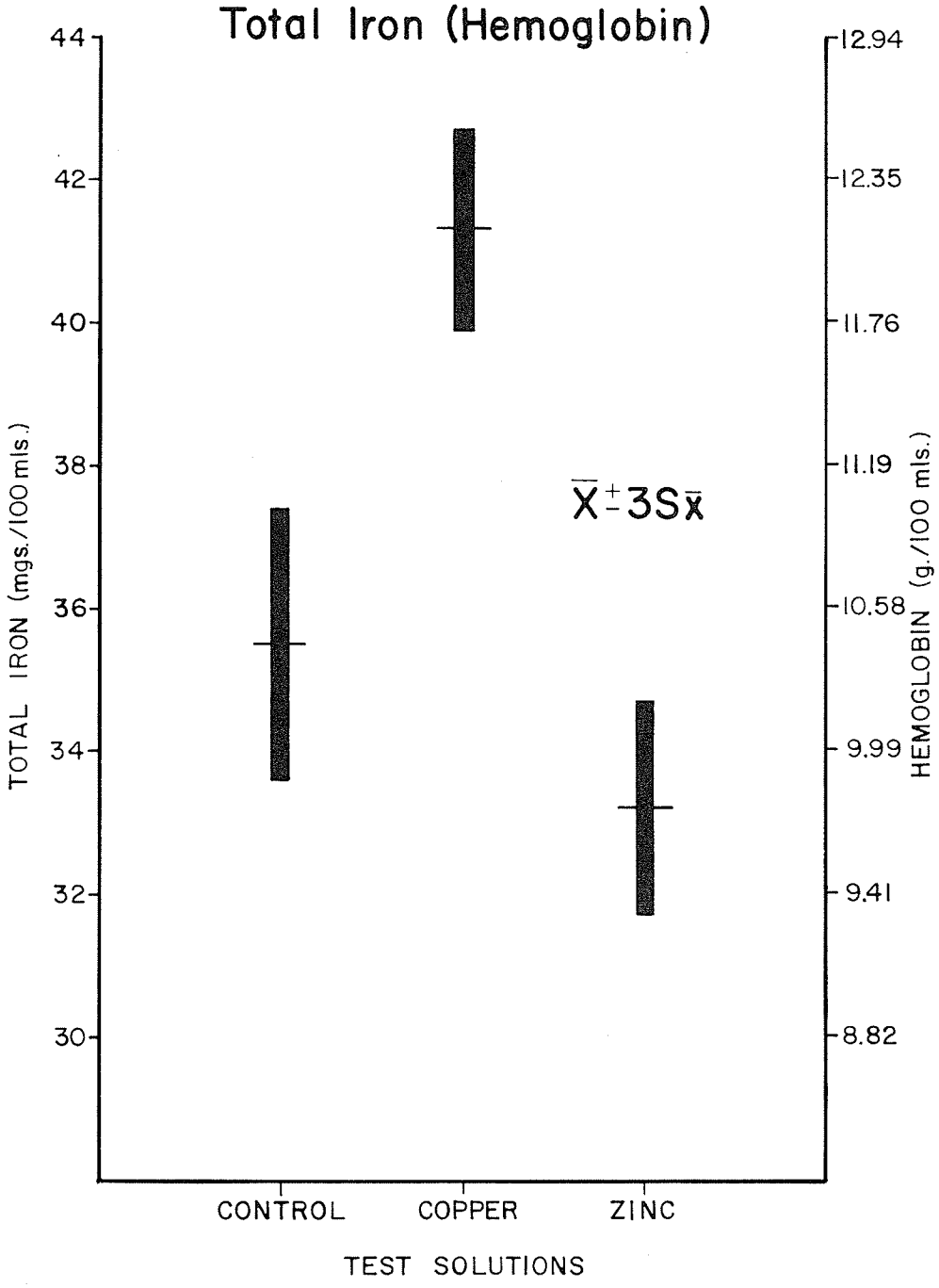
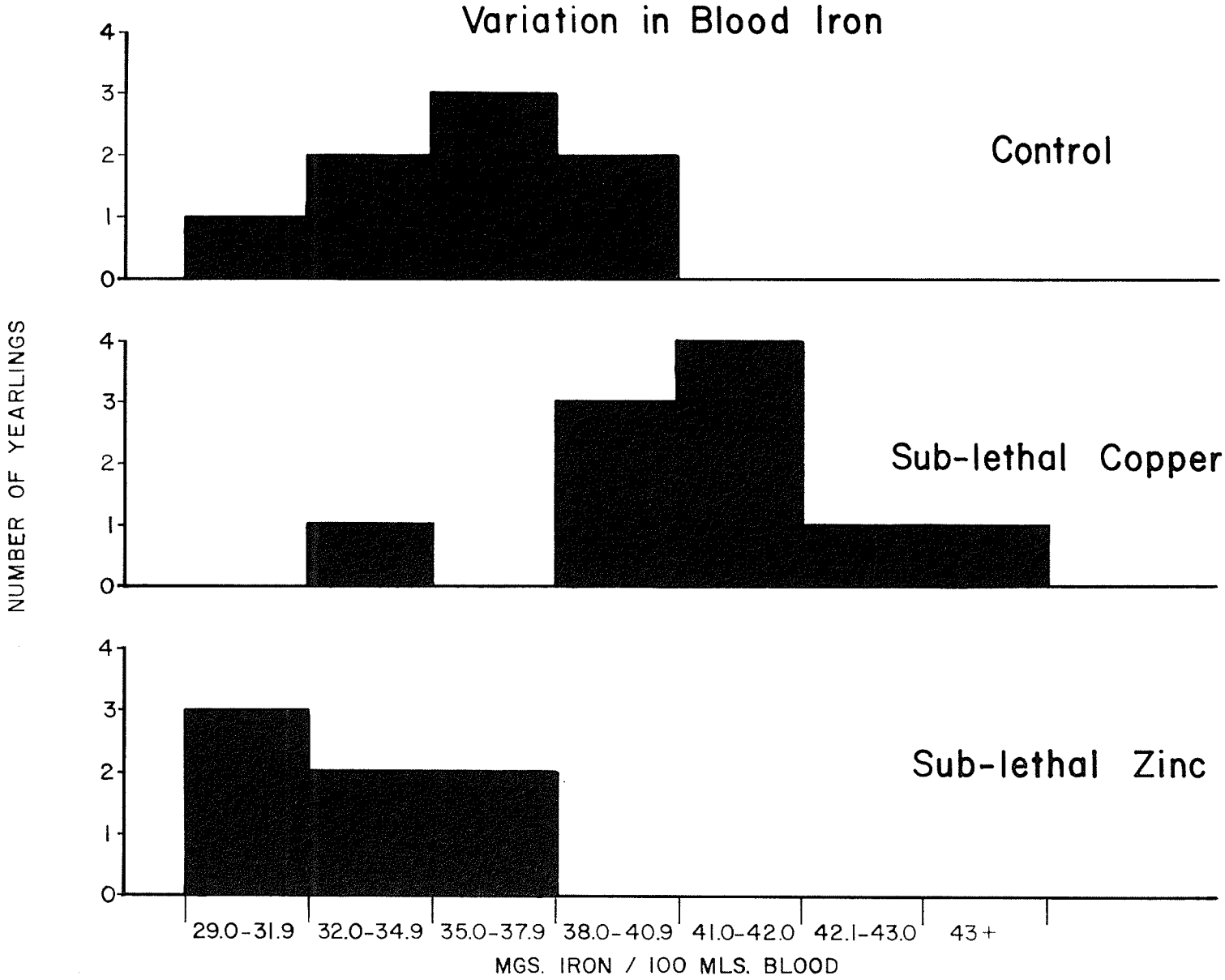

Total Iron (Hemoglobin)
44
42
40
38
36
34
32
30
12.94
12.35
11.76
11.19
10.58
9.99
9.41
8.82
TOTAL IRON (mgs./100 mls.)
HEMOGLOBIN (g./100 mls.)
$\bar{X} \pm 3S_{\bar{x}}$
CONTROL
COPPER
ZINC
TEST SOLUTIONS

Figure 12

The variation in blood iron content among individual brook trout held in control, sublethal copper (.3 mg. per l.), or sublethal zinc (2.9 mg. per l.) solutions for 21 days.


Variation in Blood Iron
Control
Sub-lethal Copper
Sub-lethal Zinc
NUMBER OF YEARLINGS
0
1
2
3
4
29.0-31.9
32.0-34.9
35.0-37.9
38.0-40.9
41.0-42.0
42.1-43.0
43+
MGS. IRON / 100 MLS. BLOOD

For purposes of comparing these results with those of other authors who have expressed their results in grams hemoglobin per 100 ml. of blood, the factor

0.294 X mg. iron = grams hemoglobin per 100 ml.

These results, including the significance limits $\bar{X} \pm 3S_{\bar{x}}$, are shown in Table IX, graphical representation Fig. 11.

(d) Mean Corpuscular Volume

The mean corpuscular volume (MCV) was determined for purposes of ascertaining variations in the mean size of erythrocytes resulting from the influence of test solutions of zinc and copper on yearling trout. Data on MCV is recorded on Table XI.

The result would indicate that the erythrocytes of trout in chronic copper solutions have an appreciably reduced volume while those of animals subjected to sublethal zinc are relatively unchanged. Graphical representation is presented in Fig. 13.

(e) Mean Corpuscular Iron Content

The mean corpuscular iron content (MCIC) was determined in order to assess the quantity of hemoglobin available to each corpuscle and to determine whether or not this was influenced by subjection of the fish to copper and zinc solutions. Analysis of the data has been recorded in Table XII, with graphical representation on Fig. 14.

From Table XII, it would appear that there is a decrease in iron and, therefore, a decrease in hemoglobin per erythrocyte in the blood of specimens subjected to sublethal solutions of copper, however, mean corpuscular iron content of the erythrocytes in the blood of specimens held in

Table XI

Mean volume of erythrocytes (MCV), in cubic microns, from yearling brook trout held in sublethal levels of copper and zinc for 21 days.

Test Solution	MCV	S. D.	S. E.	$\bar{X} \pm 3S_{\bar{x}}$
Control	267	16.1	5.7	249.9-284.1
Copper	207	10.4	3.3	197.1-216.9
Zinc	262	18.9	5.7	244.9-279.1

Table XII

Mean corpuscular iron content (MCIC), in picograms (1 picogram = 10^{-12} grams), from yearling brook trout held in sublethal levels of copper and zinc for 21 days.

Test Solution	MCIC	S. D.	S. E.	$\bar{X} \pm 3S_{\bar{x}}$
Control	0.288	0.024	.0082	.263-.313
Copper	0.264	0.012	.0038	.252-.274
Zinc	0.314	0.018	.0073	.292-.336

Figure 13

Variations in the mean corpuscular erythrocyte volume (MCV) of yearling brook trout produced by 21-day immersion in sublethal copper or zinc solutions.

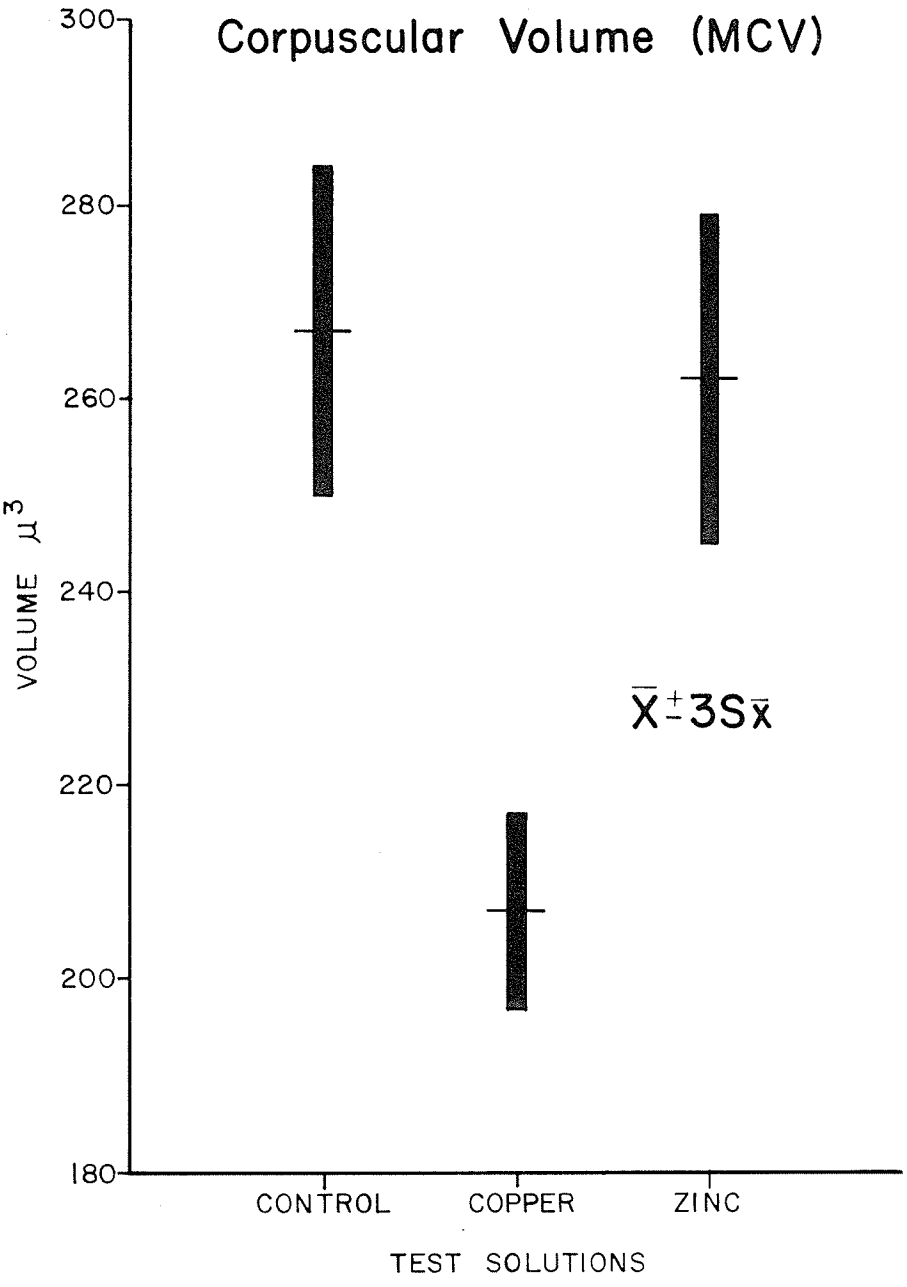
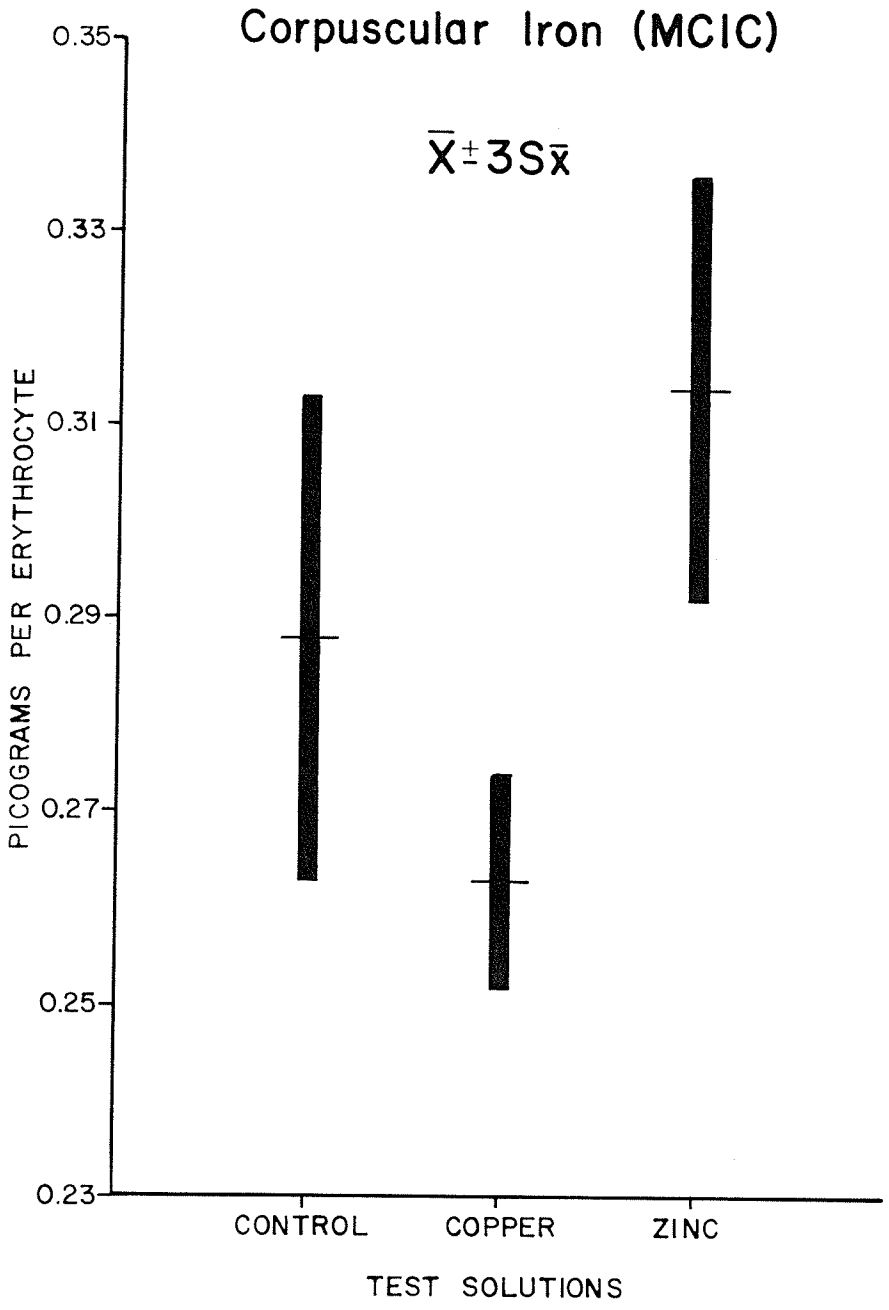

Corpuscular Volume (MCV)
300
280
260
240
220
200
180
VOLUME μ3
$\bar{X} \pm 3S_{\bar{x}}$
CONTROL
COPPER
ZINC
TEST SOLUTIONS

Figure 14

Determinations of the differences in the mean erythrocyte iron content (MCIC) of brook trout subjected to control, sublethal copper or sublethal zinc solutions for 21 days.

one picogram = 10^{-12} gm.


Corpuscular Iron (MCIC)
$\bar{X} \pm 3S_{\bar{x}}$
0.35
0.33
0.31
0.29
0.27
0.25
0.23
PICOGRAMS PER ERYTHROCYTE
CONTROL
COPPER
ZINC
TEST SOLUTIONS

sublethal solutions of zinc remains unchanged or slightly increased.

Because of the differences in mean erythrocyte size in various test solutions, the mean iron concentration per 100 cubic microns was calculated to determine the various hemoglobin densities per erythrocyte (Table XIII).

It is apparent from Table XIII and Fig. 15 (a graphical representation) that the iron content per unit volume in erythrocytes is not drastically different among the lots of yearlings subjected to the test solutions. Iron and, therefore, concentration of hemoglobin appears slightly higher in fish poisoned in chronic copper than in sublethal zinc or in control solutions.

The net result of chronic copper poisoning is an increase in the number of erythrocytes of reduced volume and mean (corpuscular) iron content, however, the mean corpuscular iron content per unit volume and the total iron content are increased. There is some indication that a reciprocal relationship exists to a lesser extent for sublethal zinc specimens.

(f) Histochemical Studies

(1) Tissue Iron Concentration

Determinations of iron in spleen and liver sections by means of an histochemical densitometer revealed the relative concentration variations between specimens in different test solutions listed in Table XIV, Fig. 16.

Although these histochemical analyses can only be regarded as semi-quantitative, it would appear that the liver from specimens

Table XIII

Mean corpuscular iron concentration (MCIConc.) in picograms iron per 100 cubic microns (1 picogram $=10^{-12}$ grams) of erythrocyte in sublethal copper and zinc-poisoned, brook trout.

Test Solution	MCIConc.	S. D.	S. E.	$\bar{X} \pm 3S_{\bar{x}}$
Control	0.110	.008	.0091	0.101-.119
Copper	.128	.011	.0035	.118-.138
Zinc	.114	.011	.0045	.101-.127

Table XIV

Histochemical tissue iron content of sublethal copper and zinc-poisoned, brook trout. Assays performed on an electrodensitometer. (Iron concentration = 100/galvanometer Deflection)

Test Solution	Tissue	Iron Concentration	M.D.D.[1]	%M.D.D.T.D.[2]
Control	Liver	4.7	1.5	7.0
Copper		4.2	4.0	16.7
Zinc		8.2	1.2	9.8
Control	Spleen	13.2	0.6	7.9
Copper		15.0	0.7	10.4
Zinc		12.0	0.7	8.5

1/ Maximum Deflection Deviation of galvanometer from mean.
2/ Percent Maximum Deviation Deflection is of Total (mean) Deflection.

Figure 15

Determination of the mean iron density per erythrocyte (picograms per 100 ^{3}) in brook trout subjected to sublethal copper or zinc solutions.

one picogram = 10^{-12} gm.

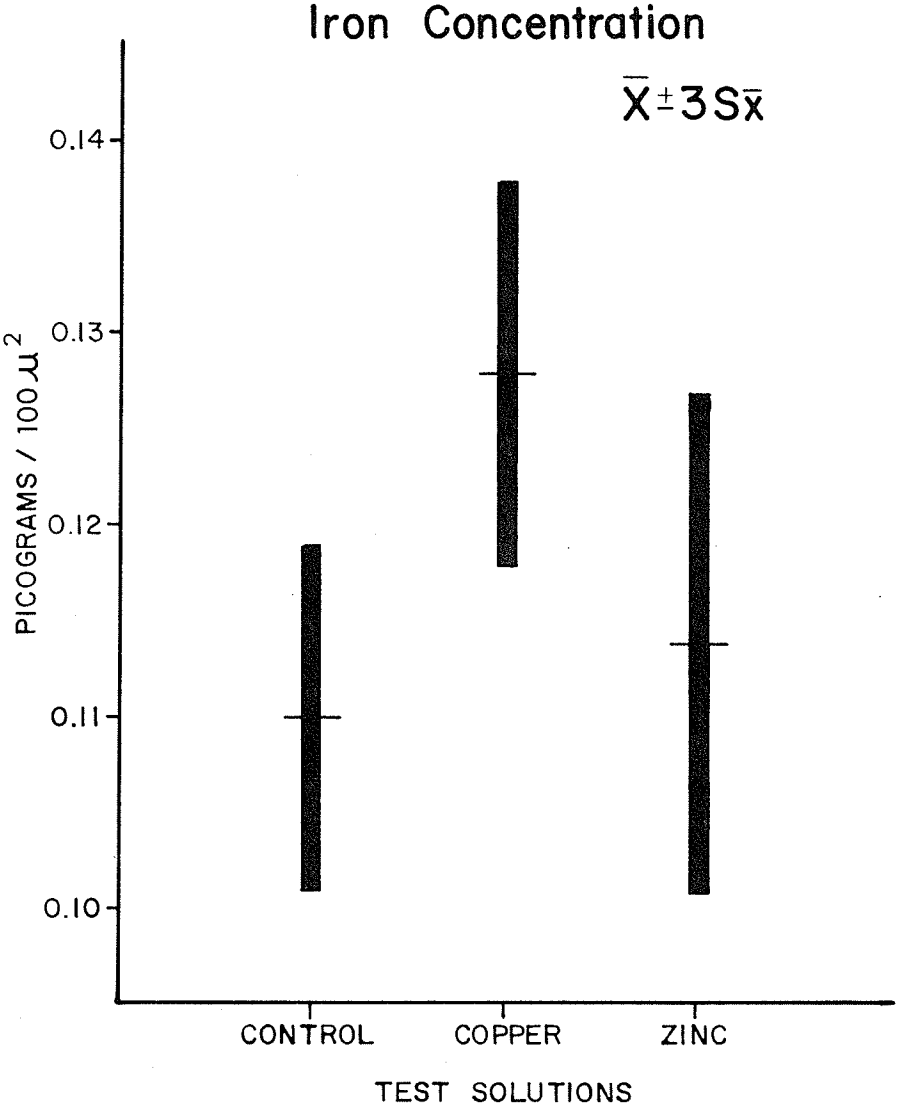
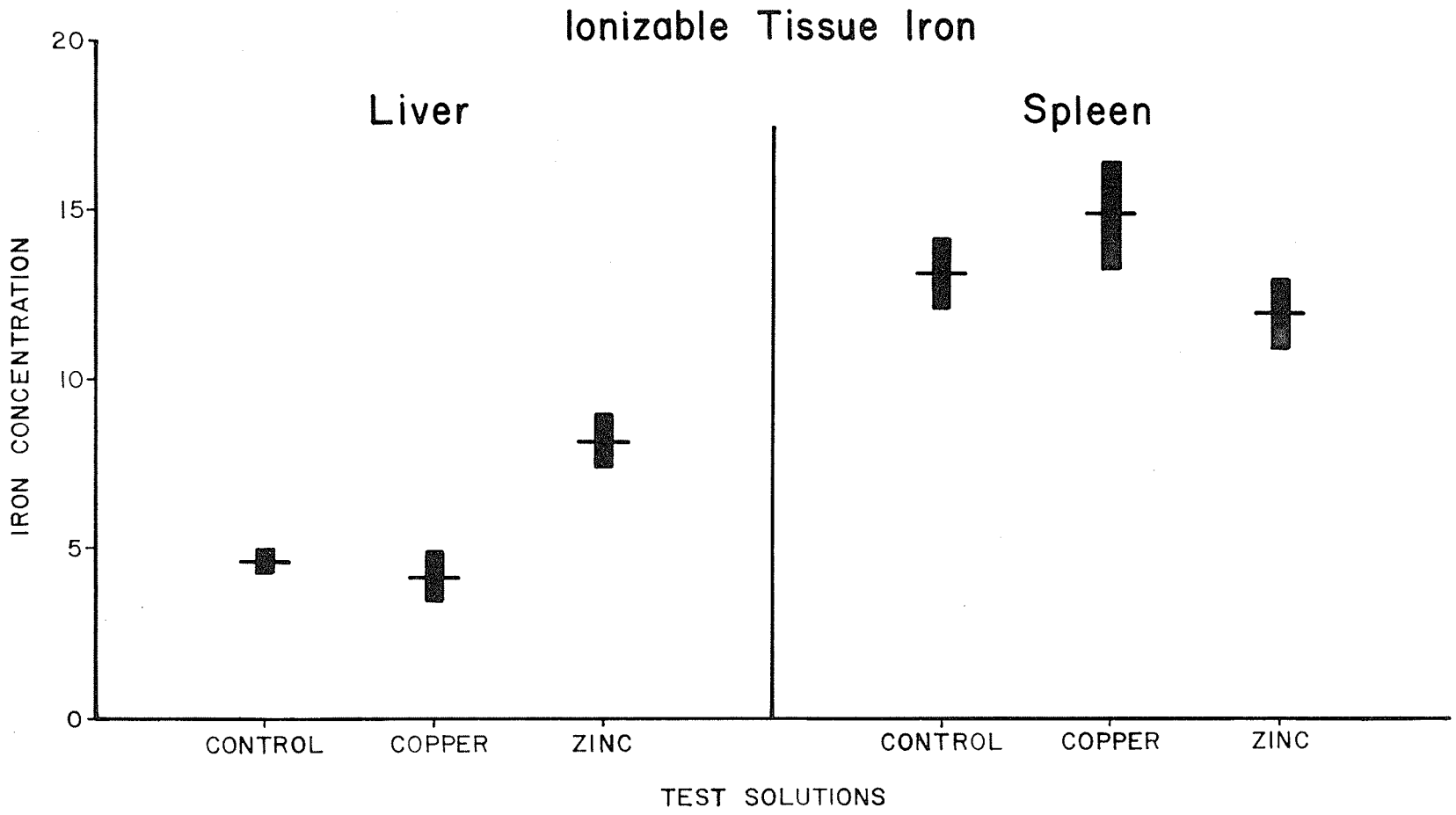

Iron Concentration
$\bar{X} \pm 3S\bar{x}$
0.14
0.13
0.12
0.11
0.10
PICOGRAMS / 100 μ^2
CONTROL
COPPER
ZINC
TEST SOLUTIONS

Figure 16

Spectrophotometric histochemical estimation of the relative quantities of ionizable tissue iron in the liver and spleen of brook trout subjected to sublethal copper or zinc solutions for 21 days. Significance limits are based on the following value:

$$\overline{X} = \pm \frac{\text{Maximum deflection deviation - mean deflection}}{\text{mean deflection}}$$

where deflection refers to spectrophotometer galvanometer deflection


Ionizable Tissue Iron
Liver
Spleen
20
15
10
5
0
IRON CONCENTRATION
CONTROL
COPPER
ZINC
CONTROL
COPPER
ZINC
TEST SOLUTIONS

held in sublethal solutions of zinc contains more iron than specimens from either the control solution or sublethal copper solution. Livers from fish in both control and sublethal copper solutions contain about the same amount of iron. The iron content of the spleen does not appear to vary greatly among the trout held in the three test solutions. A slightly higher level of iron in the spleens of fish held in sublethal copper solutions might be indicated and, a slightly reduced level in the spleens of fish from solutions of zinc might also be indicated.

(2) Tissue Copper

Semi-quantitative assays of tissue copper were made by determining the number of squares occupied by copper rebeanate granules in a portion of a Whipple counting occular. Tissue copper results are recorded on Table XV, (Fig. 17).

Although the results in Table XIV offer only a rough estimate of the copper concentration of liver, spleen and kidney, the copper content of the livers of trout having zinc poisoning appears appreciably greater than that found in controls. Copper content of livers in yearlings held in sublethal levels of copper also appears slightly increased.

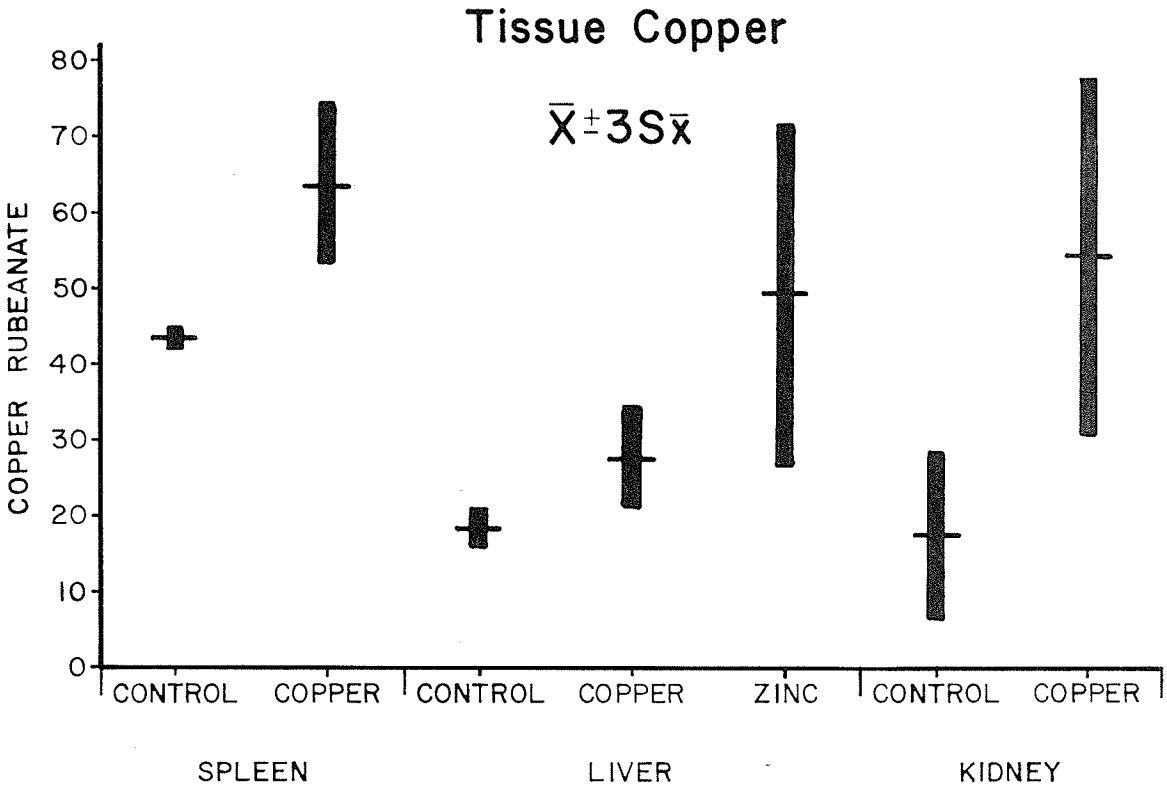
Table XV

Histochemical tissue copper content in tissue of sublethal copper and zinc-poisoned yearling, brook trout. Assays performed on basis of the number of squares of a Whipple occular (eye-piece) micrometer in a field of 240 squares which were occupied by copper rubeanate granules. Concentration recorded is the mean of three or more fields of view.

Test Solution	Tissue	Mean Iron Concentration	S. D.	$\bar{X} \pm 3S.D.$
Control	Liver	18.5	0.8	16.1-20.9
Copper		27.8	2.2	21.2-34.4
Zinc		49.2	7.5	26.7-71.7
Control	Spleen	43.5	0.5	41.0-45.0
Copper		63.8	3.9	53.1-74.5
Control	Kidney	17.5	3.9	6.8-28.2
Copper		54.7	8.0	30.7-78.7

Figure 17

Histochemical estimation of the tissue copper content in the liver, spleen and kidney of brook trout subjected to sublethal copper or zinc solutions for 21 days. Quantitative determination was established by counting the number of copper-rubeanate-stained granules in a given area in tissue of each organ, the sections having previously been stained with rubeanic acid.


Tissue Copper
$\bar{X} \pm 3S_{\bar{x}}$
COPPER RUBEANATE
80
70
60
50
40
30
20
10
0
CONTROL
COPPER
CONTROL
COPPER
ZINC
CONTROL
COPPER
SPLEEN
LIVER
KIDNEY

DISCUSSION

I. Survival in Zinc or Copper

(i) Tolerance Variation Based on Age

The possibility of a minimum lethal threshold (tolerance level) for zinc or copper solutions in fish, and for heavy metals in general, has not been adequately explored to permit any conclusion. It is entirely possible, for example, that heavy metal poisoning could upset the balance of two or more factors and thus be capable of killing in at least two ways. To illustrate specific possibilities, most solutions containing lethal zinc or copper kill fish by inflicting severe damage to the branchial epithelium. In more dilute concentrations, however, branchial degeneration does not appear serious although inhibited sodium absorption and accelerated excretion or inhibited erythropoiesis and accelerated hemolysis might prove fatal. Assuming that the mortality due to heavy metal poisoning results from a sustained imbalance of any one of several essential systems, the 'effective time' (Fry, 1947) will be proportional to the rapidity of the first vital disablement. Thus in tests effecting mortality in two to seven days mortality will result from branchial damage. The maximum tolerated concentration, however, causes no serious branchial degeneration, presumably because it is below the threshold (lethal) level for branchial degeneration (Discussion II. Histological Studies). Maintenance of fish in this concentration for one to two weeks, however, might produce mortality because sustained sodium loss reached a lethal deficiency

level. Meyer (1952) reported inhibited sodium absorption and accelerated sodium excretion in goldfish subjected to mercury poisoning. Previously he (Meyer, 1951) had presented evidence indicating that death resulted from sustained net sodium loss. Meyer (1952) observed that sodium absorption inhibition and excretion acceleration were proportional to mercuric ion concentration. Below the concentration required to trigger imbalance of the sodium homeostatic mechanism mortality in fish should disappear. If the fish are maintained longer than three months, however, at a concentration just sublethal to the sodium homeostatic system, mortality might once again appear as a result of severe hemolytic anemia. Dawson (1935) reported anemia from chronically lead poisoned brown bullhead (Ictalurus nebulosus). Finally, concentrations not directly lethal might have a lethal effect because they inhibit or eliminate reproduction. Crandall and Goodnight (1962) observed that, in solutions of lead, of zinc and of sodium pentachlorophenate, development of the male secondary sex characteristics (gonopodium and pigmentation) in the guppy was severely inhibited. When a statement is made that fish tolerated a certain concentration of a heavy metal for a given length of time, two things are suggested therefore: 1) that the concentration is less than the threshold level to inactivate certain systems; and 2) that the elapsed time appears to be less than the effective time due to inactivation of other systems.

The increased tolerance to heavy metal poisoning with advancing age among fry, fingerlings and yearlings, observed in brook trout agrees with the findings of Crandall and Goodnight (1962). They noted that smaller guppies (Lebistes reticulatus), were less tolerant of sublethal or of

incipiently lethal solutions of lead, of zinc and of sodium pentachlorophenate than were larger (older) individuals. Jones (1939) also indicated greater susceptability among very small sticklebacks (Gasterosteus aculeatus) to lead poisoning as compared to adults. This was partly because of attack and injury by larger fish but was also due to lower tolerance in the juveniles. Earlier, in 1938, he had stated that small sticklebacks (18-20 mm) were less tolerant than adults. He compared tolerance and resistance of each group, however, dividing the average survival time, in a given concentration, by the average survival time for the controls of that group. Unfortunately his adult control group survived for only one-third of the length of time of the juvenile controls, and less than the average survival time of some of the adults in test aquaria. An outside factor, such as hypo-osmotic stress or disease, was undoubtedly responsible for the poor survival of the adult controls. Jones' (1938) age-based comparisons and conclusions are therefore totally unreliable. It is possible, however, as a guide, to use his data to compare survival time of juvenile and adult directly, remembering that the test adults presumably suffer the same stress as the controls. With this technique, juvenile survival time (resistance) exceeds that for adults at all recorded concentrations. It is impossible to predict the highest tolerated concentration in his experiment. The juveniles, he indicates, would tolerate a concentration slightly less than 0.07 mg per l.

Data for brook trout, in the present experiment, indicate that fry are appreciably less tolerant to zinc poisoning than fingerlings and yearlings. Tolerance differences between fingerlings and yearlings are

only approximate from this data, however, because of the limited number of test concentrations in the area of tolerance.

Tolerance data for copper poisoning in brook trout agree closely with tolerance data for zinc. Fry are definitely less tolerant than yearlings and fingerlings. It would also appear that fingerlings are more tolerant than yearlings although the difference is slight. Since the tolerance relationships, especially of fingerling and yearling, are not totally parallel between copper poisoning and zinc poisoning, subtle pathological differences might exist. Histological examination left no doubt that mortality in all cases resulted from necrosis and desquamation of the branchial epithelium. Small differences in the susceptability of this epithelium of the two age groups could well be present.

The direct significance of this part of the study has been to determine whether or not Shelford (1918), Cole (1941) and Hynes (1960) were justified in insisting that age be considered in setting allowable limits for toxic substances. The results of this study indicate that age is a very prominent factor in the tolerance of fish to heavy metals. In addition, it is to be hoped that this study indicates the weakest link, in the post-alevin stage at least, in bio-assay for ions of heavy metals.

Tolerance limits determined in this study are in no way assumed to be safe even within experimental conditions of temperature and water quality. The test period was of a limited duration (264 hours). On the basis of the degree of 'safeness' these tests indicate concentrations tolerated only in the sense that they appear to be less than the threshold for fatal branchial damage. If factors such as sustained net sodium loss, fatal hemolytic anemia and reproductive failure, can also be attributed to

chronic zinc or copper poisoning then these tolerated concentrations are not 'safe' even under the experimental conditions recorded.

(ii) Resistance Variation Based on Age

Previous comparisons of age-based resistance measurements to heavy metal salt solutions are limited to one study (Jones, loc.cit.). The weaknesses of this study have already been discussed, however, direct comparison of the survival time indicates the juveniles are more resistant in all cases than the adults. In the present study, resistance appears to have maintained the same relationship to age in both zinc and copper solutions. Fingerlings succumbed to the metal ions before fry and both groups were killed much sooner than yearlings. The relationship of fingerling resistance to fry resistance for zinc or copper does not appear to change appreciably even with substantial concentration decreases such as from 3.5 to 2.5 mg per l. of zinc or 0.4 to 0.3 mg per l. of copper. In both zinc and copper solutions, however, once the fingerling resistance began to increase it did so very rapidly, becoming almost vertical. The vertical increase in survival marks the maximum concentration tolerated, temporarily at least. Mortality up to this point results from branchial damage which produces either asphyxiation or lethal fluid loss.

The pattern of resistance of fingerlings in zinc or copper indicates that they are more sensitive to short term 'spills' of a heavy metal toxicant into a body of water than are fry and much more sensitive than yearlings. Thus a slug of zinc or copper solution dumped into a stream would form a toxic plug that would be carried along by the current. Where

contact with the plug was sufficiently prolonged, fingerlings would be the first to succumb, followed by fry and then yearlings. Carpenter (1925) observed a case of this nature on the Rheidol River in Wales. Runoff rain water leached small amounts of lead and zinc from abandoned mine dumps and exposed working sites and carried these into the Rheidol contaminating it briefly. It is worth noting that between 1921 and 1924 Carpenter found no fish or molluscs in the Rheidol although other forms of plant and animal life, characteristic of the area, were reported including trout food organisms. Since the Rheidol had been a trout stream prior to the mining, and since certain of its tributaries contained trout, the seasonal and short-term contamination accompanying the rains was apparently adequate to clear the river of trout the year around. Resistance, as well as tolerance, to heavy metals is therefore also of significance to fish in streams where there is any possibility of heavy metal contamination. Furthermore, the least resistant age group toward a toxicant, in this case the fingerling, is not necessarily the least tolerant, in this case the fry. Toxicity studies considering tolerance alone are thus inadequate, except in highly specialized situations.

II. Histological Studies

(i) Acute Copper and Zinc Poisoning

(a) Branchial Degeneration

In acutely lethal copper and zinc solutions, branchial degeneration was the only observed histological disturbance which could be the cause of mortality. The branchial damage noted parallels, very closely, Schweiger's

report on carp after exposure to toxic levels of ions of mercury, cadmium, nickel, cobalt and manganese; and Parry's results after zinc poisoning in rainbow trout. However, the results are completely contrary to the concept held by Carpenter (1925, 1926, 1927, 1930), Dilling, Healey and Smith (1926), Jones (1935, 1938, 1939, 1957), Ellis (1937), Westfall (1945) and Doudoroff and Katz (1953), which suggests that mortality results from filming of the gill surfaces by precipitation of a mucous-metal complex. Employment of the moderately specific Periodic Acid Schiff staining technique indicated no greater mucous cell activity in gill tissue among zinc and copper poisoned trout than in control fish. Furthermore the absence of mucous on the platelet epithelium indicated that there was little tendency for it to adhere to the epithelium.

Concentrations of zinc and copper of 50 mg per l. and 10 mg per l. respectively, produced a rapid necrosis and desquamation of the branchial epithelium. Death, mainly by asphyxiation, was produced within twelve hours in all animals in both solutions. Complications in osmoregulatory balance probably arose as well but would be only secondary to the total loss of epithelium as the cause of mortality.

In 1 mg per l. and 0.01 mg per l. of zinc and of copper, respectively, total mortality resulted within forty-eight hours. The resulting branchial damage displayed a lesser degree of desquamation than above indicating the epithelium had been more able to tolerate the caustic action at these concentrations. Cloudy swelling and necrosis was general in the epithelium of both platelet and interplatelet areas and would therefore

severely restrict or prevent gaseous exchange. Asphyxiation or anoxia would again be the cause of mortality.

Jones (1947) observed oxygen consumption in sticklebacks in rapidly lethal solutions of mercury, lead and copper. He noted that although irrigation of gills increased, consumption of oxygen rapidly declined. Once initiated, the decrease became continuously greater until the animal succumbed.

Recovery of sticklebacks which were removed before death from solutions of heavy metals was reported by Jones (1938) in some cases. It was further found that immersion in a solution of mercury ions until oxygen consumption was reduced to 37 per cent of normal did not always produce mortality. Explanation probably lies in the branchial degeneration such as that observed in trout subjected to sublethal zinc for three weeks. The generalized necrosis and desquamation caused by lethal solutions is an irreversible cellular reaction. Necrotic cells would have to be replaced before the normal function of the gills would be restored. By the time necrosis is general an inadequate number of live cells remain to carry out normal respiration. Asphyxiation results before dead cells are replaced. Survival is further jeopardized because desquamation leaves gaps in the walls of the gill platelets so that blood and the toxic solution are separated only by the basement membrane and the wall of the blood sinus. In sublethal solutions of zinc cloudy swelling was the only observed form of degeneration and this is completely reversible throughout its range of

severity provided necrosis remains absent. If the irritation is continued the reaction will eventually lead to necrosis of the epithelium but if it is removed, the cells will slowly return to normal appearance and function. Recovery of fish immersed in sublethal solutions of heavy metal salts should be complete after transfer to fresh water provided the damage provoked was limited to cloudy swelling. Schweiger's observation that, after prolonged exposure to heavy metals, live fish transferred to fresh water invariably died, indicates they were already necrotic.

It is now apparent that mucous film did not produce anoxia in heavy metal poisoning. What is probable is that the mucous film detected by proponents of the theory of the so-called "coagulation film anoxia" was in fact composed of necrotic branchial epithelium. The loose epithelial sheets around the platelets (Figs. 3 and 4 in Schweiger, 1957) probably appeared like a pale mucoid coating from a macroscopic observation. None of the proponents of that theory has indicated that any histological (microscopic) examinations were in fact conducted to support the theory.

(b) Visceral Pathology

No degeneration of any form was identified definitely from histological section of the heart, liver, kidney and spleen of acutely zinc or copper-poisoned yearling trout. Pigmentary accumulations resulting from phagocytosis of erythrocytes in the spleen, kidney and liver were at a very modest level. Death, therefore, does not appear to have resulted from hemolysis of erythrocytes or of necrosis in the liver or kidneys,

frequent sites of metal poisoning in mammals. The minor swelling in a few glomeruli in several zinc-poisoned specimens would appear to be an isolated response to an unknown factor, since the response is not general even in the amimals affected.

The abnormally severe distention of the gall-bladder and the discolored brownish bile, observed in many specimens, is commonly symptomatic of an hepatic inflammation. However, no inflammation or degeneration was apparent in histological sections of the affected animals. Furthermore, the chronically poisoned yearlings displayed neither gall bladder distention nor bilary discoloration. The hepatic disturbance would, therefore, appear to be a local one affecting acutely poisoned fish coincidently with testing.

These results all pointing to an absence of visceral pathology ascribable to heavy metal poisoning in acute lethal tests concur with those of Dawson (1935). Dawson (1935) observed no pathological changes until about the fifteenth day in chronic lead poisoning of brown bullheads.

(ii) Chronic Copper and Zinc Poisoning

(a) Branchial Degeneration

Branchial degeneration observed in fish subjected to the sublethal zinc solution, illustrates why fish poisoned by sublethal quantities of heavy metal ions are sometimes distressed. Moderate cloudy swelling is very apparent in most cells in the epithelium of the gill platelets but no necrosis. As previously discussed (Acute Branchial Degeneration) cloudy swelling is a reversible reaction whereas fatty degeneration and necrosis are not. Thus any fish subjected to heavy metal poisoning, whose respiration is impaired by cloudy swelling of the platelet epithelium,

should recover after transfer to fresh water provided anoxia does not develop first.

The branchial damage suffered in sublethal zinc solution for twenty-one days did not appear to produce any apparent behavioral limitations on the fish. It is unlikely, therefore, that the fish suffered any oxygen lack.

The absence of branchial damage in sublethal copper-poisoned trout does not indicate that the copper is unable to produce branchial degeneration. Branchial degeneration was observed in gill sections from acute copper-poisoned specimens which suggests that the sublethal solution was simply too dilute to effect even cloudy swelling.

(b) Visceral Pathology

Neither macroscopic nor microscopic examinations revealed any abnormality in the gall-bladder or any discoloration in the bile. These facts suggest that the previously-observed bladder distention and biliary discolorations were due to factors other than copper and zinc poisoning.

The absence of extensive pigmentation in the spleen of sublethal copper-exposed trout is attributable either to the non-hemolytic action of copper or to the insufficiently-high level of copper in contact with the blood cells. The consistent autopsy reports of pronounced anemia in long term lethal copper poisoning in sheep strongly suggest the latter, (Boughton and Hardy, 1934). Excess accumulations of phagocytosed erythrocyte pigments in the spleen of sublethal zinc exposed trout is evidence of the increased hemolysis produced by zinc ions.

Histological sections of the kidney of chronic copper-poisoned trout appear to contain no more phagocytosed erythrocyte pigments than similar sections from control animals. From this observation it is concluded that this copper solution in the duration of the tests is inadequate to produce hemolysis.

Pronounced pigmentation in histological kidney sections of chronic zinc-poisoned trout indicates an accelerated hemolysis of erythrocytes in this organ. Thus, zinc ions in the blood damage erythrocytes. The most active site of phagocytosis is the peritubular region of the kidney. No phagocytes or pigment were observed within the kidney tubules or Bowman's capsules. These results agreed with those of Dawson (1935). Dawson, however, observed phagocytosed hemoglobin in the spleen, kidney and liver of lead-poisoned bullheads. No pigmentation was observed in the liver of any of the test groups of trout.

Histological cross-sections of the stomach indicate that the gastric submucosa of brook trout is not a particularly active site of erythropoiesis as only a few small lymphoid hemoblasts were observed in the vascular lumina and none in the gastric vascular endothelium. No damage to the gastric mucosa was apparent in the histological sections from specimens in chronic copper or zinc solutions. Neither did there appear to be any abnormal gastric mucous cell activity among the three groups. These results agree with the finding by Joyner (1962) that heavy metals enter fish predominantly by way of the gills and only slightly, if at all, through the digestive tract.

Most workers, whether monophyletic or polyphyletic in their leanings (Catton, 1951), regard the small round cell that Catton terms the small lymphoid hemoblast (s.l. hemoblast) as the stem cell of the fish erythrocyte. Small lymphoid hemoblasts in brook trout were found in the regular hemopoietic organs, the spleen and kidney, in the endothelial lining of the atrium, and ventricle, in the atrial and ventricular lumina and in the large hepatic sinuses. Catton (1951) reported that s.l. hemoblasts occurred in the kidney and spleen of the brown trout, (Salmo trutta). He also observed that small lymphoid hemoblasts were practically indistinguishable from blood lymphocytes which occurred in the general circulation. It could be possible, therefore, that the observed small lymphoid hemoblasts were not hemoblasts but blood lymphocytes. Lymphocytes could leave the regular vascular channels by diapedesis, which could explain their presence in the interior of the trabeculae, however, it appears highly unlikely that the large number of cells observed in the atrial trabeculae would all leave by diapedesis. A more plausible explanation would be that the endothelium possessed the hemopoietic potency to produce s.l. hemoblasts which, in turn, arose from the reticular cell of the reticulo-endothelium. Catton (1951) suggests an alternate path in which endothelial cells give rise to s.l. hemoblasts. This endothelial hemopoietic potency concurs with the observed s.l. hemoblast concentrations in and around the trabecular endothelium of the ventricle and especially the atrium. No large lymphoid hemoblasts were identified in any examined sections of the atrial trabeculae. It would appear, therefore, that s.l. hemoblasts can, and do, arise from the reticular cells and large lymphoid hemoblasts.

The number of proliferative s. l. hemoblast sites in chronic copper-poisoned animals exceeds the number in control fish suggesting that branchially-absorbed copper stimulates erythropoiesis. Trace dietary copper given to anemic rats coincident with normal iron intake has long been known to increase erythropoiesis (Hart et al, 1928). Conversely, the very reduced number of proliferative sites in the endothelium of chronic zinc-poisoned trout relative to control fish is indicative of erythropoietic retardation by zinc. Similar sites with similar activities were observed in the liver, kidney, spleen, blood and to a lesser extent, in the ventricular endothelium.

The observed reduction in numbers of the s. l. hemoblasts from the endothelium and blood of trout subjected to chronic zinc poisoning and the slight increases observed in chronic copper poisoning support Catton's view that they are precursors of erythrocytes. On the other hand, Jordan and Speidel (1924) believed erythrocytes were derived from so-called medium lymphoid hemoblasts, and that thrombocytes and lymphocytes arose from s. l. hemoblasts. This older hypothesis is unworkable unless copper stimulates the production of some leukocytes, derived from s. l. hemoblasts (according to their theory) as well as erythrocytes. As far as is known, only erythrocytes or their precursors have ever been reported to increase in number as a result of the stimulation by copper. It is very likely, therefore, that s. l. hemoblasts would appear in increased numbers when erythropoiesis is active, and in decreased numbers when erythropoiesis is relatively inactive.

If the medium lymphoid hemoblast was the sole stem cell for

erythrocytes, it is most likely that the numbers of these cells would preceed and parallel erythropoietic activity. No change in the numbers of medium lymphoid hemoblasts was observed in sections of spleen or kidney in any of the trout chronically poisoned by copper or by zinc. These results, therefore, support the hypothesis proposed by Catton (1951) that s. l. hemoblasts are the precursors of erythrocytes. If this is the case, the formation of blood cells in trout must be diphyletic, or polyphyletic, but not monophyletic as suggested by Jordan and Speidel (1924).

(c) Hematological Studies

(1) Fingerlings

Fingerling brook trout subjected to sublethal zinc or copper solutions for eleven days all displayed marked polycythemia. The degree of polycythemia increased with increasing concentration in all cases but one; a copper test. These results indicate that, within certain limits at least, polycythemia, due to chronic zinc or copper poisoning, is proportional to the metal concentration in the blood.

The effect of copper in facilitating the incorporation of inorganic iron into hemoglobin has long been known (Hart et al, 1928). Later, Elvehjem and Sherman (1932) showed that although the liver and spleen of anemic rats readily absorbed inorganic iron, replenishing depleted stores, they were unable to incorporate the iron into hemoglobin in the absence of copper. Small traces of orally administered copper, however, rapidly effected such incorporation.

Increases in the iron content of the blood of yearling brook trout in sublethal solutions of copper and of fingerlings in sublethal solutions of copper and zinc strongly suggest that environmental solutions

can substitute for dietary traces. The absorption studies of zinc^{65} on the brown bullhead indicates that extremely little, if any, environmental zinc solution enters fish by way of the gastro-intestinal tract; presumably the situation is the same for copper.

The physiological mechanism of copper and other heavy metals accelerating erythropoiesis is poorly understood. According to Ruch and Fulton (1961, p. 514), the main influence of copper is believed to be as a "catalyst in a non-specific manner in the utilization of iron stores in the liver..." The effect of other heavy metals on erythropoiesis is even more vague and is summed up by Ruch and Fulton (p. 515) as follows:

> "From time to time claims have been made that various minerals are essential for blood formation. Of these, manganese, zinc, nickel, vanadium, molybdenum, and germanium may be mentioned, although there is little to support the contention that deficiency in any of them may cause a failure of hemopoiesis."

(2) Yearlings

Certain features of the blood counts of yearling trout subjected to sublethal copper or zinc solutions for 21 days differed markedly from the fingerling counts. Yearling trout in sublethal zinc showed a mild anemia in blood counts and excessive phagocytosis of erythrocytes in the spleen and kidney. These results signify that the zinc enhanced erythro-

poiesis is either short term or that the yearling concentration tested elevated the blood zinc concentration sufficiently to exceed a threshold hemolytic level. In either case, it appears questionable that zinc will maintain polycythemia. Yearlings held in sublethal copper solutions continued to display pronounced polycythemia. This observation is clearly indicative that trace quantities of copper, branchially absorbed, can accelerate erythropoiesis in trout with normal erythrocyte levels. Histological examination of the branchial epithelium revealed that they were identical with those of controls, showing clearly that polycythemia was not due to anoxia. In addition, pronounced cloudy swelling in the branchial epithelium of sublethal zinc-poisoned trout occurred without inducing polycythemia.

Although differentials occur in the erythrocyte counts of the three test groups, the hematocrit value for each is practically identical. Thus, the mean erythrocyte volume (MCV) for fish subjected to chronic copper poisoning is much smaller than for fish in control or sublethal zinc solutions. The smaller size signifies greater activity in the erythropoietic tissues. Catton (1951) found that a progressive increase in the size of teleost erythrocytes occurred paralleling maturation.

Distinctly greater concentrations in the total blood iron of trout in sublethal copper over those in control or sublethal zinc solutions leaves little doubt that the copper accelerates iron incorporation into hemoglobin. The amount of iron in the average erythrocyte (MCIC), however, is somewhat less in trout subjected to sublethal copper than it is in the other two groups. But when compensation is made for the differential in

the MCV by determining the mean corpuscular iron concentration per 100 cubic microns the iron concentration is highest in fish subjected to sub-lethal copper solution. This last result implies that trace quantities of copper ions in the blood not only tend to facilitate conversion of iron to hemoglobin and the acceleration of erythropoiesis but also greater hemoglobin density in erythrocytes.

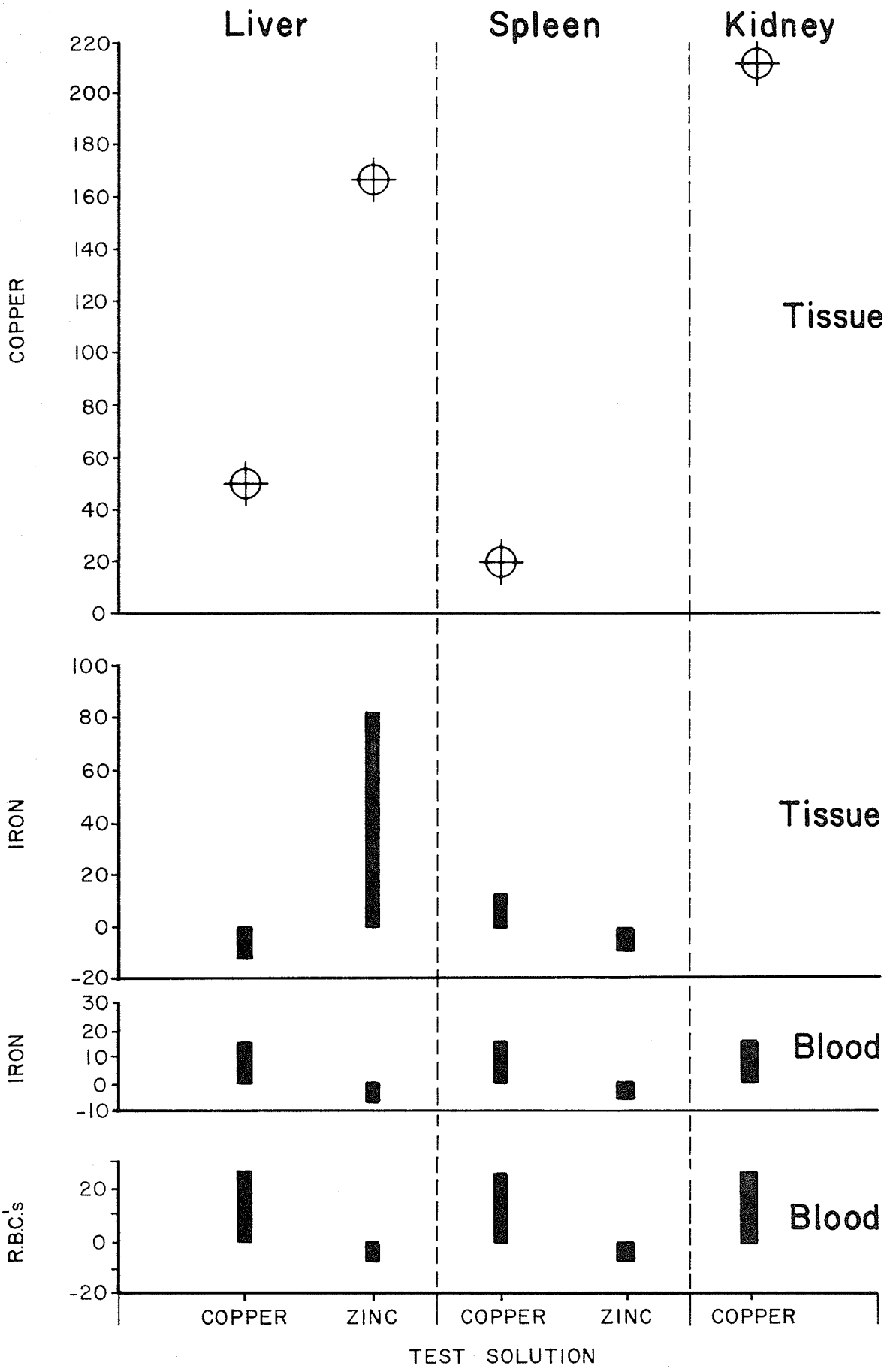
(d) Histochemical Studies

(1) Tissue Iron Concentration

The use of potassium ferrocyanide in the spectrophotometric determination of iron in histological sections proved very satisfactory because the iron appeared quite homogeneously distributed in the tissues examined. The analyses were thus not subject to the errors of spotty and incomplete staining of the microscope field. Certain small localized sites were denser than the surrounding area but generally the cells in any section appear homogeneously stained. In addition to the above-mentioned homogeneity spectrophotometric determination of iron has several other desirable attributes. The technique is rapid, sensitive and sections so stained can also be used to pinpoint histological areas of high or low density when they occur. On the other hand, the technique has a number of weaknesses or deficiencies. For one thing, it would be difficult, if not impossible, to prepare satisfactory standards to make the determinations totally quantitative. Also, histochemical analyses all suffer a distinct disadvantage when they are used as a representative sample of the whole organ because they are only two dimensional. To overcome this two-dimensional disadvantage, a number of sections should be analysed and each

section over several microscope fields. In the current tests, only one section was determined from each organ per fish but two fish were examined per test. Normally two representative microscope fields were determined on each section. Because of the single section determined per fish, iron results might, therefore, not be a good representation of the iron content in each organ. One further potential source of error has been the assumption that iron, in the amounts found in the liver and spleen, combines with potassium ferrocyanide according to Beer's Law. No reference was found in the literature on the subject. Because careful attention was observed to avoid contamination by foreign iron in the preparation of the tissue and histological sections contamination is not considered a likely source of error. A final source of error was found in utilizing potassium ferrocyanide for two different batches of slides. Apparently the initial batch of sections caused the decomposition of some of the ferrocyanide ions complex and oxidized some of the ferrous ions, because the succeeding batch appeared noticeably darker than it should have. Fresh ferrocyanide solution was, therefore, used for each batch of sections reported. Because of these weaknesses in the technique, the tissue iron results should be regarded only as a guide and not as a substitute for classical chemical analysis of iron in tissue.

With the above reservations in mind, the histochemical analyses indicate the following: hepatic iron appears to be reduced about 15 percent below control levels in sublethal copper while erythrocyte count is increased about 20 percent and blood iron about 15 percent (Fig. 19). Elvehjem and Sherman (1932) observed a similar relationship between hepatic and blood


Liver
Spleen
Kidney
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220
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Tissue
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Tissue
IRON
30
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10
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-10
Blood
R.B.C.'s
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Blood
COPPER
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COPPER
ZINC
COPPER
TEST SOLUTION

iron after trace copper administration to anemic rats, provided the rats had adequate hepatic iron reserves. It appears likely that branchially absorbed copper is also able to stimulate the conversion of hepatic iron to hemoglobin and to promote erythropoiesis in trout even in the absence of anemia. In contrast to the reduced hepatic iron, splenic iron showed approximately a 15-percent increase. Such an increase is difficult to explain unless it represents iron transferred to the spleen from the liver as a ready reservoir for the increased erythropoiesis. Catton (1951) found the spleen to be the main erythropoietic organ in the brown trout (Salmo trutta). It is not surprising, therefore, that trout held in sub-lethal zinc solutions, where erythropoiesis is slightly retarded, display the reverse liver-spleen-blood iron relationship to that found in copper. Hepatic iron showed an 80-percent increase above the control level indicating either a transfer of iron to the liver or an error in histochemical analysis. Slightly reduced iron concentrations appeared in the spleen and blood, the former suggests the possibility of a feed-back from spleen to liver in reduced periods of erythropoiesis. Blood iron might also feed back to the liver under these conditions but histological examination of the spleen and kidney suggest the cause is increased hemolysis. Since no kidney sections were available for iron determinations, the corresponding changes in renal iron remain unknown.

(2) Histochemical Determination of Copper

Analysis of tissue copper with rubeanic acid was not possible spectrophotometrically because copper sites were very localized and sparsely scattered as small green-black, crystal-like granules. Analysis

was modified, therefore, and performed on the basis of the number of small squares occupied by the rubeanate granules in a Whipple eye-piece grid. This technique suffers from certain of the defects of the iron histochemical technique such as the two-dimensional sampling error. In addition, however, no allowance could be made for variation in the density of copper rubeanate granules nor size differences in the granules. For example, if a small square contained a small, pale granule, it was considered occupied, if it contained two or more larger, darker granules, once again only one square was counted as occupied unless the granules overlapped into other squares. The copper results must, therefore, be regarded with a considerable degree of caution.

Copper ions in the liver and spleen of sublethal copper-poisoned trout were moderately above corresponding control levels but copper in the kidney was over two times the amount in the control kidney. These accumulated copper ions may stimulate conversion of hepatic iron to hemoglobin, but on the other hand, a relatively very high level of copper was detected in the liver of sublethal zinc-poisoned trout and these animals displayed slightly reduced blood iron and erythropoiesis. It would appear that copper, although facilitating the conversion of hepatic iron to hemoglobin, may not act directly on the liver to do so.

The elevated renal copper concentration indicates that copper accumulated to a large extent, like most heavy metals, in the kidneys. Perhaps after extended accumulation in sublethal copper, the tubules might show degeneration characteristic of lead and mercury, and the blood, the characteristic damage. In the three weeks sublethal immersion, no renal or blood damage was apparent.

(3) Tissue Zinc Concentration

Zinc in the tissues was not histochemically analysed for two reasons: (a) Hibiya and Oguri (1961) and Joyner (1961) had examined the zinc^{65} accumulation in goldfish and brown bullheads respectively and with minor exceptions were in agreement, (b) histological sections from control and sublethal zinc-poisoned trout were in short supply and were required for the tissue iron determinations. In addition, no satisfactory histochemical technique was found for zinc.

The differences between the results of Hibiya and Oguri and of Joyner are probably largely attributable to the method of zinc uptake. Hibiya and Oguri injected the zinc into the air-bladder whereas Joyner allowed the fish to absorb the zinc solution through the gills for over a week. Because Joyner's absorption more accurately describes natural immersion in zinc contaminated waters, his results would appear more likely to show the natural accumulations by fish. According to both studies, the greatest accumulations occurred in the gastro-intestinal tract. Joyner observed, however, that renal zinc accumulation is approximately equal to that in the gut. Hibiya and Oguri counted opisthonephros and pronephros separately, summing the two together the zinc activity might well have approximated that of the intestinal accumulation. Branchial accumulation of zinc was only slightly less than that of the kidneys according to Joyner. Hibiya and Oguri, who were only measuring the zinc carried to the gills by the blood found it appreciably less. Both investigations found hepatic zinc accumulation to be about half that of the intestine and Joyner found the splenic zinc level only a little less.

Consequent to these results, it is apparent why zinc can be an effective hemolytic agent. Neither investigation reported blood zinc levels but because of the large zinc accumulations in such highly vascularized organs such as the gastro-intestinal tract, gills, kidney, liver and spleen, circulating erythrocytes would be drawn into close proximity with these cells. Lysis of erythrocytes and retardation of erythropoiesis would be a constant threat to the circulatory system even if the blood itself did not reach hemolytic zinc levels. Examination of the histological sections of kidney and spleen of sublethally-poisoned trout clearly shows that zinc induces increased phagocytosis. Similarly, histological examination of erythropoietic tissue indicates exogenous zinc ions within the animal reduce erythropoiesis.

III. Concluding Remarks

Information leading to a satisfactory explanation of differential susceptibility of fish of various ages to poisoning by heavy metals is extremely limited, partly faulty and frequently contradictory to previous information, therefore, a hypothesis is proposed as a starting-point for such an explanation.

Experimental observations on carp (Schweiger, 1957), rainbow trout (Parry, 1960) and brook trout in the present study all clearly indicate that mortality induced by higher levels of heavy metals results from branchial degeneration. Factors controlling resistance to poisoning must, therefore, be associated with the branchial epithelium. The exact nature of the mechanism is totally speculative but could probably consist of selective membrane impermeability to salts of heavy metals and resistance by such a membrane to the lytic action of these ions.

The acceptance of the existence of a branchial defence mechanism leads to a discussion of tolerance variations with age of the fish. If the branchial epithelium became more resistant to chemical damage with age and normal trace metal contact, the overall tolerance of the fish should increase from hatching to maturity. No totally conclusive evidence indicates that fish become acclimatized to heavy metals in their environment. Paul (1952) observed that wild fish survived in certain California streams, polluted by copper, which hatchery-reared fish of the same species were unable to tolerate because of the copper. Affleck (1952) reported that rainbow trout transported in galvanized (zinc-lined) containers for a second time suffered fewer mortalities than in the initial exposure. Goodman (1951) had also observed fish acclimatized to streams polluted by zinc. In all of these cases, however, it is not certain whether individual acclimatization or population acclimatization, by natural selection, occurred.

If acclimatization to heavy metals occurs by contact with traces of heavy metals in natural waters, variations in tolerance with age are quite simple. Tolerated concentrations of copper or zinc would increase with age until peak acclimatization levels had been reached. The degree of acclimatization possible (peak acclimation potential) and the rate at which it could occur probably would show some differences among various metals. Increases in tolerance limits were observed to follow this pattern. In zinc poisoning, yearlings displayed a markedly higher tolerance than fingerlings and fingerlings rather more than fry. This pattern would indicate that acclimation to increasing zinc concentrations, by increasing

branchial degeneration resistance, develops continuously from hatching to maturity. The pattern also indicates that considerable acclimation to zinc is possible in terms of increasing branchial resistance. In copper poisoning, tolerance by fry and fingerlings is more widely separated than is similar tolerance to zinc poisoning. Fingerling tolerance to copper appears very slightly greater than that of yearlings, however, without an experiment on yearlings at a level of 0.15 mg. per l., the fingerling tolerance level, and in view of the point width indicated by the 95 percent confidence interval for the preceeding resistance-concentration points, the difference between yearling and fingerling tolerance limits is not significantly different. It is entirely possible, therefore, that yearling and fingerling maximum limits of tolerance are the same. Acclimation to copper appears to proceed for a very limited period accompanying fish development and is complete at the fingerling stage. Although natural acclimation proceeds for a very limited time, tolerance differences between fry and the two older groups are appreciable indicating considerable acclimation has occurred.

The nature of the resistance to branchial degeneration is almost certainly that of a fixed resistance. That is, the defence mechanism possesses a conditioned membrane hardness, impermeability or other similar mechanism which operates at peak level at all times. Thus, increases in the concentration of a toxicant such as copper or zinc does not increase the resistance (immediately, at least). It merely increases the possibility that the stress will be increased beyond the mechanism's ability to exclude it.

High concentrations of salts of heavy metals exerts an intense and rapidly destructive action on the epithelium. The fixed resistance available in the epithelium is quantitatively different at various ages, but its influence is minimal at intensive concentrations of copper or zinc, therefore, survival times, for all age groups, are closest in this range. As concentration decreases, the potential of the toxicant to destroy epithelium also decreases, fewer ions are available for destruction. The greater magnitude of the fixed resistance of older groups neutralizes a much larger portion of the potency of the metals for such branchial degeneration at any given concentration than that of the younger groups. With decreased branchial damage, oxygen consumption is less inhibited resulting in longer survival. Consequently, older groups, such as yearlings, have longer survival times with decreasing concentration while younger groups continue to show little change in survival time (the resistance component of the latter groups remain insignificant in comparison to the branchial degeneration component of the metals). When the concentration of heavy metal decreases to the point where the resistance component is significant, relative to the branchial degeneration component, the younger groups will also display increasing resistance with decreasing concentration. Hypothetical curves are shown in Fig. 18 which indicate this proposed relationship of survival times to concentrations. It is to be expected that copper and zinc survival curves would follow this pattern and basically they do, however, a second factor is believed to deform part of the relationship. Suppose group 3 of Fig. 18, representing the fingerlings, required a greater oxygen intake per unit

weight than the other groups. As oxygen consumption was reduced by branchial damage, in higher concentrations, group 3 animals would begin to die before the others were seriously affected. Wherever oxygen consumption was forceably reduced, points for group 3 animals would be displaced to the left and below their hypothetical position on the curve (Fig. 18) before the other groups were affected.

Zeuthen (1953) presents an argument with some evidence that indicates that some animals including Bufo, Rana, Mytilus, Artemia and Asterias show triphasic metabolic rate versus development curves. These curves indicate that, in the growth of the animal, at some intermediate stage in development, oxygen consumption will increase above what it was for a younger animal and what it will be as an adult. Shepard (1955) determined the tolerance of three age groups of brook trout to hypoxia and found the tolerance to oxygen deficiency increased with age. The youngest group examined were fry approximately two months after hatching and averaging 4.3 cm. in length and 0.9 gm. in weight. These are close to the age of fingerlings in this study. Similarly, his second group of five month-old fingerlings were 6.8 cm. and 3.8 gm., appears to fall midway between the age of fingerlings and yearlings used here. His oldest group, large fingerlings, 10 to 11 months were 9.9 cm. and 12.1 gm., are younger but larger than most of the yearlings used. Shepard's results, therefore, very clearly indicate that tolerance of fingerlings to hypoxia is lower than that of yearlings. No conclusions can be taken from his results for the tolerance of fry used in this study to lower levels of

oxygen. The hypothesis developed depends for its explanation of the reduced overall fingerling tolerance on the assumption that the tolerance of hypoxia by young fry exceeds that of small fingerlings.

Summarizing the hypothesis briefly, requires two assumptions. First, tolerance to branchial degeneration from heavy metal salts increases with age from fry to some older age. Secondly, that tolerance to hypoxia shows a triphasic relationship between young fry and yearling stages.

Using this hypothesis to predict the resistance and tolerance of the three age groups of brook trout tested to copper and zinc poisoning, we would expect the following: tolerance to poisoning by heavy metals should increase from fry to fingerling to yearling unless acclimatization is completed at an early age. Curves of survival should generally adhere to the hypothetical model for survival times, shown in Fig. 18. If small fingerlings have a higher oxygen requirement and lower tolerance, their overall tolerance will be lower than the hypothetical value shown in Fig. 18.

When survival time for fingerlings begins to increase, it will do so at a greater rate than that of fry or yearling because of reduced stress to both phases of its sensitivity. Deviation from the hypothetical curves in Fig. 18 would include a displacement of the fingerling curve to the left and below its indicated position. Such a displacement would place fingerling and fry curves in closer proximity to one another or, in concentrations producing rapid limited branchial damage but slower lethal damage, fingerlings would appear more sensitive. This latter situation appears to occur in the upper concentration range of both zinc and copper tests.

At high concentrations of heavy metals, producing extensive epithelial necrosis and desquamation, there is little doubt that mortality results from asphyxiation or hypoxia. Mortality in lower concentrations, however, inducing lesser branchial damage, might result from or be complicated by factors other than asphyxiation. Meyer's observation (1952), that mercuric ions reduce or eliminate sodium absorption in fish, coupled with his previous observation that sustained sodium or chloride loss, which results in mortality, can occur, lends credence to the probability that mortality, due to heavy metals, can result from electrolyte imbalance. The action of mercuric ions on the absorption of sodium or chloride by the gills might be specific to mercury alone. On the other hand, heavy metals are known to be capable of poisoning enzyme systems (Davson, 1959, p. 244) especially those with the sulph-hydryl, S-H group (Randall and Seeler, 1949). An enzyme with a free sulph-hydryl group has been identified pharmacologically (Meyer, 1952) and histochemically (Cafruny et al, 1954) as the enzyme responsible for active sodium absorption. Presumably other heavy metals are capable of inhibiting sodium absorption.

Mortality, resulting from inadequate absorption of sodium and chloride, due to poisoning by heavy metals if it occurs, must do so as a result of permeability of the branchial epithelial or secretory cell membranes to these metals. Furthermore, this penetration must be at a sufficiently low level not to effect generalized necrosis or mortality would result from asphyxiation. The degree of penetration and the time over which it occurred would control the extent of the enzyme poisoning. This would, in turn, directly control the inhibition of sodium absorption;

increased penetration increases poisoning which increases inhibition (Meyer, 1952). Where fish are fasting or fed infrequently, as they were in this study, salt balance must have been dependent on branchial absorption of sodium and chloride. Restriction of such absorption in conjunction with continued renal sodium clearance would result in a sodium deficit. Such a deficit would continue as long as the fish was held in the solution of heavy metal ions. The greater the rate of poisoning of the transport mechanism for sodium, the more rapidly would mortality result.

Previously, it was assumed that branchial epithelial resistance to heavy metals increased with age. If this resistance rises as a consequence of selectively reduced permeability (towards heavy metals) of the cellular membrane then the extent of a sodium deficit should decrease with age. The tolerance results for brook trout poisoned by zinc or copper tend to substantiate this assumption provided that mortality, in the longer term acute poisoning,is due to sodium deficiency.

SUMMARY

Tolerance to acute copper and zinc poisoning was found to increase with age from fry (0.12 g.) to fingerling and yearling. Increased tolerance from fingerling to yearling appears dependent upon:

(i) metal ions contacted

(ii) the rate of acclimation with growth

(iii) the extent to which acclimation can be carried

Natural acclimation, resulting from continuous contact with trace metals of the normal environmental waters, is suggested as the cause of the age-based increase.

Resistance to copper and zinc was also observed to increase with age. Fingerlings, however, displayed equal or greater sensitivity than fry in both copper and zinc rapidly lethal concentrations. Evidence is presented to suggest a second factor, increased sensitivity to oxygen lack, operating in the case of the fingerlings to a greater extent than in fry. It is assumed that when allowance is made for this fingerling sensitivity, resistance to branchial degeneration (and percentage oxygen reduction would be age-based.

Histological examination of the gills of acute copper and zinc-poisoned, speckled trout clearly confirmed the observations of Schweiger (1957) and Parry (1960). Mortality results from asphyxia produced by branchial degeneration of the platelet epithelium not muco-metallic platelet filming. Heavy metal solutions which prove lethal within 24 hours produce complete desquamation. Solutions lethal after longer

periods display a transition from total to slight desquamation, reduced necrosis and increased cloudy swelling corresponding to extending survival times. Severe cloudy swelling alone apparently does not produce asphyxiation, although there is some evidence that it may be associated with inhibited sodium absorbtion. Sustained inhibition of sodium absorption could lead to a lethal electrolyte imbalance.

Asphyxiation or electrolyte imbalance produces mortality in acutely-poisoned fish before degeneration in the visceral organs becomes apparent.

In chronic copper-poisoning, erythropoiesis is stimulated resulting in increased blood iron, erythrocytes/m.m.^{3}, and mean corpuscular iron/100 cubic microns. Reductions occur in mean erythrocyte volume and mean corpuscular iron. Corresponding tissue analyses indicate a catalysing effect well known in mammals (Elvehjem and Sherman, 1932). Hepatic iron concentrations are reduced corresponding to blood iron increases. Contrary to previous observations in rats, however, trout splenic iron appeared in apposition to hepatic stores and increased after copper immersion. Phagocytosis in the spleen and opisthonephros appeared identical in control and copper-poisoned animals.

Chronic zinc poisoning resulted in increased R. B. C. lysis, evident in reduced count and increased splenic and renal pigment deposits. Total blood iron was reduced almost proportional to the R. B. C. reduction. Mean corpuscular iron content was slightly greater than in the other groups, but mean corpuscular iron per unit volume was less than in copper and almost identical to control animals.

Evidence was noted which was indicative of reduced hemopoiesis in both the organs and the general circulation of chronic zinc-poisoned trout.

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Appendix Ia

Age-based variation in survival time for brook trout subjected to copper or zinc poisoning.

(a) Fry - Zinc

Survival time (Y), in hours for indicated zinc concentration in mg. per l.

No. Surviving (X)	.16	.32	.64	1.28	2.50	3.84	5.12
9	24	24	12	36	24	12	12
8	96	60	12	36	24	12	12
7	108	72	24	36	36	24	12
6	132	108	24	48	36	24	12
5	168	168	132	72	36	24	24
4	288	168	132	108	36	24	24
$X = 39$							
$X^2 = 271$							
$Y =$	816	600	336	336	192	120	96
$\bar{Y} =$	136	100	56	56	32	20	16
$Y^2 =$	150048	77472	36288	23040	6336	2595	1728
$XY =$	4524	3360	1704	1944	1200	7320	576
$m =$	-44.57	-30.86	-27.43	-13.21	-2.74	-2.74	-2.74

(b) Fingerling - Zinc

Survival time (Y), in hours for indicated zinc concentration in mg. per l.

No. Surviving (X)	1.25	1.88	2.20	2.50	2.80	3.40
9	48	36	32	14	8	8
8	72	60	56	14	8	8
7		120	80	14	8	8
6		120	92	14	20	8
5		192	92	14	20	8
4		228	104	14	20	8
X 39						
X^2 271						
Y		756	456	84	84	48
$\bar{Y}$		126	76	14	14	8
Y^2		122544	38304		1392	
XY		4236	2816		492	
m		-38.74	-13.71		-3.09	

Appendix Ib

Age-based variation in survival time for brook trout subjected to copper or zinc poisoning (continued).

(c) Yearling - Zinc
Survival time (Y), in hours
for indicated zinc concentration in mg. per l.

No. Surviving (X)		2.16	2.52	2.88	3.60	7.20
5		40	36	24	24	24
4			36	36	24	24
3			48	36	24	24
2			60	36	36	24
X	39	Y	180	132	108	96
X^2	271	$\bar{Y}$	45	33	27	24
		Y^2	8496	4464	3024	
		XY	588	444	360	
		m	-8.40	-3.60	-3.60	

(d) Fry - Copper
Survival time (Y), in hours
for indicated copper concentration in mg. per l.

No. Surviving (X)		.007	.014	.028	.057	.115	.230	.460
9		36	36	24	24	12	12	12
8			36	60	24	24	12	12
7			48	72	48	48	36	12
6			48	72	48	60	36	12
5			72	88	48	72	36	24
4			168	108	72	72	36	36
X	39	Y	408	424	264	288	168	108
X^2	271	$\bar{Y}$	68	60.6	44	48	28	18
		Y^2	40608	33950	13248	16992	5472	2448
		XY	2268	2504	1560	1644	996	624
		m	-21.94	-14.40	-8.91	-13.03	-5.49	-4.46

Appendix Ic

Age-based variation in survival time for brook trout subjected to copper or zinc poisoning (continued).

(e) Fingerlings - Copper
Survival time (Y), in hours
for indicated copper concentration in mg. per l.

No. Surviving (X)	.150	.175	.200	.300	.400
9	216	144	17	5	5
8		192	29	17	5
7		216	41	17	5
6		216	41	17	17
5		252	41	17	17
4		264	41	17	17
X 39	$\underline{Y}$	1284	210	90	66
X^2 271	$\underline{Y}$	214	35	15	11
	Y^2	284112	7854	1470	942
	XY	7956	1287	555	375
	m	-22.29	-4.46	-1.71	-3.09

(f) Yearling - Copper
Survival time (Y), in hours
for indicated copper concentration in mg. per l.

No. Surviving (X)	.173	.202	.230	.345	.460
5	72	84	68	36	20
4	84	84	80	60	20
3	120	96	116	60	20
2	132	108	164	60	32
X 39	$\underline{Y}$ 408	372	428	216	92
X^2 271	$\underline{Y}$ 102	93	107	54	23
	Y^2 44064	34992	51376	12096	2224
	XY 1320	1260	1336	720	304
	m -21.6	-8.4	-32.4	-7.2	-3.6

Appendix IIa

Erythrocyte count in incipiently lethal zinc and copper-poisoned fingerling brook trout. Erythrocytes X 1000 per mm^3.

(a) Zinc Solutions

Fish No.	Control	0.625 mg/l	1.28 mg/l	1.88 mg/l
1	870	910	1390	1290
	950	890	1360	1380
2	850	1160	1040	1300
	770	1240	1240	1410
3	950	1030	1040	1380
	960	1040	1120	1220
4	1060	860	1250	1180
	1030	900	1370	1110
5	970	990	1100	1120
	1070	940	1090	1150
6	880	870		
	800	960		
7	1090	1040		
	1120	1050	———	———
$\bar{X}$	884	991	1200	1250

Appendix IIb

Erythrocyte count in incipiently lethal zinc and copper-poisoned fingerling brook trout. Erythrocytes X 1000 per mm^3.

(b) Copper Solutions

Fish No.	Control	0.100 mg/l	0.150 mg/l	0.175 mg/l
1	920	1350	990	1030
	950	1060	990	1006
2	900	1170	1070	1270
	920	1090	1080	1300
3	970	1170	1140	1130
	970	1130	1150	1160
4	900	1010	1240	1260
	780	950	1200	1320
5	800	990	1030	990
	970	1180	1130	920
6	1020	1280	1060	1120
	810	1270	1110	1060
7	870	1110	1160	1120
	920	1080	1170	
8	860	1060	1060	
	910	1100	1100	----
$\bar{X}$	851	1121	1105	1135

Appendix IIc

Erythrocyte count in chronic copper and zinc-poisoned yearling brook trout. Erythrocytes X 1000 per mm^3.

	Solution					
Fish No.	Control (X)	$X-\bar{X}$	Copper (X)	$X-\bar{X}$	Zinc (X)	$X-\bar{X}$
1	1030	211	1520	38	1040	160
	1080	161	1740	182	930	270
2	1260	19	1700	142	830	370
	1280	39	1770	212	870	330
3	1390	149	1540	18	540	660
	1520	279	1480	78	530	670
4	1170	71	1400	158	740	460
	1130	111	1640	82	1110	90
5	1090	151	1670	112	1360	160
	1170	71	1590	32	1670	470
6	1290	49	1470	88	1530	330
	1200	41	1320	238	1300	100
7	1300	59	1710	152	1240	40
	1180	61	1480	78	1250	50
8	1330	89	1760	202	1580	380
	1440	199	1610	52	1630	430
9			1350	208	1430	230
			1310	248	1450	250
10			1610	52	1080	120
			1480	78	1110	90
11					1540	340
					1430	230
12					1340	140
					1260	60
$\bar{X}$	1241		1558		1200	

Appendix III

Packed cell volume (Hematocrit) of yearling brook trout subjected to sublethal copper or zinc poisoning for 21 days.

Test	Animal	Hematocrit (X)	$X - \bar{X}$
Control	1	39.0	6.1
	2	31.0	1.9
	3	33.9	1.0
	4	30.4	2.5
	5	28.1	4.8
	6	32.2	0.7
	7	27.0	5.9
	8	41.7	8.8
	Mean ($\bar{X}$)	= 32.9	$(x-\bar{x})$ = 31.7
	S = 2.65	$S_{\bar{x}}$ = 0.94	$(x-\bar{x})^2$ = 183.9
Copper	1	33.3	0.8
	2	36.6	4.1
	3	35.2	2.7
	4	31.4	1.1
	5	33.4	0.9
	6	31.4	1.1
	7	28.8	3.7
	8	33.9	1.4
	9	32.7	0.2
	Mean ($\bar{X}$)	= 32.5	$(x-\bar{x})$ = 21.7
	S = 1.71	$S_{\bar{x}}$ = 0.54	$(x-\bar{x})^2$ = 76.2
Zinc	1	22.2	9.1
	2	22.0	9.3
	3	32.6	1.3
	4	32.7	1.4
	5	29.7	1.6
	6	34.0	2.7
	7	30.8	0.5
	8	32.7	1.4
	9	36.5	5.2
	10	33.3	2.0
	11	32.2	0.9
	12	36.7	5.4
	Mean ($\bar{X}$)	= 31.3	$(x-x)$ = 40.8
	S = 2.99	$S_{\bar{x}}$ = 0.86	$(x-\bar{x})^2$ = 246.0

S - Standard Deviation
$S_{\bar{x}}$ - Standard Error

Appendix IV

Total iron in the blood of brook trout held in sublethal copper or zinc solutions for 21 days.

Test	Animal	Concentration (X) mg/100 ml.	$X - \bar{X}$
Control	1	39.8	4.3
	2	36.1	0.6
	3	38.0	2.5
	4	30.4	5.1
	5	34.4	1.1
	6	32.7	3.8
	7	36.6	1.1
	8	35.8	0.3
	Mean ($\bar{X}$)	= 35.5	$(x-\bar{x})$ = 18.8
	S = 1.78	$S_{\bar{x}}$ = 0.63	$(x-\bar{x})^2$ = 68.1
Copper	1	42.5	1.2
	2	41.0	0.3
	3	41.0	0.3
	4	41.9	0.6
	5	39.8	1.5
	6	39.6	1.7
	7	38.0	3.3
	8	55.2	13.9
	9	32.5	9.0
	10	41.5	0.2
	Mean ($\bar{X}$)	= 41.3	$(x-\bar{x})$ = 32.0
	S = 4.36	$S_{\bar{x}}$ = 1.4	$(x-\bar{x})^2$ =292.3
Zinc	1	30.8	2.4
	2	29.2	4.0
	3	33.4	0.2
	4	36.5	3.3
	5	34.8	1.6
	6	30.3	2.9
	7	37.2	4.0
	Mean ($\bar{X}$)	= 33.2	$(x-\bar{x})$ = 18.4
	S = 1.30	$S_{\bar{x}}$ = 0.49	$(x-\bar{x})^2$ = 59.7

S - Standard Deviation
$S_{\bar{x}}$ - Standard Error

Appendix V

Mean corpuscular volume (MCV) for yearling brook trout subjected to sublethal copper or zinc solutions for 21 days. S-Standard Deviation, $S_{\bar{x}}$ - Standard Error

Test	Animal	Hematocrit	RBC Count (1000's)	MCV cubic microns	$X-\bar{X}$
Control	1	39	1055	370	3
	2	31	1270	244	23
	3	33.9	1455	233	34
	4	30.4	1150	264	3
	5	28.1	1130	249	18
	6	32.2	1245	216	8
	7	27.0	1250	216	51
	8	41.7	1385	301	34
				mean $\bar{X}$ = 267	
				$(x-\bar{x})$ = 174	
				$(x-\bar{x})^2$ = 5848	
		S = 16.1	$S_{\bar{x}}$ = 5.7		
Copper	1	33.3	1630	204	3
	2	36.6	1735	211	4
	3	35.2	1510	233	26
	4	31.4	1520	207	0
	5	33.4	1630	205	2
	6	31.4	1395	225	18
	7	28.8	1595	181	26
	8	33.9	1685	201	6
	9	32.7	1480	221	14
	10	27.8	1545	180	27
				mean $\bar{X}$ = 207	
				$(x-\bar{x})$ = 126	
				$(x-\bar{x})^2$ = 2666	
		S = 10.4	$S_{\bar{x}}$ = 3.3		
Zinc	1	22.2	985	225	37
	2	22.0	850	259	3
	3	32.6	535		
	4	32.7	925	354	8
	5	29.7	1515	196	66
	6	34.0	1415	240	22
	7	30.8	1245	247	15
	8	32.7	1105	296	34
	9	36.5	1410	259	3
	10	33.3	1095	304	42
	11	32.2	1485	217	45
	12	36.7	1300	282	20
				mean $\bar{X}$ = 262	
				$(x-\bar{x})$ = 295	
				$(x-\bar{x})^2$ = 11861	
		S = 18.9	$S_{\bar{x}}$ = 5.7		

Appendix VI

Mean corpuscular iron content (MCIC) for yearling brook trout held in sublethal copper or zinc solutions for 21 days.

Test	Animal	Iron mg/ 100 ml.	RBC Count (1000's)	MCIC Picograms ($\bar{X}$)	$X-\bar{X}$
Control	1	39.8	1055	.377	.089
	2	36.1	1275	.284	.004
	3	38.0	1455	.261	.027
	4	30.4	1150	.264	.024
	5	34.4	1130	.301	.013
	6	32.7	1245	.263	.025
	7	36.6	1250	.293	.015
	8	35.8	1385	.258	.030
			mean ($\bar{X}$) =	.288	$(x-\bar{x})_2$ = .227
					$(x-\bar{x})^2$ = .112
		S = 0.024	$S_{\bar{x}}$ = 0.0082		
Copper	1				
	2	42.5	1630	.261	.002
	3	41.0	1735	.236	.027
	4	41.0	1510	.272	.009
	5	41.9	1520	.276	.013
	6	39.8	1630	.244	.019
	7	39.6	1395	.284	.021
	8	38.0	1595	.238	.025
	9	55.2	1685	.328	.035
	10	32.5	1484	.220	.043
	11	41.5	1545	.269	.006
			mean ($\bar{X}$) =	.263	$(x-\bar{x})_2$ = .200
					$(x-\bar{x})^2$ = .055
		S = 0.012	$S_{\bar{x}}$ = 0.0038		
Zinc	1				
	2	30.8	850	.362	.089
	✱ 3	29.2	535	.546	
	4	33.4	925	.361	.047
	5				
	6				
	7	36.5	1245	.293	.021
	8	34.8	1105	.315	.001
	9	30.3	1440	.210	.004
	10	37.2	1095	.340	.026
			mean ($\bar{X}$) =	.314	$(x-\bar{x})_2$ = .147
		S = 0.18	$S_{\bar{x}}$ = 0.0073		$(x-\bar{x})^2$ = .056

(✱) Not used in calculation

S - Standard Deviation; $S_{\bar{x}}$ - Standard Error

Appendix VII

Mean corpuscular iron concentration per 100 cubic microns of erythrocyte for yearling brook trout subjected to sublethal copper or zinc solutions.

Test	Animal	MCIC	$\frac{MCV}{100}^3$	$\frac{MCIC}{\text{per } 100}^3$	$X-\bar{X}$
Control	1	.284	3.70	0.077	.033
	2	.261	2.44	.107	.003
	3	.264	2.33	.133	.023
	4	.301	2.64	.114	.004
	5	.263	2.49	.106	.004
	6	.293	2.59	.113	.003
				mean $\bar{X}$ = .110	$(x-\bar{x})_2$ = .079
					$(x-\bar{x})^2$ = .0017
		S = .008	$S_{\bar{x}}$ = .003		
Copper	1	.261	2.04	.128	.000
	2	.236	2.11	.112	.016
	3	.272	2.33	.117	.011
	4	.276	2.07	.133	.005
	5	.244	2.05	.119	.009
	6	.284	2.25	.126	.002
	7	.238	1.81	.131	.003
	8	.328	2.01	.163	.035
	9	.220	2.21	.100	.028
	10	.269	1.80	.149	.021
				mean $\bar{X}$ = .128	$(x-\bar{x})_2$ = .130
					$(x-\bar{x})^2$ = .0029
		S = .011	$S_{\bar{x}}$ = .0035		
Zinc	1	.362	2.59	.140	.026
	2	.361	3.54	.102	.012
	3	.293	2.47	.119	.005
	4	.315	2.96	.128	.014
	5	.210	2.59	.081	.033
	6	.340	3.04	.112	.002
				mean $\bar{X}$ = .114	$(x-\bar{x})_2$ = .092
					$(x-\bar{x})^2$ = .0021
		S = .011	$S_{\bar{x}}$ = .0045		

S - Standard Deviation
$S_{\bar{x}}$ - Standard Error

Appendix VIII

Spectrophotometric determination of the zinc or copper concentration in sublethal 21-day tests on yearling brook trout. Zinc was determined by dithizone extraction while copper was determined by carbon tetrachloride extraction of the cupric diethyldithiocarbazone complex.

Test Day	Zinc Aquarium mg. per l. Zn	Copper Aquarium mg. per l. Cu
1	2.75	0.33
3	2.90	.34
5	3.15	.32
7	2.90	.32
9	3.00	.28
11	3.00	.30
13	2.75	.33
15	3.25	.34
17	2.75	.26
19	3.00	.30
21	2.90	.30
Tap Water		.03

Appendix IXa

Histochemical iron analysis of liver and spleen from sublethal copper or zinc-poisoned yearling brook trout. The potassium ferrocyanide staining technique was used to identify ferric iron. Quantitative estimates were obtained using a histochemical spectrophotometer.

Test	Section Type	Section Thickness	Galvanometer Deflection	Animal
Control	Liver	8	20 amps	1
			22	1
			22	2
			22	2
		mean $\bar{X}$	21.5	
Copper	Liver	8	26	1
			25	1
			25	1
			20	2
			24	2
		mean $\bar{X}$	24	
Zinc	Liver	8	12	1
			12	1
			11	1
			13	2
			13	2
		mean $\bar{X}$	12.2	

Continued . . .

Appendix IXa

Test	Section Type	Thickness	Galvanometer Deflection	Animal
Control	Spleen	7	8 amps	1
			8	1
			7	1
			7	2
			7	2
			8	2
		mean $\bar{X}$	7.6	
Copper	Spleen	7	6	1
			7	1
			7	1
			6	1
			7	1
			7	1
		mean $\bar{X}$	6.7	
Zinc	Spleen	7	8	1
			8	1
			8	1
			9	2
			9	2
			8	2
		mean $\bar{X}$	8.3	

Appendix Xa

Histochemical copper analysis of liver, spleen and kidney of sublethal copper or zinc-poisoned brook trout. Copper sites were stained with rubeanic acid (dithio-oxamide). Quantitative estimates were made counting the granules in a given area with a Whipple occular in a microscope.

Test	Fish	Section Type	Thickness	Squares occupied (X)	$X-\bar{X}$
Control	1	Spleen	7	46	2.5
				42	1.5
	2			41	2.5
				45	1.5
				mean $\bar{X}$ = 43.5	$(x-\bar{x})_2$ = 8.0
					$(x-\bar{x})^2$ = 17.0
		S = .5			
Copper	1	Spleen	7	67	3.2
				55	8.8
	2			77	13.2
				56	7.8
				mean $\bar{X}$ = 63.8	$(x-\bar{x})$ = 33.0
					$(x-\bar{x})^2$ = 332.8
		S = 3.9			
Control	1	Liver	8	18	0.5
				21	2.5
	2			18	0.5
				17	1.5
				mean $\bar{X}$ = 18.5	$(x-\bar{x})_2$ = 5.0
					$(x-\bar{x})^2$ = 9.0
		S = 0.8			
Copper	1	Liver	8	26.0	1.8
				21.5	6.3
				26.0	1.8
				38.4	10.6
	2			22.6	5.2
				17.0	10.8
				30.5	2.7
				53.1	25.3
				14.7	13.1
				mean X=27.8	$(x-\bar{x})_2$ = 77.6
					$(x-\bar{x})^2$ = 121.2
		S = 2.2			

Continued . . .

Appendix Xa

Test	Fish	Section Type	Thickness	Squares occupied (X)	$X-\bar{X}$
Zinc	1	Liver	8	74	24.8
				44	5.2
				46	3.2
	2			62	12.8
				39	10.2
				30	19.2
			mean $\bar{X}$ =	49.2	$(x-\bar{x})$ = 75.4
					$(x-\bar{x})^2$ = 1288.8
		S = 7.5			
Control	1	Kidney	7	20	2.5
				14	3.5
				10	7.5
	2			14	3.5
				19	1.5
				16	1.5
	3			32	14.5
				15	2.5
			mean $\bar{X}$ =	17.5	$(x-\bar{x})$ = 37.0
					$(x-\bar{x})^2$ = 308.0
		S = 3.9			
Copper	1	Kidney	7	58	3.3
				68	13.3
				68	13.3
	2			57	2.3
				31	23.7
				57	2.3
				44	10.7
			mean $\bar{X}$ =	54.7	$(x-\bar{x})$ = 68.9
					$(x-\bar{x})^2$ = 1051.4
		S = 8.0			