

THE ACUTE TOXICITY OF MOLYBDENUM TO THE BLUEGILL  
" (LEPOMIS MACROCHIRUS)

by

Richard L.<sup>c</sup> Easterday  
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## ABSTRACT

The ninety-six hour TL<sub>50</sub> value for molybdenum as sodium molybdate to bluegills (Lepomis macrochirus) was determined as 1320 ppm molybdenum in ion-free water. Using Blacksburg tap water the toxicity of 1400 ppm molybdenum was decreased. Supplementation of 1400 ppm molybdenum with 3,000 ppm sulfate or 0.125 ppm copper did not appear to effect molybdenum lethality.

Treatment with 650 ppm molybdenum for two weeks showed rapid uptake of molybdenum by stomach, intestine, liver, muscle, and blood. After nine days blood levels plateaued near 400 ppm molybdenum, and bile levels reached 2100 ppm molybdenum. The spleen iron concentrations of treated fish with 1400 ppm molybdenum for five days was increased significantly. In addition, this treatment appeared to increase the water content of the muscle an effect which was partially reversed by adding sulfate with the molybdenum.

Dialyzing whole fish homogenates against ion-free water removed 90% of the molybdenum. Only a trace of molybdenum was present in the ether-extracted lipids from fish treated with 500 ppm molybdenum.

Blood of fish exposed to 1400 ppm molybdenum in ion-free water showed a 21% increase in hemoglobin concentration, crenated red blood cells, and changes in the relative proportions of the plasma proteins. Gross examination of molybdenum-treated fish showed hemorrhages (intramuscularly, in the iris of the eye, along the dorsal fin, and the pectoral fins), and blue stomachs; histologic examinations showed thrombi present in vessels of the kidney, spleen, gut, and meninges.

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#### IV. REVIEW OF LITERATURE AND INTRODUCTION

##### A. Molybdenum

The toxicity of molybdenum has been studied in several classes of animals, including mammals, avians, and pisces, and to a more limited extent in the class of plants Schizomycetes. Although much work has been done with molybdenum in animal systems, the mechanism or mechanisms of action by which molybdenum is toxic or serves an essential function are poorly understood. On the other hand, the functions and essentiality of molybdenum for plants have been firmly established.

The first report of a biological role for molybdenum came in 1930 when Bortels (10) showed that the element was important for the fixation of gaseous nitrogen by Azotobacter chroococcum. The experiments and conclusions of Bortels have since been confirmed by many workers (14, 64). In 1935 Carner (41) showed that molybdenum is an essential nutrient of Aspergillus niger. It now appears that molybdenum is necessary for nitrogen fixing organisms and higher plants (4, 41, 71, 75). Molybdenum has also been identified as an essential constituent of nitrate reductase from the fungus Neurospora and soybean leaves (66, 67). The role of molybdenum in plants must, however, not be limited to the above two processes because plants grown with combined nitrogen sources other than nitrate still require molybdenum (32). This fact and the possible roles of molybdenum as a

necessary part of a hydrogenase from Clostridium pasteurianum, as a necessary part of a flavoprotein electron carrier, as an inhibitor of plant phosphatases, and as a factor in iron metabolism have been reviewed by Evans (32) and Nason (68).

Even though Karantassis (49) had studied the toxicity of molybdenum and tungsten in guinea pigs as early as 1920, attention was not focused on molybdenum as a toxic agent until 1938 when Ferguson (36, 37) in England established that excessive amounts of molybdenum were the cause of an "age old" disease in grazing cattle called "teart." This disease in cattle is characterized by severe scouring (diarrhea), loss of condition, and harsh staring discolored hair coats. These symptoms can develop from within twenty-four hours to a few days after moving an animal onto a teart pasture or drenching the animal with soluble molybdate. Similar diseases have been reported in various parts of the world including California and New Zealand (12, 20). It was readily noticed that the symptoms of teartness in England resembled those of a copper deficiency seen in Holland. It was found that copper salts were effective in alleviating the "teart" of cattle. Underwood (84) recently reviewed the literature on this disease in cattle.

In New Zealand it had been observed that high concentrations of molybdenum existed in the tissues of sheep suffering from copper toxicity, "red water." This fact led to the successful use of

molybdenum salts in the treatment of chronic copper toxicity, and again implied an interrelation between copper and molybdenum.

After a treatment was found for "teart" or "peat scours," the disease per se was no longer a problem, but interest in the mechanism of action and possible biological functions of molybdenum in animals was greatly stimulated. The toxicity of molybdenum has been studied extensively in mammals and avians, and a species difference in tolerance appears to exist. Cattle and sheep (24) appear to be the most sensitive to molybdenum, while the pig and horse appear to be the most tolerant. Guinea pigs, rabbits, (5), chickens, (25), and fish (81) fall somewhere in between the two other groups. From these studies with different animals evidence for interrelations of molybdenum with copper, sulfate sulfur, protein level of the diet, tungsten, and iron metabolism has appeared and has been reviewed by Miller et al., (60), Dick (28), and Underwood (87).

Some evidence also exists for the essentiality of molybdenum in animals based on work done with chickens (72, 73) and rats (82). Final proof has not been obtained because it has thus far been impossible to prepare a molybdenum-free diet sufficient in all other respects and produce a deficiency which can be reversed by the addition of molybdenum to the diet. Molybdenum contamination of so-called molybdenum-free diets and body stores at birth appear to prevent the deficiency from occurring.

Biochemical and histological evidence for the mechanism or site of molybdenum action is very limited. In rats and rabbits bone abnormalities have been described and recently reviewed (47). Fairhall (34) et al. administered orally 25-200 mg of molybdenum daily as molybdenum trioxide over periods of 4-25 days and studied the histological changes. They found indications of fatty infiltration in livers and kidneys, moderate swelling of kidney epithelial cells, increased pulp reticulum cells of the spleen, and hemosiderin laden phagocytes in liver and spleens. They concluded, however, that "... there were no significant histopathologic findings in animals—either rats or guinea pigs, given molybdenum sulfide and calcium molybdate by inhalation and ingestion or in the same species given ammonium molybdate by ingestion or molybdenum trioxide fume by inhalation, aside from some bronchopneumonia in the inhalation groups." (34).

The first indication of a biochemical function and thus an implied essentiality for molybdenum in animals came in 1953 when it was identified as a necessary part of the flavoprotein xanthine oxidase (23, 74) which contains iron, flavin adenine dinucleotide, and molybdenum in the approximate molar ratio of 4:1:0.7, respectively. (39). Xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and uric acid. Tungstate, a molybdenum antagonist, was fed to rats by Higgins et al (44) causing almost complete elimination of molybdenum and xanthine oxidase from the rats without producing any overt symptoms of deficiency. The evidence seems to indicate that

the animal can replace xanthine oxidase with another system to produce uric acid, the xanthine oxidase level in the depleted animals was still high enough to function catalytically, or the animal does not have to produce uric acid.

Besides bone abnormalities, decreases in bone phosphatase have been reported and reviewed (47), Johnson (47) showed that by restricting the feed of control rats so that they grew at the same rate as experimental rats being fed molybdenum, no difference in the femur phosphatase activity of the molybdenum-fed rats compared to controls could be detected. This indicated that the reduced rat femur alkaline phosphatase activity was not due to molybdenum per se.

The chemistry, function, and metabolism of many cations and anions in biological systems are well known. Perhaps the baffling and highly complex chemical properties of molybdenum make it easier to understand why the action of molybdenum in living systems are difficult to ascertain. Killeffer and Linz (50) attribute some of the difficulties of molybdenum chemistry to several reasons. Molybdenum atoms can exist at six different oxidation states, 0, + 2, + 3, +5, and + 6 and possess coordination numbers of 4, 6, and 8. Molybdenum compounds disproportionate to form complex mixtures in which all valence states may exist and slight alterations in the external conditions can cause the coordination numbers to shift. By a polymerization condensation reaction, molybdate ions readily aggregate in solution to form polyions. Thus, in many cases the products of a simple reaction are comprised of a mixture of compounds

whose identity and concentration are dependent on the pH, temperature, pressure, redox potential, and proportions of original constituents.

The only highly soluble salts of molybdenum in aqueous solutions are the molybdates of sodium, potassium, ammonium, rubidium, lithium, magnesium, beryllium, and thallium. Acids readily dissolve the insoluble molybdate salts to supposedly form polymolybdate ions. The aqueous soluble salts have been found to be the most toxic as would be expected if one assumes solubility is necessary for the functioning of absorption mechanisms in animal systems.

Molybdenum can, however, react with organic compounds and these compounds can form salts. Molybdic acid reacts with carboxyl-hydroxy and polyhydroxy organic compounds including phenols, alcohols, sugars, and organic acids (50). These condensation products again are complex and in many cases the composition of the products is in doubt. No molybdenum organic compounds have been isolated from biological systems except the molybdenum bound to certain enzymes (23, 66, 69, 74).



### B. Fish Toxicology

Many fish toxicology studies have been conducted because of natural and man-made pollution of waterways. Other toxicity studies have been performed from a pharmacological, physiological, or biochemical aspect. A great deal of the work has been directed at establishing the toxic levels of metal salts under various conditions and at finding methods for rendering them nontoxic. The actual mechanisms by which these toxic agents cause death, and the histological, physiological, and biochemical changes which they produce, have been investigated only to a limited extent.

The literature on the toxicology of industrial wastes to fish has been critically reviewed by Doudoroff and Katz (29, 30, 31). Generalizations as to the suitability of water for fish based on simple chemical and physical criteria are extremely difficult. The water requirements of different species may vary greatly, and the number of factors which can effect the water quality are great. The natural water in which a fish lives is a dynamic system. The introduction of another factor, the toxic agent, into this system will elicit a response from the fish to the whole system, and not just to the toxic agent. The other metal ions in the water may have an antagonistic or synergistic effect on the toxic material, probably both. Thus, the response of a fish to a toxic material will vary according to the effects of the other environmental factors on it. Some of the factors which will control the fishes response to his medium are:

temperature, concentration and proportion of cations and anions, oxygen tension, volume, flow rate, pH, hardness, total alkalinity, size of fish, and light. It is at once evident that a set of standard conditions is necessary to evaluate the toxicity of any material to fish. Much of the old literature on this subject is not useful because the authors did not standardize their conditions or at least did not describe them fully. Several articles have been published recently recommending certain standard conditions for evaluating toxic agents or industrial wastes (18, 30, 42). In order to determine the true toxicity of one particular metal ion or its toxicity irrespective of other ions, the problem of establishing standard conditions is more complex.

Based on studies with different fish involving varying conditions, the relative toxicities of a number of metal ions have been determined and reviewed (13, 31). Silver, mercury, copper, zinc, aluminum, nickel, chromium, and lead are considered highly toxic metals. Cobalt, iron, manganese, barium, and lithium are considered to be of intermediate toxicity, while potassium, magnesium, calcium, strontium, and sodium are considered least toxic.

The only metal ions for which a mechanism of acute toxic action has been agreed upon are the salts of the heavy metals lead, zinc, copper, and mercury (30, 31). It is believed that death is the result of suffocation brought on by the formation of insoluble metal-protein compounds precipitating on the respiratory epithelium of the gills.

Wood (94) has emphasized the use of pathologic procedures in conjunction with bio-assay techniques and biological studies in order to better diagnose the cause of fish mortalities.

The bluegill (Lepomis macrochirus) has been recommended as an experimental animal for toxicity studies (24), and has been used to determine the effects of several metal ions and salts (81,84,85,86,92). The bluegill is a fresh water fish of some game importance and provides food for larger game fish such as bass. It can adapt to laboratory conditions of high water temperature, restricted volume, artificial food, varying pH, changes of water composition, and handling quite well. Unlike other laboratory animals, growth cannot be used as a criterion of toxicity in studies of less than 50 days because it is so slow.

Williams (92) determined the 24 hour TLM\* of a variety of salts to bluegills. He tested these salts in a standard reference water of known composition. The reference water was of such a nature, however, that it by itself could not be tolerated by the fish for more than a few days without severe distress. He determined the following 24 hour TLM values: Calcium chloride, 8,350 ppm, sodium chloride, 14,125 ppm, and sodium sulfate as 17,500 ppm.

Using bluegills in a reference water based on the data of Clark (1924), and the 24 hour TLM values determined by Williams (92), Abegg (1) found that after 24 hours or less sodium chloride, potassium chloride, and calcium chloride caused a decrease in the percentage of tissue fluids. Sodium chloride caused a decrease in blood specific gravity and a decrease in blood density.

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\* 24 hour TLM (medium tolerance limit) is that quantity of material which will kill 50% of the fish in 24 hours.

Trama (84) determined the 96 hour T<sub>1m</sub> value of copper to bluegills as 0.74 ppm in Chu's (15) reference water. He found it necessary to keep the pH of the test solutions close to pH 5.3 in order to keep the copper salts in solution. Chelating agents would keep the copper in solution at higher pH values and greatly reduce the toxicity.

Trama et al (86) determined the 96 hour T<sub>1m</sub> of chromium as dichromate to bluegills as 110 ppm chromium, and as chromate as 170 ppm chromium.

Fromm and Schiffman (39) determined the 48 hour T<sub>1m</sub> of hexavalent potassium chromate to large mouth bass as 195 ppm chromium. Exposure of bass to 94 ppm chromium as potassium chromate caused a 27% decline in oxygen consumption after six hours, and after 36 hours, caused widespread destruction of the intestinal epithelium below the pyloric caeca. No changes in the gills were noted. These workers hypothesized that the chromium entered via the gills and was excreted by the liver via the bile.

Knoll and Fromm (51) found that exposure of rainbow trout to 2.5 ppm chromium as potassium chromate caused the accumulation of chromium in concentrations exceeding that of the water in the spleen, posterior gut, pyloric caeca, stomach, and kidney. They hypothesized that the concentrations in the tissues was correlated with excretion. The probable route of entry was the gills since fish with esophageal occlusions had accumulated the same amount of chromium as those

without. They also found that chromium given by stomach tube was accumulated only to a very limited extent in other tissues. When fish that had accumulated chromium were placed in fresh water, there was a rapid loss of the chromium from the blood, liver, stomach, and pyloric caeca. The kidney and spleen even after twenty-four days still contained significant concentrations of chromium.

Schiffman and Fromm (77) determined that rainbow trout exposed to 2-4 ppm chromium as potassium chromate for 24 hours had increased hematocrit values. They explained this increase as the result of an increase in cell number, cell volume, and a probable decrease in plasma volume.

Tarzwell and Henderson (81) in an exploratory study determined the toxicity of some of the less common metals to flathead minnows in a hard and soft reference water. The 96 hour TLM for molybdic oxide in soft and hard water was tentatively placed at 70 ppm and 370 ppm of molybdenum, respectively. The author is unaware of other studies on the toxicity of molybdenum to fish.

The fish is a less complex animal than the higher classes of animals in which most of the studies of molybdenum toxicity have been made. In view of this, it was hoped that the response of the bluegill to toxic amounts of molybdenum might be of a more basic and discernable nature than the responses of other animals. The introduction of molybdate into animals hitherto studies had been dependent upon the gastrointestinal tract for absorption, or absorption and diffusion from some injected site. In general, the concentration of molybdenum

in animal tissues had been relatively low, possibly because of the difficulty in obtaining a continuous intake of molybdenum by feeding and the rapid excretion of molybdenum. It was hoped by placing molybdate ions in the water a continuous concentration gradient would be established at the gills tending to force the molybdenum directly into the blood stream. The ability of the gills to absorb metal ions has been demonstrated and reviewed (13).

The objectives of these studies were: (1) to determine the TLm of molybdenum as sodium molybdate to bluegills, (2) to see how Blacksburg, Virginia tap water and the addition of copper and sulfate ions effected the toxic level of molybdate, (3) to determine what histological changes occurred with acute molybdenum toxicity, (4) to determine if changes in the blood accompanied exposure to molybdenum, and (5) to gain an insight as to the mechanisms of action of molybdenum as an acute toxic agent to the bluegill.

## V. GENERAL EXPERIMENTAL PROCEDURE

Bluegill sunfish (Leponis macrochirus) weighing between 2.5-6 grams live weight were secured from the Virginia State Fish Hatchery at Marion, Virginia, the United States Federal Fish Hatchery at Wytheville, Virginia, and from ponds in the Blacksburg area. The fish were transported to the laboratory in five gallon tin cans. It was found that fish losses were kept to a minimum if the cans of water were oxygenated with pure oxygen forced through cinkered glass aerators during transportation, and the fish were allowed to remain in the aerating cans for several hours after arriving at the laboratory. This allowed the temperature of the water in the cans to reach that of the holding tank, 20-22° C, in the air conditioned laboratory. The fish were then placed into a 500 gallon plastic holding tank containing aerated Blacksburg tap water. The chemical composition of this water is stated in Table 1. The fish were held in this holding tank until they were to be acclimated to aquariums. The water of the holding tank was kept clean of fecal material and uneaten food by intermittent sweeping of the tank bottom with the aid of an electric water pump. Slow additions of Blacksburg tap water were made to the holding tank as needed. During the holding period which lasted up to two and one-half months, the fish were conditioned to eat twice daily a commercially prepared pelleted fish food. The composition of this diet is found in Table 2. The fish appeared to do well on this food.

Fish were transferred as needed in groups of 20 to 10 gallon glass aquariums with slate bottoms which had been previously sealed with plexiglass. The tanks contained tap water which had previously been aerated for several hours and had reached a temperature of 21-23° C.

Table 1

Composition of Blacksburg Tap Water<sup>\*</sup>

Component	ppm
Carbonate	43.0
Sulfate	10.0
Chloride	3.0
Silicon dioxide	9.0
Total phosphate	0.9
Hardness as calcium carbonate	46.0
Calcium	13.0
Magnesium	3.0
Orthophosphate	0.1
Metaphosphate	0.8 <sup>+</sup>
Molybdenum	< .002 <sup>+</sup>
Iron	0.07 <sup>+</sup>
Copper	2.2 <sup>+</sup>

<sup>\*</sup> Personal communication, Christiansburg-Blacksburg-Virginia Polytechnic Institute Water Authority.

<sup>+</sup> Analyses done by the author.



Table 2

\*  
Pelleted Fish Diet

Component	Percent of Total
Dried skim milk	10.00%
Cottonseed meal	24.00%
Fish meal	18.00%
Wheat flour middlings	24.00%
Corn distillers solubles	12.00%
Dried brewers yeast	10.00%
Cod liver oil	2.00%
300 AOAC Vitamin D units/gm.	
2250 USP Vitamin A units/gm.	
Ether extract	9.32%
Protein	35.34%
Crude Fiber	5.58%
Ash	6.76%
Molybdenum	1.66 ppm <sup>+</sup>
Copper	21.0 ppm <sup>+</sup>
Iron	159.0 ppm <sup>+</sup>

\* Red Fish Food, prepared by Sherwood Feed Mills, Inc. Baltimore, Maryland.

<sup>+</sup> Analyses done by the author.

An oil air compressor supplied pressurized air which was bubbled through water traps to remove any extraneous material and then forced through glass cintered aerators. Each tank had an activated charcoal spun glass filter which had a flow rate of approximately 50 ml./minute. The tanks were illuminated twenty-four hours a day with fluorescent lights. The laboratory contained incandescent lights which were automatically controlled to give twelve hours of light per day. Since the laboratory was air conditioned and thermostatically controlled, the acclimation tanks maintained a temperature of 21-23° C without heaters. The fish were fed daily, and the tanks were cleaned daily by removing approximately half of the twenty-four liters of water by suction through a sweeper. The water placed back in the tanks to make up the volume was distilled water. Thus, the original water was gradually depleted of its ions and a very soft water resulted. The only ions entering the water were from the food. This procedure continued for a period of ten days. The fish showed no signs of distress or gave any indications of an inability to adjust to this procedure.

In all experiments, except those of more than five day duration, the fish were fasted 24 hours, before transfer to experimental tanks. This eliminated a large amount of the fecal material which would have been excreted while the fish were in the experimental tanks with the addition of ions to the water.

Fish were randomly selected from five ten-gallon acclimating tanks for experimental and control groups. The lots of fish were made up so that the largest fish was never more than twice the weight of the smallest fish. All groups had an average weight of between 2.5-3.5 grams unless otherwise noted. Before the fish were transferred to the experimental tanks, they were rinsed twice in ion-free water. Weighing was done in a tarred beaker of water on a triple beam balance to the nearest 0.1 gram. Fifteen fish were used in each experimental groups unless otherwise noted.

The experimental tanks were 15 gallon, glass with metal frame, aquariums with slate bottoms which had been sealed with plexiglass. They were calibrated to hold 45 liters of water at 24° C, and all experiments were run in this volume unless otherwise noted. The tanks were aerated with forced air which had been cleaned by a water trap and filtered through a micronite filter. The air was forced into the water through a glass cinkered aerator near the bottom of the tank. The tanks were completely covered, but not sealed, with plexiglass tops with built-in fluorescent lights in aluminum reflectors. All metal exposed to the inside of the tank was coated with a thin layer of plexiglass. Each tank was supplied with an electric heater which maintained the temperature at 24° C  $\pm$  1°, and a plexiglass filter containing spun glass and operating at a rate of 75 ml./minute. The tanks were washed and scrubbed with a stiff brush before each experiment with a few drops of ammonium hydroxide in tap water and

and then rinsed several times with distilled and ion-free water. The tanks were then assembled, filled with 40 liters of ion-free water, and aeration begun. The weighed salts were then dissolved and the volume of water made up to 45 liters. The tank was allowed to remain (with the fluorescent light on, filter in operation, heater on, aerators in operation) for twenty-four hours before the experiment was started. Before each experiment and daily during the experiment the pH of the water was determined with a Beckman pH meter. Before and after each experiment a sample of water was analyzed for molybdenum and copper.

All samples of tissue and whole fish which were to be analyzed for metals were dried in a forced draft oven at 85° C for 48 hours, cooled in a desiccator, and weighed on an analytical balance. The samples were then wet digested with perchloric acid and redistilled nitric acid until only a white ash remained. The samples were then dissolved in dilute hydrochloric acid and made up to a known volume. If iron analysis were to be run, the samples were made up in redistilled hydrochloric acid.

Copper was determined by the carbamate method of the AOAC (6) molybdenum by the thiocyanate method of Evans et al. (33), iron by the method of the AOAC (7), and hemoglobin by the acid hematin method (16).

Preliminary studies showed that the oxygen concentration in experimental tanks never dropped below 6 mg./liter as determined by the standard Winkler method (78). The fish in all experiments had at least 3 liters of water per fish, and thus it was assumed that with the aeration procedure used, dissolved oxygen never became limiting.

The chemicals used in the various tests were the following unless otherwise noted: Molybdenum as sodium molybdate dihydrate, copper as copper sulfate pentahydrate, and sulfate as sodium sulfate.

The 96 hour  $TL_{50}$  value was determined by plotting percent survival versus log concentration of the toxic agent in ppm, according to recommendations made by Henderson et al. (43) and Boudoroff et al. (30).

## VI. DETERMINATION OF ACUTE TOXIC LEVELS OF MOLYBDENUM

Methods

Experiment one was designed to determine the 96 hour TLM level of molybdenum as sodium molybdate dihydrate. In accordance with the recommendation of Anderson et al. (3) and Doudoroff (30), levels of molybdenum were selected from a logarithmic series. The levels of molybdenum used were 200 ppm, 500 ppm, 650 ppm, 870 ppm, 1150 ppm, 1500 ppm, and 2100 ppm. As a positive control, sodium chloride was added to provide 500 ppm, 1150 ppm, and 2100 ppm of sodium. The fish and tanks were prepared as described previously. The fish were observed at least once during each twenty-four hour period and the number of fish dead at the end of each twenty-four hour period was recorded. All fish were removed from the tanks when they were observed to be dead.

Experiment two was designed to determine what effect Blacksburg tap water in place of ion-free water had on the toxicity of 1400 ppm molybdenum. Three tanks were used containing the following: (1) 1400 ppm molybdenum in Blacksburg tap water, (2) 1400 ppm molybdenum in ion-free water, and (3) tap water control. The experiment was run for 96 hours and the deaths were recorded every twenty-four hours.

Experiment three was designed to determine what effect 3,000 ppm sulfate as sodium sulfate had on the toxicity of 1400 ppm molybdenum. Three tanks were used containing the following: (1) 3,000 ppm sulfate in ion-free water, (2) 3,000 ppm sulfate plus 1400 ppm molybdenum in

ion-free water, and (3) 1,400 ppm sodium as sodium chloride in ion-free water. The tank containing sodium chloride was used as a control.

Experiment four was designed to establish what effect copper as copper sulfate would have on the toxicity of 1400 ppm molybdenum as sodium molybdate. The experiment was run with four tanks containing the following solutions: (1) 1400 ppm molybdenum in ion-free water, (2) 0.25 ppm copper in ion-free water, and (3) 0.125 ppm copper in ion-free water, and (4) 0.125 ppm copper plus 1400 ppm molybdenum in ion-free water. The pH of the water was adjusted to 5.3 with sulfuric acid in order to keep the copper in solution.

#### Results and Discussion

The results of Experiment one are depicted in Figure one and the 96 hour TLm value for molybdenum was determined as 1320 ppm. Concentrations of 500 ppm molybdenum or less produced no deaths within 96 hours. At concentrations of 500 ppm molybdenum and above some fish were observed to hemorrhage along the dorsal fin, the pectoral fins, and in the muscle below the skin in the area of the tail. At the 500 ppm level of molybdenum hemorrhaging was first observed after twenty-four hours along the dorsal fin in about 20 percent of the fish. The incidence of bleeding increased and its onset became more rapid as the concentration of molybdenum was increased. At 2100 ppm molybdenum, hemorrhaging was observed within fifteen minutes. Hemorrhaging was not observed in the control fish.

As the toxicity progressed the fish became sluggish and usually remained near the surface with their bodies at about a 45 degree angle to the horizon. At times some fish excreted white mucus-like material, but none appeared on the gills or on the outside of the body. The fish became hypersensitive to motions and vibrations such as tapping on the side of the tank which under normal conditions would not disturb them. At times this type of stimulus would produce convulsions. The fish would swim rapidly about the tank convulsing and shaking while at other times they would apparently lose control of their equilibrium and swim backwards tail over head. Generally the fish would recover and appear quite normal, but occasionally they died in this manner. Most fish died quietly as they appeared to lose their strength and their ability to swim or maintain equilibrium. The fish first lost the use of their tails and were forced to swim using only their pectoral fins. The pelvic spines were rigidly extended a great deal of the time, always during convulsions, and always before death. When the fish became extremely weak, they would swim towards the surface in an almost vertical position. As they fatigued they would sink towards the bottom of the tank, and when exhausted they would lay on their sides on the bottom on the tank, breathing with an increased rate of opercular movements.

Opercular movements of the fish in the molybdenum tanks were observed to be greater than those of the control fish, particularly as the toxicity progressed and the fish neared death. Six control fish were observed to have a rate of opercular movements of forty to fifty per minute, while six fish in 870 ppm molybdenum for ten hours



had a rate of eighty-five to one hundred opercular movements per minute.

The toxicity due to 1400 ppm molybdenum was greatly decreased in Blacksburg tap water. Only 15.5% mortality was observed in the Blacksburg tap water while the same amount of molybdenum in ion-free water produced 50% mortality after four days. It has been known that a toxic agent is usually less toxic in a harder water, presumably due to the presence of other ions. It is not known at this time what in the Blacksburg water has produced the decrease in fish mortalities or how it does so.

Jones (48) found that the toxicity of lead and zinc to sticklebacks could be reduced greatly with small additions of calcium to soft water. In vitro studies with eel slime showed that calcium prevented the precipitation of the protective slime material by lead and zinc. It is also known that calcium effects cell permeability and is necessary to maintain the integrity of the cell. Other workers have suggested that in some cases the ions of the water effect the solubility of the toxic agent along with a simple dilution effect.

The addition of 3,000 ppm sulfate to 1400 ppm molybdenum did not significantly affect the lethality of the molybdenum. After five days in the molybdenum tank, 60% of the fish survived, and in the molybdenum plus sulfate tank 66.8% survived. This percentage represented a difference of only one fish and is not considered significant. It required five days exposure to this level of molybdenum to produce 60% mortality while in previous studies, it usually took four days to

produce 50% mortality. The fish for this particular experiment were obtained from a holding pond into which the Blacksburg-Christiansburg-Virginia Polytechnic Institute Water Authority drains the washings from their filters. Although the water in the pond appeared clear, the bottom of the pond was covered with approximately two and one-half feet of silt. It is believed that the toxicity to molybdenum may have been effected by the nature of the water in which the fish were raised and lived prior to the experiments.

The alleviation of molybdenum toxicity by sulfate in other animals seems to be due, at least in part, to an increased rate of excretion of molybdenum, and replacement of sulfate due to increased loss of sulfate (62). Perhaps any increased excretion of molybdenum in fish is overshadowed by an increased rate of absorption since a constant concentration gradient is present. It could also be that the excretion mechanism for molybdenum is already working at maximum capacity and is not effected appreciably by increased sulfate.

Copper alone proved to be much more toxic than expected; 0.25 ppm, and 0.125 ppm copper all killed one-hundred percent of the fish in three and one-half hours or less. Trama (84) determined the 96 hour TLm of copper as copper sulfate in Chu's (15) water as 0.76-0.72 ppm to bluegills. The addition of 1400 ppm molybdenum to 0.125 ppm copper (approximately 50% survival), was much less toxic than copper alone (0% survival). Approximately 50% of the fish survived in water containing 1400 ppm molybdenum alone. Thus, it appears as if molybdenum alleviated the copper toxicity, but the copper did not effect the toxicity of molybdenum. In order to determine if copper will

alleviate the toxicity of molybdenum, concentrations of less than 0.125 ppm copper must be used. Fish in the copper-containing tank rapidly produced a considerable amount of mucus which covered the body and particularly the gills of the fish. The fish in the copper-containing tanks died with their mouths open and gill flaps extended as is typical of death caused by asphyxiation.

## VII. MOLYBDENUM ABSORPTION STUDIES

Methods

In order to determine the rate and amount of absorption of molybdenum in various tissues of bluegills, fish were exposed to a range of concentrations of molybdenum for various periods of time extending to as long as five weeks. The first experiment was designed to determine the rate and amount of absorption of fish exposed to 650 ppm molybdenum as a function of the length of time the fish were exposed to molybdenum. The fish were acclimated as described previously, except that they were fed prior and during the experiment. Fish were offered twice daily as much food as they would readily eat. The tanks were cleaned of fecal material and any excess food by sweeping. Approximately ten liters of water were removed in the cleaning process and was replaced with freshly prepared water corresponding to that which was placed in the tank at the beginning of the experiment.

Blood samples of 0.01 ml. were taken by puncture of the bulbus arteriosus. The stomach, liver, gall bladder, intestine (including the pyloric caeca), and dorsal muscle were isolated, dried, cooled in a desiccator, weighed, and wet digested. The part of the fish remaining after the isolation of these various parts and hereafter referred to as the fish residue, was prepared for analysis in the same manner. Three fish were sacrificed at 1/2, 1, 2, 3, 4, 5, 6, 7, 9, 11, 13, and 14 days.

Total fish molybdenum concentrations were also determined for fish exposed to 200 ppm, 500 ppm, 990 ppm, and 1075 ppm molybdenum for five days in ion-free water.

### Results and Discussion

Fish exposed to 650 ppm molybdenum showed a relatively rapid uptake of molybdenum compared to other animals. The blood appeared to curvilinearly increase after which the concentration plateaued (see Figure two). Liver, muscle, and residue molybdenum concentration was highly correlated with blood molybdenum concentration (see Table 3). Although smooth curves or lines have been calculated (see Figure 2-6) statistically to fit the data the mechanisms by which the fish handles increasing concentrations of molybdenum in the various tissues might be interpreted in the following way. There are three possible modes of entry for molybdenum into the fish; through the skin by endosmosis, by absorption in the intestinal tract, and by absorption directly into the blood at the gills. There is no apparent destruction of the epidermis and the concentration of molybdenum in the muscle on a fresh weight basis is low compared to other tissues (there is 60 ppm molybdenum in muscle versus 541 ppm molybdenum in blood). Thus, absorption through the skin would not seem a likely route of entry when treated with 1400 ppm molybdenum for five days unless the molybdenum is transported from the muscle at an exceedingly rapid rate against a concentration gradient.

Table 3

Correlation Coefficients of Molybdenum Concentrations between Tissues from Molybdenum-treated Fish\*

	Stomach	Intestine	Gall Bladder	Muscle	Residue	Liver	Blood
Stomach	1.000	.28145	.12364	.16850	.16412	.26686	.15248
Intestine	.28145	1.000	.49707	.77094	.76494	.78059	.72335
Gall Bladder	.12364	.49707	1.000	.78433	.73227	.75984	.73668
Muscle	.16850	.77094	.78433	1.000	.98405	.94001	.95620
Residue	.16412	.76494	.73227	.98405	1.000	.90015	.94254
Liver	.26686	.78059	.75984	.94001	.90015	1.000	.88359
Blood	.15248	.72335	.73668	.95620	.94254	.83359	1.000

\* Correlation coefficients calculated from samples taken during two-week exposure, of the fish to 650 ppm molybdenum.

For many years it was assumed that fresh water teleosts drank little if any water because of their disadvantageous osmotic pressure relationship with fresh water. Allee and Frank (2) have reviewed the literature and have reported evidence that goldfish do drink water. The high concentration of molybdenum found in the stomach (1840 ppm dry weight) after only six hours of exposure to 650 ppm molybdenum would seem to corroborate their findings. Since the fish were not fed while treated with molybdenum until after the sixth hour and twenty-four hour samples were taken, the high concentration in the stomach cannot necessarily be associated with the swallowing of food. It will be noted (see Table 3) that the concentration of molybdenum in the stomach did not correlate well with any other tissue. In fact, statistically the concentration in the stomach was after six hours constant ( $1795 \pm 482$  ppm molybdenum) to the the end of the experiment. It has been established that fish can absorb metal ions directly into the blood via the gills and in fact, can do it selectively. With high concentrations of molybdenum in the water (a concentration not noticeably altered by absorption), it seems that absorption of molybdenum directly into the blood via the gills and through the intestine are the most likely routes of entry of molybdenum. Analysis of bile and bile plus gall bladder indicate that over 97% of the molybdenum is actually in the bile. The very high increasing concentration of molybdenum in the gall bladder and the rapidly increasing concentration of molybdenum in the intestine after

five days would seem to indicate that the bile may be an important route for the elimination of molybdenum. The concentrations of total fish molybdenum for five day treatments with 200-1400 ppm molybdenum are given in Table 4.

The aquariums were cleaned of fecal material and replenished with water on the third, seventh, and eleventh days. Analysis for liver copper showed a curious relation to these water changes, (see Figure 7). The cleaning of the tanks and the dilution back to 45 liters with ion-free water plus 650 ppm molybdenum undoubtedly caused the concentration of copper present from the excess food and fecal material to decrease. This apparent drop in water copper appeared to be reflected in a drop in liver copper. The copper content of the residue was also determined and found not to differ significantly from the controls at the end of the experiment. It, therefore, seems that the fluctuation in the liver copper is not a simply shift of body copper between the liver and residue. At the end of two weeks the liver copper had increased from  $19.68 \pm 3.78$  to  $31.70 \pm 1.80$  ppm.



Table 4

Molybdenum and Copper Concentrations of Fish  
Related to Level of Molybdenum Treatment and Time

Treatment	Metal	No. of Samples	Mo and Cu Concentration ppm
25 ppm Mo 5 weeks ion-free water	Mo	10	92.56 $\pm$ 19.00
	Cu	10	4.66 $\pm$ 0.34
Control 5 weeks NaCl in ion-free water	Mo	6	1.73 $\pm$ 0.29
	Cu	3	2.92 $\pm$ 0.33
50 ppm Mo 5 weeks ion-free water	Mo	8	131.97 $\pm$ 34.50
	Cu	8	6.20 $\pm$ 2.20
200 ppm Mo 5 days ion-free water	Mo	4	174.0 $\pm$ 44.3
	Cu	4	3.43 $\pm$ 0.29
500 ppm Mo 5 days ion-free water	Mo	3	251.5 $\pm$ 18.8
	Cu	4	3.93 $\pm$ 1.60
1075 ppm Mo 5 days ion-free water	Mo	4	899.0 $\pm$ 96.2
990 ppm Mo 5 days ion-free water	Mo	4	733.0 $\pm$ 57.9
Tap water control	Cu	10	1.350 $\pm$ 0.556
1400 ppm Mo 5 days ion-free water	Mo	3	999.7 $\pm$ 61.4
	Cu	3	2.99 $\pm$ 1.03
1400 ppm Mo plus 0.25 ppm Cu in ion-free water 4 days	Mo	6	742.0 $\pm$ 26.3
	Cu	6	6.70 $\pm$ 3.41

## VIII. INFLUENCE OF MOLYBDENUM ON COPPER AND IRON LEVELS

### Methods

Studies were designed to determine if the total copper concentration of the fish changed with exposure to molybdenum and to determine if liver and spleen iron concentrations were effected by exposure of fish to molybdenum. Fish were treated with 25 ppm and 50 ppm molybdenum in ion-free water for five weeks with feeding. Fish were also treated with Blacksburg tap water, 1400 ppm molybdenum in ion-free water, 1400 ppm molybdenum in Blacksburg tap water, and 1400 ppm molybdenum plus 3,000 ppm sulfate, and 1400 ppm molybdenum plus 0.25 ppm copper. The later experiments were run for a duration of five days except where copper was added—then it was four days.

### Results and Conclusions

Increases in the total fish copper were observed when fish were exposed to low levels of molybdenum for five weeks. The copper concentrations of the total fish increased from  $2.92 \pm 0.33$ , the control value, to  $6.20 \pm 2.2$  ppm when the treatment was 50 ppm molybdenum, and to  $4.66 \pm 0.34$  ppm when the treatment was 25 ppm molybdenum (see Table 4).

Treatment of fish with Blacksburg tap water and 1400 ppm molybdenum in ion-free water resulted in a copper concentration in the total fish of  $1.35 \pm 0.56$  ppm and  $2.99 \pm 1.03$  ppm, respectively. Analysis of the ion-free water plus 1400 ppm molybdenum indicated that the copper present must be less than 0.01 ppm. The source of the

increased copper may have come from small amounts of residual food left in the stomach of the fish when the treatment with molybdenum was started or by leaching of the copper from the fecal material which accumulated during the five-day period of the experiment. The fish had been fasted twenty-four hours before the experiment, but some food usually remained in the stomach or intestinal tract because fecal material was always excreted when the fish were placed in the molybdenum-containing tanks. Fish treated with 1400 ppm molybdenum plus 0.25 ppm copper increased their total body copper to  $6.70 \pm 3.41$  ppm.

Analysis of fish treated with Blacksburg tap water showed iron concentrations in the liver to be  $822 \pm 243$  and the spleen as  $2,666 \pm 1,643$  ppm (see Table 5). Fish treated with 1400 ppm molybdenum in tap water showed spleen iron concentrations of  $4,988 \pm 1,741$  ppm and liver iron concentrations of  $1,245 \pm 181$  ppm. The spleen values were significantly higher than the controls ( $p < .05$ ) but the livers were not ( $p < 0.2$ ). Fish treated with 1400 ppm molybdenum in ion-free water resulted in a spleen concentration of  $6,704 \pm 1,815$ , but was not significantly different from the control value (see Table 5). This insignificance is likely due to small sample size (3 samples). The excessively high concentration of iron in the spleen caused by treatment with molybdenum indicates an anomaly in the metabolism of iron.

The addition of 3,000 ppm sulfate to 1400 ppm molybdenum, it will be remembered, did not significantly effect the lethality of

molybdenum. Analysis of spleen, however, indicated a moderation of the effect of molybdenum on increasing spleen iron concentrations. The molybdenum supplemented with sulfate combination resulted in a spleen iron value of  $3,265 \pm 1,030$  ppm which was not significantly different from the control value or the 1400 ppm molybdenum spleen value. The sulfate plus molybdenum treatment also decreased the blood molybdenum significantly ( $p < 0.001$ ) from  $541 \pm 87.2$  ppm observed with the treatment of fish with 1400 ppm molybdenum in ion-free water to  $314 \pm 67.1$  ppm molybdenum. The sulfate plus molybdenum treatment also caused a significant drop in muscle molybdenum from  $448.2 \pm 171.3$  to  $131.9 \pm 16.8$  ppm on a dry weight basis ( $p < 0.01$ ), (see Table 5). It appears as though 3,000 ppm sulfate did have an ameliorating effect on some of the effects produced by treatment with molybdenum, though it did not increase the percent survival of fish treated with 1400 ppm molybdenum.

Table 5

The Effects of Molybdenum and Sulfate Supplementation upon Fish Hemoglobin, Muscle Water and Molybdenum Concentration, and Liver and Spleen Iron Concentration

Treatment	No. Samples	Hemoglobin g./100 ml.	Blood Mo ppm	Muscle Water	Muscle ppm	No. Samples	Liver Iron ppm	Spleen Iron ppm
A Control in tap water	10	9.12 +0.65		81.07 +1.95		6	822 +243	2,666 +1,586
B 1400 ppm molybdenum in ion-free	8	11.62 +2.34	541 + 87.2	85.07 +2.00	448.2 +171.3	3		6,704 +1,815
C 1400 ppm molybdenum in tap water	6			81.59 +1.46		6	1,245 + 181	4,988 +1,741
D 1400 ppm molybdenum plus 3,000 ppm sulfate	8	11.63 +1.96	314.2 +67.2	83.60 +1.96	31.9 +16.8	3		3,265 +1,030
p value		A B p < .05	p < .001	A p < .001 B	B p < .01 D		A p < .02 B	A p < .05 C A N.S. D A N.S. B

## IX. DIALYSIS OF WHOLE FISH HOMOGENATES

### Methods

The object of this experiment was to determine how much of the molybdenum present in the bluegill exposed to 1400 ppm molybdenum in ion-free water for five days was easily dialyzable with ion-free water. Two fish were separately homogenized in the cold for six, thirty-second periods in 50 ml to 75 ml of ion-free water in a Virtis homogenizer. The homogenate was made up to 100 ml with ion-free water and three milliliters of this solution dialyzed against one liter of ion-free water in the cold for nine hours. The contents of the dialysis sac and the water were digested and analyzed for molybdenum.

### Results and Conclusions

It was found by analysis that an average of 10.2% of the molybdenum could not be dialyzed away from a homogenate of whole fish with ion-free water. It appears from this information that little of the molybdenum in the fish is tightly bound. It is possible, however, that the molybdenum which is not readily dialyzable could represent the amount of molybdenum necessary to saturate the sites of a complexing system.

## X. ETHER EXTRACTION OF WHOLE FISH

### Methods

An experiment was designed to determine if any of the molybdenum present in fish exposed to 500 ppm molybdenum could be removed from the fish by ether extraction. Four fish which had been exposed to 500 ppm molybdenum for five days without feeding and three control fish which had been exposed to 500 ppm sodium as sodium chloride for the same period were dried for 48 hours at 85° C and then ground in a mortar with a pestle. One gram samples of the ground fish were weighed, placed in extraction thimbles, and then extracted with diethyl ether over night in a continuous extraction apparatus. The ether was then evaporated from the extract and the weight of the lipid material remaining in the flask was determined. The lipid material was then wet digested and analyzed for molybdenum.

### Results and Conclusions

Only a trace of molybdenum was found in the lipid fraction extracted from the fish with ether. From this data it appears that molybdenum does not combine or complex to any significant degree with any ether extractable lipids. Detoxification of molybdenum by association with lipids thus seems unlikely.

The lipid material extracted from molybdenum-treated fish and control fish represented in both cases about 2.2% of the total wet weight of the fish. Analysis of many species of fish for total lipid

content by other workers (13) indicates that a range of from 2-20% fat is found, the amount of fat present being dependent upon the time of the year, nutritional history of the fish, and the nature of available food.

Analysis of the material remaining after ether extraction for nitrogen by the Kjeldahl method and determination of the free water content of the bluegill as 78.34%, indicated that the fish was approximately 14% protein, assuming that 16% of fish protein is nitrogen. Approximately 64% of the dry material remaining after ether extraction from four molybdenum-treated and four control fish was protein.



## XI. TISSUE FLUIDS

### Methods

In order to determine the effects of acute toxic levels of molybdenum on tissue fluid concentrations, a strip of dorsal muscle was removed from bluegills exposed for five days to either 1400 ppm molybdenum in ion-free water, 1400 ppm molybdenum plus 3,000 ppm sulfate in ion-free water, 1400 ppm molybdenum in Blacksburg tap water, or Blacksburg tap water alone. Dorsal muscle free of skin, ribs, and spines was removed according to the procedure described by Abegg (1). Each sample was weighed wet in a sealed tarred flask, dried at 90° C for 48 hours, placed in a dessicator to cool, and then reweighed. The amount of free water was calculated and expressed as a percentage of the wet tissue weight.

Thirty fish were exposed to Blacksburg tap water without feeding for three days after acclimation. The fish were blotted dry and weighed in groups of three in tarred beakers. The fish were then dried for several days at 90° C and cooled in a desiccator. They were then reweighed and the amount of free water and percentage of free water were calculated.

### Results and Conclusions

Fish treated with molybdenum in tap water showed no significant change in the percentage of tissue water in the dorsal muscle, but molybdenum in ion-free water caused a significant increase of 4.93% in tissue water. The presence of sulfate with molybdenum in ion-free

water caused a 3.12% increase in tissue water (see Table 5). The effect of the sulfate in decreasing the hydrating effect of molybdenum is in keeping with the finding of Abegg (1) that sulfate causes a dehydration of bluegill dorsal muscle. Apparently, however, the effect of the molybdenum was stronger because a slight hydration still occurred when both were present.

Chromium is one of the few metals which can exist as an anion under physiological conditions and whose effect on dorsal muscle tissue fluids has been studied. Abegg (1) found that 728 ppm chromium as sodium dichromate caused a 2.4% increase in tissue fluids in the dorsal muscle when bluegills were exposed for twenty-four hours. He suggested that the chromium effected the mucus covering of the fish possibly causing a change in cell permeability.

Treatment with 1400 ppm molybdenum in ion-free water resulted in a concentration of 60 ppm molybdenum on a fresh weight basis in the muscle and a concentration of 448 ppm molybdenum on a dry weight basis. The concentration of molybdenum in the blood, assuming a specific gravity of 1.0 was 541 ppm or about nine times that of fresh muscle. If one considers the molybdenum in the fish to be mainly ionic along with the observation that the muscle has undergone hydration, it appears as if no simple ionic concentration mechanism can explain the hydration. It appears as though water is leaving the blood to enter the tissue fluids against a concentration gradient. The hydration of the muscle could be explained if the concentration of particles present in the tissues was greater than the concentration of particles in the blood.

This could occur if molybdenum caused metabolites to build up in the muscle tissue or if the molybdenum effected the permeability of the cells so that ions other than molybdenum moved from the blood and into the tissues. It is also possible that the molybdenum in the blood is loosely bound to blood proteins whereas in the cell it might be free. This could cause an osmotic pressure favorable for hydration of the tissues. Brinkman (10) (using ammonium sulfate precipitation) found that 27.6% of the total blood molybdenum from rats precipitated with the plasma proteins.

Electrophoresis studies indicate that a protein which might correspond to albumin may decrease (see page 59, electrophoresis study). Decreases in albumin in humans cause tissue edema and could explain the muscle hydration seen here.

Ten fish exposed to Blacksburg tap water were found to have a free body water content of  $78.34 \pm 1.11\%$ .

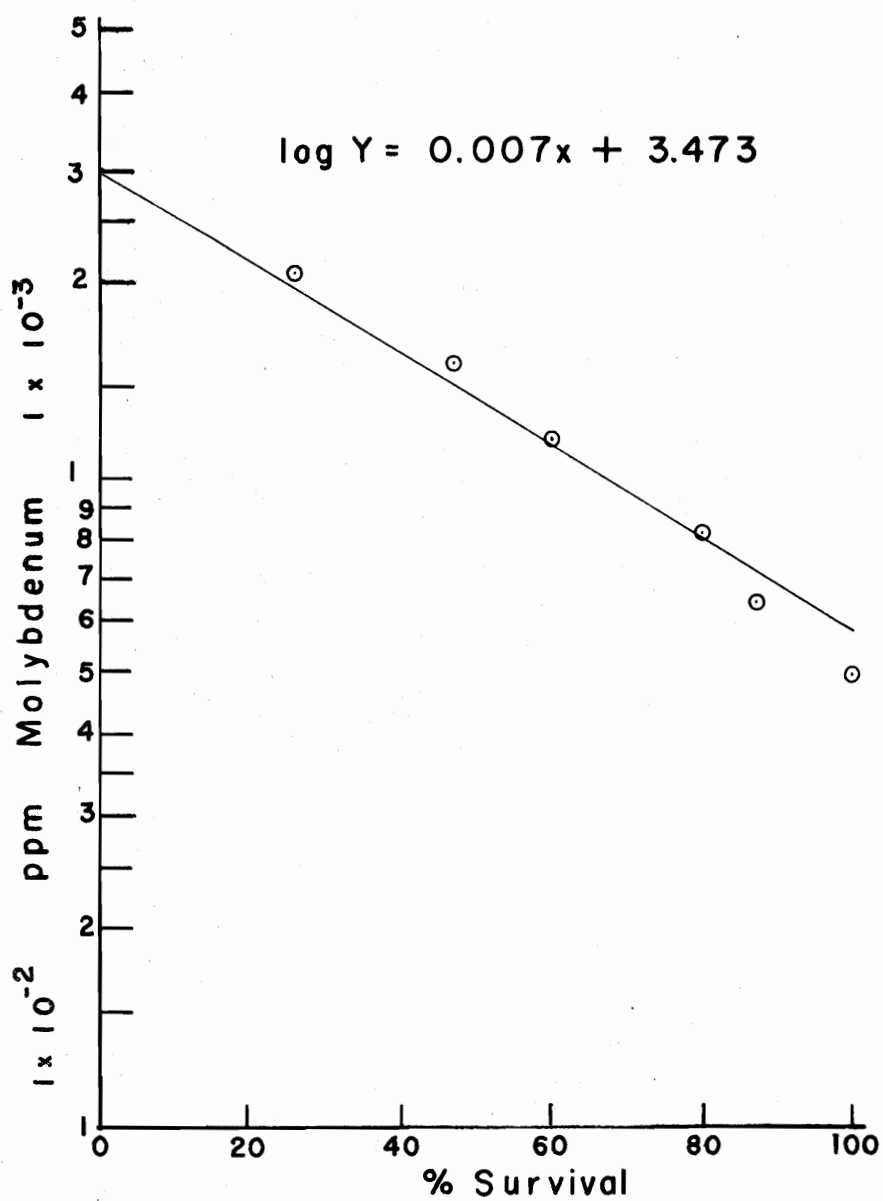


Figure 1. 96 Hour Dose Response of Fish to Molybdenum.

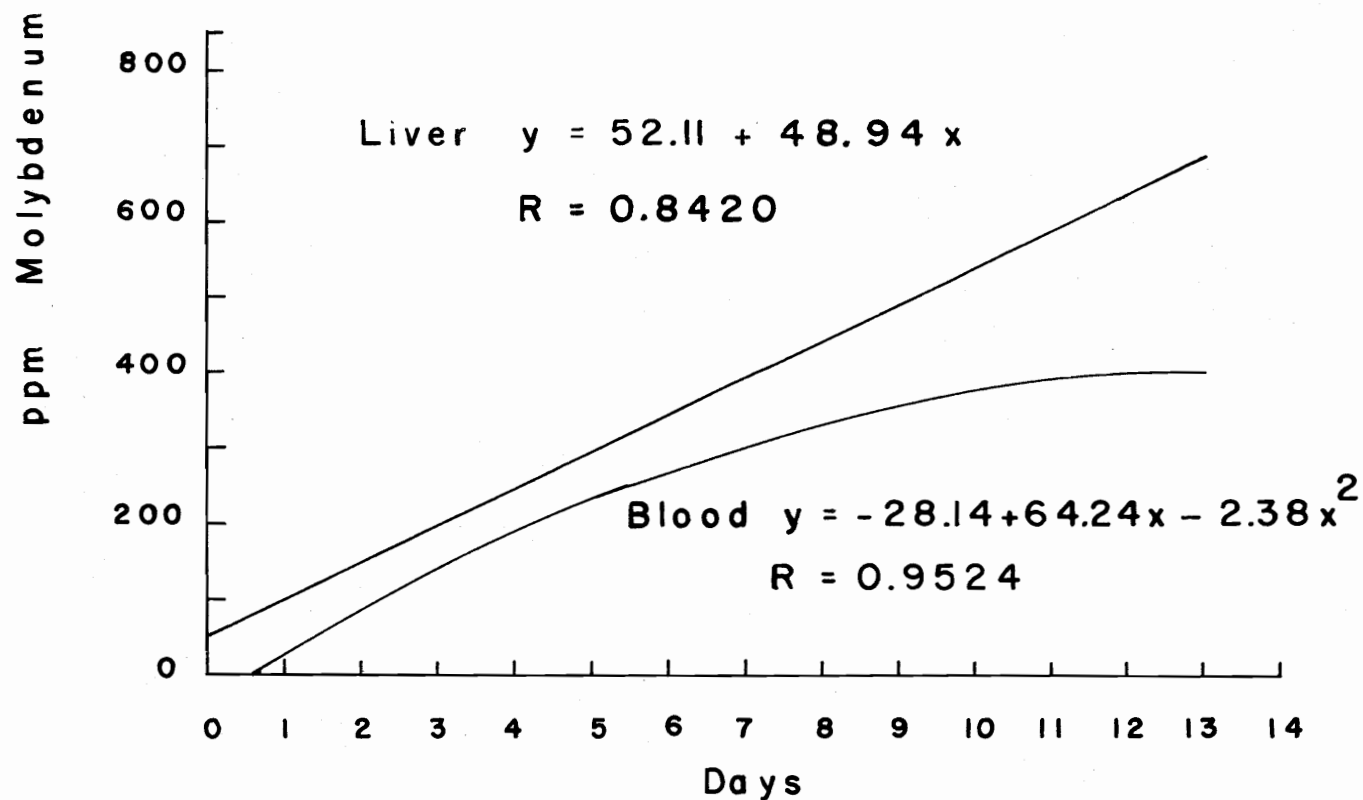


Figure 2. Blood and Liver Molybdenum Concentration with respect to Time of Fish Treated with 650 ppm Molybdenum.

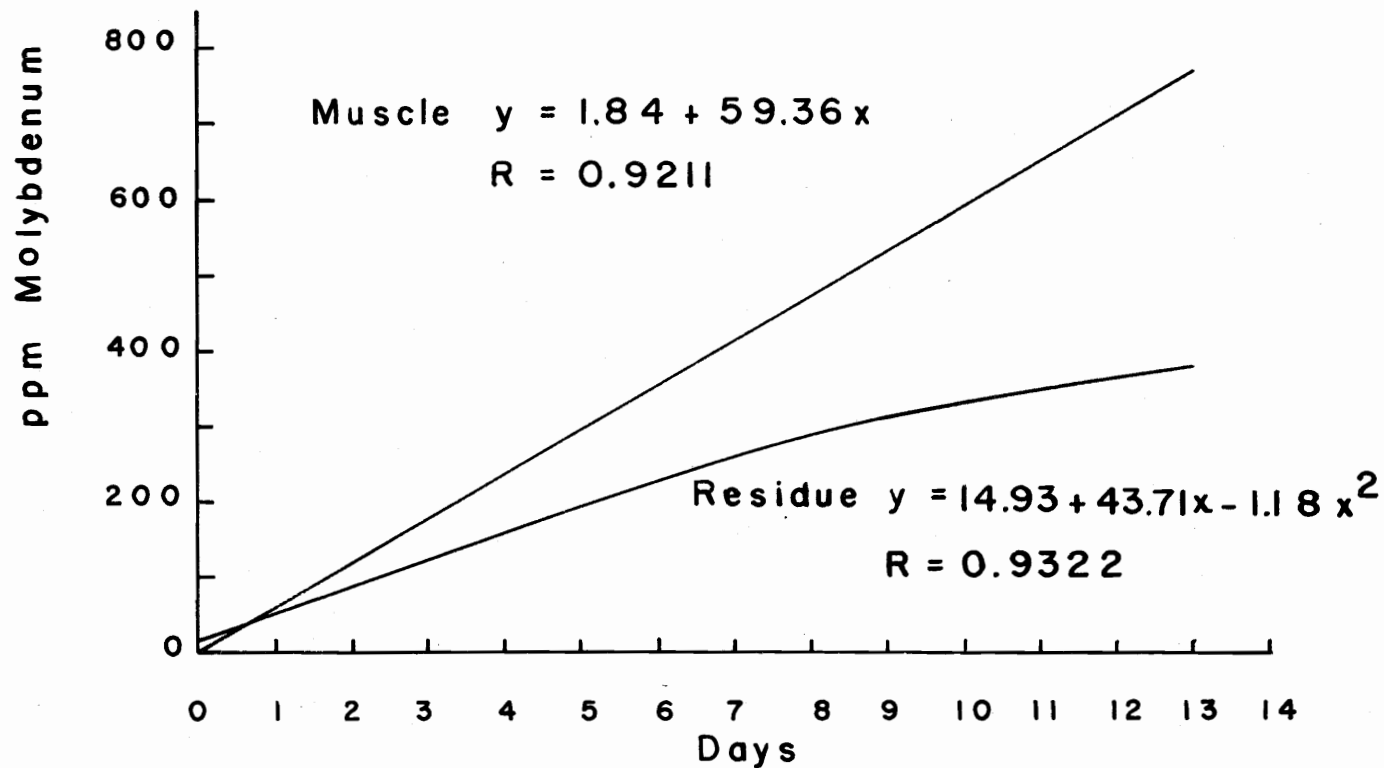


Figure 3. Muscle and Residue Molybdenum Concentration with respect to Time of Fish Treated with 650ppm Molybdenum.

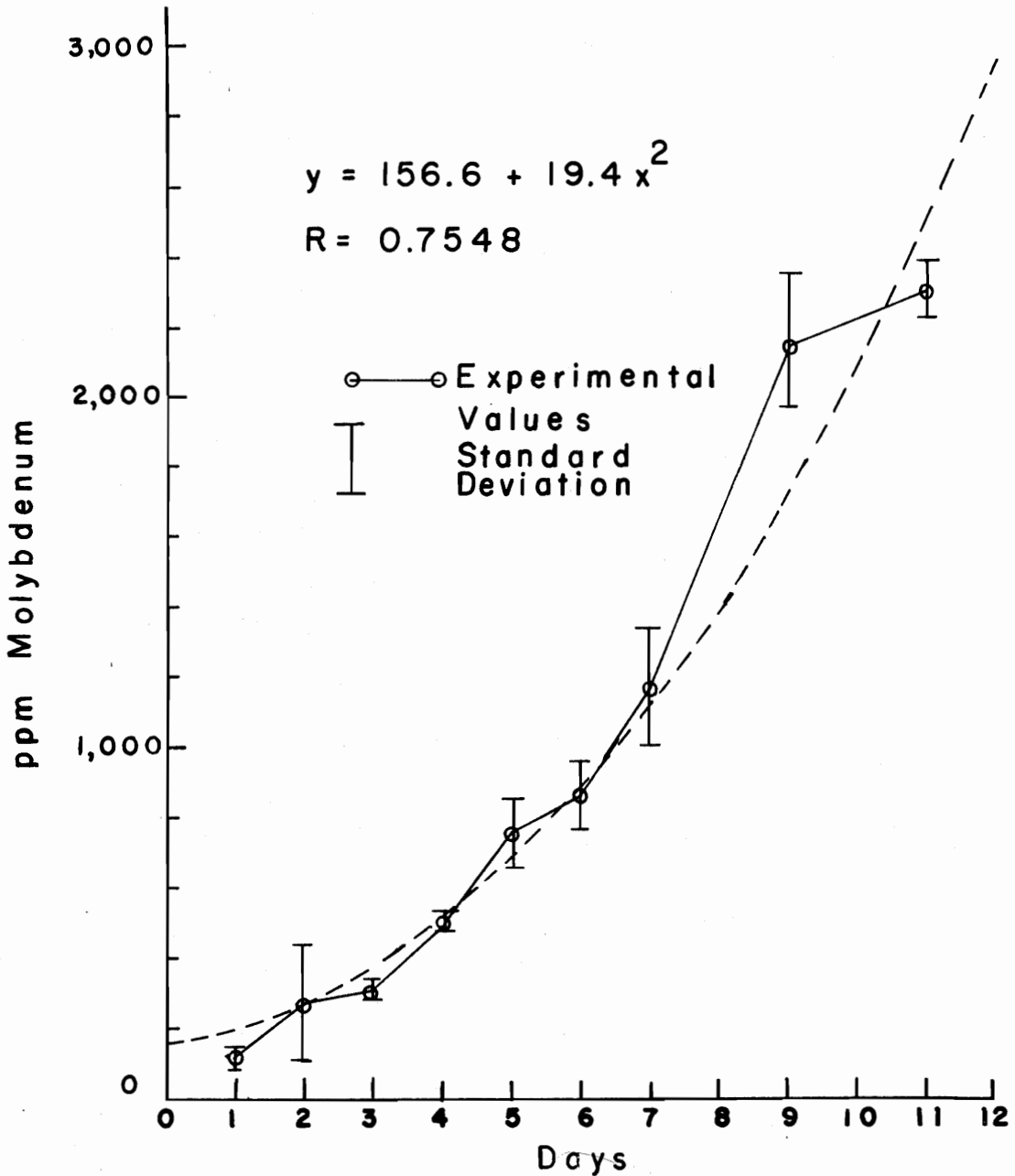
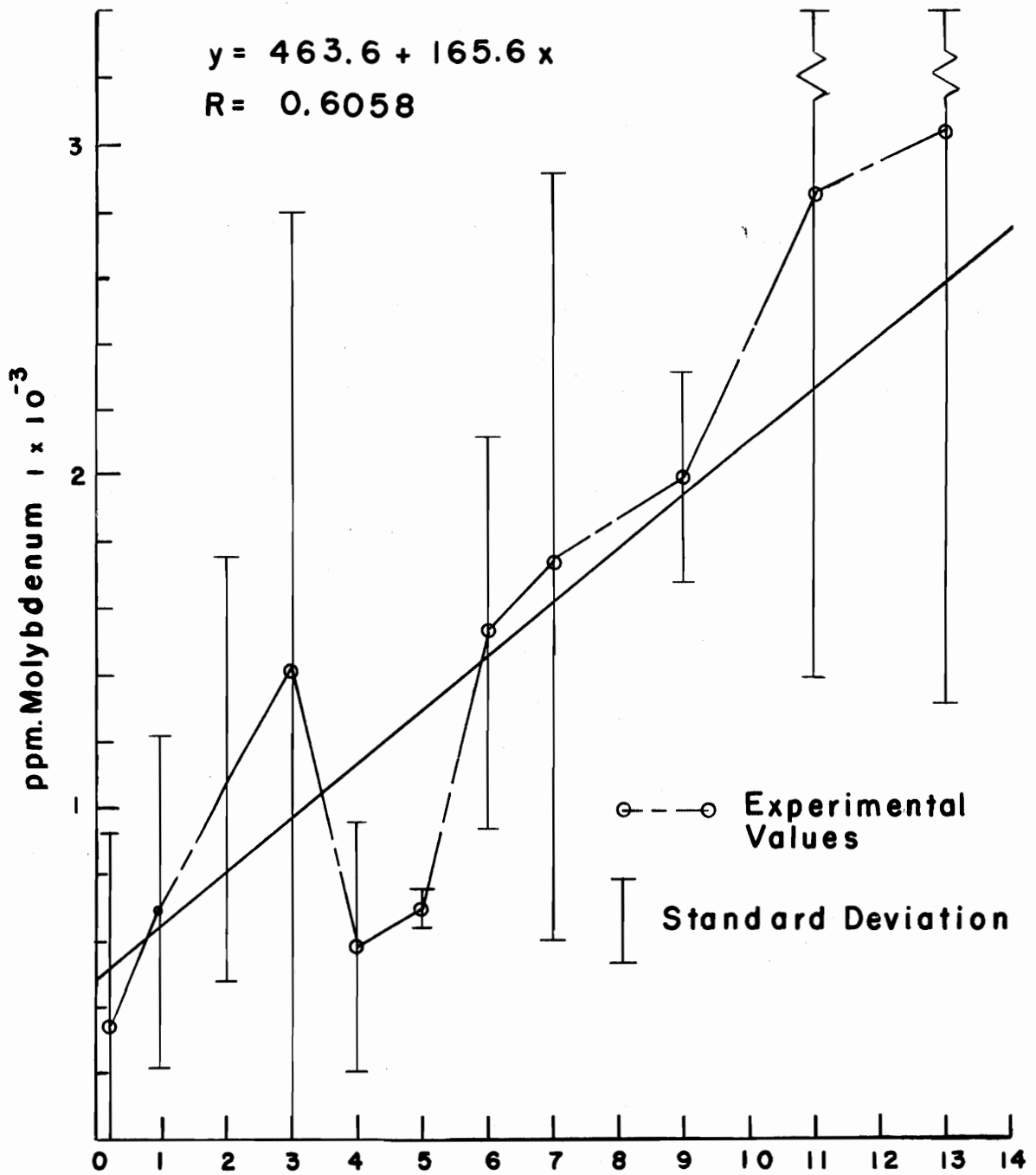


Figure 4. Gallbladder Molybdenum Concentration with respect to Time of Fish Treated with 650 ppm Molybdenum.



**Figure 5. Intestine Molybdenum Concentration with respect to Time of Fish Treated with 650 ppm Molybdenum.**



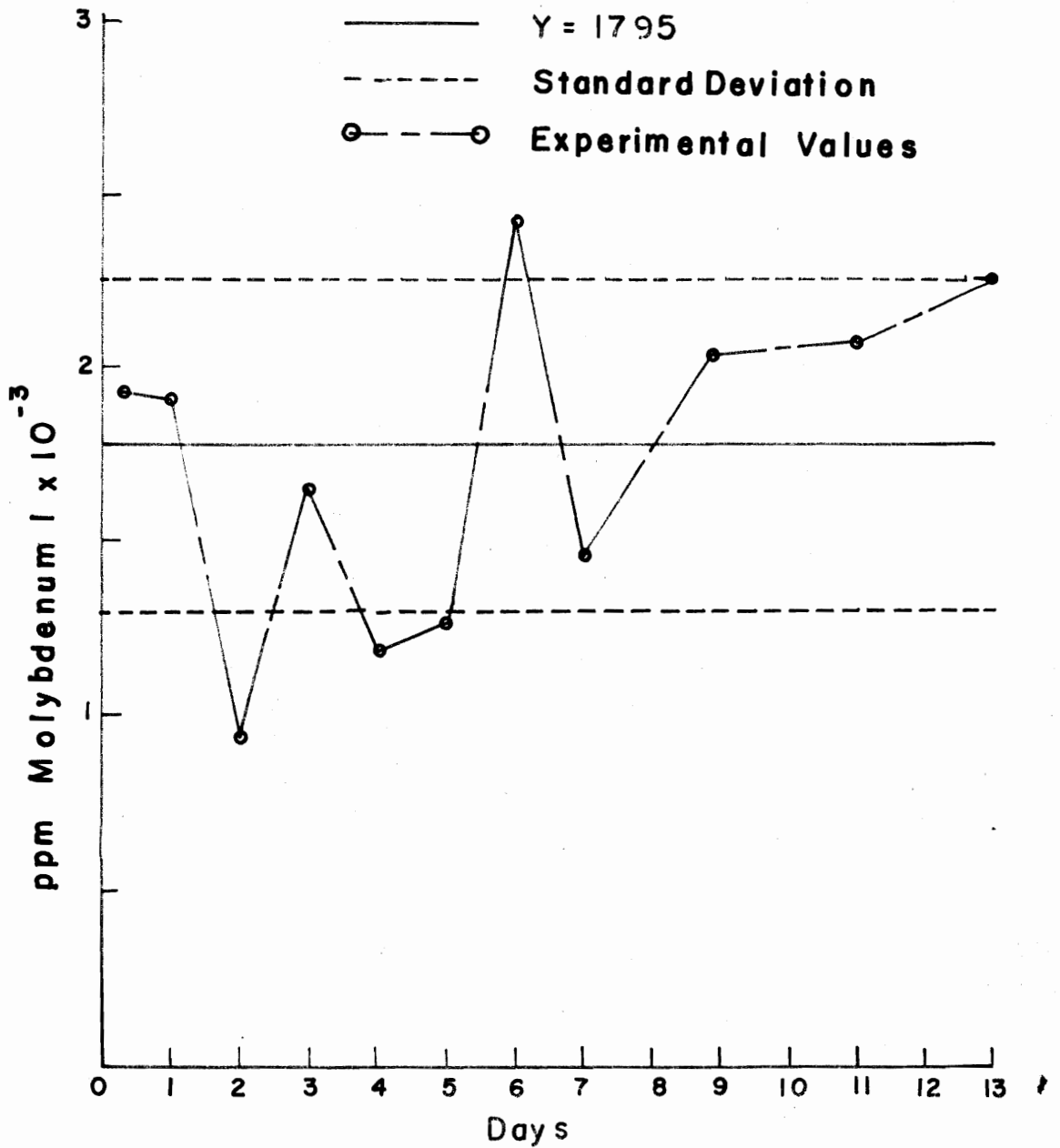
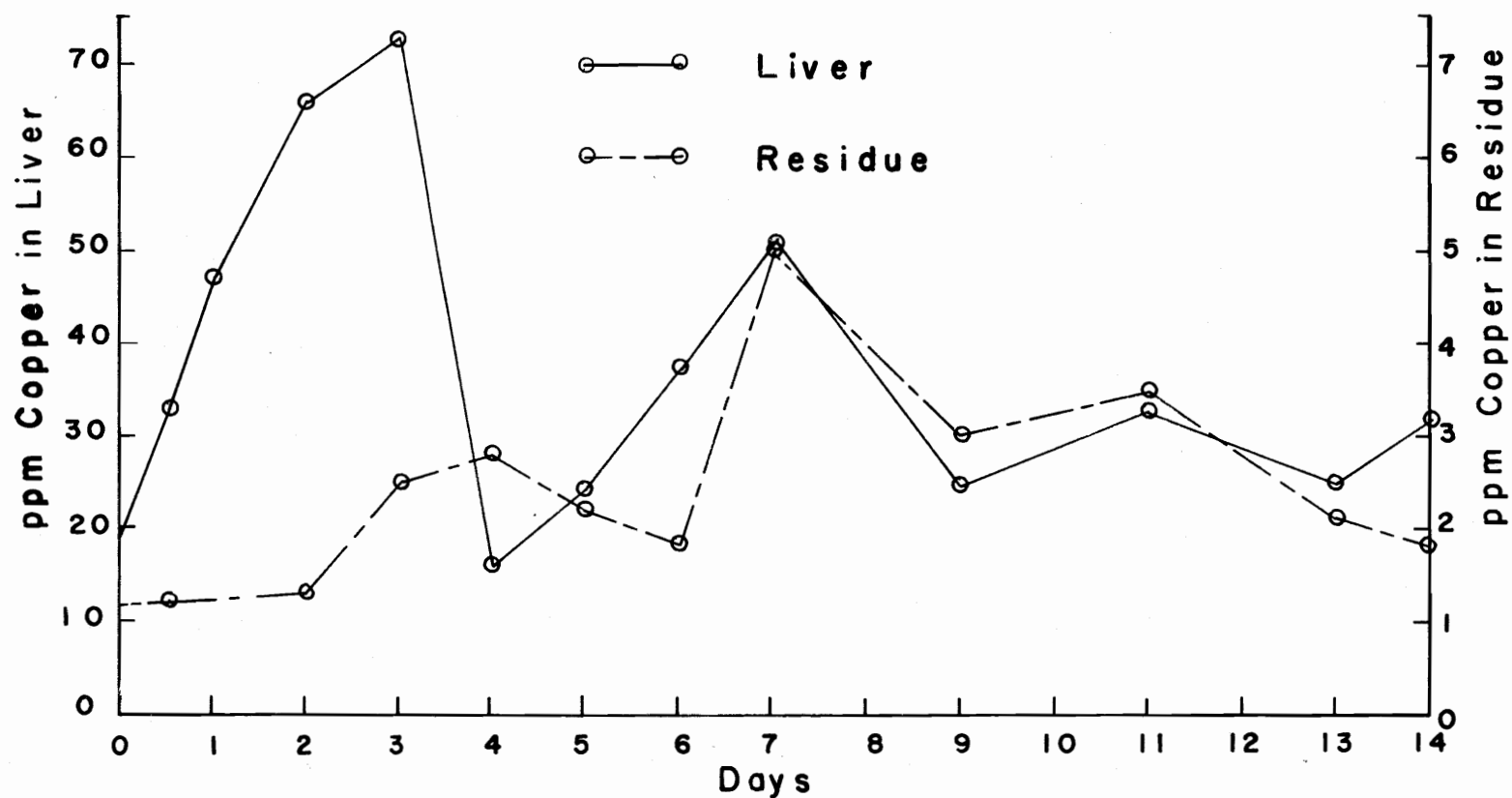
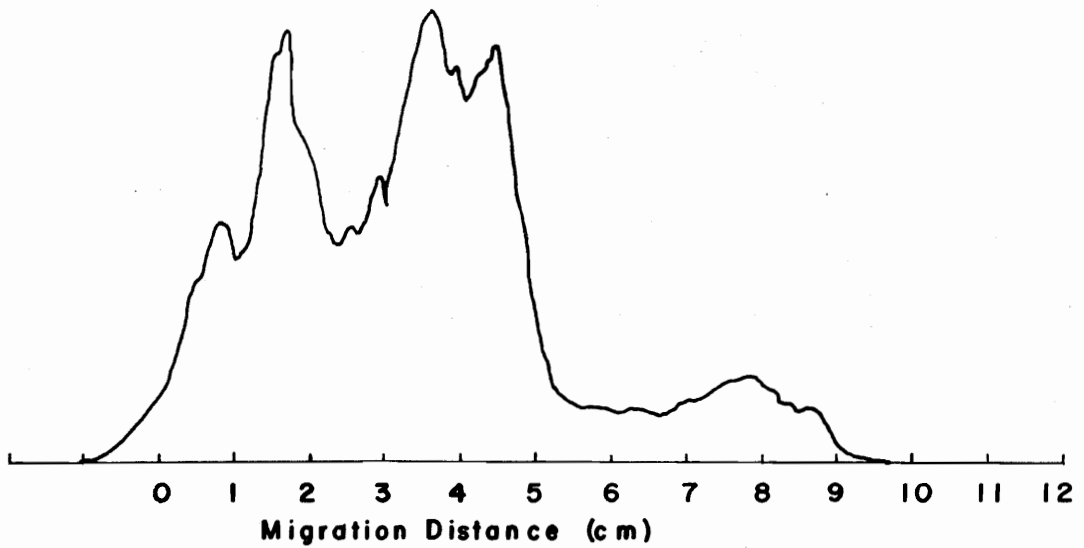


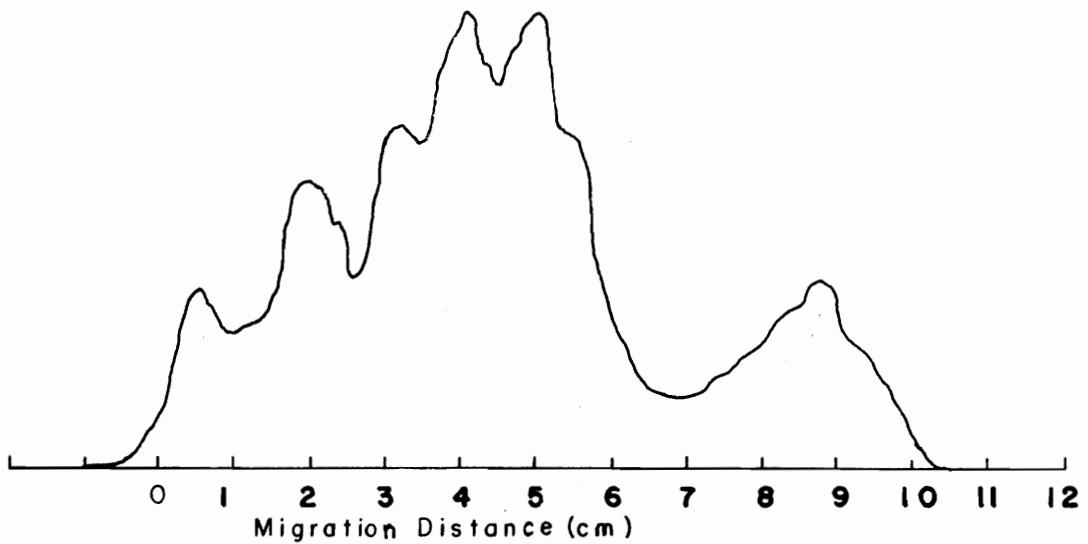
Figure 6. Stomach Molybdenum Concentration with respect to Time of Fish Treated with 650 ppm Molybdenum.



Liver and Residue Copper Concentration with respect to Time of Fish Treated with 650 ppm Molybdenum. Figure 7.



Treated with 500 ppm Molybdenum for Two Weeks  
with Feeding.



Two Week Tap Water Control Pair Fed to Molybdenum  
Treated Fish.

**Figure 8. The Influence of Molybdenum on the  
Relative Concentration of Fish Plasma Proteins.**

## XII. DISTRIBUTION OF MOLYBDENUM IN THE BLOOD

### Methods

The experiment was designed to determine the distribution of molybdenum in the blood between the red blood cells and the plasma. Blood samples were collected in oxalated capillary tubes from three fish which had been exposed to 500 ppm molybdenum in Blacksburg tap water for two weeks. Erythrocytes were separated from plasma by centrifugation. Whole blood samples and plasma samples were digested and analyzed for molybdenum.

### Results and Discussion

Assuming a plasma volume of 56.3% of whole blood from hematocrit determinations, the molybdenum in the red blood cells was calculated to be  $22.2\% \pm 2.2\%$  of the total blood molybdenum. These results are similar to those reported by Brinkman (11). He found that the erythrocytes of blood from rats treated with 400 ppm molybdenum contained  $25.6\% \pm 5.8\%$  of the total blood molybdenum.

### XIII. EFFECTS OF MOLYBDENUM ON BLOOD HEMATOCRITS, ERYTHROCYTE SHAPE AND HEMOGLOBIN CONCENTRATION

In view of the fact that blood molybdenum levels in the bluegill can be as much as ten times the highest levels reported in other animals such as rabbits and rats, studies were designed to see if molybdenum had altered blood hematocrits, erythrocyte shape, or hemoglobin concentration of bluegill blood. Blood was obtained by puncture of the bulbus arteriosus from fish which had been exposed for five days without feeding to 1400 ppm molybdenum in ion-free water, 1400 ppm molybdenum plus 3,000 ppm sulfate in ion-free water, and Blacksburg tap water.

Hemoglobin concentrations were determined by the acid hematin method of Cohen and Smith (15). The treatment of fish with 1400 ppm molybdenum in ion-free water resulted in a mean hemoglobin concentration of 11.6 g./100 ml. which was a 27.52% increase above the control mean hemoglobin value of 9.1 g./100 ml. The difference in hemoglobin concentration between the molybdenum-treated fish and the controls was significant ( $p < 0.05$ ) (see Table 5). The presence of 3,000 ppm sulfate with 1400 ppm molybdenum did not cause the hemoglobin concentration to differ significantly from the value determined for 1400 ppm molybdenum alone. Even though sulfate decreased the concentration of molybdenum in the blood (see Table 5), it did not alter the effect of molybdenum on the hemoglobin concentration. The increase seen in hemoglobin concentration caused by the molybdenum could represent a true increase in total blood

hemoglobin or it could simply be a reflection of dehydration of the blood. Since increased iron concentrations were seen in the spleen and hematocrit levels appear to have increased, the latter seems more probable.

Fish treated with 500 ppm molybdenum in Blacksburg tap water for two weeks had a mean hemoglobin value of 12.36 g./100 ml. which was not significantly different from the value obtained from the fish treated with 1400 ppm of molybdenum in ion-free water for five days.

Steffens (79) has reported that fish blood, which is unlike most mammalian blood in that it remains nucleated, gives values higher than actual values for hemoglobin concentrations if the determination is done by the acid hematin method.

Hematocrits were determined for 18 fish which were exposed to 650 ppm molybdenum in ion-free water for two weeks and for ten control fish kept in Blacksburg tap water. Blood was collected in oxalated capillary tubes, and centrifuged for 20 minutes. The percentage of red blood cells was determined by measuring the relative amounts of plasma and red blood cells with a millimeter rule. The hematocrit value determined for the control fish was  $40.6\% \pm 4.1\%$  and for the fish exposed to molybdenum it was  $43.7\% \pm 4.4\%$ . There seems to be a trend of increased red blood cell concentration, but the difference between the hematocrit values was insignificant due to large variations between samples.

Blood smears made from blood taken from fish exposed to different concentrations of molybdenum ranging from 300 ppm to 1400 ppm showed crenation of the red blood cells which was not seen in the blood smears from control fish.

## XIV. EFFECT OF MOLYBDENUM ON FISH ELECTROPHORETIC PATTERNS

Methods

The experiment was designed to determine if the exposure of bluegills to a non-acute toxic level of molybdenum for an extended period of time would effect the electrophoretic patterns of the blood plasma proteins. Five fish weighing between 8-10 grams were exposed to 500 ppm molybdenum in Blacksburg tap water for a period of two weeks. The fish were fed twice daily as much as they would readily eat. Five control fish in Blacksburg tap water were fed the same amount of food consumed by the fish on molybdenum treatment. The tanks were cleaned as was necessary. At the end of two weeks duplicate blood samples were taken in capillary tubes containing ammonium oxalate by puncture of the bulbus arteriosus. The capillary tubes were centrifuged for 20 minutes in order to separate the red blood cells from the plasma.

The Spinco Model R paper electrophoresis system using the Durrum type cells and the Model RD-2-Duostat was utilized for the separation, and the Model RB Analytrol was used for the determination of the relative percentages of the blood plasma proteins. The electrophoretic patterns were determined on all fish using 0.01 and 0.02 ml. of plasma. The experiments were run on paper strips (Beckman No. 320046) in the presence of Veronol buffer at pH 8.6 with an ionic strength of 0.075. The cells were operated at a current of 2.5 ma. per cell for sixteen hours. At the end of this

time period the strips were dried in an oven at 120° C and then stained with bromphenol blue according to the standard procedure described in the Beckman Spinco Electrophoresis Manual.

Analysis of blood plasma from the fish treated with molybdenum in the experiment indicated that approximately 5 µg./0.01 ml. of molybdenum was present in 0.01 ml. of plasma. An attempt was made to detect the molybdenum on the electropherograms by spraying them with 10% ammonium thiocyanate, 1% ferric chloride, and 10% stannous chloride. The first acetic acid rinse from the bromphenol blue staining procedure as well as the stained strips were also digested and analyzed for molybdenum.

### Results and Discussion

After two days there was a noticeable decrease in the appetite of the molybdenum-treated fish. After four days they refused to eat food almost completely and showed outward signs of the toxicity. After five days had elapsed one fish treated with molybdenum died and on the eleventh day another died.

The electrophoretic patterns revealed six distinct components to be present in the plasma of control fish. Good resolution was observed with this technique and typical electropherograms are shown in Figure 8. The peak with the least mobility was designated Component I, and the other peaks were numbered accordingly. It was immediately evident that changes had occurred in the plasma proteins



of the fish which had been exposed to the molybdenum (see Table 6). Component II from the plasma of fish treated with molybdenum was observed to be increased 75.5% above that in the controls. The difference was highly significant ( $p < 0.001$ ). Component III from the fish treated with molybdenum was found to be decreased 37.5% below the control value. The difference was highly significant ( $p < 0.01$ ). A decrease in Component IV from the molybdenum-treated fish was observed, but was not significant ( $p < 0.1$ ). This component, if one assumes a correlation to human plasma proteins, would represent albumin. No differences between molybdenum-treated and control fish were seen in Components I, IV, or V. Fujiya (40) studied the electrophoretic patterns of fish exposed to toxic agents such as copper and found that the electropherograms of these fish generally showed fewer peaks and a slight decrease in migration compared to controls. In his studies, however, he could resolve the plasma proteins into only four components.

Although total plasma protein concentration was not determined with these fish, it is possible that an alteration of the plasma proteins as described above might have pronounced effects on blood osmotic pressure and on the amount of water in the blood and tissues.

Attempts to stain for molybdenum by the thiocyanate method on electropherograms which had not been put through the bromphenol blue staining process proved unsuccessful. Apparently the molybdenum was below the level of detection because of its low total concentration or because it was diffused throughout too large an area of the paper strip. Since it is known that approximately 5 to 10  $\mu\text{g}$  of molybdenum

were present in the plasma spotted originally on the paper, a low total concentration on the paper at this point would indicate that the molybdenum had migrated off the paper or was washed off with buffer. Digestion and analysis of electropherograms which had been stained with bromophenol blue indicated no molybdenum present. Thus, it appears that either molybdenum was not tightly bound to any of the proteins or that it was washed off in the staining procedure. Analysis of the first acetic acid wash, which had been used in the rinsing of six molybdenum electropherograms, however, did contain 1.5  $\mu\text{g}$  of molybdenum. It is also possible that some molybdenum may have been removed from the strips by the rinsing in the absolute methanol solutions. It can be concluded, however, that since 5 to 10  $\mu\text{g}$  of molybdenum were spotted on the strip and since no molybdenum was detected in analysis of the stained electropherograms, the molybdenum did not become attached strongly to any particular protein or group of proteins.

Table 6

The Influence of Molybdenum Treatment on the Relative Concentrations of Fish Plasma Proteins

Component	% of Total	Standard Deviation	P	% Difference	*
I No control	11.13 11.34	$\pm 1.36$ $\pm 3.28$	N.S.		$\gamma + \delta$
II No control	28.49 16.23	$\pm 5.52$ $\pm 4.78$	<.001	+ 75.5%	$\beta_2$
III No control	9.03 14.44	$\pm 3.48$ $\pm 2.89$	< .01	- 37.5%	$\beta_1$
IV No control	19.90 18.94	$\pm 6.43$ $\pm 4.22$	N.S.		$\alpha_2$
V No control	23.73 26.99	$\pm 4.95$ $\pm 6.41$	< .4		$\alpha_1$
VI No control	7.45 12.04	$\pm 1.22$ $\pm 3.69$	< .1	- 38.2%	albumin

\* Human plasma proteins with the same relative mobility as the fish components.

#### IV. GROSS PATHOLOGY AND HISTOLOGY OF FISH TREATED WITH MOLYBDENUM

##### Methods

Fish treated with 650 ppm molybdenum for seven days and fifteen days as described in the absorption experiment and fish treated five days with 1400 ppm molybdenum as described in the determination of the 96 hour TL<sub>m</sub> experiments were sacrificed, dissected, and fixed in neutral buffered formalin. Tissues were imbedded in paraffin and stained with hematoxylin and eosin. Livers and spleens were also stained with Perl's stain for ferric ions (hemosiderin). Frozen sections of fresh gut were made and stained by using 10% ammonium thiocyanate, 10% ferric chloride and 10% stannous chloride. Tissues fixed in absolute methanol and imbedded in paraffin were also stained with this procedure.

##### Results and Discussion

Gross examination of fish treated with 500 ppm molybdenum or more for periods of three days or more occasionally showed focal areas of hemorrhage in muscle and subcutaneous tissues. Hemorrhages were also seen in the iris of the eye and frequently noted in fish which appeared to be near death. Hemorrhaging and a redened area were observed in the area of the pectoral radials or the base of the pectoral fins. No precipitation of mucus on the skin or gills was evident though occasionally a white mucus-like material was excreted. The water in tanks containing molybdenum always was more cloudy than control tanks.

The stomach of fish exposed to 500 ppm molybdenum or more showed a distinct blue color (see Plate 1). The intensity of the color did not appear to increase with an increase in the concentration of the molybdenum in the water. The intensity of the color seemed to be related to three factors: the amount of molybdenum present in the water, the length of exposure to the molybdenum, and length of time elapsed from the last feeding. If the fish were allowed to eat a small amount of food, very intense blue colored stomachs were observed after several days treatment with 500 ppm molybdenum. The blue color could also be produced by feeding the fish a mixture equal parts by weight of dry sodium molybdate dihydrate and regular diet in gelatin capsules. It is believed that the blue color is molybdenum blue, a complex mixture of molybdenum polyoxides. It has been observed that molybdate in acid media and in the presence of reducing agents will produce a blue color. The chemical nature and structure of this blue-colored compound has not been determined with certainty and has not been prepared in crystalline form. In vitro studies made by the author showed that sodium molybdate added to dilute hydrochloric acid and sucrose or glucose at room temperature would produce a blue color. The necessary acid pH and reducing agents such as sugars would exist in the fish stomach especially if a small amount of food was present, so it is felt that the blue color is a result of the formation of a molybdenum complex such as molybdenum blue. No attempt was made to identify the blue material present in the stomach because of the difficulty in identifying molybdenum blue even when it is isolated.

Molybdenum blue prepared by the author showed one very broad absorption spectrum between 500 to 800 m $\mu$  with a maximum in the region of 675 m $\mu$  using the Beckman DB ultraviolet spectrophotometer. No blue color was observed in the intestine and thus, if it moves to the intestine it is destroyed, perhaps due to the change in pH. Opening of the stomach showed the blue color to be contained in a mucus-like material covering the epithelium of the stomach, and the color was distinct on the serosal surface of the stomach. Histologic examination of the stomach showed a slight lymphocytic infiltration of the mucosa.

The stomach and intestine of the fish treated with molybdenum were almost always completely empty of any food or fecal material when sacrificed whereas control fish often were found with food in the stomach and fecal material in the gut. A diarrhea developed rapidly when fish were placed in the molybdenum-containing tanks.

Histologic examination of fish treated with 650 ppm molybdenum for seven to fifteen days showed thrombi to be present in the muscular layers of the gut wall.

The gall bladder of molybdenum-treated fish were usually full and generally much larger than those observed in the control fish. The color of the bile varied from yellow to dark green and occasionally showed a reddish tinge. A few fish which were examined immediately after death were found to have ruptured gall bladders. Histologic examination was not done to determine if the common bile duct was obstructed.

In most cases the livers of molybdenum-treated fish had a pale yellow color unlike the red livers of the controls. Histologic

examination of the livers indicated fatty infiltration.

In fish where the treatment was 650 ppm molybdenum for seven to fifteen days heavy deposits of an iron positive golden pigment (hemosiderin) were observed in the spleen with a small quantity of the same pigment in the Kupfer cells of the liver. Hemosiderin is considered a crystalline macromolecule or aggregate of ferritin (91) and by others it is considered a colloidal form of iron oxide (58). It can be formed locally as a result of hemorrhage and generally in the reticuloendothelial cells with repeated hemolysis. It is also believed that iron occurring in excess of the capacity of the ferritin storage mechanism accumulates in the liver and spleen as hemosiderin. It was noted in the study of the blood plasma proteins (see Table 6) that Component III decreased with exposure to molybdenum. If one assumes a similarity between the blood proteins of fish and humans, this fraction would correspond to a  $\beta$ -1 globulin. Transferrin (siderophilin) which is the iron protein complex responsible for the transport of iron is a pseudo- $\beta$ -1 globulin. If such a protein is one of the proteins which was observed to decrease in the electrophoretic patterns of the bluegill, it might account for the high concentration of iron as hemosiderin in the spleen and liver. Under experimental conditions iron in the diet (28.8 ppm) was available for absorption, so whether the excess iron in the liver and spleen came from an increased absorption or a breakdown of other iron compounds in the body is not known. Also, under these conditions thrombi were seen in the vessels of the spleen and arteries of the kidney (see plates 3 and 4).

Fish exposed to 1400 ppm molybdenum showed lesions similar to those exposed to the lower level. In addition, there was a slight hyperplasia of the gill epithelium and in one case thrombi were noted in the cerebral meninges (see plate 4).

Frozen sections of gut from fish exposed to 650 ppm molybdenum and stained by the thiocyanate method produced a light yellow orange color which was uniformly distributed through the tissue. Thus, it is not known if the molybdenum is truly distributed uniformly in the intact fish tissue or if the water present or the staining with aqueous solutions has redistributed the molybdenum.

Attempts to stain paraffin sections of tissue fixed in absolute methanol by the thiocyanate method for molybdenum were unsuccessful. Analysis of the tissue slices for molybdenum indicated that most of the molybdenum had been leached out by the fixing or imbedding process. Microincineration or radioautograms produced by radioactive molybdenum used in conjunction with conventionally stained hematoxylin and eosin slides might help to locate the site of molybdenum in the tissues.

Histologic examination of fish treated with molybdenum as well as control fish revealed that both groups at times were diseased. In all cases the diseases were slight and never caused death to any control fish used in an experiment. Minute lesions which appear to be foci of a myxosporidian or microsporidian infection were observed in a few fish kidneys.

Roundworms, Camallanus sp. (Nematoda: Camallanidae) were recovered from the intestine of both groups of fish. No male specimens of this worm were recovered.



Black spots observed occasionally on the skin and in the intramuscular connective tissue were fibrous nodules which were accompanied by pigmentation. They enclosed larval forms (metacercariae) of trematode parasites.

While all fish used in experiments outwardly appeared to be vigorous and healthy, the extent to which these diseases may have effected the results of these experiments is believed to be unimportant. However, it is impossible to make a statement of certainty.



Plate 1. Blue stomach of fish treated with 500 ppm molybdenum with feeding for two weeks (3x).

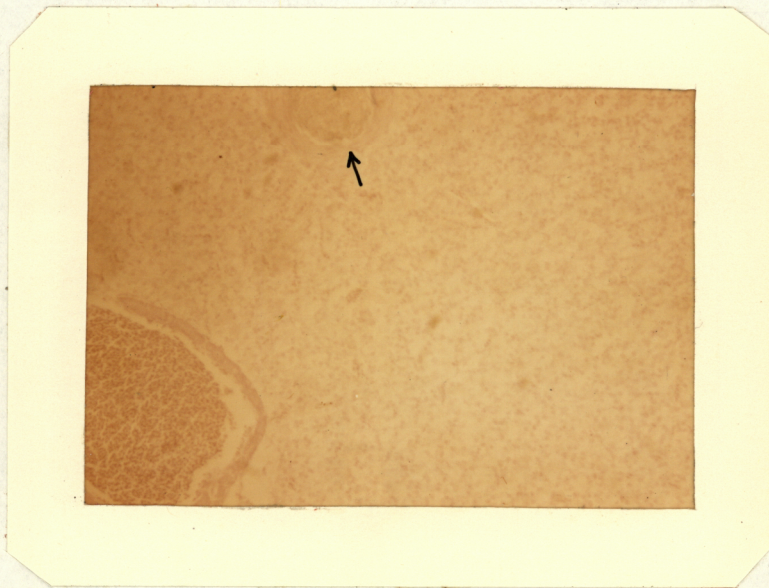


Plate 2. Thrombosed vessel in spleen of fish treated with 650 ppm molybdenum for two weeks with feeding (100x).



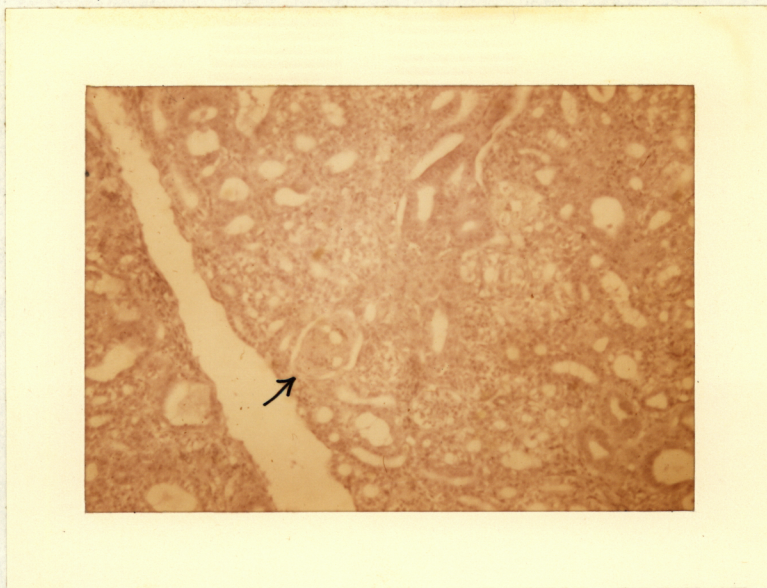


Plate 3. Thrombosed artery in kidney of fish treated with 650 ppm molybdenum for fifteen days with feeding. Foci of a myxosporidian can also be seen. (430x).

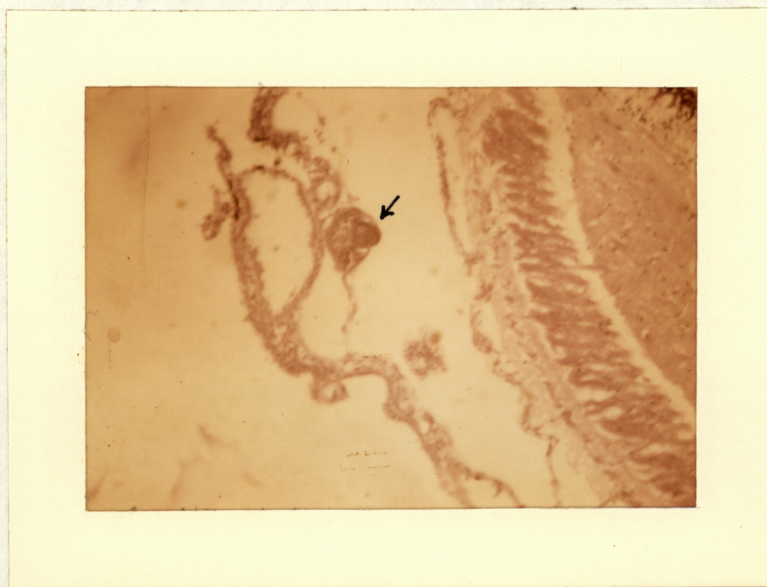


Plate 4. Thrombosed vessel in meninges of fish treated with 650 ppm molybdenum for fifteen days with feeding (430x).

## XVI. SUMMARY

The 96 hour TLm of molybdenum as sodium molybdate in ion-free water was determined as 1320 ppm for bluegills. The percent survival of fish exposed to 1400 ppm molybdenum was increased in tap water, but was not effected by the addition of 3,000 ppm sulfate or 0.25 ppm copper to 1400 ppm molybdenum in ion-free water.

The absorption of molybdenum by fish as a function of the length of time exposed to 650 ppm molybdenum indicated linear uptake in gall bladder, blood, and residue, and a constant level in the stomach after 6 hours. The mode of entry of molybdenum whether by absorption at the gills or from the intestine is discussed.

Treatment of fish for two and five weeks with low levels of molybdenum and short periods with high levels of molybdenum indicated an increase in total body and liver copper concentration.

Treatment of fish with 1400 ppm molybdenum in tap water and in tap water caused an increase in spleen iron concentration from 2,666 ppm to 4,988 ppm.

It was determined that 90% of the molybdenum present in fish treated with 1400 ppm molybdenum for four days could be removed by dialysis of whole fish homogenates against ion-free water.

Analysis of ether extracts from dried fish treated with 500 ppm molybdenum for five days showed only a trace of molybdenum to be present in the lipid extracted.

Treatment of fish with 1400 ppm molybdenum in ion-free water for five days showed a 5% increase in dorsal muscle free water. The



addition of 3,000 ppm sulfate reduced the hydration to a 3% increase above controls.

Analysis of blood from fish treated with molybdenum showed a tendency for increased hematocrits, significant increase in hemoglobin concentration (21.5%) which was not alleviated by sulfate, and crenated erythrocytes. The molybdenum was distributed between the erythrocytes and plasma as 22.2% and 77.8%, respectively. Electrophoresis of blood plasma proteins showed significant changes in two components of fish treated with 500 ppm molybdenum in tap water for two weeks as compared to controls.

Gross examination of fish treated with 500 ppm to 2100 ppm molybdenum showed hemorrhages in the muscle below the skin, and the areas at the base of the dorsal and pelvic fins. A blue color believed to be molybdenum blue was observed in the stomach.

Histologic examination of fish treated with 650 ppm to 2100 ppm molybdenum showed thrombi in vessels of the muscular layers of the gut wall and in arteries of the kidneys and meningeal vessels. Heavy deposits of hemosiderin were seen in the spleen with a small quantity in the Kupfer cells of the liver.

Fatty infiltration of the liver was observed when fish were treated with molybdenum.

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## XVIII. VITA

The author was born on April 2, 1938 in Green Bay, Wisconsin and is the eldest son of Mr. and Mrs. M. R. Easterday. The author moved to Racine, Wisconsin with his family in 1940. Attendance in the Racine Public Schools led to a diploma from Washington Park High School in June, 1955. The author attended North Central College and received a B. A. degree with a major in Chemistry in 1960.

In September of 1960 the author came to Virginia Polytechnic Institute and began work leading to a M. S. degree in Biochemistry and Nutrition with a minor in Physiology.

*Richard Lee Easterday*

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