HISTOPATHOLOGIC, HISTOCHEMICAL

AND

DISSOLVED OXYGEN TOLERANCE STUDIES ON

THE MINNOW (PHOXINUS PHOXINUS),

IN ACUTE AND SUBACUTE

HEAVY METAL TOXICITY.

by

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1. SUMMARY OF PROJECT

Using the minnow (Phoxinus phoxinus) as a test animal experiments were conducted to investigate the toxicity of copper, lead, nickel and zinc.

The emphasis of the investigation was upon subacute (chronic) toxicity although experiments were performed to assess the relative acute toxicity of the four metals. In acute toxicity the metals could be arranged thus: Cu > Zn > Pb > Ni, in order of decreasing toxicity (5.1).

Fish were found to react more slowly to lethal concentrations of copper than of the other metals (5.2). Acutely toxic concentrations of the metals were considered to kill the fish by direct non-specific gill damage resulting in tissue hypoxia (7) and large amounts of the metals were always detectable in the gills (8). Normally acutely toxic concentrations of the metals were found to be non-toxic when tested in sewage effluents (6).

Fish were exposed to subacute concentrations of the metals for periods in excess of a hundred days. Histopathologic studies revealed little or no gill damage but the internal organs were affected especially after copper exposures (9). Histochemical and analytical studies revealed a continued uptake and storage of copper in the internal organs (10,12). Lead and nickel were accumulated to a lesser extent. Zinc was also detected in the fish but there was no continued accumulation. Enzyme histochemical studies revealed some changes in the enzyme activity in the internal organs of exposed fish (13). The subacute toxic effect of all metals tested was reduced when they were present in sewage effluents (11).

Survival rates at low oxygen levels in the presence of subacute concentrations of the metals were similar to those for control fish above 2 mg/l. oxygen, but below this more fish died (14.1). Exposure to subacute concentrations of zinc and copper for fifty days reduced the ability of fish to survive at low oxygen levels (14.2).

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2. INTRODUCTION

The biological effects of the discharge of toxic effluents of industrial origin into the rivers and streams of Britain is now receiving more attention than at any time past.

At the time of writing most of the investigations conducted with respect to fish and water pollution have sought to establish the "lethal limits" for various known toxicants in laboratory tests of short duration.

The most convenient method of expressing the "acute" toxicity of a substance is the $L.C._{50}$ - which is that concentration of the substance in water which kills half the test animals in a specified time, e.g. 48 hours.

Whilst it is important to decide which substances are potentially toxic to fish and also to gain some idea of their lethality, this approach does not point to the highest concentration of a poison which will not have a significant adverse effect on a fish population exposed to the poison more or less continuously.

Many pollutants, including heavy metals, occur in rivers in fluctuating amounts or at concentrations below those which in the laboratory have been found to be rapidly lethal. In recent years it has become increasingly obvious that low but consistent concentrations of toxins in fresh and sea water can be potentially harmful to living organisms.

The need for investigations of the chronic effects of long-term exposure of fish to low concentrations of poisons has been pointed out by Edwards and Brown (1967). Most of the experiments described and discussed in the present study were designed to assess chronic toxicity particularly with respect to histopathologic and biochemical changes occurring within fish.

The avenues of approach in studies of chronic toxicity are many. In addition to straightforward survival time data, records of growth, reproduction, acclimatisation, behaviour and general physiological condition can be made. It is hoped that in the near future more attention will be turned to investigations involving long-term laboratory exposures of fish to assess chronic toxicity although in the final analysis the effects of water pollution can only be judged ecologically.

3. REVIEW OF THE LITERATURE

Heavy metals are commonly occurring pollutants of streams and rivers and their presence in natural waters and possible harmful effects on living organisms have attracted attention for some time.

Copper, zinc and nickel occur in high concentrations in the wastes produced by metal plating industries. Zinc is used in the manufacture of rubber and in galvanising processes. The manufacture of accumulators results in lead wastes and many paints are lead-based. Copper has been widely used as an algicide in lakes and reservoirs.

Most of the work concerning the toxicity of heavy metals to freshwater organisms has been directed towards fish and of the metals zinc has been most thoroughly studied. Doudoroff and Katz (1953) have reviewed the early literature on metal toxicity and later reviews will be discussed in the present text.

1. Factors affecting the toxicity of heavy metals to fish

The lethal concentration of a heavy metal for a given species of fish is affected by water quality. Reviews of the factors affecting the tolerance of toxic metals (and other poisons) by fishes have been compiled by Weiss and Botts (1957), Lloyd (1963), Skidmore (1964) and Allan (1967).

Because of the effect on heavy metal toxicity of the physical and chemical properties of the water in which assays are performed a great variation in lethal limits for the metals has been reported. No attempt will be made in the present summary to enumerate the vast number of observations which have been made with respect to lethal limits but a brief review of the factors affecting the toxicity of heavy metals is included below.

i) Effect of Calcium concentration

It has long been known that as the calcium content of the water is increased the toxicity to fish of heavy metal salts is reduced.

Jones (1938) demonstrated that 1 ppm of lead was not immediately toxic to minnows (Phoxinus phoxinus) and sticklebacks (Gasterosteus aculeatus) in water containing 50 ppm of calcium, whereas the same concentration in very soft water was fatal to sticklebacks in 38.5 hours. Ellis (1937) demonstrated a reduction in the toxicity of copper to goldfish (Carassius auratus) when the calcium concentration of the water was increased.

Lloyd (1962) points out that so far as is known the calcium content of the water affects only the toxicity of metals to a marked degree.

Although part of the reduction in toxicity can be ascribed to the precipitation of insoluble basic salts, thus lowering the concentration of metal in solution, a further protection is afforded by the presence of calcium in solution.

Jones (1938) concluded that the reduction in the toxicity of a metal was due to the fact that the calcium prevented the precipitation of mucus on the gills of the fish. This, however, does not hold in many cases, e.g. Lloyd (1962) did not observe precipitated mucus on the gills or in the gill chamber of rainbow trout (Salmo gairdnerii) poisoned by acutely toxic concentrations of zinc sulphate. Lloyd (1963) reported data which indicated a possible internal protective mechanism afforded by calcium. When rainbow trout were exposed to zinc in soft water the toxicity of the metal was greater where the fish had been reared in soft water rather than hard. Fish reared in hard water and acclimatised to soft water for three days reacted in the same way as the "hard-water fish". Acclimatisation for five days to soft water, however, resulted in similar sensitivity to the "soft-water fish". It thus seems possible that the fish lost calcium after immersion in soft water for an adequate period. Further evidence that the calcium content of the cells of fish varies with the calcium concentration of the water has been presented by Houston (1959) using steelhead trout (Rainbow trout).

In view of the effect of calcium concentrations on the toxicity of heavy metals to fish the calcium hardness of all test solutions employed in the present study was carefully controlled.

ii) Effect of salts of other heavy metals and other toxicants

Using mixtures of ammonia and zinc, Herbert and Shurben (1964) demonstrated that if the concentration of each toxicant was expressed as a proportion of its lethal threshold value,

i.e. $\frac{A_S}{A_T}$, $\frac{B_S}{B_T}$ etc.

where A_S = concentration of toxicant in solution

A_T = lethal threshold concentration,

then when $\frac{A_S}{A_T} + \frac{B_S}{B_T} = 1$, the mixture should be at a threshold concentration for the fish.

Lloyd (1961) tested the toxicity of copper - zinc mixtures to rainbow trout and plotted the concentrations of the metals as:

$$\frac{Zn_S}{Zn_T} + \frac{Cu_S}{Cu_T}$$ against median survival time on a logarithmic scale. At lower concentrations the data fitted a curve which compared closely with that drawn from data where the metals were tested separately, but at higher concentrations the points did not fit the same curve and the mixtures were more toxic than the single salts. This indicated that low concentrations of zinc and copper were simply additive but at higher concentrations there was a synergistic effect.

A study by Sprague and Ramsay (1965) indicated similar results for copper - zinc mixtures with juvenile Atlantic salmon (Salmo salar). At lower concentrations when addition of the "toxic units" contributed by each metal totalled 1.0 the fish died at the expected rate, whereas when the "toxic units" totalled 2 and 5 potentiation occurred and the fish died faster than would be expected.

Bandt (1946) investigated the toxicity of mixtures of heavy

metals and reported that for trout and roach, copper and zinc, copper and cadmium, and nickel and zinc displayed synergism whilst cadmium and zinc, and nickel and cobalt appeared simply additive.

Jones (1938) demonstrated that there was a reduction in the toxicity of copper to freshwater invertebrates in the presence of lead.

In the present study the toxicity of the metals was examined separately except in one case where fish were exposed to an effluent containing a mixture of metals.

iii) Effect of temperature

It is known that for fish in general a decrease in temperature increases survival time in toxic solutions.

Lloyd (1962) demonstrated a 2.35-fold increase in survival time for trout exposed to acutely toxic concentrations of zinc when the temperature was reduced from 22°C to 12°C. Lloyd (1963) suggested that if temperature affected only survival time then the lethal threshold concentration would remain unchanged by temperature since at that concentration the survival time curve would be parallel to the time axis. Data were presented to confirm this.

In the present study all laboratory exposures of fish were conducted at the same temperature (15°C) and thus variations in toxicity were independent of this parameter.

iv) Effect of dissolved oxygen

When the dissolved oxygen concentration of the water is reduced there is a general increase in the toxicity of poisons to fish. Weiss and Botts (1957) demonstrated changes in time of response to Sarin (isopropyl methylphosphorofluoridate) relative to decreased dissolved oxygen.

Lloyd (1963) expressed the increase in toxicity by dividing the lethal threshold concentration obtained with oxygen at an air saturation value (X_{SAT}), with that obtained at the lower dissolved oxygen concentration (X). When the resulting $^{X}SAT/_{X}$ fractions for zinc, lead

and copper obtained with rainbow trout were plotted against the dissolved oxygen concentration of the water the points obtained fitted a similar curve indicating a similar effect for all metals tested. Curves for phenol and ammonia were found to be similar to those obtained with heavy metals. Lloyd considered that as phenol and ammonia exert their toxic effects in different ways from metals it appeared that the increase in toxicity was due to a physiological response by the fish to water of low oxygen content. The most probable response was an increase in the rate at which water was pumped over the gills (opercular rate). Fish generally react to a deficient oxygen supply by breathing more rapidly, (Ellis 1937, Shepard 1955, Weiss and Botts 1957 and Jones 1964). Assuming that the gill epithelium is the site of the toxic action of heavy metals, Lloyd put forward the following hypothesis, "When the rate at which the heavy metal ions enter the gill epithelial cells is less than the rate at which they are removed into the blood-stream, no build-up of metal ions will occur in the epithelial cells and the fish will survive. If the rate at which the heavy metal ions enter the gill epithelial cells is greater than the rate at which they are removed into the blood system, then a build-up will occur and the fish will die". Thus the increased ventilation rate initiated by low dissolved oxygen concentrations would cause an increase in the rate at which metal ions reach the gill surface and death would result more quickly.

If the above hypothesis is sound it would seem possible that under conditions of low dissolved oxygen normally subacute levels of heavy metals would prove to be acutely toxic. This possibility is investigated in Section 14.

Increased activity and stress also increase the opercular rate and thus the rate at which toxic ions would reach the gills. Herbert and Shurben (1963) demonstrated that when rainbow trout were forced to swim at 85% of their maximum sustainable velocity there was an increase of 0.7 in the 48 HR $L.C._{50}$ value for zinc sulphate and 0.81 in the 24 HR $L.C._{50}$ value for ammonium chloride.

In the field high and low oxygen levels alternate throughout the twenty-four hour cycle and Cairns and Scheier (1957) showed a significant decrease in the tolerance of bluegills (Lepomis macrochirus) to zinc chloride when the dissolved oxygen concentration was varied from saturation down to 2 mg/l throughout the day (96 hr. $L.C._{50}$). Pickering (1968) studied the effects of low dissolved oxygen concentrations on the subacute toxicity of zinc to bluegills. Dissolved oxygen concentrations of 1.8, 3.2 and 5.6 mg/l were tested. The dissolved oxygen concentration of the water was lowered by 1 mg/l per day until the test level was reached, in order to acclimatise the fish. Fish were then maintained at the test dissolved oxygen level for one day before the zinc was added. 20 day tests indicated $L.C._{50}$ values ranging from 7.2 to 12.0 mg/l of zinc and were significantly different at the 95% level of confidence.

It seems very likely that low dissolved oxygen is an important factor causing the absence of fish from rivers and streams. Alabaster (1959) considered that fish would live in a particular sewage effluent which normally proved toxic if the dissolved oxygen concentration was high and field studies revealed that the dissolved oxygen was always low in fishless regions. A recent study by Libosvarsky et al (1967) indicated that low oxygen was the main factor causing death and compelling fish to leave an area.

v) Carbon dioxide

Low dissolved oxygen concentrations in rivers due to organic pollution are often accompanied by elevated carbon dioxide levels.

A number of workers have demonstrated that increased carbon dioxide concentrations reduce the tolerance of fish to low oxygen. Alabaster et al (1957) showed that rainbow trout died at higher dissolved oxygen levels where high levels of carbon dioxide were present. They stated that the lethal effect of carbon dioxide is exerted by reducing the oxygen carrying capacity of the blood.

Presumably the effect of high carbon dioxide concentrations would

also be to decrease the lethal limits for heavy metals, but little information is presently available.

The carbon dioxide concentration in all tests in the present study is considered to have been an insignificant factor.

2. Toxic effects of heavy metals

The toxicity of heavy metals to fishes can be conveniently described as acute or chronic.

Acute toxicity is due to higher concentrations of metals and is manifested very quickly usually within two days and after five to seven days at the extreme.

The term "sub-lethal concentration" is often applied where the effects of long-term chronic toxicity are investigated. The definition is something of a misnomer as such concentrations of metals may eventually prove lethal. The term "subacute" is better applied in such cases.

Most of the toxicity tests conducted with freshwater fishes in relation to river pollution have lasted only for a relatively short period (usually a few days) and have sought to establish the lowest concentration of a given poison which would kill fish of average sensitivity in this time. Edwards and Brown (1967) have pointed out the need for tests involving long term exposures to assess the chronic effects of low concentrations of poisons, as although it is presently possible to state for many poisons which concentrations will kill fish in a few days there is relatively little information available as to the highest concentration which is harmless to fish after long-term continuous exposure.

Some workers have suggested permissible concentrations of toxicants by derivation from observed $L.C._{50}$ values. Jones (1964) quotes a factor of 0.1 of the $L.C._{50}$ value but also quotes Beak (1958) who considers this to be little more than an intelligent guess. Rachlin and Perlmutter (1968) however, using fish cell cultures to assess toxicity found that the concentration of zinc which exerted no appreciable

toxic effect was in fact 0.1 of the 96 HR. $L.C._{50}$ value. Edwards and Brown (1967) suggested a factor of 0.3 - 0.4 on the basis of field data. Sprague (1969) recommends the incipient $L.C._{50}$ (after long-term exposure) as the most useful criterion of toxicity. If impracticable the 96 HR. $L.C._{50}$ was considered a useful substitute and often the equivalent; the acute lethal process in most cases ceasing within four days. Some toxicants, however, are known to exert delayed lethal effects upon fish, e.g. pesticides (Alabaster and Abram 1965), cadmium and acrylonitriles (Notes on Water Pollution 1969) and these must be taken into account when determining permissible levels. Herbert (1961) suggested that a practicable permissible level in rivers receiving sewage effluents and industrial discharges would be that concentration which under low flow conditions would not kill more than a fixed percentage of fish (1 - 5%) in a three month period.

The chronic effect of some poisons is only evident as changes in the physiological response of the organism and attempts have been made to document these changes, e.g. Tamura and Yasuda (1963); however as Mount (1967) points out, although death is obviously adverse, the ecological significance of many of the physiological changes is less evident.

Wilber (1965, 1969) has suggested the use of organ : body weight ratios as a method for evaluating the effects of toxicants at sublethal levels.

In the present study the toxic effects of copper, lead, nickel and zinc will be considered under the two headings acute and chronic; although the emphasis will be on the chronic effects of long-term exposure.

i) Acute Toxicity

(a) Toxic action

Early workers attributed the acute toxicity of heavy metals to the precipitation of mucus on the gills of fish, thus interrupting respiratory flow. It was later suggested that the gill epithelium

might be damaged by the formation of insoluble metal - protein complexes.

Carpenter (1927) exposed minnows (Phoxinus phoxinus) to acutely toxic concentrations of lead nitrate and she noticed that fish died with symptoms of acute respiratory distress. The body became covered with a bloom of coagulated mucus and the gills were also covered by a thicker bloom. When the mucus was treated with a dilute solution of ammonium sulphide it turned black, indicating the presence of lead. Further work (Carpenter, 1930) indicated a similar effect with zinc, copper, cadmium and mercury. Ellis (1937) recorded comparable results with copper sulphate and goldfish and Jones (1935, 1938) reported the same reactions of heavy metals with the stickleback. Dilling et al (1926) had recorded similar observations when investigating the effects of lead on the growth of plaice (Pleuronectes platessa) and Westfall (1945) used the term "coagulation-film anoxia" in studies where the precipitation of mucus due to lead by goldfish was seen.

In all these cases no histopathological studies appear to have been made. Later work revealed a definite course of events in the destruction of the gills by heavy metals. Damage to the gill epithelium by heavy metals has been reported by Kuhn and Koecke (1956), Schweiger (1957), Lloyd (1960), Haider (1964, 1965), Mount (1965) and Brown et al (1968). Similar damage due to detergents has been reported by Cairns and Scheier (1962), Schmid and Mann (1962), Lemke and Mount (1963), Bock (1965) and Lang (1967); and due to phenols by Christie and Battle (1963) and Mitrovic et al (1968).

Gill damage caused by heavy metals is characterised by swelling and eventual sloughing of the respiratory epithelium covering the secondary lamellae. The underlying pilaster cells are sometimes fused and the blood spaces formed between their processes contracted. A detailed account of gill damage to Phoxinus caused by copper, lead, nickel and zinc is given in Section 7 and comparisons with other toxicants and species are made.

The characteristic pattern of gill damage incurred after acute exposures to metals seems to be the most likely cause of death, although precipitation of mucus on the gills may play a part in some cases. Lloyd (1960) and Skidmore (1970), however, have both reported the absence of precipitated mucus on the gills of rainbow trout killed by zinc sulphate.

Skidmore (1970) pointed out that the destruction of the respiratory epithelium had not been shown to cause death through any breakdown of gill function. In a study using rainbow trout, physiological measurements were made on fish exposed to rapidly toxic solutions of zinc. The rate of routine oxygen uptake by lightly sedated, quiet trout did not alter on exposure to a rapidly toxic solution of zinc sulphate. However, oxygen utilisation decreased seven times, gill ventilation volume increased six times, heart rate was halved and coughing rate (reversal of water flow over the gills) increased eighteen times. Measurements of the $P.O_2$ of aortic blood indicated a decline. The respiratory changes were considered generally similar to changes in the same fish when under hypoxia and the results suggested that damage to the respiratory epithelium decreased the permeability of the gills to oxygen. Zinc was not found to be a rapid internal poison and death of the fish was ascribed to tissue hypoxia.

It would thus seem that exposures of fish to acutely toxic concentrations of heavy metals cause death by direct damage to the gills. Lloyd's (1965) hypothesis postulated a build-up of metal in the gills during acute exposures due to the inability of the fish to remove incoming metal into the bloodstream at a great enough rate. Thus where fish have been killed by acutely toxic metal concentrations a measurable uptake of metals by the gills would be expected.

This subject will now be further considered.

(b) Uptake of the metal

Detection of heavy metals in the gills of fish has been attempted in four ways:

A. Direct chemical analysis
Kariya et al (1968, 1969)

B. Spectrophotometric analyses
(and Polarography)
Mount (1964)
Mount and Stephan (1967)
Eisler (1967)

C. Use of radioisotopes
Hibiya and Oguri (1961)

D. Histochemical methods
Haider (1964)

Lloyd (1960) measured the zinc content of two trout killed by a 20 ppm solution of zinc as 7.4 and 12 ppm wet weight respectively. The gills of these fish contained 63 and 60 ppm of the metal. The relationship between whole body zinc : zinc in water was 0.5 : 1 whereas the gill zinc : zinc in water ratio was 3 : 1 indicating a concentration of zinc in the gills after acute exposure.

Using bluegills (Lepomis macrochirus), Mount (1964) described an autopsy technique for zinc-caused mortality. The technique was based on the difference in metal uptake by the gills with respect to a bone sample in acute and subacute exposures. Thirty to ninety day subacute exposures indicated that the opercular bone accummulated zinc at approximately the same rate as the whole gill and that the bone always contained more zinc. Histochemical preparations (method unstated) revealed that most of the "normal" zinc was present in the bony gill arch and not in the soft tissues. Since the gill arch was very similar in composition to the opercular bone the uptake would be expected to be the same for both. The lower concentrations of zinc found in the gills of control fish and fish exposed to subacute zinc concentrations were due to the low concentration of zinc in the soft parts which reduced the overall concentration in the gills. During acute exposures the soft parts of the gills accumulated zinc in amounts far in excess of the opercular bone and the overal zinc in gill : zinc in bone ratio increased significantly compared with control fish.

When bluegills were used this ratio was always less than 1 when control fish or fish exposed to subacute zinc concentrations were tested.

After acute exposures the ratio increased to values between 2 and 5. Variation was found in the ratio in live fish taken from the field and the maximum ratio for each species was considered as an acceptable dividing line between a positive and negative autopsy result. Carp (Cyprinus carpio) presented autopsy problems as firstly the zinc content of the gills was highly variable (50 mg/g - 2500 mg/g) and secondly the average zinc content was very high (500 mg/g) and thus any increase would be small on a percentage basis.

In the present study large though not very variable amounts of zinc were detected in the gills of control minnows and thus similar problems arose.

Mount and Stephan (1967) investigated the uptake of cadmium by bluegills using techniques similar to those employed for zinc. After acute exposures that resulted in death the cadmium content of the gills was markedly higher than after exposures which were not lethal. Similar results were obtained using bullheads (Ictalurus nebulosus).

Eisler (1967) used atomic absorption spectrophotometry to determine uptake of zinc at acutely toxic concentrations, by Fundulus heteroclitus. Groups of adult fish were exposed to different concentrations of zinc as zinc chloride at 24‰ salinity and pH 8 at 20°C. Fish exposed to 43 ppm or less of zinc were all alive at 192 hours and there was no appreciable increase in the zinc content of the gills regardless of the initial zinc content of the solution. At higher concentrations where the fish died between 24 and 48 hours whole fish and gill arch contained seven and eight times respectively more zinc than control fish. These figures thus afford an index of zinc caused mortality for the species.

Kariya et al (1968) employed the dimethylgloxime method to determine the nickel content of goldfish killed by solutions of nickel sulphate and nickel-plating solutions. No nickel was detected in control fish when skin, muscle, viscera and gills were examined. Exposure to 50 ppm of nickel resulted in death and measured concentrations of the metal in the gills of dead fish ranged between 89.4 and

166.7 ppm. It is not clear whether these were wet or dry figures (paper in Japanese) but due to their magnitude the figures probably relate to ppm Ni per dry weight of tissue. With the exceptions of the skin (which presumably secreted mucus which combined with the nickel in solution) where the measured concentrations of nickel varied between 31.7 and 62.5 ppm, the other tissues showed only very slight increases in nickel content.

Using rainbow trout Kariya et al (1969a) employed the diphenylthiocarbazone method to determine the levels of lead in fish after acute exposure. The lead content of control fish was stated to be less than 0.34 ppm on average. After acute exposures (5 ppm Pb) that resulted in death the lead content of the gills rose to 494 ppm and the skin to 89.3. This large increase in the metal content of the gills should serve as an indicator of acute lead toxicity. Similar results were obtained by Kariya et al (1969b) when solutions of tin salts were assayed.

Haider (1964) using a modified silver-sulphide technique (see Section 10) was able to identify deposits of lead in the gills of fainbow trout killed in lead acetate solutions.

To summarise, present evidence indicates that acutely toxic concentrations of heavy metals kill fish by direct damage to the gills. At the same time there is a significant increase in the concentration of the metal within these organs. The gill damage causes death by tissue hypoxia when maximum gill ventilation is no longer sufficient to supply the oxygen needs of the fish (Skidmore 1970).

ii) Chronic Toxicity

The relative lack of information on the effects of subacute concentrations of heavy metals to fish has already been discussed. The greater part of this thesis is concerned with the chronic toxicity of copper, lead, nickel and zinc to Phoxinus phoxinus.

(a) Toxic Action

Histopathologic studies

Dawson (1935) examined the effects of long-term exposure of the

catfish (Ameiurus nebulosus) to subacute levels of lead acetate. Evidence of blood cell injury was seen and deposition of pigmented material resulting from the breakdown of erythrocytes was noted in the spleen, kidney, liver and intrahepatic pancreatic connective tissue. Damage to the gall bladder occurred in some cases and there was a general reduction in the erythropoietic activity of the spleen.

Ellis (1937) suggested the possibility of cumulative poisoning of fish with insoluble lead sulphide.

Calventi and Nigretti (1961) demonstrated that Funduli subjected to concentrations of copper above 0.4 ppm (copper citrate) showed significant histological changes. The liver cells developed numerous vacuoles and the kidney exhibited extreme granulocytopoietic activity. There was also evidence of increased erythrocyte fragility.

Using goldfish as a test animal, Vickers (1962) found that three week exposures to $^{M}/_{600}$ cobalt solutions resulted in vacuolisation of the liver cells together with fatty degeneration. The intestine exhibited increased mucous cell production under the influence of cobalt and manganese though zinc and nickel produced less effect.

Cusick (1968) examined the effects of various heavy metals on the lingual epithelium of the guppy (Lebistes reticulatus) and observed a measurable drop in the mucous cell populations (due to depletion?). This decrease reached significant proportions and was considered to provide a suitable index to specific heavy metal damage. Cusick also observed that zinc induced mortality was associated with a depletion of lingual mucous cell populations whereas copper was not. He further considered that the different effect on the lingual epithelium of copper and zinc lent support to the view that the mechanisms of heavy metal toxicity to fish could be internal and not necessarily identical for each metal, and that differential changes in the mucous cell population and in the lingual epithelium were evidence of varied sites of activity for different metals. This, as was pointed out, is in some disagreement with the conclusions of Lloyd (1965) that both copper and zinc exert their toxic effect in the

same way. Cusick went so far as to suggest that the observed synergistic effect where copper and zinc are tested together is a consequence of the fish being poisoned at two different sites simultaneously. Results reported in the present study (Section 7) indicate that in acute toxicity there is probably a greater mucous cell response to zinc than to copper. This was not quantified however and both metals exerted a similar damaging effect on the respiratory epithelium.

Crandall and Goodnight (1963) conducted a comprehensive histopathologic study of the effects of subacute concentrations of lead, zinc and sodium pentachlorophenate on the guppy. Fish were exposed to solutions containing 2 and 5 ppm of lead nitrate. The authors calculated the lead content of the solutions as 1.24 and 2.48 ppm respectively (Jones(1964) points out that the 5 ppm solution of lead nitrate would actually contain 3.12 ppm lead). In both solutions after exposure periods of up to 60 days fish differed little from controls. After this time the kidneys exhibited distended lumens containing debris, there was reduced mesenteric fat and examination of blood cells revealed a relative increase in granulocyte and lymphoid cells. The liver and pancreas, however, did not show any consistent or marked alteration. Evidence for failure of maturation of the gonads and even gonadal degeneration was found in some cases.

In 5 ppm solutions of zinc sulphate (1.15 ppm as zinc) after between 22 and 49 days exposure of newborn guppies little histological change occurred except that the livers exhibited some vacuolation. After 55 - 65 days in 1.15 ppm zinc liver blood vessels were poorly developed and the mesenteries were practically devoid of fat. Kidney tubules and glomeruli were distended and the lymphoid tissue of the kidneys was reduced. Some retardation of gonadal development was also evident. Five fish sectioned after 95 days exposure had livers containing large vacuoles filled with granular material. The kidney tubules were again expanded and the spleen was consistently small and

contained large amounts of pigment macrophages. Only one of the four male fish examined was mature. After exposure to 10 ppm of zinc sulphate (2.3 ppm as zinc) for 58 - 70 days the livers of ten fish examined showed marked degenerative changes and contained large vacuoles and irregular dark staining muclei. The pancreas was undersized and vacuolated, and the kidneys were haemorrhaged and possessed expanded tubules. The skeletal muscles were small and appeared highly vacuolated compared with control fish. An interesting feature of these studies is that generally no gill damage was observed. Crandall and Goodnight considered that the most evident abnormalities observed were probably secondary more or less non specific results of the toxicants. The inhibition of growth and sexual maturation and some of the histological changes suggested severe inanition. Such inanition could have been caused by inadequate food intake but all fish except the zinc groups were observed to feed normally. It was considered that a more probable explanation was the production of metabolic lesions resulting in poor food utilisation. The authors also discussed the related problem of stress reactions. Skidmore (1964) has pointed out that the histological changes observed by Crandall and Goodnight correspond well to Deevey's (1959) description of the later stages of the stress syndrome in mammals. Skidmore also mentions that it is the interrenal tissue in fishes (which is homologous to the adrenal cortex in mammals) which is believed to initiate changes of the sort observed by Crandall and Goodnight, and that studies of this tissue with respect to chronic toxicity would seem profitable.

Other histopathologic studies on chronic toxicity are few and concern other toxicants. The observations of Mitrovic et al (1968) on the chronic toxicity of phenol to rainbow trout are of interest, however, as a source for comparison. When fish were exposed to subacute levels of phenol in hard water for seven days the skin became thickened (both epidermis and corium) and mucous cells were full and enlarged. Many empty mucous cells were present at the surface of the skin and in some fish the outer layers of the epithelium were destroyed. Many leucocytes were present in the epithelium evidencing chronic inflammation. The kidneys

spleen and liver had swollen outer membranes. Swelling of the kidneys was noted in some cases and Malpighian bodies were enlarged with swollen capillaries. The spleen was also swollen. Hepatic blood vessels were enlarged so that groups of cells were separated by swollen capillaries. Possible hepatic necrosis was also observed. Finally damage to the mucous coat of the small intestine was seen along with a reduction in mucous cell numbers.

Physiological effects

Studies on the physiological effects of exposure to chronic concentrations of heavy metals have followed three lines:

1. Effects on growth, maturation and feeding.
2. Detection of acclimatisation or increased sensitivity after prolonged exposure.
3. Effects on enzyme systems.

Crandall and Goodnight (1962) found that newborn guppies reared in subacute zinc solutions grew more slowly than fish reared in zinc free water. After 90 days the median weight of the experimental fish was about half that of controls.

Pickering (1967), however, found that with bluegills (Lepomis macrochirus) exposures of 20 days to subacute levels of zinc did not significantly affect growth, and tin as stannous ion in concentrations of the order of 0.000005 M. was found to accelerate the rate of growth of goldfish (over 20 days) by Finkel and Allee (1940).

Where growth rate is reduced inadequate food consumption may be the cause. Edwards and Brown (1967) report that when rainbow trout were kept in concentrations of zinc above about 1.6 mg/l they did not appear to take food as readily. Fish were fed a standard amount of pelleted food (15 gms/50 fish) and at about 0.6 of the lethal threshold for zinc 20% of the food was not taken; whereas control fish left much less. When maggots were fed, however, equal weights were taken in all concentrations of zinc tested.

Cairns and Loos (1967) exposed zebrafish (Brachydanio rerio) to 3.7 and 6.7 ppm Zn^{++} under controlled conditions. The time taken for

each fish to consume ten out of twenty pieces of Tubifex worms was determined at 0, 24, 48, 72 and 96 hours. Response varied markedly between individuals but after 96 hours the majority of exposed fish required more time to consume the allotted food than did the majority of controls.

After exposure to very low concentrations of heavy metals the fish may become partly acclimatised to them. Edwards and Brown (1967) report that when the 48 HR. $L.C._{50}$ for zinc of fish kept at 0.5 of the zinc threshold for 60 days was determined a 40% increase was found compared with controls. The apparent increase in tolerance resulted in part from the death of the more sensitive individuals during the initial exposures. However, mortalities during the initial period were too low to account for the whole of the increased tolerance and it seemed likely that acclimatisation to zinc had occurred. When fish were exposed to 0.6 of the threshold there was no increased tolerance indicating probably physiological distress caused by chronic poisoning.

These results are of importance as they demonstrate the very small difference in zinc concentrations which results in either acclimatisation to higher concentrations and thus some benefit, or conversely the onset of the symptoms of chronic toxicity. They also demonstrate that when field populations of fish are examined varied responses due to past exposure to metals can be expected.

Brown et al (1968) compared the toxicity of alkylbenzene sulphonate (A.B.S.) detergent to rainbow trout which had been chronically exposed (100 days) to 0.8 ppm zinc, with untreated control fish. When tested alone the toxicity of A.B.S. was similar for both groups, but when tested in the presence of 0.8 ppm zinc the mixture was more toxic to fish that had been exposed previously to zinc. Chronic exposure to zinc thus seemed to increase susceptibility to A.B.S. toxicity. This conflicts somewhat with the observations of Edwards and Brown (1967) where acclimatisation in subacute levels of zinc was demonstrated.

Cairns and Scheier (1964) exposed sunfish (Lepomis gibbosus) to A.B.S. for 30 days at "a concentration sufficient to damage the gills."

The fish were then exposed to zinc chloride. Results indicated that the exposure to A.B.S. did not cause a gross change in the tolerance of fish to zinc.

In the present study experiments are described which were conducted to ascertain whether exposure to subacute concentrations of the metals for 50 days impaired the ability of minnows to survive at low levels of dissolved oxygen (Section 14.2)

Only one attempt has been made to investigate the effects of heavy metals on enzyme systems in fishes. Jackim et al (1970) exposed Killifish (Fundulus heteroclitus) to 96 HR. $L.C._{50}$ concentrations of six metals (Pb, Cu, Hg, Be, Cd, Ag). Livers were taken from fish surviving the exposures, homogenates prepared and assays for five enzymes were performed. Metal salts were also added directly to tissue extracts from unexposed fish. The changes in enzyme activity observed were not necessarily the same in magnitude and direction in the two cases. This work is further discussed in Section 13 where enzyme histochemical studies of fish exposed to heavy metals were made.

(b) Uptake and accumulation of metals

Having established some pathological and physiological changes which occur after long term exposure to subacute metal concentrations it becomes necessary to discuss how the metals may get into the fish (if at all), and whether any uptake results in accumulation of the metal in the internal organs of the fish.

Entry of metals

Carpenter (1925, 1927 and 1930) considered that the death of fish exposed to heavy metal solutions did not result from any penetration of metal into the body of the fish, but that (in acute toxicity) there was an interaction between the metallic ion and mucus secreted by the gills. The coagulated mucus thus impaired respiratory efficiency andcaused death by hypoxia. Behrens (1925) and Ellis (1937) were in general agreement although Behrens, using a radioisotope, was able to detect some penetration of lead into the internal tissues of the fish. Jones (1937, 1939) also considered the reaction to be external. It has however been pointed out

earlier in this summary that metals do enter the bodies of fishes. There are three possible routes by which metals can gain entry, namely via the skin, intestine and gills. Carpenter (1927) noted a film of mucus on the bodies of fish exposed to lead nitrate. This mucus response has since been recorded for a number of fish and metals. Scheier (1957) working with bluegills detected zinc in the mucus covering of fish after exposure for one hour to 1 ppm. After 24 hours most of the zinc and mucus had been precipitated to the bottom of the container but some of the zinc was absorbed by the fish. It is known that in freshwater fish the body wall allows the passage of water, and Joyner (1961) considered that zinc combined with mucus and was subsequently taken up by the fish.

Freshwater fishes are generally considered not to "drink" water as their body fluids are hypertonic to the external medium there is a constant osmotic inflow of water. Abegg (1959), however, considered that freshwater fish do drink some water and ingestion of colloidal thorium dioxide from the external medium was demonstrated by Frank and Allee (1950). Joyner (1961) exposed brown bullheads (Ictalurus nebulosus) to dilute solutions of zinc chloride labelled with $zinc^{65}$. Groups of three fish were exposed for 96 hours to 2 litre samples of lake water (75 ppm $CaCo_3$) containing initially 0.25, 0.5, 1.0, 3.0 and 6.0 ppm of zinc. Skidmore (1964) points out that the ratios of wet weight fish : weight of zinc varied from 16000 : 1 to 660 : 1 so that detoxification of the solution would be rapid. There was a rapid initial uptake of zinc followed by a short period of decline (possibly due to detoxification). Over half the total zinc absorbed was detected in the gills and gut with lower levels in the kidney, spleen, muscle, bone and skin. Joyner further observed that when the oesophagus of the bullhead was plugged with petroleum jelly the fish absorbed as much zinc as controls indicating that the intestine was not important in that instance. After 24 hours return to zinc free water, fish exposed to 6 ppm lost 43% of accumulated zinc and after 6 more days only 11% of the remainder had been lost.

Saiki and Mori (1955) exposed carp (Cyprinus carpio) to solutions containing low concentrations of zinc labelled with $zinc^{65}$. Twenty-two

days' exposure resulted in the highest concentrations of metal appearing in the kidney whereas the gills contained a little less. The zinc content of the gut was much lower and does not compare with the results of Joyner (1961). On return to zinc free water 42% of accumulated metal was lost in two days.

Joyner and Eisler (1961) exposed 25 Chinook salmon (Oncorhyncus tshawytscha) for 24 hours to 5 litres of lake water containing 0.2 ppm of zinc labelled with 18.54 microcuries of Zn^{65}. Periodic assays of the gamma radioactivity of the fish showed that rapidly growing fish had removed approximately 2% of the zinc^{65} from the medium and had retained nearly all of it for 63 days after transfer to flowing zinc-free water. This is at variance with the results of other experiments with Bullheads where nearly half the accumulated zinc was lost in 24 hours after transfer to zinc-free water.

Hibiya and Oguri (1961) using the goldfish investigated the uptake and accumulation of various radionuclides including Zinc^{65}. When the isotope was injected into the "air-bladders" (presumably swim bladders) radioactivity counts indicated that the greatest accumulation of zinc was in the intestine. The authors proposed that zinc was being actively excreted into the gut but they also quoted the work of Miura and Tzunazima (1956) and Saiki et al (1958) who considered that zinc was readily accumulated from the external medium by the digestive tract as well as the gills.

The main point of entry of heavy metals into the body of fishes in acute and subacute exposures is probably the gills although there is no concrete evidence at present. The constant uptake of water by fresh water fishes results in the production of copious amounts of urine which although very dilute constitutes a serious drain on salt reserves. This loss is made good by active uptake of ions via the gills and it is there themetals probably gain access during chronic exposures.

Where fish have been exposed to metals in solution high levels of metals have always been detected in the gills. (Saiki and Mori 1955, Joyner 1961, Mount 1964, Mount and Stephan 1967, Kariya et al 1969a).

Using the gills of the Japanese eel (Anguilla japonica) Hibiya and Oguri (1961) have demonstrated uptake of zinc and the rate was measured at $0.7 - 4.1 \times 10^{-6}$ mg/hr. Although at acutely toxic levels damage to the gills occurs, at subacute levels the fish can probably transport metal ions from the gill tissues into the blood stream fast enough so that a lethal build-up does not occur (Lloyd 1963).

Accumulation of metals in internal organs

If metals enter the body of the fish from the external medium and are not immediately excreted, storage in various organs will occur and may cause damage to the tissues. Uptake of heavy metals by fish was demonstrated nearly sixty years ago by Thomas and White (1912) and later by Thomas (1924). Subsequently their uptake has been demonstrated by several workers mostly dealing with zinc.

The only experiments involving really long-term exposures have been reported by Mount (1964) and Mount and Stephan (1967). In the first study when Bluegills were exposed to subacute concentrations of up to 5 ppm of zinc for 90 days (120 - 250 ppm $CaCo_3$), the eyes, kidneys and skin were found to accumulate large amounts of the metal. The gills and gut contained a lesser amount and the zinc content of the liver, muscle and spleen was little changed. Very high levels of zinc were measured in the kidneys (approximately 300 ppm) and the eye (approximately 750 ppm) of control fish whereas the control levels for other tissues fell between 10 - 80 ppm zinc per dry weight of tissue. Where high control levels of metals occur these are often variable and difficulties can arise in assessing significant accumulations. Mount found that the gills of carp presented this problem (Section 3.2.i). It is thus important to have data from a wide range of control fish before valid conclusions can be reached concerning metal uptake. Where a fish kill occurs in the field the "normal" metal content of the fish from the specific location must thus be known.

Mount and Stephan (1967) investigated acute and subacute cadmium toxicity to Bluegills and Bullheads. Analyses were made of bone, liver, muscle, gut, spleen, gills and kidneys of living fish. After subacute

exposure of up to 90 days there were substantial accumulations of cadmium in the liver, kidneys, gills and gut and lesser amounts in the spleen (no figures given in the paper). No significant accumulation was measured in the bone or muscle. The liver accumulated up to 500 µg/g cadmium per dry weight of tissue and was considered to be a useful monitor of past exposure to the metal. Comparison of the uptake curves for liver and gill after 30, 60 and 90 days indicated that an equilibrium was established between the cadmium in the tissue and that in the external environment between 60 and 90 days.

The use of trace element analysis was employed by Stapleton (1968) in a study of two populations of calico bass (Paralabrax clathratus). One population came from an unpolluted environment and the other from an effluent pipe containing heavy metals. Although the trace element concentration of tissues was found to vary within each population there was a significant degree of variation between the two populations.

Slater (1961) has demonstrated a gradation in the absorption rate of zinc by different species of trout. Brown trout (Salmo fontinalis) absorbed the metal more quickly than cut-throat trout (Salmo gairdnerii). Thus as well as individual variations in control metal levels it is important to assess uptake in separate species and not to rely on mixed data.

Gustafson et al (1967) investigated the Zn^{65} and Mn^{54} content of euryhaline and stenohaline fish taken from seawater polluted by these isotopes. Retention of Zn^{65} was demonstrated only in euryhaline fish from seawater and the authors considered that the complex osmoregulatory systems inherent in these animals in addition to their feeding habits were casual agents in the uptake.

Using pike (Esox lucius) Johnels et al (1967) were able to demonstrate significant accumulations of mercury by fish inhabiting contaminated waters in Sweden.

Kariya et al (1969) employed the diphenylthiocarbazone method to detect lead in rainbow trout after exposures of up to 96 hours only. The

experiments were mainly concerned with acute toxicity but in fish surviving at 96 hours the liver, kidney and gut contained 40, 104 and 61.8 ppm of lead respectively (control levels were less than 0.34 ppm on average).

Haider (1964) was able to demonstrate the uptake of lead by rainbow trout from subacute solutions of lead acetate. Using a modified silver-sulphide technique lead deposits were histochemically demonstrated in the liver, kidney, spleen and bone (ribs).

Summary

The relative lack of information with respect to the effects of chronic heavy metal toxicity to fish has been pointed out.

The acute toxicity of heavy metals to fish is well documented and death appears due to direct gill damage resulting in tissue hypoxia.

Chronic toxicity has been investigated to a limited extent and some damage to internal organs accompanied by uptake and storage of heavy metals has been noted. In only very few cases have experiments investigating chronic toxicity been conducted for more than two to three weeks.

4. GENERAL - MATERIALS AND METHODS

The minnow (Phoxinus phoxinus) was used as a test animal firstly because of its small size and secondly because of ease of availability. All fish were from Lake Windermere and were obtained via the Freshwater Biological Association. Batches of fish were received four or five times a year during the project.

No attempt was made to breed stock fish. Fish not immediately required for bioassay were maintained out of doors in a fibreglass tank, containing 900 litres of Birmingham tap water. The water was filtered through two towers of activated charcoal to remove chlorine and then passed to the holding tank. The rate of flow into the tank was 25 - 45 litres per hour and this was found sufficient to keep the holding water clear. At all times the water in the tank was well aerated.

Water was taken from the tank for analysis on four occasions and the results are shown below:

ppm metal	1	2	3	4
Cu	0.025	0.040	0.070	0.020
Zn	0.035	0.015	0.05	0.015
Pb	nil	nil	nil	nil
Ni	nil	nil	nil	nil
Ca	X	10	12	10

The observed levels of copper and zinc were a cause for concern and because of this fish were never maintained in the tank if there were facilities available in the laboratory. No lead or nickel was measurable in the stock tank. The levels of calcium measured were below those employed in the laboratory except where the toxicity of lead was studied.

When fish were maintained in the laboratory and when bioassays were conducted an artificial stock medium was prepared using glass distilled water and various salts. The recipe used was that for B.O.D. water (see chemical analysis as applied to sewage and sewage effluents H.M.S.O. 1956).

The solution was prepared using water from an all-glass still and the distillate contained none of the four metals by analysis. All metal

analyses were carried out using a Unicam S.P.900A atomic absorbtion spectrophotometer except where stated to the contrary.

In the case of copper, zinc and nickel tests, the artificial medium was prepared with a final calcium concentration of 80 ppm Ca (200 ppm as $CaCo_3$). In the case of lead the medium used had a calcium concentration of 10 ppm Ca (25 ppm as $CaCo_3$).

The lower calcium levels in the case of lead were employed because of the effect of calcium in the water of reducing the solubility of lead. The reduction in lead solubility in waters of high calcium content is due mainly to the precipitation of insoluble lead carbonate from the bicarbonate in the water. In the artificial medium employed in the present study calcium was added as calcium chloride due to the relative insolubility of the carbonate. The reduction in the solubility of lead would thus be less significant although bicarbonates would be present in the water due to the presence of carbon dioxide in solution.

Exposures of fish to the four heavy metals were made in 5 litre all-glass tanks except where otherwise stated. Heavy metal salts were added from 1 litre concentrated stock solutions. The solutions were prepared from the following salts: copper sulphate, lead nitrate, nickel chloride and zinc sulphate. The solutions were made up in volumetric flasks slightly acidified with the appropriate acid in order to maintain their concentrations and stored in polythene bottles. The addition of acid did not significantly affect the pH of the final assay solution. The stock metal solutions were replaced every three weeks except for lead which was made up freshly once a week.

Fish were fed daily on "Tetra-Min" tropical fish food and occasionally on Daphnia. Details of feeding and of particular methods of acclimatising and exposing of fish to metal solutions will be given in each section.

In the laboratory all stock fish and those under experimental conditions were maintained at $15^{\circ}C \pm 1^{\circ}C$. Where 5 litre glass tanks were used they were immersed in a temperature-controlled water bath to a depth of 5 - 6 cms (see Plate 1). The water in the tanks was aerated

Plate 1

Cooled waterbath containing all-glass aquaria.


STOCK
Zn
100 ppm
pH 8
Zn 3
Zn 2
Zn 1
Cu 3
Cu 1
Cu 2
Pb 3
Pb 2
Pb 1
Ni 3
Ni 1
Ni 2
Ni 1
Pb 1

and the constant agitation of the water was found sufficient to maintain a steady temperature throughout each tank. Other methods of maintaining a steady temperature will be described in the appropriate sections.

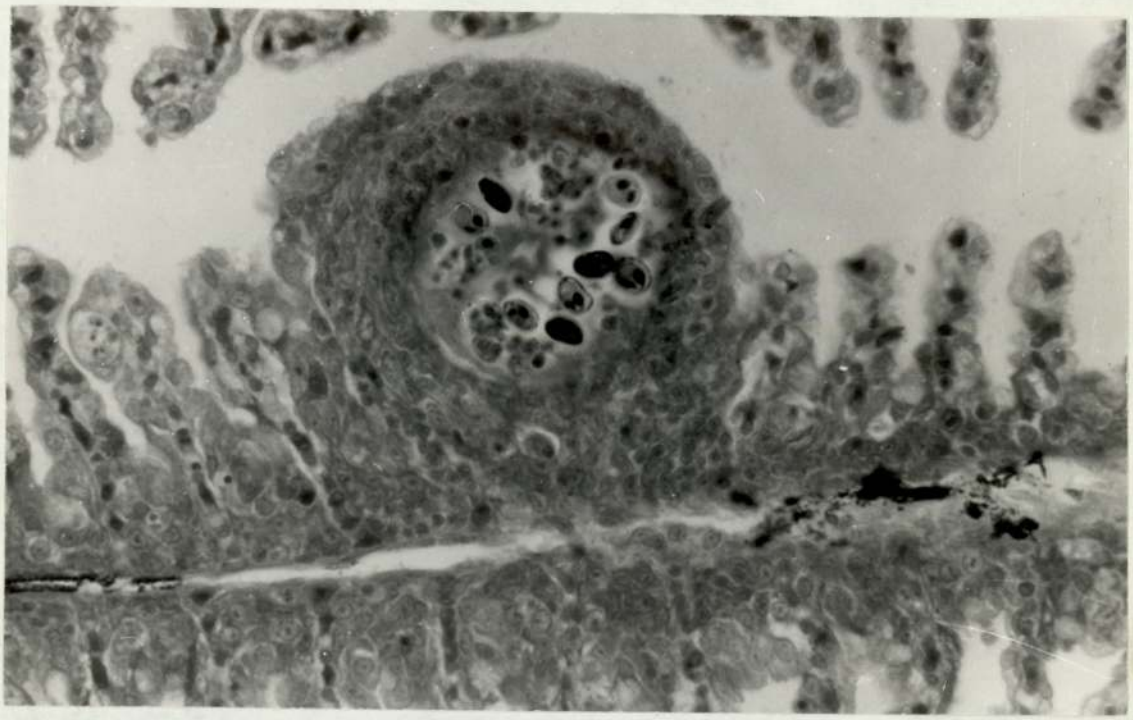
Disease caused very great problems during the course of the project. The ciliate parasite Ichthyophthirius multifilius causing "white spot" disease occurred from time to time. Mercurochrome preparations were not used as a cure because of their mercury content and methylene blue was found to be unsatisfactory. The most effective treatment was found to be the use of quinine sulphate which was added to the water at the rate of 0.2 gms/5 litres. The treatment was repeated after 5- 7 days. Fish suffering from whitespot were immediately isolated and were never used in acute toxicity tests, though fish cured of this disease were used in chronic toxicity tests. This precaution was taken because the cysts caused by the parasites occur upon the gills as well as on the general body surface (see plate 2) and this would interfere with normal respiratory function thus invalidating results.

Fin and tail-rot were occasionally noted and as these are usually secondary infections fish suffering from them were killed and disposed of. Saprolegnia ferax which is also a secondary infection appeared very infrequently and on two occasions red spot was noted; in both cases fish were killed and discarded.

The greatest disease problem was associated with the development of lesions of the skin which very rapidly developed into large open wounds. The time span from the first sign of infection to the final stages where deep wounds occurred was sometimes only three days. The muscles of the pelvic and caudal region were most affected and often in the later stages the spine was exposed. The particular disease was not indentified but it is considered that "ulcerative dermal necrosis" (U.D.N.) is a satisfactory diagnosis. Fish succumbed very rapidly to the disease

Plate 2

7 microns gill section (Bouin's fixed)
stained in haemotoxylin and eosin
showing an advanced whitespot cyst.
(X 550)



and no cure was found. The disease appeared each summer and was at its worst between early June and the beginning of September. Whole stocks of fish were lost together with many experimental animals. After the first epidemic no cure was attempted and all fish infected were killed and disposed of. All tanks and nets etc., which had been in contact with diseased animals were sterilised in "Chloros".

The only other pathologic condition which deserves mention is that where fish with twisted spines were observed. The cause was uncertain but was probably a result of inbreeding. These fish were generally healthy but appeared to be slightly less resistant to heavy metals and low dissolved oxygen than control fish and were thus not used in toxicity tests.

Because of the problems of disease new stocks of fish were quarantined for up to two weeks wherever possible.

5. THE ACUTE TOXICITY OF INORGANIC SOLUTIONS OF THE METALS

(1) Survival times

In order to investigate survival times of minnows in inorganic metal solutions static tests were employed. Due to lack of space only six fish were tested at each concentration. Five litre all-glass tanks were used and each tank contained three fish. The temperature was maintained at $15^{\circ}C \pm 1^{\circ}C$ by immersing the tanks in the temperature-controlled water bath. The tanks were aerated for the duration of each test and the dissolved oxygen concentration of the water varied from 8.5 to 9 ppm as determined by the Winkler technique. Fish were not fed for 24 hours before testing and were acclimatised to the artificial medium for seven days at $15^{\circ}C$ before the introduction of metal salts. Minnows for testing were caught in large numbers from a stock tank and then taken as randomly as possible. Fish of similar size were used (5 - 6 cms fork length) and the average weight of fish calculated from a sample of 20 was 2.35 g. The sex of individuals was not determined.

In the interest of accuracy tests were started early in the morning as it was found that when deaths occurred most were during the first 12 - 15 hours. During the first day observations were made at regular intervals and the time of death was noted very accurately. On the second and subsequent days the time of death was noted to the nearest hour. During the first night accuracy was difficult to maintain and when fish died during this period an estimation of the time of death was made. The error thus incurred was always less than 20%.

Detection of Survival time.

The point of death was taken as the cessation of opercular movement and lack of response when the fish was touched. Often when opercular movement had ceased, stimulation of the fish with a glass-rod would bring on a spasm of movement.

Fish - Metal Ratios

An important factor in any toxicity test is the amount of toxin available to each fish in the test solution and thus the degree of detoxification of the test medium which can occur. It will be evident from the results described in this section that under the conditions of the experiments very low concentrations of heavy metals are rapidly toxic to Phoxinus. Because of this the "wet weight of fish" : metal weight ratios were not very satisfactory. The ratios for zinc and copper varied between 1870 : 1 and 18700 : 1. Those for lead varied between 280 : 1 and 1220 : 1 and those for nickel from 140 : 1 to 280 : 1. Because of the likelihood of significant detoxification of the solutions they were replaced as near as possible every twelve hours during each test. This was accomplished by gently transferring the fish by net to a fresh tank of solution at the same temperature.

Choice of Metal Concentrations

Metal concentrations were employed on a logarithmic scale (Doudoroff et al 1951).

1. Copper

The concentrations of copper tested (ppm as Cu) were as follows:- 0.75, 0.42, 0.24, 0.18, 0.135, 0.075 ppm.

An acidified solution on 100 ppm of copper was prepared with $CuSO4\ 7.H_2O$ and stored in a polythene bottle. The appropriate amount of stock solution was then included in each 5 litres of test solution (e.g. 12 ccs of stock made up to 5 litres = 0.24 ppm Cu).

2. Zinc

The concentrations of zinc tested were the same as those for copper. An acidified stock solution of 100 ppm zinc was prepared with $ZnSO_4.5H_2O$.

3. Lead

The concentrations of lead tested were as follows: 4.9, 3.7, 2.8, 2.1, 1.8 and 1.55 ppm.

An acidified stock solution of 500 ppm lead was prepared with $Pb(NO3)_2$

4. Nickel

The concentrations of nickel tested were as follows:- 10.0, 8,7, 7.5, 6.5, 5.6 and 4.9 ppm nickel. An acidified stock solution of 2000 ppm nickel was prepared with $NiCl_2$.

RESULTS

Survival times in all metal salt solutions are indicated in table 10 (see Appendix). Average survival times were calculated as the geometric mean = antilog $\left\{ \frac{\log T}{N} \right.$

T = survival time

N = number of fish

1. Copper

All concentrations of copper down to 0.18 ppm killed all the fish in 13 hours or less.

At 0.075 ppm copper all fish survived for the ten day experimental period.

Under the conditions of the experiment copper was the most toxic of the four metals studied.

2. Lead

At the lower calcium level of 10 ppm all concentrations of lead down to 2.1 ppm killed all fish within 6 hours. At concentrations below this the fish survived for much longer periods until at 1.15 ppm they survived for the whole of the 10 day experimental period. At the higher metal concentrations (4.9, 3.7, 2.8 ppm) there was a definite white precipitate when the lead stock solution was added to the artificial medium indicating a probable precipitation of large amounts of the added lead before an equilibrium was reached. The tanks were aerated and carbon dioxide absorbed from the air would precipitate lead carbonate. This period before the attainment of equilibrium may have been responsible for the longer survival time (on average) in 4.9 lead than in 3.7 ppm lead. Although it is possible that at the higher concentrations of lead a threshold reaction time had been reached (Jones 1964).

3. Nickel

Nickel was the least toxic of the four metals tested. Concentrations of 7.5 ppm upwards killed all fish in less than 6 hours but at 6.5 ppm the fish survived the experimental period of 10 days.

4. Zinc

The response to zinc solutions was very rapid; concentrations of 0.24 ppm and above being lethal in an average time of 1 hour or less. On average fish survived for slightly longer at 0.75 ppm than at 0.42 ppm and it again seems possible that as in the case of lead a threshold reaction time had been reached.

Comparison of Metal Toxicities

On the basis of the above observations the metals can be arranged thus, in order of decreasing toxicity: Cu > Zn > Pb > Ni. All the metals tested exhibited threshold concentrations. When the results were plotted as log concentrations against log survival time the result was a series of curves which eventually became parallel to the survival time axis. See Fig. 1.

The main point of interest apart from the difference in toxicities was the slower response to toxic levels of copper. Possible reasons for the slower reaction to toxic levels of copper are discussed in Section 7.

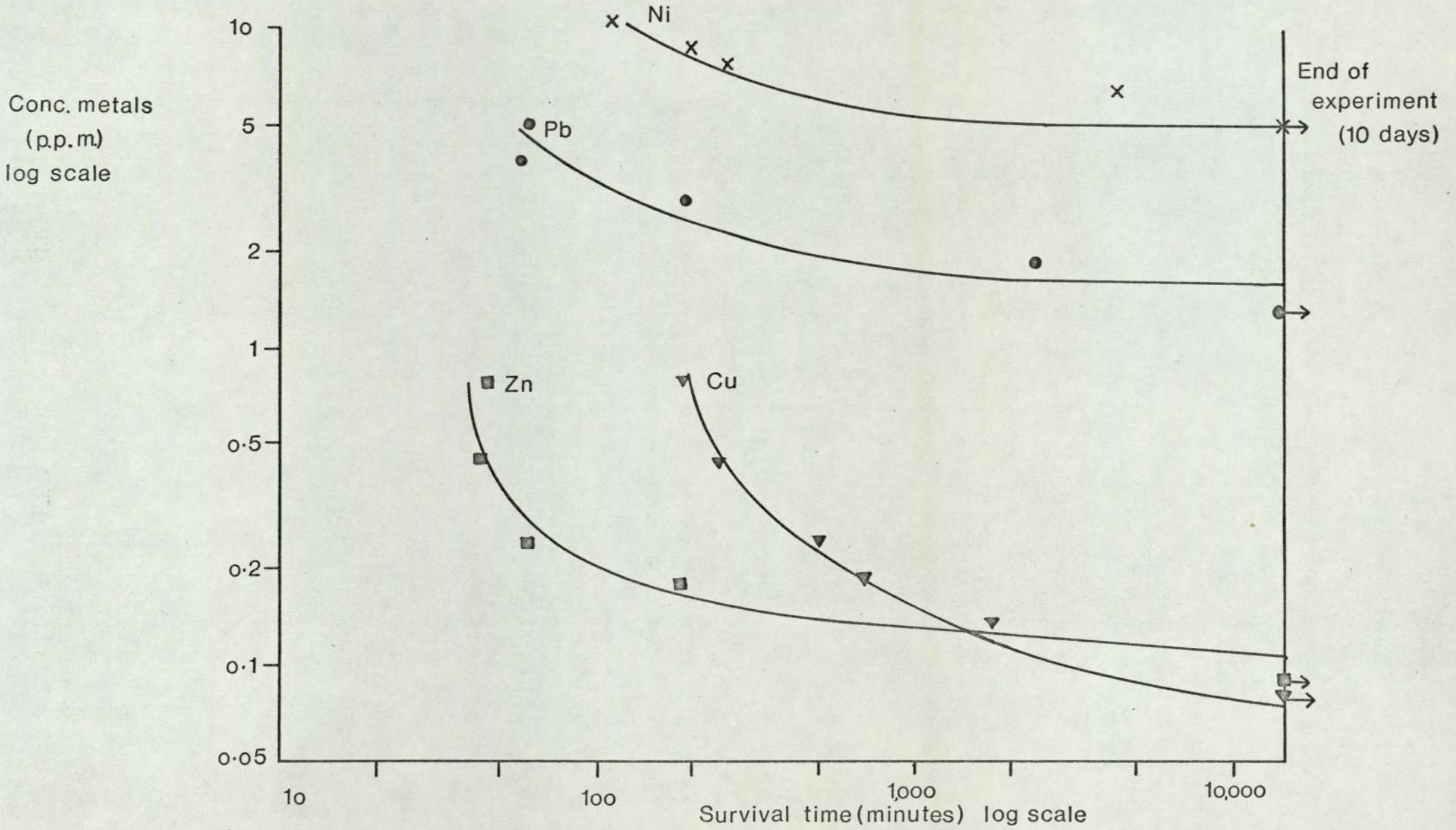
Lloyd (1963) showed that when the concentrations of heavy metals were expressed as a multiple of their lethal threshold concentrations i.e., $^{MS}/_{MT}$, where MS = Conc. metal in solution

MT = Lethal threshold conc.

the log concentration - log survival time curves for copper and zinc salts with rainbow trout were idential (in both hard and soft water). Lloyd also replotted Schweiger's (1957) data for the toxicity of cadmium, nickel, cobalt, mercury and manganese in the same way and the results showed that although the points for cadmium cobalt, and nickel lay on the same line those for mercury and manganese were different, indicating a possible difference in

Figure 1

Survival of minnows in different concentrations of copper, lead, nickel and zinc. Each symbol represents the median value for six fish.


Conc. metals
(p.p.m.)
log scale
10
5
2
1
0·5
0·2
0·1
0·05
Ni
Pb
Zn
Cu
End of
experiment
(10 days)
10
100
1,000
10,000
Survival time (minutes) log scale

toxic action.

Further experiments were carried out in the present study to obtain 48 hour $L.C._{50}$ values and these were then incorporated in MS/MT data after the fashion of Lloyd.

2. Determination of 24 and 48 hour $L.C._{50}$ Values

The $L.C._{50}$ for any toxicant is that concentration which under the conditions of a particular experiment kills 50% of the fish within a set period. The term $L.C._{50}$ was preferred to $L.D._{50}$ as the metal was not supplied as a "dose" but as an "external" concentration. The $L.C._{50}$ is only of value when comparing the toxicity of different toxicants, or the same toxicant under differing conditions and does not indicate safe concentrations on a long term basis.

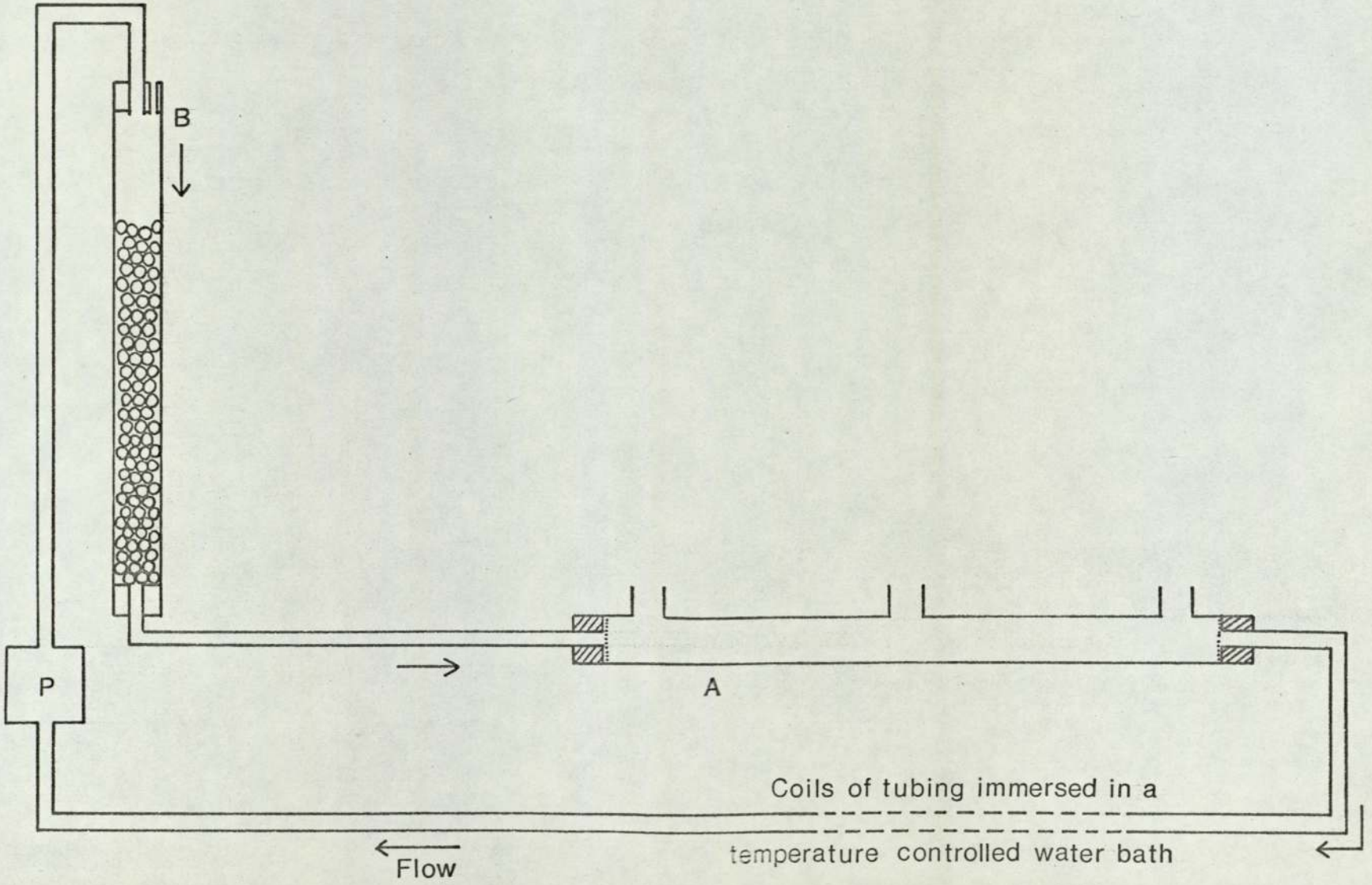
For the determination of $L.C._{50}$ values static tests involving the use of 5 litre all-glass tanks were not employed, as all tanks were in use to determine the effects of chronic toxicity. The apparatus used for $L.C._{50}$ determinations was intended primarily for use in the experiments described in Section 14, where the effects of low dissolved oxygen concentration on the toxicity of the four metals was investigated. For the purpose of the experiments described in the present section the apparatus consisted of a closed circuit around which the toxic solution was pumped. Circulation was achieved by the use of a "Fontan" magnetic pump. Although these pumps have a magnetic core over which the solution flows, it is encapsulated in polypropylene and no metal part came in contact with the water.

A simplified diagram of the apparatus is shown in Figure 2.

The fish were confined to a four foot long glass tube (A). Nylon meshes were incorporated at each end of the tube to prevent escape of the fish. The direction of flow is indicated by arrows. Thevertical tower (B) was essentially part of the apparatus for maintaining steady levels of dissolved oxygen and its full use will be described in Section 14.

Figure 2

Apparatus used for 24 and 48 hour $L.C._{50}$ determinations


B
P
A
Coils of tubing immersed in a
temperature controlled water bath
Flow

In the present experiments it was employed simply as a means of ensuring aeration of the assay solution. An "air-stone" was placed at the bottom of the column and the tube was filled with 2 cm diameter hollow plastic spheres ("All-plas"). A slit was cut in the side of the bung at the top of the column (B) to allow excess air to escape during aeration. The dissolved oxygen concentration within the system was 8.5 - 9 ppm as determined by the Winkler technique. All the plastic tubing used in the apparatus was of non-toxic specification and only silicone-rubber bungs were employed. The tube (A) was held by a system of clamps and by adjusting the level of these a satisfactory level of water in the tube was achieved. In these experiments the three ports on top of the tube (A) were not closed. The magnetic pump (P) would only pump water and could not deal with water containing air pockets. Consequently the pump was placed at the lowest point in the apparatus.

The rate of water flow around the system was governed by adjusting the height of the column (B). The flow rate was in fact very slow and at no time did the fish have to set themselves against a current. Herbert and Shurben (1963) have shown that rainbow trout forced to swim at 55% of their maximum sustainable velocity were not more sensitive to acute zinc poisoning and in the present study the conditions provided never approached that situation.

Two sets of apparatus were assembled. One set was accommodated in a temperature controlled room at 15°C ± 1°C. The other set was not in a temperature controlled environment and a steady temperature was maintained by immersing coils of the tubing between C and D in a water-bath. The temperature of the water bath was thermostatically controlled and was set between 8-10°C depending upon the ambient temperature. This gave a temperature of 15°C ± 1°C when measured over 24 hours with a recording electrical thermometer.

Ten fish were exposed to each metal concentration. As the volume of solution contained in the apparatus was 8.5 litres the Fish : Metal ratios were again unsatisfactory. The test solutions were thus replaced

at 6, 12, 24, 30 and 36 hours during the 48 hours duration of each test. This was accomplished by stopping the pump and connecting the input tube to the pump to a 10 litre polythene aspirator of fresh toxic solution at the same termperature. The fresh solution was then pumped into the apparatus thus displacing the old. The pump was then stopped, the system restored to its former state and the pump restarted.

An attempt was made with both sets of apparatus to control periods of light and darkness during the test period. In the "cold-room" a time switch was incorporated into the lighting circuit so that the hours of light and darkness matched as closely as possible those of the other set up. Approximately 8 hours of darkness and 16 hours of light were supplied to both sets of apparatus in 24 hours. The control of light may be a significant factor in some toxicity tests. Shepard (1955) showed that fish exposed to light, and presumably having a higher oxygen demand due to greater activity, tended to be less resistant to hypoxia than did fish maintained in the dark. He also demonstrated, however, that brown trout acclimatised to low dissolved oxygen more quickly in the light.

Fish for testing were caught in large numbers from the stock tank and were then collected as randomly as possible. All fish were acclimatised for 7 days to the artifical medium before exposure to metal salts. Fish were placed in the Chamber (A) via one of the open ports with the aid of a wide diameter plastic filter funnel. For the first few minutes the fish were agitated but they soon became settled in the tube. Numbers of fish dead were counted at 24 and 48 hours and these were expressed as a percentage of the whole. Dead fish were removed as soon as possible by using a long wire with a hooked end with which they were manoeuvered under one of the ports where they were removed with forceps.

Due to the very narrow range of concentrations which caused either 0% or 100% kills over a period of 48 hours, the concentrations of metals tested were not arranged on a logarithmic scale. Errors inherent in

reading the 50% mortality concentration from the resulting curves were however very slight as the range of concentrations was so narrow.

At the end of each experiment the apparatus was washed well and then dried out by passing a stream of air through it.

As in earlier experiments the calcium concentration of the stock solution was 80 ppm where the toxicity of copper, zinc and nickel was investigated and 10 ppm for lead.

RESULTS

The collected results are given in table 11 (see appendix)

1. Copper

The range of concentrations tested fell between 0.1 and 0.16 ppm. At 48 hours 0.14 ppm killed all ten fish in the sample but at 0.10 ppm none died. The difference between the 24 and 48 hour L.C. 50's was very slight and the values obtained were:-

24 hr. $L.C._{50}$ = 0.13 (Estimated graphically)

48 hr. $L.C._{50}$ = 0.125 (Estimated graphically)

2. Lead

The range of concentrations tested fell between 1.6 and 2.0 ppm. At 48 hours 2.0 ppm killed all fish but 1.6 ppm killed none.

The 24 and 48 hr. $L.C._{50's}$ were identical at 1.85 ppm and were estimated graphically.

3. Nickel

Even with nickel which was the least toxic of the four metals tested there was a relatively narrow range of concentrations between an all or nothing effect.

7.25 ppm of the metal resulted in a 50% kill at 24 and 48 hours and was thus taken as the $L.C._{50}$ value for both.

4. Zinc

Zinc proved only slightly less toxic than copper and again it was possible to test only a very narrow range of concentration. At 0.18 ppm all fish were killed and at 0.12 ppm none died. The difference

between the 24 and 48 hours $L.C._{50's}$ was again very small and the values obtained were as follows:

24 hours $L.C._{50}$ = 0.16 (From table)

48 hours $L.C._{50}$ = 0.15 (Estimated graphically)

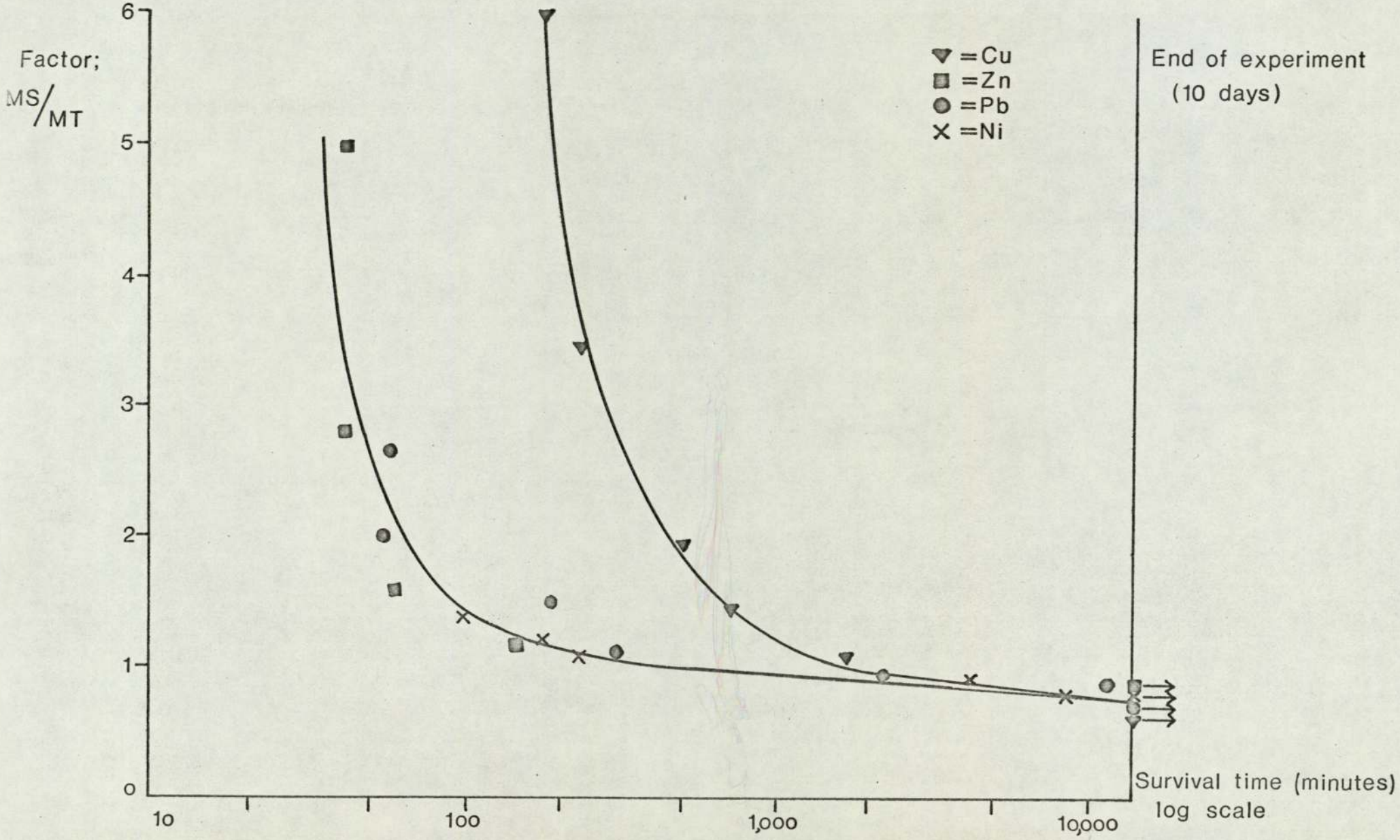
It can be seen from the above results that the effect of acutely toxic concentrations of heavy metals to Phoxinus is relatively rapid and thus the 24 and 48 hour $L.C._{50's}$ are very similar.

Taken together with the results from part 1 of this section it would seem that in acute toxicity the toxic process ceases within a few days at the most and thus that fish dying after prolonged exposure to subacute concentrations of the metals would be succumbing to a different type of damage.

When the 48 hour $L.C._{50}$ values obtained in these experiments were included in the MS/MT data as described at the end of part 1, the resultant plot showed a difference between copper and the other metals. (Fig. 3) The graph demonstrates that minnows react more slowly to lethal levels of copper than to any of the other metals tested, the points for lead, zinc and nickel fitting a similar curve whereas those for copper are situated to the right of the main group. The reasons for this difference are not clear but are discussed in the light of histopathologic observations in Section 7.

Figure 3

Survival of minnows in toxic metal solutions. Metal concentrations expressed as a proportion of their 48 hour $L.C._{50}$ values (i.e. MS/MT).


Factor;
MS/MT
6
5
4
3
2
1
0
▼ =Cu
■ =Zn
● =Pb
× =Ni
End of experiment
(10 days)
10
100
1,000
10,000
Survival time (minutes)
log scale

6. THE ACUTE TOXICITY OF THE METALS IN COMPLEX ORGANIC SOLUTIONS (i.e. IN SEWAGE EFFLUENTS)

1. Field Experiment - Minworth

It is possible that when fish are exposed to toxic metal salts in polluted waters or where there is a high content of organic matter, some complexing of the metals will occur and consequently the lethal limits for the metals will be higher.

A sewage effluent containing heavy metals seemed a reasonable medium in which to expose fish to examine this phenomenon.

The effluent taken from four trickling filter beds at the Minworth Sewage Works of the Upper Tame Main Drainage Authority, was used in a field experiment.

The effluent from the beds was sampled by the Authority via a conduit leading to a pumping house and it was there that an experimental tank was installed. A plastic tank of dimensions 3' x 6" x 8" was used and a flow-through system was arranged tapping from a sampling tube used by the Authority. The tank was filled to a depth of 6" and thus held 21.25 litres. Twenty fish were placed in the tank, thus allowing a little over 1 litre of effluent per fish. There were, however, no detoxification problems as the medium was constantly renewed. The experiment was run for three weeks and the fish were fed daily on commercial dried food. The tank was aerated using a "Hyflo" Major piston pump and four air-stones and this was found sufficient to maintain an adequate supply of dissolved oxygen. Nylon netting grids were fastened over the inflow and outflow tubes and a cover of similar material was fitted to the top of the tank to prevent the escape of fish. The inflow and outflow tubes were installed at the same level. The outflow led into a smaller plastic tank the outflow from which led to a drain. The reason for the small tank was that an automatic liquid sampler (North Hants. Engineering, Type 2) was employed to collect two-hourly samples over the three week period of the experiment and the

tubes leading to it were placed in the smaller tank so that no disturbance or damage to the fish in the main tank occurred. The samples contained twelve glass bottles which were connected separately by a tube to the secondary tank. A partial vacuum was created in all the bottles by means of a hand pump and the vacuum was then broken at two hour intervals by a trip-hammer connected to a rotary clock mechanism. The sampler took twelve samples before it needed attention and was therefore emptied and the bottles and tubes thoroughly washed every morning for the three week test period. The effluent samples were analysed for the four metals in solution using a Perkin-Elmer atomic absorption spectrophotometer at the trade effluent laboratories of the Upper Tame Main Drainage Authority. The treatment of the samples was as follows:

A 1. 100 ml of settled effluent taken

2. 5 ml concentrated nitric acid added

3. Acidified sample evaporated to dryness on a hotplate.

4. Sample cooled to approximately 50°C.

5. 1 ml concentrated hydrochloric acid added plus 10 ml of deionized glass distilled water.

6. Boiled for a few minutes until clear.

7. Made up to 25 ml (i.e. 4 x concentration of the original).

B During the first 24 hours the samples were shaken and thus included all suspended solids which presumably had metal ions adsorbed on to them. The treatment was then the same as described above.

Results

Of the 20 fish exposed to the effluent for three weeks only one died. The results of the analyses for heavy metals are given in Table 12. Other analytical data for the appropriate period are shown in Table 13. (For both tables see appendix)

In the case of both copper and zinc, fish survived for the whole of the test period at concentrations of the metal which in laboratory tests (in inorganic solutions) proved toxic. (See Section 5)

The range of copper concentrations measured during the test period was 0.10 - 0.55 ppm (Mean = 0.24 ppm) whereas the 48 hr $L.C._{50}$ value as determined in laboratory experiments was 0.13 ppm. The range of zinc concentrations measured during the test period was 0.31 - 0.96 ppm (Mean = 0.55 ppm) whereas the 48 hr. LC_{50} value as determined in laboratory experimenting was 0.15 ppm. The concentrations of lead and nickel in solution in the effluent did not approach the 48hr $L.C._{50}$ concentrations determined by experiment.

Where suspended solids were included for analysis the mean copper content measured over the initial 24 hr. period was 1.38 ppm, that for zinc was 5.26 ppm, for nickel 0.69 ppm and for lead 0.27 ppm. The concentrations of nickel and lead were again well below the levels found to be toxic in laboratory experiments. The very large amounts of copper and particularly of zinc found in the suspended solids were apparently not acutely toxic to the fish and it appears that adsorbtion of the metals by organic particulate matter in the effluent is an important agent in reducing toxicity.

The reduction in the toxicity of copper and zinc in solution was presumably due to chelation of the metals by organic compounds in solution in the effluents it may be that chelated metals are not readily available to the fish due to the decreased permeability of the gills to the larger molecules. It is unlikely that increased calcium levels played a part in the reduction in toxicity of the metals as although no measurements were made the calcium content of the effluent was certainly less than the 80 ppm employed in laboratory tests.

An attempt to quantify the reduction in the toxicity of the metals was made with an artificially produced effluent in the laboratory and the results are reported in part 2 of this section.

2. Determination of the 24 and 48 hour $L.C._{50}$ values for the metals when present in an artificially produced sewage effluent.

Evidence for a reduction in the toxicity of the metals when present in an organic effluent has been furnished in part 1 of this section.

In the present experiment an attempt was made to put the reduction in toxicity on a quantitative basis.

When attempting to compare the toxic effects of low concentrations of heavy metals in sewage effluents with their effect in simple solutions the main problems are to supply an effluent which:-

1. Contains a specified amount of the particular metal ion.
2. Does not contain other toxic metals in significant quantities.
3. Does not contain other toxic materials e.g. phenols, cyanide etc., in significant quantities.

In the following experiments the effluent used was artificial in origin as this was necessary to fulfil the three criteria set out above. Another method of preparing an effluent in the laboratory will be described in section 12 but this does not satisfy the second point outlined above.

The effluent used in the present experiments was derived from a synthetic sewage. The sewage medium with added metal was circulated by a magnetic pump of the type described in the previous section and passed down a column containing the requisite microorganisms rather after the fashion of a trickling filter on a sewage works. The stock sewage solution was prepared from a recipe supplied by the Water Pollution Research Laboratories at Stevenage.

Stock sewage solution

13.0 g Beef extract (Oxoid Lab. Lemco Code No. L.30)

13.0 g Nutrient broth (Oxoid Code No. C.M.1)

* 4.27 g

(13.0 g) Dipotassium hydrogen phosphate

13.0 g Glucose

2.5 g Ammonium sulphate

* 4.27 g of added salt gives an equal amount of phosphate

to that in the artificial medium prepared for all other experiments.

The reagents were dissolved in 1 litre of almost boiling distilled water and the cooled solution was stored in the refrigerator in a polythene bottle. The stock solution was diluted 100x with distilled water to produce synthetic sewage.

The apparatus in which the sewage was circulated in the presence of microorganisms so as to produce an effluent is shown in Plate 3. The medium for the attachment of microorganisms was 2 cm. diameter "All-plas" polythene spheres which were housed in the glass column. The apparatus was initially innoculated with the appropriate organisms by circulating a settled "mainly domestic" sewage in it for 7 days until a reasonable growth of film had built up. The sewage used was obtained from the Langley Mill works of the Upper Tame Main Drainage Authority. The synthetic sewage and added metal were then circulated in the apparatus for 24 hours before use. The apparatus was maintained in a cold room and treatment was carried out at $10^{\circ}C \pm 1^{\circ}C$. This temperature was chosen as one at which the growth of the film did not increase at a rate where blockage of the system would occur.

After 24 hours the resultant effluent was diluted 10x with artificial stream water in order to approximate the conditions in a stream receiving an effluent discharge. Due to the tenfold final dilution relatively large amounts of metal salts had to be added to the sewage initially. This did not cause problems in most cases but at some of the higher concentrations of copper (8 - 10 ppm in the sewage) some breakdown of the film occurred, presumably due to the toxic effects of the metal on the microorganisms. When metal containing effluents were not in preparation the system was kept supplied with metal-free sewage. This sewage was renewed every few days otherwise some breakdown of the film occurred due to lack of nutrients.

The diluted effluent plus metal was used for bioassay in the same way as previously described in Section 5 . 2. Ten fish were used for

each test concentration at a temperature of 15°C ± 1°C. The dissolved oxygen concentration was not less than 8 mg/litre as determined by the Winkler technique. No experiments were conducted with lead salts as no fish were available due to the development of ulcerative dermal necrosis amongst stock fish.

Results

The collected results are shown in table 14 (see Appendix)

1. Copper

Copper was the only metal which produced strikingly different results. The 48 hr. $L.C._{50}$ value rose from that of 0.125 ppm in simple solution to 0.65 ppm.

2. Nickel

The 48 hr $L.C._{50}$ for nickel remained the same at 7.25 ppm, although 7.5 ppm did not kill all the sample as in the case of previous experiments.

3. Zinc

The 48 hr $L.C._{50}$ for zinc was a little higher at 0.17 ppm than when tested in simple solution (0.15 ppm).

The reduction in the toxicity of copper compares favourably with that observed in Minworth effluent. It is known that copper readily forms complexes with organic matter in solution in effluents and this must account for the sixfold increase in the 48 hr $L.C._{50}$ concentration when compared to that measured in simple solutions. The very slight reduction in zinc toxicity was probably not significant. Zinc was however found to be considerably less toxic in Minworth effluent and it seems probable that chelating agents were present there which were not supplied in the artificial effluent.

Attempts to quantify any reduction in the toxicity of heavy metals in a particular effluent are of limited value and it seems more important to ascertain why the metals are less toxic under such conditions. In an attempt to answer this question and to investigate any differences

in the pathology of heavy metal toxicity in sewage effluents long term experiments with limited numbers of fish were performed. Exposed fish were examined histopathologically and their heavy metal content investigated. The results of these experiments are reported in Section 12.

7. HISTOPATHOLOGIC STUDIES OF THE GILLS

A total of 24 fish were used in this study; six for each metal tested. Two concentrations of each heavy metal were employed.

Metal	Test Conc.	Survival times (mins)	Test Conc.	Survival times (mins)
Cu	0.2 ppm	590 - 700	0.4 ppm	240 - 340
Pb	2.0 ppm	220 - 350	4.0 ppm	40 - 85
Ni	8.0 ppm	140 - 170	10.0 ppm	70 - 120
Zn	0.2 ppm	60 - 115	0.4 ppm	30 - 70

Tests were conducted in 5 litre all-glass tanks at 15°C ± 1°C. Test solutions were aerated for the duration of each test.

Fish were removed for fixation at the point of immobilisation and at intermediate times before loss of equilibrium occurred. The behaviour of the fish exposed to the metal solutions and the external symptoms of acute heavy metal toxicity will be discussed in part 1.ii of this section.

After removal from the test solution live fish were wrapped in a small tissue, held firmly and the back of the head struck against a hard surface to kill the fish. This had to be done carefully to prevent damage to the gill regions. The four gill rakes were removed from each side of the animal after the operculum had been carefully cut away. The gills were removed with extreme care to prevent bleeding and were handled with fine forceps, gripping only the bone at the base of each rake. The gills were fixed in cold (4°C) Lillies neutral buffered formalin for twelve hours or overnight (McManus and Mowry P.19)

40% Formalin (Commercial)	100 ml
Distilled water	900 ml

Sodium Acid Phosphate
Monohydrate ($Na\ H_2\ PO_4 .\ H_2O$) 4.0 grms

Disodium phosphate, Anhydrous
($Na_2H\ PO_4$) 6.5 grms.

The use of this fixative gave dependable protection against the acidity of formalin and prevented the production of formalin pigment in the sections.

After fixation tissues were washed for 3 - 4 hours in 3 changes of 70% ethanol, then for 2 hours in three changes of 90% ethanol, for one hour in three changes of 95% ethanol and thence to a number of changes of absolute ethanol. The relatively short time employed for dehydration was feasible due to the small size and delicate nature of the gills. Tissues were then cleared in 3 changes of xylene for 1 hour and then infiltrated and embedded in 2 changes of paraffin wax (Melting point 54°C).

Decalcification was not practised as it was found to be easy to cut sections without it and the use of decalcifying fluids such as formic acid may have had a detrimental effect on the delicate structure of the secondary lamellae.

Sections were cut at 5 - 7 microns (the primary lamellae being cut longitudinally across the secondary lamellae), and stained with Ehrlich's haematoxylin and eosin.

Structure of the gills.

The gills of the minnow are essentially similar in structure to those of trout which have been described by Brown, Mitrovic and Stark (1968). They consist of four arches on either side of the fish, each arch bearing two adjacent rows of flattened gill filaments (primary lamellae) The primary lamellae bear on both surfaces a number of thin parallel folds, the secondary lamellae. Towards the tip of each primary lamella the secondary lamellae are lower but their structure appears the same. The epithelium of the secondary lamellae is very low and consists of virtually a single layer of cells in the form of a pavement epithelium. The endothelial tissue of the secondary lamellae consists of connective tissue cells (pilaster cells) which assist in the support of the lamellae

and form the walls of the lacunae along which erythrocytes pass. (See Plates 4 and 5) and figure 4.

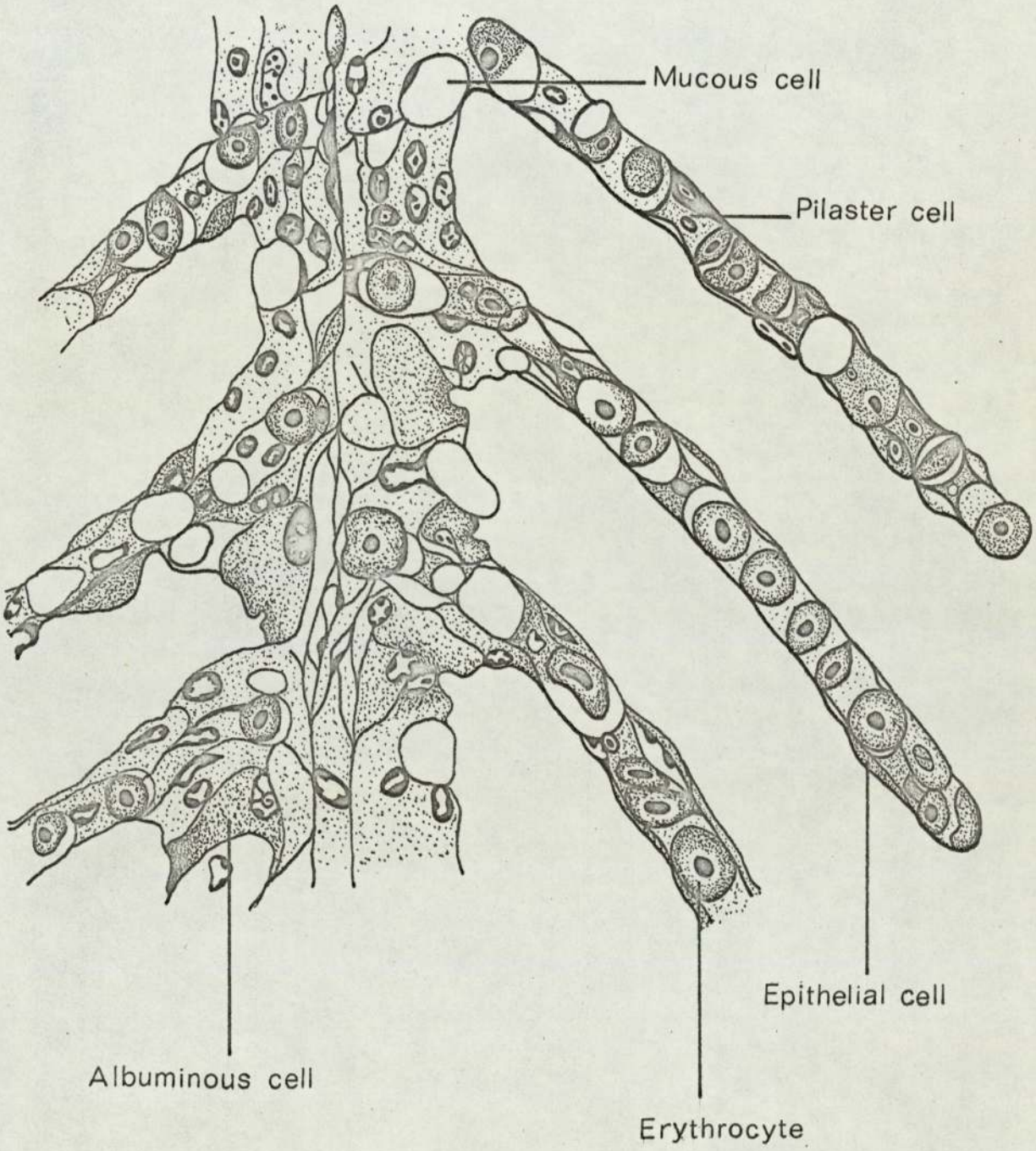
Between the bases of the secondary lamellae the epithelium is much thicker and contains secretory cells, albumen cells and possibly the so called "chloride cells" which are concerned with salt balance (Keys and Willmer, 1932).

Results

The effect of acutely toxic concentrations of the four metals was essentially similar. In the early stages mucous cell discharge was evident in most cases and especially with zinc. Lead and nickel exposed animals did not exhibit such a rapid mucus response either histologically or externally. Copper produced least mucus response and this is in agreement with the observations of Cusick (1968) (See section 3. 2.) No special methods were used to study mucous cells (e.g. P.A.S. technique) but discharged mucous cells were clearly visible. The second response of the gills was the development of oedema of the epithelium of the secondary lamellae and also amongst the cell masses between them. (See plates 6 and 7). The respiratory epithelium covering the secondary lamellae became very swollen but remained attached to the underlying endothelium. As exposure continued haematomas were observed (See plate 8). The thickening of the cell mass between the secondary lamellae seemed to be entirely due to oedema and not to cell proliferation. In all cases the final stages of gill damage were the same. The swollen epithelium became disorganised and began to separate from the endothelium. The sloughing of the epithelium appeared to start at the tips of the secondary lamellae and to continue towards the base (See Plate 9). The respiratory epithelium together with the swollen mass of mucous and glandular cells from between the lamellae eventually became completely detached and occluded the interlamellar spaces. In many cases the secondary lamellae were collapsed at this stage presumably due to the loss of vascular function which assists in the maintainance of rigidity in the

Figure 4

Structure of the gills of Phoxinus (primary lamellae cut longitudinally across the secondary lamellae), partly after Schöttle.


Mucous cell
Pilaster cell
Epithelial cell
Albuminous cell
Erythrocyte

gills. See Plate 10). The naked secondary lamellae were often coalesced. Although most of the pilaster cells appeared intact fusion of the processes of some of them was noted. As previously mentioned (Section 3 . 2) Skidmore (1970) has pointed out that the destruction of the respiratory epithelium might not cause death through breakdown of gill function. Indeed, the removal of the respiratory epithelium places the gill capillaries in closer contact with the external environment! Skidmore however did demonstrate that death results from tissue hypoxia and presumably that the less of the respiratory epithelium reduced the permeability of the gills to oxygen. It is considered that in the present investigation the collapse of the secondary lamellae together with the loss of the respiratory epithelium and occlusion of the interlamellar spaces with cell debris would be sufficient to account for the death of the fish.

At the point of immobilisation the respiratory epithelium was sloughed off from approximately one third of the total gill surface although intermediate stages of damage were evident on parts of the remainder. The extent of sloughing of the respiratory epithelium may determine the rate of survival of different species of fish in heavy metal solutions, those which survive longer being able to withstand a greater loss of epithelium.

Brown, Mitrovic and Stark (1968) pointed out that studies on the histopathology of gills in acute toxicity necessitate the observation of large areas of tissue. The reason for this is that dmaage is not uniformly distributed over the whole of the respiratory surface. Some gill arches are relatively undamaged whereas the damage to adjacent ones is severe. Adjacent primary lamellae may even exhibit great differences in the extent of damage, (See Plate 11) primarly lamellae with naked capillaries lying next to relatively undamaged ones.

A summary of the treatment and results obtained from the 24

fish is given in table 1.

In this part of the present study only the gills were examined. Attempts were also made to histochemically locate heavy metals in the gills and these will be discussed in Section 10.

Conclusions

The pattern of gill damage seemed to be similar for all metals tested and was in agreement with the results of other workers in most respects. The production of large amounts of mucus associated with acute zinc toxicity is however different from the observations of Lloyd (1960) who found no precipitated mucus on the gills of rainbow trout killed by zinc sulphate.

The overall response to heavy metals seemed inflammatory causing oedema of the respiratory epithelium and consequent breakdown of the cells. Mucus secretion was considered to be a secondary contributor to the cause of death. In the case of the minnow it would seem that a loss of well under half of the respiratory epithelium proves fatal. The overall effect seemed to be non-specific so far as the different heavy metals were concerned and because of this a series of short experiments were conducted with three other toxicants to see if they produced a similar pattern of gill damage. The methods and results of these experiments will be described in part 2 of this section.

(ii) The behaviour and external symptoms of minnows in acute heavy metal toxicity.

During exposure of the fish to the toxic solutions their behaviour was closely followed. A summary of the observations for each of the metals is given below.

Copper 0.2 ppm

Immediately upon introduction to the toxic solution the fish showed signs of distress. These did not parallel the typical signs of stress which occur when fish are netted and transfered from one tank to another. On transference to the copper solution the fish darted

about the tank and then hung motionless for a moment, tail downwards, in the body of the water. After 30 minutes or so these signs of distress would lessen but during the first 8 - 10 hours of exposure the pattern would set in again from time to time and occasionally the fish would jump clear of the water. During the whole of this period the fins were maintained in an erect position and no mucus secretion either in the gill chamber or over the body surface was observed. For the whole of the exposure period except for the time approaching the point of overturning the fish appeared very excited and almost stimulated. Between 8 and 10 hours after the initial exposure a slightly granular brownish-white mucus secretion appeared on the pelvic fins and around the operculum and mouth. At about this time the fish began swimming very rapidly and repeatedly from the top to the bottom of the tank. The rate of opercular movement increased enormously at this time although no quantitative measurements were made. Soon after the rapid increase in opercular rate the fish began to lose equilibrium and to roll over. At the same time the fins began to collapse (dorsal fin first) and a small amount of stringy white mucus was usually visible hanging from the ventral surface. After the initial loss of equilibrium the fish appeared to recover temporarily and to hang at the surface of the water gulping air. The time taken to immobilisation after the initial overturn was between 60 - 90 minutes. During this time bleeding around the mouth and at the bases of the fins was seen. The oral haemorrhage was particularly noticeable in the case of copper. In the latter stages the edges of the fins became lined with white mucus.

At the terminal stage the bleeding of the fin bases and mouth was very pronounced (much more than with the other metals). Within the last 20 - 30 minutes before death the fish seemed to lose control of their chromatophores, expanded patches appearing at random on the sides of the body. In the last few minutes before death dark areas appeared on top of the head and below and between the opercula in all cases.

TABLE 1

Fish No.	Metal	Conc ppm.	Time taken and condition.	Appearance of gill on removal.	Histopathologic condition of gills.
1	Zn	0.2	Alive 60 mins.	Patchy white mucus cover - gills red.	Some oedema - no sloughing.
2	Zn	0.2	+ 77 mins.	Thick white mucus cover - gills pale	< 1/3 of epithelium sloughed off.
3	Zn	0.2	+ 84 mins.	As 2 " "	As 2 " "
4	Zn	0.4	Alive 30 mins.	As 1	As 1
5	Zn	0.4	+ 60 mins.	As 2 and 3	As 2 and 3
6	Zn	0.4	+ 65 mins.	As 2 and 3	As 2 and 3
7	Cu	0.2	Alive 550 mins.	No mucus evident - gills red	Very slight oedema
8	Cu	0.2	+ 610 mins	Small amount of brownish mucus - gills pale	½ epithelium sloughed off.
9	Cu	0.2	+ 700 mins	More mucus than 8 - gills pale	As 8
10	Cu	0.4	Alive 158	No mucus evident - gills red	Oedema noticeable
11	Cu	0.4	+ 24 mins	As 8	> ½ epithelium sloughed off.
12	Cu	0.4	+ 340 mins	More mucus than 11	½ epithelium sloughed off.
13	P6	2.0	Alive 190 mins.	No mucus evident - gills red	Slight oedema
14	P6	2.0	+235 mins.	Gills with white mucus but less than with zinc - gills pale.	½ epithelium sloughed off
15	P6	2.0	+350 mins.	As 14	As 14
16	P6	4.0	Alive 20 mins.	A few white patches of mucus - gills red	Oedema present and some sloughing of respiratory epithelium
17	P6	4.0	+ 45 mins.	As 14 and 15	As 14 and 15
18	P6	4.0	+ 85 mins.	Considerably more mucus than 17 - equivalent to zinc	As 14 and 15
19	Ni	8.0	Alive 90 mins.	No evident mucus - gills red	Very little oedema

TABLE 1 (continued)

Fish No.	Metal	Conc. ppm.	Time taken and condition	Appearance of gill on removal.	Histopathologic condition of gills.
20	Ni	8.0	+ 140 mins	White mucus present < P6	$\frac{1}{3}$ Epithelium sloughed off.
21	Ni	8.0	+ 170 mins	As 20	As 20
22	Ni	10.0	Alive 45 mins.	Slight mucus precipitate only	Slight oedema
23	Ni	10.0	+ 70	As 20 and 21	As 20 and 21
24	Ni	10.0	+ 110	As 20 and 21	As 20 and 21

A reliable diagnostic feature of imminent death was the fact that the pectoral fins became motionless and stuck out at an angle of ninety degrees to the body. After opercular movements had ceased for up to 90 seconds the fish still exhibited violent muscular spasms sufficiently strong to lift them clear of the water. These spasms could be induced by agitating the water or by prodding the animal with a glass rod. The spasms ceased at about 2 minutes after the cessation of opercular movement. At death the mouth was open to its widest extent. On examination of the gills on their removal relatively little mucus was seen and that which did occur appeared brownish and not white as was the case with the other metals.

Lead 2.0 ppm

Little immediate reaction was observed when the fish were placed in the toxic solution. After 35 minutes however the fish became very passive, lying on the bottom of the tank and exhibiting very irregular opercular movements. At this time they also seemed to lose equilibrium, rolling slightly from side to side. This behaviour contrasted strongly with that in copper solutions where the fish were very active for the greater part of the exposure time. After 45 minutes one fish was lying completely on its side breathing slowly and irregularly and them swimming off very slowly in a rather serpentine manner. No mucus secretions were noted at this stage and none appeared until 120 minutes after the initial exposure when a fine white mucus lining was noted around the edge of the fins. At 160 minutes, the fish became agitated, mucus secretion became more evident and appeared as a bluish-white "bloom" over the whole body surface. The fish exhibited a series of rapid wriggling movements in the body of the water and then fell tail downwards to the bottom of the tank. At this time slight bleeding of the fin bases was noted. At 200 minutes the fins were all collapsed and strings of mucus appeared all over the body surface. This behaviour continued for about another two hours after

which time opercular movements became very irregular and finally ceased. The first stages were the same as those described for copper involving loss of chromatophore control, violent spasms and immobilisation of the pectoral fins.

The main difference in the course of events leading up to death between copper and lead was that with acute lead toxicity the initial loss of equilibrium occurred well before death (after only 1/10 of the total exposure time) where with copper the fish did not overturn until much nearer the point of immobilisation. Haemorrhage was very much less advanced after lead treatment but mucus secretion was far greater over the body surface and the gills on removal were found to be covered with a noticeable mucus "bloom".

Nickel 8.0 ppm.

The initial stages of exposure to nickel resulted in symptoms similar to those observed in lead solutions, in that shortly after exposure to the toxic solution the fish became very quiescent and began to lose equilibrium. Whitish mucus appeared on the underbelly and below the mouth after 40 - 50 minutes. After one hour there was apparent loss of co-ordination of swimming movements when the fish began to exhibit typical serpentine motions. At this time breathing became very irregular and the fins of all fish were collapsed. At 70 - 75 minutes haemorrhaging of the fin bases appeared and was more extensive than that caused by lead solutions. Mucus strands were seen trailing from the gill chamber and soon complete loss of equilibrium occurred. The situation then remained the same until about 160 minutes after the initial exposure when haemorrhage increased noticeably around the mouth and the fish became very weak. All fish attempted to gulp air from the surface and were also seen to spin slowly around in a perpendicular position at the water surface. The final stages of poisoning at about 170 minutes resembled those of copper and lead. More bleeding was noticeable than

with lead but the secretion of mucus was a little less pronounced.

Zinc 0.2 ppm

Immediately upon transference to zinc solutions the fish became very distressed. After only 20 minutes the fins began to collapse and very pronounced mucus section was seen on the underbelly and around the mouth. After 40 minutes the mucus secretion was even more pronounced and appeared upon the fins and around the mouth and operculum as well as a general well defined "bloom" over the body surface. Equilibrium was lost at about this time. After 1 hour all fish were extremely weak and exhibited the typical serpentine swimming motion characteristic of heavy metal poisoning. Immobilisation occurred at between 70 and 90 minutes and resembled that where other metals were tested. There was, however, very little haemorrhage noticeable in the case of zinc but mucus secretion was intense and the gills upon removal were thickly covered in grey-white mucus.

A summary of the external symptoms of acute heavy metal poisoning are given in the following table.

	Haemorrhage	Mucus Secretion	Loss of Chromatophore co-ordination
Cu	Intense	Slight	Intense
Pb	Slight	Moderate	Moderate
Ni	Slight (more than Pb.)	Moderate	Moderate
Zn	V. slight	Intense	Slight

Conclusions

The symptoms exhibited under the influence of all four metals were basically similar. Death appeared to be due to asphyxiation. Mucus secretion was considered to play a greater part in the toxicity

of zinc than in the case of the other metals and especially copper. However the more rapid response to zinc toxicity compared with the slightly more toxic copper was presumably not due to mucus secretion as sloughing of the respiratory epithelium occurred more quickly in zinc solutions than in copper. No explanation is offered for the intense haemorrhage caused by copper but the increase in activity and the very noticeable loss of control of chromatophores suggests a possible neurotoxic effect for the metal.

2. Histopathology of the gills after exposure to acutely toxic concentrations of other toxicants.

The following short experiment was performed in order to assess whether the histopathologic effects of heavy metals upon minnow gills are specific or whether other toxicants exert similar effects.

In all cases experimental conditions including processing of tissues were the same as those described in part 1 of this section.

The three toxic materials chosen were as follows:-

(i) Alkylbenzenesulphonate detergent, supplied at a concentration of 10 ppm.

(ii) Phenol (carbolic acid) supplied at a concentration of 18 ppm.

(iii) A solution of pH4 was prepared by adding concentrated sulphuric acid to the artifical stock solution. pH was measured using a standard instrument.

The concentrations of toxicants were chosen as those giving suitable survival times as a result of preliminary experiments.

(i) A.B.S. - detergent

Three fish were exposed to 10 ppm solution. One fish was removed alive after 45 minutes exposure and the other two at immobilisation (140 and 145 minutes respectively).

Results

Gill damage was essentially similar to that caused by heavy

metals in that the respiratory epithelium was swollen and sloughed off in parts. The sloughing of the epithelium however appeared to commence at the bases of the secondary lamellae and proceed upwards, which was the reverse of the pattern observed with heavy metal poisoning. The oedema also seemed less marked. The extent of damage over the whole of the gill surface was more uniform than with metals and adjacent primary lamellae exhibited similar degrees of damage. (See Plate 12) The pilaster cells always appeared intact and little or no haemorrhage was observed. Damage was only slightly more severe at the tips of the primary lamellae than at the bases.

An interesting phenomenon was that the time between the point of overturning (in fact the first noticeable symptom) and the point of immobilisation was extremely short, being between 5 - 10 minutes. Mucus secretion was evident upon the body of the fish and parallelled that where nickel and lead toxicity were studied.

(ii) Phenol (Carbolic Acid)

Three fish were exposed to 18 ppm of phenol. One fish was removed alive after 50 minutes and the other two at immobilisation (170 and 185 minutes respectively).

Results.

The gill damage observed was quite different from that which was seen in acute heavy metal toxicity. No swelling (oedema) or sloughing of the respiratory epithelium was observed and the overall picture was one of erosion of the tissues. (See Plate 13). The interlamellar cell masses remained in place but were obviously damaged and exhibited a "fuzzy" appearance. The respiratory epithelium was not sloughed off but was broken at many points as was the endothelium. Erythrocytes were seen in the interlamellar spaces and separated pilaster cells were obvious with escaped erythrocytes around them. Little mucus secretion was seen upon the body of the fish or upon the gills and the fins of the fish remained erect throughout the whole exposure. Some

bleeding at the bases of the fins and around the mouth was noted.

(iii) pH.4.

Three fish were exposed to artifical stock medium in which the pH had been reduced to 4. with sulphuric acid. One fish was removed alive at 30 minutes and the other two at immobilisation (83 and 95 minutes respectively).

Results.

At death gill damage was found to be widespread and severe affecting 75% of the respiratory surface (See Plate 14). The respiratory epithelium and the interlamellar cell masses were sloughed off and cell debris occluded the spaces between the lamellae. Oedema however was less marked than in the case of heavy metals. The tips of the primary lamellae were noticeably more damaged than the bases. The pilaster cells were more damaged than with detergent or heavy metals and a noticeable amount of adhesion had occurred. Some haemorrhage was also noted. The sloughing of the respiratory epithelium resembled that occurring under the influence of A.B.S. detergent more than heavy metals. Mucus secretion was very obvious on the body of the fish and as a covering over the gills. The interval between initial overturn and immobilisation was again very short being between 15 and 20 minutes.

Conclusions.

A.B.S. detergent and pH4 produced histopathologic effects essentially similar to those produced by the four metals tested. Phenol however exerted a much less subtle effect and appeared to simply erode the tissues causing a non specific breakdown of all the soft parts of the gills. This is in some disagreement with the results of Mitrovic et al (1968) where the acute toxicity of phenol to rainbow trout resulted in sloughing of the respiratory epithelium.

Overall it seems that heavy metals, detergent and low pH exert a more or less non-specific action upon the gills of the minnow at acutely toxic concentrations. No satisfactory explanation is available as to the straightforward "caustic" effect of phenol.

Plate 3

Apparatus for the production of a metal-containing effluent from synthetic sewage. The growth of bacterial film etc. can be seen on the medium in the glass tube.

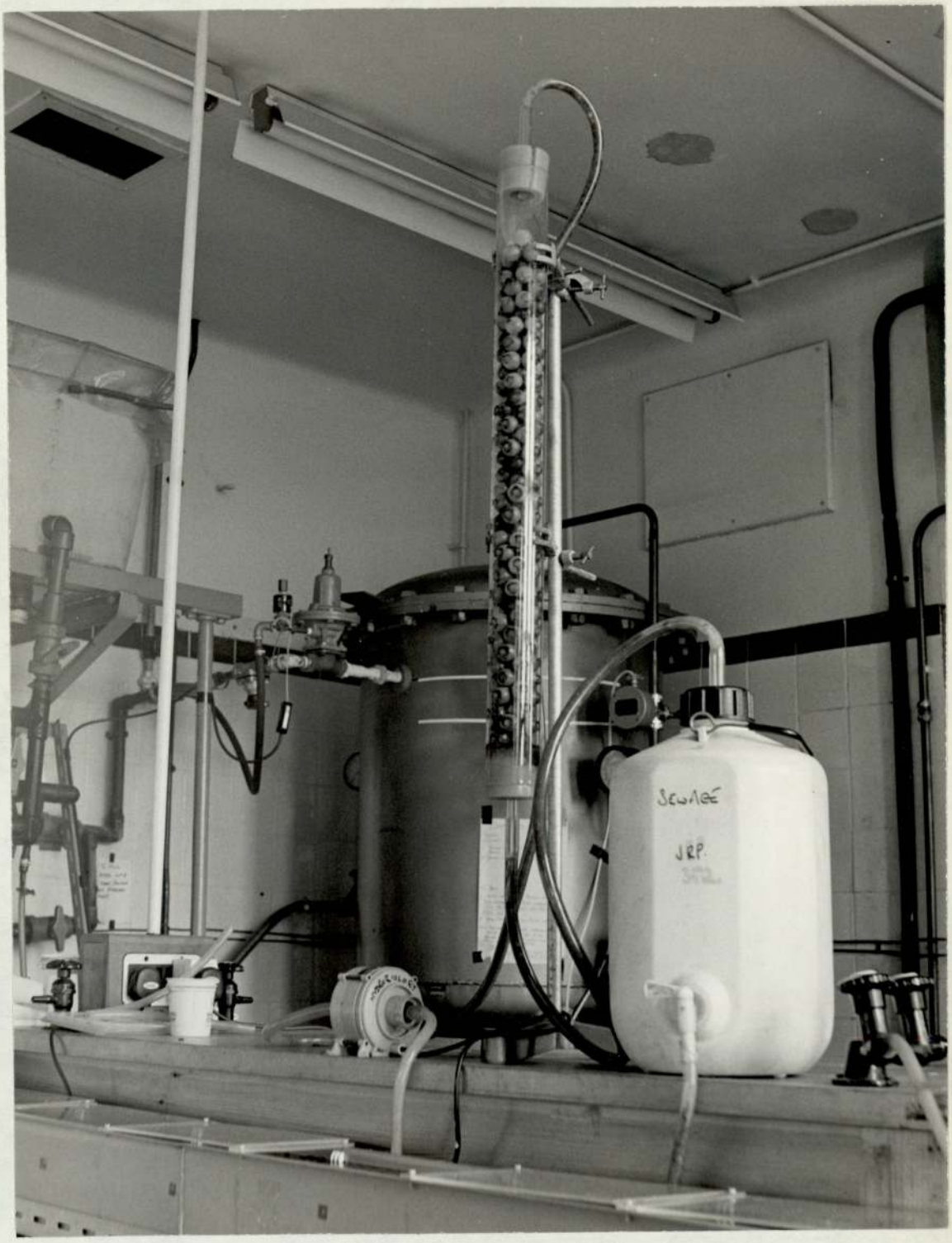

SEWAGE
JRP

Plate 4.

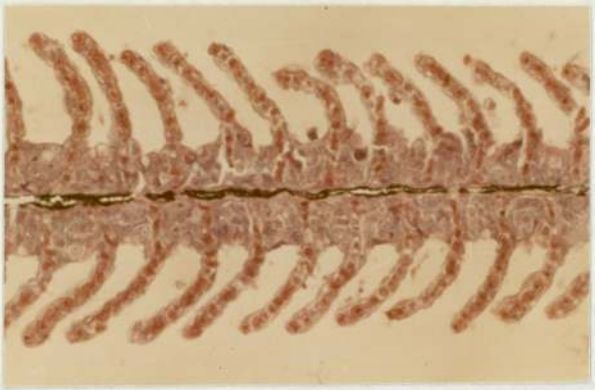
7 microns gill section (Buffered formalin fixed, stained in haematoxylin and eosin) from a control fish. (X230)

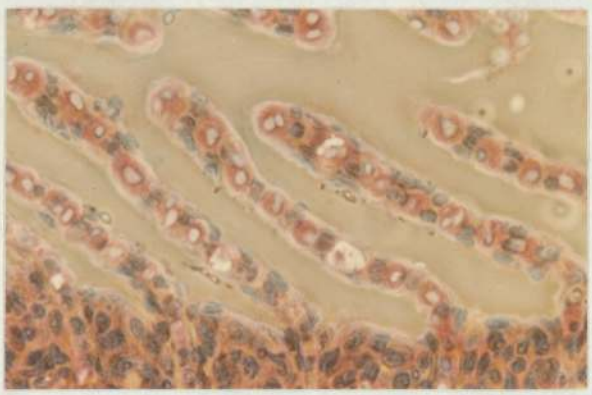
Plate 5

As above, but phase contrast (X 590)

Plate 6

Gills of a minnow taken alive from a 0.4ppm copper solution after 158 minutes exposure. (X 230)





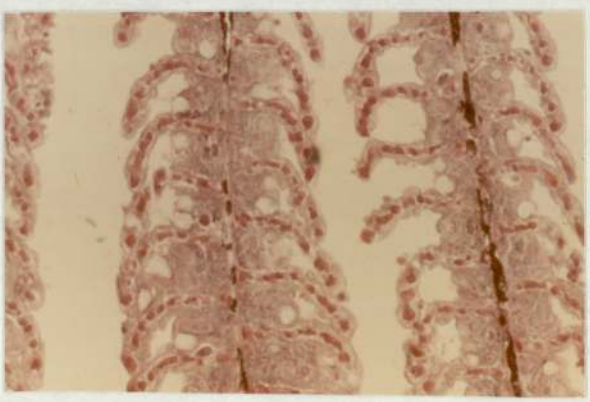


Plate 7

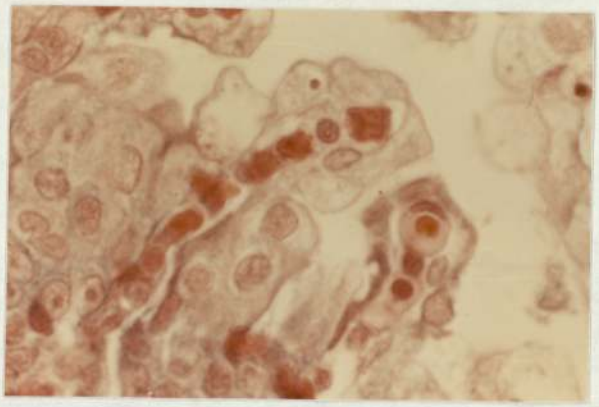
Later stage of acute copper poisoning (Cu = 0.4 ppm)(x920). Note the extreme swelling of the respiratory epithelium (compare with plate 5) and the apparent oedema of the cells between the secondary lamellae.

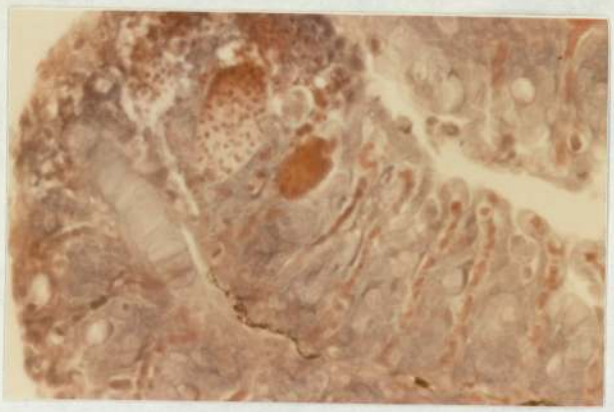
Plate 8

Primary lamella of the gills of a minnow taken alive from 0.4 ppm copper after 158 minutes exposure showing a haematoma and erosion of the tip. (X 300)

Plate 9

Secondary lamellae exhibiting sloughing of the respiratory epithelium. (X 920). The fish was taken dead from a 0.4 ppm solution of copper after 240 minutes exposure. Note that the sloughing of the respiratory epithelium is proceeding from the tips of the secondary lamellae towards their bases.





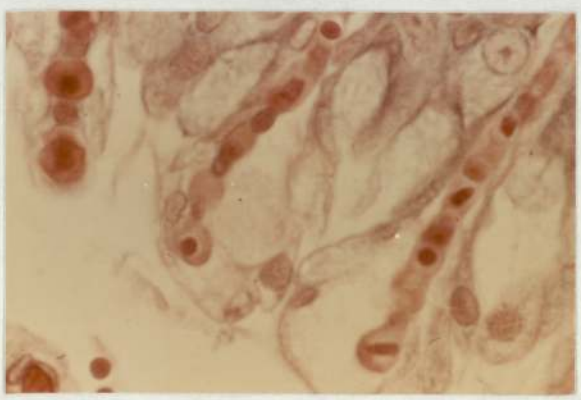


Plate 10

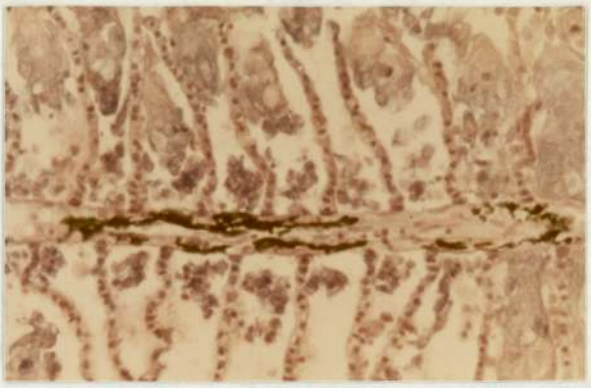
Primary lamella of a fish taken dead from a 0.2 ppm solution of zinc after 84 minutes. (X 300). Note the loss of both the respiratory epithelium and the interlamellar cells. The secondary lamellae are collapsed in many places and the processes of the pilaster cells contracted.

Plate 11

Gills of a fish taken dead from a 2.0 ppm solution of lead after 350 minutes exposure. (X 115). Note the great difference in the damage suffered by adjacent primary lamellae.

Plate 12

Gills of a fish taken dead from a 10 ppm solution of alkylbenzene sulphonate detergent after 140 minutes exposure. (X 230). The damage is similar to that caused by heavy metals.(See plate 10).



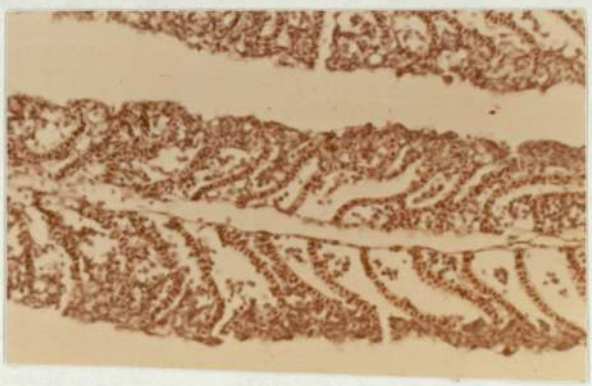
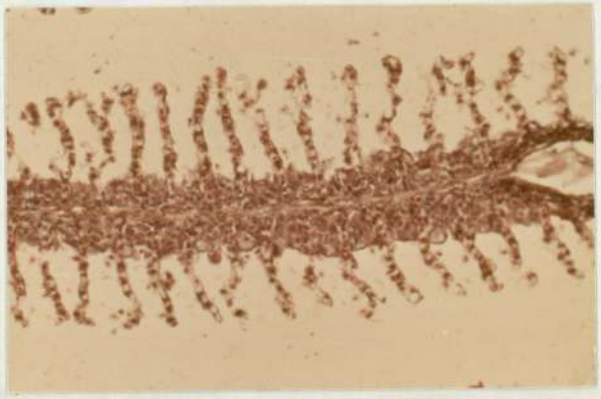


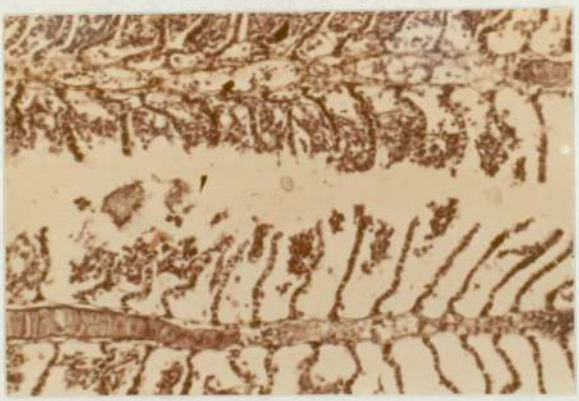
Plate 13

Primary lamella of a fish taken dead from an 18 ppm solution of phenol after 185 minutes exposure (X 230). The damage is dissimilar to that caused by heavy metals. The inter-lamellar cells remain in place and the respiratory epithelium appears simply eroded. Note the complete breakdown of some of the secondary lamellae.

Plate 14

Gills of a fish taken dead from water at pH 4 after 95 minutes exposure. (X 230). The damage is similar to that caused by heavy metals. (See plate 10).





8. GRAVIMETRIC ANALYSES FOR HEAVY METALS IN ACUTE TOXICITY.

The aim of the following experiment was to determine uptake and accumulation of the four metals by the gills during acute exposures.

Uptake by the whole gill was compared with a bone sample (opercular bone) and expressed as a ratio after the fashion of Mount (1964) (See Section 3.2). The observed ratios were then compared with those of control fish and with those observed for subacute exposures (See Section 11). In the present section only gill and opercular bone samples were analysed but in the experiments in Section 11 where chronic toxicity was studied liver and kidneys were also examined.

1. Experimental Exposures

Exposures to toxic metals solutions were performed in 5 litre all-glass tanks under the conditions already described in Sections 4 and 7. Control fish were taken only from stock which had been maintained in the laboratory in the artifical stock medium for at least three weeks.

2. Preparation of Tissue Samples.

Due to the small size of the minnow pooled samples of at least three fish were used to prepare each tissue extract. Gill and opercular bone samples were removed and placed in preweighed porcelain crucibles. All crucibles and glass-ware used in the analyses were washed in a non-ionic detergent, rinsed thoroughly in tap water, soaked in 1:1 nitric acid for at least twelve hours and then rinsed exhaustively in deionized glass-distilled water.

Opercular bone samples (with associated skin) were rinsed in dilute nitric acid from a wash-bottle. This follows the recommendation of Mount (1964) (Mount however used a small toothbrush dipped in nitric acid) in that the removal of loose mucus from the gills and skin is essential because heavy metals combine with the mucus and if not removed would impair the accuracy of the zinc gill/zinc bone ratios.

Gill arches were removed by cutting as close to the first primary lamella as possible so as not to include the large amounts of bone and

cartilage found at the ends of the arches. The gill samples were not acid-washed before being placed in the crucibles.

Wet weights of samples were not recorded as due to the small size of the samples rapid dehydration occurred immediately after removal and satisfactory accuracy was not attainable.

Samples were dried for 48 hours in the crucibles at 105°C. They were then transferred quickly to a desiccator until cool enough to handle and then weighed upon a balance containing a tray of silica gel desiccant. Provided the silica gel was kept fresh no increase in weight due to rehydration was noted in the time taken to weigh the crucible and sample.

Dry weights of gill samples varied between 7.5 and 18 mg and of bone between 10 and 24 mg.

The weighed, dried samples were then ashed at 520°C ± 10°C for 14 - 16 hours or until a completely white ash was obtained. Each ashed sample was then dissolved in 0.15 ml of normal hydrochloric acid (AnalaR) after the method of Hu and Friede (1968), and then heated to dryness to remove excess hydrochloric acid. This reaction converted the alkali phosphate to biphosphate and alkali chloride. 0.03 ml of normal hydrochloric acid was then added to each sample and the whole content was repeatedly washed with deionized glass-distilled water into a 5 ml volumetric flask and made up to the mark. Samples were only dissolved in acid and transferred to volumetric flasks immediately prior to analysis as storage in glass vessels for extended periods can cause absorption of some of the metal ions on to the glass thus affecting the accuracy of the results.

Heavy metal analyses were performed using a Unicam S.P.900A atomic absorption spectrophotometer. Standard solutions of the heavy metals were prepared from 100 ppm stock solutions. The stock solutions were prepared by dissolving 0.1 g of the appropriate AnalaR metal in the minimum amount of nitric or hydrochloric acid and then making the solution up to 1 litre in a volumetric flask. Stock solutions were stored

in polythene bottles. Standard solutions were freshly prepared immediately before each analysis. A range of standards was prepared as follows:-

Copper and Zinc

0.1, 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 ppm

Lead

1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 ppm

Nickel

0.5, 1.0, 1.5, 2.0, 2.5 ppm

5 ml of sample was found to be more than adequate for each determination and for control samples two and sometimes three determinations could be made from one 5 ml aliquot. The sensitivity to copper and zinc of the instrument was good the limits being in the region of 0.05 ppm. The detection limits for nickel and lead were not as satisfactory being 0.5ppm and 1 ppm respectively. Due to difficulties in the analyses for lead six fish were pooled to produce each sample.

All heavy metal concentrations in tissues were expressed as ppm metal per dry weight of tissue.

3. Results.

(i) Copper

The results for copper fish are shown in table 15 (see Appendix).

A total of 27 fish representing 9 samples of both whole gill and bone were analysed. The average copper content of the gills was 62.6 ppm (Range 54.3 - 71) and the average copper content of the bone samples was 39.4 ppm. (Range 23.7 - 49.5). The gills always contained larger amounts of copper than the bone samples and the average copper in gill/copper in bone ratio was 1.64 (Range 1.1 - 2.45).

Experimental exposures of fish were made at 0.2, 0.3, 0.5 and 0.75 ppm of copper. Three fish were tested at each concentration. Survival times varied from 600 minutes at 0.2 ppm to 95 minutes at 0.75 ppm.

The maximum uptake of copper by the gills was observed at the

highest concentration tested (0.75 ppm) and was 152.4 ppm (approximately 2.5 x the average control level). The gill/bone ratio in this case rose to 2.21. Only at 0.3 ppm and 0.75 ppm did the ratio appear greater than the control average (2.12) and in both cases the ratios fell within the range of controls. The very limited rise in the gill/bone ratio is considered to have been due to insufficient removal of copper bearing mucus from the opercular samples thus lowering the ratio. The bone sample at 0.75 ppm copper contained 1.75 x more copper than the average control figure and it is probable that most of this increase was due to copper combined with mucus. If adequate removal of copper had been achieved the gill/bone ratio may have been in the region of 3.2 (taking the average figure for control bone samples). Unfortunately no fish were available to repeat this experiment which was carried out at the end of the project.

The results described above will be further considered in Section 11 in the light of results obtained for long term subacute exposures.

(ii) Zinc

The results for zinc are shown in table 16 (see appendix).

The 27 fish used as controls were the same as those used for copper.

In control fish the average zinc content of the whole gill was 366 ppm (Range 280 - 401) and that for the bone samples was 409 ppm (Range 382 - 425). In most cases the bone sample contained more zinc than the gills and the average gill/bone ratio was 0.986. In two cases however there was more zinc in the gills (401/394 ppm and 382/370 ppm respectively).

When compared with copper both gill and bone samples contained much higer concentrations of zinc and when gill sections were stained for zinc (see Section 10) the test revealed that most of the zinc was in the gill arch and not in the soft parts. This was in agreement with the observations of Mount (1964).

Experimental exposures of fish to zinc were made at 0.2, 0.3, 0.5 and 0.75 ppm. Three fish were tested at each concentration. Survival times varied from 85 minutes at 0.2 ppm to 25 minutes at 0.75 ppm.

The maximum accumulation of zinc by the gills was noted at 0.3 ppm and was 818 ppm. (Average control level = 366 ppm). This represented an increase of 2.24 x. This increase is very large when the high control level is considered.

In the case of zinc considerable care was taken with the nitric acid rinse of the opercular bone sample in view of the difficulties encountered with copper. As a result the average increase in the zinc concentration of the bone was only 1.08 x that of the controls.

The average gill/bone ratio for all lethal exposures was 1.6 (Range 1.27 - 1.84) as compared with 0.896 for controls. Thus, provided adequate care is taken with the removal of zinc bearing mucus from the skin surrounding the bone sample, the increase in the ratio seems to be a reliable indicator of acute zinc toxicity.

(iii) Copper-zinc mixture.

As toxic metals rarely occur singly in polluted waters it was decided to test a mixture of copper and zinc. Only three fish were available and so only one exposure was made. The fish were exposed to a solution containing 0.1 ppm of copper and 0.1 ppm of zinc. Taken separately minnows would survive indefinitely in these concentrations but when added together they represent a value greater than the lethal limit for both.

Results.

The three fish exposed to the mixture survived for 295, 345 and 385 minutes respectively. Analytical results are included in tables 15 and 16 (see Appendix).

Copper

The concentration of copper measured in the whole gill was 75.25 ppm and this probably represents a slight increase over the control level (Ave.

Control level = 62.6, Range = 54 - 71). The bone samples were rinsed exhaustively in dilute nitric acid but still exhibited a copper concentration higher than the controls. The resultant gill/bone ratio was in fact 1.41 which was less than the average control value of 1.64.

Zinc.

The concentration of zinc in the whole gill was measured as 526 ppm which represented a 1.44 x increase over the average control figure. The bone sample was almost within the range of control levels. The resultant gill/bone ratio of 1.09 represented only a slight increase over the control level of 0.896 but was outside the range of controls.

It is obviously not possible to form any valid conclusions as a result of one experiment of this type. However the zinc concentration of the gills increased with respect to the controls to a greater extent than did copper and it seems possible that zinc was contributing more toxic effect.

(iv) Lead

A total of 12 fish representing 2 groups of 3 and one of 6 were taken as controls for lead analyses.

Results

All control gill and bone lead determinations attempted were negative even where six fish were pooled.

Exposures of fish to lead were made a 2, 4 and 6 ppm and 5 fish were tested at each concentration. Survival times varied from 285 mins at 2 ppm to 45 mins at 6 ppm. The amount of lead accumulated by the gills increased with the increase of lead in the toxic solution and the highest measured level of lead in the gills was 404 ppm at 6 ppm external concentration. Bone samples exhibited lead concentrations of between 72 and 88 ppm. These concentrations were not considered to represent true levels of lead in bone but were due to inadequate removal of lead bearing mucus. The results of the analyses are given in table 17 (See Appendix).

(e) Nickel

A total of 12 control fish representing 2 groups of 6 were analysed for nickel content and were negative in all cases.

Results

Experimental exposures of fish to nickel were made at 6, 8 and 10 ppm and 5 fish were tested at each concentration. Survival times varied from 75 hours at 6 ppm to 65 mins at 10 ppm.

The gills were found to accumulate the metal and the maximum uptake was measured after lethal exposure to 8.0 ppm of the metal (93 ppm). As with the other metals the bone samples proved positive and the average concentration of nickel measured was 38 ppm. The results of the analyses are given in table 18 (see Appendix).

Discussion.

The purpose of the experiments described in this section was to determine whether or not the gills of the minnow accumulated heavy metals during lethal exposures and to compare any gill uptake of metals measured with the concentrations in bone samples which in short term acute toxicity tests would not be expected to accumulate a significant amount of the metal. The gill/bone ratios could then be used as an index of specific acute heavy metal toxicity and would be of practical use in assessing toxicity in the field.

For copper and zinc it was possible to compare gill/bone ratios although in the case of copper inadequate removal of copper-bearing mucus from the opercular bone sample affected the result. The copper content of the gills did however increase significantly and this alone is probably a good enough indicator of acute copper toxicity. Zinc exposures produced results which could be more successfully expressed using gill/bone ratios. The ratio rose from an average control level of 0.9 to an average experimental level of 1.6. Although the difference in the ratios is not very great it represents a large difference in concentrations as the control zinc levels were very high.

Nickel and lead gave negative control results and thus any metal uptake measurable in the gills was significant. The highest levels of the two metals found in the gills of dead fish were 93 ppm nickel and 404 ppm lead.

An important aspect of gravimetric analysis is that it is a reliable method for indicating heavy metals responsible for fish kills in the field. Histopathology is of little or no use with fish which are not freshly killed. Even where gill/bone metal ratios do not give satisfactory results the straightforward increase in metal content of the gills of fish should serve as a sufficient indicator of the particular metal responsible for a fish kill.

9. HISTOPATHOLOGIC STUDIES OF CHRONIC HEAVY METAL TOXICITY.

1. Methods.

For histopathologic studies groups of three fish were maintained in 5 litre all-glass tanks at 15°C ± 1°C. Fish were fed at least every two days on "Tetra-Min" tropical fish food and occasionally on live Daphnia. Fish used as controls had been maintained in the laboratory in the artifical medium for at least two weeks. Exposures of fish to the heavy metal solutions were continued for prolonged periods, often well over 100 days. One subacute concentration of each of the metals was tested. The concentrations employed are indicated in the following table together with the fish/metal ratios and the proportion of the observed 48 hr L.C.50 which they represent.

Metal	Conc. Tested	Fraction of 48 hr. $L.C._{50}$	Wet weight Fish/ wt metal
Cu	0.1 ppm	0.81	14000/1
Zn	0.1 ppm	0.65	14000/1
Pb	1.0 ppm	0.55	1400/1
Ni	4.0 ppm	0.55	350/1

The concentration of copper tested was the nearest to a 48 hr. $L.C._{50}$ value of any of the metals and probably would be expected to exert the most effect because of this. The different proportions were however unavoidable. For instance if the concentration of zinc had been raised to 0.12 - 0.13 ppm it would have represented a similar proportion of the 48 hr. $L.C._{50}$ to the concentration employed for copper. This was not possible however as it was found that 0.13 ppm of zinc was found to be fatal to some fish within four days, death resulting from direct gill damage. The lower levels of nickel and lead were employed for the same reason.

The problems of detoxification of the test solutions are obvious when the fish/metal ratios are inspected. In order to overcome this the experimental solutions were replaced every 4 - 6 days during the exposures.

Analyses for metals in solution were made on one occasion taking water from experimental tanks each containing three fish after one and five days. The results are indicated in the table below.

Metal	Initial Conc.	1 day conc.	5 days conc.	% loss at 5 days
Cu	0.1 ppm	0.09 ppm	0.04 ppm	60%
Zn	0.1 ppm	0.08 ppm	0.04 ppm	60%
Ni	4.0 ppm	2.7 ppm	2.4 ppm	40%
Pb	1.0 ppm	0.5 ppm	0.3 ppm	70%

Samples were concentrated ten times and analyses were performed on a Unicam SP.900A atomic absorbtion spectrophotometer.

The actual metal concentrations tested were thus lower on average than the initial concentrations. Lead showed the greatest loss of concentration falling by 70% in 5 days.

As with earlier experiments the calcium content of the water where copper, zinc or nickel were tested was 80 ppm and that where lead was tested was 10 ppm.

Fish were removed for sectioning after different periods of exposure and compared with control animals.

2. Treatment of tissues

Liver, spleen, pancreas, kidneys, intestine, gills and skin were routinely examined.

Fish were not fixed as whole animals but were dissected and the organs fixed individually. Fish were starved for 48 hours before sacrifice so that the gut would be empty on removal.

Gills were removed from both sides of the animal by cutting carefully at the ends of the gill arches preventing bleeding as much as possible. Liver, spleen, pancreas and intestine were removed as one unit by cutting the gut at the anterior and posterior ends. Pieces of liver however were often separately fixed. The kidneys were removed by carefully pulling away the peritoneum and then by gently breaking the adhesion between the organs and the dorsal abdominal wall. Kidneys were placed on strips of filter paper for fixation in order to retain their shape. The skin was examined on some occasions and to facilitate this whole sections of the trunk region were cut from the fish and fixed.

The gills were fixed in cold (4°C) Lillie's neutral buffered formalin (see section 7) or sometimes in Bouin's fixative (Pantin 1964). The viscera as a block were fixed in Bouin's, formol-saline and sometimes Helly's fixative (see Humason 1967). The kidneys like the gills were fixed in neutral buffered formalin. The blocks of trunk muscles and skin were fixed and decalcified simultaneously in Schmidt's fluid (1956) after Humason (1967).

Schmidt's fluid 4% formalin + 1 gm sodium acetate - 100 ml

Disodium versenate (EDTA) - 10 gms.

In all cases except where Helly's fixative was employed tissues were transferred to 3 or 4 changes of 70% ethanol for 12 hours or overnight. They were then placed in 3 or 4 changes of both 90% and 95% ethanol for 12 hours total time and thence to 3 or 4 changes of absolute ethanol for 1 - 3 hours depending on size. Tissue blocks were cleared in two changes of xylene for one hour and impregnated and embedded in 2 changes of 54°C paraffin wax.

Fixation of the liver.

Satisfactory fixation of the liver caused great problems. The use of routine fixatives such as formalin, Bouin's, Zenker-formol (Helly's) and even glutaraldehyde resulted in a very unsatisfactory picture.

In all cases the contents of most of the hepatic cells streamed and collected either at one pole of the cell or around the nucleus, frequently obscuring it. The problem of fixation was eventually attributed to the high glycogen content of the hepatic cells. After many attempts alcoholic - formalin was found to be the most satisfactory fixative.(See plates 15 and 16).

Alcoholic-formalin

Neutralised commercial formalin - 10 ml

95% Ethanol - 90 ml

The use of this fixative was something of a compromise as although the glycogen was fixed, considerable shrinkage of the tissue samples occurred due to the high ethanol content. The fixative was best used at 4°C or even at deep freeze temperatures (-20°C). After fixation liver samples were transferred directly to absolute ethanol after which the treatment was the same as that already described for other tissues.

Sections were cut at 4 - 7 microns, floated on distilled water and mounted on albumenised slides. Sections were routinely stained in Ehrlich's haematoxylin and eosin (Humason 1967) and Mallory's triple stain (Pantin 1964).

Sections were mounted in DePeX synthetic resin.

3. Normal structure of the organs

Gills The structure of the gills of Phoxinus has already been described (see Section 7).

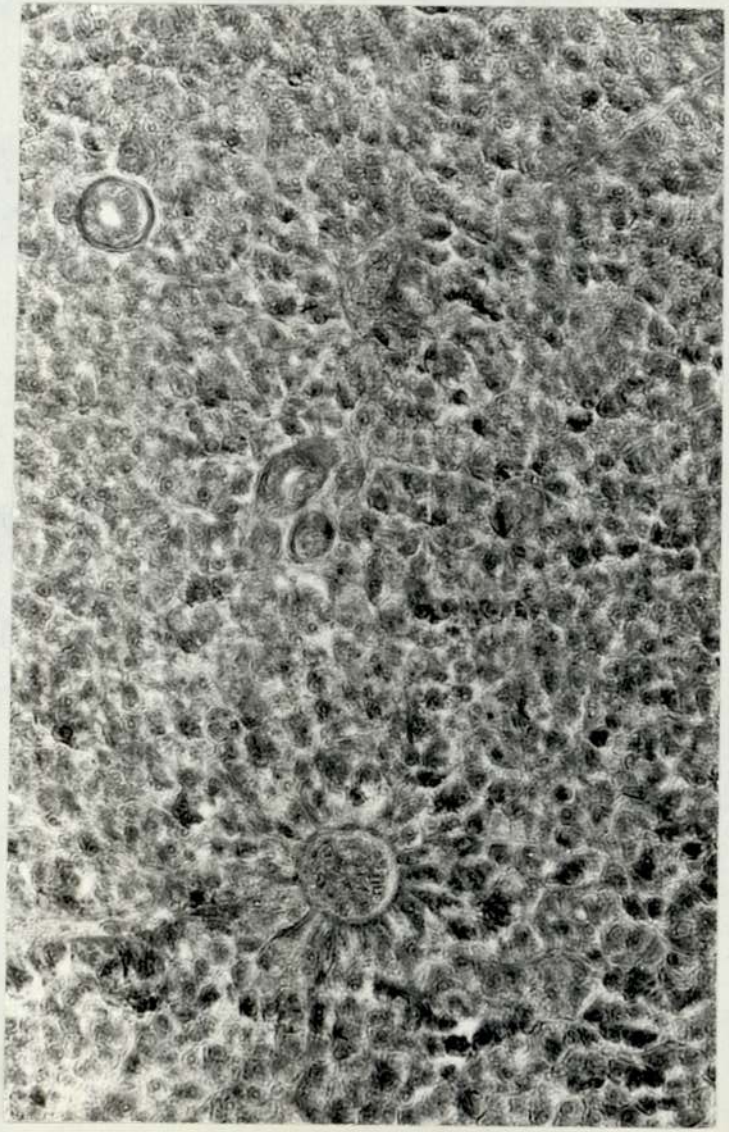
Skin In the region examined (posterior part of the body) the skin has a simple two layered structure, the outer stratified squamous epidermis and the inner dermis containing the scales. The epidermis of control fish contains numerous unicellular glands (goblet cells) responsible for the secretion of mucus on to the surface of the skin. The goblet cells develop at the base of the epidermis and move up to the surface where they discharge. In control fish most of the goblet cells appear discreet and are not always easy to distinguish. Sensory receptors

Plate 15

Liver fixed in cold neutral buffered formalin. (Mallory's triple stain) (X 345). The glycogen within the hepatic cells has streamed and collected around the nuclei and cell membranes.

Plate 16

Liver fixed in cold alcoholic formalin. (Mallory's triple stain). (X 550). The glycogen is preserved in the hepatic cells but the section is still difficult to view.



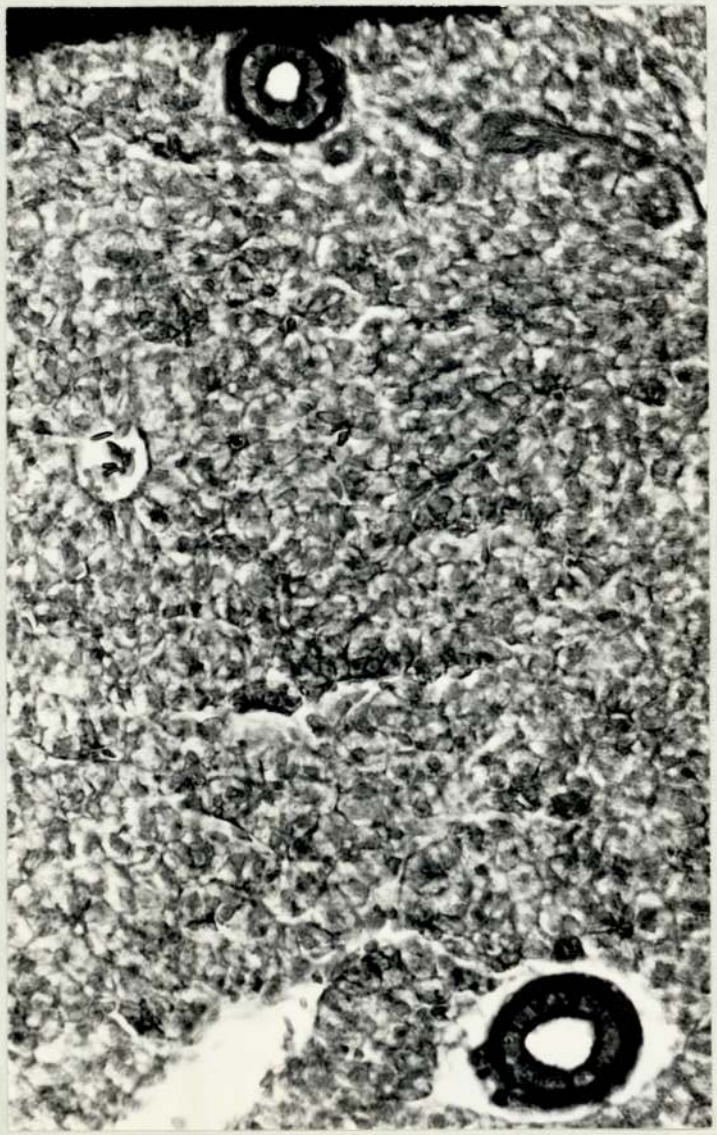
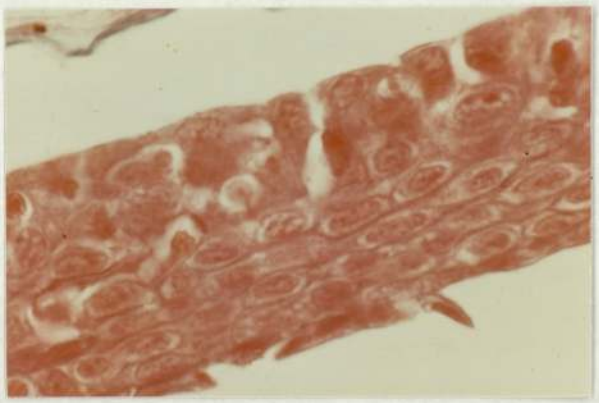


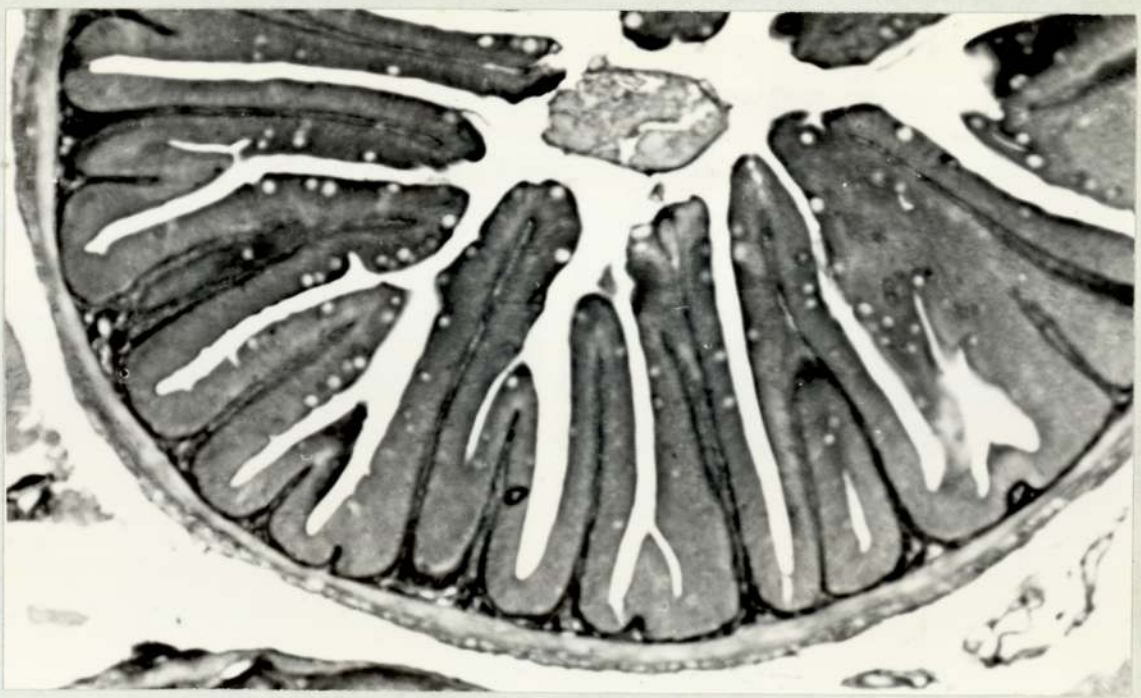
Plate 17

Skin of a control fish (Mallory's triple stain) (X920). The epidermis consists of 5-6 layersof cells. Mucus secreting cells are discreet in both layers.

Plate 18

Transverse section of the midgut of a minnow. (Mallory's triple stain). (X 110). Discharged mucous cells can be seen in the epithelium being more numerous near the tips of the rugae.





with nervous supplies were also seen in the epidermis. For control skin see Plate 17.

Digestive tube

The digestive tubes of freshwater teleosts have been described by Rogick (1931), Ahsan-ul-Islam (1949), Al-Hussaini (1949) and Vickers (1963).

The intestine of Phoxinus resembles closely that described in Campostoma by Rogick (1931). In the present study only the anterior part of the mid-gut was examined. The intestine as a whole forms an "S" shape and in the region of the mid-gut consists of four layers. The epithelium consists of simple columnar cells. The cells are relatively tall and rest upon a thin basal membrane which is supported by a sheet of submucosa. The elongate slender nuclei are located in the basal third of the epithelial cells and contain a large amount of chromatin. There is a well-marked striated border at the outer edge of the epithelial cells. Very small amoeboid cells having small dark nuclei were often seen in the mucosa. The whole of the mucosa and submucosa in the minnow is thrown up into a series of zig-zag folds (rugae). When the gut is empty these are very prominent (see Plate 18) but they are much reduced when the gut is distended with food. Mucous goblet cells are common in the intestine increasing in numbers towards the posterior region. In the mid-gut the mucous cells are formed at the bases of the rugae and migrate up the sides moving to the surface and discharging as they approach the apex (Vickers 1962). There are no multicellular glands of the gastric or intestinal type along the whole length of the intestine. The submucosa consists of a thin sheet of very vascular fibrous connective tissue. It is present in every fold in the gut wall and is somewhat thicker between the bases of the rugae. Cells with a granular appearance were often seen in the mucosa and may have been of the same type as those considered to be mast cells by Bolton (1933). The muscularis consists of two definite layers, an inner circular muscle layer and an outer smaller

longitudinal layer. Between the muscle layers there is a thin layer of connective tissue. The serosa consists of a thin layer of squamous epithelium. Loose pancreatic tissue surrounded by adipose tissue is attached to the intestine in the mesenteries along much of its length.

Liver.

The structure of the liver in fishes has been described by Elias and Bengelsdorf (1951). In Phoxinus the liver fills the visceral cavity surrounding the gut. Histologically no lobules are present and the continuous mass of parenchymatous hepatic cells form walls or laminae between which are found blood sinuses. The hepatic cells are however arranged somewhat radially around small veins. The nuclei of the hepatic cells are vesicular and the nucleoli are relatively large and easily seen. Large bile capillaries are quite evident having walls consisting of more cuboid cells and having a smaller lumen than blood capillaries. The cytoplasm of the hepatic cells appeared very granular and sometimes vesicular. The granular appearance was due to heavy deposits of glycogen which were verified using a P.A.S. stain. Slight vacuolation was noted in the livers of some control fish but using fat tests (Pearse Oil Red "O") no neutral fat was detected. Cells of Kuppfer were difficult to see but their typical elongated nuclei were present amongst the parenchymatous cells.

The liver was surrounded by a thin sheet of fibrous connective tissue.

Spleen.

The general histological features of the spleen have been described by Yoffey (1929). The spleen of the minnow is relatively large lying between the liver and intestine. It is characterised by its deep red colour. The large size presumably means that it is as important as the intertubular tissue of the kidneys as a blood forming organ. In control fish vast numbers of differentiating erythrocytes could be seen, accounting for 80% of the total area of the section. Pigment and pigment macrophages

were relatively uncommon in control fish although wide variations occurred.

Kidneys

The structure of the nephron in fishes has been described by Grafflin (1937). In Phoxinus the paired kidneys are located retroperitoneally along the dorsal body wall occupying the length of the body cavity. They are partly fused along the mid-line except at the anterior end. The thin fibrous coat is heavily pigmented with melanin. The renal tubule of the minnow appears to consist of at least four of the five basic segments characteristic of many freshwater teleosts (Grafflin 1937) and noted by Crandall and Goodnight (1963) in the guppy (Lebistes reticulatus). There is a short neck region followed by the proximal convoluted tubule which as in Lebistes is divided into two histologically distinct sections. The first region has tubular cells with a higher brush border and many of the epithelial cells have clear areas in their cytoplasm. The second region has high epithelial cells but a less well developed brush border and the cytoplasm of the tubular epithelium is more regular. The existence of an intermediate section is doubtful. The distal convoluted tubules are recognisable as having a distinctly cuboidal epithelium. (See Plate 21).

The peritubular lymphoid tissue which in the minnow as in many teleosts has an erythropoietic function is much more developed at the anterior end of the kidney. Pigment and pigment macrophages were often seen in regular but local accumulations in the intertubular tissue.

4. Results

A total of 65 fish were examined including 59 experimental animals.

i) Copper

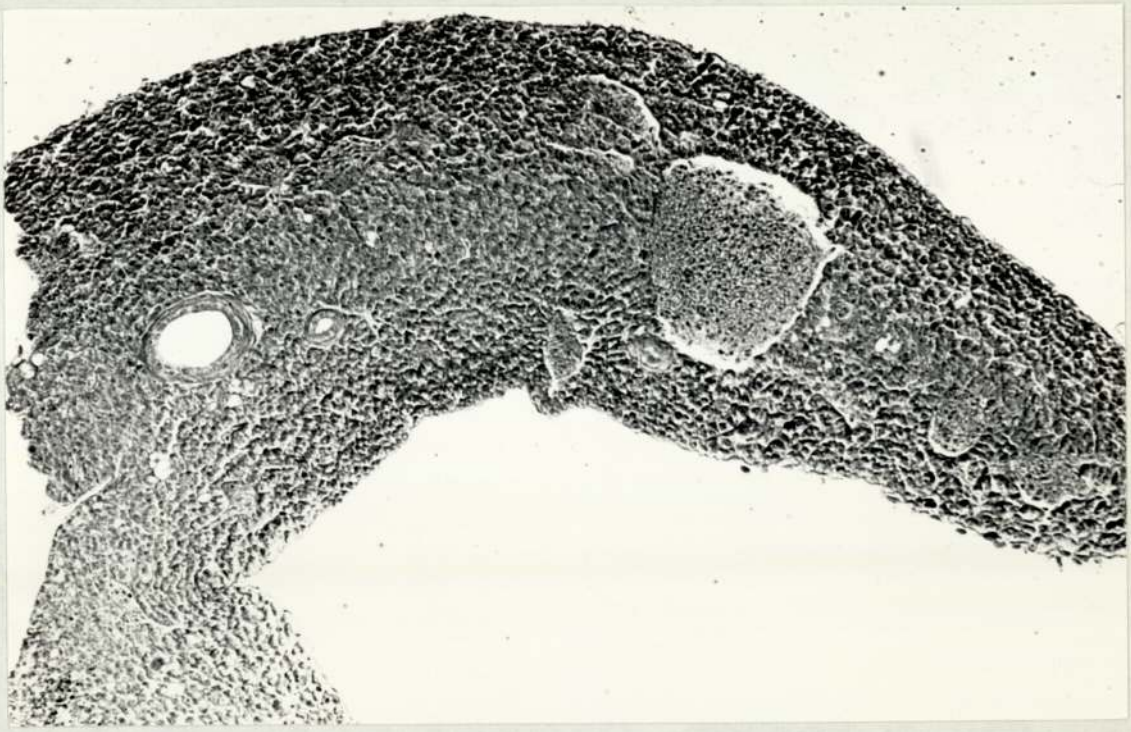
A total of 16 fish were examined after exposures to 0.1 ppm copper for up to 130 days. The histopathologic changes occurring as a result of chronic copper toxicity were by far the most striking of all the metals. Of 5 fish examined for up to 30 days exposure all showed no

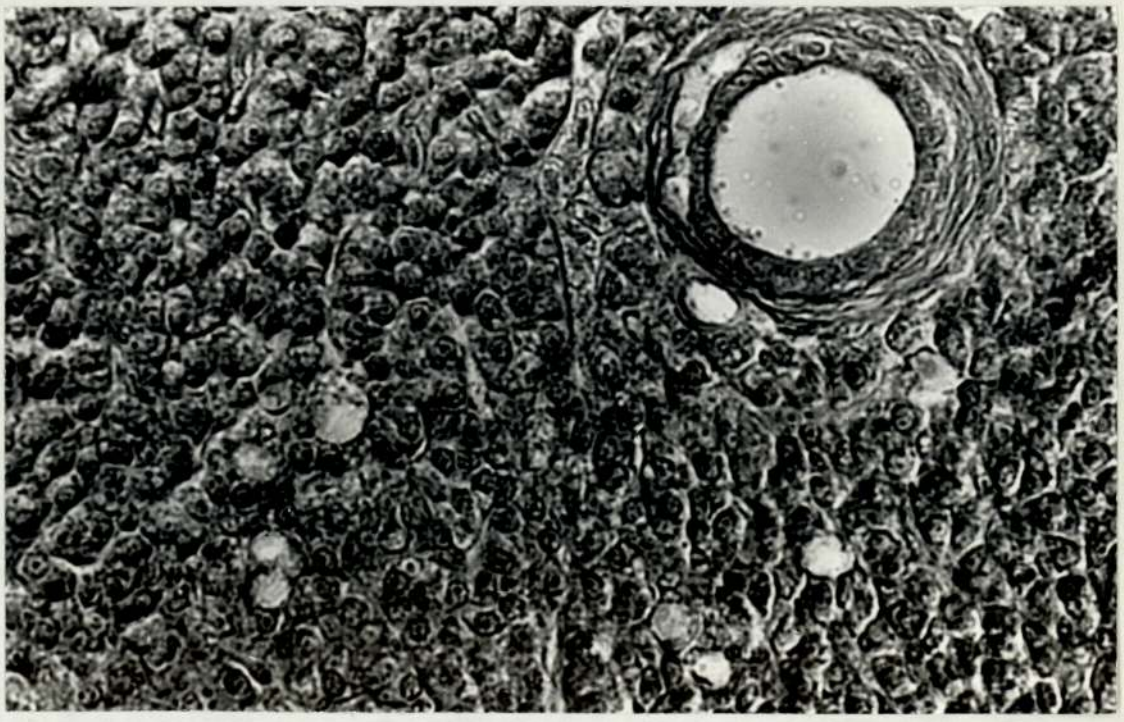
Plate 19

Liver of a fish taken moribund from a solution of 0.1 ppm copper after 120 days exposure. (Alcoholic formalin fixed, Mallory's triple stain) (X 215). Note the small size of the organ and the apparent increase in hepatic blood vessels due to the decrease in numbers of parenchyma cells.

Plate 20

As above. (X 880). Note the clear areas amongst the hepatic cells, possibly indicating dead cells.





external symptoms except that food was not taken as readily as by controls. The skin of two fish was examined and appeared to be thinner than that of the control fish although at this stage the difference was not very marked. A slight increase in active mucous cells was seen in the skin. The gills of three fish were examined and did not show any consistentor marked histologic changes. The liver appeared normal in all five cases except that some vacuolation was noted and the organ was possibly smaller. At this stage, however, histochemical and analytical tests demonstrated large increases in the copper content of the organ (see Sections 10 and 11). The spleen appeared normal except that increased pigment deposition was apparent in four cases. No changes were noted in the pancreas or kidneys at this stage.

Seven fish were examined after between 54 and 90 days' exposure to 0.1 ppm copper. The gills did not appear to be altered in any way. The skin in the two specimens examined was noticeably thinner than in the control fish, apparently consisting of only three to four layers of cells as opposed to seven to nine layers in the controls. Mucous cell activity however was not much greater than in control fish. The liver was examined in all seven cases and in six marked changes had occurred. Over all the organ was smaller and redder than usual. There appeared to be a great change in the ratio of hepatic cells to blood sinuses, the numbers of hepatic cells decreasing quite dramatically (see Plates 19 and 20). Vacuolation of the hepatic cells was not very extensive and neutral fat was only sparsely deposited in the three fish where fat tests were carried out. In three of the seven fish areas of disorganised cells without nuclei were seen. The nucleoli of many of the cells were less evident than in the controls, possibly indicating a slowing down of protein synthesis. The glycogen content of most of the cells appeared to be the same as in control fish. In two cases the fibrous coat of the liver appeared swollen. The kidneys of five out of the seven fish examined showed marked changes. The glomeruli were noticeably swollen and appeared very prominent. The brush borders of the proximal tubules

appeared to be larger than the controls and granules under the brush border were noted. Two fish sacrificed at 80 days had a very definite lack of intertubular tissue and the kidneys were very small and difficult to handle on removal from the fish (see Plates 21 and 22). Damage to the renal tubules was also noted and many cells appeared shrunken. Some evidence of swelling of the tubules was also noted and debris was often contained in them. Many of the nuclei of the tubular epithelial cells appeared rounded and densely staining. Some slight vacuolation of the acinar pancreas was observed and in one case fat deposits were stained. There was an increase in the deposition of pigment in the renal intertubular tissue and in the loose connective tissue in and about the intrahepatic pancreas. The spleen did not exhibit a marked increase in pigment.

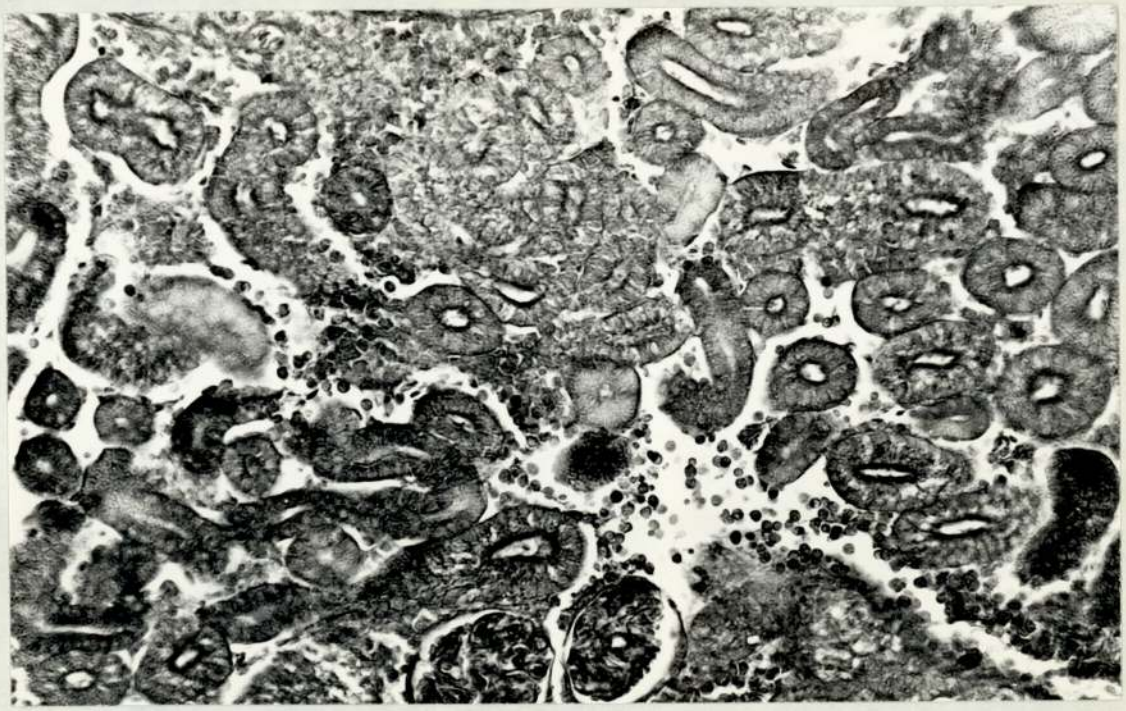
Of four fish examined after 90 days all were extremely moribund. In fact, counting other experiments a total of 17 fish were maintained at 0.1 ppm Cu for more than 70 days and in 9 cases the same symptoms set in between 70 and 130 days. The fish lost weight and became "hollow-gutted". The muscles of the trunk region were noticeably wasted and the fish became "hump-backed," and had difficulty in swimming. Feeding activity was extremely limited in all fish at this stage although some food was taken. Fish eventually lost equilibrium and after this initial loss the time to death was often 3 - 4 days. In some fish a marked darkening of the dorsal body surface occurred in the later stages of copper poisoning. The gills of the four fish examined showed hardly any abnormalities, the only change being that some of the secondary lamellae were swollen at their tips. No fusion of secondary lamellae was seen. The skin appeared to be in the same condition as 30-60 day fish, being thinner but possessing only slightly more active mucous cells than control fish (see Plate 23). In all cases the liver was exceedingly small and difficulty in obtaining pieces for fixation was experienced. In most cases the hepatic cells appeared relatively unchanged and vacuolation and fatty degeneration were only slight. Many of the nuclei were densely staining and presumably pycnotic and in one case a large area of disorganised cells with no

Plate 21

Kidney. (Neutral buffered formalin fixed, Mallory's Triple stain). (X 275). Control fish.

Plate 22

Kidney of a fish taken moribund from a 0.1 ppm solution of copper after 80 days exposure. (X 275). Note the severe damage to the kidney tubules and their distended lumens. There is also a noticeable loss of intertubular tissue.



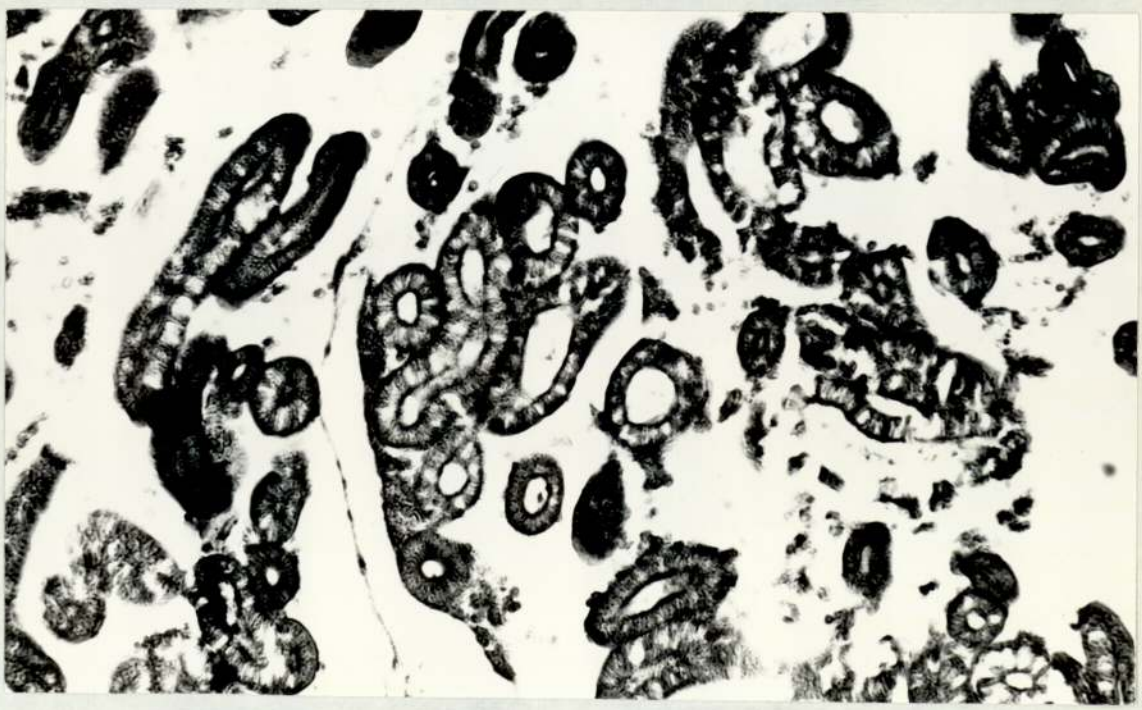
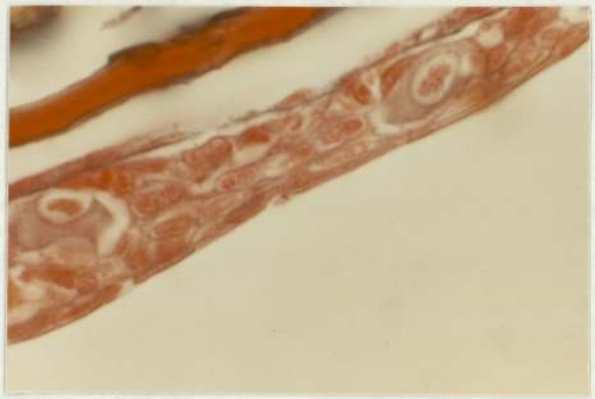


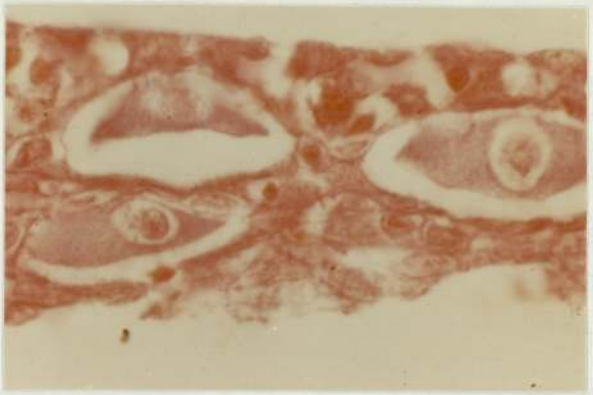
Plate 23

Skin of a fish taken moribund from a 0.1 ppm solution of copper after 120 days exposure. (X 920). Note the reduction in the thickness of the skin and the presence of active mucous cells. (Compare with plate 17).

Plate 24

Skin of a fish taken in apparently healthy condition from a 1.0 ppm solution of lead after 110 days exposure. (X 920). Note the swelling of the skin and the large active mucous cells. (Compare with plate 17).





nuclei was seen. The pancreas was unchanged apart from some vacuolation of the acinar cells. The spleen was little changed in all cases and developing erythrocytes were numerous in the pulp, although the organ appeared paler and somewhat smaller than usual. No excessive pigment deposition was noted in the spleen. The kidneys were very shrunken and friable in all cases presumably due to the loss of intertubular tissue. Great difficulty was experienced with the removal of the kidneys from these individuals as due to the wasting of the muscles of the dorsal region the ribs were almost protruding into the visceral cavity beneath the kidneys. In all fish the peritubular lymphoid tissue was extremely sparse. Extensive damage to the renal tubules was evident and many of the tubular epithelial cells were shrunken and contained an excessive amount of clear cytoplasm. Pycnotic nuclei occurred abundantly and the brush borders of the proximal tubules were very well defined. Pigment accumulation was again noted in the kidneys in an intertubular position.

No serious damage to the mid-gut was noted at any stage of copper poisoning but vacuoles were noted in the epithelium of the mucosa and these were shown to contain copper. (See Section 10).

The evidence presented here and in Sections 10 and 11 indicates a delayed response to subacute levels of copper, the symptoms reliably appearing after 50 days' exposure. A summary of the histopathologic changes during chronic copper toxicity is given in Table 2.

ii) Lead

A total of 14 fish were examined after exposure to 1 ppm of lead for up to 345 days. The observed histopathologic changes due to lead toxicity were probably the least marked of the four metals studied.

Four fish were examined after 30 days' exposure. The gills in all cases showed no degenerative changes, but there was evidence of increased mucous cell activity. Active mucous cells were observed in the respiratory epithelium between the secondary lamellae. The skin, however, was markedly altered at this stage and remained in the same condition after all periods of exposure. In all cases the skin appeared inflamed and in places the outer epidermis was damaged. (See Plate 24) Mucous

0 = No observation
X = No change

SUMMARY OF CHRONIC COPPER TOXICITY

TABLE 2

Fish No.	Exposure time (Days)	Condition when fixed	STATE OF						
			SKIN	GILLS	LIVER	PANCREAS	SPLEEN	KIDNEYS	MID-GUT
1	28	Active	0	0	Very slight vacuolation	X	Slight increase in pigment	X	X
2	28	Active	0	X	"	X	X	X	X
3	28	Active	Thinner and increased mucus activity	X	"	X	Very slight increase in pigment	X	X
4	30	Active	"	X	"	X	"	X	X
5	30	Active	0	0	"	X	"	X	Vacuoles visible in mucosal epithelium
6	54	Inactive	0	X	Marked decrease in size. Some vacuolation	X	X	X	X
7	54	Active	0	X	"	X	X	Some swollen glomeruli granules under brush borders	X
8	54	Active	0	0	" plus necrotic areas	X	X	X	X
9	54	Inactive	0	X	As No.6	Very slight vacuolation	Slight increase in pigment	Swollen glomeruli, shrunken epithelial cells + loss of intertubular tissue	X

TABLE 2 (Continued)

Fish No.	Exposure time (Days)	Condition when fixed	STATE OF						
			SKIN	GILLS	LIVER	PANCREAS	SPLEEN	KIDNEYS	MID-GUT
10	60	Active	0	X	As No.6	Very slight vacuolation	Slight increase in pigment	Swollen glomeruli, shrunken epithelial cells and loss of intertubular tissue	X
11	80	Inactive Wasting	Thinner and increased mucus activity	X	Less change but very small	X	"	" and pycnotic nuclei	X
12	80	Inactive Darkened Wasting	"	X	As Nos. 8/9	Slight vacuolation and fat cells	"	"	Vacuoles visible in mucosal epithelium
13	95	Moribund Very wasted	"	Slightly swollen epithelium	Very small Some vacuolation and necrosis	X	"	Small and friable Extensive damage	X
14	106	Overturned very wasted	0	X	" but no necrosis	X	"	"	X
15	120	Moribund Very dark	0	As No.13	"	X	X	"	Vacuoles visible in mucosal epithelium
16	130	Almost dead; very dark	0	X	"	Slight vacuolation	X	"	X

cells in the skin were much more active than in controls. In all four fish the liver was unchanged as was the pancreas. The spleen appeared healthy but there was an increase in pigment scattered throughout the organ. The kidneys were undamaged at 30 days although an increase in granulocytopoietic activity was noted in two cases.

Six fish were examined after exposures of between 30 and 90 days. The gills and skin exhibited the same changes as the 30 day fish. In some cases the liver was vacuolated although it was large and appeared healthy on removal. No fat deposits were detected in the liver. The pancreas appeared normal in four cases but two fish had a marked accumulation of brownish granular material in the connective tissue between the pancreas and the liver. The spleen was heavily pigmented in five fish but was otherwise normal. (See plates 25 and 26) The kidneys appeared normal in all cases.

Four fish were examined after over 90 days' exposure. Two fish were taken at 110 days and a further two were maintained for 345 days. In all cases the skin and gills appeared the same as in fish after shorter exposures. The liver was vacuolated in all cases particularly at 345 days but fat tests revealed only slight deposits. The pancreas and spleen appeared as before. No tubular damage or lack of intertubular tissue was noted in the kidneys although a few of the nuclei of the proximal tubules appeared rounded and densely staining. The brush borders of the proximal tubules of the 345 day fish were very well defined.

Even after a period of almost one year the histopathological effects of lead exposure appeared only slight. The fish grew well (although no measurements were made) and appeared outwardly healthy throughout the whole exposure period.

A summary of the histopathologic effects of chronic lead toxicity is given in Table 3.

0 = No observation
X = No change

SUMMARY OF CHRONIC LEAD TOXICITY

TABLE 3

Fish No.	Exposure time (Days)	Condition when fixed	STATE OF						
			SKIN	GILLS	LIVER	PANCREAS	SPLEEN	KIDNEYS	MID-GUT
1	30	Active	Swollen and increased mucus activity	Slight increase in mucus cell activity	X	X	Slight increase in pigment deposits	X	X
2	30	Active	"	X	X	X	"	Granular material in intertubular tissue	X
3	30	Active	0	X	X	X	X	"	X
4	30	Active	As No.1	X	X	X	As Nos. 1/2	X	X
5	45	Active	0	As No.1	X	X	"	X	X
6	60	Active	As No.1	X	Slight vacuolation	Pigment in surrounding connective tissue	Heavy pigmentation	X	X
7	65	Active	0	X	X	X	"	X	X
8	73	Active	0	X	As No.6	X	"	X	X
9	88	Active	0	X	Vacuolation more evident	As No.6	Some pigment deposit increase	X	X
10	88	Active	0	X	As No.6	X	As No.6	X	X
11	110	Active	0	As No.1	Increase in vacuolation; fat test negative	X	"	X	X

TABLE 3 (Continued)

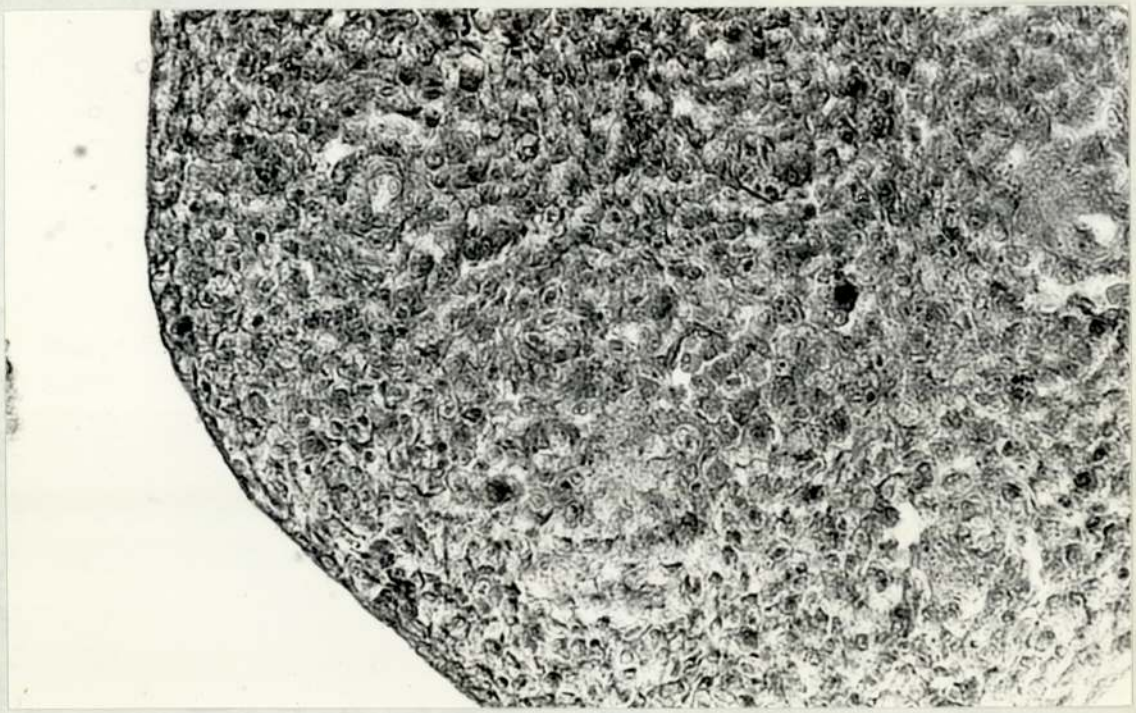
Fish No.	Exposure time (Days)	Condition when fixed	STATE OF						
			SKIN	GILLS	LIVER	PANCREAS	SPLEEN	KIDNEYS	MID-GUT
12	110	Active	As No.1	As No. 1	Increase in vacuolation fat test negative	X	As No.6	Granular material as in 2 & 3	X
13	345	Active	"	X	"	As No.6 but more granular	"	" plus large brush borders	X
14	345	Active	"	X	"	"	"	"	X

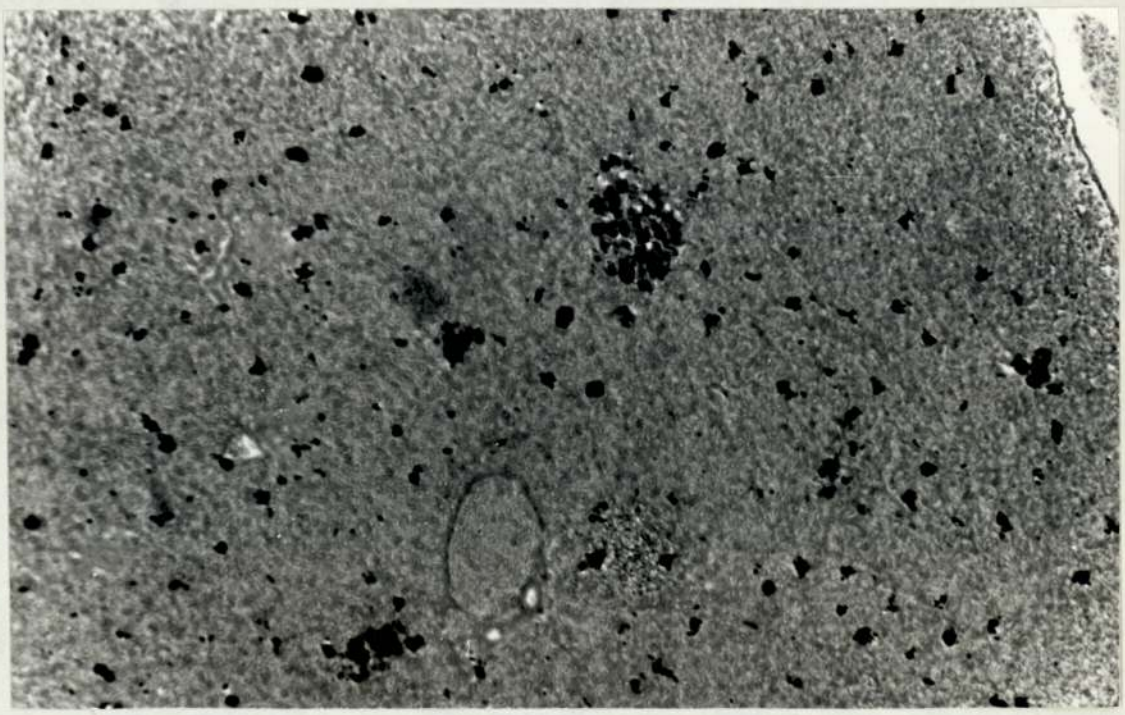
Plate 25

Spleen of a control fish. (Neutral buffered formalin fixed, Mallory's triple stain) (X 550).

Plate 26

Spleen of a fish exposed to 1.0 ppm of lead for 110 days. (Stain as above) (X 215). Note the increase in pigment deposition.





iii) Nickel

A total of 14 fish were examined after exposure to 4 ppm of nickel for up to 209 days.

Four fish were examined after 30 days' exposure. The gills showed little change except that the mass of mucous and albuminous cells between the secondary lamellae appeared enlarged due to cell proliferation. The skin showed a marked mucous cell response (see Plate 27) with many active goblet cells and many exhausted cells at the surface of the epidermis. No damage to the epidermis was evident. No damage to the liver, kidneys, spleen or pancreas was noted at this time.

Five fish were examined after between 30 and 90 days' exposure and the histopathologic changes were slight, approximating to those of the 30 day fish. One difference was that in two cases vacuolation of the liver was quite evident.

Five fish were examined after exposure periods of between 90 and 209 days. In three cases the fish were moribund (110, 145 and 209 days).

Where other experiments are included, of 12 fish maintained in 4 ppm nickel solutions for over 90 days, six developed similar symptoms. The fish became very much darker dorsally and swimming motions became rather serpentine. The loss of co-ordination of swimming activity progressed over a period of up to 25 days. At the time when the first loss of equilibrium occurred the fish would be quite unable to control their direction when swimming and in fact swam round in circles for days! Considerable weight loss occurred at this stage, although it was never as marked as in the case of copper and the fish appeared to take some food from the bottom of the tank. After overturning the time to death was very protracted, being five days in one case. The loss of co-ordination and darkening of the skin suggest a possible neurotoxic effect for nickel. On examination of the gills one fish (145 days) had swollen secondary lamellae and fusion of the lamellae had occurred in some places. (See Plate 28.) The mass of cells between the secondary lamellae had also proliferated. The condition of the skin paralleled

0 = no observation
X = No change

TABLE 4

SUMMARY OF CHRONIC NICKEL TOXICITY

Fish No.	Exposure time (Days)	Condition when fixed	Skin	Gills	Liver	Pancreas	Spleen	Kidneys	Mid-Gut
1	30	Active	Marked increase in mucus activity	X	X	X	X	X	X
2	30	Active	"	Apparent proliferation of inter-lamellar cells	X	X	X	X	X
3	30	Active	0	0	X	X	X	X	X
4	30	Active	0	X	X	X	X	X	X
5	48	Active	0	X	X	X	X	X	X
6	55	Active	As No.1	As No.2	X	X	X	X	X
7	65	Active	0	"	Slight vacuolation	X	X	X	X
8	65	Active	0	X	X	X	X	X	X
9	90	Active	As No.1	As No.2	Vacuolation evident	X	X	X	X
10	104	Inactive Darkened	"	"	" plus positive fat test	X	Increase in pgiment deposits	Slight damage to tubules and intratubular debris	X
11	110	Moribund Darkened	"	X	Very vacuolated and positive fat test	X	X	"	X
12	123	Active	"	X	Slight vacuolation	X	As No.10	X	X

TABLE 4 (Continued)

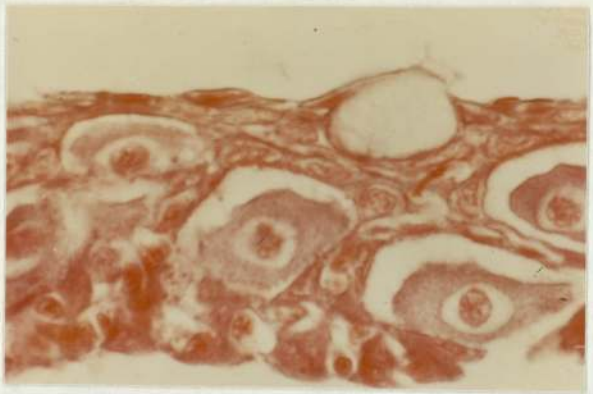
Fish No.	Exposure time (Days)	Condition when fixed	SKIN	GILLS	LIVER	PANCREAS	SPLEEN	KIDNEYS	MID-GUT
13	145	Moribund Darkened	0	2^{o} lamellae swollen and some fusion	As No.1	X	X	As No.10	X
14	209	Moribund Darkened	As No.1	As No.2	"	X	As No.10	More severe than above	X

Plate 27

Skin of a fish taken moribund from a 4.0 ppm solution of nickel after 110 days exposure. (X 920). Note the increase in active mucous cells. (One can be seen discharging at the surface).

Plate 28

Gills of a fish taken moribund from a 4.0 ppm solution of nickel after 145 days exposure. (X 500). The secondary lamellae are slightly damaged and fusion of adjacent lamellae is evident in places. The interlamellar cells appear increased in numbers.
(Compare with plate 4).



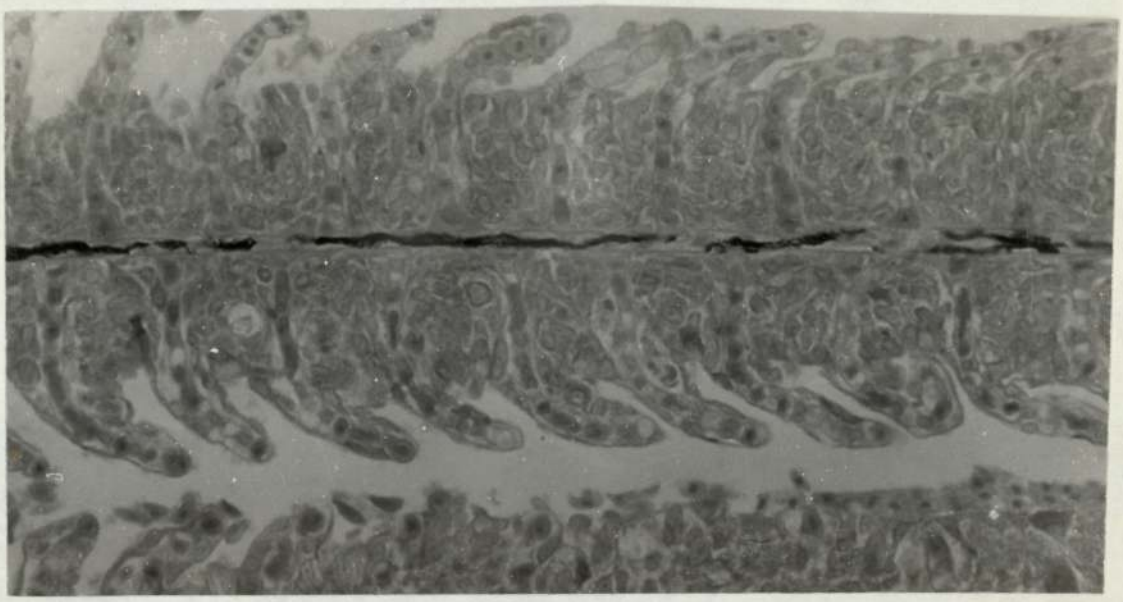
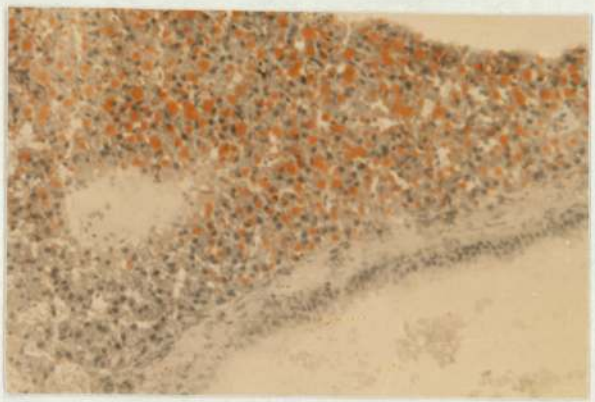


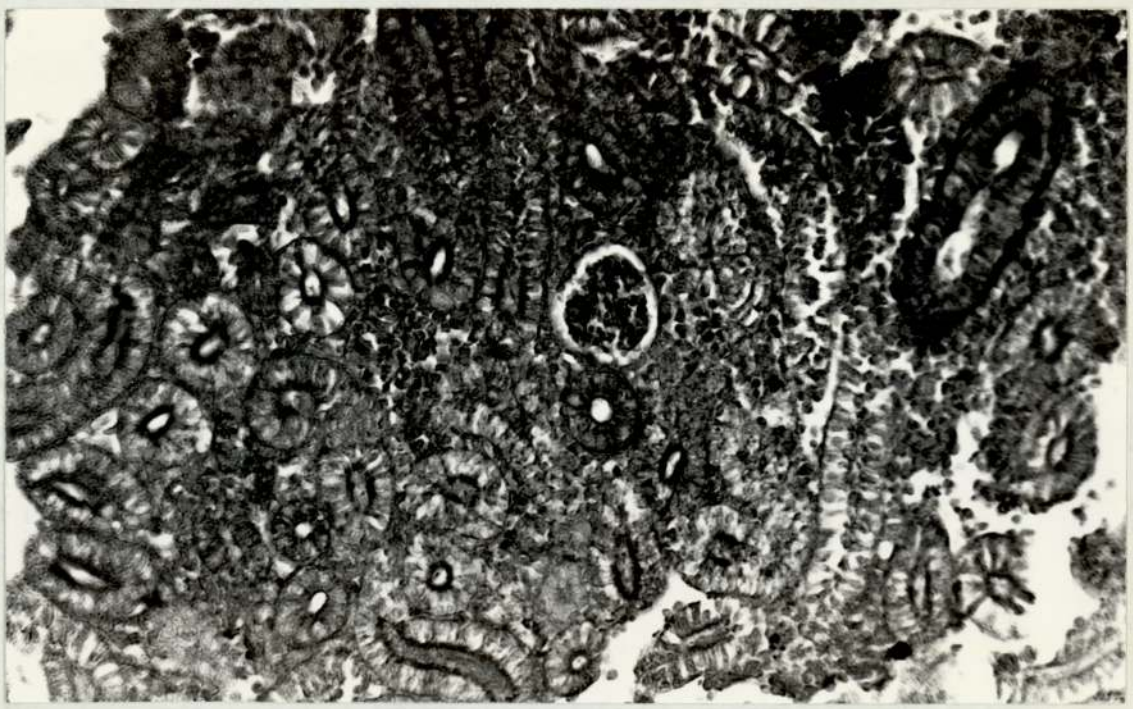
Plate 29

Frozen (cryostat cut) section of liver (12 microns) from a fish taken moribund from a 4.0 ppm of nickel after 145 days exposure. Extensive fatty degeneration (red areas) is indicated using Oil red "O" stain (X 150).

Plate 30

Kidneys of a fish taken moribund from a 4.0 ppm solution of nickel after 110 days exposure. (Mallory's triple stain) (X 275). The cells of some tubules appear shrunken.





that in shorter exposures. The liver was extremely vacuolated in the three moribund fish and noticeably so in the other two. In two cases fat tests revealed extensive fatty degeneration although some parts of the liver were affected much more than others. (See Plate 29)

No evident necrotic areas were seen in the liver and the nuclei appeared normal. The spleen possessed increased pigmentation in three cases and pigment was seen around the pancreas. The kidneys had some swollen tubular epithelial cells (i.e. with more cytoplasm). However, some shrunken cells were also seen. (See Plate 30) The amount of peritubular lymphoid tissue did not seem significantly decreased. An increased amount of brown pigment was seen in an intertubular position in all five fish. No changes were seen in the gut of any of the fish.

A summary of the histopathologic effects of chronic nickel toxicity is given in Table 4.

iv) Zinc

A total of 15 fish were examined after exposure to 0.1 ppm zinc for up to 110 days. It was intended to examine fish after longer exposure periods but the occurrence of disease (ulcerative dermal necrosis) prevented this.

Five fish were examined after between 25 and 30 days' exposure. The gills of two fish (30 days) had secondary lamellae with swollen tips. These, however, accounted for less than 10% of the whole gill surface examined. The cells between the bases of the secondary lamellae were increased in size and possibly in numbers thus reducing the respiratory surface. No damage to the respiratory epithelium was noted. The skin showed a marked mucous cell response in all cases similar to that observed for nickel. Active mucous cells were abundant and could be seen discharging at the surface. The skin appeared inflamed and some damage to the epidermis was evident (see Plate 33). The livers of three of the five fish examined were vacuolated and in one fish the fibrous coat was swollen. No changes were seen in the spleen and pancreas. The kidney tubules had granules under the brush borders which did not appear in the controls and also the brush borders of many proximal tubules were

enlarged. No damage to the tubules was noted at this stage.

Six fish were examined after between 30 and 90 days' exposure. The gills of two fish appeared to have a reduced number of cells between the secondary lamellae (see Plate 32). Discharged mucous cells were evident in the cell mass and the apparent loss of cells from this area may have been due in part to the exhaustion of mucous cells. The skin of the two fish examined resembled that of the 30 day fish. The liver had highly vacuolated patches in all six cases but the extent of vacuolation was never great. No pycnotic nuclei or necrotic areas were seen. The spleen appeared normal in all cases and no excessive pigment was seen. The kidneys of three fish had some expanded tubules and some of the glomeruli were swollen. Well defined granules were again noted below the brush border. In one fish the cytoplasm of many of the tubular epithelial cells was shrunken and the peritubular lymphoid tissue in the anterior part was reduced. The mid-gut in three cases had an increased number of discharged mucous cells at the tips of the rugae although no damage to the mucosal epithelium was seen.

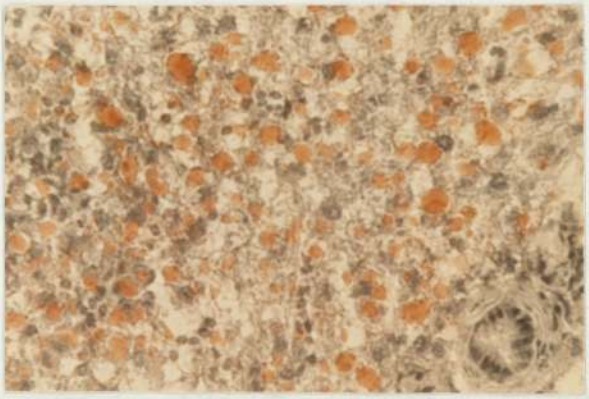
Four fish were examined after 90 - 110 days' exposure. One fish (99 days) was moribund. Including other experiments of a total of 20 fish maintained for over 90 days this was the only one which appeared to be at the point of death although many fish appeared weak and exhibited unco-ordinated swimming movements. The gills of the dying fish were relatively normal and no sloughing of the respiratory epithelium was observed. The skin of this fish and one other after 110 days' exposure was examined and found to be extremely inflamed in patches. It may be that the observed susceptibility of fish exposed to zinc for long periods to ulcerative dermal necrosis is connected with the inflammation of the skin produced by the metal. The liver was damaged in all cases. The vacuolation of the parenchyma cells was more widespread, than after shorter exposures and tests for fat revealed widespread fatty degeneration (see Plate 31). No necrotic areas were seen in the liver but some nuclei appeared very dense and nucleoli were not as evident. The glycogen content of the liver cells appeared normal and the organ was not

Plate 31

Liver of a fish taken moribund from a 0.1 ppm solution of zinc after 99 days exposure. Cryostat section (12 microns), Oil red "O" fat test. (X 370). Note the extensive deposits of neutral fat.

Plate 32

Gills of a fish exposed to 0.1 ppm zinc for 80 days. (X 500) There is an apparent decrease in the numbers of interlamellar cells, possibly due to mucous cell depletion.



significantly reduced in size. The condition of the spleen was similar to that in control fish. In two cases fat cells were found in the acinar tissue of the pancreas. This may, however, have been a normal phenomenon although it was not seen in control fish. The kidneys of the moribund fish were most damaged. Expanded tubules were seen and blood cells were found around them in large quantities. The kidneys of the other three fish were less damaged but some evidence of tubular damage was seen and many tubules contained debris. In no case was there a great reduction in intertubular tissue.

A summary of the histopathologic effects of chronic zinc toxicity is given in Table 5.

5. General Discussion

The purpose of the preceeding experiments was to determine histologically the effects of chronic copper, lead, nickel and zinc poisoning in the minnow. Evidence for chronic copper toxicity appears to be overwhelming, the same symptoms setting in in the majority of fish between 90 and 130 days. The symptoms were loss of weight, cessation of active feeding, darkening of the skin and loss of co-ordination and equilibrium. Extensive damage to the liver and kidneys was noted together with some effects on other organs. The darkening of the skin and apparent loss of co-ordination of swimming movements suggests a neurotoxic effect for copper. Copper has been suggested as having an effect upon the nervous system of fish by Malacea and Gruia (1964). The extensive damage to the liver and great reduction in the size of the organ may be due in part to reduced food intake, however some feeding was observed and the general wasting of the fish may be due to incomplete utilisation of food due to metabolic lesions induced by copper.

Zinc and nickel resulted in some pathologic conditions after extended exposure, but the position with respect to lead is less clear. After long term exposure to 4 ppm of nickel (> 100 days) weight loss, darkening of the skin and loss of co-ordination set in in half the exposed fish. Here as with copper there may be a neurotoxic effect.

0 = No observation
X = No change

SUMMARY OF CHRONIC ZINC TOXICITY

TABLE 5

Fish No.	Exposure time (days)	Condition when fixed	SKIN	GILLS	LIVER	PANCREAS	SPLEEN	KIDNEYS	MID-GUT
1	25	Active	Marked mucus cell response; epithelium swollen	X	Vacuolation evident; fibrous coat swollen	X	X	Granules under brush borders	X
2	25	Active	"	X	Slight vacuolation	X	X	X	X
3	28	Active	0	0	"	X	X	As No.1	X
4	30	Active	0	A few secondary lamellae with swollen tips	"	X	X	"	X
5	30	Active	0	X	Vacuolation plus positive fat test	X	X	X	X
6	42	Active	0	Reduction in no. of cells between secondary lamellae	Highly vacuolated in patches	X	X	As No.1	X
7	65	Active	0	0	"	X	X	X	X
8	65	Active	As No.1	X	"	X	X	Some swollen tubules and glomeruli	X
9	80	Active	0	As No.6	Some vacuolation	X	X	No damage but noticeable brush borders	Apparent increase in discharged mucus cells
10	80	Active	0	X	Highly vacuolated in patches	X	X	Some swollen tubules - reduction in peritubular tissue	X

TABLE 5 (Continued)

Fish No.	Exposure time (Days)	Condition when fixed	SKIN	GILLS	LIVER	PANCREAS	SPLEEN	KIDNEYS	MID-GUT
11	90	Active	As No.1	X	Highly vacuolated in patches	X	X	Swollen glomeruli and tubules	As No.9
12	99	Moribund	Very inflamed in patches	X	Vacuolation widespread; positive fat test	X	X	Swollen tubules slight haemorrhage	X
13	105	Inactive	As No.1	X	Extremely vacuolated	X	X	Swollen tubules and debris	X
14	105	Inactive	0	X	"	X	X	"	As No.9
15	110	Inactive	As No.12	X	"	X	X	"	X

Damage to the liver and kidneys was extensive but not as severe as with copper. Zinc and nickel appeared to cause a considerable amount of damage to the liver particularly with respect to fatty degeneration. However, out of all the fish exposed to zinc for over 90 days only one became distinctly moribund although others were obviously distressed. Exposures to zinc for longer periods are indicated but as already pointed out U.D.N. disease precluded this possibility in all attempts. The disease only seriously affected fish exposed to zinc and it seems worth repeating that the inflammation of the skin caused by the metal may be a contributory factor in the onset of the disease.

No fish died as a result of exposure to subacute concentrations of lead and any symptoms recorded were generally slight.

In all cases the skin was affected and this in itself is considered to be hazardous, possibly interfering with osmotic balance and with protection against pathogens.

In all but a few cases the gills appeared normal and extensive damage or sloughing of the respiratory epithelium was never observed. The lack of gill damage whilst damage was inflicted upon the internal organs is in agreement with the findings of Crandall and Goodnight (1963) concerning chronic zinc and lead toxicity to the guppy. A tentative conclusion can thus be made as a result of these studies, i.e. that the toxic effect of the metals can be exerted in two ways. Firstly that the effect of acutely toxic concentrations is to kill the fish by direct gill damage and secondly that the effect of chronic toxicity is to destroy the functions of the inner organs and to cause metabolic lesions resulting in the wasting of the fish due to poor food utilisation.

In order to investigate possible causes for tissue damage experiments were designed to attempt to locate any accumulated heavy metal in the tissues by histochemical means and also to measure any uptake of metals using gravimetric analysis. The methods and results of these experiments are described in the following two sections.

10. THE USE OF HISTOCHEMICAL TECHNIQUES TO ASCERTAIN UPTAKE AND STORAGE OF HEAVY METALS IN CHRONIC TOXICITY

1. Introduction

The use of histochemical techniques in studies of heavy metal toxicity to fish has been reported only by Haider (1964) (1965). Using a modified silver-sulphide technique he demonstrated uptake of lead by rainbow trout during protracted exposures to subacute levels of the metal. Silver-sulphide techniques were amongst those used in the present study and their theory and application will be discussed in parts 4 & 6 in this section.

The advantage of histochemical techniques for the detection of accumulated toxicants is that as well as indicating their mere presence in tissues the topography of localisation is indicated, which is of great use when interpreting their toxic effects.

The methods used for the demonstration of metals in tissues need to satisfy two main criteria:-

(1) To demonstrate the smallest possible amount of metal.

(2) To show its exact location.

In order to satisfy the first point only highly sensitive reagents can be employed and all materials used in the process must be metal free. In order to prevent loss of metal from the tissue and to retain its "in vivo" location the treatment of tissues prior to staining must be carefully controlled so as not to cause mobilisation or at worst complete loss of the stored metal. The use of fixatives with a high water content is thus avoided and often absolute ethanol (or methanol) is employed as a simultaneous fixing and dehydrating medium.

2. Exposures of fish

Exposures of fish to subacute concentrations of heavy metals were made at the same four levels and under exactly the same conditions as described in the previous section.

3. Copper

Histochemical methods for the demonstration of copper in tissues have been described by Okamoto and Utamura (1938), Mallory and Parker (1939), Green (1955), Uzman (1956), Howell (1959), Wilkins (1960) and Scheuer (1967). The early work of Mallory depended on the use of non-specific stains such as haematoxylin. Later workers used specific organic reagents. In the present study three reagents were used for the detection of copper.

i) Reagents for copper detection

(a) Rubeanic acid (Dithio-oxamide)

Rubeanic acid is a red crystalline compound insoluble in water but soluble in ethanol. It was first used for the histochemical demonstration of copper by Okamoto and Utamura (1938). It forms an olive green - black precipitate with copper which is believed to consist of the imido internal salt of copper rubeanate. The reaction has a sensitivity limit of 0.006 microgrammes and a concentration limit of 1 in 2,500,000 in vitro. The reaction is most specific at neutral or slightly alkaline pH levels especially in ethanolic solutions. Cobalt and nickel also form inner salts with rubeanic acid but these are soluble if the reaction is, (a) acid at the beginning of the procedure and (b) if the medium in which the reaction is carried out contains acetate ions.

Two basic methods were attempted using rubeanic acid.

Uzman's method (1956)

The method employed by Uzman was designed to avoid loss or mobilisation of stored copper by fixing, dehydrating and staining the tissue for copper simultaneously.

Small pieces of fresh tissue were cut with a clean scalpel into pieces not more than 2 mm. thick. These were immediately placed in a bottle containing 0.1% rubeanic acid in 70% ethanol. All glassware used in the procedure was soaked in 50:50 nitric acid overnight, rinsed

in tapwater and exhaustively rinsed in deionized glass distilled water. AnalaR ethanol (copper free) was used to dissolve the reagent. After 10 - 15 minutes sodium acetate (AnalaR) was added to the ethanolic rubeanic acid solution in the solid form at the rate of 200 mg. per 100 ml. of reagent. The bottle was securely stoppered and the contents mixed by shaking. The tissue was left for 24 - 48 hours in the reagent and then transferred to three changes of 70% ethanol in which it was left overnight. After the excess rubeanic acid had been washed out with ethanol no stringent precautions were necessary to prevent copper contamination. The tissue was then transferred to three changes of absolute ethanol for 6-8 hours, cleared in xylene, impregnated and embedded in 54°C paraffin wax and cut in the usual manner. After cutting the sections were dried on to slides without adhesive, dewaxed in xylene and mounted. Mounting was achieved in cellulose caprate (tricaprate, tridecanoate) after the method of Lillie (1965). This is a pale yellow resin of refractive index 1.47 (when dry). It was used as a 50:50 mixture with xylene. The use of this mountant made unstained sections more readily visible, nuclei and cell granules etc. being readily perceived.

The technique described above resulted in a fine green-black precipitate at the sites of copper storage in the tissues.

Howell's method (1959)

In this technique sections were cut after fixation in cold (4°C) AnalaR methanol or ethanol and affixed without adhesive to clean glass slides. The slides had been washed in a non-ionic detergent, rinsed in tapwater, left in 50:50 nitric acid overnight and rinsed exhaustively in deionized glass-distilled water. A stock solution of 0.1 gms rubeanic acid in 100 ml of absolute ethanol was prepared. The solution keeps well. 5 ml of the stock solution was added to 100 ml of a 1o% aqueous solution of AnalaR sodium acetate. Sections were placed in a sealed Coplin jar and incubated at 37°C overnight. This technique gave better results

than the first, copper staining reliably as green-black granules. One disadvantage was that if the sections were not adequately fixed to the slides detachment occurred during the long incubation period.

(b) p - Dimethylaminobenzylidinerhodanine

This is a pink crystalline substance soluble in ethanol but not in water. Its use is described by Howell (1959). In acid media it yields a violet colour with cuprous ions and in neutral media it reacts with cupric ions forming a red-violet complex. The complex is formed with cuprous ions at a concentration of 3×10^{-7}.

A saturated alcoholic stock solution was prepared and 3 -4 ml of this were added to 100 ml of deionized glass-distilled water. Sections treated as for Howell's rubeanic acid technique were placed in the medium in a Coplin jar, sealed and incubated overnight at $37^{o}C$.

The technique was used infrequently as the results were disappointing. No red or violet granules were ever observed but in many cases a pink background was seen in sections indicating diffusion of the complex.

(c) Sodium diethyldithiocarbamate

This is a yellowish-white crystalline substance soluble in water. A yellow-brown colloidal suspension is produced in combination with copper. The reagent can detect copper down to a concentration of 2×10^{-8}.

Alcohol fixed sections were brought to water and flooded with a filtered saturated aqueous solution of the compound. They were left for 30 minutes after which they were washed in water and mounted either in glycerine jelly or dehydrated in ethanol, cleared in xylene and mounted in cellulose caprate. Results were intermediate between those achieved with the first two reagents. Copper stained as a brownish somewhat amorphous mass and discreet granules were not easily seen. The brown stain was also somewhat misleading as it was easily confused with naturally occurring pigments.

Of the four methods Howell's rubeanic acid method was considered to be

superior and was most used.

Fresh frozen sections cut using a "Slee" Pearse-type cryostat were used on occasions. The results obtained with these were inferior to those where tissue was fixed in cold alcohol and there never appeared to be any significant increase in the amount of copper retained in the tissues compared with fixed material.

ii) Results

Tests for copper were performed on control fish and also on tissues from 9 fish exposed to 0.1 ppm of copper for periods between 20 and 120 days. Skin, gills, liver, spleen, pancreas, kidneys and mid-gut were all examined.

(a) Control fish

Copper was never demonstrated in any tissues of control fish using the methods described although the concentrations of copper measured in various organs ranged from 40 to 65 ppm copper per dry weight of tissue. (See Section 11). One explanation for the negative reactions obtained may be that in the tissue the copper is tightly bound to proteins and is not free to react. In order to attempt to release this "bound-copper" mounted sections were cleared in xylene and then placed face down over a beaker one third filled with concentrated hydrochloric acid for 15 minutes. After exposure the sections were rinsed in absolute ethanol for 10 - 15 minutes and then transferred to the rubeanic acid medium. Uzman (1956) found that this method increased the intensity of staining of copper in nervous tissue. In the present study hydrochloric acid treatment resulted in a largely negative reaction although in the liver a greyish background was seen which may have been due to particles of submicroscopic dimensions.

(b) Experimental fish

Skin

A positive raction for copper was never observed in any of the skin preparations.

Gills

A positive reaction for copper was never seen in gill preparations.

Liver

Using all three techniques a rapid accumulation of copper was demonstrable in the liver. With rubeanic acid green-black granules were visible in the liver at 20 days and a progressive increase in the amount of stored copper was observed in subsequent preparations. The copper was confined to the parenchymatous cells and was never seen in the endothelial cells of blood vessels or in the lining of bile ducts. (see plates 34, 35 & 36). Rubeanic acid gave by far the best results. Sodium diethyldithiocarbamate produced a yellowish brown colour over the whole of the organ and p-dimethylaminobenzylidinerhodanine gave only a faint pink colour to the tissue. The accumulation of copper in the parenchyma cells was noticeably uniform, no area seeming to contain more copper than others, except that around some blood vessels the accumulation seemed to be a little greater. In fish exposed to the metal for long periods where shrinkage of the liver had occurred the concentration of copper deposits in the organ was very high and a greenish colour was noticeable in the stained tissue without the aid of a microscope. Erythrocytes in the hepatic blood vessels were examined but they never showed a reaction for copper. In every case examined the picture was the same, large amounts of copper appearing rapidly and filling out the hepatic cells. It is also of interest to note that the liver of a goldfish (Carassius auratus) which had been maintained in 1 ppm of copper sulphate (1 ppm as the salt) for 28 days, when tested also contained a similarly large amount of demonstrable copper (see plate 37). The uptake of copper by the liver demonstrated by this technique is confirmed by analysis in the next section.

Spleen

No copper was ever detected in the spleen.

Pancreas

Copper was never observed in the pancreatic tissue proper but in the connective tissue between the pancreas and the liver some of the stored pigments which normally appeared brownish-black appeared to

Plate 33

Skin of a fish after 50 days exposure to 0.1 ppm zinc (X920). Note the extreme inflammation and the active mucous cells.

Plate 34

Liver from a fish exposed to 0.1 ppm of copper for 120 days (X 150). Rubeanic acid stain showing the even distribution of stored copper.
N.B. The deposits are in fact dark green but the colour has been altered during printing.

Plate 35

As above, but showing detail of the copper containing cells. (X 920).

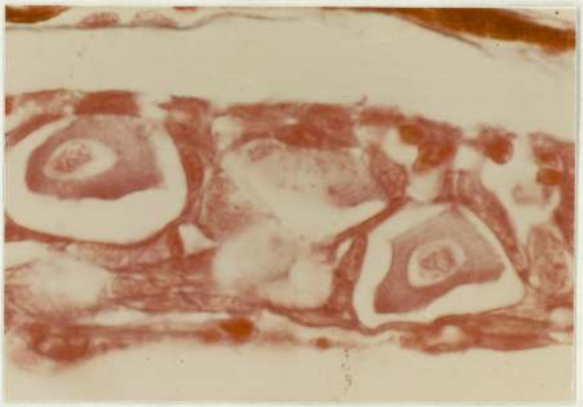
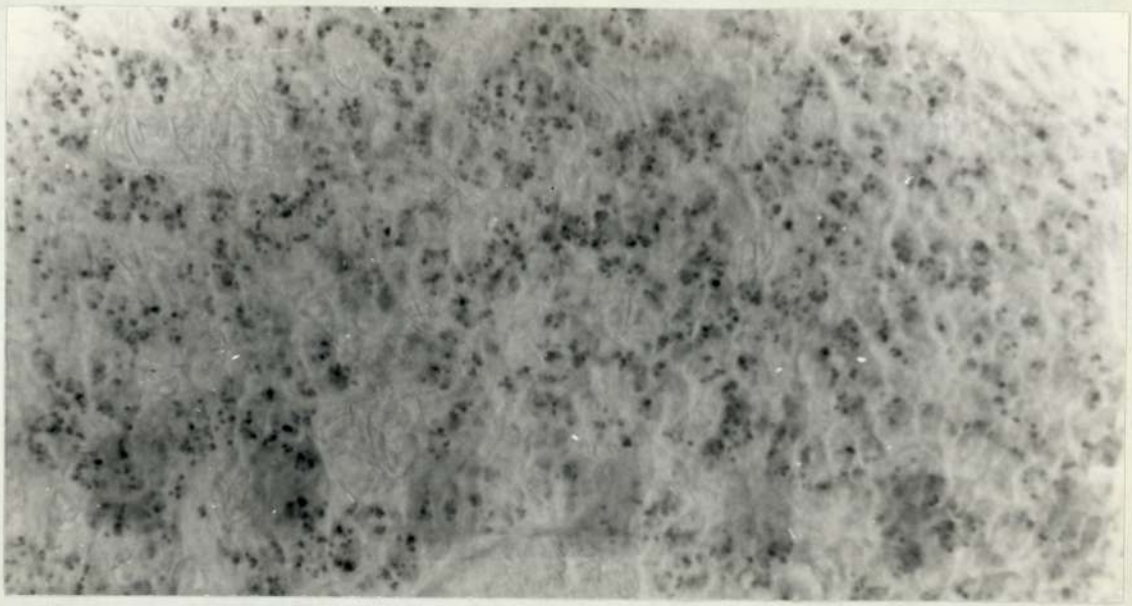


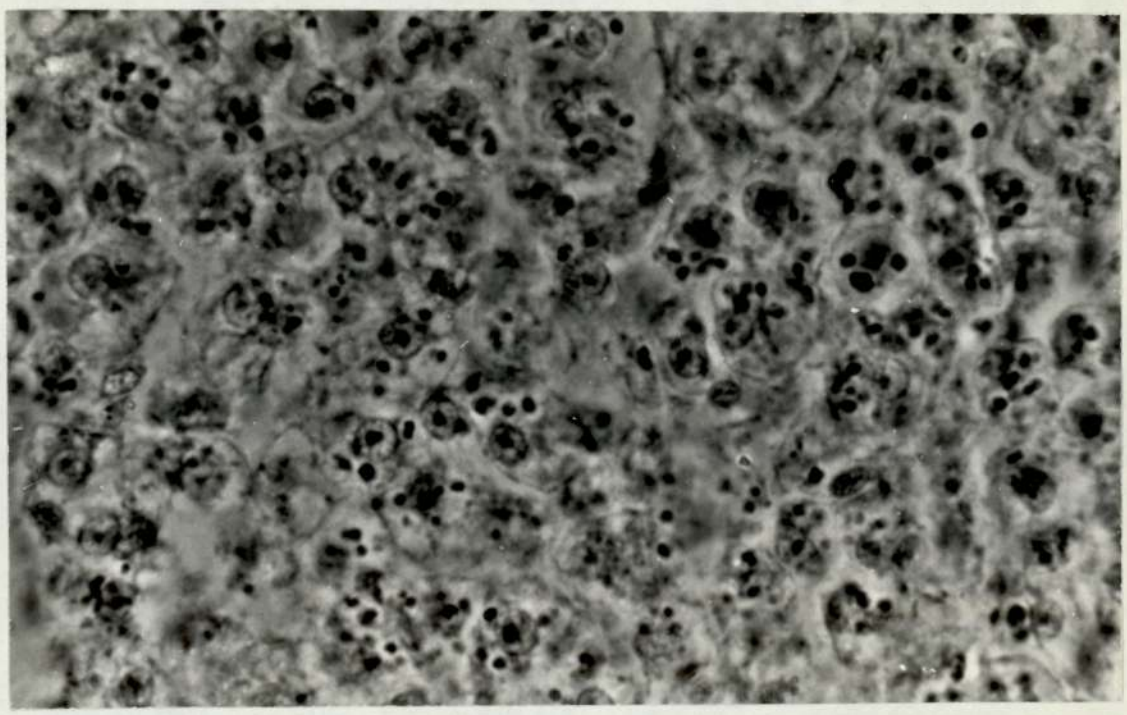
Plate 36

As plates 34 and 35 (X 420) showing the even distribution of stored copper in the liver.

Plate 37

Section of the liver of a goldfish maintained in 1.0 ppm of copper for 28 days. (X 690). Rubeanic acid technique. Note the extensive deposits of copper (darkest granules) in the hepatic cells.





stain lightly for copper.

Kidneys

In the six fish examined after exposures of from 20 to 58 days no reaction was observed in any kidney tissue. In the three remaining fish copper was never detected in the tubular epitheliun by any of the techniques used, but using the rubeanic acid method a slight reaction was detected in patches in the intertubular tissue in two cases and in all three stored pigment stained an olive green colour. In two cases (98 and 120 days) the contents of some tubules stained for copper with rubeanic acid and sodium diethyldithiocarbamate but not with p-dimethylaminobenzylidinerhodanine. The stain was not associated with granular debris but with an amorphous mass within the lumen. The intensity of the stain was however slight. Gravimetric analyses (see section 11) indicated an almost threefold increase in the concentration of copper in the kidney after 90 days exposure. The only explanation for the lack of stained copper in the kidneys (apart from reaction insensitivity) would seem to be that the copper is present mainly in the urine and as such is not easily demonstrated histochemically.

Intestine

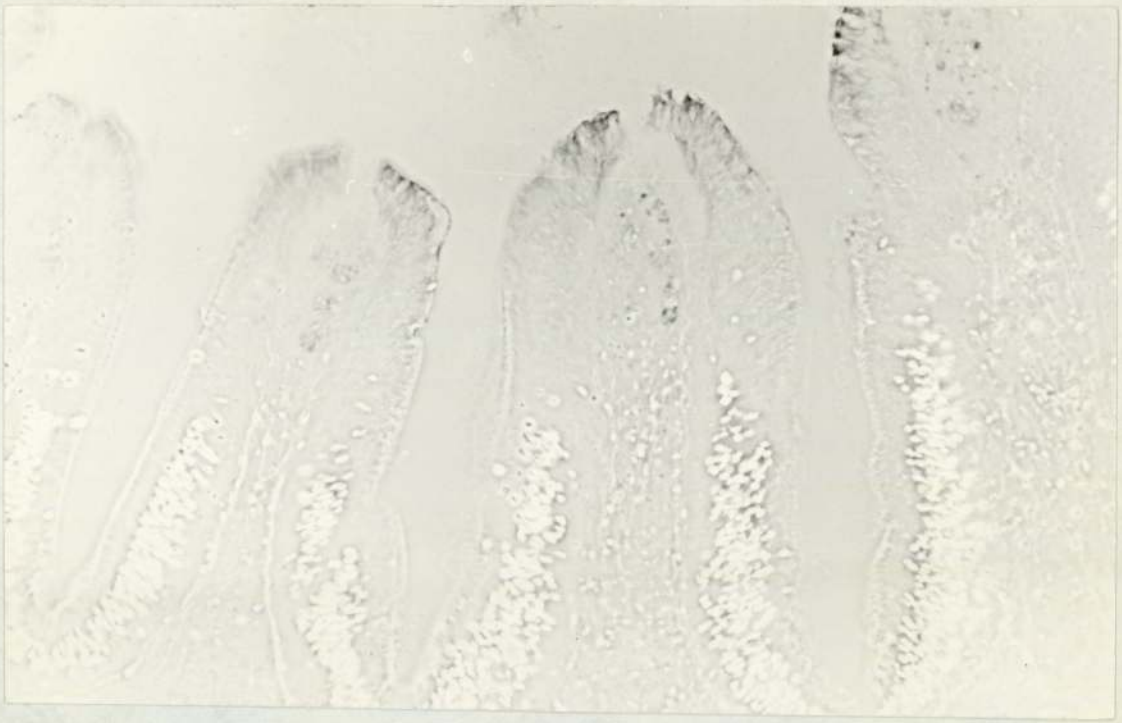
The anterior part of the midgut was examined in all nine cases. In five cases copper was detected in the gut using Howell's rubeanic acid technique. One fish preserved at 36 days had deeply staining areas at the tips of the rugae. (see plate 38). Three fishes exposed for between 40 and 60 days had large goblet mucous cells in the mucosa which were outlined by a greenish black stain (see Plate 39). A fish taken at 55 days exhibited "vacuoles" in the mucosal epithelium which stained very deeply for copper. (see Plates 40 and 41). The copper containing vacuoles did not appear to be mucous goblet cells and some copper-staining bodies were also noted in the loose connective tissue of the submucosa. Freshwater fish do not normally "drink" their external medium although some drinking had been demonstrated in fish including

Plate 38

Midgut of a fish exposed to 0.1 ppm of copper for 36 days. (X 600). (Phase contrast). Rubeanic acid technique. The tips of therugae are strongly positive for copper.

Plate 39

Midgut of a fish exposed to 0.1 ppm of copper for 40 days. (X 880). (Phase contrast). Rubeanic acid technique. Mucous goblet cells are outlined with the stain.



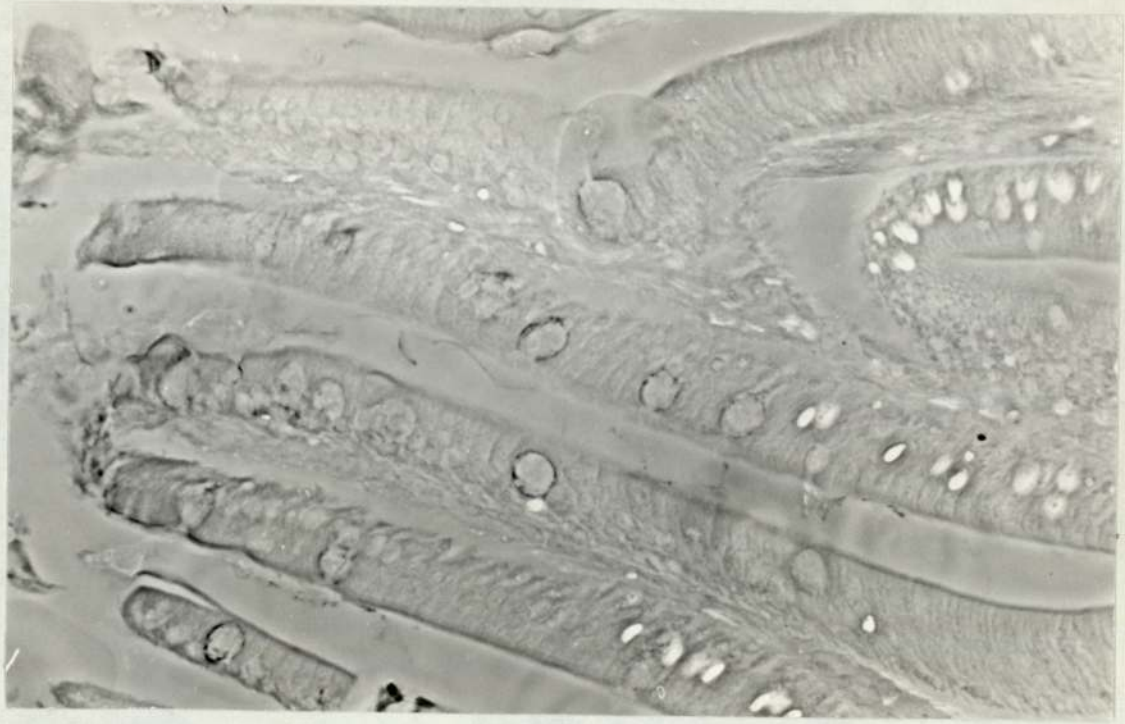
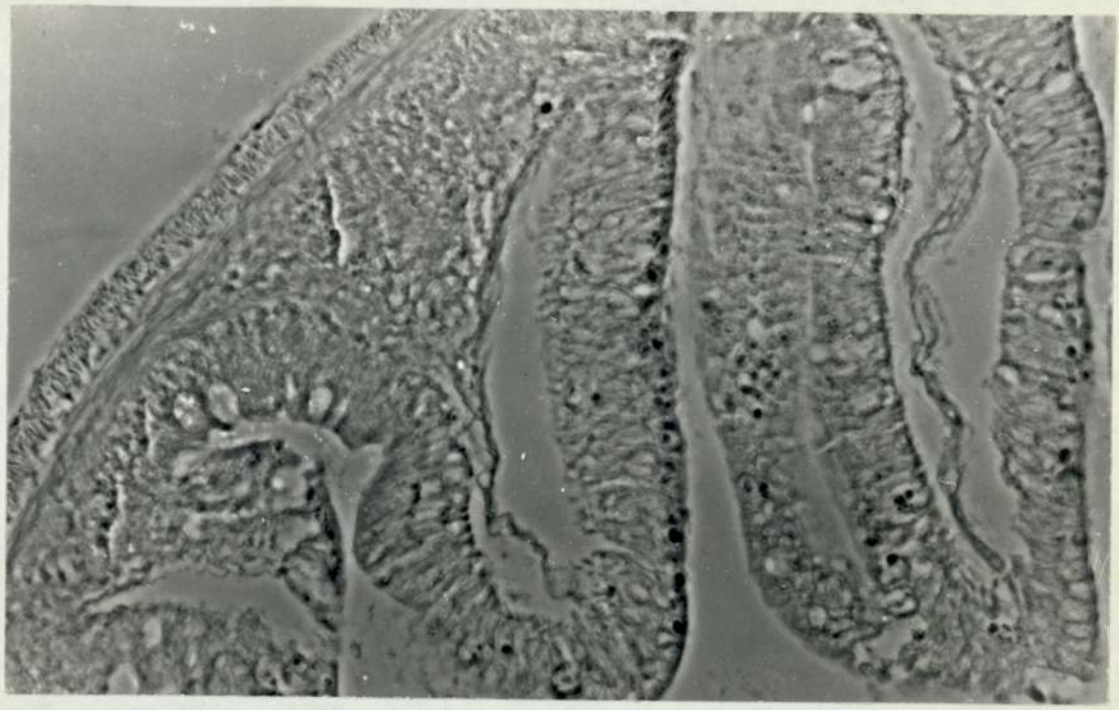


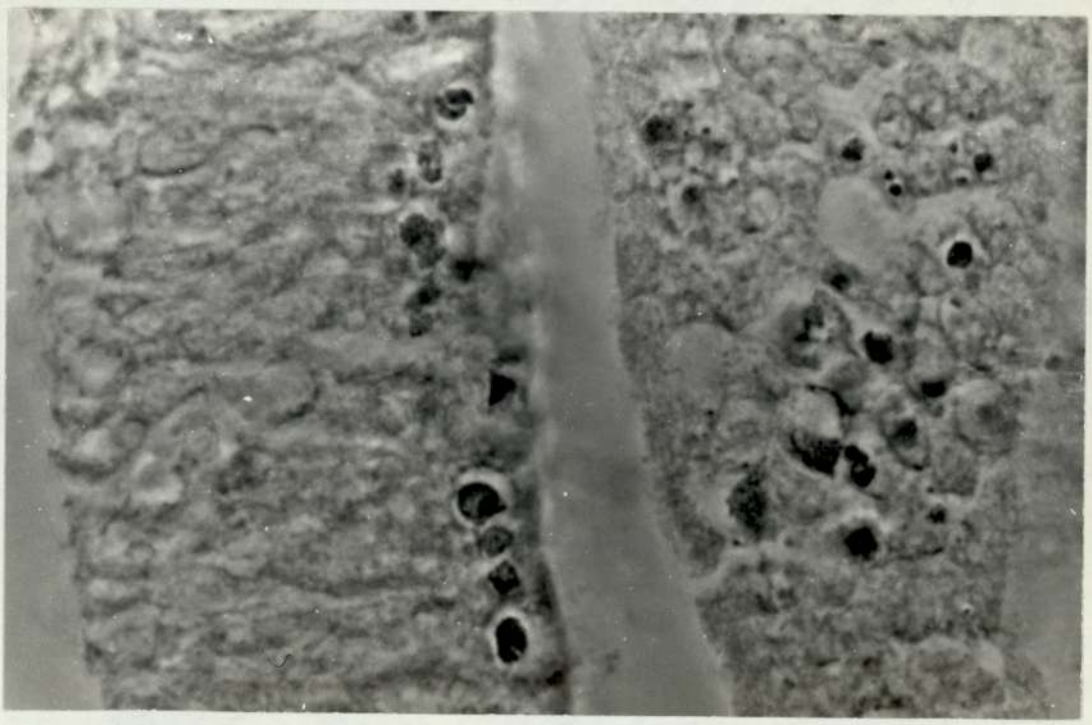
Plate 40

Midgut, 55 days exposure to o.1 ppm of copper.(X 440). (Phase contrast). Rubeanic acid technique. "Vacuoles" exhibiting a positive reaction for copper are evident in the mucose particularly in the centre of the field of view.

Plate 41

As above. (X 1720). The "vacuoles" are shown not to be mucous cells.





Cyprinids by Frank and Allee (1950) and Abegg (1949). The uptake of copper from the external medium by the gut may be due to drinking or due to the fact that the food taken in by the fish was copper contaminated. Contamination of food which had lain in the toxic solution for some time before being eaten was evident when the gut contents when present gave a positive response with rubeanic acid.

4. Methods for lead

i) Histochemical tests for the detection of lead in tissues have been described by Mallory and Parker (1939), Timm (1936, 1958, 1960, 1963) and Voigt (1959). The method of Mallory and Parker relied on non-specific staining with haematoxylin. The silver sulphide-technique described initially by Timm is considered to be the most sensitive for the detection of heavy metals. Modifications of the method for use in electron microscopy have recently been described by Okamoto and Kawanishi (1966) and Pihl (1967). A methodological study of the silver sulphide technique has been made by Brunk et al (1967).

The technique relies on the fact that heavy metals in tissues, when transformed into sulphides, catalyse the reduction of silver ions into molecular silver. The reaction is performed in a solution of Ag^+ ions with a reducing substance, usually hydroquinone, in the presence of a protective colloid such as gum arabic. With suitable concentrations of the three reagents in a weakly acid mixture a redox - reaction takes place and the sulphides become coated with a steadily enlarging shell of metallic silver. The process is largely analogous to the development of a photographic film. The initial production of metal sulphide in the tissues is achieved by treating either small pieces of tissue or sections with hydrogen sulphide gas dissolved in 70% ethanol or with an alcoholic or aqueous solution of ammonium sulphide. The method chosen should be the one which converts the metals to sulphides as quickly as possible. It is equally important to prevent the loss of sulphides when formed. It is well known that many metal sulphides are easily oxidised by atmospheric oxygen in the presence of water, water soluble products

being formed, (Timm 1963, Brunk et al 1967, Pihl 1967). Because of this, the interval between sulphide and silver treatment should be as short as possible and the tissue should not be mounted in an aqueous medium after initial sulphide treatment. Brunk and Sköld (1967) showed that some metal sulphide is oxidised and lost in paraffin embedded material during the steps used for deparaffinization and thus this stage must proceed as rapidly as possible.

The silver-sulphide technique as described above does not demonstrate a specific metal but merely gives a picture of all heavy metals which form insoluble sulphides. In order to attain specificity some workers (Timm 1963, Falkmer et al 1964, Haider 1964) have tried to dissolve out all metal sulphides except for the one under investigation. In the present study similar methods were applied, however the techniques are not easy and their absolute effectiveness is questionable. Sulphides are difficult to remove from the hydrophil colloid of the cell substance and false negative results may occur if part of the cytoplasm including metal sulphide is removed by treatment with strong acids etc.

Materials and methods

Either:-

(1) Small pieces of tissue were taken (not more than 3 - 4 mm thick) and fixed in 70% ethanol saturated with hydrogen sulphide gas for a maximum of 15 hours. Tissue pieces were then washed in 3 changes of 70% ethanol for 3 - 4 hours and transferred to 90% ethanol overnight. Dehydration was completed in three changes of absolute ethanol for two hours and clearing in two changes of xylene for one hour. Tissue blocks were then impregnated and embedded in 54°C paraffin wax and cut at 5 microns. Ribbons of sections were floated out on glass-distilled water at 45°C and mounted on clean glass slides without adhesive. Cleaning of the slides was as described for copper techniques. After drying at 37°C for two hours sections were quickly deparaffinized in two changes of xylene (30 seconds each) and rehydrated as far as 70% ethanol. The sections were then placed in the 70% ethanol/hydrogen sulphide solution

in a sealed Coplin jar and left overnight.

Or:-

(2) Pieces of tissue were frozen with solid carbon dioxide and sections cut at eight microns on a "Slee" Pearse-type cryostat. Sections were mounted on clean warm slides and allowed to dry for 30 minutes at room temperature. Sections were then placed in the 70% ethanol - H_2S solution for 12 - 15 hours.

At this stage the treatment for both paraffin and frozen sections was identical. Sections were transferred from H_2S - alcohol to two changes of 70% ethanol and thence to double distilled water. Following the method of Haider (1964) the sections were immersed in 50% AnalaR sulphuric acid for 5 - 10 minutes. This treatment was intended to remove zinc and iron which are soluble in mineral acids. The sections were then washed in distilled water with extreme care to prevent detachment from the slides. They were then transferred to 0.5% potassium cyanide solution for 5 minutes in order to remove any copper sulphide. The sections were then again washed carefully in distilled water.

The "development" of the sulphide particles in the tissues was carried out using the following solution after Lillie (1965).

40% gum arabic	-	60 ml	
10% silver nitrate	-	0.5 ml	
Reduction fluid	-	5 ml	
<u>Reduction fluid</u>		0.4 g	Hydroquinone
		0.9 g	Citric acid
		20 ml	Double distilled water

All solutions were stored separately until needed. The reduction fluid and the silver nitrate solution were made up freshly when required and stored in the refrigerator in brown bottles in the dark. The gum arabic solution was prepared 14 days in advance and stirred with a glass rod every day in order to obtain the correct colloidal properties. A few thymol crystals were added to prevent the growth of moulds and the suspension was stored at 4°C. Gum arabic solutions over ten weeks

old were discarded. All reagents except the gum arabic were of AnalaR purity. In some later tests a developing solution after Brunk et al (1967) was used. This consisted of,

20% gum arabic	- 100 ml
10% silver nitrate	- 1 ml
5 g citric acid	
and 2 g of hydroquinone	
in 100 ccs of distilled water	- 10 ml

This developing solution appeared to give better results probably due to the greater ease of removal of the thinner gum arabic suspension after development.

The gum arabic and silver nitrate solution were mixed and shaken well for five minutes. The reduction fluid was then added and the whole shaken well for another 30 seconds. This was most important as it ensured even dispersal of the reagents in the colloid. The mixture was then poured over the sections and left to develop for 1.5 - 2 hours. Development both in light and dark was performed.

Light

All solutions were prepared in the light and after flooding the sections with the reaction mixture they were exposed to the light from a 60 watt bench lamp.

Dark

The reagents were mixed under a photographic safe light in a dark-room and the development left to proceed in total darkness.

In general material developed in the dark gave better results. In artificial light and daylight the developer turned rapidly brown and then black due to the accumulation of reduced silver. In the dark the developer remained clear even though metallic silver was attached to the sulphide particles. After development periods of approximately 100 minutes in the light fine non-specific precipitates of silver appeared in the tissue sections although silvering of the sulphide

particles was not sufficient. No silver precipitates of this sort occurred where development was carried out in the dark-room. Sections were developed in the dark for 90, 105, 120 and 135 minutes. After 135 minutes the silver precipitate was very intense and merging of individual granules occurred. 120 minutes was considered the optimum time for all tissues.

After development the sections were washed in several changes of distilled water. Washing overnight or for at least eight hours was found necessary to remove excess gum arabic. Sections were then stained lightly in Ehrlich's haematoxylin, "blued" in tap water, dehydrated in graded baths of ethanol, cleared in chloroform and mounted in Gurr's Refrax. This mounting medium has a high refractive index (1.666 when dry) and rendered the silver particles more easily visible.

ii) Results

(a) Normal silver-sulphide picture (S.S.P.)

The normal S.S.P. is the term applied to the picture obtained when a tissue section is stained using the technique without attempting to remove other metals. Control sections, both frozen and paraffin embedded were treated thus in order to ascertain the general heavy metal reaction of the tissues for comparison with experimental fish. Gills, liver, spleen, pancreas, mid-gut and kidneys were routinely examined.

Gills

The normal S.S.P. for the gills was in all cases virtually negative. Some darkening of the bony arch was noticeable which may have been due to submicroscopic particles but no reaction was ever observed in the soft parts.

Liver

The normal S.S.P. for the liver was a little varied. Paraffin sections gave superior results to cryostat cut material. In two cases out of seven examined a fine granulation was noticeable in the parenchyma cells. No granules appeared in the lining of bile

capillaries or in the endothelia of blood vessels. In the other five cases no granulation was seen but the cytoplasm of the parenchyma cells had a grey-brown appearance whilst that of other cells did not.

The reason for the lack of reaction in the control liver may be due to binding of the metals present. It is questionable whether all the heavy metals in the liver can be transformed into sulphides. It is probable that heavy metals in prosthetic groups have such a complex binding that sulphide linking is prevented. Also steric factors may be important. The metal atoms may be hidden in macromolecules and thus be less accessible or inaccessible so far as conversion to sulphides is concerned.

The reason for the inferior results with cryostat sections may be that some of the tissue metal complexes are water soluble. During both the freezing and thawing of the tissues movements of fluid could remove the complexes from their original sites. The associated increased membrane permeability caused by freezing and thawing would presumably facilitate this.

Spleen

The pigmented and granular material within the spleen did not exhibit a convincing reaction. The pulp had no black granules but a brown background colour always developed again possibly indicating particles of submicroscopic dimensions.

Pancreas

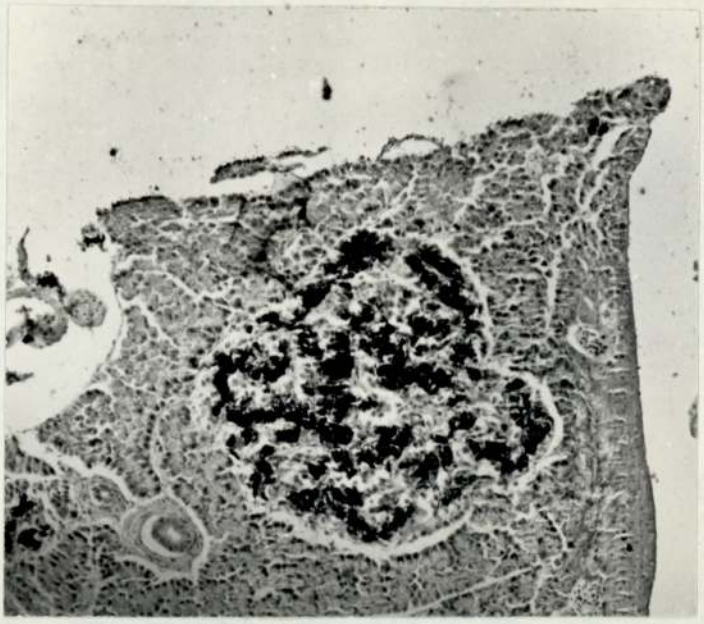
The pancreas of the minnow has a few giant islets of Langerhans. In all cases the islet tissue exhibited a very strong reaction. (see Plate 42). This reaction was considered to be due to their high zinc content. Zinc is known to be present both in mammalian and fish islets in association with the enzyme carbonic anhydrase. Where zinc removal from the tissue was attempted using 5% sulphuric acid a large measure of success was achieved as the blackening of the islet tissue was considerably reduced although a slight reaction was still seen. The acinar tissue was

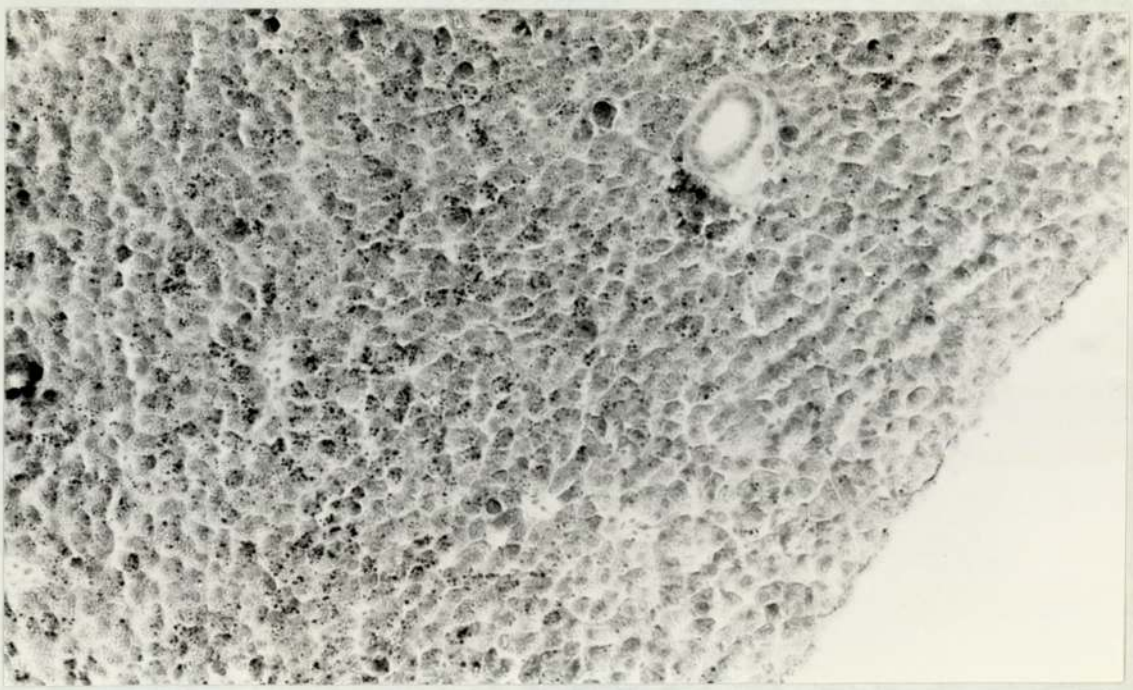
Plate 42

Normal silver-sulphide picture of the pancreas of a minnow. (X 430). Note the very intense positive reaction for zinc (black granules) in the islet tissue.

Plate 43

Liver of a fish exposed for 243 days to 0.1 ppm of lead. (X 275). Silver-sulphide technique (for lead). Note the extensive deposits of silver in the organ with increased aggregations near to blood vessels.





always negative.

Midgut

The mucosal epithelium of the midgut was examined in seven cases and in all a positive reaction was noted. The typical S.S.P. was one of extremely fine granulations in the upper part of the cells. This granulation was absent in three cases but a grey-brown colour was always noticeable in the epithelial cells and not in the cells of the submucosa. The presence of heavy metal in the mucosal epithelium may be associated with the high acid phosphatase activity found there. (see Section 13).

Kidneys

The kidneys were almost negative in the seven cases studied and no conspicuous black granulation was seen. In four cases however a distinct darkening of the area immediately below the brush borders of some of the tubules was evident. Very fine granulation was seen in some cases but was difficult to observe. Like the intestine the heavy metal in the kidney may be associated with phosphatase activity.

(b) Experimental fish

Tests for lead were carried out on tissues from 9 fish exposed to 1.0 ppm of lead for periods of from 30 to 345 days.

Gills

Results obtained in all cases were directly comparable with those from control fish.

Liver

In fish examined after 30 days exposure the liver contained no noticeable granulation other than that observed in control fish. Three fish examined at 65, 70 and 78 days respectively had definite black granules in some of the parenchyma cells. The deposits were not uniformly distributed and some cells contained large amounts of silver where others had none. The cells with a strong reaction were generally in clusters and were always proximal to blood vessels. Deposits were not observed in the lining of bile capillaries or blood vessels.

In a fish examined at 105 days the picture was similar to that described above. Two fish were examined at 243 days and in both cases the liver reacted strongly. Extensive silver deposits were found in most of the parenchyma cells. (see Plate 43). Dense accumulations were evident in some groups of cells especially in those near to blood vessels. Where some of the heavy deposits occurred there appeared to be limited necrotic patches. Again no granules were seen in the lining of bile ducts and blood capillaries, neither were they ever seen in an intranuclear position. Kupffer cells also contained granules but due to the treatment of the sections these cells were difficult to see, the most reliable characteristic being the somewhat elongate nucleus. Two fish were examined at 345 days. The livers appeared similar to those of the fish taken at 243 days. The extent of the silver deposits was no greater and the liver was of normal size. The liver cells were however more vacuolated than in earlier cases. (see Plate 44).

Spleen

The spleen was examined in 8 of the 9 experimental fish. In all cases there was an increase in stored pigment as noted in the last section. The S.S.P. was negative in most cases although in both fish at 345 days and one at 243 days some of the brownish granular material deposited in the organ showed a faintly positive reaction.(see Plate 45).

Pancreas

Apart from a reaction in the islet tissue due to insufficient removal of zinc, the pancreas was negative in all cases.

Midgut

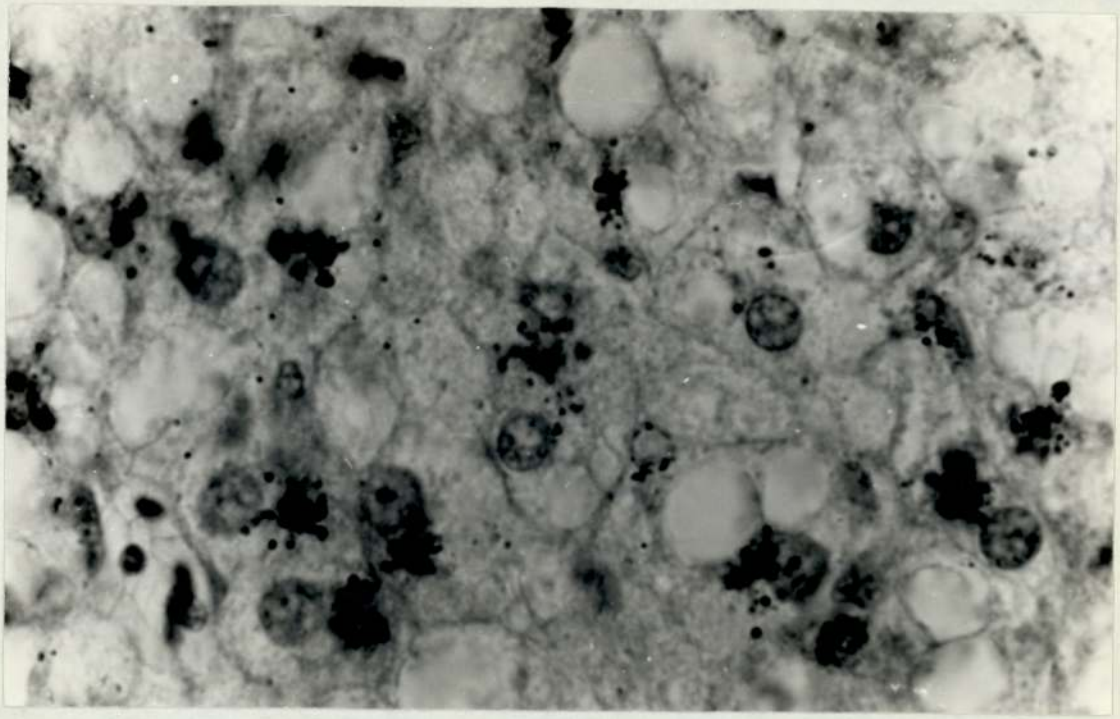
In four fish (70, 78, 105 and 345 days) a positive reaction was seen in the mucous epithelium of the midgut. Granules were found in the upper part of the cells. (see Plate 48). The distribution of the granules was very uniform in the sections examined. They never appeared in an intranuclear position and were always in the upper part of the epithelial cells and never below the positions of the nuclei. Granules

Plate 44

Liver of a fish exposed for 345 days to 1.0 ppm of lead. (X 1720). Silver-sulphide technique. Detail of silver deposits within the hepatic cells. Vacuolation probably due to alcohol fixation is also evident.

Plate 45

Spleen of a fish exposed to 1.0 ppm of lead for 345 days. (X 920). Silver-sulphide technique. Silver granules associated with pigment deposits.



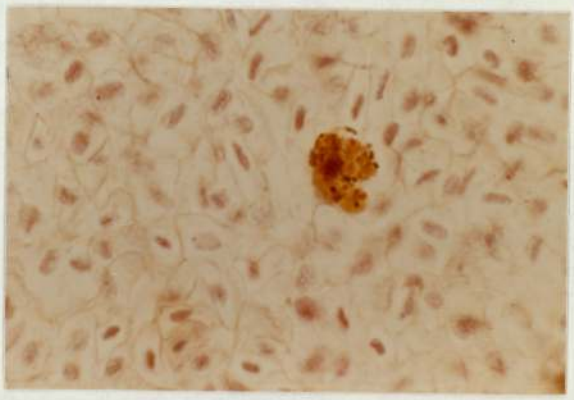
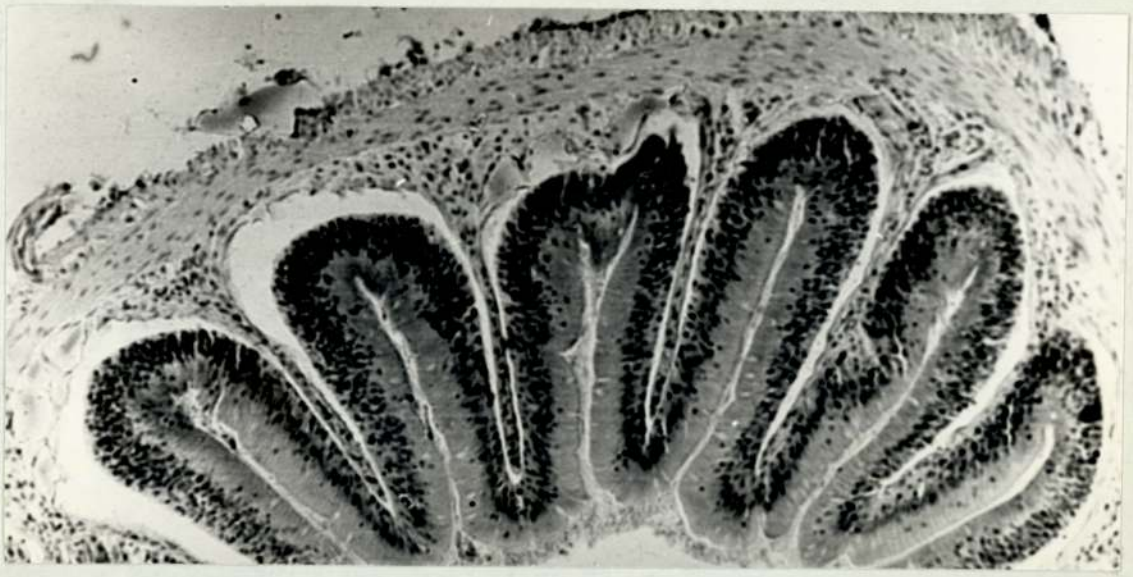


Plate 46

Midgut of an untreated fish. (X 172). Silver-sulphide technique, haematoxylin counterstain. Compare with plate 47.

Plate 47

Midgut of a fish exposed to 1.0 ppm of lead for 78 days. Silver-sulphide technique, haematoxylin counterstain. Dense aggregation of silver granules can be seen in the mucosa mainly below the paler more elongate nuclei. Silver deposits are also visible in the submucosa.



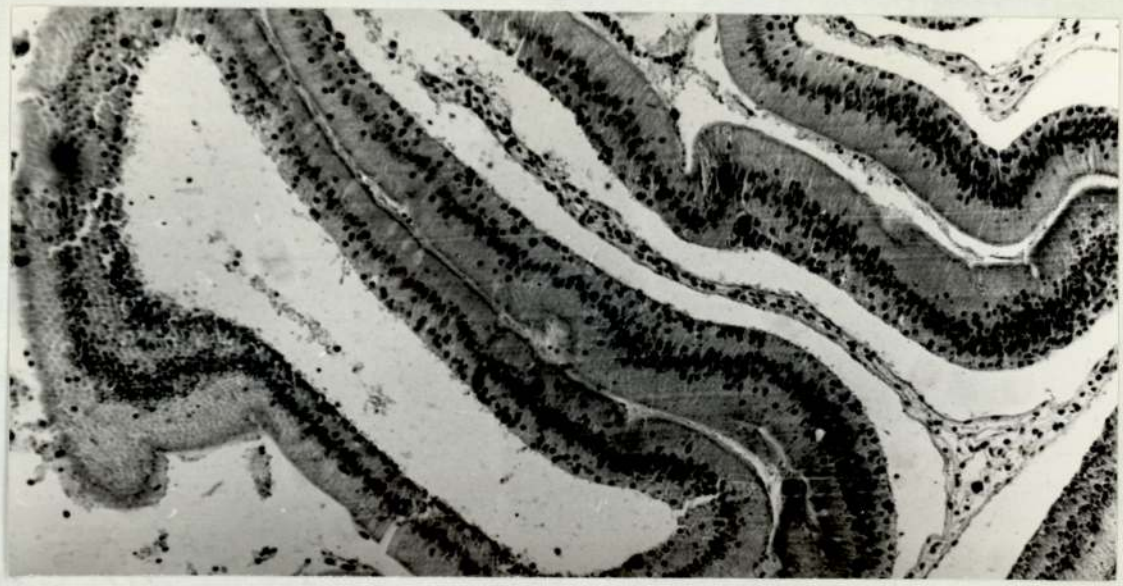
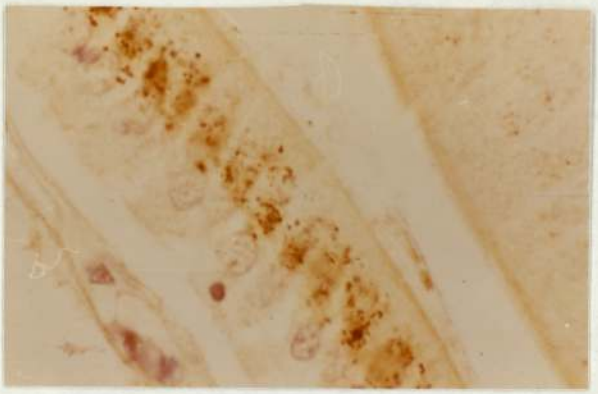


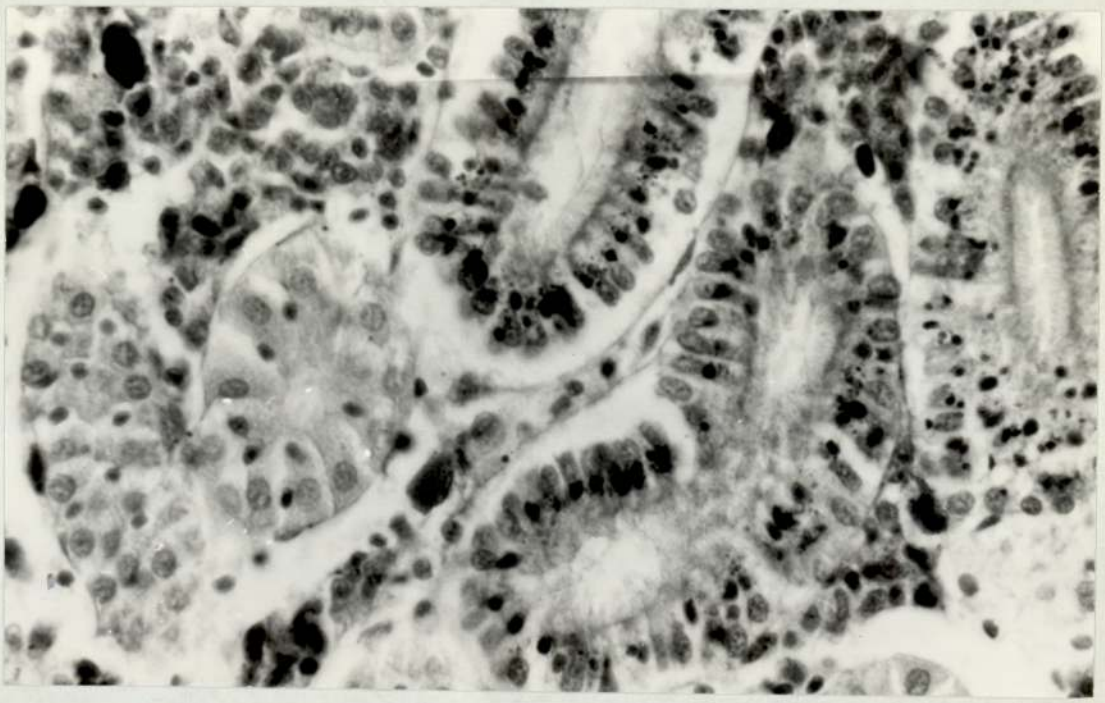
Plate 48

Silver deposits in the mucosa of the midgut of a fish exposed to 1.0 ppm of lead for 345 days. (X 920). Silver-sulphide technique, haematoxylin counterstain.

Plate 49

Kidneys of a fish exposed to 1.0 ppm of lead for 345 days. (X 1720). Silver-sulphide technique, haematoxylin counterstain. Marked deposits of silver can be seen in the tubular epithelial cells of the proximal tubules but not in the more cuboid cells of the distal tubules.





of equivalent size were never seen in control fish and it is thus presumed that they were indicators of lead absorbed by the gut wall. In one case (78 days) an unusual observation was made. Great numbers of very densely staining bodies appeared towards the base of the columnar epithelial cells. With very few exceptions these bodies were situated lower in the cells than the nuclei. They were also observed in the submucosa. (see Plates 46 & 47). On closer inspection they appeared to be the extremely dense nuclei of many small amoeboid cells. They were never observed in any other fish, control or experimental and no explanation could be found for their presence or for the apparent positive reaction of their nuclei.

Kidneys

The kidneys of fish exposed for 70, 78, 105, 243 and 345 days exhibited similar reactions . In the first two cases (70 & 78 days) the only reaction was that some of the tubules had a darkened appearance . The tubules which reacted thus were considered to be proximal tubules, the more cuboidal epithelial cells of the distal tubules remaining clear. At 243 and 345 days as well as darkening of the tubules positive granules were noted below the brush borders of some tubules. (see Plate 49). In many cases large aggregations of granules obscured the nuclei. The basement membrane of the tubules exhibited a slight reaction in some cases but it was never extensive. This observation is in disagreement with that of Haider (1964) who observed initial storage of lead around the basement membrane when trout were poisoned by chronic concentrations of lead acetate. Silver deposits were also noted in some cases in the peritubular tissue.

Tests for lead with sodium rhodizonate

Sodium rhodizonate is recommended by Pearse (1961) as a reagent for the histochemical detection of lead.

Using tissue from fish exposed to 1.0 ppm of lead for 78, 243 and 345 days both frozen sections and paraffin material were tested.

Some tissue was fixed in cold (4°C) absolute AnalaR methanol

overnight, cleared in xylene, impregnated and embedded in 54°C paraffin wax, cut at 8 microns mounted on clean slides, dried, deparaffinized and rehydrated.

Frozen sections were cut at 10 microns on a cryostat, mounted on warm slides and air dried.

Fixed material was placed in a 0.2% solution of sodium rhodizonate in 1% acetic acid. Frozen sections were placed in the same solution to which formalin had been added in order to fix them.

After one hour the sections were removed, washed quickly in distilled water and mounted in glycerine jelly.

The livers of the 243 and 345 day fish stained a pale red colour in places. The stain was very unsatisfactory and red granules were never seen. All other tissues were negative.

5. Methods for Nickel

i) Reagents for Nickel detection

The literature relating to histochemical methods for the detection of nickel is sparse.

According to Lison after Lillie (1965) a fresh alcoholic pure haematoxylin solution stains nickel salts lilac to blue.

Choman (1962) adapted the dimethylglyoxime method for nickel from Feigl (1958) for the histochemical demonstration of the metal. This method was attempted in the present study.

Rubeanic acid reacts with nickel to give a blue-violet insoluble inner complex salt. (Feigl 1958). For the demonstration of nickel in tissues Lillie (1965) quotes Romeis as using a solution of 2-5 ccs of 0.1% alcoholic rubeanic acid solution in 100 ccs of 10% aqueous sodium acetate solution. The sections are incubated in this medium at 37°C for 24 hours. According to Uzman (1956) if the medium is acid at the beginning of the procedure and if it contains ethanol and acetate ions the nickel rubeanate produced goes into solution. Because of this danger, in the present study a slightly ammoniacal 1% ethanolic solution

of rubeanic acid was used. (pH approx. 7.8 - if too much ammonia is added the sections are destroyed). This method is based on the spot test for nickel recommended by Feigl (1958).

Materials and methods

Two methods were attempted for the histochemical detection of nickel.

(a) Dimethylglyoxime technique

Tissues were either,

a) Fixed in small pieces (2-3 mm thick) in 4°C absolute AnalaR methanol overnight, cleared in xylene, impregnated and embedded in 54°C paraffin wax, cut at 8 microns, floated on distilled water and fixed without adhesive to clean glass slides. Sections were then deparaffinized in xylene and taken via absolute alcohol to 70% alcohol.

or,

b) Frozen sections were cut on a cryostat at 10 microns, affixed to clean warm slides and air dried for 15-20 minutes.

Sections of both types were then exposed face down to the fumes of concentrated ammonic water in a small beaker. The slides were then removed and a drop of 1% dimethylglyoxime in 95% alcohol placed on each section. After one minute the reagent was washed off gently with 70% ethanol and the sections were dehydrated in 90%, 95% and Absolute ethanol. They were then cleared in xylene and mounted in cellulose tricaprate. Nickel is demonstrated as red acicular crystals and the sensitivity limit according to Choman (1962) is 1 in 10,000,000.

(b) Rubeanic acid technique

Frozen sections and alcohol fixed material were prepared as described for the dimethylglyoxime technique. Fixed material was completely rehydrated.

Sections were placed in a Coplin jar containing a 1% ethanolic solution of rubeanic acid to which had been added enough ammonia to raise the pH of the solution to approximately 7.8. The jar was

sealed and the sections left to incubate at 37°C overnight.

After staining the sections were washed for 10 minutes in 3 changes of 70% ethanol, transferred to 3 changes of absolute ethanol, cleared in xylene and mounted in cellulose caprate.

(ii) Results

(a) Control fish

Using both techniques all control fish gave completely negative results.

(b) Experimental fish

Eight fish were examined after exposure to 4 ppm of nickel for periods from 40 to 203 days.

In the five fish examined after exposures of between 40 - 108 days all tissues proved negative with both tests. With the three remaining fish only the liver gave strongly positive results.

The dimethylglyoxime technique of Choman was always negative. Choman's experiments with the technique involved the detection of nickel absorbed into skin from external nickel solutions and it seems likely that the concentrations of nickel in the tissue would thus be considerably higher than in the present study.

Using the rubeanic acid method the presence of nickel was indicated by a violet-blue coloured precipitate. The coloured complex was not as granular as that where copper was detected presumably due to the greater diffusion velocity of the nickel salt. (Feigl 1958). The distribution of the nickel rubeanate complex in the liver cells was very uneven. The great majority of the parenchyma cells were negative but at 126 days nickel was detected intermittently in some of them. The fish examined at 175 and 203 days presented a changed picture with nickel deposits appearing in very concentrated areas in a few cells whilst other cells contained very small amounts and most of the parenchyma cells were negative in reaction. The large accumulations of nickel were found very close to blood vessels. Sometimes only one cell would be

stained heavily for the metal whilst others about it would be almost negative. (see Plates 50 and 51).

The kidney produced positive results in the fish taken after exposures of 126, 175 and 203 days. The stain was however very weak, a pale violet colour appearing in places in the intertubular tissue. The reactive cells were very scattered but were definitely positive for nickel. No violet colour every appeared in the kidney tubules.

6. Methods for zinc

(i) Reagents for zinc detection

The histochemical demonstration of zinc has received more attention than that for any other heavy metal. The use of silver-sulphide techniques for zinc identification has been widely used and the method has been reviewed under the section dealing with lead identification.

The use of the organic dye diphenylthiocarbazone (dithizone) was first reported by Okamoto (1943) and Sebruyns (1946). Chauncey and Lionetti (1952), Mager et al (1953) (1960), McNary (1954), Rixon (1959), Haumont (1961), Kattenbach (1966) and Okamoto and Kawanishi (1966) have all reported and discussed techniques utilising the dye for the histochemical detection of zinc.

Dithizone is a violet-black solid prepared by condensing carbon disulphide with phenylhydrazine followed by oxidation. It is not water soluble but dissolves readily in carbon tetrachloride, chloroform and acetone to give dark green solutions which are photosensitive and break down in sunlight to a yellow dye diphenylthiocarbodiazone. Dithizone reacts with many heavy metals to form coloured complexes. Earlier methods involving the use of the dye were not absolutely specific relying only on the red colour reaction with zinc to differentiate it from the complexes formed with other metals. Mager et al (1953) were the first to use and test a complex-forming buffer which formed non-reactive complexes with other metals. In the present study this technique was employed.

Plate 50

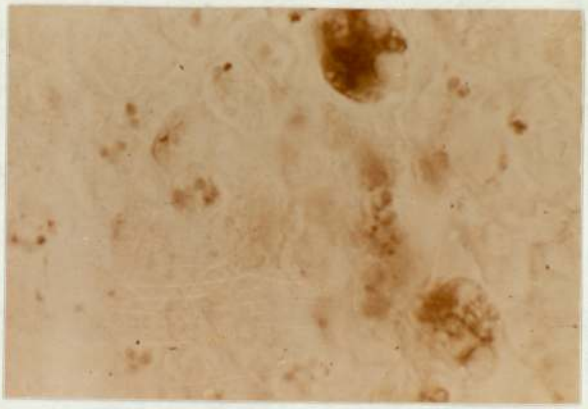
Localised nickel deposits in the liver of a minnow exposed to 4.0 ppm of the metal for 175 days. (X 920). Rubeanic acid method. (N.B. The deposits are in fact a dark violet colour.)

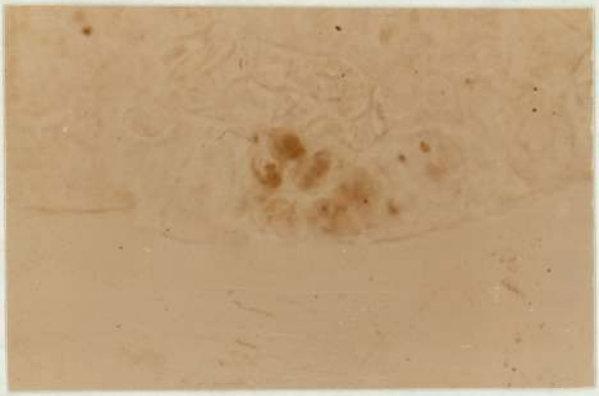
Plate 51

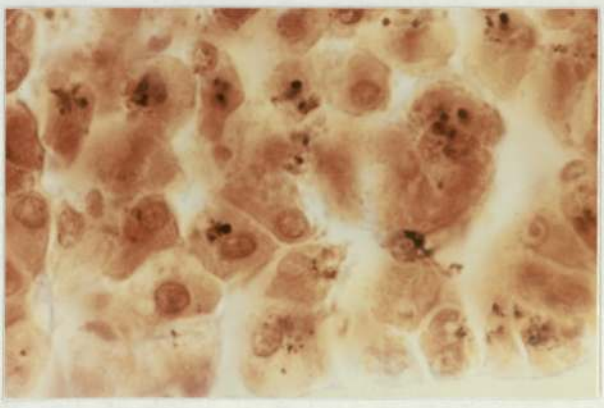
As above. (X 920). The few local nickel deposits were usually associated with blood vessels as illustrated.

Plate 52

Liver of a fish exposed to 0.1 ppm of zinc for 110 days.(X 920). Silver-sulphide technique (for zinc). Detail of an area of heavy silver deposition in the hepatic cells.







(a) Dithizone techniques

All reagents used in the techniques were of AnalaR purity. Only deionized glass-distilled water was used and all glassware was washed in a non-ionic detergent rinsed in tap water, soaked in 50:50 nitric acid overnight and repeatedly rinsed in deionized glass-distilled water before use.

A solution of 0.01% dithizone in acetone was prepared. This solution is light sensitive and was stored at 4°C in an amber bottle. Even under these conditions the solution did not last and was replaced every two months. This solution can be diluted 1.5 : 1 with zinc-free water and used directly but as such it is not specific for zinc. In order to attain specificity the complex-forming buffer of Mager et al (1953) was used. This was prepared by dissolving 550 g of sodium thiosulphate pentahydrate, 90 g of sodium acetate trihydrate and 10 g of potassium cyanide in approximately 1 litre of zinc-free water. The pH was then adjusted to 5.5 with glacial acetic acid using a standard meter. The volume was then made up to exactly two litres with zinc-free water. The solution was then extracted with dithizone in carbon tetrachloride in a separating funnel until a clear green colour indicating complete removal of zinc was attained. 24 ml of the 0.01% dithizone in acetone solution were added to 18 ml of deionized glass-distilled water and the pH adjusted to 3.7 with 1.N acetic acid. 5.8 ml of the complex-forming buffer and 0.2 ml of 20% aqueous sodium potassium tartrate solution were added slowly with stirring. This solution was then using for staining. When used thus dithizone combines specifically with zinc to form the bright red dithizonate without interference from other dithizone-reactive metals.

Frozen sections were cut on a cryostat at 10 microns and fixed in cold (4°C) AnalaR methanol for 10 minutes. They were then stained in the dithizone-buffer solution.

Paraffin embedded material was fixed in two changes of cold (4°C) AnalaR methanol for 12 hours, cleared through two changes of xylene (1 hour)

and impregnated and embedded in 54°C paraffin wax. Sections were cut at 5 - 8 microns, mounted on clean slides without adhesive and dried at 37°C for 12 - 15 hours. Sections were deparaffinized and allowed to dry after which they were stained in the dithizone-buffer solution.

Sections of both types were left to stain for 10 minutes or until the colour of the dye turned to clear yellow. Excess dye was then removed by washing quickly in chloroform. The sections were then rinsed briefly in distilled water and mounted in glycerine jelly. Dehydration, clearing and mounting in a synthetic resin was attempted but interfered with the dithizonate precipitate. No counterstaining was attempted.

(b) Silver-sulphide technique

The silver-sulphide technique used for zinc detection was essentially similar to that used for lead. The only difference was that the treatment of the tissues with dilute sulphuric acid (to remove iron and zinc) prior to development was omitted. Treatment with 0.5% potassium cyanide solution (to remove copper) was however included in the method.

ii) Results

(a) Control fish

Silver-sulphide technique

The results of the normal S.S.P. described under the section relating to lead are equally applicable to zinc.

Dithizone technique

Gills

No red granulation was evident but the erythrocytes in the gill capillaries stained red-orange without granules. This is in agreement with the observations of Chauncey and Lionetti (1952). The bony arch of the gills also exhibited the same colour.

Liver

Six control fish were examined and were completely negative in all cases.

Spleen

A pale red-orange non-granular colouration was detectable in the

mass of erythrocytes but the rest of the organ was negative in reaction.

Pancreas

The islet tissue of all six fish examined was strongly positive. The islet cells were stained a pronounced red colour and fine granulation was noted. Frozen sections gave a stronger reaction than the paraffin material but the reaction was never as intense as that achieved with the silver-sulphide technique.

Midgut

In three of the six fish examined a positive reaction was obtained in the mucosa and submucosa. The reaction in the mucous epithelium was only slight, the lumenal parts of all epithelial cells having pink-orange colour. The submucosa exhibited a definite red granulation but the reaction was never strong. This reaction only occurred where frozen sections were used and because of the poorer histological detail inherent in this material difficulty in determining the sites of reaction was encountered. Histologically the submucosa consists of areolar connective tissue. It is well supplied with blood and contains granular cells which may be mast cells or macrophages. The red granulation observed was not comparable to that seen in the erythrocytes and it is possible that the cells with a granular cytoplasm exhibited the zinc reaction, however no explanation of the significance of this is offered.

Kidneys

Attempts to detect zinc in the kidneys were disappointing. By analysis the kidney contained an average of just over 700 ppm of zinc per dry weight of tissue (see Section 11) and on this basis the histochemical demonstration of zinc in the organ would seem a straightforward matter. Using the dithizone method, in all cases no red granulation was ever observed although most of the section took on a pink colouration. Some tubules were more intensely stained than others but the difference was not great. The intertubular tissue and glomeruli reacted similarly. Erythrocytes observed in the kidney sections had the same pink-orange colour as in other organs.

(b) Experimental fish

Nine fish were examined after exposure to 0.1 ppm of zinc for periods from 30 to 110 days.

Gills

Silver-sulphide technique(S.S.T)

At all stages the gill preparations compared exactly with those of control fish.

Dithizone

The reaction was similar to that seen in controls except that in three cases (78, 105, 110 days) there was a slight increase in the positive reaction of the bony gill arch.

Liver

S.S.T.

In all cases the picture was different from that seen in control fish. In two fish examined after 30 days exposure there were coarse deposits of silver in some of the parenchyma cells. Less than 10% of the cells were affected but in some cases aggregations of cells packed with granules could be seen. Only the parenchyma cells reacted and no precipitate of silver was ever seen in the linings of bile capillaries or blood vessels. Fish examined after exposures in excess of 30 days did not exhibit a greatly altered picture (see Plate 52). There did not appear to be a progressive accumulation of zinc in the liver as was the case with copper and one fish (65 days) was negative. No silver deposits were observed in an intranuclear position.

Dithizone

Paraffin embedded material never gave positive results with dithizone. Frozen sections were employed in four cases and all were positive. A granular red deposit was scattered throughout the organ. The stain appeared restricted to the parechyma cells and vacuoles which were common in the sections contained accumulations of red granules. (see Plate 53). As with the S.S.T. there did not appear to be a progressive accumulation of zinc after protracted exposures.

Plate 53

Frozen section (cryostat-cut) of the liver of a fish exposed to 0.1 ppm of zinc for 105 days. (X 370). Dithizone technique. Scattered red deposits in the hepatic cells.

Plate 54

Mucosa of the midgut of a fish exposed to zinc (0.1 ppm) for 110 days. (X 920). Silver-sulphide technique, haematoxylin counterstain. Note the fine silver deposits immediately below the striated border.

Spleen

S.S.T.

No change.

Dithizone

No change.

Pancreas

S.S.T.

No change.

Dithizone

No change.

Midgut

S.S.T.

In five of the nine cases examined very prominent deposits of silver granules were seen in the mucosal epithelium. (In all cases the fish were starved for 24 - 48 hours before sectioning). The granules were more prominent than those observed with lead and their distribution was very uniform. They were situated in the upper part of each epithelial cell above the nucleus and just below the striated border. (see Plate 54). No "vacuoles" of the type found after copper exposures were seen.

Dithizone

An increase in the intensity of the stain in the mucous epithelium was observed in all four cases examined. (Frozen sections only). The increase was not however accompanied by the appearance of distinct granules. The red granular precipitates noted in the submucosa of control fishes were again present and in three of the four fish were definitely more intense. (see Plate 55).

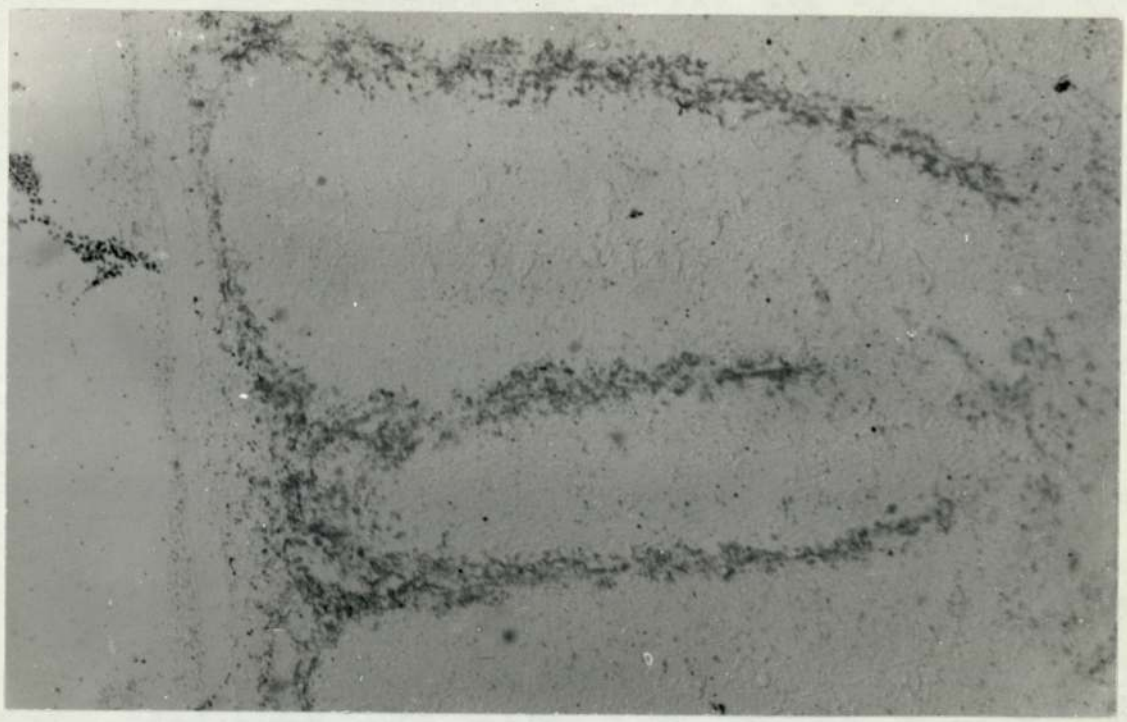
It may be that the increase in zinc in the midgut after subacute zinc exposures is due to uptake of the metal. It is known that freshwater fishes do drink some of their external medium (see Section 3.2) and Miura and Tsunazima (1956 and Saiki et al (1958) considered that fish can take up zinc into the digestive tract from the external medium as well as by the gills. The fish in the present study may have taken food contaminated with zinc (from the external solution) into the intestine and Hoss (1964) has demonstrated greater accumulation of Zn^{65} from food than water by a

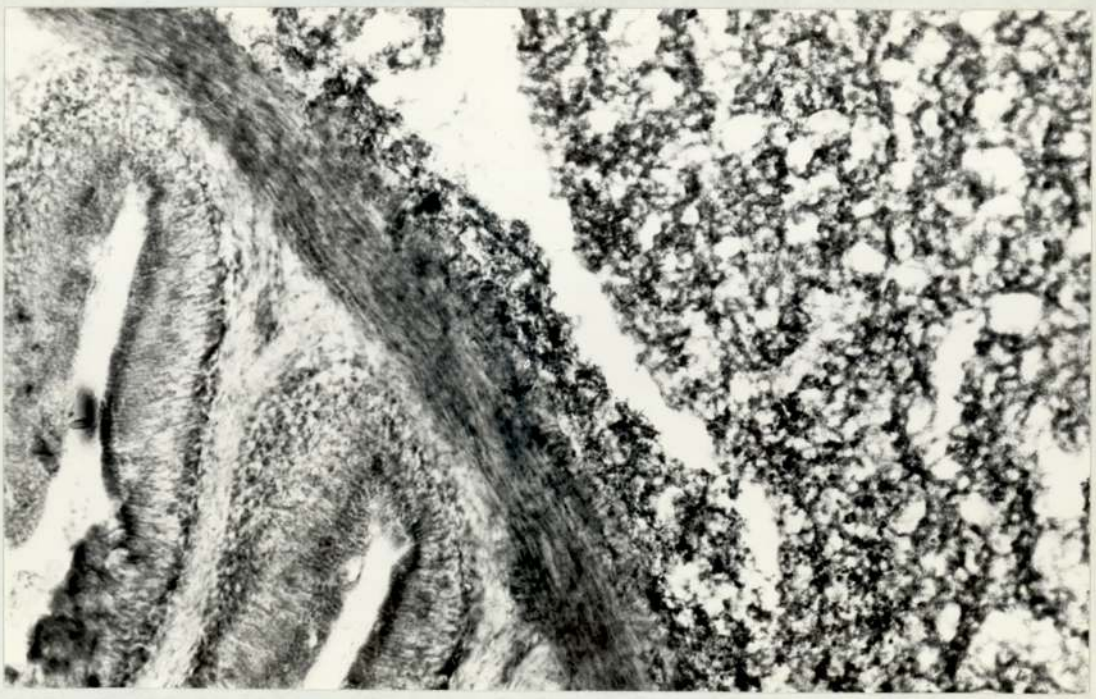
Plate 55

Frozen section (cryostat cut) of the midgut of a fish exposed to 0.1 ppm of zinc for 105 days. (X 275). Dithizone technique. Zinc dithizonate deposits can be seen in the submucosa extending into the rugae.

Plate 56

Malic dehydrogenase activity (Tetrazolium-Formazan technique) in the liver and midgut of an untreated minnow. (X 275).





flounder of the genus Paralichthys. Conversely Hibya and Oguri (1961) found that when Zn^{65} was injected into goldfish the greatest accumulation was found in the intestine and they suggested that the gut was responsible for active excretion of the metal.

Kidneys

S.S.T.

In all cases examined there was an increase in the darkening of some of the tubular epithelial cells (30,45,78,85,110 days). The darkening was not granular but was presumed to consist of particles of submicroscopic dimensions. The increase in silver precipitate was located primarily just below the brush borders of the proximal tubules and the distal tubules appeared unchanged. No granular silver deposits were seen in the intertubular tissue and there did not appear to be a continual build up of zinc in the organ. as exposure time increased. The weak overall reaction of the kidney is surprising in the light of the gravimetric analyses reported in the next section where 1600 ppm zinc per dry weight of tissue was measured in the kidneys of fish exposed to the same concentration of zinc for 30 days. It may be however that a very large proportion of the metal was present in the urine and was lost during the histologic processing.

Dithizone

The kidneys of four fish (30,45,65 and 105 days) were stained using dithizone. The results obtained were less significant than those from the silver-sulphide technique. Most of the tissue appeared little altered from the controls but the proximal tubules in general gave a more intense reaction. No red granular deposits were seen.

Discussion

The purpose of the experiments described in this section was to investigate histochemically any uptake of metals occurring during possible chronic intoxication. The methods developed were designed to give some insight into the metabolic changes proceeding within the fish in an attempt

to explain the mechanisms of chronic toxicity and are not intended for use in the field. When histochemical tests are conducted the tissue must be very fresh and this is rarely the case in the field where fish kills are not discovered until at best a number of hours after the death of the individuals.

There is only one reported study involving histochemical techniques (Haider 1964) and in the absence of other data it is obvious that further experiments need to be conducted with other fish before any firm conclusions can be made. The case of copper is however virtually beyond doubt. The data from Section 9 and also from this Section confirm the chronic toxic effects of very low concentrations of the metal. Analytical data to be presented in Section 11 will further confirm this effect. The rapid uptake and accumulation of copper by the liver must be injurious to the fish and the subsequent wasting of the animals is probably due to impaired liver function. The lack of histochemical reaction in the gills is not altogether surprising as if Lloyd's (1963) hypothesis is correct, at subacute levels the copper ions moving into the gills are immediately removed into the blood-stream and no build up occurs. There is however some build up of copper in the gills (see Section 11) but it occurs slowly and does not cause any apparent cell damage. Theweak reaction observed in the kidneys is probably due to the fact that the increased copper content of these organs is due to copper in the urine which would be difficult to detect histochemically.

The picture with the other metals investigated is less clear. Exposure to subacute concentrations of lead seems to have little effect up to 60 - 70 days. After this time lead was observed in the liver and kidneys but it was not until exposure periods of 200 days or more that large amounts of the metal were detected (see also Section 11) and even then there seemed to be little effect upon the fish. The evidence for nickel uptake is slight but observed nickel in the liver at exposure periods of over 120 days is correlated with the results of experiments in Section 9 where three out of five fish died during exposure periods of

90 to 210 days. The amount of nickel stored by the liver was however very small being found in very local accumulations. The data obtained for zinc is somewhat incomplete although no continued build up of the metal appeared to occur. Longer exposure periods are needed to ascertain any chronic affects of the metal but as already mentioned this was precluded by the development of ulcerative dermal necrosis which may in fact have been aggravated by the metal.

The problem of the point of entry of heavy metals into the body of fishes has already been discussed in section 3. From the limited data derived from the present studies little more can be said except that in the case of copper, lead and zinc, metals were detected in the gut wall. It may be that these metals were being excreted by the intestine and so far as lead and zinc are concerned there is no real indication pointing to excretion or absorption. The case of copper looks more like absorption as vacuoles were found in the mucosa which appeared to have concentrated the metal. Attempts were made to stain for metals in the skin but pretreatment of the hard tissues prior to sectioning may have leached any metals out as all attempts proved negative. Reasons for the lack of positive results in the gills have already been discussed.

The results presented in this section go some way to elucidating the paths of heavy metals in the tissues of the minnow. One of the greatest problems with histochemical techniques of this sort is to maintain all the metal in the tissue so that some sort of quantitive as well as topographical comparison can be made. Obviously losses of metal will occur due to tissue processing and in order to gain a more accurate picture of the amounts of metals in the tissues analyses of tissue samples were made after subacute exposures to heavy metals. The results of these experiments are reported in the next section.

11. GRAVIMETRIC ANALYSES FOR HEAVY METALS IN CHRONIC TOXICITY

The aim of the experiments described in this Section was to quantify any metal uptake, to ascertain whether or not any metal accumulation was continuous or whether an equilibrium was reached and to compare the metal content of the gills in acute and chronic toxicity.

1. Materials and methods

Exposures of minnows to subacute concentrations of the four metals were conducted for up to 90 days (Exposure of four fish to lead was continued for 160 days and to nickel for 136 days). Fish were taken for analysis at 30, 60 and 90 days. Due to the small size of the minnow pooled samples of three or four fish were used. Exposures were made under identical conditions to those described in Sections 9 and 10 and at the same metal concentrations.

Gills, opercular bone, liver and kidneyswere analysed for their heavy metal content. The preparation of tissues and analytical procedures were exactly as described in Section 8. The concentrations of metals in the tissues are all expressed as parts per million of metal per dry weight of tissue.

For the gills and bone samples the figures derived from the groups of control fish in Section 8 are used for comparison. Analyses were performed simultaneously on liver and kidneys and the results obtained are used as controls for the experiments described in the present section.

Dry weights of pooled liver samples for experimental and control fish varied between 8.3 and 30.7 mgs (the lower level is from fish exposed to copper for extended periods where reduction in the size of the liver occurred) and the weights of kidney samples from 4.0 to 14.1 mgs.

2. Results

i) Copper

A total of 27 control fish constituting 9 batches of 3 were analysed for copper content. The results are shown in table 19 (see appendix).

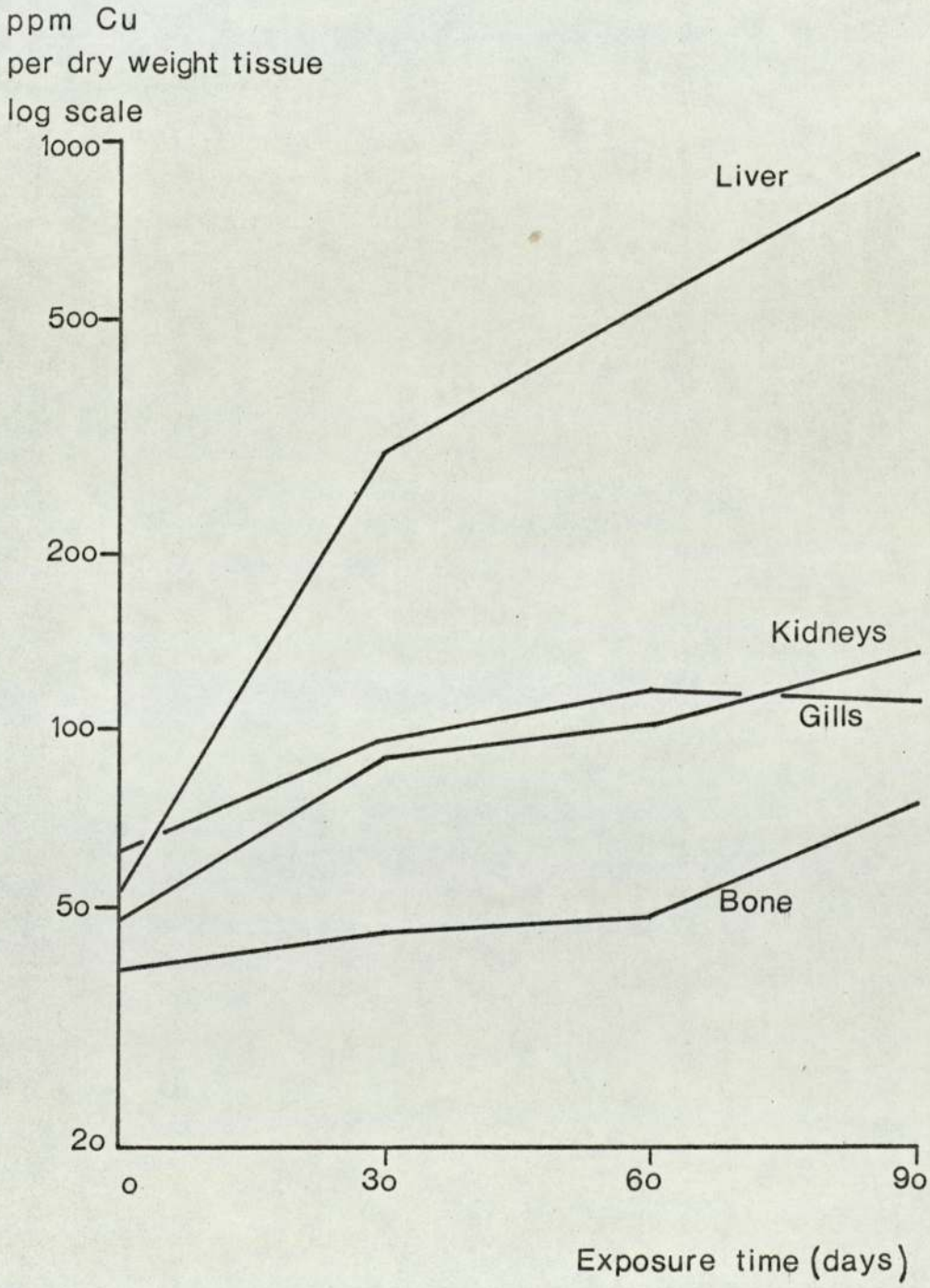
The average copper content of the gills was 62.6 ppm (54.3-71.0), that of the bone samples was 39.4 ppm (23.7-49.5), that of the liver was 55.4 ppm (44.6-64.0) and that of the kidney 47.9 ppm (36.0-54.0).

The most notable aspect of copper uptake was the continued increase in all organs except the gills. The copper content of the liver rose from 55 ppm in the control fish to 960 ppm in the sample taken from three fish exposed for 90 days. The uptake of copper by the various organs is expressed graphically in figure 5. This continued rise correlates well with the histochemical tests described in the previous section. The copper content of the kidneys rose from an average control level of 48 ppm to 138 ppm after 90 days exposure and that of the bone sample from 39 to 74 ppm. Unlike the results obtained by Mount and Stephan (1967) with cadmium where an equilibrium concentration appeared to develop some time between 60 and 90 days, in all cases except the gills the accumulation of copper continued to increase. The copper content of the gills increased from an average control level of 63 ppm to 119 ppm after 60 days. At 90 days the measured level was 112 ppm. This small difference is probably accounted for by individual variation and did not mean that the copper content of the gills had reached a maximum.

The gill/bone copper ratios (see Section 8) at 30, 60 and 90 days were 2.1, 2.4 and 1.51 respectively compared with a control average of 1.64. These do not compare well with the observed ratios for acute exposures to copper which averaged 1.86. (Range = 1.41 - 2.21). The highest level of copper observed in the gills after subacute exposure was 119 ppm andthis concentration was higher than the level observed after fish had been killed at the acutely toxic copper concentration of 0.2 ppm (103.8 ppm) and equal to the level where fish were killed at 0.5 ppm of copper. It was pointed out in Section 8 that the low gill/bone copper ratios are in part due to insufficient removal of copper-bearing mucus from the opercular bone sample and that if this had been achieved the gill/bone ratio would probably have been in the region of 3.2. This however does not account for the similarity in the copper content of the

Figure 5

Copper uptake by minnows in subacute toxicity.


ppm Cu
per dry weight tissue
log scale
1000
500
200
100
50
20
Liver
Kidneys
Gills
Bone
0
30
60
90
Exposure time (days)

gills after acute and chronic exposures. The similarity is also very unlikely to be due to variation amongst individuals as the nine groups of control fish had gill copper levels which were all very similar and in any case did not exceed 71 ppm. It may be that the fish acquire a measure of resistance to the presence of copper in the gills as the exposure continues and that consequently a slow build up occurs reaching levels comparable with those observed after acute exposures. In the absence of other published work it is difficult to comment further on this phenomenon at present but acquired resistance to zinc has been demonstrated by Lloyd (1960) although no similar effects with copper have been reported.

Overall it would seem that examination of the liver with respect to its copper content would serve as a reliable and useful indicator of sub-acute exposure to copper.

(ii) Lead

A total of 12 control fish representing one group of 6 and two groups of 3 were analysed for lead content. In all cases the results were negative.

Lead is the most difficult of the metals to analyse by atomic absorbtion spectrophotometry and the very small tissue samples aggravated the problem. It is possible to state with some accuracy however that any lead present in the various organs was below the following levels:-

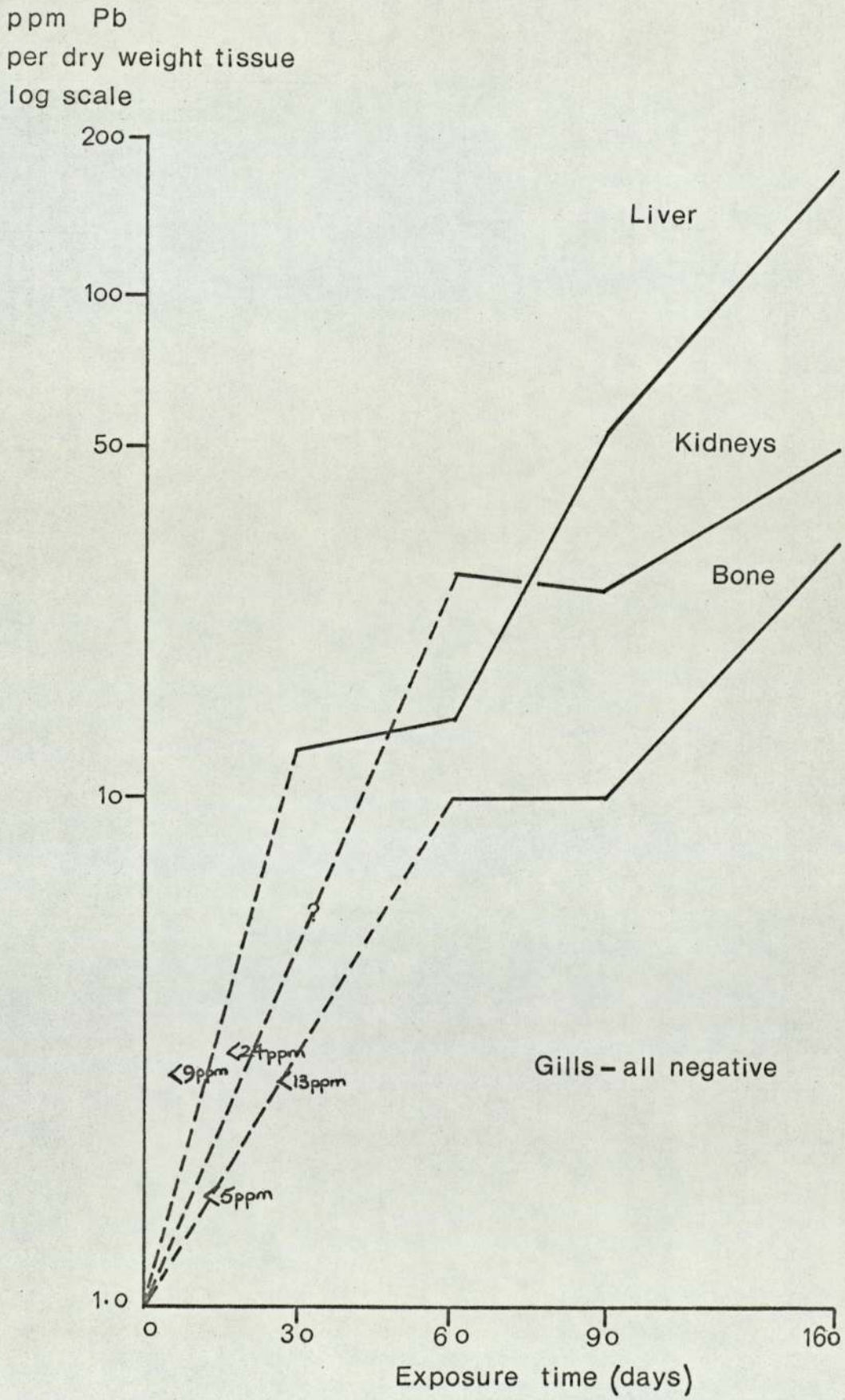
Liver	9 ppm
Kidney	24 ppm
Gills	14 ppm
Bone	5 ppm

Analyses were performed on four batches of experimental fish totalling 15 in all. The results are shown in Table 20 (see Appendix). A batch of three was examined at 30 days, four at 60 days, four at 90 days and four at 160 days. The results are expressed graphically in figure 6.

The inclusion of a batch of fish exposed to the metal for a period well over 90 days was deemed necessary on the basis of the histochemical results described in the previous section where it appeared that lead began to be

Figure 6

Lead uptake by minnows in subacute toxicity.


ppm Pb
per dry weight tissue
log scale
200
100
50
10
1·0
Liver
Kidneys
Bone
<9ppm
<24ppm
<13ppm
<5ppm
?
Gills – all negative
0
30
60
90
160
Exposure time (days)

stored in the liver in large amounts only after very extended exposures.

In all cases the gills proved to be negative at the sensitivity of the method. The kidneys exhibited some uptake of lead the maximum amount of 49 ppm occurring after 160 days exposure. This observation correlates with the histochemical results described in the previous section. The kidney extract from the three fish exposed for 30 days was unfortunately spilt and no substitute was available! A slight accumulation of lead was measured in the bone sample reaching 31.5 ppm after 160 days. No histochemical tests were made on bone samples but Haider (1964) was able to demonstrate histochemically accumulated lead in the bones of rainbow trout exposed to subacute concentrations of lead salts. The results obtained with the liver parallelled those of the histochemical tests. At 30 days 12.5 ppm was detected but it should be noted that this was very near the limits of sensitivity for the instrument and is something of an approximation. The lead content of the liver did not rise dramatically until 160 days where 173.5 ppm was detected. Exposure periods of longer than 160 days are desirable as at 345 days extensive deposits of lead were stainable in the liver. This was not possible however owing to the lack of time remaining. I would like to point out that the results obtained for lead content of the various organs at 160 days may be somewhat inaccurate. The hollow cathode lamp used for lead determinations at this stage was emitting only a low signal and was due for replacement. Because of this the instrument sensitivity was somewhat lowered but the results are unlikely to be more than 10% in error as the calibration curve drawn from standard solutions appeared satisfactory.

As with copper it would seem that analysis of the livers of fish would indicate past exposure to lead. In the present study any detectable lead in the liver could be regarded as such an indicator although substantial amounts did not appear after exposures of less than 90 days.

Due to the negative reaction of the gills in all cases no comparison of gill/bone lead ratios could be made with fish from acute exposures.

iii) Nickel

A total of 12 control fish representing two groups of six were analysed for nickel content. In all cases the results were negative at the sensitivity of the method.

The nickel content of the various organs may not have been detectable but was in any case less than the following levels:-

Liver	7.5 ppm
Kidney	20 ppm
Gills	12 ppm
Bone	6 ppm

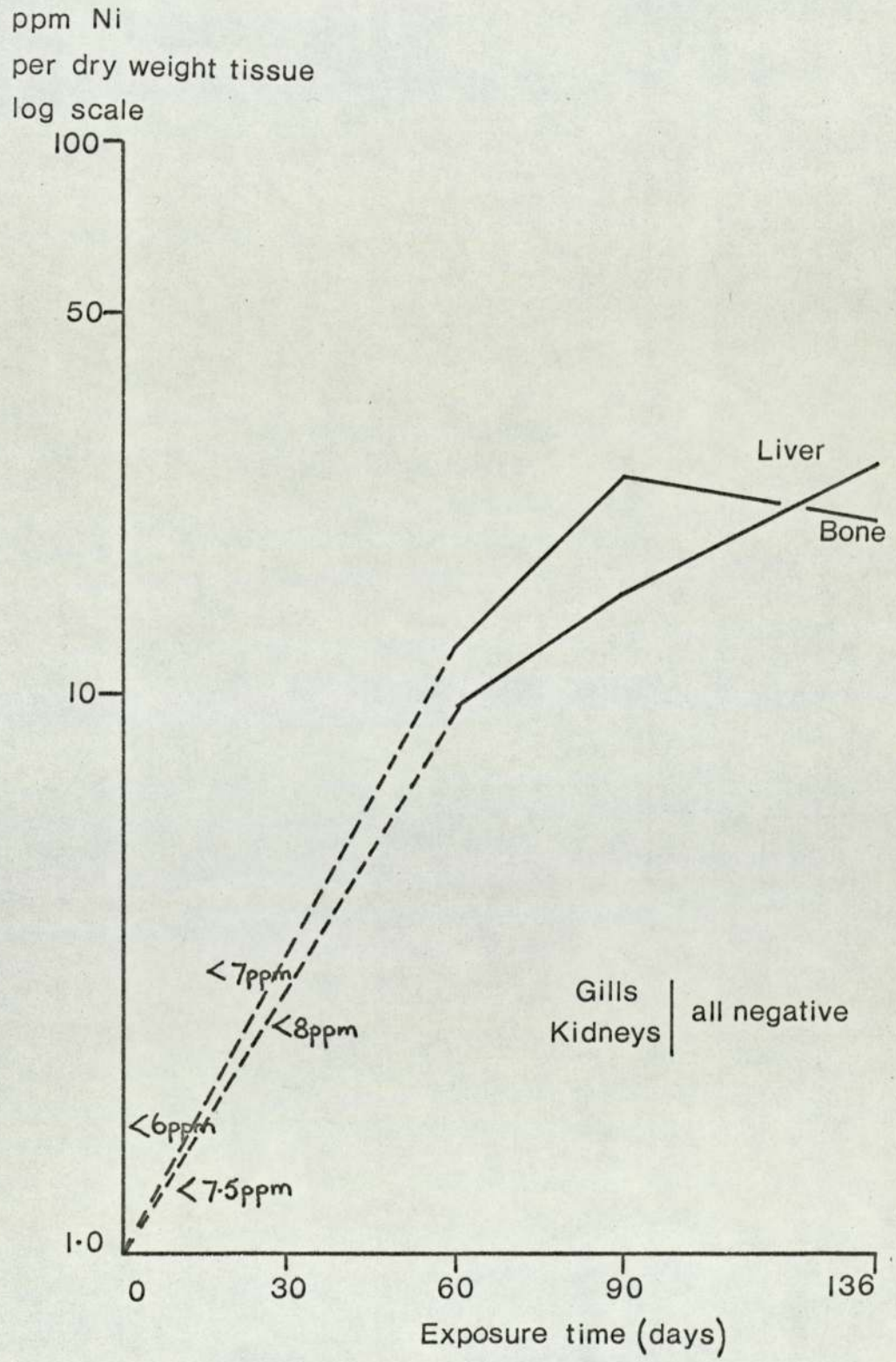
Analyses were also performed on four groups of fish after 30,60,90 and 136 days exposure to 4.0 ppm of nickel. Each group except the last represented a pool of three fish, four being pooled after 136 days exposure. Results of the analyses are shown in table 21 (see Appendix) and expressed graphically in figure 7. At 30 days all tissues were negative. After 60 days exposure only the bone sample gave a positive result (12 ppm). The determination was performed at the limits of sensitivity of the machine and is possibly somewhat inaccurate. At 90 days a positive result was obtained for the liver (15 ppm) but the same conditions apply as for the bone sample at 60 days. The concentration of nickel measured in the 90 day bone sample was 25 ppm and at 136 days was 20.5 ppm. At 136 days the nickel content of the livers had increased to 26.5 ppm. This result is in some agreement with the histochemical observations where only local accumulations of nickel were detected in the organ.

The relatively small uptake of nickel by the liver is difficult to measure accurately where small fish such as the minnow are used. It may serve however as an indicator of long term chronic exposure where a larger species such as the trout is used.

Due to the negative results with the gills no comparison of gill/bone ratios with fish from acute exposures could be made.

Figure 7

Nickel uptake by minnows in subacute toxicity.


ppm Ni
per dry weight tissue
log scale
100
50
10
1·0
Liver
Bone
<7ppm
<8ppm
<6ppm
<7·5ppm
Gills
Kidneys
all negative
0
30
60
90
136
Exposure time (days)

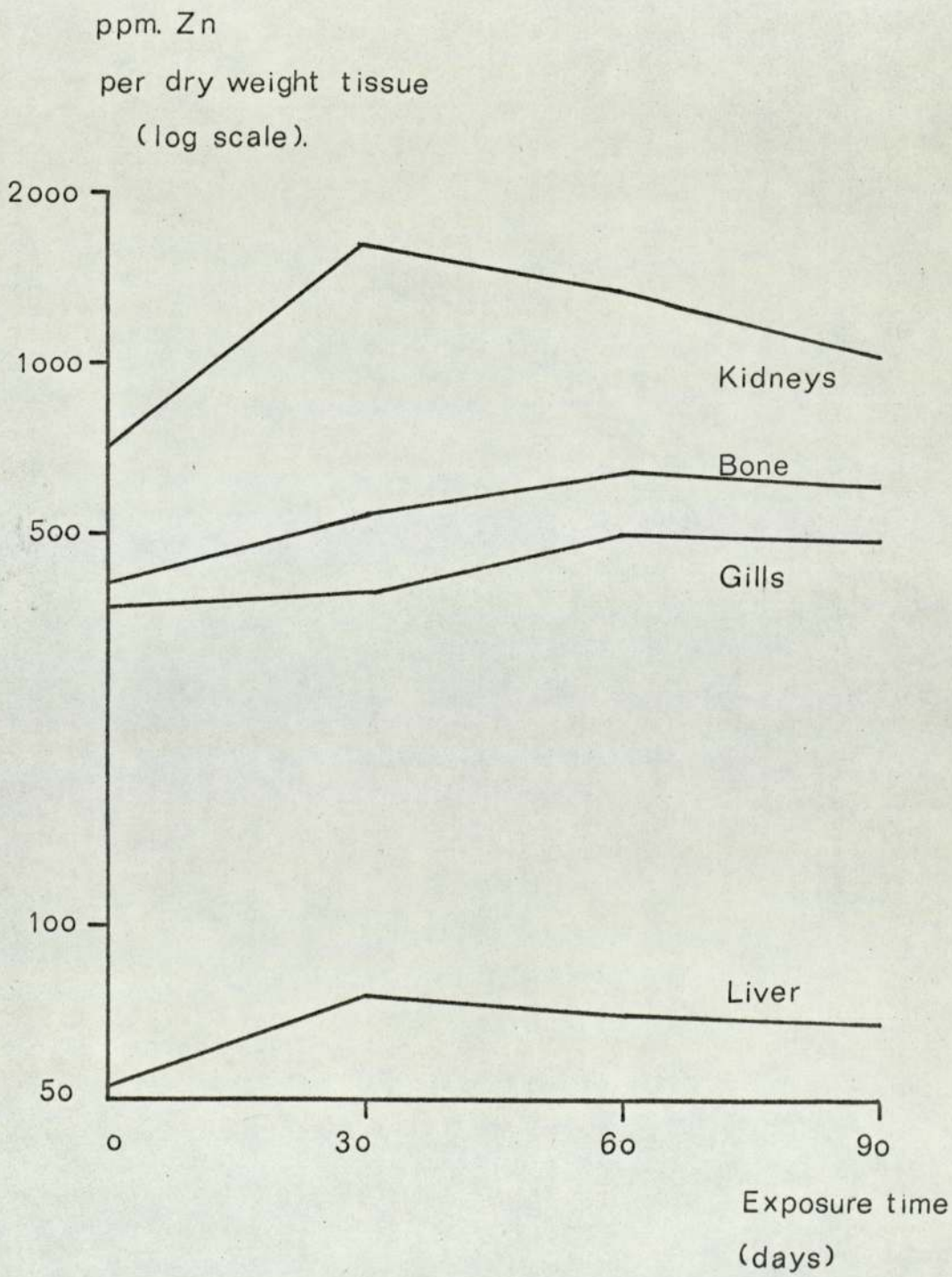
iv) Zinc

A total of 27 control fish representing 9 batches of three were analysed for zinc content. The average zinc content of the gills was 366 ppm (280-401 ppm), that of the bone 409 ppm (370-425 ppm), that of the liver 52.7 ppm (38-67 ppm) and that of the kidneys was 712.8 ppm (646-826 ppm). The very high level of zinc detected in the kidneys is not surprising as control zinc levels of 300 ppm rising to 900 ppm after zinc exposures were detected by Mount (1964) using Lepomis.

Three groups of fish were exposed to 0.1 ppm of zinc for 30, 60 and 90 days. After 30 days the zinc content of the kidneys had risen from the average control level of 713 ppm to 1628 ppm. The fact that this enormous increase in the zinc content of the kidneys was not satisfactorily detected histochemically in fish from similar treatments is somewhat surprising, although most of the zinc may have been in the urine and would thus have been lost during histologic processing. The zinc content of the kidneys fell after 60 and 90 days exposure possibly indicating that a state of equilibrium had been reached after the initially large uptake. This observation is quite opposite to the reaction occurring with copper where a small but continued increase occurred. Both the gills and the bone sample reacted similarly although there was not such a great initial rise in zinc concentration, the 90 day concentrations of zinc in both tissues being slightly lower than those at 60 days exposure. The concentration of zinc in the liver was much lower than that in the other tissues but a similar pattern developed with the initial rise in concentration falling off as the exposure continued. This result is supported by the histochemical evidence where only slight zinc uptake was detected in the liver cells. The liver in chronic zinc toxicity unlike the case with the other metals does not appear to be an indicator of past exposure to the metal, although the kidney may well be. Mount's (1964) results with bluegills and zinc indicate a similar result, the liver accumulating only small amounts of the metal. The results for control and zinc exposed

Figure 8

Zinc uptake by minnows in subacute toxicity.


ppm. Zn
per dry weight tissue
(log scale).
2000
1000
500
100
50
Kidneys
Bone
Gills
Liver
0
30
60
90
Exposure time
(days)

fish are given in table 22 and presented graphically in figure 8.

The gill/bone ratios at 30, 60 and 90 days were 0.73, 0.78 and 0.79 respectively. These compare favourably with an average control figure of 0.89. Where fish killed after acutely toxic zinc exposures were examined (see Section 8) the ratios varied between 1.27 and 1.84. Thus for zinc at least, the gill/bone ratio seems to be a reliable indicator of acute toxicity, the control and chronic exposure ratios being very similar.

Whilst the gill/bone ratios seemed satisfactory it should be noted that at 60 days exposure the zinc concentration in the gills was 495 ppm which is a higher level than that observed in fish taken dead from a solution of zinc (471 ppm) at the acutely toxic level of 0.2 ppm. Individual variation in the case of zinc (control gills varied from 280-401 ppm Zn) may however be responsible and other acute exposures resulted in far higher gill zinc concentrations.

3. Discussion

One of the main purposes of the experiments described in this section was to ascertain whether any measurable accumulation of heavy metals by the minnow during subacute exposures was continuous or whether a state of equilibrium occurred after a set period. Mount and Stephan (1967) detected levelling off of cadmium accumulation in the gills and liver of bluegills (Lepomis macrochirus) after exposure periods of up to 90 days. In the present investigation only zinc gave similar results. (see fig. 8). This apparent state of equilibrium indicates that the fish may be able to exert some control over the storage of zinc in the body. Zinc is found widely in higher concentrations than copper in animal tissues generally and analyses of the tissues of the minnow revealed the expected picture. Zinc is an essential component of the enzyme carbonic anhydrase which catalyses the hydration of carbon dioxide in many tissues such as the gills, red blood corpuscles and the kidneys. It is also found in kidney phosphatases and this may go some way to

explaining the high zinc content in the kidneys of the minnow. (Histochemical preparations reveal high phosphatase activity in the kidneys of the minnow - see Section 13). The initial increase in the zinc content of the kidneys may have been due to active excretion by them. Other routes of zinc excretion are possible although the heavy metal metabolism of fish is poorly understood (Vallee 1962). Hibiya and Oguri (1961) using goldfish (Carassius auratus) which had been injected with Zn^{65} considered that zinc was excreted actively into the digestive tract. There is no reason why the gills should not also be able to excrete the metal.

Although continued accumulation of zinc appeared to be arrested after 30 - 60 days, damage due to the metal was noted in the liver and after the longest exposures particularly in the kidneys of fishes. (see Section 9). So far as zinc is concerned the liver does not seem to be a useful indicator of past exposure to the metal and analyses of the kidneys would seem more profitable.

Copper, lead and nickel appeared to accumulate continuously in the liver. Analyses of the liver for copper should indicate past exposure to the metal. After subacute copper exposures all tissues but the gills steadily accumulated the metal and the results compared well with the histopathologic and histochemical studies described in Sections 9 and 10. On the basis of the evidence so far presented it seems certain that copper acts as a cumulative toxicant to the minnow at subacute levels. The uptake of both nickel and lead presented a different picture to that of copper although in both cases there appeared to be a continued accumulation in the liver. The storage of significant amounts of the metals however seemed to be delayed until exposure times of over two months had been reached. This may have been due to the eventual poisoning of enzyme systems and investigations along these lines are described in Section 13. No gill/bone metal ratios could be compared for lead and nickel as in both cases only in acute toxicity did the gills contain measurable amounts of the metals. It may be that there

was a substantial intake of metals via the intestine but no measurements were made.

12. THE SUBACUTE TOXICITY OF THE FOUR HEAVY METALS TO THE MINNOW WHEN PRESENT IN A SEWAGE EFFLUENT.

Evidence was presented in Section 6 to show that when heavy metals were present in sewage effluents they were generally less toxic than in simple inorganic solutions. An attempt to quantify this was described in Section 6.2 where the 48 hr. $L.C._{50}$ values for groups of ten minnows exposed to the metals in an "artificial effluent" were compared with the values obtained where fish were tested in inorganic solutions. Only copper gave a significantly different result, the 48 hr. $L.C._{50}$ rising from 0.12 to 0.65 ppm. The field experiment described in Section 6.1. however pointed to a reduction in the toxicity of zinc as well as copper.

In the following experiments fish were maintained for over 100 days in a metal - containing effluent prepared in the laboratory from a mainly domestic sewage by the activated sludge process.

1. Materials and methods

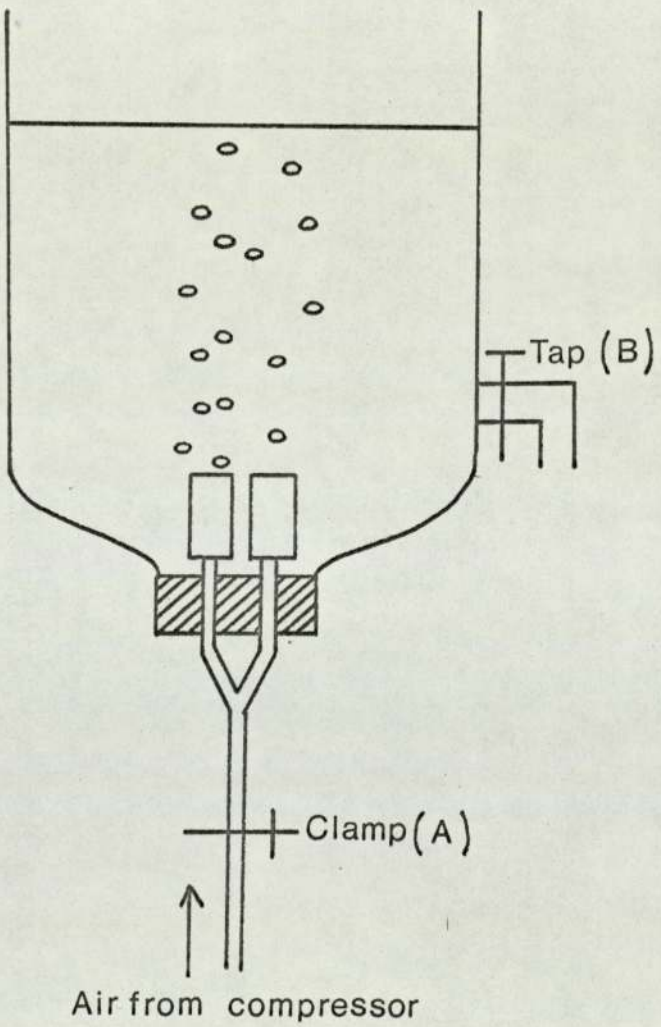
Effluents containing the individual metals were prepared separately by aerating settled sewage obtained from the Langley Mill works of the Upper Tame Main Drainage Authority with activated sludge from the Coleshill works of the same authority. Sewage and activated sludge were collected weekly. The apparatus used to perform the process is shown in plate 57 and figure 9. It consisted of four identical polythene vessels each of which was supplied with two air diffusers in the base. A strong flow of air was provided by a compressor. The clamp (A) was closed and 4.8 litres of settled sewage were added to the container. The air compressor was then switched on the clamp (A) opened and the airflow adjusted to a suitable rate. An appropriate amount of stock metal salt solution was then added to each container. After twenty minutes mixing of the sewage and added metal salt 1.2 litres of shaken activated sludge was added, making 6 litres in all. The liquor was left to aerate for 18-20 hours. The aeration and consequent agitation allowed the micro-organisms in the sludge to breakdown the sewage and lower its oxygen demand at the same

Plate 57

Apparatus for the production of a metal-containing sewage effluent by the activated sludge technique.

Figure 9

Apparatus for the production of a sewage effluent by the activated sludge technique.


Tap (B)
Clamp (A)
Air from compressor

facilitating chelation of the metals by organic compounds in the sludge. The production of an effluent from a sewage with added metal was considered to be far more satisfactory than simply adding known amounts of metal salts to an effluent where sufficient complexing of the metals may not have occurred. After aeration the tap (A) was closed, the air supply cut off and the activated sludge allowed to settle out for 30-60 minutes. The metal containing effluent was then run off from the tap (B) and diluted as necessary with the artificial stock medium for bioassay. Each effluent sample was analysed for metal content using a Unicam S.P.900.A atomic absorbtion spectrophotometer. It was not necessary to concentrate the samples prior to analysis and after the settling out of any particulate material the effluent samples were aspirated directly into the burner. After analysis the dilution factor for each batch of effluent was easily calculated and the appropriate dilution made. The initial concentrations of metal salts added were: copper 2.5 ppm, zinc 1.1 - 1.3 ppm, nickel 30 ppm and lead 110 - 130 ppm. The initial concentrationswere based on the data of Stones (in Klein , 1966 , Vols 3 pp 145-146) where the expected removal rates of metals from sewage by activated sludge are quoted as:

Copper	-	80%
Zinc	-	60%
Nickel	-	30%
Lead	-	90%

The observed rates of removal in the present study correlated closely on average with those above although some wide individual variations occurred. The average removal rates from 17 treatmentswere:

Copper,	78.6%	(60.6 - 84.4)
Zinc,	56%	(26.1 - 76.2)
Nickel,	35.7%	(13.3 - 45)
Lead,	90.2%	(76.7 - 96.6)

The analytical record of all treatments is given in table 23.

The high initial metal concentrations in the liquor are considered to have caused some of the wider variations due to their toxic effect on

the sludge organisms. With lead and especially nickel a rather cloudy effluent was produced on some occasions presumably because the sludge was partially inactivated. Aeration periods of much over 20 hours were found to cause "autolysis" of the sludge due to the depletion of available nutrients in the sewage by the sludge organisms.

The untreated settled sewage was analysed for heavy metals on three occasions and the results were as follows:-

		1	2	3
Copper	(ppm)	0.11	0.06	0.25
Zinc	(ppm)	0.12	0.12	0.17
Nickel	(ppm)	Nil	Nil	Nil
Lead	(ppm)	Nil	Nil	Nil

By calculation (assuming no great variation in the sewage metal content) the maximum amount of zinc likely to be encountered in prepared solutions of other metals would be between 0.02 and 0.05 ppm and that for copper between 0.03 and 0.06 ppm. The presence of other metals at this low concentration was unavoidable and it was because of this that the method described in section 6.2. was used for acute toxicity tests.

The calcium levels of the final solutions were measured on three occasions and were 65, 68 and 63 ppm (Ca) respectively. When diluted 4 : 1 with the artificial stock medium (Ca = 80 ppm) the difference in calcium levels was thus negligible. Where lead solutions were employed the stock solution calcium level was 10 ppm and thus resulted in final solutions with calcium levels varying from 13 -30 ppm.

It was originally intended to use 4 : 1 dilutions of stock solution and effluent but due to variation in the metal content of the different samples different dilutions were employed from week to week. The extremes of dilution for the four metals were as follows:

Cu	790 ccs	—	in 5 litres
	1667 ccs	—	"
Zn	781 ccs	—	"
	1923 ccs	—	"
Ni	1154 ccs	—	"
	1818 ccs	—	"
Pb	352 ccs	—	"
	2060 ccs	—	"

The effluent stock solution mixture was aerated and the temperature brought to 15°C by placing the tanks in the temperature controlled water bath before the fish were added.

The metal concentrations chosen for testing in the case of copper, zinc and lead were the 48 hr. $L.C._{50}$ levels determined in Section 5.2. namely 0.12, 0.15 and 1.8 ppm respectively. At these concentrations all fish tested survived for over 100 days. The 48 hr. $L.C._{50}$ as determined for nickel in Section 5.2. was 7.25 ppm. When 6 fish were exposed to an effluent with a similar nickel content all died within two days. It is possible that this was due to incomplete complexing of the metal as the resultant effluent was very cloudy, indicating damage to the sludge by the initially high concentration of metal. As a result of this an effluent containing 6 ppm of nickel was subsequently prepared and fish survived in this for over 100 days.

Six fish were exposed to each of the metal containing effluents for up to 124 days and the solutions were replaced every 5-7 days. After the exposures three fish from each metal were used for gravimetric analysis, one fish was examined histochemically for heavy metal deposits and two fish were examined histopathologically.

2. Results

i) Gravimetric analyses

Opercular bone, gills, liver and kidneys were analysed after exposure to all four metals. Treatment of tissue samples was the same as that

already described in Sections 8 and 11 except that storage of the samples was necessary. On removal from the fish pieces of tissue were quickly frozen by quenching in iso-pentane at the temperature of liquid nitrogen. They were then quickly transferred to sealed polythene specimen tubes and stored in a deep-freeze at -20°C. The storage period for all tissues was up to 17 months. On removal from the deep-freeze the still frozen tissue samples were quickly placed in clean pre-weighed porcelain crucibles and dried for 48 hours at 105°C. The dry weight was then calculated by reweighing and the procedure then continued as described in Section 8. Although the period of storage was long it is considered that no loss of the metals from the tissues could occur as from the time of removal from the animal until the commencement of drying the tissue samples were always frozen at a temperature of at least -20°C.

(a) Copper

The levels of copper detected in the tissues were as follows (in ppm. metal per dry weight tissue);

Organ	Concentration	Range of Controls.
Liver	213.6	(44.6 - 64.0)
Kidneys	67.0	(36.3 - 69.6)
Gills	74.3	(48.2 - 71.0)
Bone	49.4	(23.7 - 49.5)

Only the liver exhibited a significant rise in its copper concentration although the level measured was below that observed at 30 days in fish exposed to 0.1 ppm Cu in inorganic solution (296.8 ppm, see Section 11). The levels measured in kidney, gill and bone were comparable with the highest control levels noted earlier.

(b) Lead and nickel

All analyses attempted gave negative results at the sensitivity of the method.

(c) Zinc

The zinc content of the liver was measured at 60.3 ppm. This was within the range of the controls and is thus not significant. The

level of zinc in the kidneys was only slightly above the highest control level detected earlier, 844.1 and 826 ppm respectively. Results obtained with the gills were similar (382 compared to the highest control level of 401 ppm). Analysis of the bone sample revealed a zinc concentration of 327 ppm. which was considerably less than the lowest control level of 370 ppm.

Overall the picture for zinc indicated no significant increase in metal content compared to control fish.

ii) Histochemical tests for the metals

One fish only was examined from each treatment. In all cases except copper the results were similar to those observed for control fish (see Section 10).

Copper

The liver of the fish exposed to copper exhibited a noticeable positive reaction with Howell's rubeanic acid method. Green-black granules were uniformly distributed throughout the organ but as described in Section 10 were located only in the parenchyma and possibly the Kupffer cells. The kidneys, gills and midgut of the same fish appeared similar to controls except that in the midgut a reaction was seen lining some discharging goblet cells.

iii) Histopathology

Two fish were examined from each exposure.

(a) Gills

No changes were observed.

(b) Liver

In all eight cases examined some vacuolation of the parenchyma cells was seen. Otherwise the organs appeared normal sized and healthy. This is of particular interest with respect to copper where reduction in the size of the organ occurred regularly after prolonged exposure to the metal in inorganic solution. The observed vacuolation was never extensive and fat tests were not performed.

(c) Other organs

No changes were observed in any other organs examined.

3. Discussion

It has been shown in Section 6 that heavy metals in organic effluents do not appear to be as toxic to Phoxinus as when they are in simple solution. Data to confirm this has been presented in this section. Fish survived for over 100 days at concentrations of the metals which in simple solution would probably prove fatal in a few days, death resulting from direct gill damage. Uptake of metals was found to be less and damage to other tissues was reduced.

The reduction in the uptake of the heavy metals may be due to the fact that the large molecules in which the metals may be complexed cannot pass through the cell membranes into the gills so easily. It is obvious that laboratory investigations of the chronic toxicity of the metals using simple solutions with no organic matter although assessing the effects of metals at subacute levels do not indicate safe concentrations for the field. Polluting metals usually occur in rivers and streams with a high organic content and because of this observed laboratory safe-concentrations can probably be raised in most cases. The concentrations tested in this study would have been expected to kill half the fish in 48 hours when tested in simple solution (except Ni). The histopathologic studies employed in this section although indicating no positive result are nevertheless somewhat invalid as no account was taken of other toxicants which may have been present in the sewage.

The exact fate of metal ions in organic effluents is a chemical problem which requires some study as the nature of the organic material will obviously govern the toxicity of metals to fish.

13. ENZYME HISTOCHEMICAL STUDIES

1. Introduction

Many of the chronically toxic effects of heavy metals to fish may be manifest as physiological or biochemical changes and not as changes in the gross histopathology. The histochemical demonstration of enzyme activity in fish tissues after exposure to subacute levels of metals was thus undertaken in order to ascertain broadly any biochemical changes occurring as a result of metal poisoning.

A great many enzymes which depend upon thiol groups for their activity are sensitive to low concentrations of heavy metals; especially mercury, but also of copper, silver, lead, zinc, cadmium and nickel, the metals forming complexes with the -SH groups. Other mechanisms of heavy metal inhibition do occur. Many metal-activated enzymes may be inactivated by the fact that an alternative metal occupies the active site but cannot bind the substrate to protein. An enzyme which requires a certain metal may be inhibited by it at concentrations above the optimum. The cation may form complexes with the enzyme, the substrate and the coenzyme, if any. These complexes are likely to be more stable than mixed complexes in the presence of excess cation and inhibition results (Kornberg et al 1948). Heavy metals can also form complexes with imidazole groups, (eg. Zn) and carboxyl groups (eg.Pb). (Gurd et al 1952,4,6). The inhibition of enzyme systems by heavy metals is reviewed by Hochster and Quastel (1963) and Albert (1968).

As well as inactivating enzymes heavy metals are general protein precipitants. The protein denatured by heavy metals is known to be a much better metal-complexing agent than undamaged protein and this may explain the local concentrations of heavy metals found during the histochemical observations reported in Section 10 where cells where protein denaturation had occurred may have been attracting more heavy metal. Adenosine triphosphate is also known to chelate heavy metals. (Neuberg and Mandl, 1949).

Hartman and Kalnitsky (1950) have shown that copper inhibits the kidney enzyme complex by inhibiting the activating manganese. Inhibition of carboxypeptidase by copper has been demonstrated by Coleman and Vallee (1961) and copper inhibition of carbonic anhydrase activity has been demonstrated by Lindskog and Malmstrom (1962). Copper together with lead, zinc and silver has been demonstrated to inhibit saccharase activity (Myrbäck 1926), and copper and zinc have both been found to exert a powerful inhibitory effect on the enzyme phosphoglucomutase (in muscle) by competing with the activating Mg^{++} ions (Millstein 1961). Sadasivan (1951, 1952) and Van Reen (1953) studied the effects of excess copper on various enzymes in the rat. Excess dietary zinc was found to induce abnormally high liver alkaline phosphatase activity and at the same time there was a decrease in intestinal phosphatase associated with decreased phosphate absorption and poor mineralisation of bone. Excess zinc also decreased cytochrome oxidase and to a lesser extent catalase activity in the liver. Both these phenomena were symptoms of copper deficiency.

Nickel has been shown to inhibit enzyme activity in some cases (Ceresa 1947), but Speck (1949) demonstrated that many polyvalent cations and particularly Zn^{++} and Ni^{++} were very effective in accelerating the decarboxylation of oxaloacetate in the tricarboxylic acid cycle. Ohlmeyer (1947) demonstrated strong inhibition of acid phosphatase activity by nickel and Smith (1951) found nickel to inhibit carnosinase. Other examples of metal ion inhibition of enzymes include silver and urease, (Sumner and Myrbäck 1930, Ambrose et al 1951) and the inhibition of malt amylase by heavy metals (Olsson 1921).

Sexton and Russell (1955) demonstrated succinic dehydrogenase activity in excised goldfish gills. When 10^{-3}g mercuric chloride was added to the substrate inhibition occurred. The authors considered that succinic dehydrogenase is involved in active sodium transport across the gills as Meyer (1952) inhibited active uptake of sodium by goldfish gills with mercuric chloride.

The only published work relating directly to metal toxicity and enzyme systems of fish is that of Jackim et al (1970) although Holland et al (1962) were able to relate acetylcholinesterase inhibition and organophosphorus insecticide toxicity and Leduc (1966) and Bell (1968) have demonstrated changes in some fish enzymes as a result of exposure to cyanide or injections of carbon tetrachloride and bromobenzene. Jackim et al examined the activities of five liver enzymes (acid and alkaline phosphatase, catalase, xanthine oxidase and ribonucease) from killi fish (Fundulus heteroclitus) which had survived exposure to 96 hr. $L.C._{50}$ concentrations of Pb, Cu, Hg, Be, Cd and Ag. They also observed the changes in enzyme activity produced when metal salts were introduced directly into enzyme preparations. The changes induced by exposure of fish to metal salts were in many cases opposite to those induced by adding metal salts to tissue homogenates. The significance of this will be discussed later.

In the present study enzyme distribution and activity was studied histochemically. It is believed that this preliminary study is the only one at present relating enzyme histochemistry and subacute heavy metal toxicity.

2. Materials and methods

Five enzymes were chosen for study. Alkaline phosphatase was chosen as a metal-requiring enzyme, acid phosphatase and ATP-ase because of their connection with mineral metabolism, and lactic and malic dehydrogenases because of their known inhibition by heavy metals.

Fish were exposed to heavy metals at the same concentrations and under the same conditions as already described in Sections 9 and 10. Liver, kidneys, gills and midgut were routinely examined. Pieces of tissue were removed from the fish and frozen rapidly with solid carbon dioxide (Dry-ice). Fish for sectioning were not removed from the tank until immediately required. All fish were starved for two days prior to sacrifice.

Extreme care was taken not to thaw tissue pieces once frozen. All sections were cut frozen on a Pearse-type "Slee" cryostat at 10 microns

and -20°C. Sections were mounted directly on to clean glass slides.

Sections from untreated fish were always cut and mounted on the same slides as experimental sections so that comparisons could be made. When examining and comparing enzyme histochemical preparations, it is most important that sections are of the same thickness as if differences occur a false impression of a change in enzyme activity may be given. In order to avoid this only the third section taken at each "cutting session" was used. (The first section is often thicker than those following when cutting is delayed for a few minutes as the tissue block tends to expand a little).

Pretreatment of the sections before incubation will be described as necessary under the section dealing with each enzyme.

i) Dehydrogenases

Lactic and malic dehydrogenase were demonstrated using a modified Tetrazolium-Formazan method. It was considered worth investigating both enzymes as all reagents except the substrates were identical and L.D.H. is concerned with the reduction of pyruvate to lactic acid (anaerobic cycle), whereas M.D.H. catalyses the transformation of malic acid to oxaloacetic acid in the tricarboxylic acid cycle.

The demonstration of these enzymes is facilitated by the presence of their cofactor diphosphopyridine nucleotide (D.P.N.)(N.A.D.). Cyanide is introduced to prevent oxidation (dehydrogenation) by the alternative cytochrome system. Colourless, soluble, tetrazolium compounds are thus converted by the addition of hydrogen into water insoluble deeply coloured pigments known as formazans.

For both L.D.H. and M.D.H. frozen sections mounted on clean glass slides and allowed to air dry for 10 - 15 minutes were then incubated without fixation. (Cold acetone or alcohol fixation destroys the enzymes).

Method proper

Two stock solutions were prepared and stored at 4°C in the fridge in brown bottles.

(1) 0.20M. Tris. HCl. pH 7.2 - 25 ml.
0.05M. $MgSO_4$ - 10 ml.
0.10M. KCN (pH 7.2 with 1.N HCl) - 10 ml.
Distilled water - 10 ml.

(2) 25 mg of nitro-blue tetrazolium salt (Nitro B - T) were dissoved in 2.5 ml of dimethylformamide and made up to 25 ml. with distilled water.

The solutions were not kept in excess of two months and were not mixed until required.

. Due to the high cost of Nitro-B.T. sections were not incubated in Coplin jars, but wax walls were built around the sections with a glass rod dipped in melted histologic wax and the incubating fluid run into the resultant enclosure.

4.4 ml of Tris. HC[1] buffer was added to 2.0 ml of nitro-B.T. in dimethylformamide in a 10 ml beaker. 2 mg of D.P.N. were then added. The solution was then divided equally between two 10 ml beakers (3.2 ml in each).

For L.D.H. 0.3 ml of 1.M. sodium lactate was added and for M.D.H. 0.3 ml of sodium malate. The lactate and malate substrates were stored in a deep freeze at -20°C when not in use. The pH of each solution was then adjusted to 7.2 with 0.1N NaOH. The media were then poured over the sections and left to incubate for 45 - 60 minutes at 37°C.

After incubation the sections were washed carefully in tapwater for five minutes, fixed in 10% formalin overnight, washed again in tap water and mounted in glycerine jelly. Sites of dehydrogenase activity appeared blue. Controls were produced by omitting the malate and lactate substrates.

ii) Alkaline phosphatase

Alkaline phosphatase was demonstrated using a modified azo dye technique.

Cryostat sections 10 microns thick were affixed to clean slides, air dried for 10 minutes and fixed for a further 10 minutes in acetone at 4°C. After fixation the slides were removed and the sections dried in

the air for 15 minutes. Sections were then incubated.

Incubating medium

A.S.B.1. - Phosphate (Sigma)	---	5 mg.
(Dissolved in 3 -5 drops dimethylformamide).		
Veronal acetate buffer pH 8.2	---	50 ml.
Fast Blue B. B.	---	40 mg.

Before the addition of the sections to the incubating medium it was filtered and the temperature adjusted to 37°C by immersing the staining jar in a water bath. Incubation was continued for 45 minutes.

(The substituted napthol substrate (A.S.B.1) is hydrolysed by the enzyme releasing ∝ napthol which combines with a diazonium salt (Fast blue) to form a coloured azo dye at the sites of enzyme activity).

After incubation the sections were washed for 10 minutes in tap water and fixed overnight in 10% formalin. They were then again washed in tap water and mounted in glycerine jelly.

Sites of alkaline phosphatase activity appeared blue.

Controls were produced by omitting the A.S.B.1 substrate.

iii) Acid phosphatase

An azo dye technique was again employed. Cryostat sections cut at 10 microns were affixed to clean slides and after air drying were fixed in cold (4°C) Bakers Calcium-formalin fixative in a refrigerator. (Humason 1967). Sections were then washed for 10 minutes in tap water and placed in the incubating medium for 12 - 14 hours at 4°C.

Incubating medium

0.1 M	Sodium acetate	----	50 ml.
Hexazotized fuchsin*		----	3.0 ml.

* (1 part basic fuchsin (400 mg fuchsin in 8 ml of boiling distilled water + 2 ml conc. HCl) and 1 part 4% sodium nitrate freshly made).

The pH was then adjusted to 5.5 with a standard meter. 20 mg of A.S.B.1. - phosphate dissolved in a few drops of dimethylformamide were then added.

The mixture was filtered before use.

After incubation the sections were washed well in water for 10 - 20 minutes and then fixed overnight in 10% formalin. They were then washed for two hours in tap water and then mounted in glycerine jelly.

Sites of acid phosphatase activity appeared red-brown.

Controls were produced by omitting the A.S.B.1. substrate.

iv) Adenosine triphosphatase (Mg^{++} activated).

A.T.P - ase was demonstrated by a modified Wachstein technique.

Cryostat sections were cut at 10 microns, attached to clean slides, dried for 5 - 10 minutes and fixed in cold ($4^{\circ}C$) anhydrous acetone for 10 minutes. Sections were removed from the fixative, air dried and placed in the incubating medium at $37^{\circ}C$ for up to one hour.

Incubating medium

Adenosine triphosphate (Sigma)	---	20 mg.
Distilled water	---	22 ml
Tris maleate pH 7.2	---	20 ml
2% lead nitrate in distilled water	---	3 ml
2% magnesium sulphate in distilled water	---	5 ml

If necessary the pH was adjusted to 7.2. The solution was then placed in an incubator at $37^{\circ}C$ and left to ripen for one hour after which it was filtered and used.

During incubation the enzyme removes the terminal phosphate bond from A.T.P. and the released phosphate is captured by lead nitrate to form lead phosphate. Activating Mg^{++} ions are supplied as magnesium sulphate. The lead phosphate is later visualised as lead sulphide by treatment with dilute ammonium sulphide solution.

After incubation the sections were washed in running tap water for 10 minutes and then placed in a 1% aqueous solution of yellow ammonium sulphide for 1 minute. Sections were then washed in water for 5 - 10 minutes, fixed overnight in 10% formalin, washed in tap water and mounted in glycerine jelly.

Sites of A.T.P. - ase activity were demonstrated as dark brown-black deposits.

Controls were produced by omitting A.T.P. from the incubating medium.

3. Exposure of fish

Experimental exposures of fish to heavy metals in this investigation were disrupted by the occurrence of ulcerative dermal necrosis. Due to this the times of exposure and numbers of fish examined vary from metal to metal. No fish with any symptoms of the disease were ever employed for histochemical purposes.

Four fish were examined after exposure to copper. (Two at 65 days, one at 70 and one at 75 days). Only three fish were examined after exposure to lead. (40, 70 and 95 days). Five fish were examined after exposure to nickel. (78, 85, 105, 120, 130 days), and five fish were examined after exposure to zinc. (70, 75, 85, 105 and 120 days). The results of all exposures are summarised in tables 6, 7, 8 and 9.

4. Results

i) Control fish

(a) Malic dehydrogenase

Liver

After 45 minutes incubation M.D.H. activity was evenly distributed throughout the parenchyma cells. The epithelium of blood vessels exhibited a higher activity and a positive reaction was also seen in the endothelium. The epithelium of bile canals was also positive.(see Plate 56).

Midgut

After 45 minutes high concentrations of enzyme activity were seen in the mucosal epithelial cells, whereas activity in the submucosa was noticeably lower. Mucus goblet cells did not show increased activity. Both the circular and longitudinal muscle layers exhibited higher activity than any other part of the gut. (see Plate 56).

Kidneys

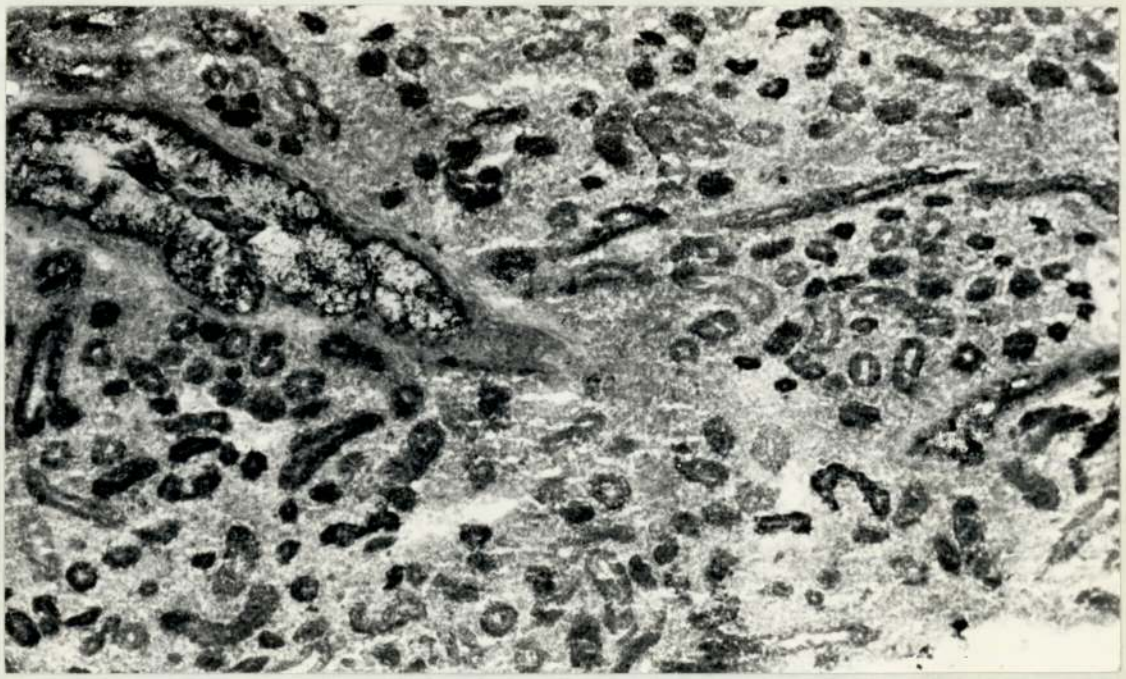
After 60 minutes incubation the highest enzyme activity was seen in the proximal tubules. The distal tubules exhibited lower activity and although the intertubular tissue was positive the reaction was much reduced. The glomeruli were also positive but detailed location of the activity was not possible. The observable reaction in the tubular

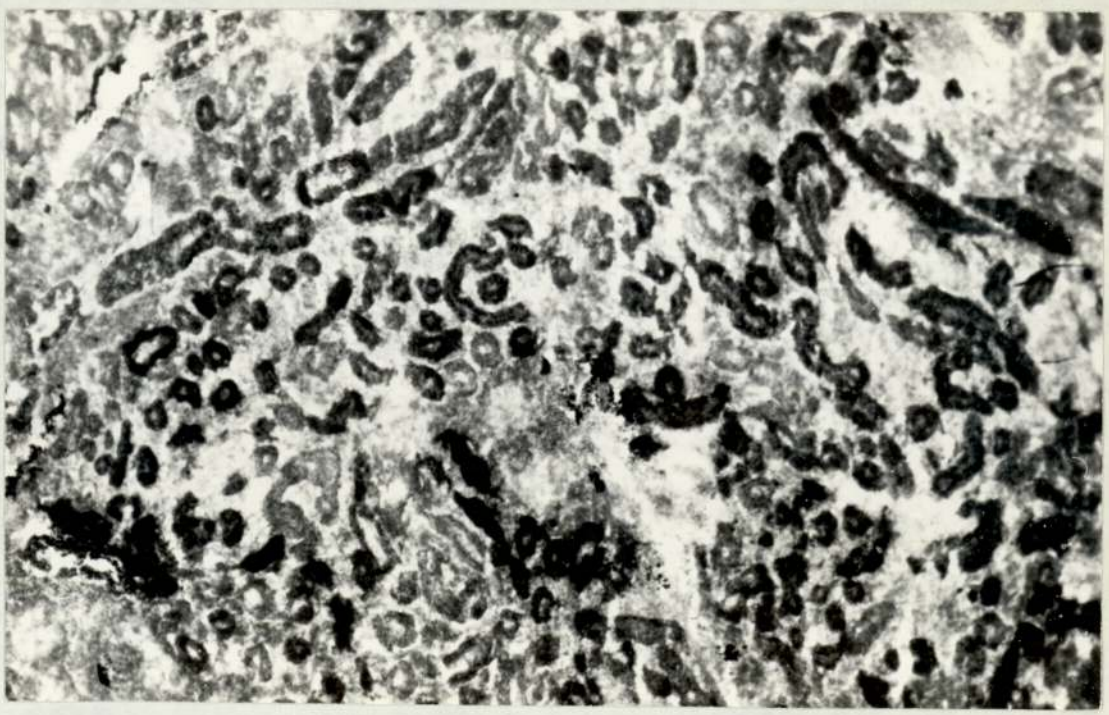
Plate 58

Lactic dehydrogenase activity in the kidneys of an untreated fish. Cryostat section, Tetrazolium-formazan technique. (X 108).

Plate 59

As above, but fish exposed for 75 days to 0.1 ppm of copper. (X 108). Note the reduction in enzyme activity in the intertubular tissue.





epithelial cells appeared uniformly distributed, (ie. not concentrated below the brush borders.)

Gills

After 60 minutes incubation a weak reaction was observed throughout the gills. Longer incubation times did not intensify the reaction. M.D.H activity appeared distributed throughout the epithelium of the secondary lamellae. A slightly stronger reaction was noticeable in the cartilage rays of the primary lamellae otherwise activity was relatively uniform.

(b) Lactic dehydrogenase

Liver

The distribution of L.D.H. activity was comparable with that of M.D.H.

Midgut

As M.D.H.

Kidney

As M.D.H. (possibly somewhat higher)

Gills

A somewhat more intense reaction indicating L.D.H. activity was seen in the gills. The fine granular blue formazan precipitate was more highly concentrated in the cells between the bases of the secondary labellae but all the epithelial cells were positive.

(c) Alkaline phosphatase

Liver

Only the endothelium of blood vessels and bile canaliculi was positive all parenchyme cells exhibiting no activity. (see Plate 60)

Midgut

After 45 minutes incubation a strong reaction was seen around the striated border of the mucus epithelium. On detailed examination activity was detected as fine blue azo granules in the upper part of the mucus epithelial cells. No activity was seen in developing mucous goblet cells. Small areas of activity were detectable in the submucosa. The inner

Plate 60

Alkaline phosphatase activity in the midgut and liver of an untreated fish. Cryostat section, simultaneous coupling azo-dye technique. (X 215).
Note the activity in the mucosa and the muscularis of the intestine. Activity in the liver is restricted to the lining of blood capillaries and bile ducts.

Plate 61

General view of alkaline phosphatase activity in the midgut of an untreated fish. (X 75). Technique as above. The activity is confined almost entirely to the lumenal part of the mucosa.

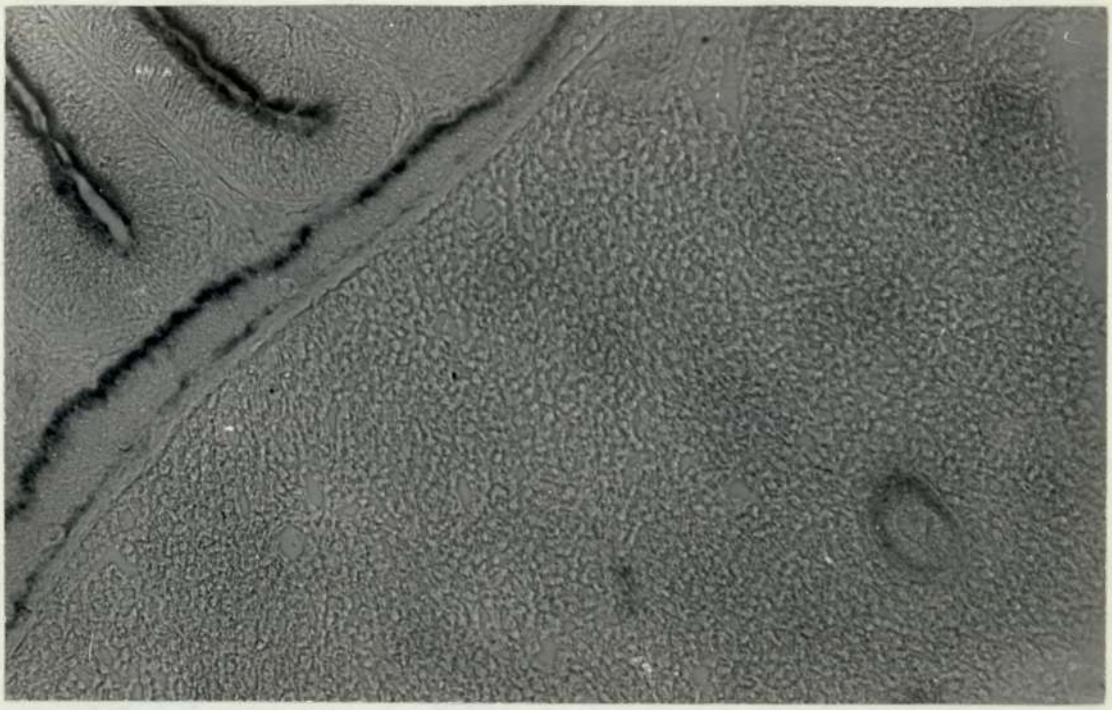
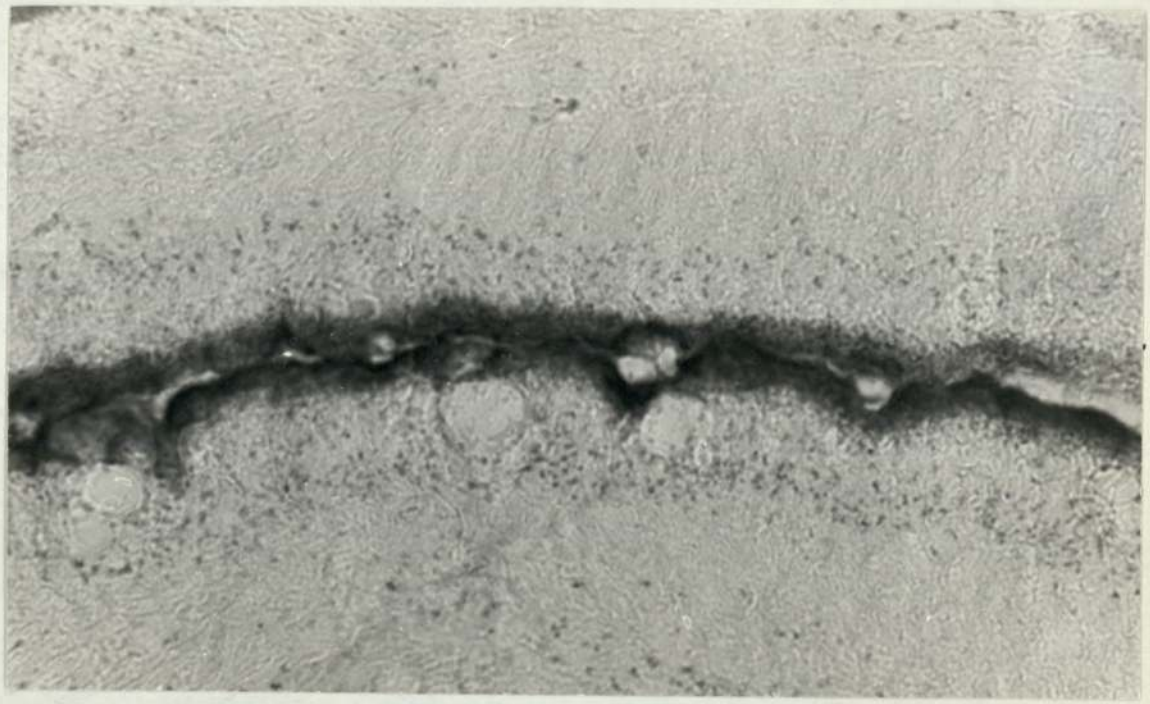


Plate 62

Detail of alkaline phosphatase activity in the intestinal mucosa of an untreated fish (X 690). Technique as plates 60 and 61. Note that the activity is very concentrated at the upper part of the epithelial cells and that it does not appear to be associated with mucous goblet cells.

Plate 63

Reduction in intestinal alkaline phosphatase activity in the intestine of a fish exposed to 0.1 ppm of zinc for 105 days. (X 92).



circular muscle layer had marked enzyme activity whilst the much thinner outer longitudinal muscle had less. (see Plates 61 & 62).

Kidneys

The kidneys exhibited consistent alkaline phosphatase activity. The proximal tubules were strongly positive, a fine blue azo dye deposit appearing just below the brush border. In many cases activity was also detected within the lumen of tubules but this may have been due to diffusion of the reaction product. Distal tubules and glomeruli exhibited little or no activity but a positive reaction was seen in the squamous epithelium of the Bowman's capsules. The intertubular tissue had very high activity the heavy azo deposits outlining the negative distal tubules. (see Plate 64).

Gills

Alkaline phosphatase activity was seen only in the cells between the bases of the secondary lanellae. The activity was evenly distributed amongst the cells presumably indicating their involvment with ion transfer. Careful observations revealed little or no activity in the respiratory epithelium. (see Plate 66).

(d) Acid phosphatase

Acid phosphatases are highly soluble enzymes and their demonstration in cryostat sections is somewhat unrealistic. The brief fixation in cold Baker's Ca-formalin employed in the present study prior to incubation prevents a certain amount of diffusion.

Liver

After 24 hours incubation enzyme activity appeared as a granular red azo deposit. Due to diffusion the deposit was difficult to associate with any particular cell inclusions but it is known that in many cells acid phosphatases are associated with lysosomes..

All the parenchyma cells of the liver reacted strongly. (see Plate 70). The epithelium of blood vessels was also possitive but the endothelium appeared negative.

Midgut

Plate 64

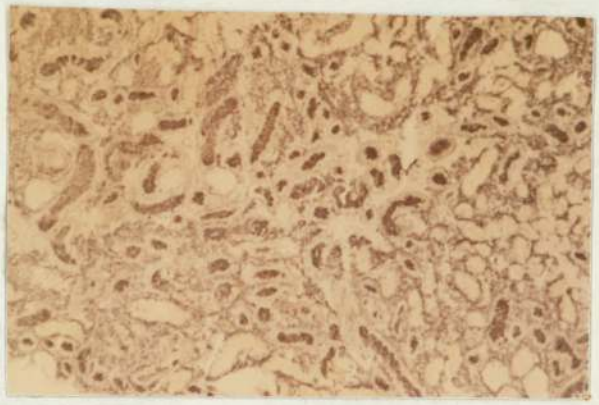
Alkaline phosphatase activity in the kidneys of an untreated fish. (X 60). Cryostat section, azo dye technique.

Plate 65

Alkaline phosphatase activity in the kidneys of a fish exposed to copper (0.1 ppm) for 65 days. (X 60). Note the large increase in activity in the proximal tubules and the decrease in activity in the rest of the tissue.

Plate 66

Alkaline phosphatase activity in the gills of an untreated fish. (X 150). Technique as above. The activity is evenly distributed in the cells between the bases of the secondary lamellae.



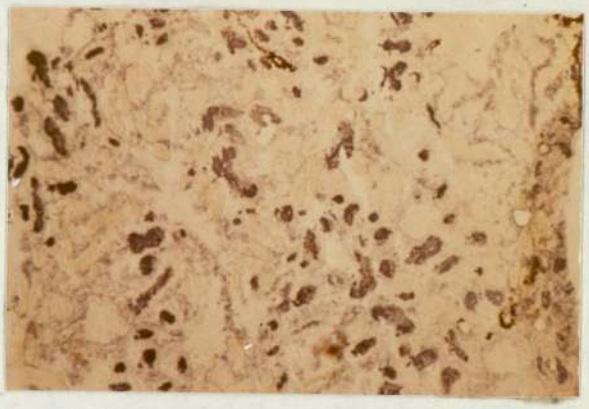


Plate 67

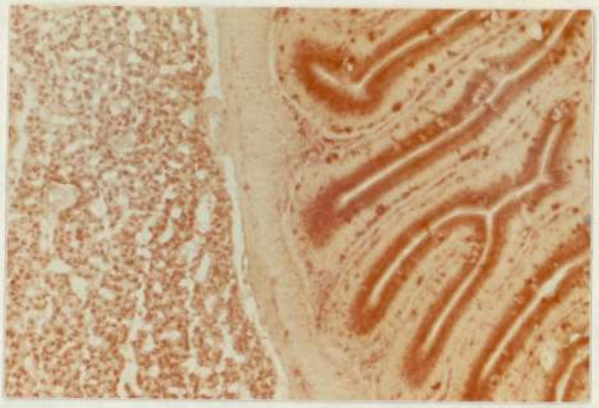
Acid phosphatase activity in the liver and mid-gut of an untreated fish. (X 92). Cryostat section, simultaneous coupling azo-dye technique.

Plate 68

As above, detail of intestinal mucosa. (X 920). Enzyme activity is high in the epithelial cells but is also very prominent in developing mucous goblet cells.

Plate 69

Acid phosphatase activity in the gills of an untreated fish (X 150). Cryostat section. The activity is restricted to specific cells between the bases of the secondary lamellae and not evenly distributed as is alkaline phosphatase. (see plate 66).



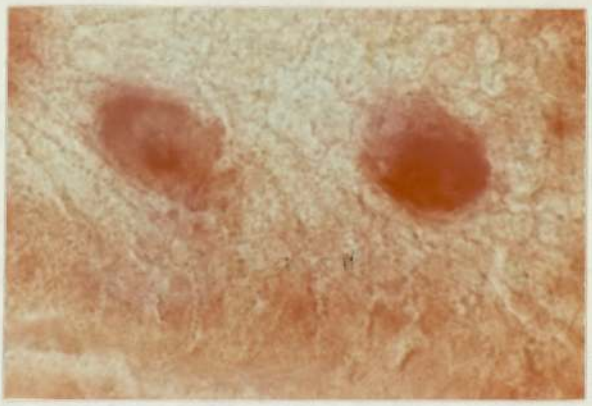
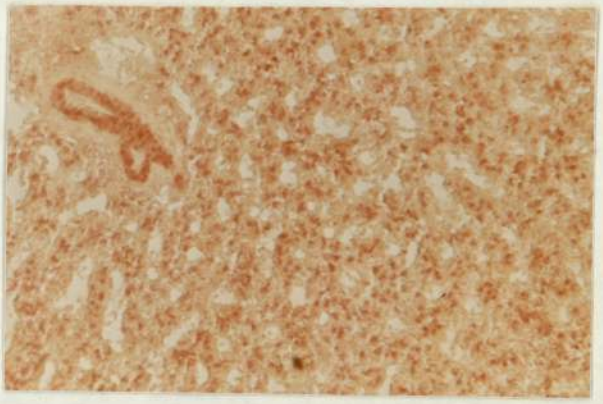


Plate 70

Acid phosphatase activity in the liver of an untreated fish. (X 150). Cryostat section, azo dye technique. The gaps in the tissue are due to tissue processing. Note the increased activity in the epithelium of the blood vessel.

Plate 71

Increased acid phosphatase activity in the liver of a fish exposed to 4 ppm ofnickel for 130 days. (X 150). Technique as above.



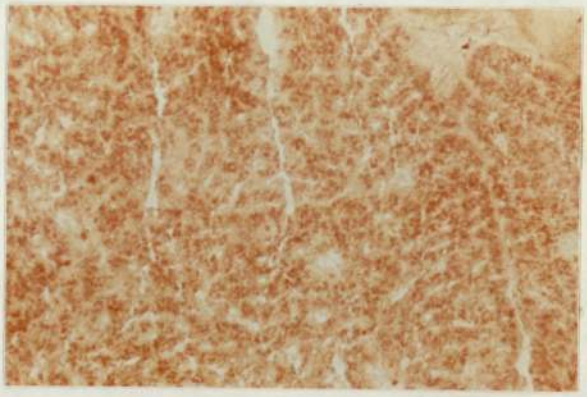
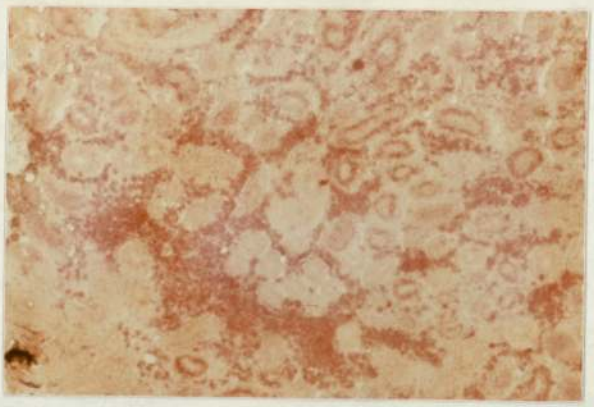


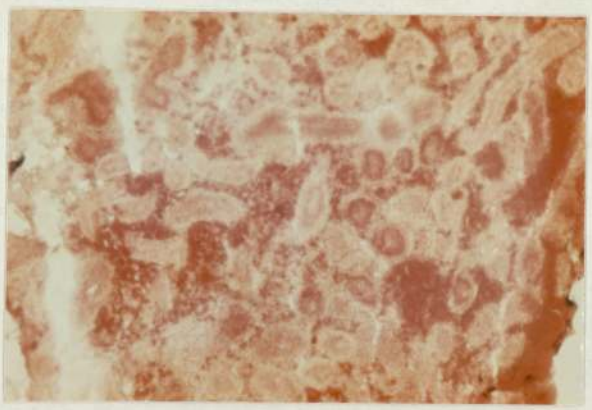
Plate 72

Acid phosphatase activity in the kidneys of an untreated fish. (X 92). Cryostat section, azo-dye technique.

Plate 73

Acid phosphatase activity in the kidneys of a fish exposed for 120 days to 0.1 ppm zinc. (X 92). Technique as above. Note the increased activity in the intertubular tissue and many of the tubules.





A strongly positive reaction was obtained in the upper part of all the mucosal epithelial cells and within the striated border. Unlike alkaline phosphatase no fine granulation denoting enzyme activity were seen below this region but compact bodies possessing high activity were seen between and towards the bases of the epithelial cells. (see Plates 67 & 68). These were considered to be developing mucous cells. Neither of the muscle coats gave a positive result.

Kidneys

The overall distribution of acid phosphatase activity in the kidneys was similar to that observed for alkaline phosphatase. Proximal tubules exhibited the highest activity the azo dye appearing as a well defined granular deposit just below the brush border. Distal tubules and glomeruli were negative but high activity was detectable in the intertubular tissue. (see Plate 72).

Gills

The distribution of acid phosphatase activity in the gills provided an interesting comparison with alkaline phosphatase. The enzyme activity was again limited to the cells between the bases of the secondary lamellae but unlike alkaline phosphatase the activity was limited to particular cells and not spread evenly. (see Plate 69). The identity or physiological function of the active cells was not determined.

(e) Adenosine triphosphatase

Liver

After 60 minutes incubation A.T.P -ase activity was only weakly demonstrated in the liver. Further incubation did not intensify the reaction In the parenchyma cells a fine brown precipitate was seen but it was only in the epithelium of blood vessels and in bile canaliculi that a strong reaction was obtained.

Midgut

60 minutes incubation revealed enzyme activity corresponding to the distribution observed for alkaline phosphatase. The lumenal poles of the mucosal epithelial cells were strongly active but no reaction was seen

in developing mucous cells. Unlike the other enzymes studied a strong reaction was obtained in the submucosa. (see Plate 74). The circular muscle layer was strongly positive but a weak reaction was seen in the longitudinal muscle.

Kidneys

After 60 minutes incubation the proximal tubules exhibited very marked enzyme activity and often the lumen was heavily stained. The distal tubules were weakly positive but glomeruli were negative. Marked enzyme activity was observed in the intertubular tissue. (see Plate 76).

Gills

The gills exhibited only very weak activity which was contained in the cells between the bases of the secondary lamellae.

ii) Experimental fish

The results of all exposed fish are summarized in tables 6,7,8 & 9.

Copper

(a) Dehydrogenases

Both L.D.H. and M.D.H. activity in the liver appeared generally lower after exposure to 0.1 ppm of the metal for 65 - 75 days. The kidneys also exhibited a general reduction in dehydrogenase activity although with L.D.H. the reduction in activity was only noticeable in the intertubular tissue. (see Plate 59). With M.D.H. the reduction in activity was generally confined to the proximal tubules. The midgut and gills did not exhibit significant changes.

(b) Alkaline phosphatase

Examination of the kidneys revealed great changes in the distribution and activity of the enzyme. In three of the four fish examined the activity in the proximal tubules was very increased whilst that in the intertubular tissue had decreased enormously. (see Plate 65). Even with cryostat sections damage to the kidney tubules comparable with that observed in Section 9 was evident. Kidney phosphatase is concerned with the reabsorbtion of sugar (Wilmer 1944). It is possible that the increase

Plate 74

A.T.P-ase activity in the midgut of an untreated fish. (X 140). Cryostat section, modified Wachstein technique. Enzyme activity is restricted to the lumenal part of the mucosa and is not associated with mucous goblet cells.

Plate 75

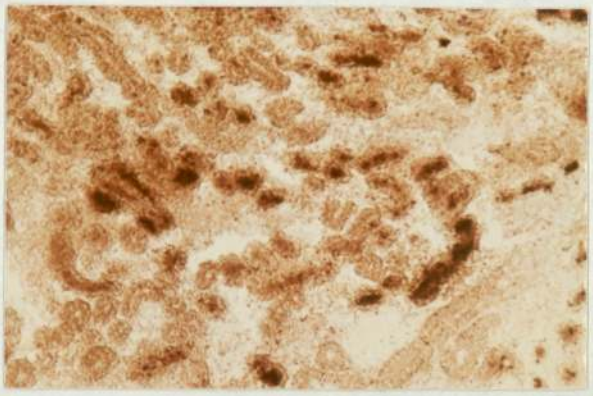
Reduced A.T.P-ase activity in the midgut of a fish exposed to nickel (4.0 ppm) for 130 days. (X 140). Technique as above.

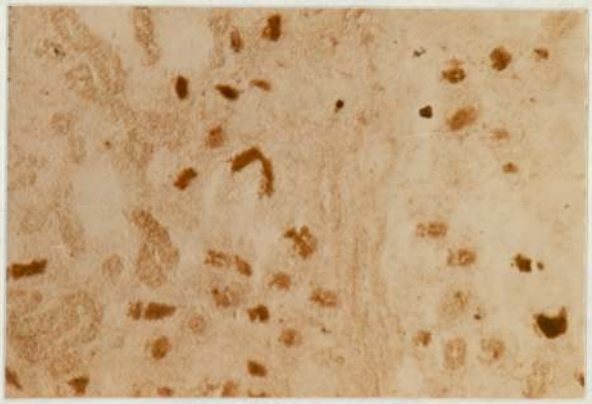
Plate 76

A.T.P-ase activity in the kidneys of an untreated fish. (X 92). Cryostat section. Modified Wachstein technique.

Plate 77

A.T.P-ase activity in the kidneys of a fish exposed to 0.1 ppm of copper for 75 days. (X 92). Technique as above. Note the increase in enzyme activity in the proximal tubules and the decrease in activity in the rest of the tissues.





in the enzyme activity in the proximal tubules was caused by an increase in urine flow due to increased permeability of the gills caused by copper. None of the other metals tested produced this overall effect. Slight changes in the activity of the enzyme in the gills and gut were probably not significant.

(c) Acid phosphatase

In all cases examined there was an increase in the acid phosphatase activity of the liver relative to control fish. There was only a slight increase in activity in the proximal tubules of the kidneys but in one case an increase in enzyme activity in the intertubular tissue appeared as very local compact areas.

The midgut and gills exhibited no changes although in one case in the gills an increase in activity was seen.

(d) A.T.P - ase.

The picture with respect to the kidney was rather confusing. In two cases a reduction in activity in both tubular and intertubular tissue was noted. In the remaining fish a reduction in intertubular A.T.P-ase activity was seen in one whilst in the other the activity in the tubular epithelial cells appeared increased. (see Plate 77).

The gut and gill sections were comparable to controls.

Lead

Due to disease problems only three fish were examined.

(a) Dehydrogenases

Liver, midgut and gills showed no significant changes. In the kidneys there was a slight reduction in M.D.H. activity in all three cases but L.D.H. activity was only reduced in one fish.

(b) Alkaline phosphatase

No significant changes in enzyme activity were observed except that a fish taken after 40 days exposure exhibited a slightly increased activity in the gills.

(c) Acid phosphatase

In all three cases the liver exhibited an increase in enzyme

activity and in one fish the increase was very marked. In one fish (95 days) the enzyme activity in the kidneys was generally very much reduced. In all fish there was a noticeable increase in enzyme activity in the gills. The cells possessing activity appeared much more deeply stained and more sharply defined than the controls. No changes were observed in the mid-gut.

(d) A.T.P - ase.

The kidneys only showed marked changes but the picture was confusing with an increase in activity in one case and a marked decrease in the activity in the proximal tubules in another.

Nickel

(a) Dehydrogenases

After 105 and 130 days exposure to 4.0 ppm of the metal there was a marked increase in the activity of L.D.H. and M.D.H. in the liver. The kidneys of all cases examined had proximal tubules with increased activity and in the distal tubules activity was very much reduced in the case of M.D.H. In four cases the gills exhibited increased L.D.H. activity but changes in the activity of M.D.H. were less pronounced. In the mid-gut L.D.H. activity was increased in two cases (105, 120 days) and decreased in one (130 days). The changes however were only slight and probably not significant.

(b) Alkaline phosphatase

Very slight changes noticed in the kidneys were considered not significant. The mid-gut of all fish appeared to have slightly lowered activity and in one fish this was very marked (130 days).

(c) Acid phosphatase

In three cases the livers exhibited a noticeable increase in enzyme activity. Increases in activity were also seen in the gut and gills though they were not as consistent. In general the kidneys exhibited minor changes although in one case there was a considerable increase in the activity of the proximal tubules.

(d) A.T.P - ase.

The kidneys of only three fish were successfully examined and in all

cases there was an overall reduction in enzyme activity. Other tissues did not exhibit marked changes, except that in one case (130 days) there was a marked reduction in enzyme activity in the intestine. (see Plate 75).

Zinc

(a) Dehydrogenases

The M.D.H. activity of the liver was reduced in four out of the five cases examined. The picture with L.D.H. was somewhat different. Three fish examined after up to 85 days exposure exhibited decreased activity which in two cases was very marked but at 105 and 120 days activity was noticeably increased. In the kidneys M.D.H. activity was slightly reduced. At 75 days L.D.H. activity was only slightly reduced, at 85 days there appeared to be a slight increase and at 105 days and 120 days the increase was marked. The activity in the proximal tubules increased to a greater extent than in the distal tubules. In most cases there was an apparent decrease in enzyme activity in the mucosa of the mid-gut. The gills exhibited no significant changes.

(b) Alkaline phosphatase

The gills in all cases exhibited reduced activity. Changes in other organs were less well marked, except that in one case (105 days) there was a considerable reduction in enzyme activity in the intestine. (see Plate 63).

(c) Acid phosphatase

There was a noticeable increase in enzyme activity in the liver, particularly in one case (120 days). (see Plate 71). The kidneys also exhibited an overall increase in activity especially in the intertubular tissue where the activity was very high. (see Plate 73).

(d) A.T.P - ase.

A varying picture was obtained with the kidneys. At 70 and 75 days the activity in the intertubular tissue was markedly lowered whereas the tubules appeared unchanged. In fish observed after longer exposure periods the drop in activity in the intertubular tissue was not apparent but there was an increase in the activity of the proximal tubules. The mid-gut

exhibited less activity in all cases but the reduction was very slight. No significant changes were seen in the gills.

5. General Discussion

The main purpose of this study was to detect enzyme changes and not to elucidate specific reasons for them. Many of the changes observed were probably due to individual variation and in tables 7 - 10 any changes symbolized by + or - are probably not significant.

All fish examined were apparently healthy except those exposed to copper where the initial symptoms of wasting were appearing. It is probable that fish in a more moribund condition would exhibit greater enzymatic changes.

The most consistent change in activity was an increase in acid phosphatase activity in the liver. Excess dietary zinc was found to induce abnormally alkaline phosphatase activity in rat livers by Van Reen (1953) and Sadasivan (1951,52). Histochemically it was not possible to assess the activity of alkaline phosphatase in the liver as it was only present in small amounts and not demonstrable in the hepatic cells generally. Both alkaline and acid phosphatase hydrolyse phosphate ester bonds and can also catalyse transphosphorylations. The increase in activity of acid phosphatase could be due to induction or stimulation by the metal. It is possible that as the stored metal concentration of the tissues increases inhibition might occur, although this was not noted with copper where it has already been shown (Sections 10 and 11) that large increases in the amount of stored copper occur. The initial increase and later decrease in L.D.H. activity in the liver after exposure to zinc may be due to initial stimulation by the metal. (L.D.H. is known to contain zinc), eventually followed by inhibition. It should be pointed out however that data from the present study does not indicate a significant build up of zinc in the liver. (see Sections 10 and 11).

The effect of copper exposure upon the alkaline phosphatase content of the kidneys is of interest. In all cases there appeared to be a great

increase in enzyme activity in the proximal tubules and a parallel decrease in activity in the intertubular tissues. This example at least demonstrates one advantage of histochemical techniques over direct bioassays. The distribution and amount of alkaline phosphatase activity of the organs did not appear to alter appreciably after exposures to lead, nickel or zinc. The acid phosphatase activity in the kidneys after copper exposures was found to increase only slightly but a greater increase was observed after zinc exposures.

From the limited amount of data presented it is obviously not possible to explain the reasons for increased phosphatase activity after copper and zinc exposures. It may be that increased reabsorption is occurring due to elevated urine output which may in turn be caused by an increase in the permeability of the gills due to the presence of the metal. It could also be that this increase and others observed are due to indirect stimulation via endocrine mechanisms.

It has been pointed out by Skidmore (1964) that the interrenal tissue of teleosts as well as possessing a haemopoietic function is homologous to the adrenal cortex of mammals and that it is believed to initiate stress syndromes. Changes in the activity of both alkaline and acid phosphatases were noted in this tissue and may be connected with hormonal changes taking place.

Studies of A.T.P - ase activity resulted in conflicting evidence. In the kidneys enzyme activity appeared reduced after copper and nickel exposures. After exposure to lead the picture was confused. Fish examined after exposure to zinc for up to 75 days had lower enzyme activity in the intertubular tissue of the kidneys whilst the rest of the organs remained unaltered. After 75 days an increase in the enzyme activity of the proximal tubules was seen but the earlier decrease in enzyme activity in the intertubular tissue was not evident. Increased activity in the proximal tubules may have been due to increased reabsorption but the reasons for the fall and subsequent recovery of activity in the intertubular tissue are not clear. Evidence for reduced activity of the enzyme in the mid-gut was

found after exposures to nickel and zinc but not to lead and copper. In one case (Ni 130 days) A.T.P - ase activity appeared almost absent from the lumenal part of the mucosa. It should be stressed that in all cases fish were starved for one day before sacrifice so that no individual variations in enzyme activity due to differences in the absorption of food from the gut would be seen.

The very low activity demonstrated in the gills in all cases is considered to have been due to lack of activation. Laurent et al (1968) histochemically studied the distribution of sodium and potassium activated A.T.P - ase in the psuedobranch of trout and found that when sodium and potassium were omitted from the incubating medium only a very weak reaction was obtained. Epstein (1967) assayed sodium - potassium activated A.T.P - ase in gill extracts from Fundulus and found a considerable increase in the activity of the enzyme when fish were adapting to seawater. The exclusion of sodium and potassium from the incubating media used in the present study is considered to have been the causal agent for the weak results obtained.

Jackim et al (1970) performed enzyme assays on liver homogenates taken from killifish exposed to six metal salts. Studies were also made on tissue extracts to which metal salts were added directly. The in vivo and in vitro exposures did not always produce similar results. It was found that copper was the strongest inhibitor of liver xanthine oxidase in vitro but that it stimulated the enzyme in vivo. Results such as these indicate the complexity of relating changes in enzyme activity to external toxic metal solutions.

Most of the enzyme changes noted in this short study were probably not of sufficient magnitude to be considered immediate causes of toxicity.

The role of the gills in the absorption of toxic metals has already been discussed. (see Sections 3, 8 and 11) and an increase in the zinc and copper content of the gills has been measured after subacute exposures of up to 90 days. (see Section 11). After copper exposures none of the gill enzymes studied appeared significantly affected after subacute exposures and after zinc only alkaline phosphatase was affected, a reduction in

activity appearing in all fish examined. Alkaline phosphatase is known to be concerned with ion exchange across the gills. Pettengill and Copeland (1948) were able to demonstrate increased alkaline phosphatase activity in the psuedobranch of Fundulus when fish were removed from seawater to freshwater. The authors considered it possible that the phosphatase activity had a relationship to the energy being expended in osmotic work (i.e. absorbing chloride). In the present study the decrease in gill alkaline phosphatase activity after zinc exposures seems likely to have been due to direct inhibition of the enzyme by the metal.

After subacute lead exposures where no measurable uptake of the metal by the gills was observed (see Section 11), acid phosphatase activity was found to be increased and in two cases alkaline phosphatase activity appeared slightly higher. It may be that the increased enzyme activity was due to increased transport of lead into the gills and thence to the blood stream. The same phenomenon was noted after nickel exposures but the evidence for increased alkaline phosphatase activity was only slight. The gills of fish after nickel exposures also exhibited a noticeable increase in dehydrogenase activity. Gill dehydrogenases are known to be inhibited by excess cation (in vitro) (Sexton and Russell 1955) and this reverse effect again demonstrates the complexity of the problem.

Rothstein (1959) points out that a knowledge of the sensitivities of individual enzyme systems in vitro does not allow a prediction of the action of metals in a cell. In the first place metals are not highly selective but are capable of inhibiting a wide range of enzymes. Secondly, the metals may never reach a particular enzyme site in the cell. Thus the localisation of metal sensitive sites in the cell is probably of greater importance than the inherent sensitivity of the sites. It is probable that the membrane of the cell which is directly accessible to the metal is the first site of damage and sometimes the only important one.

KEY TO TABLES 6, 7, 8 & 9.

+	=	Slight increase in activity (possibly not significant).
++	=	Definite " " "
+++	=	Strong " " "
++++	=	Very strong" " "
-	=	Slight decrease in activity (possibly not significant)
--	=	Definite " " "
---	=	Strong " " "
----	=	Very strong" " "
O	=	No change
X	=	No observation
P.T.	=	Proximal tubules
D.T.	=	Distal tubules
C.T.	=	Peritubular tissue

	LIVER				KIDNEYS				MIDGUT				GILLS			
	65	65	70	75	65	65	70	75	65	65	70	75	65	65	70	75
M.D.H.	---	---	-	---	-- (P.T.) 0 (D.T.) (C.T.)	-- (P.T) 0 (D.T.) (C.T.)	---	-- (P.T.) 0 (D.T.) (C.T.)	0	0	0	0	X	-	0	--
L.D.H.	--	---	0	--	--	-	-	--	0	0	0	0	X	0	0	-
Alkaline Phosphatase					++++ (P.T.) -- (C.T.)	++ (P.T.) -- (C.T.)	++++ (P.T.) -- (C.T.)	0	-	0	0	-	+	0	0	+
Acid Phosphatase	++	+	+++	++	+	+ (P.T.) + (Some C.T.)	+ (P.T.) + (Some C.T.)	+	0	0	0	0	0	+	++	0
A.T.P-ase					--	+ (P.T.) 0 (D.T.) (C.T.)	---	0 (P.T.) - (D.T.) (C.T.)	0	0	0	0	0	0	0	0
													(Activity very low in Controls.			

TABLE 6

SUMMARY OF ENZYME HISTOCHEMISTRY AFTER COPPER EXPOSURE.

	LIVER			KIDNEYS			MIDGUT			GILLS		
	40	70	95	40	70	95	40	70	95	40	70	95
M.D.H.	X	0	0	− (P.T.) 0 (D.T. C.T.)	− (P.T.) 0 (D.T. C.T.)	− (P.T.) — (C.T.)	X	0	0	X	0	0
L.D.H.	X	+	0	0	0	− (P.T.) — (C.T.)	X	0	0	X	0	0
Alkaline Phosphatase				+ (P.T.) − (D.T. C.T.)	0	0	0	0	0	++	+	0
Acid Phosphatase	++	+++	+	0	0	---	+	0	0	++	++	++
A.T.P-ase				X	++ (P.T.) 0 (D.T.) (C.T.)	---	X	0	0	X	0	0

TABLE 7

SUMMARY OF ENZYME HISTOCHEMISTRY AFTER LEAD EXPOSURE.

	LIVER					KIDNEYS					MIDGUT					GILLS				
	78	85	105	120	130	78	85	105	120	130	78	85	105	120	130	78	85	105	120	130
M.D.H.	X	0	+++	X	+++	++ (P.T.) 0 (D.T.) -- (C.T.)	++ (P.T.) 0 (D.T.) -- (C.T.)	++ (P.T.) 0 (D.T.) -- (C.T.)	X	+(P.T.) 0 (D.T.) -- (C.T.)	X	X	+	X	0	0	+	+++	0	0
L.D.H.	X	X	++	+	+++	X	++	++	++	X	X	X	++	++	--	++	++	+++	+++	0
Alkaline Phosphatase						0 (P.T.) (D.T.) - (C.T.)	0	- (P.T.) (C.T.)	0	- (P.T.) (C.T.)	-	-	-	-	--	+	+	+	0	0
Acid Phosphatase	0	++	0	+++	++++	+++ (P.T.) ++ (D.T.) (C.T.)	X	0	0	X	++	0	0	0	++	++	++	0	0	++
A.T.P-ase						X	X	--	--	----	X	-	-	-	---	X	X	0	0	-

TABLE 8

SUMMARY OF ENZYME HISTOCHEMISTRY AFTER NICKEL EXPOSURE.

	LIVER.					KIDNEYS					MIDGUT					GILLS.				
	70	75	85	105	120	70	75	85	105	120	70	75	85	105	120	70	75	85	105	120
M.D.H.	—	-	--	--	-	-	--	-	-	X	--	--	--	--	X	-	-	0	+	+
L.D.H.	---	--	---	++	+++	-	- (P.T.) 0 (D.T.) (C.T.)	+	++ (D.T.) (C.T.) 0 (P.T.)	++	--	--	--	0	0	-	-	X	+	++
Alkaline Phosphatase						0	- (P.T.) 0 (D.T.) -- (C.T.)	0	0	X	-	0	X	---	-	--	--	--	--	X
Acid Phosphatase	+	++	++	+	+++	++	+	++	++	+++ (C.T.) ++ (P.T.) (D.T.)	0	+	0	0	+	X	+	+	⊖	—
A.T.P-ase						---	--- (C.T.) 0	+ (P.T.) 0	++ (P.T.) 0	++ (P.T.) 0	-	-	-	-	-	-	⊖	-	0	0

TABLE 9
SUMMARY OF ENZYME
HISTOCHEMISTRY
AFTER ZINC

14. THE EFFECT OF SUBACUTE CONCENTRATIONS OF HEAVY METALS ON THE SURVIVAL OF MINNOWS AT LOW DISSOLVED OXYGEN CONCENTRATIONS

The effect of low concentrations of dissolved oxygen on the toxicity of heavy metals to fish has been realised for many years. In general when the oxygen concentration of the water is reduced the lethal threshold for any metal falls.

Westfall (1945) demonstrated increased toxicity of lead to goldfish in water with low dissolved oxygen and Weiss and Botts (1957) showed that changes in time of response to Sarin in a water solution decreased relative to decreases in dissolved oxygen. Lloyd (1960) tested unacclimatised fingerling rainbow trout at three sublethal concentrations of dissolved oxygen in five lethal concentrations of zinc sulphate in hard water (320 ppm $CaCo_3$). He calculated that after an exposure time of 1000 minutes the concentration of zinc necessary to kill 50% of the fish was 1.4 times greater at an oxygen concentration of 8.9 mg/litre than it was at 3.8 mg/litre. With fish acclimatised for 18 hours to the experimental dissolved oxygen concentration the toxicity of zinc was less.affected. Pickering (1968), investigating the effects of dissolved oxygen concentrations on the toxicity of zinc to the bluegill (Lepomis macrochirus) showed that on the basis of 20 day $L.C._{50's}$ significant differences occurred (at the 95% level of confidence) when the effect of three dissolved oxygen concentrations on subacute levels of zinc were tested. Lloyd (1961) exposed trout to graded concentrations of zinc sulphate at different dissolved oxygen concentrations, none of which were low enough to be lethal. From the resultant data he was able to draw a series of log. concentration/log. survival time curves. Using data from experiments with lead, copper, zinc and monohydric phenols he then graphically demonstrated the increase in the toxicity of a poison which occurs when the dissolved oxygen concentration is reduced by dividing the lethal threshold concentration obtained under air saturation conditions ($X_{SAT.}$) by the lethal

threshold concentration for the lower oxygen level (X). The values for the factor $^{X}SAT./_{X}$ were then plotted against the corresponding dissolved oxygen concentrations for the four toxicants. When plotted in this way the points for all three metals and for phenol fitted the same curve. Thus the effect of lowering the dissolved oxygen concentration was found to be similar for all four poisons. The hypothesis put forward to account for this phenomenon is that the fish increase water flow over the gills as the dissolved oxygen content of the water falls and the increase in flow consequently brings the toxicant into contact with the gills at a greater rate. As the gills are probably the main site of action (damage) or absorption of most toxins the net effect is to increase the apparent toxicity of any poison. An extension of this hypothesis is that any environmental factor which increases gill ventilation should exert the same effect as lowering the dissolved oxygen content of water. This subject is briefly discussed in section 3. 1. (iv).

The possibility that normally subacute concentrations of heavy metals would become acutely toxic at low dissolved oxygen concentrations was investigated in the first part of this section (14.1). Fish were exposed to subacute concentrations of the metals at different low oxygen concentrations and the toxic effects estimated on a 48 hour $L.C._{50}$ basis. Histological preparations of the gills were also made in an attempt to ascertain the cause of death. (i.e. Gill damage or direct hypoxia?)

The data presented in sections 9 - 13 of this thesis indicate varying effects due to exposure to subacute concentrations of the heavy metals. Damage to the internal organs, uptake and accumulation of the metals and some changes in enzyme activity have been demonstrated. Changes in the pathology of the gills were however only noted after zinc exposures and even then were very slight. When considering the ecological implications of subacute exposures to metals one of the most important aspects of the possible continued survival of fish populations is whether or not individuals will be able to survive

extremes of the environment such as low dissolved oxygen concentrations. In organically polluted waters the dissolved oxygen concentration can fall to very low levels during the latter part of the night. (Owens and Edwards (1964)) Pollution by heavy metals is often accompanied by organic pollution causing deoxygenation. The question thus arises whether fish which have been exposed to subacute levels of metals for extended periods will be able to survive the very low levels of dissolved oxygen which may occur during each 24 hour cycle. This possibility is tested in the second part of this section (14.2) where fish were exposed to subacute levels of the metals for 50 days and then subjected to low dissolved oxygen levels.

1. 48 hour $L.C._{50}$ values for the metals at reduced dissolved oxygen concentrations.

After testing fish in the absence of heavy metals at low dissolved oxygen (D.O.) it was found that even when the D.O. was lowered to the experimental level quite quickly (approximately 1 hour) from air saturation levels some fish were able to tolerate under 1 mg/litre of D.O. at 15°C. Data will be presented in part 2 of this section to show that where longer acclimatisation periods are employed, Phoxinus can be maintained at 0.5 mg/litre at 15°C for extended periods. Because of the tolerance of the fish to very low D.O. levels it became obvious that where tests at the bottom of the D.O. range were employed, great accuracy in the maintainance of D.O. levels would benecessary. The apparatus in which the experiments were carried out fulfills this criterion and was constructed as described below.

i) Materials and methods

Fish were contained in a large glass tube which was part of a circulating system similar to that described in Section 5.2. Apparatus to control dissolved oxygen levels has been described by Fry (1951), Cairns and Scheier (1958), Davison et al (1959), Mount (1964), and Pickering (1968).

The apparatus used in the present experiments depended upon the

bubbling of nitrogen gas through the test solution so as to displace dissolved oxygen. Other workers have used vacuum degassing systems and fractionating columns as well as the nitrogen displacement method. Unlike earlier methods the dissolved oxygen concentration in the present study was precisely controlled by electrical means. The apparatus is shown in Figure 10 and Plate 78.

After leaving the "fish chamber" (A) the test solution was pumped through non-toxic plastic tubing coiled in a temperature controlled water bath. A magnetic pump of the type described in 5.2 was employed. The test solution then passed to the column (B) where deoxygenation was effected. The column was filled with 2 cm diameter "All-plas" polythene spheres, and as the water passed down over them nitrogen was bubbled upwards from a diffuser stone at the base. On leaving the base of the column (B) the water passed through two chambers (C) and (D). Chamber (C) housed an electrical thermometer and chamber (D) a Mackereth type dissolved oxygen electrode. Water was caused to flow downwards over the dissolved oxygen electrode as prescribed by the makers and the flow rate was sufficient to prevent deoxygenation of the water in the chamber by the electrode. Both the electrical thermometer and the dissolved oxygen electrode were connected to an E.I.L. (Model 15.A) dissolved oxygen meter. The maintenance of constant, predetermined D.O levels was achieved as follows: a 24 hour "Fielden" pen recorder was connected to the D.O. meter. A solenoid valve (Alcon type A.C.O.2) was incorporated into the nitrogen supply from the cylinder to the column (B). The solenoid valve was controlled by the pen recorder. The required D.O. concentration was set on the recorder and when the D.O. level in the assay solution was above this level the valve opened and nitrogen from the cylinder was admitted to the system thus causing the D.O. to fall. The dissolved oxygen meter monitored the fall and this was transmitted to the recorder. When the D.O. fell below the pre-selected level on the recorder the solenoid valve was closed, cutting off the nitrogen supply. A slit was cut in the side of the silicone

Plate 78

Apparatus used to maintain fish at steady low dissolved oxygen concentrations.

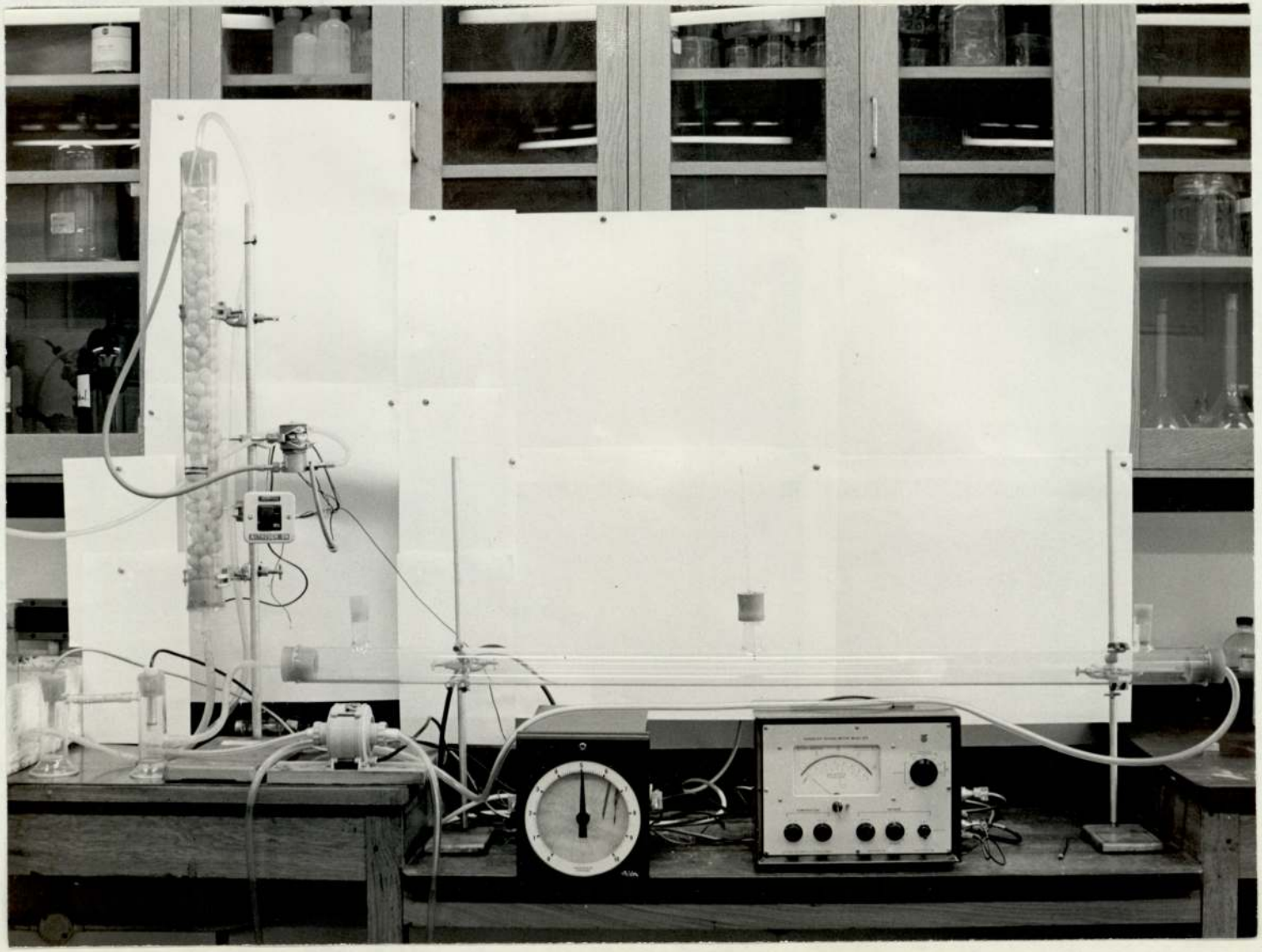


Figure 10

Apparatus for maintaining fish at constant low oxygen levels.

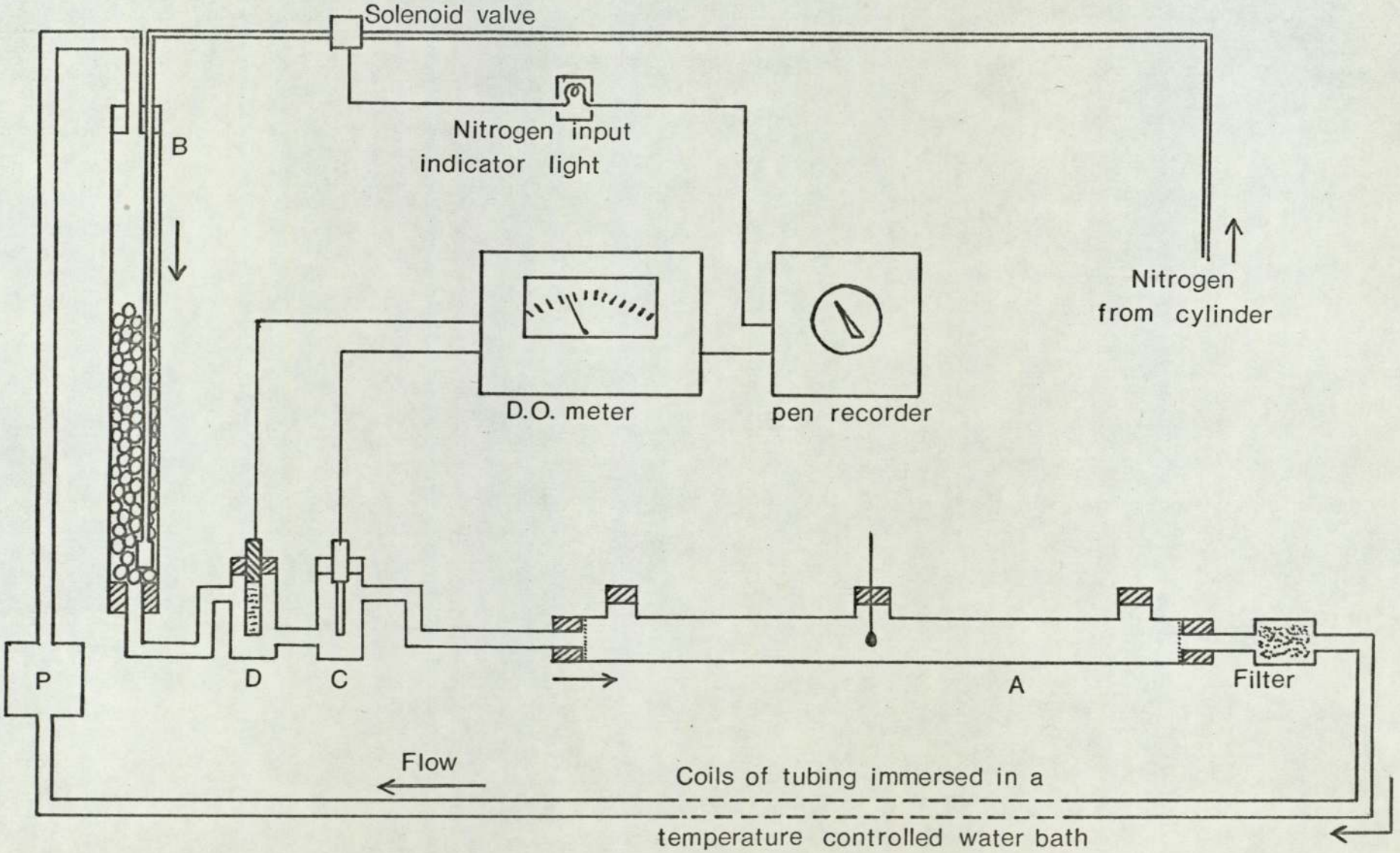
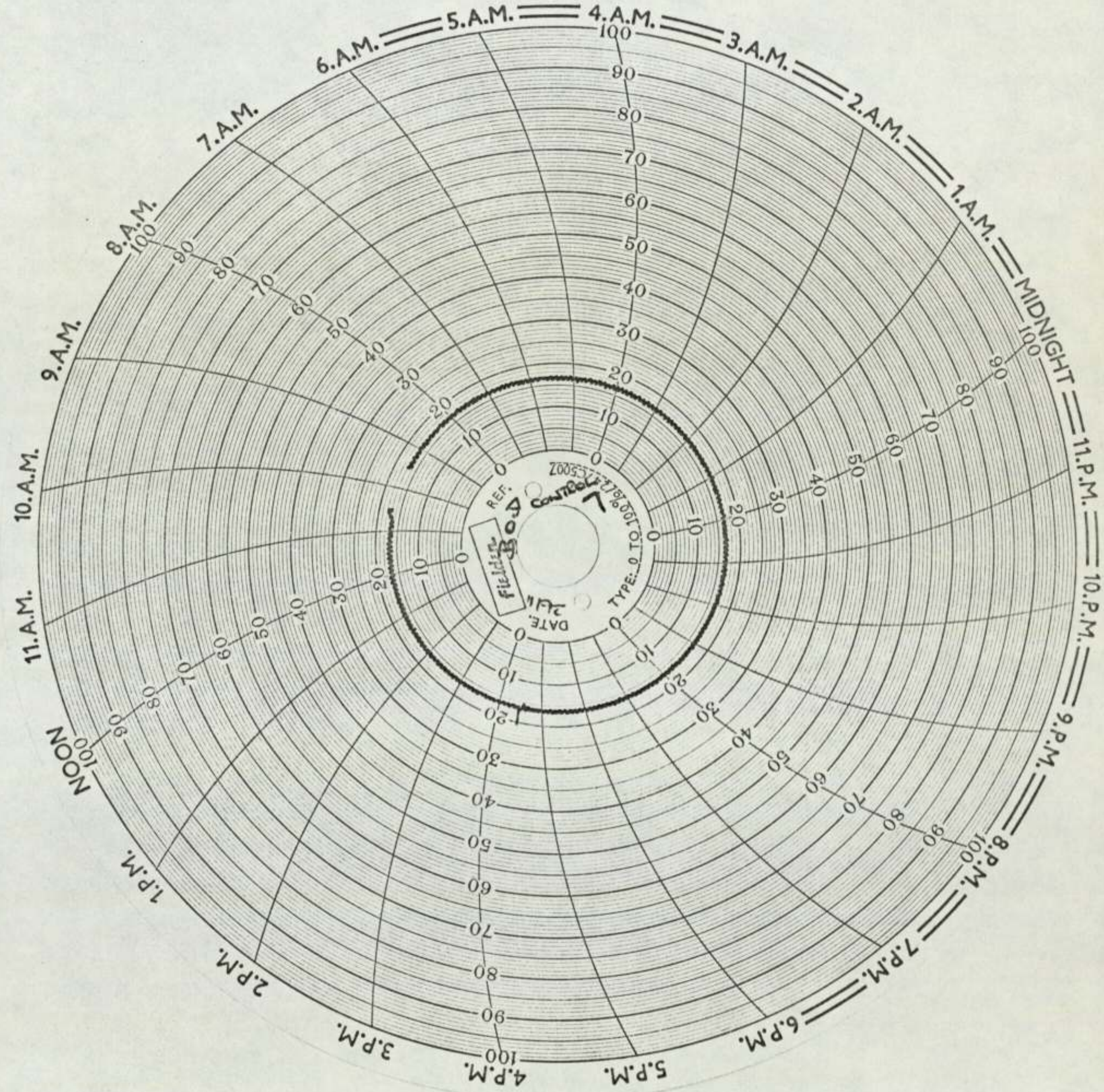

Solenoid valve
Nitrogen input
indicator light
B
Nitrogen
from cylinder
D.O. meter
pen recorder
P
D
C
A
Filter
Flow
Coils of tubing immersed in a
temperature controlled water bath

Plate 79

A typical pen recorder chart showing a steady level of dissolved oxygen (1.5 mg/l) maintained over a twenty-four hour period.


MIDNIGHT
1.A.M.
2.A.M.
3.A.M.
4.A.M.
5.A.M.
6.A.M.
7.A.M.
8.A.M.
9.A.M.
10.A.M.
11.A.M.
NOON
1.P.M.
2.P.M.
3.P.M.
4.P.M.
5.P.M.
6.P.M.
7.P.M.
8.P.M.
9.P.M.
10.P.M.
11.P.M.
0
10
20
30
40
50
60
70
80
90
100
REF.
DATE.
TYPE: 0 TO 100%
BOD CONTROL 7

rubber bung at the top of the column (B) to allow excess air to escape as the nitrogen was admitted. The downward movement of the water in the column (B) tended to drag air into the system and after the nitrogen supply was cut off the dissolved oxygen level would rise slowly. When setting the D.O. at the lowest levels, very little variation due to the "on-off" system was desirable. The initial setting caused some problems because it was difficult to tell whether the solenoid valve was open or closed except by listening for the click as it opened. Because of this a "Nitrogen on" indicator light was wired into the system. A switch was also installed so that the solenoid valve could be closed in isolation from the recorder. This was done because when the D.O. was lowered from levels at air saturation to around 1 mg/litre some "overshoot" would occur in that when the solenoid valve closed additional nitrogen in the system caused the D.O. to fall farther. By closing the solenoid valve a little time before the preselected D.O. concentration was reached, the problem was overcome. The two sets of apparatus used in these experiments were maintained at $15.5^{\circ}C \pm 1^{\circ}C$ as at that temperature the % oxygen saturation of the water (as read by the D.O. meter) corresponds to the D.O. concentration in mg. per litre, i.e. 85% saturation = 8.5 mg/litre.

A very steady level of dissolved oxygen was maintained using this apparatus together with a complete record of any variations during the test period. A typical recorder chart for a 24 hour period is shown in Plate 79.

Operating sequence

A polythene aspirator containing the test solution was connected to the system immediately before the pump inlet. The tap of the aspirator was opened and the solution allowed to pass through the pump, so as to prime it. The pump was then switched on and the system allowed to fill. The two bungs in the end ports of the fish chamber (A) had central bores which were plugged with glass rods. This made it possible completely to fill the chamber as the glass rods were inserted

only after overflowing occurred. It was considered desirable to provide no air - water surface in the apparatus as some of the fish might have prolonged their survival at low dissolved oxygen concentrations by drinking air bubbles. When the apparatus was full of test solution the aspirator tap was closed, the end of the tube clamped and then connected up to the outlet tube to complete the circuit. Just prior to the completion of the circuit the level of the water in the column (B) was allowed to rise almost to the top in order to provide the head necessary to complete the circuit as when the two ends of tubing were connected some spillage was unavoidable. Before the fish were added the level of the water in the apparatus was allowed to equilibrate by removing the glass rod from one of the bungs in the chamber (A) thus allowing excess solution from the column (B) to run out of the system. This was done in order to avoid a high pressure of water in the apparatus which may have been injurious to the fish. After setting up the apparatus was allowed to run until a steady temperature of 15.5°C had been achieved (measured by the electrical thermometer). A mercury thermometer was inserted in the central part of the fish chamber (A) to check that there was a steady temperature throughout the apparatus.

Ten fish were used for each experiment and were introduced via one of the ports in the chamber (A) with the help of a wide diameter plastic filter funnel. The bung was then replaced making sure that there was no air trapped beneath it. The flow of test solution in the system was never enough to cause the fish to have to swim to maintain their position in the chamber. After 15 minutes, when the fish had settled down, the dissolved oxygen was lowered to the desired level by setting the chart recorder. Lowering of the D.O. to the test level was accomplished in one hour $\pm$ ten minutes.

The use of a continually recycled batch of test solution presented detoxification problems especially as the weight fish : weight metal ratios were rather high, varying from 360 : 1 up to 48,000 : 1. To overcome this the test solutions were replaced at 6, 12, 24 and 30 hours during

the 48 hours duration of each test. The replacement of the test solution was achieved by the method already described in Section 5.2 except that the solutions were partially deoxygenated. Nitrogen was bubbled through each replacement batch of solution and the D.O. level was then checked using the Winkler technique. Levels as near as possible to the test D.O. concentration were produced before thenew solution was admitted to the apparatus. When the circuit was broken and the new solution added, a rise in the D.O. concentration occurred, but this was kept to a minimum by "manual" admission of nitrogen using the switch incorporated in the circuit. After replacement of the test solution the dissolved oxygen concentration was back at the preselected level within 15 - 20 minutes.

Dissolved oxygen levels and heavy metal concentrations were selected on a logarithmic scale as recommended by Doudoroff et al (1951). Numbers of fish dead at 24 and 48 hours were noted and expressed as a percentage of the whole. Dead fish were removed as soon as possible with a wire hook. The apparatus was thoroughly cleaned between test runs. Hours of light and darkness were controlled up to a point along the lines described in Section 5.2.

ii) Results

The tabulated results of all tests are shown in Tables 24 and 25 (see appendix).

Control fish

Three series of experiments were performed in the absence of heavy metals and in all cases fish survived a dissolved oxygen concentration of 1.15 mg/litre. 0.87 mg/litre O_2 killed 30 - 40 % of the fish and 0.65 mg/litre O_2 killed all of them.

The response of the minnow to lethal levels of low oxygen was essentially similar to that described by Shepard (1955) and Jones (1952). When the dissolved oxygen was first lowered the fish showed immediate signs of distress, attempting to surface and exhibiting rapid darting movements. At 0.87 mg/litre O_2 after the first 60 minutes many of the fish lost equilibrium and rolled over. Equilibrium was later regained

by some of the fish and these lay still on the bottom of the experimental chamber. The opercular movements of these fish were very exaggerated and their frequency was greatly increased. To quote Shepard (1955), "On exposure to hypoxic conditions the depth of breathing increased, the opercula described wider arcs and the movements of the jaws and floor of the mouth became exaggerated". There was a secondary loss of equilibrium with some fish at 0.87 mg/litre O_2 from which they did not recover. At 0.65 mg/litre O_2 where all the fish died there was no temporary recovery after the initial overturn. At death the mouth was generally open to its maximum extent and the opercula were often spread out, exposing the gills. Sometimes the pectoral fins stood out at right angles to the body.

Experimental fish

(a) Copper

The range of copper concentrations tested was: 0.049 (0.05), 0.065, 0.087, 0.115 and 0.155 ppm.

Apart from the experiments conducted at a copper concentration of 0.115 ppm which at air saturation dissolved oxygen concentration killed 10% of the fish in 48 hours there was no increased percentage kill until the dissolved oxygen was lowered to 1.15 mg/litre. At 0.087 ppm of copper which is not a lethal concentration at high dissolved oxygen levels, 30% of the fish died at 1.15 mg/litre O_2. No control fish (i.e. in the absence of copper) died at this oxygen level. The question thus arises whether the death of the fish was due to the increased gill ventilation rate bringing copper to the respiratory surfaces faster than it could be removed and thus causing the gill damage typical of acute toxicity, or whether the presence of the metal in the water impaired the ability of the gills to take up oxygen thus causing death by hypoxia but with no gill damage? Histological studies of the gills of two fish tested at 1.15 mg/litre O_2 and 0.087 ppm copper tend to support the second hypothesis in that no gill damage of the sort associated with acute heavy metal toxicity was observed. There were

however, parts of the respiratory epithelium where some swelling was evident but these accounted for less than 5% of the whole and similar conditions have been observed in control fish subjected neither to metal salts nor to low dissolved oxygen.

(b) Zinc

The range of zinc concentrations tested was the same as those for copper.

Apart from the experiments at a zinc concentration of 0.155 ppm, which killed 60% of the fish at air saturation dissolved oxygen in 48 hours, there was no increased kill until 1.55 mg/litre O_2, where 10% of the fish died at 0.115 ppm of zinc. At 0.087 ppm of zinc at 1.15 mg/litre O_2 30% of the fish died. Tested separately the metal concentration and the low oxygen would not be lethal. Histological examination of the gills of two of the three dead fish revealed no gill damage concurrent with acute zinc toxicity and the cause of death is assumed to be direct hypoxia aggravated by the presence of zinc in the water. Two more fish were examined histologically after death in 0.155 ppm zinc (103% of the 48 hour $L.C._{50}$ at air saturation D.O.) at 4.9 and 2.8 mg/litre O_2 respectively. In both cases the gills had a swollen oedematous epithelium and sloughing was evident. The gills also had a thick mucus coat on removal from the fish and this was not the case where fish were taken dead from the 0.087 ppm zinc solution. 0.065 and 0.049 ppm of zinc did not exert a significant effect at low D.O., the results appearing almost the same as those for control fish.

(c) Lead

The range of lead concentrations tested was 0.56, 0.75, 1.0, 1.35 and 1.8 ppm.

Apart from the experiments at a lead concentration of 1.8 ppm which killed 30% of the fish at air saturation D.O. in 48 hours, there was no increase in percentage kill until the oxygen was lowered to 2.1 mg/litre.

Histological examination of the gills of one fish taken dead

from 1.8 ppm Pb and 1.55 mg/litre O_2 where 50% of the fish died revealed gill damage typical of acute lead toxicity. Examination of the gills of three fish killed at 1.35 ppm Pb and 1.15 mg/litre O_2 revealed conflicting evidence in that the pathology of the gills of one fish appeared typical of that due to acute lead toxicity, whereas the other two preparations appeared relatively normal.

The lowest concentration of lead tested (0.56 ppm) produced results very similar to those observed for control fish.

(d) Nickel

The range of nickel concentrations tested was as follows: 4.2, 4.9, 5.6, 6.5 and 7.5 ppm

Except at 7.5 ppm where all fish died at air saturation D.O. no deaths occurred in the other nickel solutions tested until the D.O. was reduced to 2.1 mg/litre (at 6.5 ppm Ni). The lowest concentration of nickel tested (4.2 ppm) resulted in percentage kills similar to those observed with control fish.

Histological examination of the gills of two fish, one from 6.5 ppm Ni and 1.15 mg/litre O_2 and one from 5.6 ppm Ni and 1.15 mg/litre O_2 indicated no damage typical of acute nickel toxicity.

The combined effects of low dissolved oxygen and heavy metals in general

In the present study generally non-lethal levels of heavy metals were tested with non lethal and lethal levels of dissolved oxygen. Lloyd (1960) tested lethal levels of zinc with non lethal levels of dissolved oxygen and found an increase in zinc toxicity when the dissolved oxygen was progressively lowered between 8.9 and 3.8 mg/litre. The fact that in the present study no increased mortality was observed until levels of at least 2 mg/litre O_2 is probably due to the fact that normally non lethal concentrations of the metals were tested.

The ecological significance of the present results is that normally "safe" concentrations of heavy metals become lethal at low dissolved oxygen levels. The dissolved oxygen level necessary to

exert this effect is, however, very low and fish would probably not be found in such concentrations in the field, regardless of the metal content of the water. With control fish, at all dissolved oxygen concentrations below 1.5 mg/litre the fish were very sluggish and in the field would probably not survive due to their inability to search for food or escape from predators. Another factor which occurs in the field but was not tested in the present study is the fact that where low dissolved oxygen concentrations occur in polluted waters the carbon dioxide level is generally higher than normal. Carbon dioxide exerts a toxic effect upon fish by reducing the capacity of the blood to absorb oxygen (Alabaster, Herbert and Hemens 1957). This factor would obviously increase the sensitivity of the fish to low oxygen. In the tests described earlier carbon dioxide as well as oxygen was displaced from the water by nitrogen and was thus not an important factor.

Histological studies of the gills revealed slightly conflicting evidence but generally there was no damage of the type associated with acute heavy metal toxicity. Death of the animals was thus ascribed to direct hypoxia aggravated by the presence of heavy metal. Because of the apparent effects of the presence of metals in reducing tolerance to low dissolved oxygen in the absence of gill damage, experiments to determine the effect of long term subacute exposure to heavy metals on tolerance to low oxygen were carried out. This work is reported in part two of this section.

It has already been pointed out that Lloyd (1961) was able to express the increase in the toxicity of a poison at low oxygen levels by plotting the factor $^{X_{SAT.}}/_{X}$ against dissolved oxygen concentrations on a logarithmic scale.

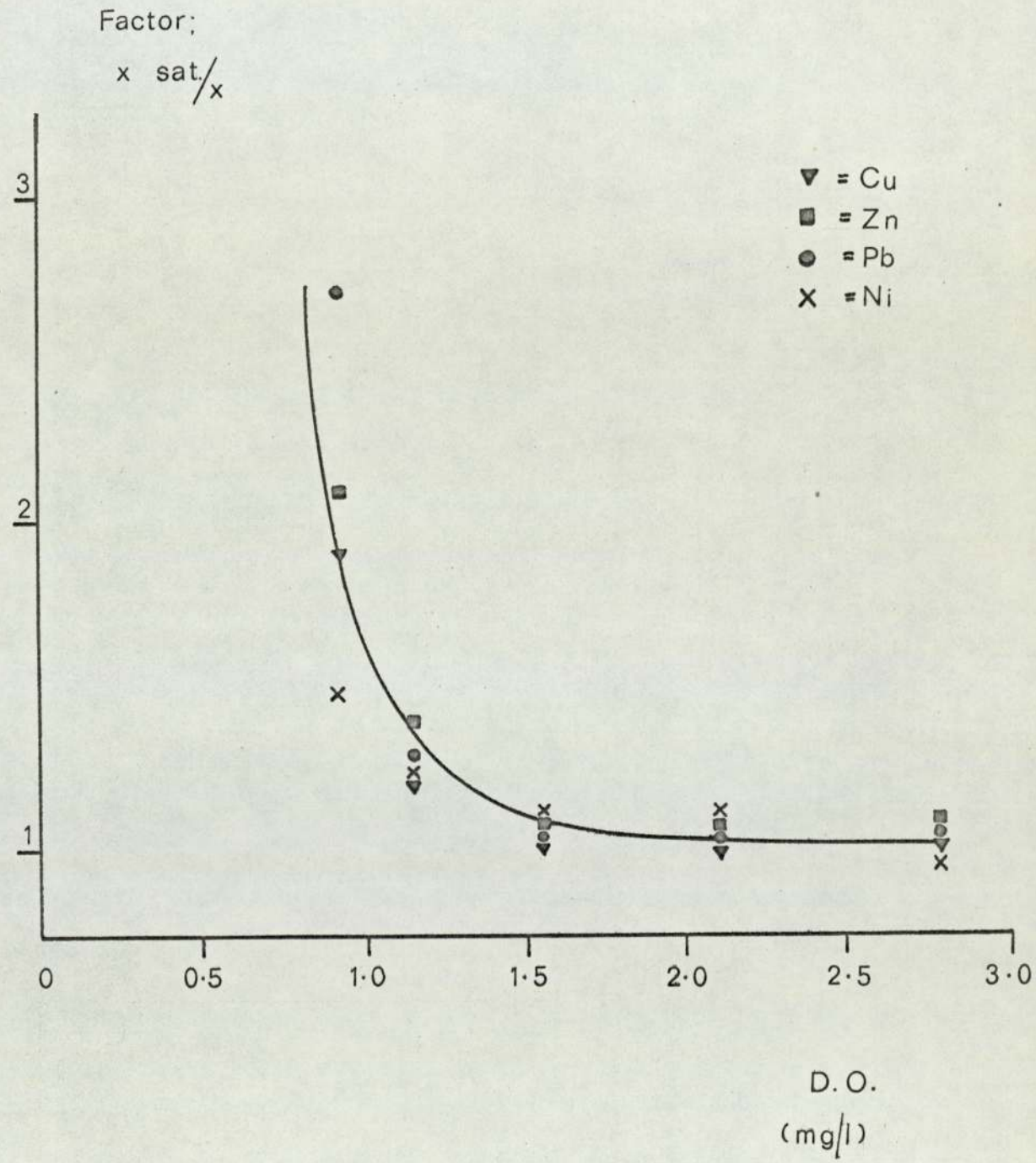
(X_{SAT} = L.C.$_{50}$ at air saturation)

(X = L.C.$_{50}$ at a particular low oxygen level)

When data from the present experiments are expressed in this way results similar to those obtained by Lloyd are found. The points for all four metals fall on the same curve indicating a similar effect for each

Figure 11

Survival of minnows at low oxygen levels in the presence of heavy metal salts. For explanation of $X.SAT/_X$ see text.


Factor;
x sat./x
3
2
1
▼ = Cu
■ = Zn
● = Pb
X = Ni
0
0·5
1·0
1·5
2·0
2·5
3·0
D.O.
(mg/l)

(see Figure 11). The $^{X}SAT./_{X}$ values are given in Table 26 (see appendix). The $^{X}SAT.$ values are derived from the data from Section 5.2 (48 hr. $L.C._{50's}$). The resultant fractions range from 1 to 1.07 at 2.1 mg/litre O_2 and from 1.48 to 2.72 at 0.87 mg/litre O_2. The large increase in the factors at 0.87 mg/litre O_2 is due in part to the 30% mortality which would occur in the absence of heavy metals (see control results, Table 24). If it were not for this the curve obtained would not be so sharply reflexed. However, the expression of the results in this way agrees with Lloyd's observations and with the general observation that any factor which increases gill ventilation increases the toxicity of heavy metals.

2. Survival at low oxygen levels after previous long-term exposure to subacute concentrations of heavy metals

i) Materials and methods

Batches of 20 minnows were exposed to low dissolved oxygen levels for up to 22 days after 50 days exposure to subacute concentrations of the metals. One control batch of fish was also exposed to low oxygen. Fish were exposed to the same metal concentrations used in the experiments described in Sections 9, 10 and 11 (0.1 ppm copper, 0.1 ppm zinc, 4.0 ppm nickel, 1.0 ppm lead). Exposures were made in five litre all-glass tanks but unlike earlier experiments five fish were placed in each tank due to shortage of space. This elevated the fish : metal ratios which were then:

Copper	23000 : 1
Zinc	23000 : 1
Nickel	580 : 1
Lead	2300 : 1

Because of the increased risk of detoxification of the test solutions they were replaced every four days. Fish were fed every two days throughout the exposure time. The calcium content of the test solutions was 70 ppm for zinc, copper and nickel and 10 ppm for lead. Control fish were maintained in heavy metal free water at 70 ppm calcium for 50 days before exposure to low oxygen.

All fish were subjected to low oxygen in the absence of heavy metals in the same apparatus as described in Part 1 of this section.

The only difference was that a small glass-wool filter was added to the circuit immediately after the fish-chamber to retain faeces and uneaten food. As in earlier experiments the temperature was maintained at $15^{\circ}C \pm 1^{\circ}C$.

The dissolved oxygen concentration was lowered very slowly and followed the same pattern for all test groups.

Day Number	Dissolved Oxygen (ppm)
1	Air saturation
2	6
3	4
4	2
5	2
6	1.5
7	1.5
8	1.0
9	1.0
10	0.75
11	0.75
12	0.75
13	0.5
.	.
.	.
.	.
20	0.5
21	0.3
22	0.3

During the first seven days the dissolved oxygen was progressively lowered to 1.0 ppm. There followed three days at 0.75 ppm when the dissolved oxygen was lowered to 0.5 ppm. Daily fluctuations in the D.O. levels at and below 1.0 ppm never exceeded 0.05 ppm.

The water in the apparatus was not replaced during the test period and the fish were fed every two days by introducing dried food into the chamber via one of the ports. Dead fish were removed as quickly as possible. Times of immobilisation were assessed asaccurately as possible and are quoted to the nearest hour. During the night estimations of the time of death had to be made but the error was never greater than 20% and then only on the first night.

ii) Results

The results are shown in Tables 27 and 28 (see appendix) expressed graphically in Figure 12.

(a) Control fish

All control fish survived exposure to 1.0 mg/litre O_2 and only one fish succumbed during the three days at 0.75 mg/litre O_2. Eleven of the remaining nineteen fish died at 0.5 mg/litre O_2 up to 59 hours after the oxygen level was reset.

The level of dissolved oxygen was maintained for a further five days and no more fish died. The D.O. was then reduced to 0.3 ppm and the remaining fish then died, the longest survival period being 26 hours.

(b) Experimental fish

Batches of fish previously exposed to copper and zinc all died within the three days' exposure to 0.75 mg/litre O_2.

Fish previously exposed to nickel behaved similarly to controls although more died at 0.5 mg/litre O_2 and the rate of death was faster.

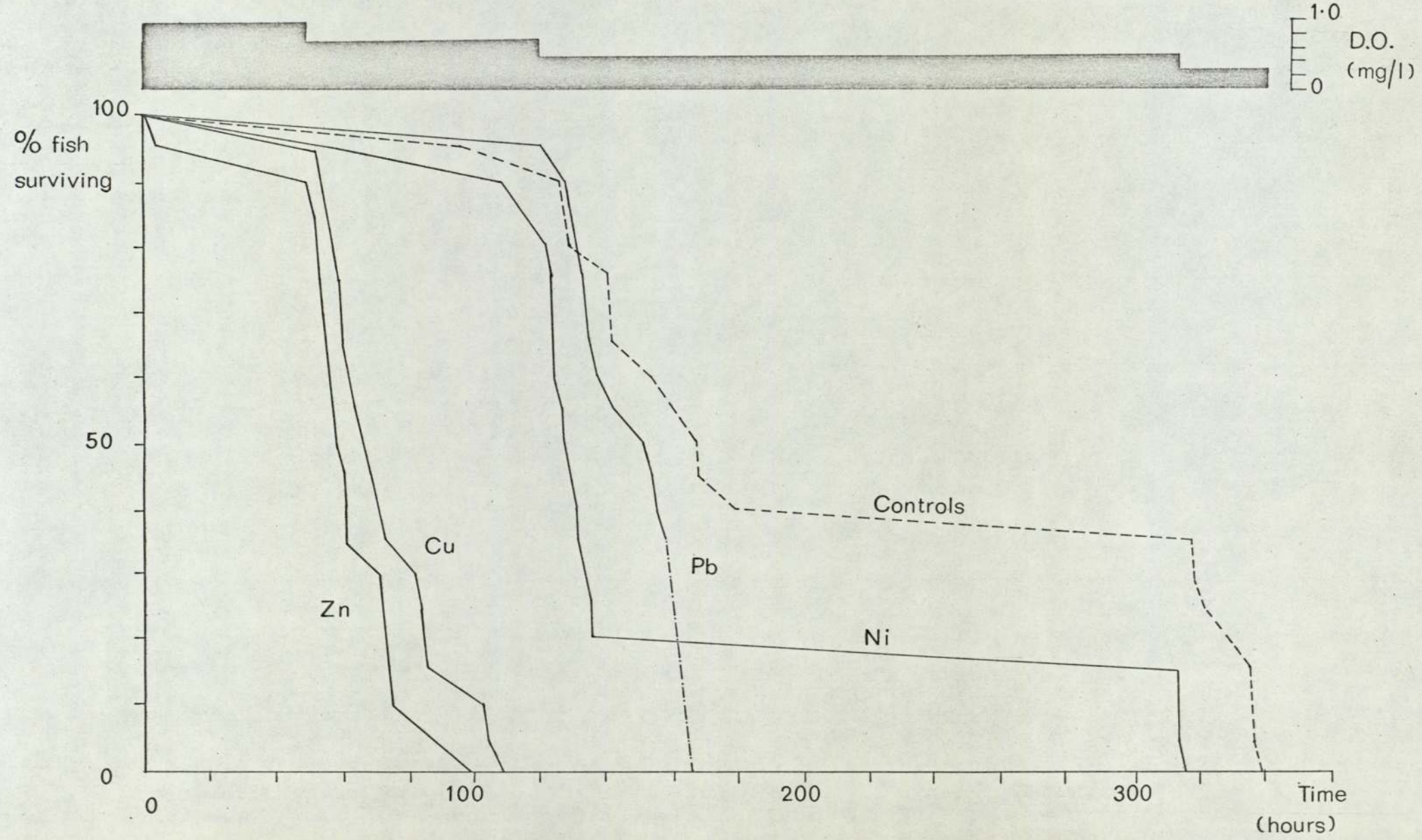
Data obtained from fish previously exposed to lead is considered invalid as "whitespot" disease appeared during the experiment. It was first noted at 142 hours into the experiment and accurate times of death were not recorded after 159 hours, when 65% of the fish had died. 100% mortality was, however, noted at 167 hours. The parasite causing "white spot" forms cysts on the gills and it is considered that fish thus affected would not survive as well as unaffected fish at low oxygen levels. It seems possible that in the absence of disease the graph drawn for lead exposed fish would have resembled that dfawn for nickel treated fish and controls. (See fig. 12).

In all cases 50% of the fish died either before or during the 0.5 mg/litre O_2 exposure. The times taken to kill 50% of the fish are indicated below. The start of the experiment is taken as the time when the D.O. had been reduced to 1.0 ppm.

Controls	168 hours
Copper	69 hours
Zinc	58 hours
Nickel	128 hours
Lead	151 hours

Figure 12

Survival of minnows at low oxygen levels after previous subacute exposures to heavy metals.


1·0
D.O.
(mg/l)
0
100
% fish
surviving
50
0
Controls
Cu
Pb
Zn
Ni
0
100
200
300
Time
(hours)

Discussion

Histological observations (see Section 9) revealed very little alteration in the pathology of the gills after extended subacute exposures. In the case of zinc, minor changes were seen, but the other metals produced virtually no effect. In the present investigation, however, fish previously exposed to both copper and zinc died at a greater rate when subjected to low oxygen, than unexposed fish. Fish exposed to nickel seemed relatively unaffected and the results recorded for lead-treated fish were invalidated by disease. In the absence of severe gill damage it would seem that the inability of copper and zinc exposed fish to survive at low oxygen was due to the generally poor condition of the fish. Fish exposed to copper were observed to develop the symptoms of wasting but exposure to zinc produced far less effect. It is possible that changes in the physiological state of the blood may be responsible for the reduced low oxygen tolerance.

The difference in low oxygen tolerance between test groups was slight, but when fish in the field are considered, any slight reduction in their low oxygen susceptibility is significant when they are exposed to the large and rapid diurnal fluctuations of dissolved oxygen occurring in polluted streams.

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16. APPENDIX

TABLE 10

N.B. Mean = geometric mean
For explanation of MS/MT see text.

Survival times of batches of six minnows at different concentrations of copper, zinc, nickel and lead.

ppm Cu	Survival Times (Minutes)	Factor MS/MT
0.75	110, 175, 205, 210, 220, 220. (Mean = 185)	6.0
0.42	205, 210, 225, 245, 260, 310. (Mean = 240)	3.45
0.24	390, 465, 480, 505, 575, 600. (Mean = 497)	1.92
0.18	535, 610, 660, 725, 770, 820. (Mean = 706)	1.45
0.135	1300, 1660, 1720, 1809, 1860, 2140. (Mean = 1714)	1.08
0.075	00 (10 days)	0.6

ppm Zn	Survival Times (Minutes)	Factor MS/MT
0.75	23, 36, 40, 52, 66. (Mean = 43)	5.0
0.42	28, 31, 38, 44, 56, 62. (Mean = 41)	2.8
0.24	46, 46, 50, 63, 70, 105. (Mean = 60.5)	1.6
0.18	90, 103, 132, 176, 180, 270. (Mean = 148)	1.2
0.135	4 alive after 10 days (1 dead - day 3; 1 dead - day 4)	0.9
0.075	00 (10 days)	0.5

ppm Ni	Survival Times (Minutes)	Factor MS/MT
10.0	69, 84, 97, 103, 126, 139. (Mean = 100)	1.4
8.7	104, 111, 201, 244, 253, 272. (Mean = 184)	1.2
7.5	168, 185, 185, 270, 316, 343. (Mean = 235)	1.04
6.5	3240, 3600, 4200, 4500, 5520. (Mean = 4200)	0.9
5.6	7200 - 10,080. (Mean 8520)	0.77
4.9	00 (10 days)	0.68

ppm Pb	Survival Times (Minutes)	Factor MS/MT
4.9	40, 46, 54, 66, 71, 78. (Mean = 58)	2.65
3.7	35, 38, 49, 63, 75, 78. (Mean = 54)	2.0
2.8	140, 170, 173, 192, 198, 265. (Mean = 186)	1.5
2.1	245, 273, 310, 340, 345, 425. (Mean = 318)	1.1
1.8	1200, 1540, 1680, 2760, 3060, 3360. (Mean = 2110)	0.97
1.55	1080 - 12,960 (Mean = 11,520)	0.84
1.15	00 (10 days)	0.62

TABLE 11

Percentage of fish dead (samples of 10) after 24 and 48 hours' exposure to different concentrations of the metals.

ppm Cu	Percentage dead at 24 hours	48 hours
0.16	100	100
0.14	70	100
0.12	30	40
0.10	0	0

ppm Zn	Percentage dead at 24 hours	48 hours
0.18	100	100
0.16	50	70
0.14	30	30
0.12	0	0

ppm Ni	Percentage dead at 24 hours	48 hours
7.5	100	100
7.25	50	50
7.0	20	20
6.75	0	10
6.5	0	0

ppm Pb	Percentage dead at 24 hours	48 hours
2.0	100	100
1.8	40	40
1.6	0	0

TABLE 12

MINWORTH SECONDARY EFFLUENT ANALYSES

5/10/67 - 26/10/67

(All figures denote p.p.m.)

SHAKEN SAMPLES

	Cu	Zn	Ni	Pb
Thursday, 5th October				
12 midday	2.05	7.10	0.91	0.38
2 p.m.	1.05	5.32	0.55	0.15
4 p.m.	0.45	4.25	0.55	0.15
6 p.m.	1.05	5.10	0.68	0.18
8 p.m.	1.03	4.90	0.64	0.22
10 p.m.	1.40	5.25	0.66	0.26
12 midnight	3.40	7.02	1.48	0.74
2 a.m.	1.50	5.31	0.63	0.26
4 a.m.	1.53	6.11	0.66	0.30
6 a.m.	1.33	4.89	0.54	0.29
8 a.m.	0.90	4.17	0.49	0.18
10 a.m.	0.75	3.76	0.46	0.15
Mean =	1.38	5.26	0.69	0.27

SETTLED SAMPLES

	Cu	Zn	Ni	Pb
Friday, 6th October				
12 midday	0.29	0.55	0.40	0.09
2 p.m.	0.35	0.59	0.40	0.08
4 p.m.	0.30	0.68	0.43	0.09
6 p.m.	0.28	0.96	0.40	0.14
8 p.m.	0.28	0.58	0.43	0.10
10 p.m.	0.23	0.54	0.40	0.13
12 midnight	0.24	0.58	0.43	0.10
Saturday, 7th October				
2 a.m.	0.55	0.49	0.40	0.09
4 a.m.	0.28	0.53	0.40	0.11
6 a.m.	0.28	0.58	0.40	0.10
8 a.m.	0.24	0.54	0.44	0.10
10 a.m.	0.25	0.55	0.45	0.10
12 midday	0.45	0.84	0.44	0.14
2 p.m.	0.40	0.86	0.41	0.13
4 p.m.	0.30	0.71	0.41	0.13
6 p.m.	0.30	0.72	0.43	0.09
8 p.m.	0.30	0.75	0.43	0.10
10 p.m.	0.28	0.62	0.43	0.10
12 midnight	0.34	0.66	0.43	0.10
Sunday, 8th October				
2 a.m.	0.29	0.52	0.43	0.10
4 a.m.	0.28	0.51	0.43	0.13
6 a.m.	0.23	0.66	0.49	0.13
8 a.m.	0.30	0.54	0.44	0.11
10 a.m.	0.40	0.60	0.46	0.11
12 midday	0.29	0.64	0.43	0.11
2 p.m.	0.24	0.60	0.43	0.10
4 p.m.	0.23	0.60	0.45	0.10

	Cu	Zn	Ni	Pb
6 p.m.	0.24	0.60	0.45	0.11
8 p.m.	0.21	0.61	0.45	0.10
10 p.m.	0.26	0.59	0.45	0.11
12 midnight	0.21	0.55	0.45	0.10
Monday, 9th October				
2 a.m.	0.21	0.49	0.45	0.11
4 a.m.	0.20	0.50	0.45	0.11
6 a.m.	0.28	0.58	0.40	0.11
8 a.m.	0.21	0.50	0.43	0.10
10 a.m.	0.21	0.53	0.43	0.10
12 midday	0.28	0.61	0.43	0.11
2 p.m.	0.19	0.56	0.40	0.10
4 p.m.	0.19	0.61	0.43	0.14
6 p.m.	0.18	0.55	0.43	0.13
8 p.m.	0.20	0.50	0.38	0.13
10 p.m.	0.19	0.48	0.38	0.13
12 midnight	0.21	0.49	0.38	0.13
Tuesday, 10th October				
2 a.m.	0.21	0.42	0.30	0.11
4 a.m.	0.26	0.45	0.33	0.10
6 a.m.	0.20	0.65	0.41	0.10
8 a.m.	0.21	0.45	0.35	0.10
10 a.m.	0.23	0.45	0.38	0.10
12 midday	0.23	0.51	0.38	0.10
2 p.m.	0.21	0.52	0.36	0.13
4 p.m.	0.20	0.55	0.43	0.13
6 p.m.	0.20	0.52	0.39	0.09
8 p.m.	0.20	0.48	0.38	0.09
10 p.m.	0.24	0.45	0.38	0.06
12 midnight	0.21	0.44	0.34	0.06

	Cu	Zn	Ni	Pb
Wednesday, 11th October				
2 a.m.	0.23	0.40	0.33	0.06
4 a.m.	0.23	0.42	0.35	0.06
6 a.m.	0.31	0.58	0.36	0.06
8 a.m.	0.23	0.58	0.39	0.09
10 a.m.	0.25	0.65	0.39	0.10
12 midday	0.20	0.78	0.38	0.10
2 p.m.	0.21	0.75	0.38	0.10
4 p.m.	0.21	0.85	0.39	0.10
6 p.m.	0.18	0.78	0.43	0.11
8 p.m.	0.20	0.84	0.43	0.11
10 p.m.	0;25	0.85	0.42	0.11
12 midnight	0.23	0.76	0.43	0.11
Thursday, 12th October				
2 a.m.	0.25	0.76	o.43	0.11
4 a.m.	0.30	0.70	0.44	0.10
6 a.m.	0.28	0.69	0.43	0.10
8 a.m.	0.25	0.65	0.43	0.10
10 a.m.	0.26	0.59	0.43	0.09
12 midday	0.25	0.79	0.43	0.09
2 p.m.	0.25	0.75	0.38	0.10
4.p.m.	0.26	0.79	0.39	0.10
6 p.m.	0.24	0.74	0.39	0.11
8 p.m.	0.28	0.69	0.38	0.11
10 p.m.	0.28	0.56	0.34	0.09
12 midnight	0.26	0.54	0.32	0.10
Friday, 13th October				
2 a.m.	0.29	0.44	0.32	0.10
4 a.m.	0.31	0.42	0.30	0.10
6 a.m.	0.24	0.41	0.32	0.10
8 a.m.	0.39	0.42	0.30	0.15

	Cu	Zn	Ni	Pb
10 a.m.	0.33	0.40	0.32	0.14
12 midday	0.31	0.60	0.32	0.13
2 p.m.	0.26	0.49	0.32	0.11
4 p.m.	0.29	0.56	0.34	0.11
6 p.m.	0.24	0.56	0.35	0.13
8 p.m.	0.29	0.55	0.38	0.13
10 p.m.	0.25	0.55	0.38	0.13
12 midnight	0.24	0.56	0.38	0.13
Saturday, 14th October				
2 a.m.	0.26	0.56	0.42	0.16
4 a.m.	0.29	0.62	0.42	0.16
6 a.m.	0.21	0.61	0.42	0.16
8 a.m.	0.28	0.54	0.42	0.14
10 a.m.	0.29	0.51	0.42	0.14
12 midday	0.45	0.71	0.42	0.10
2 p.m.	0.20	0.55	0.39	0.10
4 p.m.	0.35	0.69	0.42	0.10
6 p.m.	0.39	0.68	0.40	0.10
8 p.m.	0.39	0.59	0.38	0.10
10 p.m.	0.34	0.54	0.38	0.10
12 midnight	0.30	0.50	0.38	0.10
Sunday, 15th October				
2 a.m.	0.34	0.42	0.34	0.10
4 a.m.	0.33	0.46	0.38	0.10
6 a.m.	0.30	0.46	0.38	0.10
8 a.m.	0.31	0.46	0.38	0.10
10 a.m.	0.28	0.41	0.38	0.11
12 midday	0.30	0.51	0.40	0.11
2 p.m.	0.24	0.61	0.39	0.10
4 p.m.	0.24	0.65	0.41	0.10
6 p.m.	0.25	0.65	0.41	0.10
8 p.m.	0.28	0.69	0.41	0.10

	Cu	Zn	Ni	Pb
10 p.m.	0.28	0.60	0.41	0.10
12 midnight	0.23	0.58	0.41	0.10
Monday, 16th October				
2 a.m.	0.23	0.54	0.41	0.13
4 a.m.	0.25	0.52	0.41	0.14
6 a.m.	0.21	0.51	0.41	0.14
8 a.m.	0.23	0.49	0.40	0.15
10 a.m.	0.23	0.49	0.38	0.15
12 midday	0.16	0.58	0.38	0.15
2 p.m.	0.14	0.54	0.38	0.15
4 p.m.	0.14	0.52	0.38	0.16
6 p.m.	0.13	0.41	0.32	0.15
8 p.m.	0.13	0.41	0.32	0.15
10 p.m.	0.15	0.35	0.29	0.15
12 midnight	0.18	0.32	0.30	0.15
Tuesday, 17th October				
2 a.m.	0.18	0.31	0.28	0.14
4 a.m.	0.15	0.36	0.29	0.13
6 a.m.	0.15	0.40	0.30	0.14
8 a.m.	0.15	0.40	0.30	0.15
10 a.m.	0.13	0.45	0.33	0.15
12 midday	0.14	0.60	0.29	0.15
2 p.m.	0.13	0.60	0.32	0.15
4 p.m.	0.10	0.64	0.32	0.15
6 p.m.	0.11	0.70	0.35	0.13
8 p.m.	0.11	0.72	0.39	0.13
10 p.m.	0.13	0.68	0.39	0.13
12 midnight	0.11	0.70	0.40	0.13
Wednesday, 18th October				
2 a.m.	0.11	0.64	0.40	0.13
4 a.m.	0.16	0.66	0.39	0.11
6 a.m.	0.15	0.68	0.39	0.11

	Cu	Zn	Ni	Pb
8 a.m.	0.15	0.61	0.39	0.15
10 a.m.	0.14	0.61	0.39	0.11
12 midday	0.19	0.66	0.41	0.11
2 p.m.	0.18	0.64	0.43	0.11
4 p.m.	0.20	0.62	0.43	0.11
6 p.m.	0.20	0.64	0.43	0.11
8 p.m.	0.21	0.56	0.38	0.13
10 p.m.	0.20	0.46	0.35	0.13
12 midnight	0.19	0.41	0.34	0.13
Thursday, 19th October				
2 a.m.	0.24	0.41	0.32	0.11
4 a.m.	0.24	0.39	0.33	0.13
6 a.m.	0.20	0.38	0.33	0.13
8 alm.	0.25	0.35	0.33	0.10
10 a.m.	0.24	0.38	0.33	0.11
12 midday	0.20	0.42	0.35	0.10
2 p.m.	0.23	0.45	0.38	0.13
4 p.m.	0.21	0.48	0.40	0.13
6 p.m.	0.23	0.51	0.38	0.13
8 p.m.	0.23	0.54	0.38	0.10
10 p.m.	0.21	0.56	0.44	0.11
12 midnight	0.21	0.60	0.44	0.14
Friday, 20th October				
2 a.m.	0.21	0.48	0.38	0.13
4 a.m.	0.25	0.42	0.37	0.14
6 a.m.	0.23	0.38	0.37	0.14
8 a.m.	0.30	0.40	0.38	0.13
10 a.m.	0.26	0.41	0.39	0.13
12 midday	0.23	0.49	0.40	0.13
2 p.m.	0.29	0.51	0.43	0.13
4 p.m.	0.26	0.56	0.43	0.10
6 p.m.	0.24	0.56	0.40	0.10

	Cu	Zn	Ni	Pb
8 p.m.	0.26	0.52	0.40	0.10
10 p.m.	0.30	0.50	0.44	0.10
12 midnight	0.25	0.48	0.34	0.11
Saturday, 21st October				
2 a.m.	0.25	0.38	0.35	0.11
4 a.m.	0.29	0.46	0.40	0.13
6 a.m.	0.24	0.41	0.41	0.15
8 a.m.	0.31	0.40	0.39	0.15
10 a.m.	0.30	0.88	0.43	0.15
12 midday	0.29	0.54	0.44	0.13
2 p.m.	0.34	0.58	0.46	0.14
4 p.m.	0.33	0.65	0.52	0.14
6 p.m.	0.38	0.65	0.50	0.15
8 p.m.	0.38	0.60	0.50	0.14
10 p.m.	0.34	0.61	0.50	0.14
12 midnight	0.28	0.75	0.48	0.14
Sunday, 22nd October				
2 a.m.	0.30	0.51	0.45	0.14
4 a.m.	0.31	0.56	0.49	0.13
6 a.m.	0.29	0.59	0.52	0.13
8 a.m.	0.40	0.55	0.45	0.13
10 a.m.	0.38	0.64	0.50	0.14
12 midday	0.41	0.70	0.50	0.14
2 p.m.	0.31	0.74	0.50	0.14
4 p.m.	0.28	0.72	0.50	0.14
6 p.m.	0.28	0.80	0.52	0.15
8 p.m.	0.29	0.72	0.52	0.15
10 p.m.	0.24	0.61	0.52	0.15
12 midnight	0.23	0.49	0.46	0.15
Monday, 23rd October				
2 a.m.	0.24	0.42	0.40	0.14
4 a.m.	0.24	0.45	0.40	0.14
6 a.m.	0.21	0.44	0.36	0.14

	Cu	Zn	Ni	Pb
8 a.m.	0.30	0.42	0.36	0.13
10 a.m.	0.23	0.46	0.40	0.11
12 midday	0.28	0.54	0.40	0.13
2 p.m.	0.21	0.55	0.49	0.13
4 p.m.	0.28	0.64	0.45	0.13
6 p.m.	0.20	0.69	0.49	0.13
8 p.m.	0.19	0.70	0.49	0.11
10 p.m.	0.19	0.65	0.49	0.11
12 midnight	0.19	0.68	0.49	0.13
Tuesday, 24th October				
2 a.m.	0.16	0.59	0.45	0.13
4 a.m.	0.20	0.66	0.45	0.13
6 a.m.	0.19	0.64	0.49	0.13
8 a.m.	0.26	0.64	0.49	0.13
10 a.m.	0.18	0.60	0.49	0.11
12 midday	0.20	0.68	0.46	0.13
2 p.m.	0.19	0.68	0.46	0.13
4 p.m.	0.20	0.76	0.46	0.13
6 p.m.	0.23	0.72	0.49	0.13
8 p.m.	0.19	0.69	0.49	0.13
10 p.m.	0.18	0.61	0.45	0.10
12 midnight	0.19	0.61	0.45	0.10
Wednesday, 25th October				
2 a.m.	0.20	0.60	0.45	0.13
4 a.m.	0.20	0.60	0.43	0.13
6 a.m.	0.21	0.58	0.43	0.13
8 a.m.	0.28	0.58	0.45	0.13
10 a.m.	0.24	0.50	0.45	0.13
12 midday	0.14	0.70	0.45	0.13
2 p.m.	0.23	0.61	0.45	0.13
4 p.m.	0.23	0.65	0.44	0.11
6 p.m.	0.28	0.65	0.44	0.10
8 p.m.	0.26	0.62	0.44	0.10

	Cu	Zn	Ni	Pb
10.0 p.m.	0.26	0.66	0.45	0.13
12 midnight	0.26	0.70	0.45	0.13
Thursday, 26th October				
2 a.m.	0.25	0.60	0.45	0.13
4 a.m.	0.29	0.66	0.45	0.13
6 a.m.	0.29	0.61	0.45	0.13
8 a.m.	0.34	0.60	0.44	0.11
10 a.m.	0.28	0.52	0.44	0.11

COPPER

MEAN CONCENTRATION = 0.24 ppm

(Range = 0.10 - 0.55)

ZINC

MEAN CONCENTRATION = 0.55 ppm

(Range = 0.31 - 0.96)

NICKEL

MEAN CONCENTRATION = 0.38 ppm

(Range = 0.29 - 0.52)

LEAD

MEAN CONCENTRATION = 0.11 ppm

(Range = 0 06 - 0.16)

TABLE 13

OCTOBER 1967 - Minworth effluent; supplementary analytical data

Date	O.A. from KMnO4		B.O.D.		Ammoniacal Nitrogen (ppm N)	Nitrite and Nitrate (ppm N)	Suspended Solids	Dichromate C.O.D.
	Settled	Shaken	Settled	Shaken				
5	13.7	14.2	7.4	7.5	13.6	19.2	51	88.0
6	9.5	10.9	3.3	7.2	10.8	22.2	29	78.4
7	10	13.1	8.5	18.2	14.4	17.8	25	83.2
8	11.5	12.5	5.6	10.9	15.2	20.8	30	80.0
9	13.0	14.0	9.0	9.1	13.0	21.0	32	84.8
10	10.8	13.5	6.0	9.7	9.5	21.4	31	84.8
11	14.6	16.2	9.1	14.2	10.5	-	45	86.4
12	12.2	14.5	3.5	12.6	9.5	20.9	36	80.0
13	11.5	14.4	6.3	14.5	14.4	20.6	30	145.6
14	9.6	11.0	6.0	9.3	10.7	20.6	21	128.0
15	10.6	13.2	4.7	9.8	15.5	18.2	46	89.6
16	10.5	12.0	7.1	23.1	5.8	17.8	78	112.0
17	25.2	29.0	12.8	16.0	7.5	18.8	39	59.2
18	12.2	13.2	8.0	9.7	15.8	22.2	23	91.2
19	9.9	13.0	10.3	12.2	13.4	20.6	32	57.6
20	10.7	15.0	8.6	25.5	11.5	23.7	35	115.0
21	7.2	14.0	5.0	13.5	12.3	31.4	30	136.0
22	13.0	14.7	2.7	6.4	19.0	17.7	27	72.0
23	14.5	15.6	9.5	21.0	13.8	18.9	19	83.2
24	13.4	16.9	10.2	14.4	14.0	18.8	30	72.0
25	13.4	16.0	7.4	15.8	12.6	17.2	30	117.0
26	11.9	15.6	13.5	16.5	20.0	15.7	43	100.0

WEEKLY AVERAGE SAMPLES

Week ending	Total Phenol	Total Phosphorus	Anionic Detergent
6.10.67	2.9	4.0	0.8
13.10.67	3.3	13.2	0.5
20.10.67	2.8	8.8	2.0
27.10.67	3.2	5.2	1.2

Figures kindly supplied by U.T.M.D.A.

TABLE 14

Percentage of fish dead (samples of 10) after 24 and 48 hours' exposure to different concentrations of the metals in an artificial sewage effluent.

ppm Cu	Percentage dead at 24 hours	48 hours
0.80	100	100
0.70	60	70
0.65	50	50
0.60	20	20
0.50	0	0

ppm Zn	Percentage dead at 24 hours	48 hours
0.18	100	100
0.17	50	50
0.16	20	20
0.15	0	0

ppm Ni	Percentage dead at 24 hours	48 hours
8.0	100	100
7.5	80	80
7.25	40	50
7.0	0	0

LEAD - no experiments

TABLE 15

Copper analyses of gills and bone samples of untreated fish and fish exposed to acutely toxic copper solutions.

CONTROL FISH

ppm Cu per dry weight tissue		Cu Gill:Bone Ratio	
Gills	Bone		
59.3	42.0	1.41	
71.0	40.5	1.75	
68.2	40.4	1.69	
48.2	30.5	1.58	
58.1	23.7	2.45	
66.8	39.5	1.69	
66.5	42.4	1.57	
54.3	49.5	1.10	
70.6	46.0	1.54	
62.6	39.4	1.64	Average

COPPER EXPOSED FISH

ppm Cu in test solution	ppm Cu per dry weight tissue		Cu Gill : Bone Ratio
	Gills	Bone	
0.20	103.8	61.3	1.69
0.30	128.3	60.5	2.12
0.50	119.0	84.7	1.41
0.75	152.4	69.1	2.21
Average	125.9	68.9	1.86

MIXED Cu/Zn SOLUTION

ppm Cu in Solution	ppm Zn in Solution	ppm Cu per dry weight tissue		Cu Gill : Bone Ratio
		Gills	Bone	
0.1	0.1	76.25	54.0	1.41

TABLE 16

Zinc analyses of gills and bone samples of untreated fish and fish exposed to acutely toxic zinc solutions.

CONTROL FISH

	ppm Zn per dry weight tissue		Zn Gill:Bone Ratio
	Gills	Bone	
	397	419	0.95
	366	409	0.90
	337	420	0.80
	382	370	1.03
	280	400	0.65
	368	410	0.90
	401	394	1.02
	381	422	0.90
	390	425	0.92
Average:	366	409	0.896

ZINC EXPOSED FISH

ppm Zn in test Solution	ppm Zn per dry weight tissue		Zn Gill:Bone Ratio
	Gills	Bone	
0.2	471	370	1.27
0.3	818	445	1.84
0.5	760	480	1.58
0.75	803	472	1.70
Average	713	442	1.6

MIXED Zn/Cu SOLUTION

ppm Zn in Solution	ppm Cu in Solution	ppm Zn per dry weight tissue		Zn Gill:Bone Ratio
		Gills	Bone	
0.1	0.1	526	483	1.09

TABLE 17

Lead analyses of gills and bone samples of untreated fish and fish exposed to acutely toxic lead solutions

CONTROL FISH

All negative

Gills < 14 ppm Pb per dry weight tissue
Bone < 5 ppm " " " "

LEAD EXPOSED FISH

ppm Pb in test Solution	ppm Pb per dry weight tissue		Pb Gill:Bone Ratio
	Gills	Bone	
2	225.8	77.5	2.91
4	306	72.04	4.25
6	404	88.34	4.57

TABLE 18

Nickel analyses of gills and bone samples of untreated fish and fish exposed to acutely toxic nickel solutions.

CONTROL FISH

All Negative

Gills < 12 ppm Ni per dry weight tissue
Bone < 6 ppm " " " "

NICKEL EXPOSED FISH

ppm Ni in test Solution	ppm Ni per dry weight tissue		Ni Gill:Bone Ratio
	Gills	Bone	
6	69.31	42.85	1.62
8	93.19	36.99	2.52
10	86.40	34.1	2.53

TABLE 19

Copper analyses of liver, kidneys, gills and bone samples of untreated fish and fish exposed to a chronically toxic copper solution (0.1 ppm Cu) for extended periods.

CONTROL FISH

	ppm Cu per dry weight of tissue	
	Liver	Kidneys
	55.2	41.7
	54.1	69.6
	62.6	50.1
	58.0	48.5
	44.6	42.0
	64.0	54.0
	51.0	36.3
	57.5	49.0
	48.4	39.8
Average:	55.4	47.9

Gill average = 62.6
Bone average = 39.4 (See Table 15)

COPPER EXPOSED FISH

Exposure Time (Days)	ppm Cu per dry weight tissue			
	Liver	Kidneys	Gills	Bone
30	296.8	90.2	97.6	46.0
60	543.5	104.0	119.2	49.5
90	960.1	138.1	112.0	74.2

TABLE 20

Lead analyses of liver, kidneys, gills and bone samples of untreated fish and fish exposed to a chronically toxic lead solution (1.0 ppm Pb) for extended periods.

CONTROL FISH

All negative

Liver	< 9 ppm	Pb per	dry weight	tissue
Kidneys	< 24 ppm	"	"	"
Gills	< 14 ppm	"	"	"
Bone	< 5 ppm	"	"	"

LEAD EXPOSED FISH

Exposure Time (Days)	ppm Pb per dry weight tissue			
	Liver	Kidneys	Gills	Bone
30	12.5 (Approx)	X	< 19.5	< 13.0
60	14.0 (Approx)	28.0	< 14.0	10.0
90	52.0	26.0	< 11.0	10.0
160	173.5	49.0	< 12.5	31.5

TABLE 21

Nickel analyses of liver, kidneys, gills and bone samples of untreated fish and fish exposed to a chronically toxic nickel solution (4.0 ppm Ni) for extended periods.

CONTROL FISH

All negative

Liver	< 7.5 ppm Ni	per	dry weight	tissue
Kidneys	< 20 ppm "	"	"	"
Gills	< 12 ppm "	"	"	"
Bone	< 6 ppm "	"	"	"

NICKEL EXPOSED FISH

Exposure Time (Days)	ppm Ni per dry weight tissue			
	Liver	Kidneys	Gills	Bone
30	< 8.0	< 37.0	< 21.0	< 7.0
60	< 9.5	< 32.0	< 16.0	12.0
90	15.0 (Approx)	< 40.0	< 22.0	25.0
136	26.5	< 44.0	< 14.5	20.2

TABLE 22

Zinc analyses of liver, kidneys, gills and bone samples of untreated fish and fish exposed to a chronically toxic zinc solution (0.1 ppm Zn) for extended periods.

CONTROL FISH

	ppm Zn per dry weight tissue	
	Liver	Kidneys
	55.0	760.1
	67.2	654.7
	55.2	671.4
	61.0	826.0
	46.0	701.0
	38.4	646.8
	43.6	721.0
	48.4	660.6
	59.1	773.2
Average	52.7	712.8

Gill average = 366
Bone average = 409 (See Table 16)

ZINC EXPOSED FISH

Exposure time (Days)	ppm Zn per dry weight tissue			
	Liver	Kidneys	Gills	Bone
30	75.7	1627.5	388.1	534.5
60	69.5	1330.0	495.0	634.3
90	67.6	1021.0	475.4	605.2

TABLE 23

Production of a metal-containing effluent in the laboratory with activated sludge. Analytical data.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
ZINC																	
Initial Conc. (ppm)	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.3	1.3	1.3	1.3	1.2	1.2	1.2	
Effluent Conc. (ppm)	0.54	0.39	0.45	0.40	0.76	0.36	0.80	0.45	0.47	0.31	0.75	0.53	0.7	0.46	0.60	0.38	
% Removal	55	67.5	62.5	66.7	36.7	63.5	33	62.5	60.8	76.2	42.9	59.7	46.2	62	50	68.3	AVERAGE 56
COPPER																	
Initial Conc. (ppm)	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5	
Effluent Conc. (ppm)	0.4	0.56	0.61	0.52	0.66	0.39	0.36	0.45	0.39	0.44	0.76	0.63	0.75	0.63	0.65	0.44	
% Removal	84	77.6	75.6	79.2	73.6	84.4	85.6	82	84.4	82.4	69.6	74.7	70	74.7	74	82.4	AVERAGE 78.6
NICKEL																	
Initial Conc. (ppm)	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	
Effluent Conc. (ppm)	26	20	18	18	17	17	17	22	20	19	18.8	20	18.8	20	16.5	17.5	
% Removal	13.3	33.3	40	40	43.4	43.4	43.4	26.7	33.3	36.7	38	33.3	38	33.3	45	41.7	AVERAGE 35.7
LEAD																	
Initial Conc. (ppm)	110	110	110	110	110	120	120	120	130	120	120	130	130	110	110	110	
Effluent Conc. (ppm)	13.5	16.2	8.45	6.5	7.5	6.3	8.4	5.2	13.8	13.3	17.5	4.4	22.1	15	25.6	4.5	
% Removal	87.7	85.3	92.3	94.1	93.2	94.8	93	95.7	89.2	89	85.4	96.6	83	86.4	76.7	96	AVERAGE 90.2

TABLE 24

Control Fish

mg/litre O_2	% Fish dead (3 experiments)					
	24	48	24	48	24	48
1.55	0	0	0	0	0	0
1.15	0	0	0	0	0	0
0.87	30	30	30	40	30	30
0.65	90	100	100	100	90	100

TABLE 25

Figures indicate % fish dead
24 and 48 hours

COPPER

ppm Cu / mg/litre O_2	0.155	0.115		0.087		0.065		0.049	
Air Saturation	100	0	10						
4.9	100	10	10						
3.7	100	0	10						
2.8	100	0	20						
2.1	100	20	20						
1.55	100	40	40	0	0				
1.15	100	40	60	20	30	0	0	0	0
0.87	100	70	70	60	60	50	50	20	30
0.65	100	100	100	100	100	100	100	100	100

ZINC

ppmZn / mg/litre O_2	0.155		0.115		0.087		0.065		0.049	
Air Saturation	60	60								
4.9	60	70								
3.7	70	70								
2.8	80	100								
2.1	100	100	0	0						
1.55	100	100	10	10	0	0				
1.15	100	100	30	30	20	30	0	0	0	0
0.87	100	100	80	80	90	90	40	40	40	40
0.65	100	100	100	100	100	100	100	100	100	100

LEAD

ppm Pb / mg/litre O_2	1.8		1.35		1.0		0.75		0.56	
Air Saturation	30	30								
4.9	20	20								
3.7	40	40								
2.8	30	30	0	0						
2.1	40	40	30	30						
1.55	50	50	30	30	0	0				
1.15	80	80	40	40	10	20	0	0	0	0
0.87	100	100	70	80	60	90	60	80	40	50
0.65	100	100	100	100	100	100	100	100	100	100

NICKEL

ppm Ni / mg/litre O_2	7.5	6.5		5.6	4.9	4.2
Air Saturation	100	0	0			
4.9	100	0	0			
3.7	100	0	0			
2.8	100	0	0			
2.1	100	20	20			

... Continued ...

Table 25 (continued)

nickel

1.55	100	20	20	0	0	0	0		
1.15	100	50	60	60	60	20	20	0	0
0.87	100	100	100	70	70	50	50	40	40
0.65	100	100	100	100	100	100	100	100	100

TABLE 26

Reduction of 48 hour $L.C._{50}$'s at low oxygen levels and X. $SAT./_X$ factors

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COPPER (48 hour $L.C._{50}$ at air saturation = 0.125 ppm)

mg/litre O_2	Cu 48 hr. $L.C._{50}$	X $SAT./_X$
4.9		
3.7		
2.8		
2.1		
1.55	0.12	1.04
1.15	0.104	1.2
0.87	0.065	1.9

ZINC (48 hour $L.C._{50}$ at air saturation = 0.15 ppm

mg/litre O_2	Zn 48 hr. $L.C._{50}$	X $SAT./_X$
4.9		
3.7		
2.8	0.14	1.07
2.1	0.14	1.07
1.55	0.135	1.1
1.15	0.11	1.4
0.87	0.07	2.1

LEAD (48 hour $L.C._{50}$ at air saturation = 1.85 ppm)

mg/litre O_2	Pb 48 hr. $L.C._{50}$	X $SAT./_X$
4.9		
3.7		
2.8		
2.1		
1.55	1.8	1.03
1.15	1.45	1.28
0.87	0.68	2.72

NICKEL (48 hour $L.C._{50}$ at air saturation = 7.25 ppm)

mg/litre O_2	Ni 48 hr. $L.C._{50}$	X $SAT./_X$
4.9		
3.7		
2.8		

(Continued overleaf)

TABLE 26 (Continued)

NICKEL

mg/litre O_2	Ni 48 hr. $L.C._{50}$	X SAT./X
2.1	6.8	1.07
1.55	6.6	1.1
1.15	6.0	1.21
0.87	4.9	1.48

TABLE 27

Survival times of minnows at low dissolved oxygen concentrations after previous exposure to subacute concentrations of heavy metals

Control Fish		50 days 0.1 ppm Zinc treatment		50 days 0.1 ppm Copper treatment		50 days 4.0 ppm Nickel treatment		50 days 1.0 ppm lead treatment	
Day No.	D.O. (Mg/l) & survival times (hrs)	Day No.	D.O. (mg/l) & survival times (hrs)	Day No.	D.O. (Mg/l) & survival times (hrs)	Day No.	D.O. (mg/l) & survival times (hrs)	Day No.	D.O. (mg/l) & survival times (hrs)
1	1.0	1	1.0	1	1.0	1	1.0	1	1.0
2	1.0		+ 6	2	1.0	2	1.0	2	1.0
3	0.75	2	1.0	3	0.75	3	0.75	3	0.75
4	0.75	3	0.75		+53		+ 62	4	0.75
	+ 96		+ 49		+ 54	4	0.75	5	0.75
5	0.75		+ 51		++ 57	5	0.75	6	0.5
6	0.5		+++54		+ 59		+ 108		+ 121
	+ 126		+ 55		++ 60	6	0.5		+ 127
	++130		++ 57		+ 62		++122		++130
	+ 141		+ 58		+ 64		+ 124		+ 133
	++142		+ 60		++ 69		+++125		++135
7	0.5		++ 61		+ 71		+ 127		+ 137
	+ 154		+ 70	4	0.75		+ 128		142 hrs whitespot first seen
	++168		+ 72		+ 74		++131		
8	0.5	4	0.75		+ 82		+ 132	7	0.5
	+ 170		++ 74		+ 85		++135		+ 147
	+ 179		+ 76		++ 87		+ 136		+ 151
9	0.5	5	0.75	5	0.75	7	0.5		+ 155
10	0.5		++ 98		+ 103	8	0.5		+ 156
11	0.5				+ 105	9	0.5		+ 159
12	0.5				+ 108	10	0.5		
13	0.5					11	0.5		
14	0.3					12	0.5		
	+ 317					13	0.5		
	+ 318					14	0.3		
	+ 321						+ 314		100% +
	++335						+ 315		167 hours
15	0.3						+ 316		
	++337								
	+ 338								

+ = death