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Relationship of Secreted Mucus to Copper and Acid Toxicity in Rainbow Trout

T. G. Miller and W. C. Mackay

Department of Zoology, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

It has been suggested that excessive mucus secreted by fish gills in response to toxic levels of heavy metals and acid pH results in death by suffocation as a result of mucus coagulation and precipitation with the metal or with hydrogen ion on the gill surface (PLONKA & NEFF 1969). However histological investigation has shown that copper and zinc poisoning resulted in a separation of gill epithelium from the basement membrane (LLOYD 1960; SKIDMORE & TOVELL 1972). Such a separation could cause suffocation by increasing the diffusion distance for respiratory gases between water and blood. These observations were made on fish exposed to acutely lethal concentrations of toxicants. SELLERS *et al.* (1975) found that there was no sign of excessive mucus production when rainbow trout were exposed to copper concentrations ranging downward from the 48 h LC50.

In addition to epithelial separation PLONKA & NEFF (1969) found bits of cellular debris embedded in mucus on the gills of brook trout exposed to acutely toxic acid pH. They suggested that mucus adhering to the cellular debris of the respiratory epithelial cells was the mechanism of coagulum formation.

LABAT *et al.* (1974) observed that exposure to near lethal concentrations of copper for 24 or 48 h caused the number of mucous cells to decrease markedly. When fish were returned to uncontaminated water some restoration of the mucous cells occurred within 24 hours at a density similar to control fish. They concluded that following short exposures (24 h) to copper the mucous cells had not been destroyed but rather they had ejected all of their contents and any further manufactured mucus was immediately released.

Because mucus is primarily composed of protein (PICKERING 1976; WESSLER & WERNER 1957), it may have a significant effect on the speciation of copper (PEISACH *et al.* 1967). In most of the natural waters he examined STIFF (1971) found that when

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copper was present in toxic concentrations the most abundant species of copper was that bound by single amino acids and small peptides. However in extremely alkaline waters he found $CuCO_3$ to be the most abundant species. Because of the potential importance of proteins, peptides and amino acids in determining the speciation of copper in solution increased mucous secretion could significantly affect the species of copper present and hence alter its toxicity. The purpose of the present study was therefore to determine the effect of lethal concentrations of copper and hydrogen ion on mucus secretion by rainbow trout and to examine the effect of pH change on the ability of mucus to bind Cu^{++}.

MATERIALS AND METHODS

The rainbow trout (*Salmo gairdneri* Richardson) used for this study were obtained from the Sam Livingston Fish Hatchery, Calgary, Alberta as fingerlings (7-12 g). They were held for several months under a natural photoperiod at 13±1°C in water of pH 7.4 and total copper concentration of 2 to 5 µg/L before they were used in these experiments.

A series of toxicity test for copper and acid were initially conducted (MILLER & MACKAY 1980), using a flow-through system which had a 90% particle turnover rate (SPRAGUE 1969) of approximately 4 h.

The test water was synthetic fresh water made with distilled, deionized water. Stock solutions of $CaCl_2$ (U.S.P. grade), $NaHCO_3$ (domestic baking soda) and $KHCO_3$ (reagent grade) were added to the deionized water via a proportional diluter modified from MOUNT & BRUNGS (1967) to produce a final water hardness of 50 mg/L (as $CaCO_3$), alkalinity of 28 mg/L (as $CaCO_3$) and a pH of 7.3. This water was used in the static exposure of 75-100 g rainbow trout to copper and acid. Each of 8 fish were allowed to acclimate for 14 days to a small aquarium containing 2 L of water at 9°C and water chemical conditions which were identical to those used in the baseline ILC50. During this time about 2/3 of the water was replaced each day. The fish were fed once each day until the 12th day. Food was then withheld to reduce fecal excretion during mucus collection and yet test the fish when they were still in positive energy balance and not relying on body stores of energy. On the 15th day, the water in each aquarium was replaced 3 times to remove residual mucus and debris. Three fish were then exposed to 1.8 toxic units (TU) of copper (85 µg/L), 3 were exposed to 1.6 TU of acid (pH 4.0) and 2 fish served as controls. The values of 85 µg/L copper and a pH of 4.0 were chosen on the basis that they should be equally toxic; each caused approximately 75% mortality in 48 h of exposure in the initial toxicity tests. This is an important factor as the exposure was only continued for 8 h. The nominal pH value was obtained by using acidified water for the replacement. This water had been reduced to pH 3.6 and had been well aerated for about one hour to remove excessive CO_2. Copper was administered in 2 doses, about 5 min apart, to approximate the rate of exposure to the acid pH.

After 8 h the fish were removed, any fecal pellets were siphoned off, and the entire volume of water in each tank was homogenized in a Waring blender to suspend all mucous particles. A 100 mL aliquot was then dialyzed to reduce the volume, freeze-dried to complete dryness and weighed. This experiment was repeated using 170 µg/L copper (3.6 TU) and pH 3.8 (4 TU). However in this second experiment the trout were allowed to acclimate to being held in the small aquarium for 21 days before being tested.

The ability of mucus to bind copper was determined by changes in the copper ion acitivity, as measured by a Cu^{++} specific ion electrode when a standard solution of copper was titrated with a solution containing a known concentration of mucus. Each of 3 fish was held in 2 L aquaria and exposed to a pH of approximately 5.0 to enhance mucous secretion. After approximately 24 hours the fish were removed, the entire volume of water was homogenized and an aliquot of this solution was used as the titrant. A standard calibration curve was first prepared by adding known amounts of copper to a mucus-free sample of the test water to prepare a standard curve to insure proper performance of the specific-ion electrode (Orion model 92). When a total copper concentration of 60-70 µg/L was reached this same standard was back titrated with the solution of mucus. A reduction in the mV reading was interpreted as a reduction in the concentration of cupric ion. Mucus samples collected from each of 3 different fish were tested independently. When the effect of pH on the ability of mucus to chelate copper was tested the pH of the standard and the mucus containing solution were adjusted to the desired values before the titration was conducted. This eliminated variability in ionic activity due to pH changes when the titrations were being done.

The ability of rainbow trout to alter the pH of the water in which they were held was determined by holding each of 3 fish in 2 L of synthetic freshwater adjusted to pH 4.0 with sulfuric acid. The pH of the aquarium water was measured at regular intervals throughout the 8 h exposure period.

RESULTS

The effects of copper and acid pH on mucous secretion by trout is summarized in Table 1. Fish exposed to low pH secreted significantly more mucus than did fish exposed to copper (p=0.05, Mann-Whitney U-test). Mucous secretion of acid exposed fish exceeded that of copper exposed fish by 41% in experiment I and by 30% in experiment II. The generally low levels of mucous secretion seen in experiment II may be attributable to the longer period (21 d) of acclimation of the fish to the small aquaria. Mucous secretion following copper exposure did not differ from control levels in either experiment.

TABLE 1. Effect of exposure to copper and to acid pH on mucous secretion by rainbow trout. Each value represents the total amount of mucus secreted in 8 h by an individual fish. The fish were allowed to acclimate to the experimental conditions for 14 d in experiment I and 21 d in experiment II. In both experiments exposure to acid resulted in significantly (p=0.05, Mann-Whitney U-test) greater mucous secretion than did exposure to copper.

Experiment I mucus secretion (g)			Experiment II mucus secretion(g)		
copper (85 μg/L)	acid (pH 4.0)	control (pH 7.3)	copper (170 μg/L)	acid (pH 3.6)	control (pH 7.3)
0.550	0.678	0.395	0.237	0.311	0.249
0.300	0.616	0.341	0.282	0.336	0.309
0.608	0.756		0.259	0.360	
0.486	0.683	0.368	0.259	0.336	0.279

The effect of mucus on cupric ion activity, as indicated by the potential of the cupric ion electrode, is illustrated in Figure 1. The copper solution was titrated against 3 solutions of mucus from 3 different fish. Lowering pH decreases the ability of mucus to bind cupric ion. At near neutral pH (7.3) the addition of mucus rapidly reduced the cupric ion activity while at low pH (3.5) large amounts of mucous solution were required to reduce the potential of the cupric ion electrode and at pH 3.0 the addition of 30 mL of mucous solution had no effect on cupric ion activity.

Trout showed some capacity to alter the pH of the water in which they were held (Figure 2). Fish weighing from 75 to 100 g raised the pH of 2 L of water from 4 to approximately 6.5 in 8 h and to 7.0 after 24 h. This apparent buffering capacity was likely due to release of ammonia resulting from protein catabolism by the fish rather than to secreted mucus.

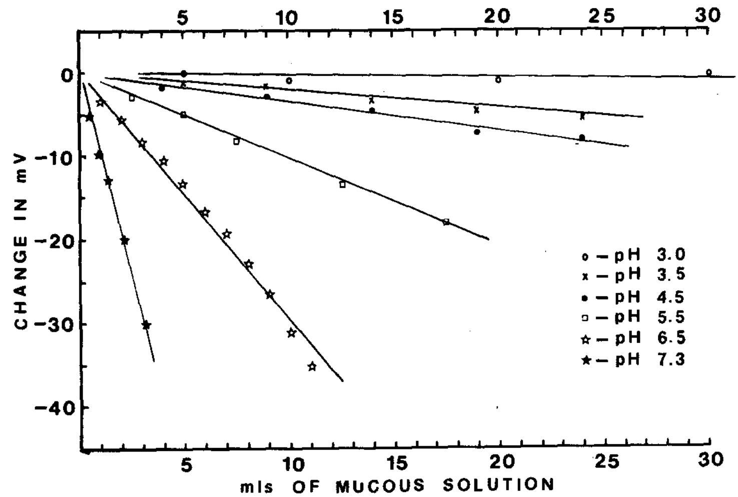

Fig. 1. Effect of pH on the copper chelating ability of mucus. The decrease in electrical potential of the Cu^{++} electrode (mV) is proportional to the decrease in Cu^{++} activity in the test solution. The mucous solution (0.4 g/L) was prepared by homogenizing 2 L of aquarium water in which an 85 g trout had been held for 24 h. For each curve a fresh copper solution (50 mL) containing 60-70 μg/L copper was used.

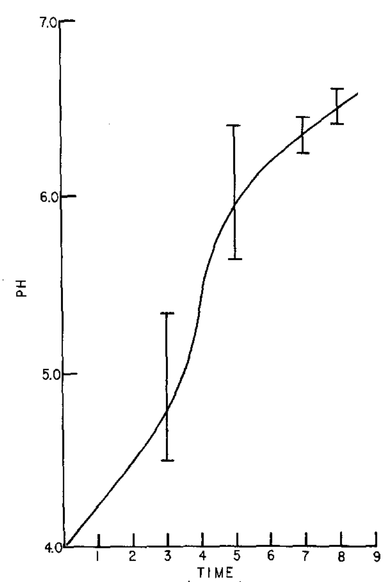

Fig. 2. Time course of the effect of 3 single fingerling rainbow trout (80-100 g) on the pH of 2 L of aquarium water which was initially adjusted to pH 4 with sulfuric acid. Vertical bars indicate one standard error.

DISCUSSION

The evidence obtained in the present study strongly suggests that the secretion of mucus in response to toxicants is not a general stress response but is a specific response to certain toxicants. Copper exposure did not affect mucous secretion however exposure to acid pH caused a considerable elevation in the rate of mucous secretion. These observations offer an explanation for the unexpected interactions of acid pH and copper in toxicity tests conducted by MILLER & MACKAY (1980). In those tests copper and acid pH acted synergistically at pH 5.4 but they acted antagonistically at pHs below 4.7. The stimulation of mucous secretion at low pH and the ability of mucus to chelate copper may explain the change from a synergistic to an antagonistic interaction of copper and acid pH at low pH.

The absence of increased mucous secretion in response to copper exposure may be supported by the histological studies of LABAT *et al.* (1974) who observed that copper exposure caused an apparent immediate ejection of the mucous cell contents. Subsequently the mucous cells remained empty suggesting to them a possible decrease in mucous secretion. The rapid release of mucus as described by LABAT *et al.* (1974) could be significant to the fish in coping with a short term acute exposure to copper such as might occur when a pulse of copper enters a river or stream.

The increased rate of mucous secretion seen in the present study during the 8 h following acid exposure is probably the result of a direct stimulation of mucus production by mucous cells rather than an increase in the number of mucous cells. PLONKA & NEFF (1969) observed an increase in the number of mucous cells in the gill lamellae following exposure to acid pH. However it is unlikely that such a response would have occurred during the 8 h of acid exposure used in the present study.

Mucus is a strong chelator of copper, particularly near neutral pH, however cupric ion binding capacity decreased with decreased pH. This is likely due to a decrease in total negative charge on the mucous molecule as a result of increased hydrogen ion concentration in the water. Other naturally occurring organic molecules such as humic acid (BROWN *et al.* 1974; ZITKO *et al.* 1973) and amino acids such as glycine, and histidine (BROWN *et al.* 1974; STIFF 1971), are effective chelators of copper and have been shown to significantly reduce copper toxicity at near neutral pHs. However, the results of the present study indicate that mucus may be an important factor in combating the toxic effects of copper. Clearly the rate of mucus secretion can change in response to specific stresses. There is also some evidence that the qualitative characteristics of the mucus secreted can change in response to stressful perturbations and these changes may provide the organism with some protection from the pertubation. In mammals a shift in the predominant type of glycoprotein, from neutral to acidic, has been found in response to infections (JONES *et al.* 1975) and to irritants (JONES *et al.* 1973).

In view of the evidence for mucus being sloughed from the body surface, except under extremely toxic conditions, an elevated rate of mucous secretion appears to be a very important defense mechanism against toxic substances. The investigation of mucous secretion in response to various pollutants would be valuable in determining the mechanisms of toxicity of various toxicants and a possible means of acclimation to them.

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