

# Hg- and Cu-Induced Hepatocellular Changes in the Mummichog, *Fundulus heteroclitus*

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To investigate mechanisms by which the mummichog (*F. heteroclitus*) successfully withstands heavy metal pollution, fish were treated with  $\text{Hg}^{2+}$  at up to 0.10 mg/L,  $\text{Cu}^{2+}$  at up to 1.0 mg/L, or combinations of  $\text{Hg}^{2+}$  and  $\text{Cu}^{2+}$ .

In earlier work, protein analysis of liver indicated that most of the cytosolic Cu is bound to the sulfhydryl-rich metallothionein, but that Hg is not associated with cytosolic proteins. Morphometric analysis indicates the Hg-treatment increases the lipid compartment of hepatocytes (ANOVA,  $F = 10.73$ ,  $p < 0.01$ ). This lipid increase is correlated with the Hg content (analyzed by atomic absorption spectrophotometry) of individual liver samples (Spearman rank correlation,  $r_s = 0.621$ ,  $p < 0.01$ ). Cu treatment causes a reduction in the lipid compartment ( $F = 10.38$ ,  $p < 0.01$ ), reduced cytoplasm in general ( $F = 18.55$ ,  $p < 0.001$ ) and an increased lysosome count ( $F = 14.21$ ,  $p < 0.001$ ). X-Ray microanalysis locates Cu in secondary lysosomes, but not in other organelles. Results of treatment with both  $\text{Hg}^{2+}$  and  $\text{Cu}^{2+}$  are similar to those of  $\text{Hg}^{2+}$  alone. Concentrations of Cu in liver varied too much to allow assessment of correlations with cytoplasmic changes.

Usual mechanisms for handling toxic heavy metals include binding to metallothionein and sequestering in lysosomes. Our findings for Cu are in agreement with this. Fish, however, can methylate Hg. (We have found >75% of killifish hepatic Hg to be methylated.) Increased cellular lipid may be a mechanism for sequestering the lipid-soluble methylmercury.

## Introduction

The killifish, or mummichog, *Fundulus heteroclitus*, survives in heavily polluted areas and can tolerate considerable exposure to heavy metals. In the course of analyzing possible mechanisms that enable the mummichog to tolerate heavy metals, their livers were analyzed because this organ is the source of metal-binding proteins (1). Earlier, the association of copper and mercury with hepatic metallothionein was studied (2). Less than 10% of the hepatic copper and none of the mercury were found to be associated with metallothionein in the cytosol. In order to gain more understanding of how these metals are tolerated, a morphological approach was employed. In this mode of investigation, substantial differences were noted among liver of  $\text{Hg}^{2+}$ - and  $\text{Cu}^{2+}$ -treated and untreated fish. The changes observed may be related to the species' ability to tolerate these metals.

## Materials and Methods

Specimens of *Fundulus heteroclitus* were collected with minnow traps from Piles Creek in Linden, NJ. Specimens 5 to 8 cm in standard length, 4 to 8 g in body weight were selected in the laboratory for 2 weeks of acclimation and depuration in an Instant Ocean aquarium. For each experimental run, five fish (both sexes) were placed in each all-glass container with 5 L of 20‰ salinity artificial seawater, to which was added 1.0 mg/L  $\text{Cu}^{2+}$  (as  $\text{CuSO}_4$ ), 0.10 mg/L  $\text{Hg}^{2+}$  (as  $\text{HgCl}_2$ ), a combination of both (0.1 mg  $\text{Hg}^{2+}$  plus 0.1 or 1.0 mg  $\text{Cu}^{2+}$ ) or nothing (control). The artificial seawater contained 0.07 mg/L Cu and no detectable Hg. The water was changed and redosed twice weekly. The fish were fed a commercial fish food (Tetramin) twice daily; this contained 22  $\mu\text{g/g}$  Cu and no detectable Hg. After 2 weeks, the fish were sacrificed. A 1-mm<sup>3</sup> piece of each liver was taken from the middle part, well away from the hilus, and fixed for electron microscopy. The remainder of the liver was divided in half, each part weighed, then prepared for analysis by atomic absorption spectrophotometry.

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Table 1. Concentrations of Hg and Cu in liver.

	Control	Concentrations of Hg and Cu after various treatments (metal ion in artificial seawater), $\mu\text{g/g}^a$			
		Cu (1.0 mg/L)	Hg(0.1 mg/L)	Hg(0.1 mg/L) + Cu(0.1 mg/L)	Hg(0.1 mg/L) + Cu(1.0 mg/L)
Hg	0.38 $\pm$ 0.12	0.42 $\pm$ 0.63	6.86 $\pm$ 2.77	7.36 $\pm$ 2.61	7.40 $\pm$ 1.36
Cu	114 $\pm$ 47	168 $\pm$ 80	152 $\pm$ 65	92 $\pm$ 46	194 $\pm$ 126

<sup>a</sup> Values are  $\bar{X} \pm \text{SD}$ ;  $\mu\text{g/g}$  wet weight.

metry, one portion for copper and the other for mercury. Copper was measured by flame absorption techniques with a Perkin-Elmer 403 instrument and mercury by the flameless cold-vapor method of Hatch and Ott (3) in a Coleman MAS-50 mercury analyzer.

For microscopic analysis, epoxy-embedded tissue metry, one portion for copper and the other for mercury. Copper was measured by flame absorption techniques with a Perkin-Elmer 403 instrument and mercury by the flameless cold-vapor method of Hatch and Ott (3) in a Coleman MAS-50 mercury analyzer.

For microscopic analysis, epoxy-embedded tissue blocks were sectioned at 0.5  $\mu\text{m}$ , stained with toluidine blue, photographed under oil immersion (1.32 N.A.) and printed at 2000X. Morphometric analysis, done in blind fashion, was performed by the method of Weibel et al. (4). Data thus collected were subjected to analysis of variance (ANOVA). The identity of organelles was confirmed by electron microscopy. Some blocks of tissue which had been embedded without  $\text{OsO}_4$  fixation, were analyzed by energy-dispersive X-ray analysis (EDXA). Sections of these blocks were mounted on titanium grids, carbon-coated and examined (CTEM mode) without any electron stain in a JEOL 100B electron microscope fitted with a Kevex X-ray microanalyzer.

## Results

The concentrations of Hg and Cu in the fishes' livers are presented in Table 1. There were no significant differences in Cu concentration by *t*-test between runs, between sexes, between treatment regimens, between controls and any treatment regimens, or, by regression analysis in relation to sizes; however, the high variance observed may preclude detection of differences. The only significant differences in the data in Table 1 are the obvious ones of Hg levels in livers of the control and  $\text{Cu}^{2+}$ -treated fish versus the  $\text{Hg}^{2+}$ -treated fish.

Microscopic analysis indicated that control hepatocytes

were characterized by considerable glycogen, some lipid and a few primary lysosomes (Fig. 1). Cu-treated fish, in contrast, had hepatocytes with reduced lipid ( $F = 10.38$ ,  $p < 0.01$  by one-way ANOVA) and glycogen, resulting in a proportionately increased nuclear compartment ( $F = 18.55$ ,  $p < 0.001$ ) and increased lysosomes ( $F = 14.21$ ,  $p < 0.001$ ). These were typically secondary and tertiary lysosomes that were sometimes larger than the nucleus (Fig. 2). Hg-treated fish had hepatocytes whose principal change from the control conditions was a significant ( $F = 10.73$ ,  $p < 0.01$ ) increase in lipid vacuoles (Fig. 3). No other changes or pathology were consistently found. Treatment with both  $\text{Hg}^{2+}$  and  $\text{Cu}^{2+}$  (either concentration) resulted in morphometric data with means which were nearly identical. However, the data from Hg + Cu (1.0 mg/L) treatment had high variance and were based on only three fish. Therefore, the data from this treatment were not considered any further. The results of the Hg + Cu (0.1 mg/L) treatment were found to be statistically similar to treatment with  $\text{Hg}^{2+}$  alone. Two-way ANOVA of the morphometric data presented in Table 2 indicated high significance (for main,  $F = 438.2$ ,  $p < 0.00001$ ; for interactions,  $F = 14.5$ ,  $p < 0.00001$ ).

The relation between metal uptake data and morphometric data from individual specimens was tested by Spearman's rank correlation test (5). There was a significant correlation between Hg uptake and lipid content ( $r_s = 0.621$ ,  $p < 0.01$ ). There was no significant relationship between Cu uptake and lysosome content; this may reflect a lack of correlation and/or the high variance encountered in Cu concentrations.

EDXA of Cu-exposed tissue demonstrated the localization of Cu within some of the dense bodies that were presumably the large lysosomes (Fig. 4). No Cu signal could be generated from either adjacent cytoplasm, presumptive nuclei, or from any area of control hepatocytes.

No attempt was made to locate Hg by EDXA, because previous experience had suggested that the small amount of Hg in the tissue is volatilized by the electron beam before a significant signal can be generated and detected.

Table 2. Distribution of subcellular compartments.<sup>a</sup>

Compartment	Control (n = 5)	Cu (n = 5)	Hg (n = 4)	Hg + Cu (n = 5)
Nucleus	8.2 $\pm$ 1.56	14.6 $\pm$ 1.04	6.0 $\pm$ 1.56	4.2 $\pm$ 0.55
Cytoplasm				
Nonlipid	72.6 $\pm$ 3.09	70.6 $\pm$ 2.41	60.8 $\pm$ 3.45	63.8 $\pm$ 3.40
Lipid	17.0 $\pm$ 2.09	6.8 $\pm$ 1.39	28.0 $\pm$ 3.37	28.6 $\pm$ 3.29
Lysosomes	2.2 $\pm$ 1.08	8.0 $\pm$ 1.84	5.3 $\pm$ 0.87	3.4 $\pm$ 0.76
Total	100	100	100	100

<sup>a</sup> Morphometric analysis by method of Weibel (3); 100 points measured per animal; means  $\pm$  SEM.

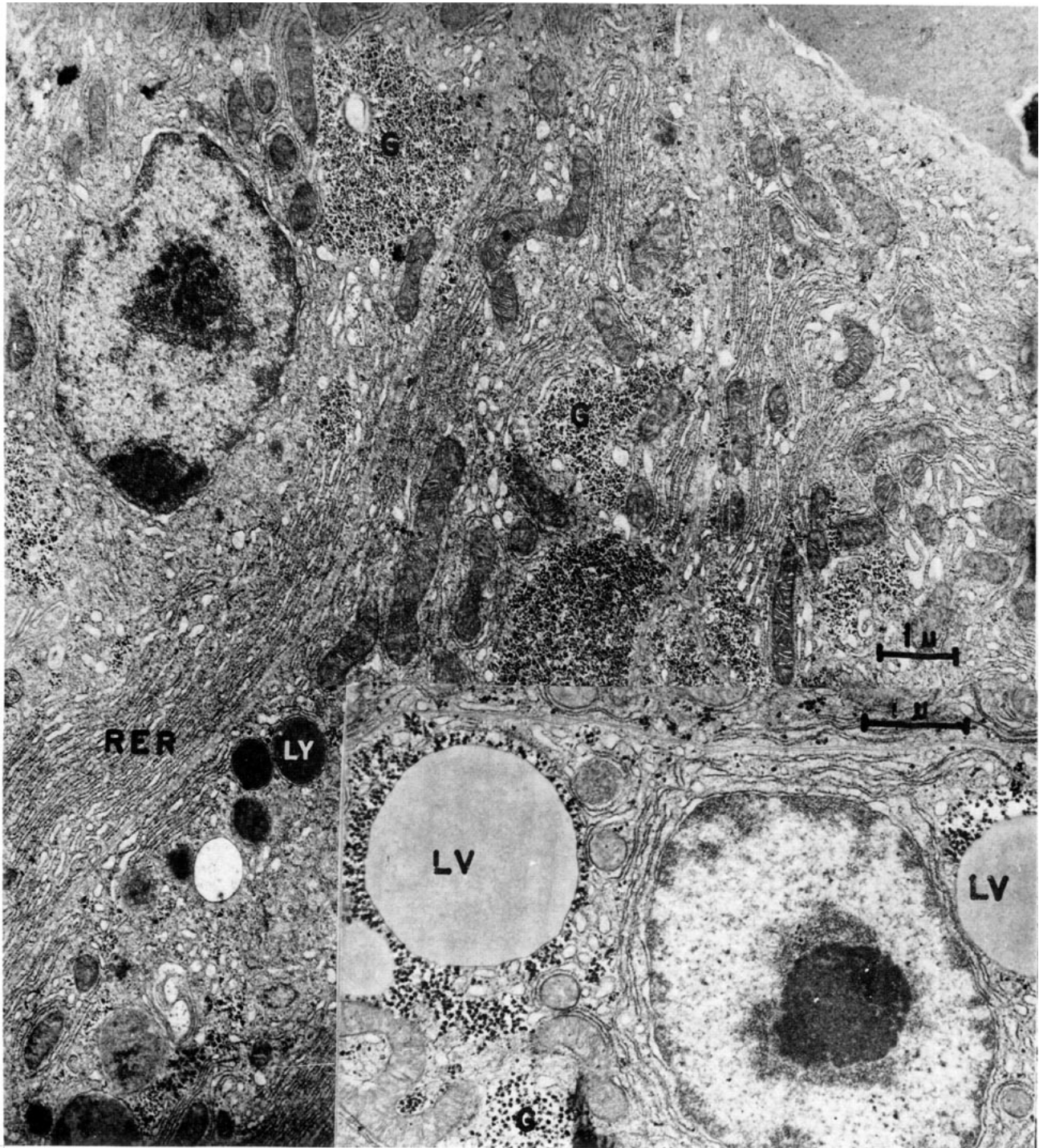


FIGURE 1. Liver of a control fish, showing glycogen (G), rough endoplasmic reticulum (RER), primary lysosomes (LY) and, in the inset, lipid vesicles (LV).

## Discussion

Mummichogs were collected from a moderately polluted estuary, acclimated in the laboratory, and then exposed to  $Hg^{2+}$  and/or  $Cu^{2+}$  in static-renewal systems. The Hg levels thus obtained were comparable to levels

found in livers of this species from the mercury-polluted Berry's Creek, NJ (Weis et al., in preparation). High Cu levels were encountered in all treatment groups; this reflects this species' ability to concentrate metals from its environment. The background level of Cu in both the water and the food probably contributed to the high tis-

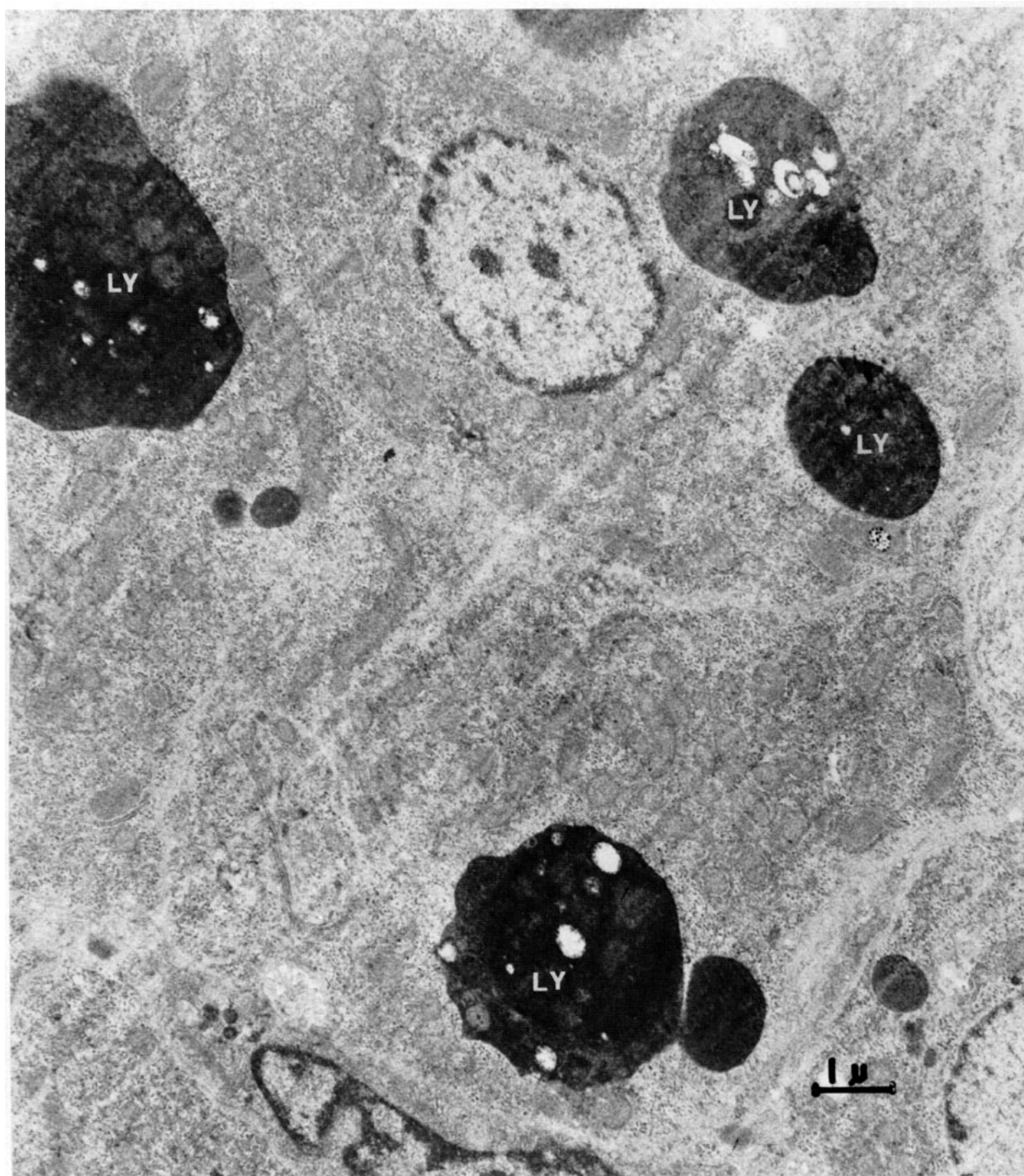


FIGURE 2. Liver of a copper-exposed fish, showing large secondary and tertiary lysosomes (LY).

sue levels. Chernoff and Dooley (6) pointed out that *Fundulus heteroclitus* has higher concentration factors for copper from lower environmental levels, resulting in a degree of equalization of tissue levels. In our laboratory system, great variance was found between similarly treated individuals in both control and experimental treatment groups. This may reflect differences in feeding

activity, thus resulting in different degrees of uptake from the food (at 22 ppm). Hg levels in Hg-treated fish were more consistent and significantly different from the controls. This reflects the lack of detectable Hg in both the food and the control water; only the  $Hg^{2+}$ -treated water was a source of Hg. No relationships were found between uptake of either metal and size or sex; this is



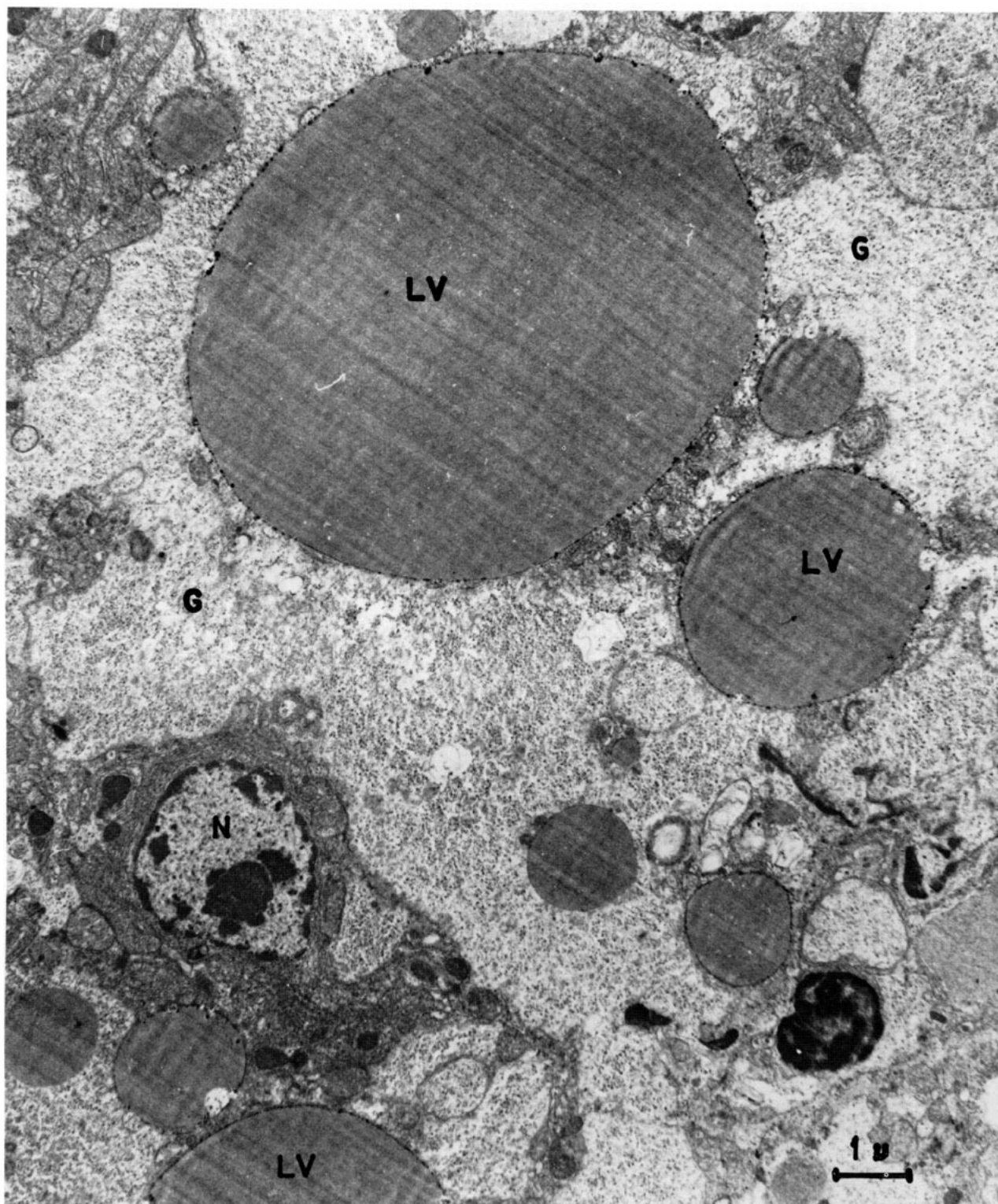


FIGURE 3. Liver of a mercury-exposed fish, showing large lipid vesicles (LV), surrounded by glycogen (G). The prominent nucleus (N) is in a ductule cell, not a hepatocyte.

in contrast to findings for Cu in this species reported by Chernoff and Dooley (6), but is in agreement with Eisler and LaRoche (7) for Cu versus sex.

Livers of  $\text{Cu}^{2+}$ -exposed fish consistently developed very large secondary and tertiary lysosomes. It is known that lysosomes accumulate metals (8), and the appear-

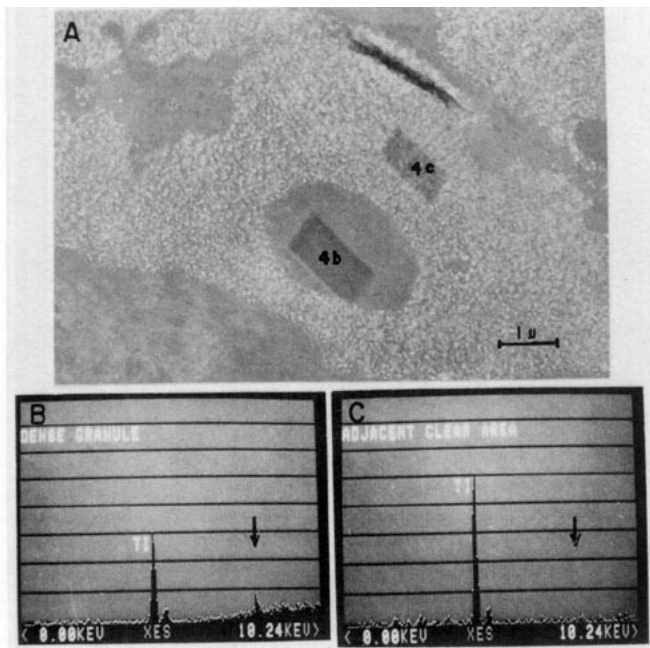


FIGURE 4. Liver of a copper-exposed fish. (A) Prepared without  $\text{OsO}_4$  postfixation, unstained, and mounted on a titanium grid. The two rectangles (4b, 4c) are the areas analyzed by energy-dispersive X-ray analysis. 4b is in a dense organelle presumed to be a secondary or tertiary lysosome, such as in Fig. 2; 4c is adjacent cytoplasm. (B,C) X-Ray spectra of areas 4b and 4c. The copper  $K\alpha$  lines are indicated by the arrows at 8.03–8.05 keV. The most obvious peak is that derived from the titanium grid (Ti).

ance of large lysosomes in carp liver was described by Rojik et al. (9) following acute exposure to copper sulfate (10 mg/L for 2 hr). Earlier, it was reported that most of *Fundulus*' hepatic Cu was in the sedimented fraction (40,000g) of liver homogenates (2). This would include lysosomes. We have now been able to confirm the presence of Cu in these lysosomes by EDXA, even though special techniques to preserve metals have not been followed. George (10,11) has demonstrated that, in the kidney of *Mytilus edulis*,  $\text{Cd}^{2+}$  is bound to metallothionein (MT) but is eventually concentrated in tertiary lysosomes. He has proposed a model in which Cd-MT is taken up into primary lysosomes, degraded in secondary lysosomes, and bound to nonspecific ligands in tertiary lysosomes. Our findings (2) that Cu-exposed *Fundulus heteroclitus* liver has increased lysosomes, Cu in lysosomes, and association of cytosolic Cu with presumptive MT suggests that a similar mechanism for sequestering Cu may be functioning in this vertebrate species.

Hg treatment did not significantly increase hepatic lysosomes as did Cu. Hg-induced lysosome increase has been reported in rats (12) and in ducks (13), but in this study, the significant change with Hg treatment (and with Hg + Cu) was the increase in lipid. Increase in lipid may be a response for sequestering lipid-soluble chemicals; in this case, methylmercury may be the eliciting agent. Fish are known to methylate mercury, prob-

ably by means of intestinal microorganisms (14), as well as in the liver (15). As a result, 70 to 90% of Hg in fish is methylated (16). We have confirmed this in *Fundulus heteroclitus* (2). Thus, the increase in hepatic lipid could be a mechanism to sequester this lipid-soluble form of the metal. Hepatic lipid was found to be increased in humans with Minamata disease (17) and was experimentally induced by treatment with methylmercury in rats (18) and in ducks (13). But, as discussed by Bhatnagar et al. (13), lipid accumulation is a typical reaction to cytotoxicity, probably related to inhibition of protein synthesis (19,20). This could be the mechanism operating in our  $\text{Hg}^{2+}$ -treated fish, and would also explain why the Hg/Cu treatments resulted in only an increase in cellular lipid. The liver may have suffered sufficient toxic reaction to be unable to respond to the Cu. If this is true, sequestering of Hg by lipid may be simply coincidental to a toxic reaction. On the other hand, we did not find pathological changes such as a focal cytoplasmic degeneration and swollen organelles as described in these other reports.

As a result of the observations described in this report, two separate mechanisms have been suggested for the sequestering of two different heavy metals in a teleost fish. How the fish then depurates the sequestered metals remains to be determined.

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