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Zinc and Copper Distribution in Excretory Organs of the Dogfish *Scyliorhinus canicula* and Chloride Cell Response Following Treatment with Zinc Sulphate

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Abstract

Zinc and copper levels in the gills, kidney and intestine of the dogfish *Scyliorhinus canicula* L. collected in a 80 to 120 m deep zone close to Barcelona (Spain) during the months of February-March 1981 were measured by atomic absorption spectrophotometry after subacute (10 ppm Zn for 3 wk) and acute (80 ppm Zn for 24 h) treatment with $ZnSO_4$. Individuals exposed to 80 ppm Zn accumulated Zn in the gills, whereas no significant increase in Zn content was found in the kidney or intestine. No differences in the Cu content of any of these tissues were found. Dogfish exposed to 10 ppm Zn for 3 wk displayed an increase in Zn content of the gills, kidney and intestine. An increase in the Cu concentration of the gills was also recorded which may suggest that Cu, and hence Zn and other heavy metals, are excreted via the gills when the kidney and intestine are overloaded. A light-microscopic study of the gill epithelium was carried out on dogfish treated with 10 ppm Zn for 3 wk, 80 ppm Zn for 24 h and 175 ppm Zn for 14 h, and chloride cell response was quantified. Chloride cells increased in number after each treatment, even appearing in the secondary lamellae following treatment with 175 ppm Zn. It is suggested that the chloride cell may excrete bivalent ions when dogfish are exposed to an excess of bivalent salts.

Introduction

Heavy metal metabolism in marine organisms has been studied intensively (see Bryan, 1979 and Coombs, 1980 for recent reviews). Mechanisms of heavy metal detoxication in invertebrates are well documented (George *et al.*, 1976, 1978, 1979; Noël-Lambot, 1976; Noël-Lambot *et al.*, 1978a; Lowe and Moore, 1979; George and Pirie, 1980; Viarengo *et al.*, 1981), yet very few data can be found in the literature on marine vertebrates (Noël-Lambot *et al.*, 1978b; Bouquegneau, 1979; Overnell and Coombs, 1979; Kito *et al.*, 1980).

The fact that acclimatization to sublethal concentrations of a metal can subsequently increase the tolerance of fish to higher concentrations (Edwards and Brown, 1967), suggests that a mechanism of detoxication is triggered by the exposure of the organism to heavy metals. The increase noted in metallothionein synthesis in fish exposed to heavy metals (Noël-Lambot *et al.*, 1978b; Bouquegneau, 1979; Kito *et al.*, 1980) may constitute such a tolerance mechanism. Many laboratories have focused their attention on metallothioneins in the last few years, yet very little attention has been given to the study of the gills as a heavy metal excretory device (Nakatani, 1966). Bivalent ions are excreted via the kidney, however, it is not unlikely that, when the kidney is overloaded, as after exposure to heavy metals, the gill may play an important role as an extrarenal route for detoxication.

The chloride cell, a mitochondria-rich cell, located in the gills of teleosts (Kessel and Beams, 1962; Munshi, 1964; Laurent and Dunel, 1978; Dunel and Laurent, 1980) as well as elasmobranchs (Doyle and Gorecki, 1961; Hughes and Wright, 1970; Wright, 1973; Laurent and Dunel, 1980), has been revealed as the main site of extrarenal excretion in teleosts (Maetz, 1971). The chloride cell undergoes cytological modifications and increases/decreases in number when environmental salinity is changed (Shirai and Utida, 1970; Karnaky *et al.*, 1976; Hossler *et al.*, 1979; Karnaky, 1980; Laurent and Dunel, 1980; Philpott, 1980). Although the main role of the chloride cell is osmoregulation, it has also been shown to excrete organic compounds (Masoni and Garcia-Romeu, 1972) and it is suggested that this cell also excretes SO_4^{2-}, Mg^{2+} (Ahuja, 1970) and Cu (Baker, 1969).

In order to verify the role played by the gill in Zn detoxication, the present investigation studied the chloride cell response as well as Zn and Cu distribution in the excretory organs of the dogfish (gills, intestine and kidney) following treatment with Zn sulphate. Our attention fo-

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cused on Zn and Cu distribution because of the close relationship in the metabolism of these metals (Cuadras *et al.*, 1981; Sutherland and Majors, 1981). The dogfish *Scyliorhinus canicula* has been shown to accumulate Zn in its gill system following subacute (Crespo *et al.*,1979) and acute (Crespo and Balasch, 1980a) exposure to the metal. Zn accumulated in the spleen, liver and pancreas following subacute contamination (Flos *et al.*, 1979), whereas no increase in Zn content in these organs was found after acute Zn treatment (Flos, unpublished data).

Materials and Methods

Dogfish (*Scyliorhinus canicula* L.) of 150 to 300 g body weight were collected in an 80 to 120 m deep zone close to Barcelona (Spain) during the months of February-March 1981. They were kept for at least 1 wk in an open circulation tank (natural seawater: 36‰ S, 13° to 15 °C) before experiments. All fish used for the determination of heavy metal levels were male, as differences in Zn content (Crespo and Balasch, 1980b) and Zn accumulation (Crespo *et al.*, 1979) due to sex have been found. Individuals for experimentation were placed in 30 litre volume tanks (2 dogfish in each) and Zn solutions were prepared by adding $ZnSO_4$ to seawater up to concentrations of 10, 80 and 175 ppm. Eight fish were treated with 10 ppm of Zn for 3 wk, and were then killed with tricaine methanesulfonate (MS-222 Sandoz). Eight fish were treated with 80 ppm of Zn, which corresponds to the LC_{50} at 48 h for the dogfish (Crespo and Balasch, 1980a), and were killed in the same way. In order to minimize the reduction of Zn concentration either by precipitation or uptake by the fish and to remove nitrogenous excretions, the Zn solution was replaced every 3 d. Nine fish were used as controls. All fish (experimentals and controls) were unfed during experimentation (3 wk). An additional group of fish (2 males and 2 females) was treated with 175 ppm Zn, which corresponds to the LC_{50} at 24 h (Crespo and Balasch, 1980a). They were killed with MS-222 Sandoz after 14 h exposure to Zn. These fish were used for histological examination only.

Zn and Cu Determination

Three of the central gills [those which accumulated the most Zn following subacute Zn treatment (Crespo *et al.*, 1979)] were removed, carefully washed in de-ionized water, oven-dried in air at 100 °C, and weighed. A piece of intestine weighing approximately 0.8 g and the kidney were also removed and handled in the same way. Each sample was digested with 15 ml concentrated HNO_3 and, after evaporation to dryness, diluted to 5 ml with de-ionized water for Cu determination. A 5× dilution was prepared for Zn. Metal levels were determined by atomic absorption spectrophotometry (Perkin Elmer 330S). The Student's *t*-test was used to check statistically significant differences between groups.

Histological Methods

Filaments were excised from the third gill arch of each fish and fixed in 5% glutaraldehyde in phosphate buffer. Samples were post-fixed in 2% OSO_4 and embedded in Araldite. Semi-thin sections were cut at 1 μm (LKB Ultratome III) and stained with toluidine blue.

For quantitative light-microscopic studies, nucleated chloride cells in the interlamellar spaces were counted on transverse sections of 3 different filaments of each fish. Four fish were analyzed for each group (controls, 10, 80 and 175 ppm-treated fish). The length of the filament was measured and the number of chloride cells per unit length was determined. The Student's *t*-test was used to check statistical differences between groups.

Results

Fig. 1 shows Zn and Cu levels (μg g^{-1} dry wt) in the gills of *Scyliorhinus canicula* following different Zn treatments. The Zn concentration after subacute treatment (10 ppm for 3 wk) was significantly higher than control levels ($P=0.05$). There was a 300% increase ($P=0.05$) in the Zn content of the gills after acute treatment (80 ppm for 24 h). Cu levels increased in the gills ($P=0.005$) after a 10 ppm exposure for 3 wk, but no significant increase occurred after acute treatment. Fig. 2 shows Zn and Cu distribution in the kidney and intestine of untreated and Zn-treated dogfish. Zn accumulated in the kidney and the intestine ($P=0.05$) following subacute treatment. No Zn accumulation was found following a 80 ppm Zn exposure for 24 h. No differences in Cu content were found following either subacute or acute treatment.

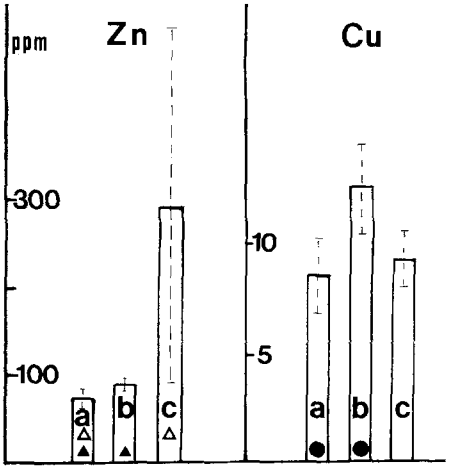

Fig. 1. *Scyliorhinus canicula.* Zn and Cu concentrations in ppm in gills of untreated (a), and treated – 10 ppm for 3 wk (b), and 80 ppm for 24 h (c) – dogfish. Levels of metals were calculated in μg g^{-1} dry wt. Means and standard deviations are shown. Student's *t*-test revealed significant differences in Zn content between a and b (▲, $P=0.05$) and between a and c (△, $P=0.05$). Cu levels increased significantly (●, $P=0.005$) following treatment with 10 ppm for 3 wk

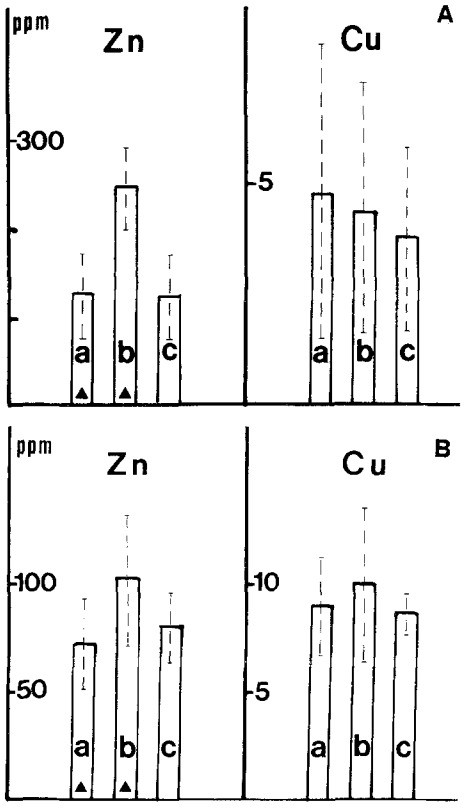

Fig. 2. *Scyliorhinus canicula.* Zn and Cu concentrations in ppm in kidney (A) and intestine (B) of untreated (a), and treated – 10 ppm for 3 wk (b) and 80 ppm for 24 h (c) – dogfish. Levels of metals were calculated in $\mu g\ g^{-1}$ dry wt. Means and standard deviations are shown. Student's *t*-test revealed significant differences in Zn content between a and b (▲, $P=0.05$)

Fig. 3a shows a cross-section of a control gill filament. Note the disposition of the primary epithelium, secondary epithelium (or lamellae) and afferent and efferent sides. Chloride cells are mainly located in the interlamellar spaces of the primary epithelium and in the afferent side; very few are visible either in the secondary lamellae or in the efferent region. Chloride cells are easily distinguishable by their great size, the basement membrane to surface orientation of their cytoplasm, and their voluminous nuclei (Fig. 3b and c).

Chloride cells in the interlamellar spaces statistically increased in number following 80 ppm exposure for 24 h ($P=0.01$) and 10 ppm exposure for 3 wk ($P=0.05$). Distance between 2 consecutive lamellae appeared to shorten, since chloride cells mainly proliferated at the base of the secondary lamellae (Fig. 3d): deep and many branched invaginations were visible in the primary epithelium (Fig. 3f). The primary epithelium of exposed individuals increased in width (Fig. 3d), and was almost entirely occupied by chloride cells of different sizes and different affinities for toluidine blue (Fig. 3f). The apical region of the chloride cells was bigger and showed more and longer protrusions of the apical membrane than controls (Fig. 3e).

Mucous cells exhibit an ovoid shape and stain heavily with toluidine blue. They are mainly located in the secondary epithelium and in the efferent side. Some can be found, however, in the afferent side, and they occasionally appear in the interlamellar spaces (Fig. 3b). No differences in the distribution and frequency of appearance of mucous cells between treated and untreated dogfish were found. No desquamation of the epithelium was observed in 10 ppm-treated individuals. Two of the 8 fish treated with 80 ppm Zn displayed desquamation of the efferent side.

Table 1. *Scyliorhinus canicula.* Toxicity symptoms displayed by Zn-treated dogfish

	Treatment		
	10 ppm, 3 wk	80 ppm, 24 h	175 ppm, 14 h
No. of fish exposed to each treatment	8	8	4
No. which showed no apparent toxicity symptoms	All	3	0
No. which "overturned"	0	3	1
No. which died in moment of sacrificing	0	2	1

In 175 ppm-treated fish, chloride cells even appeared in the secondary lamellae (Fig. 4). Desquamation of the epithelium was noted on the efferent side of the filament. The secondary epithelium, the interlamellar spaces and the afferent side also exhibited some desquamation, but this was not as marked as on the efferent side. Toxicity symptoms displayed by Zn-treated dogfish are summarized in Table 1.

Discussion and Conclusions

Exposure of *Scyliorhinus canicula* to high Zn levels (80 ppm) resulted in an increase in Zn concentration in the gills, whereas Zn levels in the intestine were unaffected. A marked increase in the Zn content of the gill system of the dogfish was described by us following treatment with 175 ppm Zn (Crespo and Balasch, 1980a). Taking into account that elasmobranchs do not need to drink water to maintain their osmotic balance and that the dogfish were unfed during experimentation, it is not probable that Zn was taken up *via* the gastrointestinal tract. It is then assumed that the main route of entry of Zn was the gill.

Previous experiments at our laboratory (Crespo *et al.*, 1979) showed that following treatment with 15 ppm of zinc, Zn accumulated in the gills, reaching a peak value on the 4th day which did not increase although treatment was prolonged up to 25 d. Zn accumulated in the storage organs (spleen, liver and pancreas) following the 7th day of treatment (Flos *et al.*, 1979). The present data revealed a marked increase in Zn content in the kidney and intestine

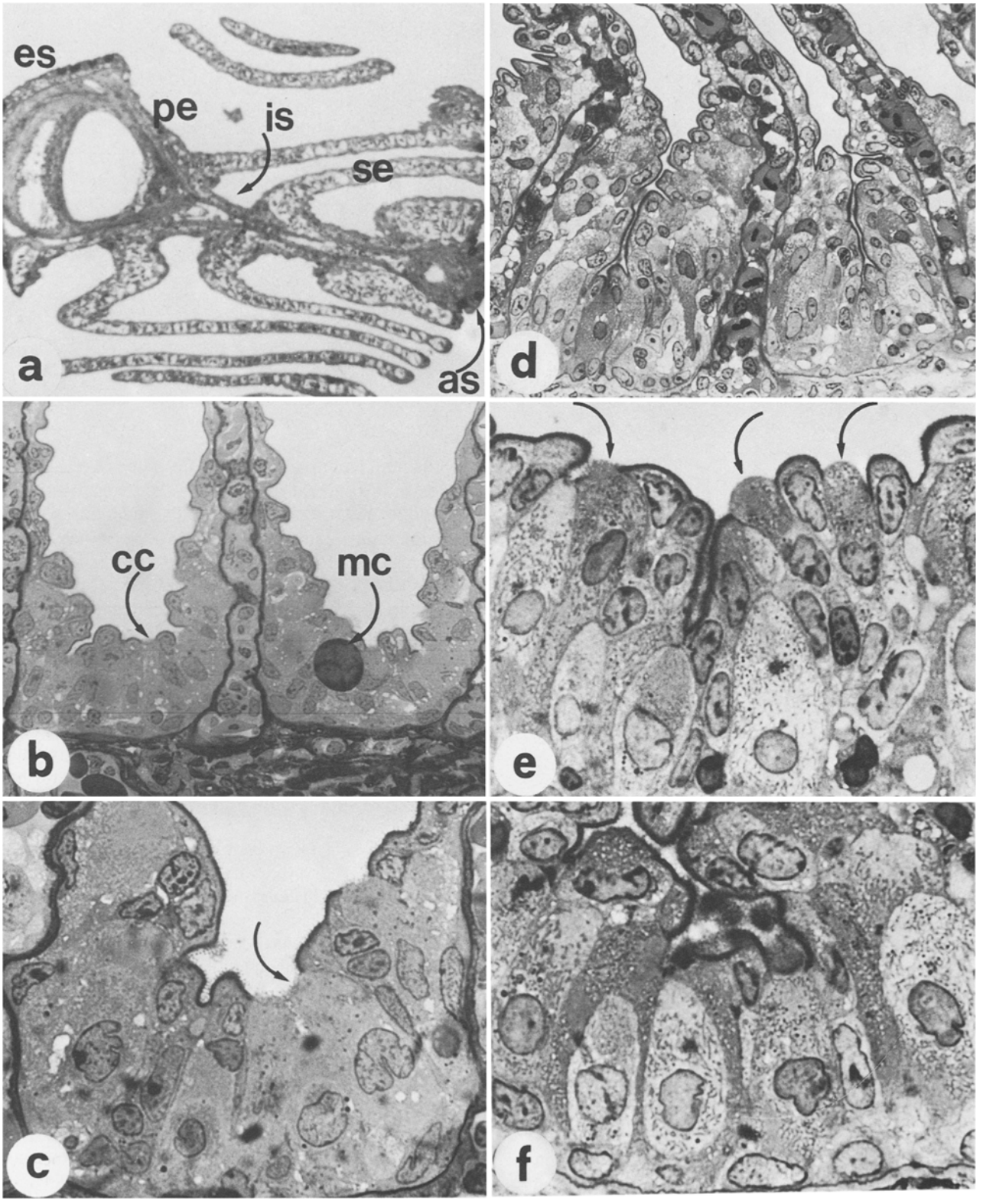

Fig. 3. *Scyliorhinus canicula.* Structure of gill filaments (a–c: controls; d–f, treated). (a) Cross-section of control filament (250×): pe, primary epithelium; se, secondary epithelium (or lamellae); as, afferent side; es, efferent side; is, interlamellar space. (b) Interlamellar spaces of control filament (1 200×: cc, chloride cell; mc, mucous cell. (c) Interlamellar space of control filament at higher magnification (3 000×); note great size of the chloride cells, basement membrane to surface orientation of their cytoplasms, voluminous nuclei and typical apical regions (arrowed). (d–f) Interlamellar spaces of gill filaments following treatment with 10 ppm Zn for 3 wk; note increase in width of primary epithelium and increase in number of chloride cells in (d), apical protuberances of chloride cells (arrows) in (e), and great number of chloride cells showing different affinity for toluidine blue in (f). (d = 1 200×; e, f, = 3 000×)

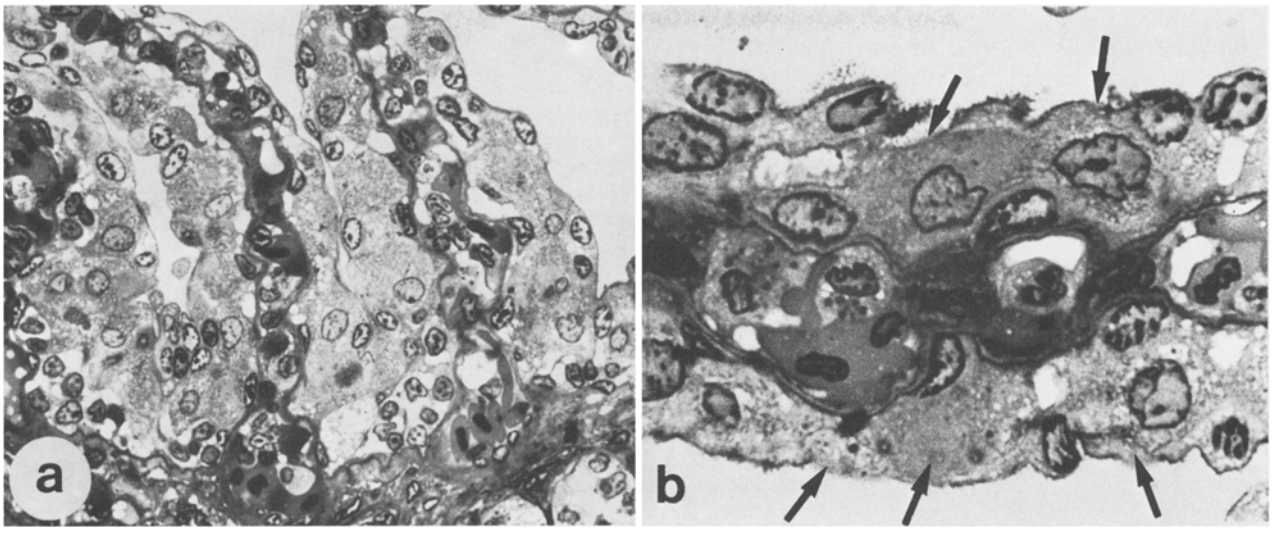

Fig. 4. *Scyliorhinus canicula.* Structure of a gill filament following treatment with 175 ppm Zn for 14 h. Note chloride cells in secondary lamellae (a), and chloride cell apical crypts (arrowed, b). (a = 1 200×; b = 3 000×)

after treatment with 10 ppm Zn for 3 wk, suggesting that these two organs may play an important role in the excretion of Zn. The kidney has been shown to be the major excretory route for zinc in bivalves (George and Pirie, 1980), whereas in crustaceans (Bryan, 1971) and fish (Nakatani, 1966) the gills and the gut are the main exit sites of this metal.

Our results demonstrate that Cu levels increased in the gills following subacute treatment (10 ppm for 3 wk). Sutherland and Majors (1981) studied the distribution of Zn following Cu contamination, and found in the gill a 300% increase of Zn which had been mainly displaced from the kidney. In the present work, levels of Cu in the kidney and intestine were unaffected; however, it is not unlikely, considering the close relationship existing between these metals, that Cu in the storage organs might be displaced by zinc. This aspect deserves increasing attention and is presently being investigated at our laboratory. Data by Sutherland and Majors (1981) and our own present results suggest the gill can act as an excretory device when the other excretory organs are overloaded, thus playing an important role in detoxication.

According to our histological observations of the gill filament, the chloride cell was the only cell type that proved to increase in number following $ZnSO_4$ treatment. Chloride cells even appeared in the secondary epithelium after a 175 ppm exposure to Zn. Baker (1969) also described an increase in chloride cell number following treatment with $CuSO_4$. As he found a decrease in the number of mucous cells, he suggested that mucous cells changed into chloride-secreting cells. Munshi (1964) also described mucous cells as being able to perform chloride cell functions. However, in the present study, no changes in mucous cell distribution were observed.

An increase in width of the primary epithelium was observed. This is in agreement with data reported by Crespo (1980) which demonstrated that the dry weight of the gills of subacutely treated *Scyliorhinus canicula* was significantly higher than control values from dogfish of equal body weight. Eisler and Gardner (1973) also described the formation of an additional epithelial layer in the gill of the teleost *Fundulus heteroclitus* exposed to cadmium. Our present observations revealed chloride cells of different sizes and of different affinity for toluidine blue in the primary epithelium of treated fish which were apparently migrating from the basal part to the free surface of the filament. We suggest that these cells are transitional stages of the typical chloride cell. Similar transitional stages have been described by Shirai and Utida (1970) in the eel *Anguilla japonica* during seawater adaptation. According to Conte and Lin (1967), chloride cells in the coho salmon *Oncorhynchus kisutch* transferred from freshwater to seawater differed from the replacement cells lying adjacent to the basement membrane: proliferation took place at the basal part of the epithelium and was followed by cell differentiation and migration.

Changes in the morphology of the apical region of the chloride cell may be related to its activity, the presence of apical pits correlating with increased activities (Shirai and Utida, 1970; Hossler *et al.*, 1979; Hossler; 1980). According to Laurent and Dunel (1980), very few apical pits occur in *Scyliorhinus canicula* gill epithelia compared to teleosts. Our own observations revealed few pits either in untreated or treated dogfish. However, the apical membrane of treated fish protrudes more than that of untreated, thus increasing contact with the external medium. A further combined transmission electron microscope and scanning electron microscope study may provide more information on chloride cell activity in treated dogfish. Unpublished results from our laboratory show that mitochondria of the chloride cells increase in number following treatment with $ZnSO_4$, suggesting increased activity of these cells.

Desquamation of the epithelium of the efferent side of the filament was noted in fish treated with 175 ppm Zn.

Some desquamation was observed in the secondary lamellae, but very little in the interlamellar spaces or the afferent side. This may be due to the fact that the junctions between chloride and epithelial cells are tight junctions. Therefore, desquamation will mainly occur in the epithelial layer which lacks chloride cells.

From heavy metal redistribution in the excretory organs of the dogfish and chloride cell proliferation following $ZnSO_4$ treatment we may conclude that the chloride cell plays an important role in detoxication. It would not be unreasonable to suppose that previous exposures to low concentrations of heavy metals might trigger not only metallothionein synthesis but chloride cell proliferation as well, thus enhancing tolerance to lethal doses. However, one of the questions that arises is whether the proliferation of chloride cells is triggered by the excess of heavy metals or is it an unspecific reaction to an excess of bivalent ions? Ahuja (1970) studied chloride cell response to chloride- and sulphate-enriched media in two species of teleosts (*Gambusia affinis affinis* and *Catla catla*). This author found that chloride cells hypertrophied normally in the chloride-enriched medium, whereas they came to a functional state only under special conditions of renal failure in the sulphate medium ($MgSO_4$). He suggested that when the kidney failed to excrete the increasing load of sulphates, bivalent ions (SO_4^{2-} and Mg^{2+}) were secreted from the gill. Taking into account Baker's (1969) and Ahuja's (1970) results as well as our own observations, it may be assumed that an excess of bivalent salts (not only heavy metals) triggers chloride cell proliferation when the other excretory organs (kidney and intestine) are overloaded.

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