Biometals (2008) 21:311–319
DOI 10.1007/s10534-007-9120-9

Autometallographic tracing of Hg–S quantum dots in frogs exposed to inorganic mercury

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Received: 22 February 2007 / Accepted: 19 September 2007 / Published online: 11 October 2007

Abstract The histochemical distribution of mercury in the kidneys and gut of frogs (*Rana ridibunda)* exposed to inorganic mercury was analyzed with autometallography (AMG). It was found that most mercury in the kidneys accumulated in the proximal convoluted tubules as Hg–S nanocrystal, while control animals were totally void of AMG grains. In the gut the highest concentration of mercury was observed in the large intestine. The AMG grains were primarily located in the apical part of the absorptive cells, although rather high concentrations of silver enhanced mercury quantum dots were also detected in a special cell type of gut epithelium and the glycocalyx. A certain amount of AMG grains were detected in the lumen of the gut. We hypothesize that this pool of quantum dots results from sloughed off epithelial cells and macrophages. Such still intact cells and red blood cells containing AMG grains were also found in the lumen of the gut.

Keywords Frog · Autometallography (AMG) · Mercury · Kidney · Gut

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Introduction

Mercury (Hg) pollution has been recognized as a potential environmental and public health problem for over 40 years. In general, the primary routes of acute and chronic Hg exposure include inhalation, dermal absorption and ingestion. In terms of exposure of aquatic ecosystems, mercury is naturally found in varying concentrations depending upon the geology and hydrology of the environment.

Mercury is a highly reactive molecule that produces toxic effects and it is distributed widely as an environmental and industrial pollutant. In humans, high mercury levels can be found in the skin, nails, hair and kidneys (Thounwonwou et al. 2003). Toxic responses such as axial malformations, stunting, neurological deficits, decreased weight, altered enzyme levels and renal failure, are all examples of the chemically-induced effects of Hg exposure (Sweet and Zelikoff 2001).

Within biological systems, mercuric ions do not exist as inorganic salts, nor are the changed ions believed to be present as free ionic state. Mercuric ions have a very high affinity for thiol-containing biomolecules, such as glutathione (GSH), cysteine (Cys), homocysteine (Hcy), metallothionein (MT) and albumin. In biological systems, mercuric ions are always bound to one or more of these molecules (Bridges and Zalups 2005).

Parenterally administered mercuric chloride accumulates primarily in the kidneys, liver, spleen, blood,

thymus, lymph nodes, bone marrow, lungs and brain of mice and rats (Hansen and Danscher 1995).

As the kidney constitutes the major excretory organ of heavy metals and is particularly susceptible to adverse effects because of high blood flow and enhanced metabolic activity associated with a number of sensitive metabolic processes, renal tissue can be considered one of the most important targets for heavy metals (Price et al. 1996). Indeed, the kidney is the primary target organ that takes up and accumulates Hg^{2+} from the blood (Zalups 1998a, b; Zalups 2000). The accumulation of this metal in the kidneys is very rapid, with as much as 50% of the nontoxic dose appearing there within a few hours of exposure (Zalups 1998c).

In humans gastrointestinal absorption, given a daily intake of inorganic mercury, make up in the range of 2–38% of the total Hg intake, depending upon the amount and type of Hg in the food (Goyer 1991; Mahaffey 1998). Inorganic mercury seems not to cross the gastrointestinal epithelium easily. However, it is transformed in marine and freshwater sediments as a result of the action of anaerobic bacteria to methylmercury (MeHg), which readily crosses the gastrointestinal epithelium.

After acute oral human intoxication with inorganic mercury salts, the immediate critical organs are the epithelial cells of the gastrointestinal tract. Due to corrosive effects of mercuric salts on the mucous membranes (Berlin 1986), gastrointestinal ulceration, perforation and hemorrhage will occur rapidly, followed by circulatory collapse. Breakdown of intestinal mucosal barriers is believed to further increase mercury absorption whereof a major part ends up in the kidneys, causing severe kidney damage (Hanley et al. 2002; Pollard et al. 2002).

Information about the exact subcellular localization of mercury in tissues has not been available, until the introduction of the autometallographic (AMG) technique for tracing mercury sulphide/selenide accumulation (Danscher and Moller-Madsen 1985; Stoltenberg and Danscher 2000). AMG allows tracing of minute accumulations of mercury, metabolized to mercury sulphide/selenide molecules that accumulates in Hg–S or Hg–Se nanocrystals or quantum dots that can be silver enhanced with AMG. The AMG grains can be traced ultrastructurally as well as in the light microscope (Danscher and Stoltenberg 2006).

AMG works by encapsulating the quantum dots in shells of silver (Danscher and Moller-Madsen 1985, Danscher and Stoltenberg 2006). In order to ensure that the metal in question is mercury it is imperative to use either chemical exclusion approaches or apply a multi element technique (Stoltenberg et al. 2002).

A number of studies describe the AMG distribution of mercury in mammals (Schionning et al. 1991, 1997; Hansen and Danscher 1997; Pedersen et al. 1999) and fish (Baatrup et al. 1986; Baatrup and Danscher 1987). On amphibians the approach has only been used once (Loumbourdis and Danscher 2003). The present study tracks the trafficking of mercury in kidney and gut of frogs exposed to mercuric chloride for 1 and 6 days respectively.

Materials and methods

Frogs from a relatively non-polluted area of northern Greece, were purchased from a local dealer and acclimatized for 5–7 days in plastic aquaria containing 2–3 cm dechlorinated water. Twelve frogs were placed in a plastic aquarium and exposed to 1 ppm Hg (as $HgCl_2$) in water. Six animals were removed from the aquarium for sacrificing after 1 day of exposure and the remaining six animals after 6 days of exposure. Another six animals, serving as controls were placed in dechlorinated water and were sacrificed one day before the sacrificing of animals exposed to Hg for 1 day. The AMG technique, used to demonstrate mercury, is based on the ability of metabolized mercury ending up in mercury-sulphur or mercury-selenium nanocrystals mercury (Danscher and Stoltenberg 2006).

The animals were subjected to the usual process of perfusion fixation, embedding in paraffin, sectioning at 6 μm, AMG developing (Danscher and Moller-Madsen 1985; Danscher 1991) and counterstaining with toluidine blue. The developer consisted of crystalline gum Arabic, citric acid, hydroquinone, and silver lactate (Danscher and Stoltenberg 2006). The specimens were placed in the developer for 70 min to ensure optimal development. During development, the specimens were kept in a light-tight box to suppress autocatalytic precipitation of silver grains in the developer. After development, the sections were rinsed in Farmer's solution (9% sodium

thiosulfate plus 1% potassium ferricyanide) for 10 s and counterstained with 0.1% toluidine blue.

Results

Kidney

AMG granules revealing the exact localization of Hg–S quantum dots was found almost exclusively in the epithelial cells of the proximal convoluted tubules (PCT) (Fig. 1). Very characteristic was the uneven distribution of AMG grains in the PCTs, some sections being crowded by Hg grains and some other sections with fewer grains (Fig. 1). The proximal tubule had a high content of AMG grains in the lysosomes, while the distal tubule was almost void of staining. An extremely small number of granules were localized in the red blood cells as well as in the endothelial cells lining the blood vessels. Very few AMG grains were observed in the fibroblasts of the connective tissue surrounding the epithelia. No AMG grains were observed in the collecting ducts or the ureter.

Stomach

Whether the animals had been exposed for one or 6 days the stomach was almost void of mercury–sulphur nanocrystals. However a few AMG silver grains were localized in the apical part of the epithelial cells of the stomach and even fewer could be traced deeply in the crypts (Fig. 2). As for the kidney, there was no detectable difference in grain concentration between the animals exposed for 1 day and 6 days respectively. Sporadically, very few Hg–S-AMG grains were observed in the endothelial cells of blood vessels.

Small intestine

After 1 day of Hg exposure, a low level of AMG grains was observed in the apical part of the absorptive cells and in the glycocalyx of the small intestine (Fig. 3). High concentrations of mercury were also observed in a special cell type located in the epithelium of the villi, closely resembling goblet cells (Fig. 3); however, closer examination revealed that they might not be goblet cells, since the latter cells were not stained at all with the AMG-technique, but with toluidine blue (Fig. 3). In animals exposed for 6 days, an indisputable increase of AMG silver grains was detected in the absorptive and goblet-like cells. In some sections, the glycocalyx resembled a thick black line (Fig. 3) and the apical part of the absorptive cells was heavily loaded with AMG grains. Staining of the endothelial cells lining the blood vessels was also much higher than after 1 day

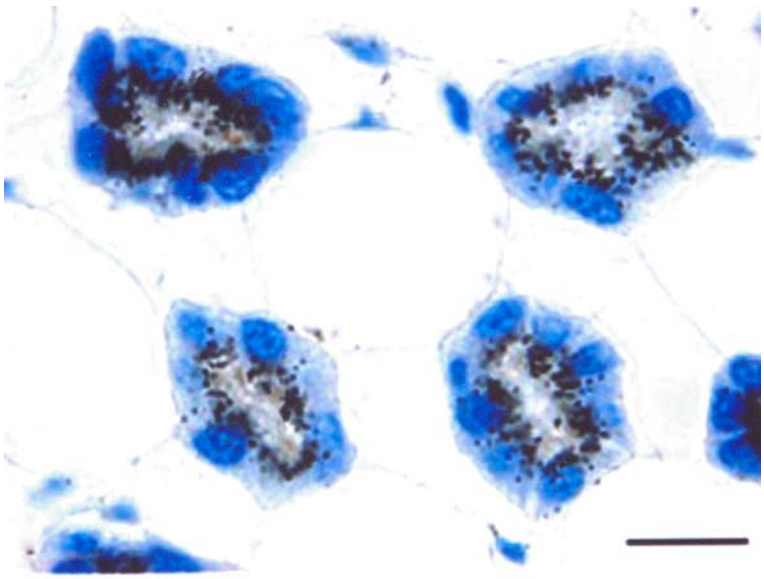

Fig. 1 AMG grains in the proximal convoluted tubules in the kidney of the frog *Rana ridibunda.* Note the differential density in various tubules

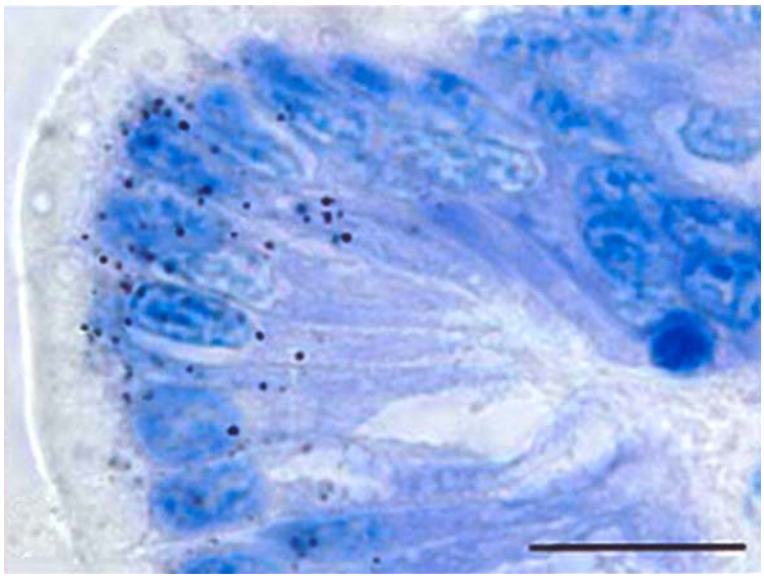

Fig. 2 Transverse section of the stomach of the frog *Rana ridibunda* stained by the AMG method. Note the low density of AMG grains in the apical part of epithelial cells. The bar equals 20 μm

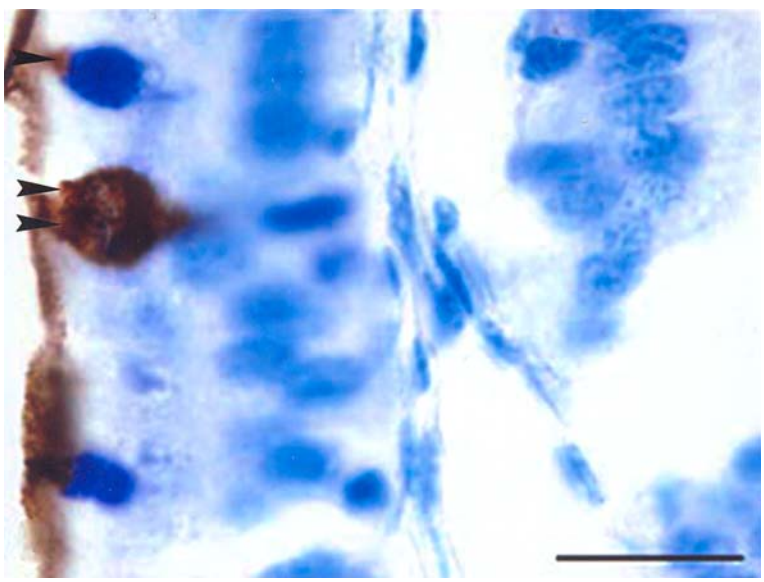

Fig. 3 First day of exposure. Note the high density of AMG grains in special type cells in small intestine (double arrow). The goblet cells (single arrow) are charged with toluidine blue-positive material. The AMG grains form a thick black line in the glycocalyx

of exposure. In the 6 days exposed animals, a constant finding was a rather high concentration of grains in the connective tissue exactly below the basement membrane of loaded epithelial cells.

Large intestine

Whether 1 or 6 days of exposure, the highest concentrations of grains were observed in the apical part of the absorptive cells of the large intestine (Fig. 4). The endothelial cells of the vessels likewise had a high content of mercury–sulphur nanocrystal caused AMG grains. As in the small intestine, high concentrations of AMG grains were observed scattered in the connective tissue below the basement membrane (Fig. 5). A narrow line above the apical part of the absorptive cells, the zone of microvilli, was void of AMG grains (Fig. 5).

Macrophages in the submucosa of both the small and the large intestine were loaded with mercury–sulphur quantum dots.

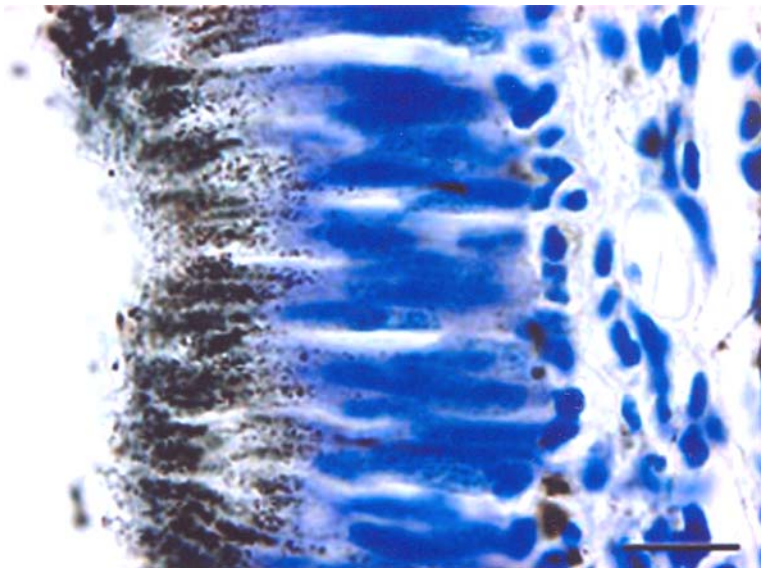

Fig. 4 Large intestine. Very high concentration of AMG grains in the apical part of absorptive cells

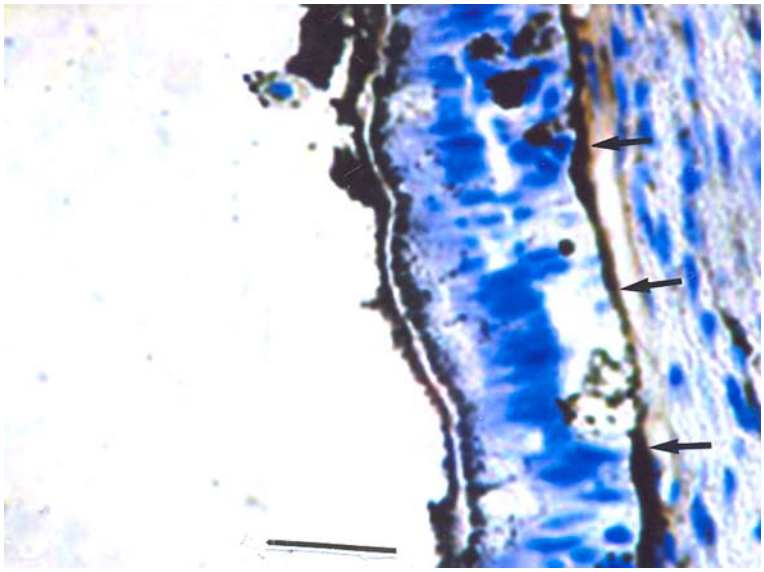

Fig. 5 Large intestine. A thick black line under the basement membrane (arrows). Note also a narrow zone over the glycocalyx devoid of AMG grains

The lumen of both small and large intestines revealed AMG staining of their content. The silver enhanced Hg–S nanocrystals were also found free in the lumen of small and large intestine and a number of red blood cells were spread into the lumen (Fig. 6).

Discussion

We have not been able to find studies that demonstrate the cellular localization of mercury in kidney and intestines of mercury exposed amphibians. AMG technique is extremely specific and sensitive if the recipe is kept to the letter (Danscher and Stoltenberg 2006). However, the technique does not provide information about the whole pool of mercury in the organism as it can reveal only mercury bound in Hg–S nanocrystals (quantum dots). The amount of mercury present in rat kidney as quantum dots has been estimated to be 30% of the total mercury (Norgaard et al. 1991) however a recent study on bismuth intoxication, a metal that is treated in many ways comparable to mercury in the organisms, suggest the real percent to be much higher (submitted).

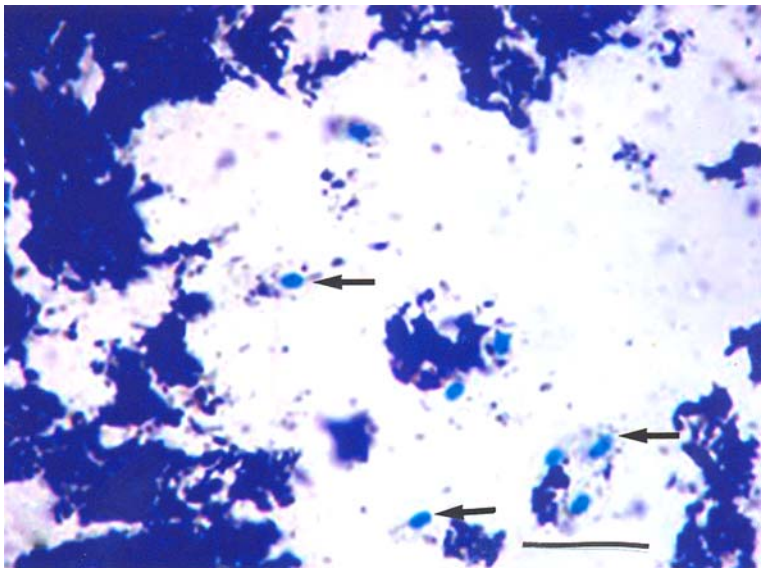

Fig. 6 Large numbers of red blood cells (arrows) spreading along the intestinal lumen. The black masses represent AMG grains mixed with feces

According to Zalups (2000), the kidney is the primary target organ for mercury. In mammals the accumulation of mercury in the kidneys is very rapid, with as much as 50% of a dose appearing there within a few hours after exposure. Similarly, Allen (1994) found that the kidney of the fish *Oreochromis aureus* also appeared to be the target tissue for mercury during acute exposures, since the kidney accumulated the highest concentrations of the mercury after 12 h of exposure.

Our study suggests that the kidney is essential in the detoxification process of *Rana ridibunda* as well, since no obvious difference in the content of AMG grains in the kidneys were seen between animals exposed for 1 or 6 days, i.e. the release of Hg–S nanocrystals in the kidney is very fast.

Our study also reveal that mercury accumulates in the lysosomes of the proximal tubules of the frog exactly as observed in the kidney of mammals (Norgaad et al. 1989; Norgaad et al. 1991) and fish (Allen 1994).

Mercuric chloride, in small to moderate doses is primarily nephrotoxic to humans and animals (Zalups and Lash 1994). In small doses, the S_3 (pars recta) segment in the cortico-medulary area is the primary target site (Magos et al. 1984;Danscher 1991; Stachiotti et al. 2003). As the dose of mercury is increased, the injury spreads to involve the S_1 and S_2 (pars convoluta) segments of the proximal tubules (Woshner et al. 2002). In whales with a low tissue Hg concentration, AMG granules reflecting presence of toxic mercury ions occurred in the cortical tubular epithelium. AMG granule deposition among whales with higher tissue Hg concentration (generally older animals) tends to extend to the epithelium of the medullary tubules and collecting ducts (Woschner et al. 2002). In old arctic sledgedogs, AMG detectable mercury quantum dots was found in the cells of the entire nephritic tubules (Hansen and Danscher 1995).

With regard to frogs, in which the architecture of the kidneys is radically different from that of mammals, there is no former histochemical data concerning the distribution of mercury. But in the time intervals that we analyzed we found nearly all the AMG grains located in the PCT. We also observed that the levels of AMG grains differed along the entire length of the proximal tubules. This uneven grain distribution may show a resemblance to the S_1, S_2 and S_3 segments of mammalian kidneys; however, more detailed histological, physiological and biochemical studies are needed to verify this hypothesis.

The AMG granules indicating the presence of Hg in the proximal tubular epithelial cells probably occur within lysosomes (Norgaard et al. 1991; Woshner et al. 2002). This lysosomal accumulation of Hg might result from autophagy of cytoplasmic Hg bound to endogenous ligands, such as metallothionein (Madsen and Hansen 1980) or from endocytosis of mercury ions bound to membrane surface molecules.

Both sources will then be metabolized in the lysosomes and finally be included in Hg–S or Hg–Se nanocrystals. This is the most likely way that the organisms have learned to detoxify mercury. As the epithelial cells are later, in mammals within 6 days, rejected into the preurin whereafter the nanocrystal bound mercury will be expelled through the urine.

Accumulation of mercury in the endothelial cells is also a common phenomenon, repeatedly observed in mammals (Hansen and Danscher 1995) and has been observed as well in the endothelial cells of the liver of frog *Rana ridibunda* (Loumbourdis and Danscher 2003). Likewise mercury grains were detected in the Kupffer cells of the frog *Rana ridibunda* (Loumbourdis and Danscher 2003) and as noted by Christensen (1996), "since mercury is accumulated in cultured macrophages and has adverse effects on the functional integrity of these cells, the presence of mercury in macrophages after in vivo treatment indicates that macrophages situated in tissues may be a critical target for tissue intoxication, leading to possible impairment of host defense".

Mercury is rarely found in the distal tubules, a situation reported in other animals (Norgaard et al. 1991). However, in belugas whales, AMG granules reflecting Hg deposition were observed in the uriniferous tubule along its whole length and in the collecting ducts (Woshner et al. 2002).

If it is true that inorganic mercury i.e. mercury ions do not easily permeate through the intestinal barrier, it is difficult to explain the high concentration of mercury present in the intestinal epithelial cells. One explanation is that given for the cadmium (Vogiatzis and Loumbourdis 1998) and copper (Papadimitriou and Loumbourdis 2003) which were detected in the gut epithelium of frogs. The high concentrations of these heavy metals was followed by high levels of metallothioneins, believed to act as a barrier against the entrance of heavy metals to the body.

As is the case with cadmium and copper, mercury can induce metallothionein synthesis in various organs, especially in the gut, the liver and the kidneys. Metallothioneins have a high cysteine content, and hence may be similar to metal chelators in providing heavy metal tolerance and in regulating Hg distribution and retention (Klaasen and Liu 1998; Yoshida et al. 1999).

It has been assumed by many researchers that the primary mechanism by which mercury enters into the gastrointestinal tract following parenteral administration of mercuric compounds, is by biliary secretion of mercury, which is bound to thiol clusters such as glutathione (O'Flaherty 1998; Bridges and Zalups 2005). Part of this Hg is reabsorbed by the gut epithelium and this absorbed mercury may be the first pool of mercury detected in the gut epithelium by the AMG method. Data from in vivo studies in rats with cannulated or ligated (Zalups 1998c) bile ducts indicated that intestinal release of Hg^{2+} from the blood into the lumen of the intestine through the intestinal epithelial cells also accounts for a substantial fraction of the total pool of Hg^{2+} that is excreted in the feces through the intestinal epithelium. This may be a second pool of mercury, detected in the gut epithelium by the AMG method.

In the stomach, the zonula occludens forms a tight junction, which comprises a continuous barrier around the cells, making the transport of metals difficult. This might be the reason for the very low concentration of mercury grains observed in the epithelium of the stomach.

At the moment we can no offer any explanation on the goblet-like cells loaded with mercury grains. Most probably it will be an object of future research.

Fecal excretion of mercury is one of the predominant means by which mercury is eliminated from the body (Zalups and Lash 1994; Tan and Perkin 2000) and, the AMG stained masses observed in the lumen of the gastrointestinal tract could be caused by mercury–sulphur nanocrystals. However in order to make this conclusion the contents have to be analyzed by a multi element approach.

It is known that, among other effects, mercury ingestion leads to gastrointestinal ulceration and hemorrhage (Eley 1997; Hultman et al. 1998). The large numbers of red blood cells observed in the gut lumen may indicate such a hemorrhage caused by the rupture of small blood vessels as a result of exposure to mercury in the frogs under study.

As with previous studies (Vogiatzis and Loumbourdis 1998; Papadimitriou and Loumbourdis 2003; Loumbourdis and Danscher 2003), this study also verifies that the frog *Rana ridibunda* is an effective biomarker of pollution by heavy metals and it could be used as such.

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