Copper in *Helix pomatia* (Gastropoda) is regulated by one single cell type: differently responsive metal pools in rhogocytes

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Copper (Cu) is an essential trace element required by all organisms as a vital constituent of cofactors, enzymes, and proteins. At the same time, Cu also is involved in toxicological interactions with cellular components, mainly originating from its ability to produce toxic hydroxyl radicals in Fenton-like reactions (52). Probably, as a consequence of this double biological role, intracellular and tissue concentrations of Cu are homeostatically regulated in many animal species (61), including mammals (60), fish (10), arthropods (43), and mollusks (57).

Terrestrial pulmonates have to match their Cu demands by metal uptake from the soil or from plants on which they feed (27). They need elevated amounts of Cu specifically as a constituent of hemocyanin, their oxygen-carrying respiratory protein (33). On the other hand, terrestrial snails may suffer from elevated Cu concentrations in their environment due to environmental pollution (19). Cu-sulfate, for example, is often used as a snail poison against certain pulmonate species that are considered pest organisms in horticulture (26). Despite the fact, however, that terrestrial pulmonates can take up Cu from their diet with a relatively high efficiency, they are able to maintain Cu concentrations in their tissues within a moderate range of concentration (16), even if subjected to exceptionally high metal loads through their food. In addition to this, they can quickly release the metal after the end of an exposure event (12). An important role in the process of Cu regulation in pulmonates has been attributed to a Cu-specific metallothionein (MT) isoform that was first isolated from the mantle tissue of the Roman snail, *Helix pomatia* (3). By virtue of the sulfur atoms of its Cys residues, the Cu-binding isoform can normally carry up to 12 Cu⁺ ions, which under reducing conditions, are tightly bound by the protein (25). The metabolic role of this isoform has been linked to the regulation of Cu in connection with the synthesis and metabolism of hemocyanin (13, 15, 16). The respiratory protein is apparently synthesized in specific cells called rhogocytes or pore cells (53, 54), which occur only in mollusks (29). The presence of hemocyanin molecules was recently demonstrated by electron microscopy in rhogocytes of the marine gastropod *Haliotis tuberculata* (1). We speculated that rhogocytes, apart from hemocyanin, may also harbor the Cu-specific MT isoform, suggested to be involved in the synthesis of the respiratory protein by acting as a donor of Cu⁺ ions for the nascent hemocyanin molecule (16). The presence of Cu-MT mRNA in rhogocytes of *H. pomatia* supports this hypothesis (11). Apart from hemocyanin synthesis, the function of rhogocytes also has been related to Cu metabolism and detoxification (37, 53), but the relationships among these different metabolic functions and pathways for Cu (hemocyanin metabolism and Cu detoxification) within one cell type have so far been speculative.

The present study was carried out to shed light on the dynamic aspects of Cu regulation in terrestrial pulmonates under physiological and superphysiological conditions of metal exposure, focusing on tissue-specific as well as cellular levels of organization. Particular attention was paid to the role of rhogocytes in Cu accumulation and Cu-MT mRNA expression. We have shown that on a tissue-specific level, the highly oxidizable Cu-specific MT isoform is present not only in the...
mantle of *H. pomatia* but also in the midgut gland, foot, and other important tissues. However, on a cellular basis, the Cu-binding MT isoform is exclusively expressed in rhogocytes that are scattered throughout all major snail tissues. These cells also are the exclusive storage sites of Cu in a granular form, which forms a separate and distinct Cu compartment from the Cu fraction present in the Cu-MT pool. The two Cu storage forms (Cu-MT-bound and granular) exhibit opposite dynamic patterns by which the Cu-MT pool remains stable, whereas the granular Cu pool can rapidly fluctuate in response to external metal supplies.

**MATERIALS AND METHODS**

**Animals and Rearing Conditions**

Roman snails (*H. pomatia* L.) were obtained from a commercial dealer (Exoterra, Dillingen, Germany) and kept in large plastic boxes on a substrate of moistened garden soil supplemented with CaCO₃ at 18°C with a 12:12-h light-dark photoperiod regimen and a humidity of ~80%. Snails were fed with fresh lettuce (*Lactuca sativa*), carrots, and potato slices three times per week. For Cu exposure, acclimatized animals of a distinct size class (18–24 g fresh weight) were transferred to plastic boxes (14 × 8 × 6.5 cm) with perforated lids and kept on a Cu-enriched diet as described below.

**Cu Exposure**

Two groups of six snails each (*groups A and B*) and one group of 20 snails (*group C*) were fed a Cu-enriched diet over a period of 15 days. The diet consisted of lettuce leaves that had been soaked for 1 h in a Cu-containing solution (CuCl₂) with a concentration of 10 mg Cu/l, prepared by dilution with distilled water from a purchasable Cu pool present in the Cu-MT pool. The two Cu storage forms (Cu-MT-bound and granular) exhibit opposite dynamic patterns by which the Cu-MT pool remains stable, whereas the granular Cu pool can rapidly fluctuate in response to external metal supplies.

**Sample Processing for Cu Analysis, Tissue Fractionation, and Cu-MT Quantification**

Five animals each from one of the Cu exposure groups (*group C*) and the control group (*D*) were dissected and processed for Cu-MT quantification. For an additional 15 animals from *groups C* and *D*, the main organs and tissues were dissected; for each of the large organs (midgut gland, mantle, and foot) tissue was split into five subsamples comprising the pooled material of three snails each, and for each of the small tissues (gut and remaining soft tissues), tissue portions were split into three subsamples containing the pooled tissue material of five snails each. One subsample of pooled mantle tissue was purified immediately after dissection and analyzed by electrospray ionization-mass spectrometry (ESI-MS) within 1 day, to prevent oxidation. The remaining tissue subsamples were stored at −70°C until centrifugation, tissue fractionation, and chromatographic separation were performed.

**Tissue Preparation for Histochemistry and In Situ Hybridization of Cu-MT**

Six snails each from one of the Cu exposure groups (*group A*) and the control group (*D*) were dissected, and organ aliquots (midgut gland, mantle, foot, and gut) were removed for different applications. One aliquot of each organ, consisting of a small tissue piece (5–10 mm³), was dissected on ice and processed for in situ hybridization (ISH), histochemical Cu precipitation, and histological observation, as described below. Additional small sample aliquots (1–3 mm³ each) of midgut gland, mantle, and foot tissues were prepared for electron microscopy. Additional tissue aliquots of snails from each treatment group (*groups A, B, and C*) were processed for wet digestion and metal analysis.

**Histology, Histochemistry, and Cu-MT Quantification**

**Tissue processing.** Tissues for ISH and histochemistry (Cu precipitation) were fixed in 4% paraformaldehyde-phosphate-buffered saline (PBS); tissues for other histological applications were fixed in Bouin’s fixative at 4°C overnight. After ethanol dehydration, tissue pieces were embedded in paraffin at 62°C. Sections were cut with a thickness of 5 μm on a precision microtome (Reichert-Jung 2040; Leica Microsystems, Wetzlar, Germany).

In situ hybridization. Digoxigenin-11-UTP-labeled sense and antisense RNA probes for ISH of Cu-MT mRNA were obtained after RNA preparation (2 μg), cDNA synthesis, molecular cloning, and RNA transcription, according to a protocol described by Chabicovsky et al. (11), based on the sequence of the coding region of Cu-MT from *H. pomatia* published in GenBank (accession no. AF399741). For ISH of Cu-MT mRNA, paraformaldehyde-PBS-fixed paraffin sections were rehydrated and incubated two times for 15 min in PBS containing 0.1% active diethyl pyrocarbonate, equilibrated for 15 min in 5× standard saline citrate (SSC; 0.75 mM NaCl, 0.075 mM Na-citrate). Prehybridization was performed for 2 h in the hybridization mix (50% formamide, 5× SSC, and 40 μg/ml salmon sperm DNA) at 53°C. After denaturation at 80°C for 8 min, probes were added to the hybridization mix (300 ng/ml digoxigenin-labeled antisense or sense transcripts). Hybridization was achieved overnight by incubation at 53°C. Hybridized sections were washed for 30 min in 2× SSC at room temperature, followed by incubation for 1 h in 2× SSC and for 1 h at 57°C in 0.1× SSC (equilibrated for 5 min in 100 mM Tris and 150 mM NaCl, pH 7.5). Sections were then incubated for 2 h with diluted anti-digoxigenin-alkaline phosphatase antibodies (1:500; Roche Diagnostics, Mannheim, Germany) at room temperature, and subsequently blocked with 0.5% blocking reagent (Roche Diagnostics). Section staining was performed for 7 h, according to the manufacturer’s recommendation by means of the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride color reaction (Roche Diagnostics). Counterstaining was achieved with methyl green according to the method of Braissant and Wahl (6). Control sections (exposed to either hybridization antisense or sense probes) were treated and incubated in the same way as samples but without the antibody. All sections were mounted with Entellan (Merck).

**Cu precipitation.** Cu precipitation was performed according to the method applied by Okamoto and Utamura (44) and Howell (30), as described by Romeis (50). Tissue sections were rehydrated and incubated by slight shaking at 37°C overnight in 5-(4-dimethylaminobenzylidene)rhodanine (Sigma, St. Louis, MO). After being washed in distilled water, sections were counterstained with haemalaun, dehydrated, and mounted with Entellan (Merck). Serial sections subjected to either ISH or Cu-specific staining were used for checking possible colocalization of Cu-MT mRNA and granular Cu deposits.

Tissue staining for histology. Additional tissue sections for histologic and routine observations were stained with hematoxylin and eosin or by the mallory-azan method according to Romeis (50). Stained sections were mounted with Entellan (Merck).

Light microscopy and photography. Tissue sections were studied with a light microscope (Reichert-Jung Polysysrav, Leica Microsystems) with ×25, ×40, and ×100 objectives. Photographs were taken with a digital Penguin 600 CL camera (Pixera, Los Gatos, CA).

**Rhogocyte quantification.** Quantification of rhogocytes with Cu-MT mRNA expression or granular Cu precipitations from control and Cu-exposed snails was achieved by screening four paraffin sections of
the main tissues (mantle, gut, midgut gland, and foot) per snail from at least four animals per group. A total number of 800–1,000 cells were counted per section. Data are expressed as percent fractions of rhogocytes with a positive signal for each organ, with means and standard deviations of four animals per treatment group.

Electron Microscopy

For electron microscopy, small sample pieces (1–3 mm³) of tissues (midgut gland, mantle, and foot) from control and Cu-exposed snails were fixed with 2.5% glutaraldehyde in 0.02 M phosphate buffer (pH 7.4) at room temperature overnight. After being rinsed in 0.02 M phosphate buffer, tissues were postfixed for 2 h in 1% osmium tetroxide in 0.02 M phosphate buffer and subsequently rinsed three times for 30 min with 0.02 M phosphate buffer. After one additional rinsing, tissues were decalcified in 0.8% sodium chloride and 1% ascorbic acid overnight (22). Dehydrated tissues were embedded in Durcupan resin (Fluka, Ronkonkoma, NY). Ultrathin sections (3–5 μm) were obtained on an Ultramicrotome (Reichert Ularutac E, Leica Microsystems) and stained with 0.8% uranyl acetate for 15 min and 2% Pb-citrate for 5 min in a CO₂-free atmosphere (48). Sections were inspected by transmission electron microscopy (TEM 902; Carl Zeiss, Jena, Germany) at 80 kV.

Quantification of Cu Ratios in Homogenate Fractions and Fractionation of Cu-MT by Chromatography and HPLC

Pooled tissue subsamples were thawed and homogenized in a threefold volume of 25 mM Tris-HCl buffer (pH 7.5) containing 20 mM 2-mercaptoethanol and 0.1 mM freshly prepared phenylmethylsulfonyl fluoride. Small homogenate aliquots (2 ml) were removed and processed for Cu analysis, and the remaining homogenate was subjected to centrifugation at 27,000 g. Small aliquots of the resulting supernatant (2 ml) and pellet (100–300 mg dry wt) were removed and used for Cu quantification in homogenate fractions. The remaining supernatants from midgut gland, gut, mantle, and foot subsamples were purified step by step by gel permeation, ion exchange chromatography, ultrafiltration, and reverse-phase HPLC, as described by Berger et al. (3), but with a slightly modified HPLC gradient over a fractionation time of 35 min instead of 30 min. After each purification step, Cu concentrations in eluate fractions were measured, and Cu-MT-containing fractions were pooled and processed for the subsequent step of purification until the Cu-MT yielded a distinct and homogeneous peak upon reverse-phase HPLC.

One mantle tissue aliquot from uncontaminated snails was processed for chromatographic purification immediately after dissection by applying exactly the same fractionation conditions as described by Berger et al. (3). The final eluate containing the Cu-MT was used for ESI-MS characterization.

Cu-MT Characterization by ESI-MS

To avoid disintegration of the oxidation-sensitive Cu-MT, the entire fractionation procedure and subsequent analysis by ESI-MS was accomplished within 1 day. Sample preparation for ESI-MS was achieved by additional fractionation of the purified holo-MT by reverse-phase HPLC in a solution of volatile ammonium acetate (25 mM, pH 6.5) with 10–15% acetonitrile and by injecting 5-μl aliquots of the eluate directly into the source of the ESI mass spectrometer (API III* triple quadrupole; PE Sciex, Concord, Ontario, Canada), as described by Gehrig et al. (25). After the first ESI-MS characterization, the purified holo-MT sample was stored under normal oxidizing air conditions at 4°C over a period of 14 days and then again subjected to ESI-MS analysis to study the influence of oxidation on protein structure and composition.

Cu-MT Quantification

For Cu-MT quantification, a modified metal saturation protocol (32) was adopted in which Cu-MT was quantified after Cu was removed from Cu-MT by means of ammonium tetrathiomolybdate (TTM) and the resulting apo-MT was saturated with Cd, as described by Dallinger et al. (18). Briefly, fresh or thawed tissue aliquots were homogenized, and non-MT proteins in the samples were denatured by addition of acetonitrile. The Cu-MT was then stripped from Cu by addition of the chelating agent TTM, and the remaining apo-MT was saturated with Cd. Molar concentrations of Cu-MT were deduced from the Cd concentrations measured in the samples by relating the molar Cd fractions to the equivalent molar fractions of Cu-MT, based on the known stoichiometric Cd ratio in snail MT of 6:1 (17, 18).

Sample Digestion and Metal Quantification

Aliquots of tissues dissected for Cu analysis were oven-dried at 60°C. After addition of 2 ml of a mixture of nitric acid (supperure; Merck) and distilled water (1:1), samples were wet-digested in screw-capped polypropylene tubes (Greiner, Kremsmünster, Austria) on a heated aluminum block at 70°C until the remaining solution was clear. Complete oxidation was accomplished by adding a few drops of H₂O₂ to the heated samples. Foot tissue samples were digested once or twice in screw-capped polypropylene tubes placed on the turntable of a microwave oven (model MLS 1200; Mikrowellen-Labor-Systeme, Leutkirch im Allgäu, Germany) at 250 W for 20 min, until the remaining solution was clear. Pellet fractions derived from centrifugation were oven-dried at 60°C and weighed. Dried pellet, homogenate, and supernatant samples were transferred to screw-capped polypropylene tubes and wet-digested in a microwave oven (model MLS 1200; Mikrowellen-Labor-Systeme) under the same conditions as described above. All digested samples were diluted with distilled water to a final volume of 10 ml and analyzed by flame atomic absorption spectrophotometry (model 2380; Perkin Elmer, Boston, MA). The instrument was calibrated with properly diluted Titrisol Cu standard solutions (Merck) containing 5% nitric acid. Measurement accuracy was checked by means of certified lobster hepatopancreas standard reference material (TORT 1, National Research Council, Ottawa, Ontario, Canada) treated in the same manner as tissue samples. Cu concentrations of standard reference material were within the range of acceptable variability (±15%) for the Cu value certified by the supplier. Cu concentrations of chromatography and HPLC eluate fractions were measured by direct aspiration of fractions into the flame of an atomic absorption spectrophotometer (model 2380, Perkin Elmer).

Cd determination in samples from the metal saturation assay for Cu-MT quantification was accomplished after wet digestion of sample aliquots with 1 ml of nitric acid (supperure; Merck) at 70°C. Digested solutions were diluted with distilled water and analyzed with either a flame atomic absorption spectrophotometer with deuterium background correction (model 2380; Perkin Elmer) or a graphite furnace instrument with Zeeman background correction (Hitachi Z-8200; Hitachi Europe, Maidenhead, UK) equipped with an autosampler, using Pd(NO₃)₂ as a matrix modifier.

Statistics

For statistical analysis, the software packages Statistica (version 6.0; StatSoft, Tulsa, OK) and SigmaStat (version 2.0; Jandel, London, UK) were used. All values were checked for normal distribution before further analysis. Metal and Cu-MT tissue concentrations of control and metal-treated snails (means and SD) were compared using two-tailed unpaired t-test. Percentage values of homogenate Cu fractions, as well as percentage proportions of rhogocytes with histochemical reaction products among control and Cu-treated animals, were compared using the Whitney rank sum test. Differences at P ≤ 0.05 were considered significant.
RESULTS

Tissue Concentrations and Subcellular Distribution of Cu and Cu-MT

Cu concentrations in organs and tissues of uncontaminated and Cu-exposed *H. pomatia* snails are shown in Fig. 1A. Cu concentrations in all important organs of Cu-treated snails were significantly elevated compared with control values, with the highest concentrations observed in the digestive tissues and the mantle.

The percent distribution of Cu in tissues of metal-exposed snails was shifted from supernatant toward pellet fractions compared with the situation in control snails (Fig. 1B). Although not always statistically significant, this shifting seems to reflect a consistent trend in all organs considered in the present study, indicating an increasing association of Cu with cellular vesicles. At the same time, Cu-MT concentrations detected in the organs of Cu-treated snails remained unaffected compared with the respective tissue levels of control snails (Fig. 1C).

HPLC Elution Patterns and Identification of Cu-MT in Single Organs

Upon fractionation of supernatants from the main snail tissues (midgut gland, mantle, gut, and foot) by gel permeation chromatography, the only detectable Cu-carrying component in the molecular mass range between 15,000 and 5,000 Da eluted in fractions subsequently identified as Cu-MT. After purification of these fractions upon HPLC, the Cu-MT eluted as a distinct, homogeneous peak. The kind of treatment of animals before fractionation (feeding on Cu-enriched or uncontaminated diet) had no influence on either the shape and size or the retention time and profile pattern of the eluted peaks. Figure 2 shows typical elution profiles of Cu-MT from the midgut gland (Fig. 2A), mantle (Fig. 2B) and foot (Fig. 2C) of uncontaminated snails. The small, Cd-containing peak eluting before the Cu-MT fractions in the HPLC profile of midgut gland (Fig. 2A) was identified, by virtue of its elution characteristics compared with our previously performed work, as Cd-MT (13). Its presence is apparently restricted to the midgut gland (14).

The identification of Cu-MT in Cu-containing HPLC peaks was based, apart from Cu analysis, on their retention times and characteristic elution profiles, as well as on amino acid sequence analysis of samples from identical peaks characterized previously (3), and on an ESI-MS spectrum performed in the present study on one of these peaks derived from the mantle of uncontaminated snails (Fig. 3). The MS spectrum of the freshly obtained sample purified within 1 day (see above) shows a prevailing relative intensity peak with a relative mass of 6,997.0 Da for the predominant constituent (Fig. 3A). As demonstrated previously (25), this compound represents the intact Cu-specific holo-MT isoform loaded with a total number of 12 monovalent Cu ions, corresponding with the expected binding stoichiometry of this peptide. Because of the high susceptibility of this isoform to oxidizing conditions, the spectrum of the same sample recorded 14 days after its purification (Fig. 3B) shows a mixture of peptides with many different relative masses, indicating the progressive disintegration of the
Cu-MT isoform and destabilization of its structure as a result of Cu oxidation during storage under normal aerobic conditions.

Visualization of Cu-MT mRNA in Snail Rhogocytes

Figure 4 shows different views of Cu-MT mRNA in rhogocytes visualized by ISH of different tissue sections from uncontaminated snails. In all samples examined, rhogocytes were exclusively identified as the cellular sites exhibiting a positive Cu-MT mRNA signal. The signal could be detected in rhogocytes of the mantle (Fig. 4A), midgut gland (Fig. 4B), gut (Fig. 4C), and foot (Fig. 4D). Rhogocytes with positive Cu-MT mRNA signals were repeatedly found in the close neighborhood of blood vessels (Fig. 4, B and C). Upon comparative inspection of serial tissue sections, both the intensity and frequency of Cu-MT mRNA precipitations in a given organ did not differ among the treatment groups (control or Cu exposed).

Localization of Cu Precipitations in Rhogocytes of Cu-Exposed Snails

Upon feeding of snails with Cu, rhogocytes apparently also become important storage sites for those fractions of the metal that enter the animal’s body because of elevated exposure. This is proven by the fact that specific Cu precipitations were nearly exclusively observed in rhogocytes of organs from Cu-treated snails (Fig. 5), appearing as granular concretions in the snail mantle (Figs. 5A), midgut gland (Fig. 5B), gut (Fig. 5C), and foot (Fig. 5D). Rhogocytes with positive Cu-MT mRNA signals were repeatedly found in the close neighborhood of blood vessels (Fig. 5, B and C). Upon comparative inspection of serial tissue sections, both the intensity and frequency of Cu-MT mRNA precipitations in a given organ did not differ among the treatment groups (control or Cu exposed).

Fig. 2. Reverse-phase HPLC elution profiles of Cu-MT from midgut gland (A), mantle (B), and foot (C) of uncontaminated individual snails of H. pomatia, with absorption peaks at 254 nm (A254), elution gradients (%B), and metal concentrations (Cu and Cd) plotted against retention time. Cd concentrations in samples from the mantle (B) and foot (C) were below detection limits.

Fig. 3. Deconvoluted electrospray ionization mass spectra of Cu-MT from the mantle tissue of H. pomatia recorded immediately after purification (A) and after 14 days of storage at −20°C (B). Assignment of the signals in spectrum in A: 6,932.9 Da, Cu11MT; 6,997.0 Da, Cu12MT; 7,060.9 Da, Cu13MT; 7,123.6 Da, Cu14MT. Assignment of the signals in spectrum in B: 6,228.9 Da, apo-MT; 6,292.7 Da, Cu1MT; 6,865.6 Da, Cu10MT; 6,928.7 Da, Cu11MT; 6,995.1 Da, Cu12MT; 7,057.5 Da, Cu13MT; 7,119.9 Da, Cu14MT; 7,182.3 Da, Cu15MT; 7,242.9 Da, Cu16MT.
Cu precipitations) could be detected in any of the rhogocytes observed throughout the sections.

**Ultrastructural Alterations of Rhogocytes Upon Cu Exposure**

With electron microscopy, rhogocytes typically appear as large cells (up to 30 μm long) scattered singly throughout the tissue or aggregated to small cell accumulations often situated around blood vessels or blood sinus (Fig. 6A). In the midgut gland, rhogocytes often can be observed in the close neighborhood of digestive, excretory, or calcium cells (Fig. 6B). In control animals, the rhogocyte cytoplasm and nucleus appear to be entirely crowded toward the cell edge by a large central vacuole exhibiting a somewhat granular internal texture (Fig. 6, A and B). A dramatic change of this general appearance occurs upon exposure of snails to Cu. As shown in Fig. 6, C and D, the large central vacuole of the rhogocytes becomes condensed and splits into several smaller vacuoles. Moreover, granular vesicles with electron-dense contents appear to be formed in the cytoplasm toward the cell periphery, with a clear tendency of granule frequency increasing with rising intensity of Cu exposure. As shown elsewhere, these granular structures are filled with debris and metal ions (especially Cu) that accumulate in these structures upon metal exposure (53).

**Quantitative Evaluation of Cellular Cu Compartments**

As demonstrated by quantitative evaluation on a cellular basis (Fig. 7), there were striking differences between the numbers of rhogocytes showing positive reaction products (Cu-MT mRNA and Cu precipitations) depending on the kind of treatment of the snails (Cu exposed or not). As shown in Fig. 7A, the Cu-MT mRNA signal was observed only in a certain proportion of the rhogocyte populations present in a given tissue, with tissue-specific quantitative differences characterizing the mantle as the organ with the highest proportion of rhogocytes exhibiting a positive reaction product (~20%), followed in decreasing order by the gut (~19%), midgut gland (~17%), and foot (~15%). At the same time, no significant differences, in terms of frequency of the observed reaction product, were found between control and Cu-exposed individuals in any of the organs examined.

Figure 7B shows that only a certain proportion of rhogocytes exhibited a positive reaction product upon Cu precipitation with the complexing dye 5-(4-dimethylaminobenzylidene)rohdanine. In contrast to the situation shown for Cu-MT mRNA (see above), however, their number increased dramatically in all tissues examined after exposure to Cu.

**DISCUSSION**

**Accumulation and Regulation of Cu at a Tissue-Specific Level**

Tissue concentrations of Cu appear to be subjected to homeostatic regulation in many animal species (4, 28, 57, 59). In terrestrial pulmonates, for example, Cu tissue levels of individuals from nonpolluted soil habitats are in the range from $10^{-4}$ to $10^{-3}$ mol/kg dry mass, whereas the respective concentrations in animals from polluted areas range from about $10^{-3}$ to $10^{-2}$ mol/kg dry mass (2, 19, 20, 27). This means that in tissues of individuals from polluted sites, Cu is biologically concentrated about 10 times with respect to the tissue levels of animals from nonpolluted habitats. As also shown by the results of feeding experiments in the present study, Cu concentrations in organs of Cu-exposed snails increased only moderately compared with those of control animals, despite the very high metal concentrations in the Cu-enriched food offered.
to the animals (see Fig. 1A). These data are consistent with the concept that *H. pomatia* and some of its helicid relatives are able to maintain Cu concentrations in their organs within a moderate range (16), even under conditions of increased Cu availability in the environment. In contrast, pulmonate snails from metal-contaminated sites accumulate the nonessential Cd about 100–1,000 times above Cd levels detected in animals from nonpolluted areas (2, 19, 20, 47). Irrespective of environmental exposure, an important fraction of Cu in snail tissues is always bound to a Cu-specific MT isoform (3). This isoform can be detected in all important organs of *H. pomatia*, as demonstrated by the tissue-specific HPLC elution profiles in the present study (Fig. 2). Its function has been related to the homeostatic regulation of Cu in connection with the biosynthesis of hemocyanin, the Cu-bearing respiratory protein present in the hemolymph of many mollusk species (3, 13, 15). A Cu-specific MT isoform involved in the synthesis and degradation of hemocyanin also was reported in marine crustaceans (8, 58). It was suggested that during the metabolic interaction with hemocyanin, the Cu-MT molecule of pulmonates may serve as a donator and acceptor of Cu$^+$ ions (16), which apparently are bound to the MT peptide in their monovalent state, at least under the reducing conditions encountered within the cell in vivo (see Fig. 3). If this assumption is true, one might expect that concentrations of both the Cu-MT and the MT-related Cu$^+$ pool should depend on the intrinsic processes governing the turnover of hemocyanin, rather than on being subjected to extrinsic stimuli due to excess amounts of Cu entering the animal tissues upon metal exposure. The present study supports this hypothesis and shows that Cu-MT concentrations in tissues of *H. pomatia* are indeed not affected by an increased uptake of Cu but remain constant in all organs, irrespective of the elevated Cu concentrations due to exposure (compare Fig. 1, A and C). The shifting of Cu in homogenate fractions from the supernatant toward the pellet indicates that under these conditions, the metal is diverted into a separate pool that is apparently associated with cellular vesicle fractions (Fig. 1B).

**Rhogocytes (Pore Cells) as Turntables of Cell-Specific Cu Regulation**

Rhogocytes are specialized cells representing modified connective tissue cells of mesodermal origin that occur in the molluscan primary body cavity, either free in the hemocoel or embedded in the connective tissue in virtually all molluscan organs (29, 36). Their involvement in hemocyanin synthesis and metal ion homeostasis has been suggested for a long time.

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**Fig. 5.** Cu precipitations with 5-(4-dimethylaminobenzylidene)rhodanine in tissue sections of different organs from uncontaminated and Cu-exposed snails. A: mantle tissue section of a Cu-exposed animal with granular precipitations of Cu (arrow) at the cell periphery of a rhogocyte. B: midgut gland tissue section of a Cu-exposed snail with granular Cu precipitations (arrow) at the cell periphery of a rhogocyte. C: gut (midgut) section of a Cu-exposed snail with granular precipitations of Cu near the cell periphery and the nucleus (arrows) in rhogocytes. D: gut (midgut) section of an uncontaminated snail with rhogocytes (Rh) devoid of reaction products. E: foot tissue section of a Cu-exposed animal with granular Cu precipitations (arrows) in rhogocytes embedded in the connective tissue. F: foot tissue section of an uncontaminated snail with rhogocytes devoid of reaction products.
Their importance in Cu metabolism and regulation also is supported by the fact that they contain the Cu-MT isoform (11). As shown by ISH, Cu-MT mRNA was exclusively localized in rhogocytes (Fig. 5). A particularly high frequency of these cells was observed in the mantle roof in close association with the dense network of blood vessels. This may explain why the mantle is the organ that nearly always yields the highest amounts of Cu-MT upon protein quantification or chromatographic fractionation (Figs. 1C and 2B). On a cellular basis, the proportion of rhogocytes with Cu-MT mRNA precipitations was rather low throughout all tissues (15–20%), irrespective of whether the animals had been exposed to Cu or not (Fig. 7A).

In the present study, rhogocytes also were the only sites where Cu precipitations of a granular appearance were observed upon histochemical staining. These granules consist of roundish membrane-surrounded structures with electron-dense contents and with an average size of 1–4 μm in diameter. The observation that Cu may occur in rhogocytes of gastropods within granular structures also has been made by other authors (37, 41, 53). In contrast to Cu-MT mRNA, however, Cu reaction products in the present study were nearly exclusively observed in tissues of metal-exposed individuals, whereas rhogocytes of control snails were almost devoid of such granules (Figs. 5 and 6). In agreement with the data available from other studies (53), we conclude that these granular vesicles must be the sites of Cu incorporation.

Inspection of other cell types, such as gut epithelial cells and midgut gland cells (calcium, digestive, and excretory cells), revealed that none of them contained the typical Cu precipitations that were seen upon histochemistry in rhogocytes (see Fig. 5). The possibility cannot be excluded, however, that small fractions of Cu also may be incorporated in other snail cell types (37, 62). Nevertheless, the present study confirms the importance of rhogocytes as main accumulation sites of Cu in *H. pomatia*. Upon inspection of serial tissue sections that had separately been processed for either ISH or histochemical Cu staining, no colocalization of Cu-MT mRNA and granular Cu deposits was found. Hence, although both kinds of Cu forms (the MT-bound Cu pool and the granular metal compartment) occur exclusively in rhogocytes, they do not seem to coexist in these cells. This suggests that rhogocytes may occur in at least two distinct metabolic states representing two different cell populations: one that is characterized by Cu-MT expression and binding of monovalent Cu" ions to the expressed MT peptide and a second one, recognizable by its granular deposits of Cu, that is not bound to MT and apparently represents a separate storage form.


Cu Speciation Among Differently Responsive Pools: Cu-MT vs. Granular Cu Deposits

The two rhogocyte populations with different Cu storage forms not only represent distinct cellular pools of this essential trace element but also respond to external Cu stimuli in opposite ways (compare Fig. 7, A and B). In the present study, the relative number of rhogocytes with Cu-MT mRNA remained constant throughout all tissues, irrespective of whether the animals were exposed to Cu or not (Fig. 7A). This suggests that the Cu$^+$ pool associated with Cu-MT is not affected by those fractions of the metal that quickly enter the animal cells upon exposure. This view is corroborated by another study (11) showing that upon quantitative real-time detection PCR, the tissue-specific concentrations of Cu-MT mRNA in snail organs did not change, even after increased Cu uptake due to metal administration. At the protein level, no differences in Cu-MT tissue concentrations could be found between control and metal-fed snails (Fig. 1C). All these results together prove that in H. pomatia, Cu is not an inducer of Cu-MT synthesis. Consequently, Cu fractions quickly taken up by the snail upon exposure do not contribute to an increase of metal concentration associated with the Cu-MT pool. This seems to be in contrast to results of several studies of other organisms in which Cu is an important inducer of MT synthesis (40, 45), giving rise to an increase of the metal fraction bound to Cu-MT (24, 35, 51, 59). In several species, however, MT induction by Cu does not follow a linear dose-dependent response pattern but may only occur when Cu tissue concentrations exceed a certain threshold level (7, 56). This is not the case for Cu-MT in H. pomatia, as repeatedly demonstrated in Roman snails exposed to rising Cu concentrations up to highly superphysiological levels (15, 18, 19). In fact, a comparative evaluation of data available in the literature demonstrates that the role of Cu as an inducer of Cu-specific MTs and its related biological function may vary in a species-specific manner (21), depending on the particular trace element demands of an organism and on the MT isoforms involved (5, 34).

Terrestrial gastropods seem to have adapted the function of Cu-MT and the role of Cu to their specific needs in connection with the turnover and metabolism of hemocyanin (3, 15, 16). This is indicated by the fact that Cu-MT is expressed in the same cell type that has repeatedly been shown to be also the site of hemocyanin synthesis in different gastropod species (1, 54, 55). In marine crustaceans, the activity of a Cu-specific MT isoform is also related to the turnover of hemocyanin (8), with close interaction of Cu-MT expression with hemocyanin fluctuations during molting (23). Intracellular fluctuations of Cu storage forms in connection with molting also were reported in terrestrial isopods (63, 64). Of course, molting does not play a role during the life cycle of terrestrial snails. There, instead, the hemocyanin turnover and its expected interaction with Cu-MT might be related to other stressful events such as, for example, hibernation, estivation, and reproduction, possibly with considerable fluctuations of hemocyanin demand.

The decoupling of Cu in the Cu-MT pool from short-term metal fluctuations confronts terrestrial pulmonates with the problem of how to cope with those nonphysiological amounts of Cu entering their tissues suddenly as a result of environmental exposure. As shown in the present study, terrestrial snails have solved this problem by establishing a second Cu compartment, also located in rhogocytes. This Cu pool is associated with metal precipitations in granular vesicles. Their number evidently increases with increasing intensity of Cu exposure (see Fig. 6). Moreover, the relative proportion of rhogocytes exhibiting granular Cu deposits was very low (0.2–0.5%) in tissues of control snails but rose to levels 100 times higher (20–27%) in organs of Cu-exposed snails (see Fig. 7B). Hence, in contrast to Cu-MT, this granular Cu pool is highly inducible by external metal stimuli and responds quickly to an increase of Cu uptake. It has been shown in earlier feeding experiments that upon cessation of Cu administration, the metal taken up during exposure is rapidly eliminated from the snail organism, with Cu levels in the animal tissues dropping to control values within a few days (12, 13).

The chemical dye used for histochemical Cu precipitation in the present study, 5-(4-dimethylaminobenzylidene)rhodanine, is not specific for any of the oxidation states of Cu (I or II) and can complex both cuprous (Cu$^+$) and cupric (Cu$^{2+}$) ions. So,
Although the question about the oxidation state of Cu in the highly responsive granular pool remains unresolved, it is clear that the chemical availability of the metal in this compartment must be significantly higher compared with the Cu fraction associated with the Cu-MT pool. We know that Cu is one of the metals that are more tightly bound by MTs, with metal-binding constants for this metal in the range of $>10^{16}$ (31). For 5-(4-dimethylaminobenzylidene)rhodanine, a binding constant for Cu$^{2+}$ of log K ([ML]/[M]-[L]) = 6.08 has been reported (where M = metal and L = ligand) (39). Hence, from a chemical point of view, the Cu ions in the highly responsive granular compartment are at least 10$^9$ times more available than the monovalent Cu$^+$ ions in the Cu-MT pool.

**Challenges for Future Research**

The mechanisms responsible for the uptake, intracellular trafficking, and elimination of Cu out of the cells are so far not known. Rhogocytes apparently possess phagocytic and endocytic mechanisms (29, 53). Moreover, their slithlike pore system has been suggested to be involved in sequestration of metal complexes from the blood plasma (37). On the other hand, it has been observed that whole rhogocytes can be eliminated from the snail’s body along with their storage products by diapedesis (9, 37). Hence, the possibility cannot be excluded that true cellular mechanisms such as endocytosis, exocytosis, or diapedesis may contribute to the processes of metal accumulation and extrusion in pulmonates. Alternatively, and possibly in addition to such specific cellular processes, Cu uptake, elimination, and intracellular trafficking among the different rhogocyte compartments may be accomplished by specific carrier proteins. As shown in other organisms, Cu-specific trafficking proteins are widespread and point to the importance that Cu has attained as an essential element for biological processes. High-affinity copper transporters (Ct) have been discovered, for example, in yeasts (49). Their activity in Cu metabolism associated with degradation and synthesis of hemocyanin. J Inorg Biochem 88: 228–239, 2002.

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**REFERENCES**

25. Gehrig P, You C, Dallinger R, Gruber C, Brouwer M, Kägi JHR, and Hunziker PE. Electrospray ionization mass spectrometry of zinc, cad-