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Experimental silver bioaccumulation in the polychaete *Pomatoceros triqueter* (L.)

A cytological and microanalytical study

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Summary. The tubicolous polychaete *Pomatoceros triqueter* was exposed for 6–7 weeks to 200 or 400 $\mu g \cdot l^{-1}$ silver introduced as the nitrate into sea water. Survival conditions and mortality were evaluated and silver bioaccumulation analysed by atomic absorption spectrometry. Characteristic morphological lesions were recognized. Histopathologic examination was performed on paraffin or semi-thin sections and at the ultrastructural level. Histochemical examination mainly concerned the metals, reducing groups and sulfur-containing proteins. Microanalytical study involved the use of a wavelength-dispersive X-ray spectrometry microprobe and ion microanalyzer, and the use of an energy-dispersive X-ray spectrometry microprobe at the ultrastructural level. Our results emphasize the role of the branchial crown for metal penetration. Its cuticle accumulates silver as a metal, in particulate form. The internal accumulation of mainly extracellular deposits concerns the basement membranes and connective tissue present in the axis of the branchial crown filaments, or surrounding the nephridial pouches and the gut sinus. The carrier role of the closed vascular system is suggested by ultrastructural observations. The silver route from transepithelial uptake to nephridial excretion involves at least two intracellular transits, plus the vascular mesothelium. Nephridia play a role in silver storage (lysosomes) and elimination (concretions). In all parts internal to the crown cuticle, silver is at least partly associated with protein SH-groups (metallothionein-like); deposits can be enriched with silver sulfide and metallic silver.

Key words: Silver bioaccumulation – *Pomatoceros* – Microanalysis – Nephridial lysosomes – Metallothionein-like

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Introduction

This study is part of a larger program on the effects of silver on mollusks and annelids. The cytotoxicity, biotransformation and bioaccumulation of the metal are investigated as well as the possibility of detoxication. Earlier publications on this topic include the mollusks *Crassostrea gigas* (Martoja R. et al. 1988) and *Chlamys varia* (Martoja M. et al. 1989), and the annelid *Sabella pavonina* (Koechlin and Grasset 1988). The project, methods and some results of these studies can be compared to previous studies by other workers, on the mollusks *Crepidula fornicata* (Nelson et al. 1983) and *Mytilus edulis* (Calabrese et al. 1984, George et al. 1986). All of these species are sedentary suspension filter-feeders, a common feature which allows experimental facilities. The importance of the problem of silver contamination has been emphasized by all the authors mentioned (e.g. Koechlin and Grasset: 'considerable concern has been expressed in recenty years about the effect of silver on the estuarine and marine environment, this metal being listed as a highly toxic potential pollutant ...').

The present study is not mainly involved in the environmental or experimental aspects of the toxic metal intervention, but the evaluation of its possible accumulation at the tissue and cell level. Regarding the biological effects of toxic metals from a cytopathological point of view, polychaete annelids have been less studied than mollusks. Also, a comparison between *Sabella* and *Pomatoceros* would appear of interest. Despite their systematic and structural closeness, the latter species is probably less sensitive to silver stress than *Sabella*. We discovered the remarkable resistance of *Pomatoceros* exposed to an effluent of the titanium dioxide industry in a previous unpublished study (Vovelle 1986). We also investigated (Vovelle et al. 1990) the sites of its normal skeleton biomineralization and mineral bioaccumulation, so we are aware of the characteristics of control animals. The animals sampled for our experimental contaminations and controls were collected by dredging in an area devoid of natural silver pollution

and at a depth which well characterizes the species *Pomatoceros triqueter*.

Materials and methods

Silver contamination. Adult worms were collected in North Brittany (Brehat and Roscoff). Specimens fixed on little stones were kept in small aquaria (1.5 l), the sea water being replaced every 2 days, oxygenated and maintained at the ambient temperaure of 15° C. Silver (as $AgNO_3$) was added at a concentration of 200 or 400 μg l^{-1}. These concentrations were chosen because they had been used in parallel experiments on *Sabella* and data from authors working on mollusks (see Discussion). Every week, three or four animals were sampled for either histological or cytological studies or for microanalysis. The experiments were stopped after six weeks and some treated animals were sampled for determination of their tissue Ag concentration by atomic absorption spectrometry as described by Amiard-Triquet et al. (1986).

Histological, histochemical and cytological methods. Material for histological purpose was fixed in concentrated formaldehyde/absolute alcohol (50:50) (McGee-Russell 1958), embedded in paraffin and sectioned at 6 μm. Histochemical studies mainly concerned the detection of reducing agents by iron-ferricyanide reduction (Schmorl 1928), SH groups by reacting with dihydroxy-2,2′ dinaphthyldisulfur (Barrnett and Seligman 1954). Cytological studies were carried out on animals fixed in 3% glutaraldehyde in 0.4 M cacodylate pH 7.2 (sometimes followed by 1% OsO_4 postfixation) and araldite embedded. Semi-thin (1 μm) or ultra-thin sections were made with a Reichert OMU2 microtome. Observations were made with a Philips EM 300 electron microscope. Non-postfixed sections were not stained.

Microanalysis. Three different instruments were used: (a) a wavelength-dispersive X-ray spectrometry MS46 Cameca microprobe on paraffin sections; (b) a SMI300 Cameca ion microanalyzer on paraffin or semi-thin sections; (c) a Temscan Jeol Asid 4DX electron microscope equipped with a Link AN 10000 energy-dispersive analytical system (see Truchet and Vovelle 1977 for details of the two former methods, Koechlin and Grasset 1988 for details of the latter).

Results

Mortality, morphological and histological lesions

Silver-ion-induced mortality was investigated after extraction of the worms from their tubes (which they were

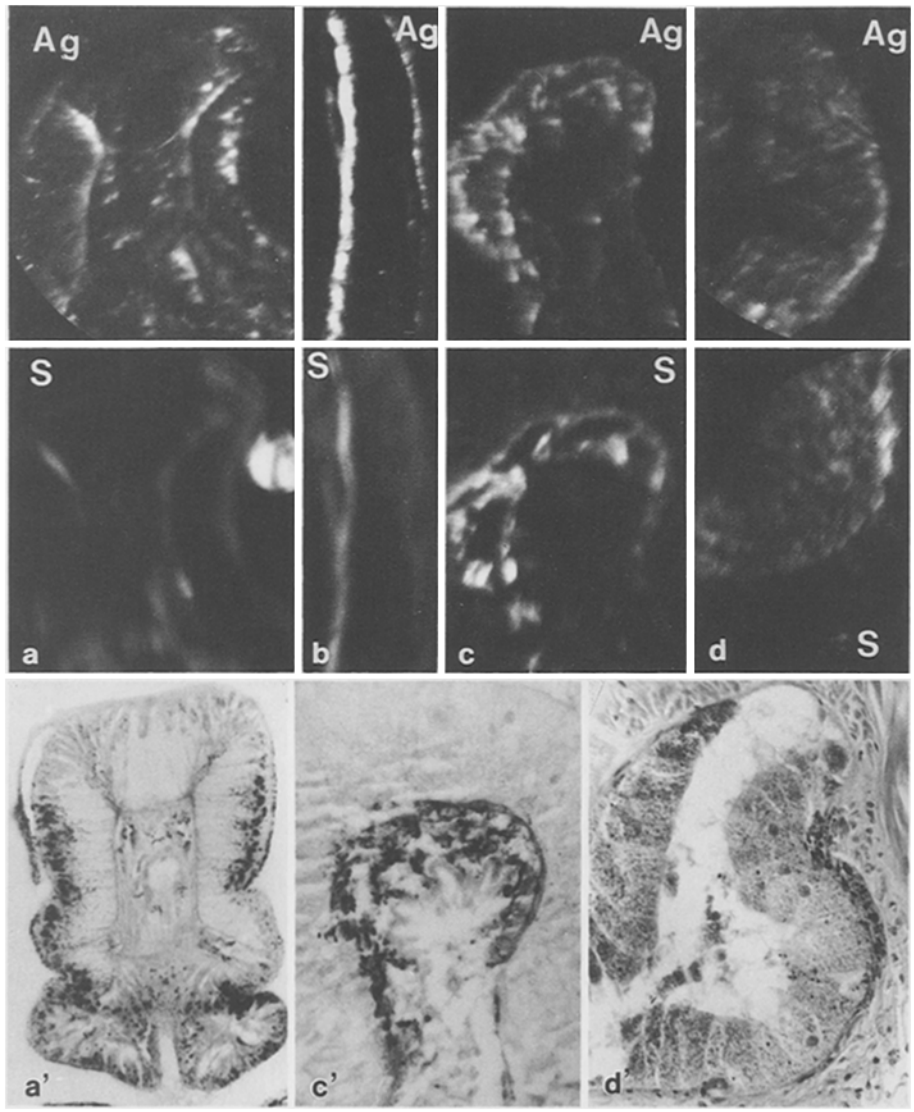

Fig. 1. Ion microanalyzer images corresponding to Ag (109^+) and S (32^-); 5-weeks exposure to 200 ppb Ag. (**a**) Filament cross section. (**b**) Filament longitudinal section (S absent from cuticle). (**c**) Connective envelope of the gut blood sinus. (**d**) Epithelium and surrounding tissues of the distal nephridia. (**a′**, **c′**, **d′**) Histology of the same tissues (semi-thin section for *d′*). Magnification 272.5

not inclined to leave). In the two series of intoxication (200 and 400 ppb), the mortality was insignificant: 4 drying animals, 1 dead, out of the 44 extracted. Metal accumulation was high: after 6 weeks of exposure to 200 ppb Ag, *Pomatoceros* concentrated silver at 83.13 ± 10.10 ppm dry mass ($n = 21$), about 700 times more than the control animals (0.109 ± 0.02 ppm, $n = 60$). Morphological alterations mainly concerned the branchial crown, which was frequently curled up and shortened, necrotic zones appeared especially on the collar.

At the tissue level, symptomatic lesions appeared, such as the histolysis of the filament tips and pinnules of the branchial crown. Internally, the progressive disorganization of the nephridial distal epithelium in some cases induced the formation of silver-rich concretions. Anatomopathological evaluation was favoured by one particular piece of evidence: silver deposits are naturally observable under the microscope. They appeared black in their superficial sites (cuticle), brown in their internal extracellular sites (connective tissue and basement membrane of the branchial crown and thorax).

Histochemistry

In the $AgNO_3$-treated animals, the detection of thiol groups by the reaction with dihydroxy-2,2′-dinaphthyldisulfur appeared positive in the connective tissue and basement membranes surrounding the gut blood sinus (adjacent to nephridiae) and in the connective frame of the branchial crown filament. The same structures were Schmorl-positive, but this latter method also detected reducing compounds in the nephridial epithelial inclusions and surprisingly the 'black grains' of the cuticle overlying the branchial crown and the mouth. This latter response can be attributed to metallic silver, because of the absence of either sulfide or sulfhydryl protein in the 'grains'.

Photonic microanalysis

The wavelength-dispersive Cameca microprobe, which permits a semi-quantitative estimation of the metal when compared to a standard (Ag_2S), detected considerable local concentrations of silver. The response reached 100 counts/s and up to 380 counts/s on the exposed parts of the branchial crown (filaments cuticle, margin of the operculum). Comparable values were obtained in intra- or extra-cellular internal localizations of the septal system (connective envelope of the gut blood sinus and nephridia, mesothelium). Values are of 80 counts/s in the nephridial epithelium, and over 650 counts/s in the nephridiopore concretions. Examination of worms sampled weekly (up to 7 weeks) indicated that the silver response becomes marked after 2 weeks of exposure. Animals placed in sea water with a high concentration of silver ions (400 ppb) were mainly contaminated on their superficial structures; animals in a lower concentration, less superficially contaminated, had a higher Ag concentration in their internal structures and organs. Sulfur was absent from cuticular grains. It became associated with silver in the basement membrane and connective tissue of filaments, and in the connective tissue and parietal cells of the thoracic septal system. Its presence in the nephridial epithelial inclusions is noticeable but ambiguous. In the concretions and cellular materials evacuated by the contaminated nephridia sulfur was abundant; this suggests that it is at least partially in the form Ag_2S and is possibly overloaded with metallic silver.

Ion microanalysis confirmed the presence of silver in the previously listed localizations, peripheral sites (cuticle and connective tissue of filaments) or internal sites (connective envelope of the gut blood sinus and nephridia, septal cells). Only one part of the nephridial epithelial inclusions contains silver. Superimposition of the silver and sulfur elemental distribution images demonstrated their association in the internal intra- or ex-

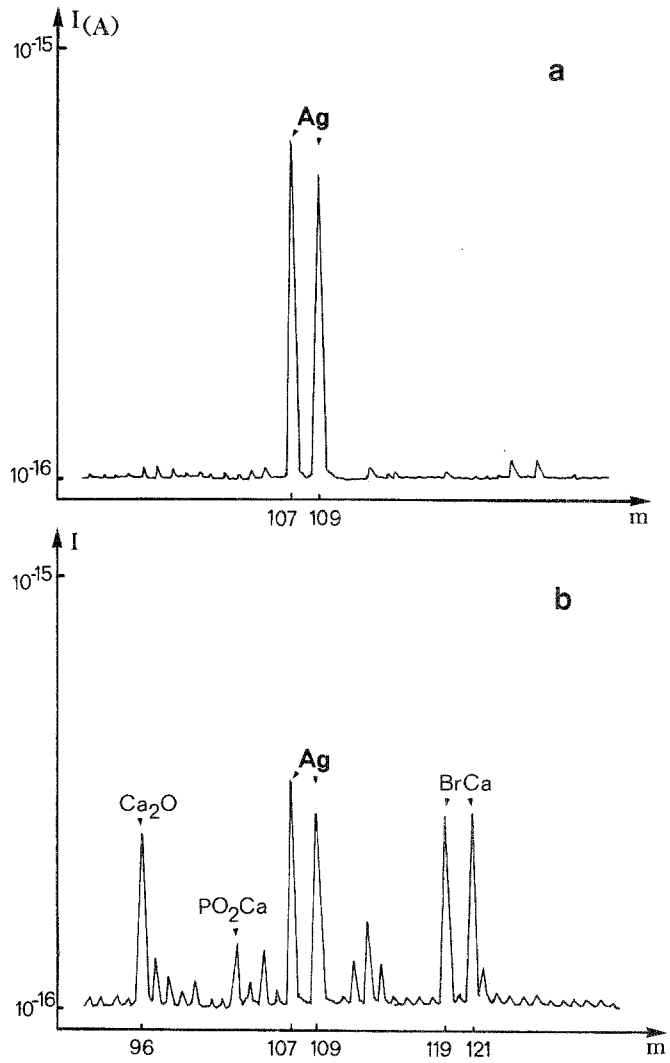

Fig. 2. Low-mass-resolution spectra (+ions) obtained with the ion microanalyzer on paraffin sections; 5-weeks exposure to 200 ppb Ag. Analyzed field 250 μm diameter. m = mass (g); I = intensity of secondary ions (A). (**a**) Buccal crossway cuticle (Ag only); (**b**) part of the distal nephridial epithelium

tra-cellular sites, and in the connective frame of the filaments (Fig. 1).

The comparison of low-mass-resolution spectra (+ions, Fig. 2) obtained from the cuticle and from the nephridial epithelium emphasized the presence of silver alone in the former and its coexistence with other elements (P, Ca, Br) in the latter. These elements are normally concentrated in the organ (Vovelle et al. 1990), but they may be partly or totally located in inclusions other than the silver-rich lysosomes.

Electron microscopy

Highly contrasted inclusions, probably due to silver, were observed on ultra-thin sections of glutaraldehyde-fixed, non-post-osmicated animals (Fig. 3). Cuticular 'black grains' appeared as homogenous and subgeometrical forms, vulnerable to electronic bombardment which deforms them and causes them to deteriorate. They could not be related to the cuticular bundles of fibrillar collagen, nor to epithelial microvilli; the metallic deposit was probably linked with the glycoprotidic cuticular component. The metal was observed passing through the filament vacuolar cells: contrasted inclusions were present first in apical multivesicular bodies and then in the vacuoles or on their walls. Many silver-rich sphaerocrystals were present in the connective tissue underlying the filament epithelium. The location of silver in the pinnules presented the same sequence of intracuticular crystals, intracellular multivesicular bodies and sphaerocrystals (in the connective tissue), even when the thickness between the cuticle and the capillar-

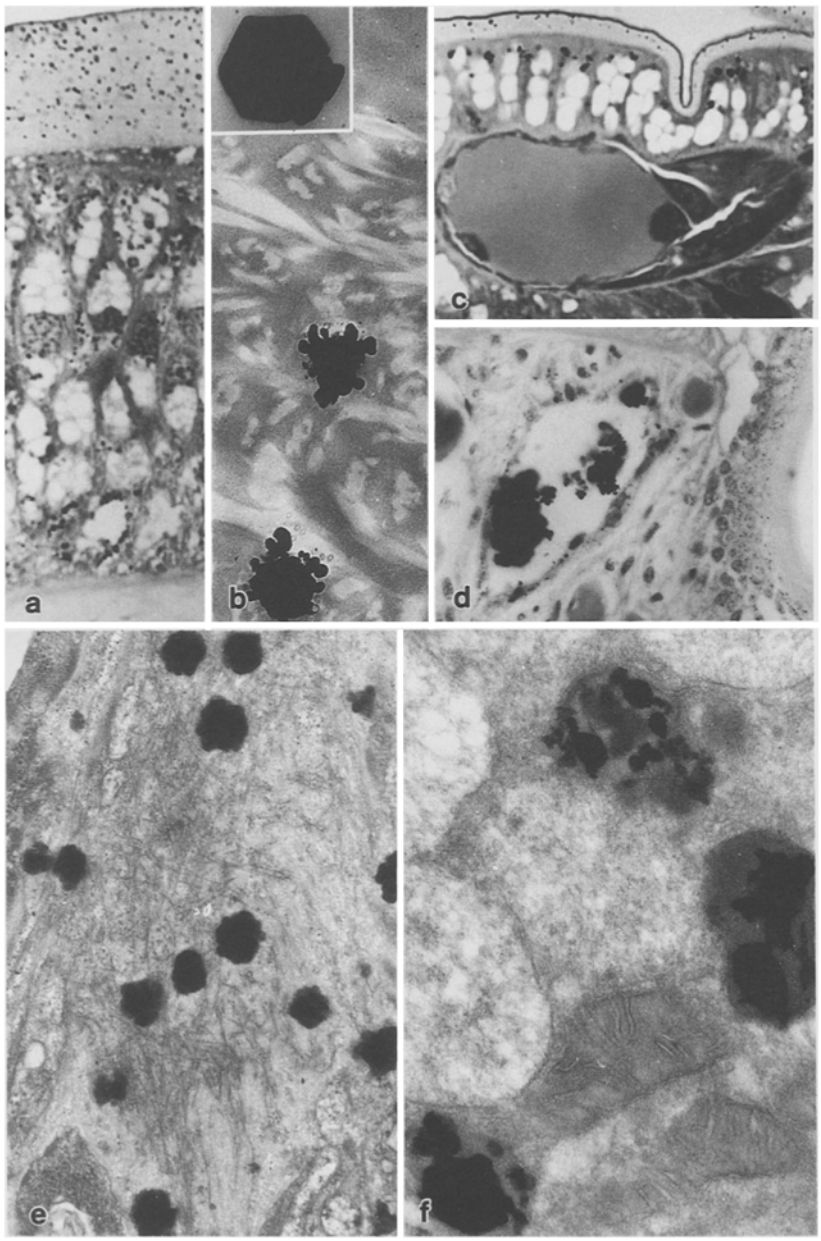

Fig. 3. Histology and electron microscopical cytology; 6-weeks exposure to 200 ppb Ag. (**a**) Vacuolar epithelium of the opercular margin and its cuticle. (**b**) 'Black grains' in the filament cuticle, electron microscopical cytology without post-osmication; inset: subgeometrical shape before electron bombardment. (**c**) Filament epithelium and capillary, semi-thin section. (**d**) Silver-rich concretion in the nephridial duct. (**e**) Silver-rich extracellular sphaerocrystals in the connective tissue underlying the filament vacuolar cells, electron microscopical cytology without post-osmication. (**f**) Lysosomes in the distal nephrocytes, electron microscopical cytology with postosmication. Magnifications: (**a**) X 1237.5; (**b**) x 15000, inset X 110000; (**c**) X 1237.5; (**d**) X 375; (**e**) X 15000; (**f**) X 30000)

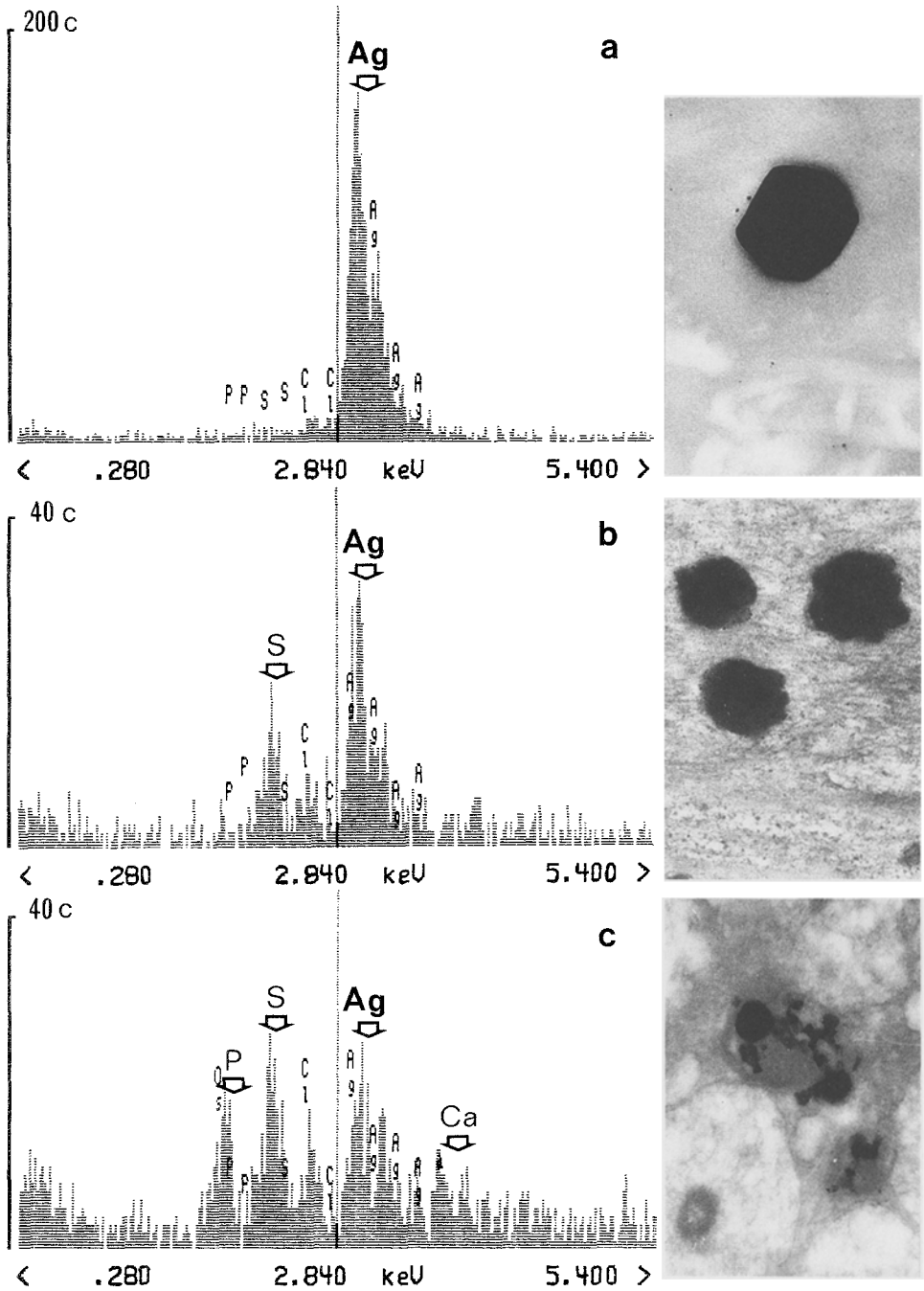

Fig. 4. Sulfur enrichment of the silver deposits as revealed by the energy-dispersive X-ray microprobe on ultra-thin sections; 6-weeks exposure to 200 ppb Ag. (**a**) 'Black grain' of the filament cuticle. (**b**) Silver-rich sphaerocrystal of the filament connective tissue. (**c**) Lysosome of the distal nephridial epithelium. Ordinate: X-ray intensity (counts); abscissa: X-ray energy (keV). Counting time 100 s (**a**) and 200 s (**b, c**); electron energy 100 kV; probe diameter 50 nm. Magnification of electron micrographs; (**a**) X 63 000, (**b, c**) X 30 000

ies was only 5 μm. The metal can probably be transported by the vascular system. Metallic deposits on the perinephridial or septal connective envelopes appeared as concretions smaller than in the branchial crown connective tissue. A high-contrast part of their matter was vulnerable to electronic bombardment. The epithelial cells of the distal nephridia contained high-contrast inclusions inside heterogeneous lysosomes which are distinct from other normal organelles. They can be interpreted as the last step of a detoxication process, leading to the formation of concretions by the excretory organ.

Energy-dispersive microprobe, at the ultrastructural level

Silver identification in the previously described locations was confirmed (Fig. 4). The cuticular 'black grains' contained silver alone, connective tissue concretions in the filaments contained silver in association with sulfur, nephridial polylysosomes contained silver and sulfur in association with Cl, P and Ca. The high responses of silver concerned the cuticular 'grains', the nephridial lysosomes having a higher sulfur ratio than the connective tissue deposits. The relative concentra-

tions of Ag and S were calculated from the X-ray data by quantitative evaluation of peak summation, with reference to silver sulfide standards. Previous data were confirmed (concretions of the filament connective tissue: Ag/S = 0.94; nephridial lysosomes: Ag/S = 0.49).

Discussion

Pomatoceros endures exposure to relatively high concentrations of silver (200–400 $\mu g \cdot l^{-1}$) for a relatively long period (6–7 weeks) better than some other marine invertebrates. As comparative references, we can recall the concentrations retained for marine mollusks by Nelson et al. (1983), Calabrese et al. (1984) (10 $\mu g \cdot l^{-1}$ during 24 months) and Martoja et al. (1988) (20 $\mu g \cdot l^{-1}$ during a shorter time). Koechlin and Grasset (1988) retained for *Sabella* a concentration of 50 $\mu g \cdot l^{-1}$ (8 weeks).

The value obtained by measurement of total Ag contamination in the soft parts of *Pomatoceros* is high, comparable to those obtained with some mollusks and four times higher than the value obtained for *Sabella* for the same length of time (but with a dilution four times lower).

Silver uptake in the surrounding medium is directly connected to water currents and anatomical systems which induce them (branchial crown, collar and thoracic membrane). Increasing superficial contamination is evident from the filament tips to the mouth, but the silver transit inside the gut is not significant.

The high concentration of silver within the operculum emphasizes its specific sealing function; this is likely to explain the remarkable resistance of the worm to contamination, even when it is long lasting.

Several types of silver deposits were identified. They represent stable bioaccumulation sites and/or stages of the metal progress up to a possible detoxication (by means of nephridial concretions). Superficial cuticular deposits are unique and without any equivalent in *Sabella*. Their appearance may be an artefact, but in any case the reducing process which induces the metallic silver deposit is not related to the use of an aldehyde fixative nor to the presence of a reducing compound in the cuticular material (SH groups and quinone-tanning processes are absent). Internal extracellular deposits related to connective tissue lack the diversity in density and shape of similar deposits found in *Sabella* or mollusks. Those were described as star-shaped or urchin-shaped granules; compared with them, the concretions of *Pomatoceros* look somewhat amorphous.

Intracellular localizations of silver were detected at the two possible ends of its transit, in the epithelial cells of the branchial crown and of the distal nephridia. The vascular system is probably involved in the extracellular transit. The presence of metal in the pinnule capillaries and in the walls of the gut blood sinus, near the nephridial pouches, accounts for this hypothesis.

Sulfur is absent from superficial metallic deposits. Its binding with silver may be different according to the different internal deposits. Protein SH-groups (metallothionein-like) are present and possibility induced by the contamination, but the thoracic extracellular concretions accumulate the metal in the form Ag_2S too. The terminal accumulations of silver are heterogeneous, as can be seen in the nephridial lysosomes and concretions.

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