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Fine-Scale Tissue Distribution of Cadmium, Inorganic Mercury, and Methylmercury in Nymphs of the Burrowing Mayfly *Hexagenia rigida* Studied by Whole-Body Autoradiography

B. Inza*, C. Rouleau,†,‡,[1] H. Tjälve,‡ F. Ribeyre,* P. G. C. Campbell,§ É. Pelletier,¶ and A. Boudou*

**LEESA, UMR CNRS 5805, University of Bordeaux 1, Place du Dr Peyneau, 33120 Arcachon, France; †Aquatic Ecosystem Protection Research Branch, National Water Research Institute, 867 Lakeshore Road, P.O. Box 5050, Burlington, Ontario, Canada L7R 4A6; ‡Department of Pharmacology and Toxicology, Swedish University of Agricultural Sciences, Biomedicum Box 573, S-751 23 Uppsala, Sweden; §INRS-Eau, Université du Québec, 2700 rue Einstein, P.O. Box 7500, Sainte-Foy, Québec, Canada G1V 4C7; and ¶Institut des Sciences de la Mer de Rimouski, 310 allée des Ursulines, Rimouski, Québec, Canada G5L 3A1*

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The distribution of inorganic ^{109}Cd(II), inorganic ^{203}Hg(II), and [^{203}Hg] methylmercury (MeHg) in nymphs of the burrowing mayfly *Hexagenia rigida* after exposure via water and sediments was studied. To better understand the mechanisms underlying the fate of Cd, Hg, and MeHg in this animal and to identify target organs, autoradiography of whole-body cryosections was used to obtain a detailed view of the distribution of the radiolabels. The gut and exoskeleton were the only structures labeled in nymphs exposed to Cd via water or sediments. After exposure to inorganic Hg via water, the Malpighian tubules exhibited a very high labeling, indicating that these organs may be a target for Hg toxicity. The distribution of Hg after exposure via sediments was similar, though the labeling of Malpighian tubules was less intense. Distribution of MeHg strongly differed between treatment groups. Nymphs were rather uniformly labeled after exposure via water, whereas in those exposed to MeHg in sediments, the intense labeling of all internal tissues contrasted with the very low labeling of the hemolymph, indicating that the translocation rate of the absorbed MeHg was faster in the latter group. This may be related to the complexation of MeHg by small thiol ligands in the gut as a result of the digestion process.

Key Words: **mercury; cadmium; methylmercury; *Hexagenia rigida*; whole-body autoradiography.**

INTRODUCTION

Insects represent one of the most common and diverse groups of freshwater animals known to readily accumulate trace metals (Hare, 1992). Benthic deposit-feeder larval stages of aquatic insects are of particular interest because they can accumulate metals via two potential routes: via the gut wall from fine-grained sediment consumed as food and via respiratory surfaces from the ambient water. Over the past 10 years, we have extensively studied the accumulation of cadmium (Cd), inorganic mercury (Hg), and methylmercury (MeHg) via both uptake routes in nymphs of the burrowing mayfly *Hexagenia rigida* (Saouter *et al.*, 1991a, 1993; Hare *et al.*, 1991; Odin *et al.*, 1994, 1995a, b; Andres *et al.*, 1998), a species that constructs burrows through which it feeds on bulk sediment (Zimmerman *et al.*, 1975; Charbonneau and Hare, 1998).

One of our objectives was to determine the distribution pattern of these trace metals within the body of mayfly nymphs to obtain information on the potential targets of metal toxicity and the mechanisms of metal exchange among nymph tissues and between the animal and its environment. We previously used microdissection techniques to study the distribution of Cd, MeHg, and inorganic Hg. However, microdissection techniques are limited to the larger body components, such as the gut and the gills, and metal distribution in organs and tissues such as the hemolymph, muscle, and the circulatory, excretory, and nervous systems remains unknown. Thus, more detailed information on the distribution of Cd, Hg, and MeHg within the body of *H. rigida* is needed to identify target organs and guide further

[1]To whom correspondence should be addressed at National Water Research Institute, P.O. Box 5050, Burlington, Ontario, Canada L7R 4A6. E-mail: claude.rouleau@cciw.ca.

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research on the toxicological consequences of metal uptake.

Microautoradiography has been recently used to study the distribution of radioactive ^{109}Cd in histological sections of chironomid larvae (*Chironomus staegeri*) exposed to the metal via water and water + sediment (Craig *et al.*, 1998). However, in addition to being rather time consuming, autoradiography of classical histological tissue sections might suffer from artifacts due to the extraction of the radioactive metal out of tissues or its displacement, given its solubility in water or other liquids used in the customary histological processes, e.g., fixation, dehydration, flotation, deparaffinization, and staining. These drawbacks can be avoided by the use of whole-body autoradiography (WBARG), as described by Ullberg *et al.* (1982). The distribution of the compound studied is preserved by freezing the animal and by preventing any liquids from coming into contact with the tissues until after the autoradiographic exposure. If a number of sections are taken at different levels in the body, most tissues and fluids can be studied. The advantage of WBARG is that the investigation is not limited to certain preselected tissues, but permits unforeseen locations to be observed, which may result in a new perspective regarding the mode of action or toxicity of the investigated substance. Though WBARG was originally designed for use with animals of the size of a mouse or a fish, the spatial resolution achieved (<100 μm) allows its use with insect larvae as small as a few millimeters in length (Rouleau *et al.*, 1998a). This paper presents the results of a WBARG study of the fine-scale distribution of $^{109}Cd(II)$, $^{203}Hg(II)$, and [^{203}Hg]MeHg(II) in nymphs of *H. rigida* exposed to these metals via water or sediment.

MATERIAL AND METHODS

Exposure to Metals

Our experimental approach has been described in detail elsewhere (Saouter *et al.*, 1993; Odin *et al.*, 1995). Experimental conditions are summarized in Table 1. Radioactive $^{109}CdCl_2$ and $^{203}HgCl_2$ were purchased from Amersham, whereas $CH_3{}^{203}HgOH$ was synthesized from inorganic $^{203}HgCl_2$ according to Toribara (1985). Nominal metal concentrations added to the water column and the sediments were a compromise between environmental realism, technical requirements for successful whole-body autoradiography, and constraints imposed by local regulations on the use of radiolabeled chemicals.

Experimental units (EUs) were held at 22 ± 0.3°C (SD) and the photoperiod was 8 h darkness/16 h light (fluorescent tubes). Each unit contained six *H. rigida* nymphs with an average total biomass per EU of 218 ± 41 mg (ww). The nymphs had background total metal concentrations of 118 ± 10 ng $Hg \cdot g^{-1}$ (ww) and 150 ± 20 ng $Cd \cdot g^{-1}$ (ww) (determined after depuration of the intragut sediment).

Sample Collection

At the end of the exposure period, nymphs were sieved from sediment and individually placed for 20 h in beakers containing natural sediment, to

TABLE 1

Parameters of the Exposure of Nymphs of *Hexagenia rigida* to Cd, Hg, and MeHg via Water or Sediments

	Cd		Hg		MeHg		Control	
	Water	Sediment	Water	Sediment	Water	Sediment	Water	Sediment
Metal added (ww)	5 $\mu g \cdot L^{-1}$	2.5 $\mu g \cdot g^{-1}$	5 $\mu g \cdot L^{-1}$	1.34 $\mu g \cdot g^{-1}$	0.3 $\mu g \cdot L^{-1}$	0.12 $\mu g \cdot g^{-1}$	—	—
Specific activity of ^{109}Cd and ^{203}Hg ($MBq \cdot mg^{-1}$ metal)	50.7	0.74	29.6		29.6		—	—
Exposure								
4 days	×	×	×	×	×	×	×	×
14 days	×	×	×	×	×	×	×	×

Replicate experimental units (EUs) were used for each set of experimental parameters (×). Each EU was filled with 1.4 L of spring water ($Ca^{2+} = 10$ mg·L^{-1}; $Mg^{2+} = 3$ mg·L^{-1}; $Na^{+} = 4$ mg·L^{-1}; $Cl^{-} = 6$ mg·L^{-1}; $SO_4^{2-} = 2$ mg·L^{-1}; $HCO_3^{-} = 34$ mg·L^{-1}, pH = 7.1 ± 0.2) and contained a 5-cm layer of natural sediment from the Garonne River, France. The latter is rich in clay (75–80%), contains 50% water by weight and $< 2\%$ organic C (Saouter *et al.*, 1993), and had background metal concentrations of 0.140 ± 0.015 μg total $Hg \cdot g^{-1}$ (wet weight, ww) and 0.300 ± 0.028 μg $Cd \cdot g^{-1}$ (ww). Four days were allowed for physicochemical conditions to stabilize before the nymphs were introduced into the EUs.

allow for clearance of contaminated substrate from the gut. After the depuration period, the nymphs were removed from the beakers and four specimens from each EU were individually weighed and used for Hg or Cd determinations. Radioactivity was measured with a LKB Wallac 1282 Compugamma counter (well-type NaI(Tl) detector). Counting times were selected to obtain a relative counting error $< 2\%$ for Hg and $< 5\%$ for Cd. After correction for decay, background, and counting efficiency, counts per minute data were converted to nanograms of Cd or Hg. Considering that different concentrations of Cd, Hg, and MeHg were used in this experiment, metal concentrations in nymphs were expressed as an accumulation factor (AF), i.e., metal concentration in nymphs/metal concentration in contaminated medium (units of ml water$\cdot g^{-1}$ wet weight (ww) for water exposure, units of g wet sediment$\cdot g^{-1}$ ww for sediment exposure). The use of AF, which expresses the affinity of an aquatic animal for a metal in terms of the equivalent quantity of water or sediment holding the same quantity of metal (Barron *et al.*, 1990), facilitates the comparison of bioaccumulation data between the different groups.

Whole-Body Autoradiography

The two remaining organisms were used for whole-body autoradiography according to Ullberg *et al.* (1982). They were embedded in a small volume (approx. 5 ml) of carboxymethylcellulose gel contained in an ice cube tray and frozen in a slurry of hexane and dry ice. The resulting blocks were embedded in a larger volume of gel (approx. 100 ml) on a microtome stage and frozen in the same way. Saggital 20-μm-thick sections were obtained with a specially designed cryomicrotome (Jung Cryomacrocut, Leica), at $-20°C$. The ^{109}Cd- and ^{203}Hg-labeled sections were freeze-dried for 24 h and applied to Hyperfilm-^{3}H (Amersham) or regular X-ray film (AGFA Structurix), respectively. Exposure period at $-20°C$ varied from 3 to 10 months, depending on the level of radioactivity that had been accumulated in each animal. After exposure, films were processed as recommended by the manufacturer. Autoradiograms and corresponding tissue sections presented in Figs. 2 and 3 were enlarged and printed on regular photographic paper.

RESULTS AND DISCUSSION

Quantitative Data

Concentrations of metal accumulated in the nymphs after exposure to contaminated water or sediment are shown in Fig. 1. Metal levels observed at day 14 were consistently higher than those at day 4, except for the group exposed to waterborne Cd. After 14 days of exposure via water, the accumulation factor for MeHg was twice that recorded for inorganic Hg (AF = 487 ml water$\cdot g^{-1}$ ww versus 237 ml water$\cdot g^{-1}$ ww). Accumulation of Cd was some 300 times lower than that for MeHg. Exposure to contaminated sediment resulted in an enrichment of larvae in MeHg compared to the sediment, i.e., AF > 1 g wet sediment$\cdot g^{-1}$ ww, but the accumulation of inorganic Hg and Cd from the same source was rather low, with AF values ranging from 0.01 to 0.05 g wet sediment$\cdot g^{-1}$ ww. These results agree with those obtained from earlier experiments conducted under similar conditions (Saouter *et al.*, 1993; Odin *et al.*, 1995b).

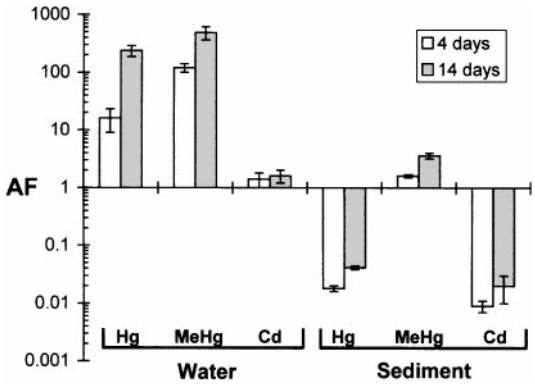

FIG. 1. Metal accumulation in nymphs of *Hexagenia rigida* exposed to ^{109}Cd(II), ^{203}Hg(II), or Me^{203}Hg(II), via water or sediment. Amounts of metal are expressed as an accumulation factor (AF), i.e., metal concentration in nymphs/metal concentration in contaminated medium. Units of AF are ml water$\cdot g^{-1}$ ww for water exposure and g wet sediment$\cdot g^{-1}$ ww for sediment exposure. Values are means $\pm$ SD, $n = 8$.

WBARG

There was no appreciable visual difference in the tissue distribution of the metals in the nymphs after 4 or 14 days of exposure. Thus, only autoradiograms from nymphs sampled at day 14 are shown (Figs. 2 and 3). Notice that the comparison of autoradiograms obtained for the different treatment groups is qualitative. Quantitative comparison is not possible because of the important differences in the quantities of metal accumulated, the specific activity of the radioisotopes, and exposure times. Semiquantitative comparisons are possible within a given group. The images presented here, selected after the

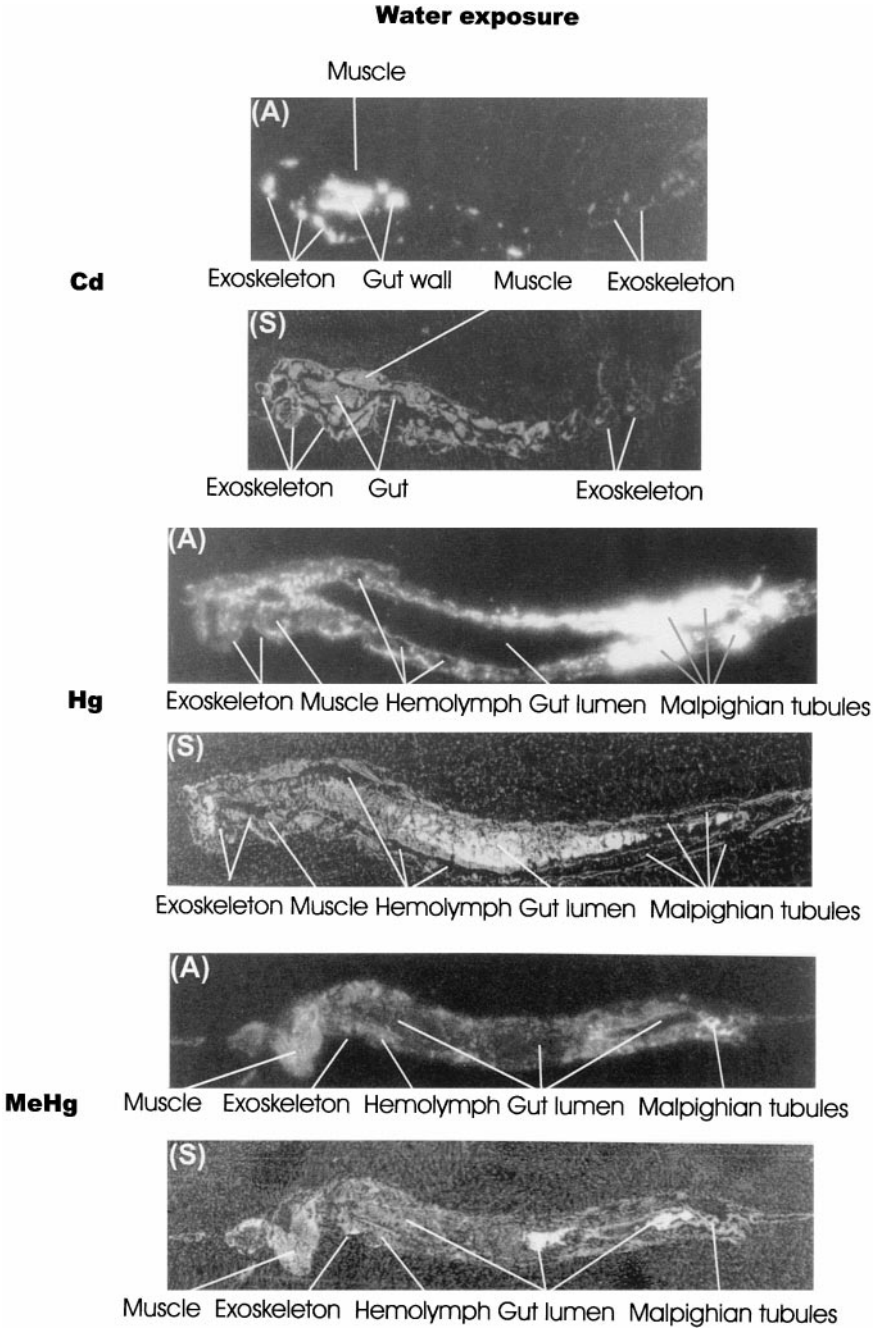

FIG. 2. Whole-body autoradiograms from nymphs of *Hexagenia rigida* after a 14-day exposure to water contaminated with ^{109}Cd(II), ^{203}Hg(II), or Me^{203}Hg(II). The intensity of tissue labeling is related to the concentration of the radiolabel, and thus white areas correspond to higher metal concentrations. (A), autoradiogram; (S), corresponding tissue section.

examination of nearly 1000 autoradiograms, are representative of the general metal distribution observed for each treatment group.

Water exposure. In autoradiograms from nymphs exposed to Cd, the gut wall and the exoskeleton were the only structures labeled (Fig. 2). This is comparable to the distributions observed in *C. staegeri* (Craig *et al.*, 1998) and *Chaoborus americanus* (Rouleau *et al.*, 1998a).

The most striking feature in nymphs exposed to inorganic Hg via water was the very strong labeling of the Malphigian tubules. The exoskeleton and internal tissues of the cephalic, thoracic, and

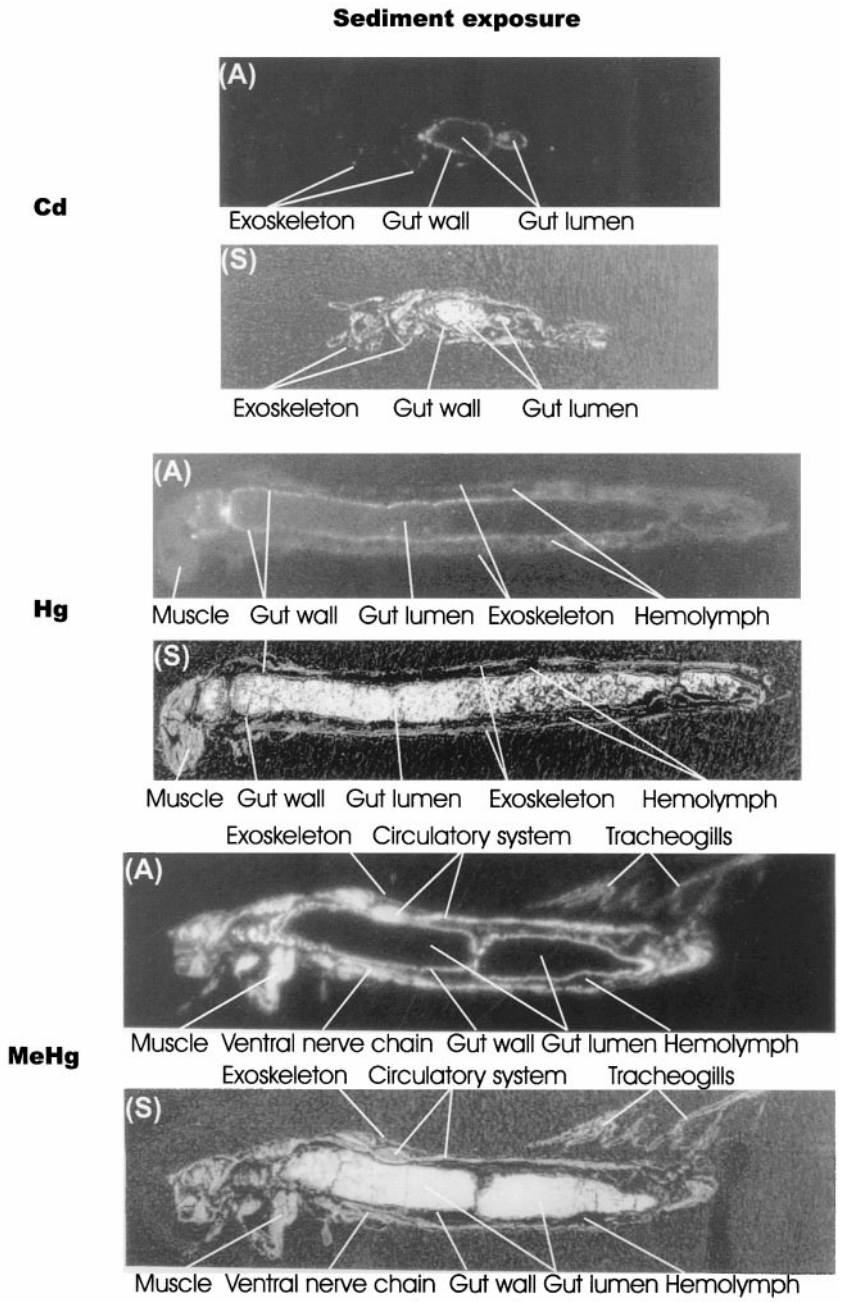

FIG. 3. Whole-body autoradiograms from nymphs of *Hexagenia rigida* after a 14-day exposure to sediment contaminated with $^{109}Cd(II)$, $^{203}Hg(II)$, or $Me^{203}Hg(II)$. (A), autoradiogram; (S), corresponding tissue section.

abdominal parts of the nymphs accumulated the metal to a lesser extent, and labeling of the hemolymph was even lower. Exposure of burrowing organisms to dissolved metals results from continuous exchanges between the overlying water column and the burrows, which are driven by the movements of the external tracheogills. Saouter *et al.* (1993) suggested that the main route of entry of inorganic Hg into *H. rigida* is via the tracheogills,[2] which accumulate high concentrations of this metal and are an important storage compartment (approx. 50% of the body burden). Autoradiograms reveal

[2]Since most saggital sections were sampled from the central part of the body of nymphs and tracheogills are located on each side of the body, the latter normally cannot be seen on tissue sections, except in a very few cases (see MeHg in Fig. 3).

that Hg concentrations in the Malpighian tubules are also very high. Malpighian tubules are implicated in the excretion of toxic metabolites and the maintenance of internal homeostasis in insects (Wigglesworth, 1972), and the very high labeling of these organs observed in the present work indicates that they may be a preferred target for inorganic Hg toxicity.

In contrast to the result obtained with inorganic Hg, the internal distribution of MeHg was quite homogenous (Fig. 2). The Malphigian tubules were the only anatomical structure with a higher labeling; the difference from the other tissues was, however, much less important than that for the nymphs exposed to inorganic Hg. The uniform distribution of MeHg in *H. rigida* is similar to that observed for many other aquatic animal species after exposure via water (Boudou and Ribeyre, 1997).

Sediment exposure. As in the case of nymphs exposed to Cd in water, the gut wall was the only internal structure labeled after exposure to Cd via the sediments (Fig. 3). This corresponds to the data of Hare *et al.* (1991), which showed that the accumulation of Cd from the sediments in the gut of *H. rigida* was 3 orders of magnitude higher than that in the other tissues. Thus, in the present experiment, radioactivity levels in tissues other than gut were probably too low to induce a blackening of the autoradiography film, despite the very long exposure time (10 months).

In nymphs exposed to inorganic Hg via the sediments, the distribution was similar to that observed in nymphs exposed to the metal via water. A weak and diffuse labeling can be seen in most tissues. Examination of the whole set of autoradiograms from this group revealed that the Malpighian tubules were also labeled, but much less intensely than in nymphs exposed to the metal via water (not shown). It is noteworthy that metal concentrations were higher in the gut wall than in other tissues. The higher labeling of the gut wall is likely related to the facts that important quantities of inorganic Hg can be accumulated at the interface between the apical part of enterocytes and the intestinal lumen of *H. rigida* and that the permeability of the intestinal barrier toward inorganic mercury is very low (Saouter *et al.*, 1991b).

The internal distribution of MeHg accumulated from the sediment was much less uniform than that in the case of water exposure. The relatively low labeling of the tracheogills and exoskeleton may reflect the accumulation of MeHg that had leached from sediment particles into the aqueous phase. Nevertheless, all internal tissues accumulated high amounts of MeHg and thus represent potential targets for MeHg toxicity. Such efficient accumulation and generalized tissue distribution for both sedimentary and waterborne MeHg have been observed for many other aquatic species (Boudou and Ribeyre, 1997; Rouleau *et al.*, 1998a, b, 1999). It is indicative of the high permeability of biological barriers to MeHg (Boudou and Ribeyre, 1997) and the ubiquitous distribution of sulfhydryl groups in living tissue, for which this organometallic compound has a very high affinity (Clarkson, 1994). An interesting observation is that the labeling of the hemolymph, the medium through which all the chemical exchanges within the insect body are effected (Wigglesworth, 1972), was very low despite the high MeHg concentrations observed in internal tissues. This contrasts with the distribution of MeHg in water-exposed animals, in which hemolymph labeling was similar to that of the other tissues (Fig. 2). The mechanism underlying this observation can only be speculated upon with the present set of data. A possible explanation is that the very low labeling of hemolymph in nymphs exposed to MeHg via the sediments indicates a short residence time of the radiolabel. Small thiol ligands play a major role in the mobility of MeHg in living organisms (Rabenstein and Evans, 1978; Ballatori *et al.*, 1991) and are likely present in the gut in much greater abundance than in the ambient water, as a result of the digestion process. The complexation of ingested MeHg with small thiol ligands in the gut might affect the rate at which it is distributed into the body after absorption (Clarkson, 1993).

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