Localizing organomercury uptake and accumulation in zebrafish larvae at the tissue and cellular level

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Using synchrotron x-ray fluorescence mapping, we have examined the uptake and localization of organic mercury in zebrafish larvae. Strikingly, the greatest accumulation of methyl and ethyl mercury compounds was highly localized in the rapidly dividing lens epithelium, with lower levels going to brain, optic nerve, and various other organs. The data suggest that the reported impairment of visual processes by mercury may arise not only from previously reported neurological effects, but also from direct effects on the ocular tissue. This novel approach is a powerful tool for directly investigating the molecular toxicology of heavy metals, and should be equally applicable to the study of a wide range of elements in developing embryos.

methylmercury | thimerosal | x-ray fluorescence mapping | eye lens

Toxic organic mercury compounds worry many communities worldwide, yet the detailed mechanisms underlying their transport and toxicity remain uncertain (1). These neurotoxic compounds are particularly insidious due to the latency in onset of toxic symptoms and have caused several devastating mass-poisonings of humans. Adults are affected, but exposure *in utero* has resulted in severe consequences such as microcephaly, cerebropalsy, seizures, and mental retardation. Methylmercury (MeHg) compounds are actively transported across cell membranes (2) although not, as originally thought, by molecular mimicry of methionine (3). Beyond this, however, our knowledge of the mechanisms underlying organic mercury (Hg) toxicity is fragmentary. MeHg-induced changes in cellular Ca^{2+} have been shown to be important (4–7), as has oxidative stress (4), and glutamate metabolism (8). In both yeast and human cells, overexpression of the ubiquitin-targeting enzyme Cdc34 confers protection against the cytotoxic effects of MeHg, which leads to the suggestion that an unknown protein containing a signal for ubiquitination by Cdc34 is involved in the development of the cytotoxic effects of MeHg (9–11).

Zebrafish (*Danio rerio*) are common model organisms for the study of embryonic development, and have found increasing use in vertebrate toxicology (12). While embryonic and larval zebrafish previously have been used to study the toxic effects of MeHg exposure at the whole body (13, 14) and at the molecular level (15), there are no available data on MeHg uptake and accumulation with respect to different tissues and organs. Such information is critical to properly integrate information on tissue and cell specific impacts of Hg with uptake and accumulation of the metal at the whole animal level. We present herein a novel approach to the investigation of heavy-metal toxicity using synchrotron x-ray fluorescence imaging (16) to directly image metal localization within zebrafish larvae and to observe remarkable differential accumulation of organic Hg using this technique. Strikingly, we find that both methyl and ethyl Hg derivatives are concentrated to the greatest degree in lens epithelium with lower levels present in the brain, optic nerve, and other organs. Our work suggests that direct effects on ocular tissue play a role in the disruption of visual processes by Hg (17). Furthermore, synchrotron x-ray fluorescence imaging is appli-

cable to any heavy metal and is expected to have broad applications in vertebrate molecular toxicology including testing of new chelation therapy drugs.

Results and Discussion

The accumulation pattern of MeHg in live zebrafish was determined by exposing larvae at 3.5 days post fertilization (dpf) to waterborne MeHg-L-cysteine [$\text{CH}_3\text{Hg-S-(L-Cys)}$] for 24 h before imaging. At the end of the exposure all larvae were alive although mostly moribund. During the imaging experiment the larvae were anesthetized with tricaine (ethyl 3-aminobenzoate methanesulfonate salt) and then immobilized in 1% low melting-point agarose gel. The elemental distribution maps were obtained by spatially rastering the live fish through a microfocused synchrotron x-ray beam at 10 μm and monitoring the Hg L_{α} , Ca and Zn K_{α} fluorescence intensities. Fig. 1 shows images of two intact MeHg-treated fish positioned with dorsal and lateral presentations to the x-ray beam. The images clearly show that Hg accumulation is neither homogenous, nor does it show the same distribution as either Zn or Ca. Regions of relatively high Hg include brain, gastrointestinal tract, and particularly the eye lens. Whereas the presence of Hg in the first two tissues is expected, the relatively high accumulation of MeHg in the eye lens is unprecedented. Both micro-Hg L_{III} near-edge spectra (18) of the lens region of treated fish and bulk spectra of large numbers of similarly treated fish were consistent with a local Hg coordination that was the same as the administered compounds (not illustrated), although we note that the high x-ray doses involved in recording near-edge spectra killed the fish. The highest Ca levels corresponded to the developing otoliths in the otic capsule. The Zn distribution reflects the general pigmentation pattern of the zebrafish, including high levels in the retina, with additional elevated Zn levels in the yolk sac.

More detailed quantitative information on Hg deposition was obtained by imaging transverse ($\approx 6 \mu\text{m}$) sections of Hg-exposed fish. We used newly hatched larvae (3.5 dpf) that were exposed to 0.2, 2, and 100 μM $\text{CH}_3\text{Hg-S-(L-Cys)}$ for 84, 36, and 24 h, respectively, or to 100 μM ethylmercury 2-thiosalicylate (thimerosal) for 20 h to additionally determine whether the accumulation pattern depends on the chemical form of the organic Hg. Following the exposures the larvae were fixed, embedded in methacrylate, and sectioned on a microtome. Of two adjacent

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Fig. 6. High resolution elemental distributions of zebrafish head. Head section from 4.5-dpf zebrafish larva treated with 100 μ M MeHg-L-cysteine for 24 h. (A) histological image, (B) mercury distribution using x-ray fluorescence imaging at 2.5 μ m resolution, and (C) Hg (green) superimposed on S (red) and Zn (blue). Quantities of the different elements are plotted on arbitrary scales.

agents (34) and can be applied to the study of essential metals and other elements of interest during normal development.

Materials and Methods

Animal Care and Embryo Collection. Adult fish were kept at 28°C in carbon-filtered tap water, with a photoperiod of 14 h. Embryos were collected and staged following standard procedures (35). After collection, embryos and larvae were reared in 25-mL Petri dishes with system water changed daily.

Reagent and Treatment Solutions. MeHg hydroxide (1M aqueous solution), L-cysteine, thimerosal, and tricaine were purchased from Strem Chemicals Inc., Alfa Aesar, and Sigma-Aldrich. The 1 M MeHg hydroxide solution was diluted in triple-distilled water to obtain a 40 mM stock solution. An equimolar stock solution of L-cysteine was prepared in 30 mM phosphate-buffered saline (PBS). A 10 mM stock solution of MeHg-L-cysteine complex was prepared by mixing suitable aliquots of 40 mM MeHg hydroxide and 40 mM L-cysteine solutions (molar ratio Hg:Cys of 1:1.2). The mixture was further diluted in 30 mM PBS as needed. Treatment solutions of MeHg-L-cysteine complex were made from dilutions of the stock in carbon-filtered tap water. A 10 mM stock solution of thimerosal was prepared in 30 mM PBS. The exposures were conducted in sterile 25-mL Petri dishes. All stock and treatment solutions were freshly made before each exposure.

MeHg and Thimerosal Treatment. For the exposure, newly-hatched, 3.5-dpf larval zebrafish were placed in 25-mL Petri dishes containing 200 nM to 100 μ M MeHg-L-cysteine or 100 μ M thimerosal. Untreated control dishes were included in each experiment. Two replicate treatments (2×25 larvae) were carried out for each compound. Exposures were at 28°C for 24–84 and 20 h for MeHg-L-cysteine and thimerosal, respectively, and included the 10-h dark period. The water/treatment solutions were changed daily for exposures >24 h. After the exposure, the larvae were rinsed several times in fresh carbon-filtered water to remove any remaining Hg.

Live Sample Preparation. Exposed and rinsed larvae were placed in a new Petri dish containing anesthetic solution (0.016% tricaine) (36). The anesthetized fish were transferred into a new dish containing 1% low melting point agarose gel (with 0.016% tricaine) for immobilization. Such mounted fish were alive for >5 h as evaluated by regular checks of heart beat using microscopy. Before imaging, a small block was cut out around the selected fish and mounted between two 6.3- μ m films of polypropylene (SPEX CertiPrep).

Preparation of Sections. For the tissue-section imaging, the larvae were fixed in 4% paraformaldehyde for 2 h at rt. The fixed larvae were dehydrated in a graded series (0%, 25%, 50%, 75%, and 100%) of ethanol in phosphate buffered saline + Tween 20 (PBST) buffer (30 mM PBS, 0.1% Tween 20) for 5 min each, and stored until needed in 100% ethanol at –20°C. For sectioning, the fixed and dehydrated larvae were rehydrated into PBST by 5 min washes in the reversed ethanol gradient. Selected larvae were properly oriented and embedded in 1% agarose gel. The blocks of gel containing the fish were cut out and dehydrated in 100% ethanol by gentle shaking for 5–8 h at 4°C. Next, the blocks were infiltrated overnight on a rotating stirrer at 4°C with JB-4 (Polysciences Inc.) catalyzed solution A (10 ml solution A: 0.125 g catalyst). The infiltration process with fresh infiltration solution continued on the following day for 5–6 h. The infiltrated samples were placed in embedding molds filled with a mixture of JB-4 solution B and fresh infiltration solution (1 ml solution B: 25 ml infiltration solution) and left overnight at 4°C to polymerize. Sections of 6- μ m thickness were cut on a microtome using glass knives. Of two adjacent sections, one was mounted on a glass slide and stained with methylene blue, while the other, intended for synchrotron x-ray fluorescence imaging, was fixed on a Thermanox plastic coverslip (Gibco BRL) without any further processing. Before imaging, the plastic coverslip was mounted between the two 6.3- μ m films of polypropylene.

X-ray Fluorescence Imaging. X-ray fluorescence images were collected at the Stanford Synchrotron Radiation Laboratory (SSRL) using beamlines 9–3 and 2–3, and at the Canadian Light Source (CLS, Saskatoon, SK, Canada) using the Hard X-ray Microanalysis Beamline (HXMA). The incident x-ray energy was set to 12.7 keV and the Hg $L_{\alpha 1,2}$ and Ca, S, and Zn $K_{\alpha 1,2}$ fluorescence lines, as well as the intensity of the total scattered X-rays, were monitored using a silicon-drift Vortex detector (SII NanoTechnology USA Inc.).

Experiments on SSRL beamline 9–3 used a Si (220) double crystal monochromator and Rh-coated silicon mirrors for focusing and harmonic rejection, with the storage ring Stanford Positron Electron Accelerating Ring (SPEAR) containing 90–100 mA at 3.0 GeV. The microfocused beam of 10 μ m was provided by a tapered glass capillary. The incident and transmitted x-ray intensities were measured with nitrogen-filled ion chambers. Samples were mounted at 90° to the incident x-ray beam and were spatially rastered in the microbeam using a Newport PM500 stage. Beam exposure was <1.5 sec per pixel for live zebrafish and $\approx$ 3.5 sec per pixel for sections.

Experiments on SSRL beamline 2–3 used a Si (220) double-crystal monochromator with harmonic rejection achieved by detuning one monochromator crystal to 50% peak intensity. The microfocused beam of 2.5 μ m was provided by a Kirkpatrick-Baez mirror pair (Xradia Inc.) with the sample at 45° to the incident x-ray beam. Other experimental details were similar to those of SSRL beamline 9–3 (described above).

Experiments at the CLS HXMA beamline used a Si (220) double crystal monochromator and Rh-coated collimating and focusing beamline mirrors. Harmonic rejection was achieved by detuning one monochromator crystal to 50% peak intensity, with the storage ring operating at 200–250 mA at 2.9 GeV. The microfocused beam of 12 μ m was provided by a Kirkpatrick-Baez mirror pair (fabricated at CLS), with the sample at 45° to the incident beam. Other experimental details were similar to those at SSRL (described above).

Data Analysis. The data were processed using SMAK software (www-ssrl.slac.stanford.edu/~swebb/smak.htm). Windowed Hg $L_{\alpha 1,2}$ and Zn $K_{\alpha 1,2}$ fluorescence data were corrected for scatter as previously described (37). The light element fluorescence signal in each image was corrected by calculating the average background intensity using the pixels outside the tissue and

subtracting it from all of the pixels in the image. Quantities of Hg per pixel were calibrated using two certified highly uniform thin film standards on 6.3 μm -thick mylar substrates (Micromatter Co.), containing 16.3 and 17.1 $\mu\text{g}/\text{cm}^2$ Au and TiCl₂, respectively. The scatter-corrected Au and Ti L _{α 1,2} fluorescence intensities were used to interpolate a Hg L _{α 1,2} fluorescence intensity and this was applied to the scatter-corrected Hg distribution maps to obtain the quantities of Hg per pixel in $\mu\text{g}/\text{cm}^2$. Quantification was not possible for images of intact fish because the thickness is not known accurately. The attenuation of the incident and fluorescent x-rays is also non-trivial and means that a signal from the back of the sample is attenuated in a complex way by the sample. Sensitivity will depend both on the beamline used and on count-times per pixel, and was estimated using standard methods (38) to be 0.02 $\mu\text{g}/\text{cm}^2$ for the quantified data collected on SSRL beamline 9–3. Similarly, signal-to-noise ratios were estimated, as peak signal intensity divided by the

full-width half-maximum noise levels, to be ≈ 25 for typical data sets (SSRL, 9–3, 3.5 sec per pixel), although this improves with longer count times per pixel.

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- Clarkson TW, Magos L (2006) The toxicology of mercury and its chemical compounds. *Crit Rev Toxicol* 36:609–662.
- Simmons-Willis TA, Koh AS, Clarkson TW, Ballatori N (2002) Transport of a neurotoxicant by molecular mimicry: the methylmercury-L-cysteine complex is a substrate for human L-type large neutral amino acid transporter (LAT) 1 and LAT2. *Biochem J* 367:239–246.
- Hoffmeyer R, et al. (2006) Molecular mimicry in mercury toxicology. *Chem Res Toxicol* 19:753–759.
- Gasso S, et al. (2001) Antioxidant compounds and Ca²⁺ pathway blockers differentially protect against methylmercury and mercuric chloride neurotoxicity. *J Neurosci Res* 66:135–145.
- Vicente E, et al. (2004) Cerebrospinal fluid S100B increases reversibly in neonates of methyl mercury-intoxicated pregnant rats. *Neurotoxicology* 25:771–777.
- Limke TL, Heidemann SR, Atchison WD (2004) Disruption of intraneuronal divalent cation regulation by methylmercury: are specific targets involved in altered neuronal development and cytotoxicity in methylmercury poisoning? *Neurotoxicology* 25:741–760.
- Johansson C, et al. (2007) Neurobehavioural and molecular changes induced by methylmercury exposure during development. *Neurotox Res* 11:241–260.
- Qu H, Syversen T, Aschnew M, Sonnewald U (2003) Effect of methylmercury on glutamate metabolism in cerebellar astrocytes in culture. *Neurochem Intl* 43:411–416.
- Hwang G-W, Furuchi T, Naganuma A (2002) A ubiquitin-proteasome system is responsible for the protection of yeast and human cells against methylmercury. *FASEB J* 16:709–711.
- Hwang G-W, Ishida Y, Naganuma A (2006) Identification of F-box proteins that are involved in resistance to methylmercury in *Saccharomyces cerevisiae*. *FEBS Lett* 580:6813–6818.
- Furuchi T, Hwang G-W, Naganuma A (2002) Overexpression of the ubiquitin-conjugating enzyme Cdc34 confers resistance to methylmercury in *Saccharomyces cerevisiae*. *Mol Pharmacol* 61:738–741.
- Hill AJ, Teraoka H, Heideman W, Peterson RE (2005) Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicol Sci* 86:6–19.
- Samson JC, Shenker J (2000) The teratogenic effects of methylmercury on early development of the zebrafish, *Danio rerio*. *Aquat Toxicol* 48:343–354.
- Samson JC, Goodridge R, Olobatuyi F, Weis JS (2001) Delayed effects of embryonic exposure of zebrafish (*Danio rerio*) to methylmercury (MeHg). *Aquat Toxicol* 51:369–376.
- Gonzalez P, Dominique Y, Massabuau JC, Boudou A, Bourdineaud JP (2005) Comparative effects of dietary methylmercury on gene expression in liver, skeletal muscle, and brain of the zebrafish (*Danio rerio*). *Environ Sci Technol* 39:3972–3980.
- Pickering IJ, George GN (2007) X-ray absorption spectroscopy imaging of biological tissues. *AIP Conference Proceedings* 882:311–315.
- El-Sherbeeney AM, Odom JV, Smith JE (2006) Visual system manifestations due to systemic exposure to mercury. *Cutan Oncol Toxicol* 25:173–183.
- Harris HH, Pickering IJ, George GN (2003) The chemical form of mercury in fish. *Science* 301:1203.
- Dahm R, Schonthaler HB, Soehn AS, van Marle J, Vrensen GFJM (2007) Development and adult morphology of the eye lens in the zebrafish. *Exp Eye Res* 85:74–89.
- Bloemendal H, et al. (2004) Ageing and vision: structure, stability and function of lens crystallins. *Prog Biophys Mol Biol* 86:407–485.
- Reddy VN (1990) Glutathione and its function in the lens – an overview. *Exp Eye Res* 50:771–778.
- Dove SG (1999) Ontological changes in the crystallin composition of the eye lenses of the territorial damselfish *Parma microlepis* and their possible effects on trace-metal accumulation. *Marine Biol* 134:653–663.
- DuVal G, Grubb BR, Bentley PJ (1987) Mercury accumulation in the eye following administration of methylmercury. *Exp Eye Res* 44:161–164.
- Kairada K, Hirayama K, Yasutake A, Matsumura A, Okamura R (1988) Mercury accumulation in lens following single administration of methylmercury. *Jpn J Ophthalmol* 32:275–280.
- Weber DN, et al. (2008) Selenomethionine reduces visual deficits due to developmental methylmercury exposures. *Physiol Behav* 93:250–260.
- Sabelaish S, Hilmi G (1976) Ocular manifestations of mercury poisoning. *Bull World Health Organ* 53:83–86.
- Harada M (1995) Minamata disease: methylmercury poisoning in Japan caused by environmental pollution. *Crit Rev Toxicol* 25:1–24.
- Saldana M, Collins CE, Gale R, Backhouse O (2006) Diet-related mercury poisoning resulting in visual loss. *Brit J Ophthalmol* 90:1432–1434.
- Sanfeliu C, Sebastia J, Cristofol R, Rodriguez-Farre E (2003) Neurotoxicity of organo-mercurial compounds. *Neurotox Res* 5:283–306.
- Corbin BD, et al. (2008) Metal chelation and inhibition of bacterial growth in tissue abscesses. *Science* 319:962–965.
- Yoon S, Miller EW, He Q, Do PH, Chang CJ (2007) A bright and specific fluorescent sensor for mercury in water, cells, and tissue. *Angew Chem Int Ed* 46:6658–6661.
- Ko S-Y, Yang Y-K, Tae J, Shin I (2006) In vivo monitoring of mercury ions using a rhodamine-based molecular probe. *J Am Chem Soc* 128:14150–14155.
- Tang B, Cui LJ, Xu KH, Tong LL, Yang GW, An LG (2008) A sensitive and selective near-infrared fluorescent probe for mercuric ions and its biological imaging applications. *ChemBioChem* 9:1159–1164.
- George GN, et al. (2004) Mercury binding to the chelation therapy agents DMSA and DMPS and the rational design of custom chelators for mercury. *Chem Res Toxicol* 17:999–1006.
- Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. *Dev Dyn* 203:253–310.
- Westerfield M (1995) *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio)* (University of Oregon Press, Eugene, OR).
- Pickering IJ, Prince RC, Salt DE, George GN (2000) Quantitative, chemically-specific imaging of selenium transformation in plants. *Proc Natl Acad Sci USA* 97:10717–10722.
- Mantler M (2006) in *Handbook of Practical X-Ray Fluorescence Analysis*, eds Beckhoff B, Kanngießer B, Langhoff N, Wedell R (Springer, Berlin).