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Dietary and tissue selenium in relation to methylmercury toxicity

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ABSTRACT

Selenium (Se) supplementation in the nutritionally relevant range counteracts methylmercury (MeHg) toxicity. Since Se tends to be abundant in fish, MeHg exposures alone may not provide an accurate index of risk from fish consumption. Molar ratios of MeHg:Se in the diets and Hg:Se in tissues of exposed individuals may provide a more accurate index. This experiment compared MeHg toxicity in relation to MeHg exposure vs. Hg:Se molar ratios in diets and tissues. Diets were prepared using low-Se torula yeast basal diets supplemented with Na_2SeO_4 to contain 0.1, 1.0, or 10.0 μmol Se/kg (~0.01, 0.08, or 0.8 ppm Se), reflecting low-, adequate-, or rich-Se intakes, respectively. Diets contained either low or high (0.5 μmol or 50 μmol MeHg/kg) (~0.10 or 10 ppm Hg). Sixty weanling male Long Evans rats were distributed into six weight-matched groups (three Se levels × two MeHg levels) that were supplied with water and their respective diets *ab libitum* for 18 weeks. No Se-dependent differences in growth were noted among rats fed low-MeHg diets, but growth impairments among rats fed high-MeHg were inversely related to dietary Se. After 3 weeks on the diet, growth impairments were evident among rats fed high-MeHg with low- or adequate-Se and after 10 weeks, rats fed low-Se, high-MeHg diets started to lose weight and displayed hind limb crossing. No weight loss or hind limb crossing was noted among animals fed high-MeHg, rich-Se diets. Methylmercury toxicity was not predictable by tissue Hg, but was inversely related to tissue Se ($P < 0.001$) and directly related to Hg:Se ratios ($P < 0.001$). Methylmercury–selenocysteine complexes (proposed name; pseudomethionine) appear likely to impair Se bioavailability, interrupting synthesis of selenium-dependent enzymes (selenoenzymes) that provide antioxidant protection in brain. Therefore, selenoenzymes may be the molecular target of methylmercury toxicity.

1. Introduction

Mercury (Hg) is listed by the International Program of Chemical Safety as one of the most dangerous chemicals in the environment (Gilbert and Grant-Webster, 1995). Humans typically encounter methylmercury (MeHg) from eating fish and seafoods. Since all forms of fish contain at least traces of methylmercury, low-level exposure is essentially ubiquitous. Although risks of very high methylmercury exposures are indisputable, the potential harm from consuming fish and seafoods containing methylmercury continues to be controversial. To protect the public from harm, federal and state agencies recommend women restrict their fish consumption during pregnancy. Although this advice will clearly limit risks associated with maternal methylmercury exposure, it may also deny beneficial effects of seafood consumption on child development (Hibbeln et al., 2007; Budtz-Jørgensen et al., 2007; Lederman et al., 2008). In this regard, selenium (Se), a nutrient that is abundant in ocean fish and known to be highly active in counteracting mercury toxicity (see reviews by Cuvin-Aralar and Furness, 1991; Yang et al., 2008), deserves special consideration.

This counteracting effect appears to arise because of the high binding affinities between mercury and selenium, whereby methylmercury covalently binds selenium in the active sites of selenium-dependent enzymes (selenoenzymes), thereby inhibiting their activity (Seppanen et al., 2004). Supplemental dietary selenium apparently replaces the selenium lost to intracellular mercury binding, thereby maintaining normal selenoenzyme activities. Maternal mercury exposure severely compromises the distribution of maternal selenium across the placenta (Parizek et al., 1971). However when maternal selenium status is enhanced by feeding rich levels of dietary selenium, toxic effects of methylmercury resulting in depressed selenium distribution and

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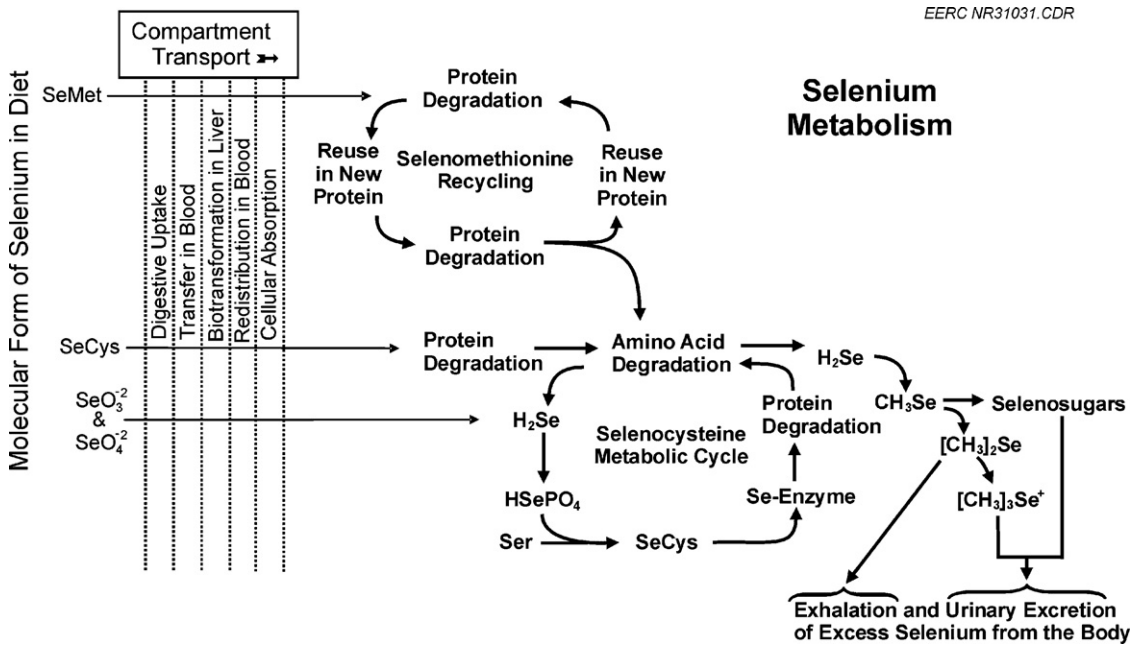

Fig. 1. Metabolic cycles of selenomethionine, selenocysteine, and inorganic selenium.

diminished selenoenzyme activities in brains of the exposed offspring are prevented and neurological signs of fetal intoxication are alleviated (Watanabe et al., 1999a,b).

The biochemical distinctions between the amino acids selenocysteine (SeCys; actively synthesized in animal tissues) and selenomethionine (SeMet; predominant form present in plants) are particularly significant (Kohrle et al., 2005). Protein synthesis cycles make no distinctions between SeMet and methionine (see Fig. 1), but upon eventual degradation, the selenium freed from SeMet becomes available for *de novo* SeCys synthesis in animal cells.

Selenocysteine is the 21st proteinogenic amino acid that is specifically encoded into unique proteins (Hatfield et al., 2006). In contrast to other amino acids, SeCys is not reused in subsequent cycles of protein synthesis, but must be degraded to release inorganic selenium for synthesis of SeCys that is specifically emplaced in the active sites of selenoenzymes (Hatfield et al., 2006). Several selenoenzymes perform apparently indispensable antioxidant functions in the brain and neuroendocrine system (Chen and Berry, 2003; Schweizer et al., 2004; Whanger, 2001; Kohrle et al., 2000, 2005) and some have newly described functions (Ferguson et al., 2006; Novoselov et al., 2007; Dikiy et al., 2007) including redox control of an abundant class of brain proteins. These functions appear to explain why all forms of animal life that possess nervous systems also express and selectively preserve selenoenzyme activities in brain and neuroendocrine tissues (Behne et al., 2000; Sun et al., 2001).

Methylmercury bound to cysteine (MeHg–Cys) appears to be the predominant form in fish tissues (Harris et al., 2003) The MeHg–Cys adduct resembles methionine biochemically, and appears to cross the placental and blood–brain barriers as a molecular mimic of methionine (Aschner and Clarkson, 1989; Bridges and Zalups, 2005). However, because selenium's binding affinity with mercury is approximately a million times greater than sulfur's (Dyrssen and Wedborg, 1991) thermodynamics promote formation of HgSe adducts. Therefore, intracellular methylmercury tends to diminish the amount of selenium that is biologically available for normal selenoenzyme synthesis, especially as Hg:Se molar ratios approach or exceed a 1:1 stoichiometry. Protective effects of dietary selenium against mercury toxicity (Ganther et al., 1972; Prohaska and Ganther, 1977; Watanabe et al., 1999a,b; Ralston et al., 2007; Yang et al., 2008), and in prevention of mercury-dependent inhibition of selenoenzyme activities (Prohaska and Ganther, 1977; Watanabe et al., 1999b), appear to occur because additional dietary selenium is able to offset the selenium sequestered by mercury and thereby maintain normal antioxidant activities of brain selenoenzymes.

This study explores the interactions between dietary methylmercury and selenium to examine dietary selenium's role in counteracting mercury toxicity. Previous studies of methylmercury–selenium interactions in animal models have not examined the role of Hg:Se molar ratios in the diets and tissues of the exposed animals. It appears that both the absolute and relative amounts of methylmercury and selenium present in the diet contribute to the associated risks or benefits of its consumption. Therefore, to determine methylmercury and selenium's interactions and effects on one another's tissue distributions and their relative influences on toxicity, this study was designed to emulate dietary co-exposures using the normal dietary range of selenium intakes and methylmercury concentrations that represent approximately normal vs. toxic exposures.

2. Methods

2.1. Diet preparation

Diets used in this study were based on the AIN-93G formula for laboratory rodents customized through the use of low-Se torula yeast as the protein source (Teklad, Madison, WI) to provide a low selenium intake (Reeves et al., 2005). The basal diets were used as received or augmented with Na_2SeO_4 to levels reflecting the nutritionally relevant range of dietary selenium concentrations. Diets were low-Se, adequate-Se, or rich-Se containing ~0.1, 1.0, or 10.0 μmol Se/kg (~0.01, 0.08, or 0.8 ppm Se, respectively). The 10.0 μmol Se/kg concentration in the rich-Se diet is well below the 25 μmol Se/kg (~2 ppm Se) level that is accepted as a high but beneficial Se concentration in nutrition studies.

Diets were specially designed by Teklad to contain 1% less safflower oil than the AIN-93 recommended levels. This purposeful

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omission allowed the methylmercury solutions to be added along with safflower oil at 1% (w/w) basis and mixed in the diet to attain the recommended 70 g safflower oil/kg diet level. Each of the Se diets was prepared with safflower vehicle or methylmercury supplemented safflower to result in either low or high amounts of methylmercury: ~0.5 or 50 μmol of MeHg/kg (~0.1 or 10 ppm Hg). On each day that diets were prepared, a fresh preparation of MeHgCl (Sigma–Aldrich, St. Louis, Missouri) was dissolved in safflower oil and continually mixed to ensure homogeneous distribution. Diets were prepared in 1.5 kg batches using 15 g of oil distributed over 1485 g powdered diet and mixed for 5 min to obtain an even distribution of methylmercury. After mixing, representative samples of these diets were set aside to be combined at the end of the study for total Hg and Se analysis. Diets that had been prepared with no added methylmercury contained 0.51 ± 0.35 μmol Hg/kg (0.10 ± 0.07 ppm Hg). This level of background Hg contamination is similar to what has previously been observed in laboratory animal diets (Weiss et al., 2005) Therefore, the actual Hg concentrations in the diet after the methylmercury additions were ~0.5 and 50 μmol MeHg/kg (~0.1 and 10 ppm Hg).

2.2. Subjects

The research protocol for this study was reviewed and approved by the Institutional Animal Care and Use Committee and animal health status was monitored twice weekly throughout the study period. Weanling male Long Evans rats (Charles River, Wilmington, MA) were individually maintained in polyethylene plastic cages in an animal care facility with room temperature maintained at 26 °C, humidity at 53%, and a 12-h light–12-h dark cycle. After 3 days of equilibration on laboratory chow, rats were weighed and distributed into six weight-matched groups (10 rats/group) with approximately equivalent mean body weights (128.2 ± 1.5 g). Groups were randomly assigned to one of six dietary treatments. Rats were provided with deionized water and their designated diets *ab libitum*. From study day 1 onward, food consumption was measured twice a week and body weights of the rats were measured weekly to recognize and quantify onset and development of anorexia, growth inhibition, and impaired growth efficiency or weight loss.

To monitor methylmercury toxicity and the effects of dietary selenium status, individual body weights were measured weekly and plotted to reveal dose- and time-dependent effects. Rats were assessed for hind limb crossing every week until signs of hind limb crossing were noted on week 10. After week 10, rat motor functions were assessed twice a week (Day et al., 2005). Hind limb crossing evaluations were done by "blinded" assessors that were given the rats in a random sequence without information that would indicate the treatment group of the animal being assessed. A score of zero was assigned to healthy animals without perceptible signs of hind limb crossing. A score of "−1" was used to indicate a noticeable spasticity of hind limb movement with one leg pulled in, a "−2" indicated both legs pulled in, but not crossed, and a "−3" indicated hind limbs were fully crossed.

On the day of termination, rats were intraperitoneally injected with ketamine–rompun (mixed 1:1.37) at a constant 1 μL/g body weight dosage. Syringes prepared with K_2EDTA were used to collect 10–15 mL of blood via cardiac exsanguination. Blood samples were mixed by repeated inversion and stored frozen until analyzed. Liver, kidney, and brains were removed, cleared of exogenous materials, rinsed in normal saline, patted dry, wrapped in pre-labeled aluminum foils, and flash-frozen in liquid nitrogen. Samples were stored at −85 °C until analyzed for mercury and selenium contents.

2.3. Sample analysis

Blood samples were analyzed on a wet-weight basis but liver, kidney, and brain samples were lyophilized and homogenized prior to sampling for analysis. Approximately 0.5 g of blood, and 0.2 g of powdered tissue samples were weighed into single-use, trace element-free 50-mL digestion tubes (Environmental Express, Mt. Pleasant, SC), with every 10th sample being prepared in duplicate and with elemental spike recovery samples accompanying each batch. Each digestion batch included analysis blanks and certified reference materials (dogfish muscle certified reference material DORM-2, National Research Council of Canada, Ottawa, Ontario, Canada). Samples were treated with 5 mL of HNO_3 (Fisher Trace Metal Grade, Fisher Scientific, www.fishersci.com) and heated at 85 °C in deep cell hot blocks (Environmental Express) for 24 h in capped tubes to preserve samples from trace element contamination. Samples were cooled, 1.5 mL of 30% H_2O_2 (Fisher Certified A.C.S., Fisher Scientific) was added, and samples were recapped and returned to heating in the dry block at 85 °C for 8 h more. Samples were cooled, and 15 mL of 12N HCl (Fisher Trace Metal Grade, Fisher Scientific) were added. Samples were heated at 90 °C for 90 min to reduce SeVI to SeIV. Samples were cooled and diluted to 50 mL with double-distilled water. Samples were further diluted into instrumental calibration ranges and analyzed for mercury content by cold-vapor atomic absorption spectrophotometry using a CETAC M-6000A (CETAC Technologies, Omaha, NE), and selenium was analyzed by hydride generation atomic absorption spectroscopy using a PS Analytical Dual Millennium Excalibur (PS Analytical, Deerfield Beach, FL). Before data from sample analysis runs were entered into the database, methylmercury and selenium concentrations in sample digestion blanks and elemental recoveries in samples of certified reference materials were evaluated to qualify the analysis batch data for inclusion.

2.4. Data comparisons and statistical analysis

In order to simplify evaluation of selenium-specific nutritional benefits in relation to potential methylmercury exposure risks presented, the Selenium-Health Benefit Value (Se-HBV) proposed by Kaneko and Ralston (2007) has been calculated for the diets used in this study. The Se-HBV incorporates the absolute and relative amounts of selenium and mercury in the diet and provides an index that is easily interpreted. The sign of the calculated Se-HBV indicates the expected health benefits (if positive values are obtained) or health risks (if negative values result) and the magnitude of the values obtained are proportional to the expected benefits or risks.

Total mercury and selenium mass concentrations (parts per million) in each sample were converted to molar concentrations (μmol/kg). Molar Hg:Se ratios were calculated by direct division of the individual molar concentrations in diets and tissues. Relative toxicity was estimated by direct subtraction of the individual rat's final body weight from the average body weight of the control (normal-Se, low-MeHg) group and times −1. The Se-HBVs for the rat diets were calculated as: Se-HBV = (μmol Se/kg × (μmol Se/kg/μmol MeHg/kg)) − (μmol MeHg/kg × (μmol MeHg/kg/μmol Se/kg)) as described in Kaneko and Ralston (2007).

Mean concentrations of mercury and selenium in tissues were estimated for each treatment group, but most of these had non-normal distributions, so 95% bootstrap confidence intervals for all treatment group means were estimated using Stata 9. Linear model ANOVAs were also estimated for selenium and mercury concentrations in blood, kidney, liver, and brain to determine the statistical significance of various own-effects of mercury and selenium dietary groups and especially to determine the impacts of

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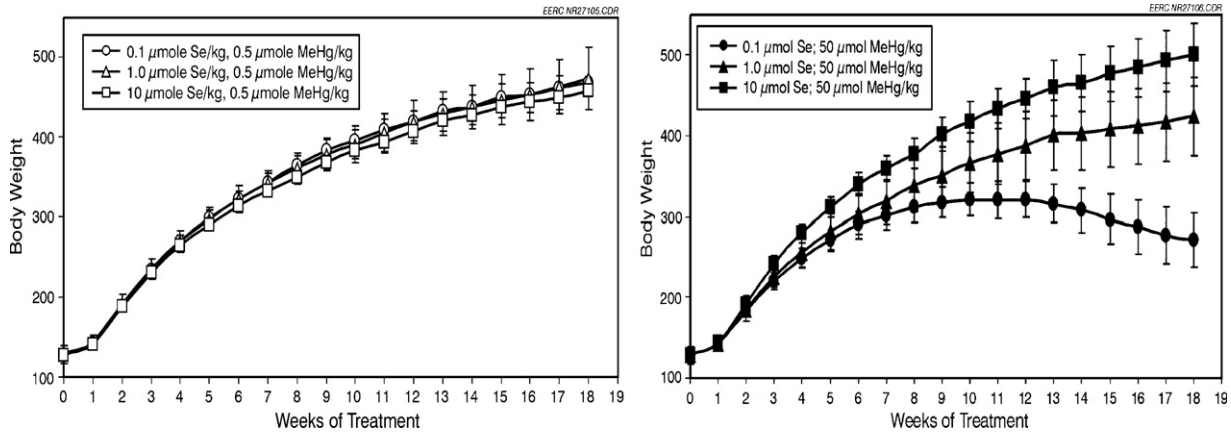

Fig. 2. Growth of groups of rats fed low, normal, or enriched dietary Se in the presence of 0.5 μmol MeHg/kg (left panel) and 50 μmol MeHg/kg (right panel). Data depicted are means ± standard deviations for group body weights (in g) at the times indicated.

Hg × Se interactions. Regression ANOVA was necessary because of substantial heteroskedasticity in many sample groups. The overall *F*-statistic for each of these ANOVAs was significant at $P < 0.0001$, and R^2s ranged from a low of 0.914 to a high of 0.981. All ANOVAs for toxicity indices influenced by mercury, selenium, and interactions were tested for joint significance using Bonferroni–Holm step-down tests.

3. Results

3.1. Effects of dietary MeHg and Se on growth and hind limb crossing

Rats fed low-MeHg, adequate-Se diets were used as the control group for comparing growth of all other groups. No differences in growth were seen among rats fed low-MeHg diets (Fig. 2), but growth of rats fed high-MeHg diets was very dependent upon their dietary Se intakes. Among rats fed high-MeHg diets, impaired growth became evident in groups fed low- or adequate-Se after 3 weeks on the diets. Growth of rats fed high-MeHg, low-Se diets was more severely affected than the other groups, and by the 10th week, rats in this group started to lose weight and individuals first demonstrated hind limb crossing (Fig. 3). Signs of hind limb crossing in this group grew increasingly severe and became uniform at the highest level from week 16 on. The growth of rats fed adequate-Se, high-MeHg diets also diminished relative to the control group, but less severely than in the case of the low-Se group and no signs of impaired motor control were evident during the study. Growth of rats fed rich-Se, high-MeHg diets was completely unimpaired and no signs of impaired motor control were evident. The study was terminated when deaths among rats fed high-MeHg low-Se diets began to occur at the start of week 18. One rat from the high-MeHg, low-Se group died 3 days before the end of the study, but since it had apparently expired just shortly before it was noticed, it was possible to collect its tissues (other than blood). These were analyzed and compared with the other rats from this group and found compatible with other data from its group. The mercury and selenium concentrations in its tissues were within 1 S.D. of the observed means, so analysis data from this rat was included in the group means. Another rat from this group that was observed to be close to death was euthanized and its tissues collected on day 124. Its blood and tissues were included in the analysis data for its treatment group. Rats were terminated over a 3-day period with the second day being day 126 of the study.

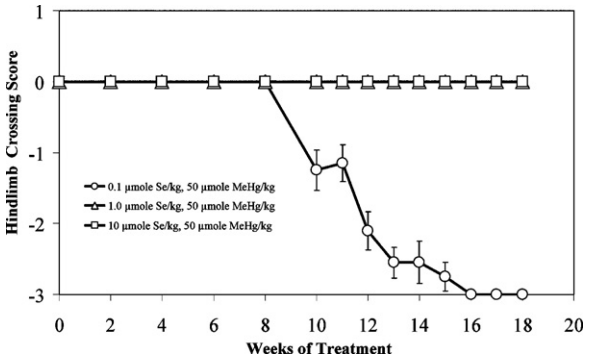

Fig. 3. Hind limb crossing scores of rats fed low-, normal-, or rich-Se diets supplemented with 50 μmol MeHg/kg. Data depicted reflect group means ± S.E.M. for hind limb crossing at the times indicated.

3.2. Effects of dietary MeHg and Se on mercury and selenium distributions in tissues

The mercury and selenium concentrations shown in Table 1 are on a wet-weight basis for blood, while those for all other tissues are on a dry-weight basis. Observed concentrations of mercury and selenium in standard reference materials analyzed alongside all sample batches were within laboratory reference ranges. The limits of detection in tissues were 0.05 ppb for total mercury and 0.1 ppb for total selenium. The mercury and selenium concentrations shown in Table 1 are on a wet-weight basis for blood, while those for tissues are on a dry-weight basis.

Consistent with findings in other studies, blood and the kidney and liver somatic tissues reflected dietary selenium intakes, but brain selenium was homeostatically protected from major changes in concentration, both up or down, in the 0.5 μmol mercury group but was sensitive to dietary MeHg in the 50 μmol MeHg/kg group. Blood selenium concentrations were influenced by dietary; Se (1.0) ($P < 0.001$), Se (10.0) ($P < 0.001$), and two significant MeHg × Se interactions, 50 × 1.0 ($P < 0.001$) and 50 × 10 ($P < 0.001$) were noted. Concentrations of selenium in kidneys were influenced by dietary MeHg (50) ($P < 0.001$), dietary Se (1.0) ($P < 0.001$), and Se (10.0) ($P < 0.001$), and MeHg × Se interactions at (50 × 1.0) ($P < 0.001$) and (50 × 10) ($P < 0.001$). Liver selenium concentrations were influenced by dietary MeHg (50) ($P < 0.002$), Se (1.0) ($P < 0.001$), Se (10) ($P < 0.001$), and MeHg × Se interactions at (50 × 1.0) ($P < 0.001$) and (50 × 10) ($P < 0.001$). Selenium concentrations in brain were influenced by dietary Se (1.0) ($P < 0.045$), Se (10.0) ($P < 0.001$), and two Hg × Se interactions at (50 × 1.0) ($P < 0.001$) and (50 × 10) ($P < 0.001$).

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Table 1
Diet-dependent effects on tissue selenium and mercury[a,b]

MeHg (μmol/kg)	Se (μmol/kg)	MeHg:Se molar ratio	Blood (μmol Se/kg)	Kidney (μmol Se/kg)	Liver (μmol Se/kg)	Brain (μmol Se/kg)
0.5	10	0.05	7.17 ± 0.27	89.84 ± 6.00	42.47 ± 1.71	6.47 ± 0.98
0.5	1	0.5	6.05 ± 0.22	67.19 ± 4.65	35.20 ± 7.64	6.31 ± 0.65
0.5	0.1	5	0.71 ± 0.22	13.36 ± 1.20	1.02 ± 0.14	5.84 ± 0.45
50	10	5	8.18 ± 0.41	992.58 ± 180.50	102.83 ± 6.21	24.90 ± 3.67
50	1	50	4.23 ± 0.18	192.12 ± 33.07	31.86 ± 1.45	5.90 ± 0.69
50	0.1	500	0.79 ± 0.14	16.30 ± 2.12	1.64 ± 0.24	2.73 ± 0.74
ANOVA			Blood	Kidney	Liver	Brain
F			$F(5, 50) = 3643.86$	$F(5, 52) = 320.95$	$F(5, 51) = 406.20$	$F(5, 50) = 37.07$
Prob > *F*			$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
MeHg (50)			n.s.	5.51 ($P = 0.017$)	0.61 ($P = 0.002$)	n.s.
Se (1.0)			5.04 ($P < 0.001$)	51.51 ($P < 0.001$)	35.90 ($P < 0.001$)	0.83 ($P < 0.045$)
Se (10.0)			6.42 ($P < 0.001$)	76.83 ($P < 0.001$)	43.92 ($P < 0.001$)	−2.60 ($P < 0.001$)
MeHg × Se (50 × 1.0)			−1.66 ($P < 0.001$)	118.71 ($P < 0.001$)	n.s.	2.57 ($P < 0.001$)
MeHg × Se (50 × 10.0)			0.98 ($P = 0.001$)	823.62 ($P < 0.001$)	50.84 ($P < 0.001$)	20.69 ($P < 0.001$)
Constant			0.83 ($P < 0.001$)	14.04 ($P < 0.001$)	1.15 ($P < 0.001$)	5.56 ($P < 0.001$)
R^2			0.981	0.964	0.954	0.914
MeHg (μmol/kg)	Se (μmol/kg)	MeHg:Se molar ratio	Blood (μmol Hg/kg)	Kidney (μmol Hg/kg)	Liver (μmol Hg/kg)	Brain (μmol Hg/kg)
0.5	10	0.05	0.02 ± 0.02	0.95 ± 0.14	0.11 ± 0.12	0.06 ± 0.02
0.5	1	0.5	0.04 ± 0.01	1.08 ± 0.25	0.08 ± 0.02	0.12 ± 0.06
0.5	0.1	5	0.03 ± 0.02	0.89 ± 0.16	0.17 ± 0.12	0.15 ± 0.14
50	10	5	384.12 ± 12.09	2444.30 ± 173.44	462.00 ± 35.73	130.24 ± 22.15
50	1	50	354.92 ± 23.30	1569.73 ± 132.05	314.78 ± 53.43	103.14 ± 15.27
50	0.1	500	358.80 ± 34.46	1008.37 ± 126.42	491.31 ± 39.93	127.91 ± 14.90
ANOVA			Blood	Kidney	Liver	Brain
F			$F(5, 50) = 470.1$	$F(5, 52) = 406.9$	$F(5, 51) = 163.1$	$F(5, 46) = 183.4$
Prob > *F*			$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$
MeHg (50)			354.12 ($P < 0.001$)	1060.65 ($P < 0.001$)	478.66 ($P = 0.002$)	130.63 ($P < 0.001$)
Se (1.0)			n.s.	n.s.	n.s.	n.s.
Se (10.0)			n.s.	n.s.	n.s.	n.s.
MeHg × Se (50 × 1.0)			n.s.	490.80 ($P < 0.001$)	−161.43 ($P < 0.001$)	−29.08 ($P = 0.009$)
MeHg × Se (50 × 10.0)			n.s.	1367.68 ($P < 0.001$)	n.s.	n.s.
Constant			n.s.	1.02 ($P < 0.001$)	n.s.	0.16 ($P = 0.003$)
R^2			0.979	0.980	0.941	0.950

[a] 95% bootstrap normal confidence intervals were estimated for total Hg and Se concentrations (dry-weight basis) in tissues collected from animals fed the indicated levels of Se and MeHg.
[b] ANOVA coefficients are differences between means of the indicated classes and the base (deleted) classes.

Mercury concentrations in blood were significantly influenced only by dietary intake of MeHg (50) ($P < 0.001$). Kidney mercury concentrations showed significant influences by the MeHg × Se interactions at (50 × 1.0) ($P < 0.001$) and (50 × 10) ($P < 0.001$). Changes in dietary methylmercury were reflected in liver mercury concentrations which showed significant effects of dietary MeHg (50) ($P < 0.002$) and one MeHg × Se interaction at (50 × 10) ($P < 0.001$). Brain MeHg concentrations were significantly influenced by exposure to dietary MeHg (50) ($P < 0.001$) and showed the influence of a MeHg × Se interaction at (50 × 1.0) ($P < 0.009$).

Table 2
Diet-dependent effects on toxicity and tissue mercury:selenium molar ratios[a]

MeHg (μmol/kg)	Se (μmol/kg)	Calculated Se-HBV[b]	Relative toxicity[c]	Blood Hg:Se	Kidney Hg:Se	Liver Hg:Se	Brain Hg:Se
0.5	10	200	3.18 ± 6.50^{1}	0.00 ± 0.00	0.01 ± 0.00	0.00 ± 0.00	0.01 ± 0.00
0.5	1	2	0.00 ± 5.12^{1}	0.01 ± 0.00	0.02 ± 0.00	0.00 ± 0.00	0.02 ± 0.00
0.5	0.1	−2	1.01 ± 6.64^{1}	0.03 ± 0.00	0.08 ± 0.01	0.19 ± 0.04	0.03 ± 0.01
50	10	−248	-5.65 ± 5.15^{1}	42.63 ± 1.28	2.64 ± 0.06	4.59 ± 0.18	5.30 ± 0.24
50	1	−2,500	10.47 ± 6.35^{2}	84.66 ± 2.09	8.59 ± 0.43	10.83 ± 0.77	17.72 ± 0.70
50	0.1	−25,000	$43.00 \pm 4.66c^{3}$	430.37 ± 36.69	55.54 ± 2.18	285.96 ± 11.49	41.19 ± 1.96
			Relative toxicity[c]	Blood Hg:Se	Kidney Hg:Se	Liver Hg:Se	Brain Hg:Se
R^2 in relation to dietary MeHg			0.158[d]	0.282	0.243	0.108	0.441
F			10.94	22.22	15.70	5.94	40.54
Prob > *F*			$P < 0.001$	$P < 0.0001$	$P < 0.0001$	$P > 0.05$	$P < 0.0001$
R^2 in relation to dietary Se-HBV			0.735	0.845	0.955	0.962	0.827
F			161.05	289.01	1138.57	1359.67	234.22
Prob > *F*			$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P < 0.0001$

[a] 95% normal confidence intervals for group means (molar ratios individually calculated using tissue mercury and selenium molar concentrations).
[b] Selenium-Health Benefit Value index results were rounded to the nearest whole number.
[c] Relative toxicity = growth inhibition times −1. Values with different superscripts are significantly different ($P < 0.01$).
[d] Regressions and *F* statistics calculated as toxicity or tissue Hg:Se relative to dietary μmol MeHg/kg or Se-HBV.

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3.3. Effects of dietary MeHg and Se on methylmercury toxicity

Observed concentrations of diet-dependent effects on tissue Hg:Se molar ratios shown in Table 2 demonstrate an essentially continuous trend of increasing Hg:Se ratios in all tissues. To some extent these tissue ratios reflect the dietary MeHg:Se ratios, but the diet ratios are not entirely consistent with the tissue results. The Se-HBV index calculated for each diet provides a far more consistent indication of the Hg:Se ratios that are observed in the tissues.

Comparisons of relative toxicity to mercury, selenium, and Hg:Se molar ratios in tissues are shown in Fig. 4. Since no toxicity would be expected and none was observed in rats fed low-MeHg diets, relative toxicity as a result of high methylmercury exposures is plotted compared to concentrations of mercury, selenium, and Hg:Se molar ratios observed in the various tissues. Graphs in the first column depict the relative toxicity (growth inhibition) observed in the animals compared to mercury concentrations in the various tissues. Blood, liver, and brain mercury concentrations demonstrated no significant relationships to relative toxicity. Although kidney mercury concentrations were significantly related to relative toxicity, the relationship has a negative slope indicating the greatest toxicity was observed in rats with the lowest kidney mercury concentrations. The relationships between

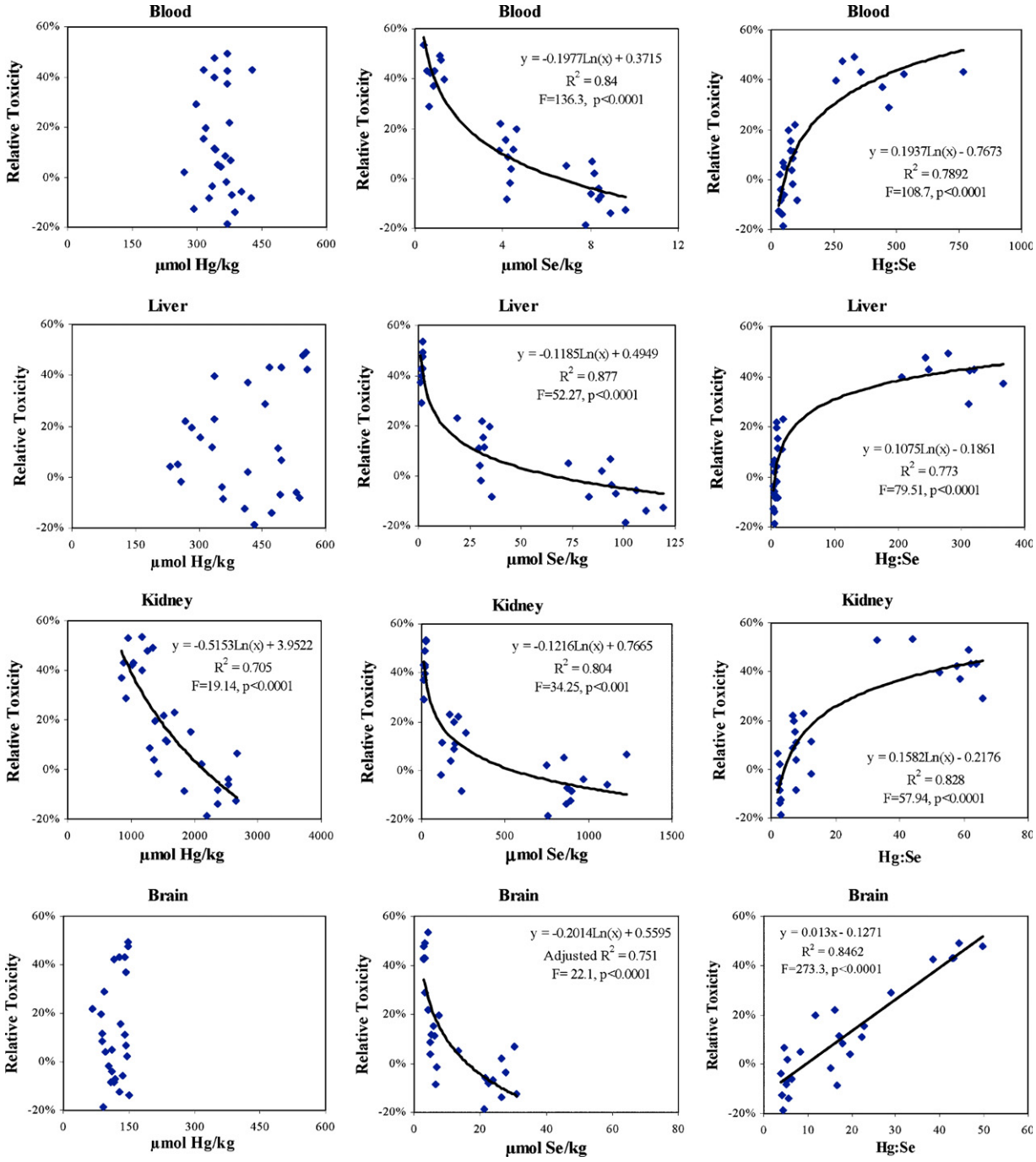

Fig. 4. Relative toxicity, calculated as % growth inhibition in rats fed 50 µmol MeHg/kg relative to growth of control animals fed diets without MeHg supplementation shown in relation to tissue mercury, selenium, and Hg:Se molar ratios.

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Table 3
ANOVAs for toxicity index as influenced by mercury, selenium and interactions in diet, blood and brain

	Coefficients	Standard error	*t*-Statistic	$P > \|t\|$	Bonferroni–Holm
Variable					
Diet[a]					
Diet Hg (50)	42.73	4.15	10.31	0.0000	$P < 0.001$
Diet Se (1.0)	−1.01	4.29	−0.24	0.8140	–
Diet Se (10)	2.17	4.63	0.47	0.6400	–
Diet Hg × Se (50, 1.0)	−32.26	5.88	−5.48	0.0000	$P < 0.001$
Diet Hg × Se (50, 10)	−51.57	5.83	−8.85	0.0000	$P < 0.001$
Constant	1.01	3.40	0.3	0.7670	–
Blood[b]					
Blood Hg	0.11	0.01	10.42	0.0001	$P < 0.001$
Blood Hg × blood Se	−0.02	0.00	−10.00	0.0001	$P < 0.001$
Constant	1.56	1.82	0.85	0.3970	–
Brain[c]					
Brain Hg	0.16	0.07	2.42	0.0190	$P < 0.0167$
Brain Se	−2.13	1.26	−1.69	0.0980	?
Brain Hg × brain Se	0.00	0.01	0.44	0.6620	?
Constant	13.26	7.96	1.66	0.1020	

[a] All coefficients are differences from the base class. The three Bonferroni–Holm *P*-values were jointly significant at those levels. $R^2 = 0.776$; $F(5, 54) = 52.45$; $P < 0.00001$.
[b] There was mild significant collinearity between blood Se and blood Hg × blood Se, but this had surprisingly little effect on either retained coefficient. $R^2 = 0.6978$; $F(5, 52) = 60.05$; $P < 0.00001$.
[c] There was extremely strong collinearity between brain Se and brain Hg × brain Se, which depressed standard errors and inflated *P*-values for those variables. Since deleting either variable would have severely biased these coefficients, they were left in the model, as suggested by Stock and Watson (2003). $R^2 = 0.5405$; $F(3, 47) = 28.23$; $P < 0.00001$.

tissue selenium concentrations and relative toxicity depicted in the middle column all show highly significant ($P < 0.001$) inverse relationships. Relationships between tissue Hg:Se molar ratios and relative toxicity were also highly significant ($P < 0.001$), but in all cases the molar ratios were directly proportional to the relative toxicity observed in the rats.

The relationships between toxicity and the concentrations of selenium and mercury in diet, blood, and brain were particularly important in evaluating causation and identifying and comparing the information value of indices that might reflect relative risk. Table 3 shows impacts of various levels of MeHg, Se, and interactions with diet, blood, and brain, respectively, on a toxicity index derived by comparing the cumulative growth of rats fed various levels of He, Se, and interactions to growth in the overall control group fed diets containing low-MeHg (0.5 μmol MeHg/kg) and adequate-Se (1.0 μmol MeHg/kg). Regression-ANOVA models were run in each case, with special interest in multiple contrasts in results. In the dietary group, there was significance at MeHg (50) ($P < 0.0001$), MeHg (50) × Se (1.0) ($P < 0.0000$), and MeHg (50) × Se (10.0) ($P < 0.000$). All these influences were also jointly significant at $P < 0.001$ in the Bonferroni–Holm test. The coefficients of these variables were 42.73 for MeHg (50), −32.26 for MeHg (50) × Se (1.0), and −51.57 for MeHg (50) × Se (10.0), strongly indicating that the positive influence of MeHg (50) on inducing toxicity was more than offset by the two MeHg × Se interactions.

In the examination of interaction between the toxicity index and blood indices, blood Hg and the interaction of blood Hg × blood Se were highly significant, and again the Bonferroni–Holm test was jointly significant at $P < 0.001$. Although the offsetting influence of the blood Hg × blood Se interactions was again present, this was not as great as in the case of the dietary model. In this case, the blood Hg coefficient was 0.114, and the interaction coefficient was −0.17.

The third panel, containing the brain model results, was problematic because of severe confounding of the brain Se own-effect and the brain Hg × brain Se interaction. Experiments with deleting first brain Se, then brain Hg × brain Se caused radical changes in coefficients of retained variables, as well as dramatic decreases in standard errors of collinear variables. This is a classic statistical dilemma: one is trapped between the knowledge that standard errors would be far smaller if a collinear variable was dropped and the knowledge that dropping them will bias coefficients of remaining variables, perhaps severely. We chose to retain all coefficients in this structural model, thus favoring avoidance of bias over efficiency.

4. Discussion

The results of this study indicate that dietary selenium has a potent influence on toxic effects of chronic methylmercury exposure. Since one of the more prominent and readily observed signs of severe methylmercury toxicity in experimental animals is growth impairment, we compared the body weights of rats exposed to high-MeHg diets to the mean body weight of the control group fed adequate-Se and low-MeHg and used the % growth inhibition as an index of relative toxicity. Based on this index, sensitivity to methylmercury-dependent growth inhibition (relative toxicity) was clearly selenium-dependent, with rats fed low-Se diets showing the greatest toxic effects from high-MeHg exposures. Dietary methylmercury exposure alone was not a reliable index of risk since observed toxic effects were also dependent on whether their selenium status was low, adequate, or rich. Similarly, blood and tissue mercury contents were excellent indicators of methylmercury exposure, but were not reliable predictors of risks of developing toxic symptoms that might accompany that exposure.

In addition to cessation of weight gain, loss of motor coordination is an advanced symptom of MeHg toxicity. The onset of this sign in rats is observed as hind limb crossing. In rats fed low-Se, high-MeHg diets, hind limb crossing and weight loss were first observed after just 10 weeks on the diet. By the end of the 18-week study, motor control in this group had deteriorated to near total disability and/or death, necessitating termination of the study. There were no signs of hind limb crossing in rats fed high-MeHg along with adequate-Se or rich-Se diets at any time during the study period, but the relationships between relative toxicity and tissue Hg:Se regressions shown in Fig. 4 suggest neurotoxicity would also eventually occur in the adequate-Se group.

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During periods of low selenium intake, blood and somatic tissues typically redistribute their selenium to preferentially supply brain and neuroendocrine tissues such as pituitary, adrenals, testes and ovaries (Behne et al., 2000; Burk and Hill, 1993). When selenium is abundant in diet, somatic tissues such as kidney and liver increase their stored reserves, but brain selenium is usually only slightly affected. In this study, dietary selenium intakes were strongly related to tissue selenium as shown in Table 1. Consistent with the findings of previous studies, low-Se, low-MeHg diets resulted in pronounced diminishments in selenium concentrations in blood, kidney, and liver. Blood selenium diminished to ~10% of normal (low-MeHg, adequate-Se group) in rats fed low-Se diets, reflecting their dietary intake. When dietary selenium increased 10-fold, blood selenium only rose ~20%, indicating normal homeostatic control. Rich-Se diets resulted in only a 2.5% increase in brain selenium concentrations compared to rats fed normal (low-MeHg, adequate-Se) diets.

However, when these dietary-Se levels were fed in the presence of high-MeHg, there was a substantial disruption of normal selenium homeostasis. Compared to selenium concentrations in tissues from rats fed normal diets, feeding high-MeHg, rich-Se diets increased selenium accumulations in kidney (~1400%), liver (~200%), and brain (~300%). The substantial increase in selenium accumulations in rats fed high-MeHg, rich-Se diets was previously observed by Newland et al. (2006). Importantly, disruptions of selenium homeostasis were most evident by the effects of high-MeHg, low-Se diets on the distribution of selenium to the brain, which coincides with symptoms of methylmercury toxicity. In comparison, feeding 60 μmol MeHg/kg to rats fed 0.1 μmol Se/kg, brain selenium diminished to 60% of normal after 9 weeks (Ralston et al., 2007), but no neurological signs were apparent. The present study found 18 weeks on a similarly high-MeHg diet caused brain selenium concentrations to drop to 43% of normal, a level of diminishment otherwise only observed by knocking out the selenoprotein P gene in mice (Hill et al., 2003).

Consistent with previous studies, the concentrations of mercury in the tissues of rats in this study generally reflected their dietary MeHg exposure levels. Mercury accumulations in kidney were directly associated with dietary selenium with the highest mercury occurring in the rats fed rich-Se diets, followed by the Se-adequate diets. In liver and brain there was a diminishment in tissue mercury when dietary Se increased from low to adequate levels, and an increase in tissue mercury when dietary Se increased from adequate- to rich-Se. However, as reported by Newland et al. (2006), brain mercury levels increase non-linearly when rats are fed rich-Se diets, resulting in greater tissue mercury accumulations.

In this study, increasing mercury levels in tissues of rats was not necessarily associated with increasing toxicity as measured by growth impairment. The total mercury contents in blood, liver, and brain were unrelated to relative toxicity as measured by growth impairment and mercury contents of kidney were inversely related to relative toxicity (Fig. 4). The highest mercury concentrations occurred in kidneys of rats with no growth impairment, and progressively less mercury was present in kidneys of rats that had increasingly severe toxicity.

There were either inverse relationships or no relationships between methylmercury toxicity and tissue mercury concentrations, but the tissue selenium and Hg:Se ratios shown in Fig. 4 were strongly related to the toxic effects observed in this study. In rats fed high-MeHg, the Hg:Se molar ratios in tissues were directly related to the severity of observed methylmercury toxicity. High Hg:Se molar ratios were associated with toxicity and lower Hg:Se ratios were associated with diminished or no observed symptoms. Therefore, Hg:Se molar ratios in blood and other tissues were the most useful statistical criteria for assessing the relationship between methylmercury exposure and toxicity.

The dietary composition of mercury and selenium was highly informative for predicting risk (Table 3), with dietary Se-HBV providing a more accurate indication of relative risk than dietary MeHg content alone. Similarly, blood mercury and mercury–selenium interactions were highly significant, supporting the concept that both elements should be measured when evaluating potential health consequences from methylmercury exposure. Brain mercury and brain selenium coefficients conformed well with the expectation that mercury disrupts selenium redistribution to the brain and this disruption was distinctly related to methylmercury toxicity.

4.1. Hypothesis regarding mechanism of chronic methylmercury toxicity

Although selenocysteine is structurally analogous to cysteine, it is genetically, biochemically and functionally unique. Cysteine's thiol is protonated ($pK_a \sim 8.5$) at cellular pH and the redox potential of cysteine's sulfur is not as great as that of selenocysteine's selenium. The catalytic activities of selenoenzymes depend upon the biochemistry of the selenocysteine present at their active sites (Behne et al., 2000). The unique capabilities of the various selenoenzymes occur because selenocysteine's high redox potential enables it to conduct reactions that cysteine cannot accomplish. Because selenocysteine's selenol (pK_a 5.7) is ionized at physiological pH, it is more active biochemically than cysteine. Unfortunately, these features that make it so valuable physiologically also make it very vulnerable to methylmercury toxicity.

Methylmercury binding to the selenium moiety of selenocysteine directly inhibits activity of enzymes that perform antioxidant functions (Seppanen et al., 2004). Just as MeHg–Cys structurally resembles methionine and is transported as a molecular mimic of methionine (Aschner and Clarkson, 1989; Aschner et al., 1990; Bridges and Zalups, 2005), MeHg–SeCys formed during inhibition of selenoenzyme activities (Seppanen et al., 2004) closely resembles methionine biochemically. The consequence of MeHg–Cys formation is that methylmercury gains easy entry into the body and is rapidly redistributed across biological barriers. Formation of MeHg–SeCys (proposed term; pseudomethionine) will not only directly inhibit selenoenzyme activities, but the selenium trapped in this form will become unavailable for reuse in future cycles of selenocysteine synthesis, as shown in Fig. 1. Just as selenium from selenomethionine is poorly available for participation in synthesis of SeCys, the selenium in MeHg–SeCys form is unlikely to release a free inorganic selenide because the Hg–Se bond has an affinity constant of 10^{45} (Dyrssen and Wedborg, 1991). Since selenide is the required metabolic precursor for selenocysteine synthesis, the consequence of extensive MeHg–SeCys formation would be a deficit of biologically available selenium for selenoenzyme synthesis.

Biochemically, an irreversible inhibitor is one that forms covalent bonds with components of the active site of an enzyme. Since selenocysteine is the principal active site catalytic component of selenoenzymes, methylmercury is by definition a highly specific irreversible selenoenzyme inhibitor since it forms covalent bonds between its mercury moiety and the selenium of the enzyme's selenocysteine. But in this case the inhibitor–enzyme complex not only abolishes the activity of the inhibited selenoenzyme, it also restricts selenium release from the MeHg–SeCys complex, severely limiting the bioavailability of that selenium for participation in future intracellular cycles of SeCys synthesis.

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4.2. Selenium's role in risk assessment related to methylmercury exposure

Based on findings of this study, blood mercury concentrations do not necessarily provide an accurate indication of risks associated with methylmercury exposure (Fig. 4). In contrast, blood Hg:Se molar ratios were proportional to observed toxicity. Since increasing blood Hg:Se ratios are indicative of increasing risk of harm, assessments of methylmercury exposure should evaluate blood Hg:Se ratios rather than just mercury levels. Impaired growth and disruption of motor function are easily observed indications of severe methylmercury toxicity. However, more sensitive biochemical (Scheuhammer et al., 2008; Basu et al., 2008) and functional (Day et al., 2005; Reed et al., 2006, 2008) indicators of neurotoxicity are needed when assessing more subtle consequences of lower methylmercury exposure.

Exposure to toxicants along with beneficial nutrients in seafoods is a classic example of statistical confounding because both types of agents affect neurodevelopmental outcomes in opposing directions. As Budtz-Jørgensen et al. (2007) indicate, if nutrient-toxicant confounding is not addressed, the harmful effects of contaminant exposures and the beneficial effects of the nutrients will both be underestimated. Choi et al. (2008) measured methylmercury toxicity and blood selenium separately without considering blood Hg:Se ratios. Although they found some protective effects using this approach, examining methylmercury toxicity using blood Hg:Se ratios as the independent variable may substantially improve their analysis.

This study finds that measuring methylmercury exposure is not sufficient to provide accurate and precise information regarding potential risks unless selenium intakes are factored into the evaluation. Blood Hg:Se ratios appear to provide more interpretable and physiologically meaningful indications of risks from methylmercury exposure than blood mercury alone. Consideration of mercury–selenium relationships in diet and tissues of exposed individuals will clarify risk:benefit relationships associated with fish consumption.

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DISCLAIMER

This article has not been subjected to review by the funding agencies and therefore does not necessarily reflect the views of these entities and no official endorsements should be inferred.

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