Methylmercury increases glutamate release from brain synaptosomes and glutamate uptake by cortical slices from suckling rat pups: modulatory effect of ebselen

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Short title: MeHg and glutamate homeostasis in rat brain

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Abstract

During the early post-natal period the brain is extremely sensitive to external agents. Here, we examined the effect of subcutaneous injections of methylmercury (MeHg; 2 mg/kg) during the suckling period – postnatal day (PND) 3-10, 3-17 or 3-24 – on glutamate release from brain synaptosomal preparations and on glutamate uptake by brain cortical slices of rat pups. The possible antagonist effect of ebselen against MeHg effect was also examined at PND 24. MeHg increased the basal (but not K⁺-stimulated) glutamate release and glutamate uptake at PND 24. A strong tendency of increase in the basal glutamate release from synaptosomes (P = 0.088) was observed at PND 17. Ebselen, which did not affect glutamate release and uptake per se, prevented both effects of MeHg. This study indicates that: i) the effect of MeHg on glutamate release could be involved in its toxicity; ii) the increase in the glutamate uptake could represent a pathophysiological response to MeHg-induced glutamate release; iii) the inhibitory effect of ebselen on MeHg-induced glutamate release could be related to its reported neuroprotective effects.

Key words: methylmercury, ebselen, glutamate uptake, glutamate release, cortical slices, synaptosomes.
1. Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system, being associated with essential brain functions, such as learning and memory, neural development, aging and responses to the environmental stimuli (Meldrum, 2000; Ozawa et al., 1998). However, glutamate at high extracellular concentrations can act as an excitotoxin, inducing neuronal injury and death (Lee et al., 1999; Meldrum, 2000; Ozawa et al., 1998). So, the modulation of extracellular levels of glutamate balances its physiological/pathological actions. This modulatory effect is exerted mainly by the high affinity sodium-dependent transporters located at astrocytic cell membranes, which remove glutamate from the synaptic cleft, keeping the extracellular glutamate levels below toxic levels (Anderson and Swanson, 2000; Danbolt, 2001; Maragakis and Rothstein, 2001).

Methylmercury (MeHg) is a highly neurotoxic compound and the mechanism underlying its toxicity is not fully understood (Clarkson, 1997). In vitro, MeHg can affect many feature of neuronal and astrocytic function. The major mechanisms involved in MeHg neurotoxicity currently explored are oxidative stress (Ou et al., 1999), impairment of intracellular calcium homeostasis (Danbolt, 2001; Sirois and Atchison, 2000) and inhibition of
glutamate uptake by astrocytes (Aschner et al., 2000; Brookes and Kristt, 1989; Danbolt, 2001). In this regard, a recent in vivo study showed that MeHg increases glutamate extracellular levels in frontal cortex of rats (Juárez et al., 2002). The increase in glutamate release has also been observed after MeHg exposure. In vitro studies have demonstrated that MeHg induces spontaneous glutamate release from mouse cerebellar slices (Reynolds and Racz, 1987). Moreover, the effect of MeHg on D-aspartate release has been observed in neonatal rat primary astrocyte cultures (Aschner et al., 1995). However, there is no substantial evidence for MeHg-induced glutamate release from presynaptic terminals after in vivo exposure.

Recently, it has been demonstrated that ebselen, a lipid-soluble seleno-organic compound that possesses glutathione peroxidase-like activity (Daiber et al., 2000; Müller et al., 1984), has a protective role against brain ischemia and stroke (Dawson et al., 1995; Lee, 1999; Yamaguchi et al., 1998). Moreover, ebselen blocks the production of thiobarbituric acid reactive species caused by intra-striatal quinolinic acid administration in rat (Rossato et al., 2002). Of particular importance, glutamate neurotoxicity in primary cultures of cerebellar neurons, which is believed to be mediated by NMDA receptor activation (Moussaoui et al., 2000; Tsuzuki et al., 1989), is significantly reduced by ebselen (Porciúncula et al., 2001).
As pointed out above, it has been observed that MeHg can modify synaptic glutamate activity of adult rats. However, studies on the effect of MeHg exposure during critical periods of brain development on glutamate uptake and release are lacking in the literature. Brain is under intense development during the early postnatal period, which may render it more sensitive to environmental chemicals toxicity, including MeHg exposure. Indeed, a substantial acceleration of synthesis of RNA, DNA, protein and myelin is observed in this period (Gottlieb et al., 1977). Of particular importance, the early post-natal period is one of intense gliogenesis (particularly of astroglia, the main site for glutamate and MeHg uptake). Considering that the exposure of pregnant women to MeHg can indirectly contaminate their children (Harada, 1995, Weihe et al., 2002) and that some studies (Aschner, 2000; Grandjean et al., 1997; McKeown-Eyssen et al., 1983) suggest that fetal exposure to MeHg is associated with further neurological deficits, the study of MeHg exposure during phases of rapid brain development becomes relevant. In fact, considering the role of glutamate in brain development, the study on how MeHg interferes with glutamatergic neurotransmission at early ages may contribute for understanding the neurotoxicity of this pollutant.
Taking into account that the increase in extracellular glutamate levels represents a relevant mechanism related to MeHg-induced neurotoxicity, and that ebselen has been shown to possess protective role against glutamate excitotoxicity, the aim of the present study was to evaluate the effects of \textit{in vivo} MeHg exposure on glutamate release from synaptosomal preparations (presynaptic terminals) and glutamate uptake by cortical slices from suckling rat pups, as well as the possible protective role of ebselen against the MeHg effects.
2. Materials and Methods:

2.1. **Chemicals:** L-[^3]H]Glutamate (48 Ci/mmol) was purchased from Amersham International (UK). Methylmercury (II) chloride was obtained from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were of analytical reagent grade and purchased from Merck (Darmstadt, Germany).

2.2. **Animals:** Wistar rats obtained from our own breeding colony were maintained at approximately 25 °C, on a 12:12-h light/dark cycle, with free access to food and water. The breeding regimen consisted of grouping three virgin females (90-120 days) with one male for 20 days. Pregnant rats were selected and housed individually in opaque plastic cages. All experiments were conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology, adopted by the Society of Toxicology in July 1989.

2.3. **Treatments:**

2.3.1. **Treatment 1:** Pups were divided in 2 experimental groups of 24 animals each: control (group A) and MeHg (group B). From postnatal day (PND) 3, they were treated daily, in consecutive days, with subcutaneous injections. Each experimental group was divided in three subgroups that were killed at
PND 10, 17 and 24 (n = 8 animals per subgroup). The period of treatment (PND 3-24) was chosen because, as mentioned above, the early post-natal period is one of intense gliogenesis (particularly of astroglia, the major site for glutamate and MeHg uptake). During the experimental treatment, pups were maintained with their dams. MeHg was dissolved in a NaHCO₃ solution (25 mM) to allow for daily subcutaneous administrations, 1 mL/kg (group B). The MeHg dose (2 mg/kg) was based on Miyamoto et al. (2001). Control group (group A) received daily subcutaneous injections of a NaHCO₃ solution - 25 mM (1 mL/kg).

2.3.2. Treatment 2: Pups were divided in four experimental groups of 10 animals each; control (group A), MeHg (group B), ebselen (group C), and MeHg plus ebselen (group D). From PND 3, pups were treated daily, for 21 days with subcutaneous injections of methylmercury and/or ebselen. Ebselen was dissolved in dimethyl sulfoxide (DMSO) to allow for subcutaneous administrations, 1 mL/kg and its dose (10 mg/kg) was based on Kurebayashi et al. (1989). Control rats (group A) received a daily injection of a NaHCO₃ solution - 25 mM (1 mL/kg) plus a daily injection of DMSO (1 mL/kg). Group B (MeHg treatment) received a daily injection of MeHg (2 mg/kg) plus a daily injection of DMSO (1 mL/kg). Group C (ebselen treatment) received a daily injection of ebselen (10 mg/kg) plus a daily injection of a NaHCO₃ solution -
25 mM (1 mL/kg). Rats treated with MeHg plus ebselen (group D) received a daily injection of MeHg (2 mg/kg) plus a daily injection of ebselen (10 mg/kg). The MeHg and ebselen injections were administered simultaneously, but at different sites to avoid the direct chemical interaction and the formation of precipitates.

In both experiments the body weight gain was significantly lower in MeHg-exposed pups when compared to the control group by Student's t–Test (experiment 1) or by one-way ANOVA, followed by the Duncan multiple range Test (experiment 2), at $P<0.05$.

2.4. Synaptosomal fraction preparation: Synaptosomal preparations were obtained by isotonic Percoll/sucrose discontinuous gradients as described previously (Dunkley et al., 1988). Briefly, homogenates (10%, w/v) from whole brain were prepared in 1.28 M sucrose, 4 mM EDTA and 25 mM DDT, pH 7.4, and centrifuged at 800×g for 10 min. The supernatant containing synaptosomes was subjected to 23, 15, 7 and 3% Percoll solution density gradient centrifugation at 24000×g for 10 min. The synaptosomal fractions were isolated from the 23, 15 and 7% Percoll bands and use for glutamate release assay and for protein measurement.
2.5. L-[³H] glutamate release by synaptosomal preparation: Release assay was carried out as Tavares et al., 2002. Synaptosomal preparation was preloaded with L-[³H] glutamate (0.1 µCi mL⁻¹ L-[³H] glutamate, final concentration 500 nM) for 15 min at 37ºC in Hank's balanced salt solution (HBSS) with low potassium (non-depolarizing medium). For removing L-[³H] glutamate not taken up, aliquots of preloaded (labeled) synaptosomal preparations (1.4 mg protein) were washed four times (at 4ºC) in HBSS by centrifugation at 16,000 g for 1 min. To further assess the basal L-[³H] glutamate release, the final pellet was resuspended in HBSS and incubated for 1 min at 37ºC. K⁺-stimulated L-[³H] glutamate release was assessed similarly, but in presence of 40 mM KCl to induce synaptosomal depolarization (NaCl decreasing). Incubation was finished by cooling the preparation followed by immediate centrifugation (16,000 g for 1 min, at 4ºC). Radioactivity present in supernatants and pellets was separately determined in a Wallac scintillation counter. L-[³H] glutamate release was calculated as a percentage of the total amount of radiolabel present at the start of the incubation period (preloaded synaptosomes). The total amount of glutamate preloaded into synaptosomes was about 9.9 nmoles/mg protein.
2.6. Lactate dehydrogenase assay: Lactate dehydrogenase (LDH; E 1.11.27) release was monitored in order to evaluate the integrity of synaptosomal preparations. The LDH activity in the incubation medium and the total LDH activity, which was determined by synaptosomal preparations disruption using 1.5% Triton X-100, were assayed using a Kit (Labtest reagents, Brazil), which measured the amount of a colored complex derived from the NADH formed by the enzymatic reaction using a spectrophotometric method (510 nm).

2.7. Preparation of brain cortical slices: Cortices were dissected and coronal slices (0.4 mm) were obtained from the parietal area using a McIlwain tissue chopper. The slices were washed with HBSS and the sections were finally separated with the help of a magnifying glass.

2.8. Glutamate uptake by brain cortical slices: Uptake was assessed by adding 0.33 µCi mL⁻¹ L-[³H] glutamate with 100 µM unlabeled glutamate in HBSS at 37°C. Incubation was finished after 7 min by two ice-cold washes with 1mL HBSS immediately followed by addition of 0.5 N NaOH, which was kept overnight. Aliquots of lysates were taken for determination of intracellular content of L-[³H] glutamate through scintillation counting and for protein measurement. Sodium independent uptake was determined by
using N-methyl-D-glucamine instead of sodium chloride, being subtracted from the total uptake to obtain the sodium dependent uptake.

2.9. Protein measurement: The protein content of synaptosomal preparations and slices was determined by the method of Lowry et al. (1951) using bovine albumin as standard.

2.10. Statistical analysis: In treatment 1, the MeHg group was compared to the respective control group (animals with same age) by Student's t–Test. In treatment 2, differences between groups were analyzed by one-way ANOVA, followed by Duncan’s multiple range Test when appropriate. Linear regression × analysis of variance was also performed to analyze the effect of MeHg treatment's length on glutamate release from brain synaptosomes and on glutamate uptake by cortical slices.
3. Results

3.1. Effect of age on L-[³H]glutamate release from synaptosomal preparation and on L-[³H]glutamate uptake by brain slices

In control suckling rats, the basal and K⁺-stimulated L-[³H] glutamate release increased, while L-[³H] glutamate uptake decreased within age (Table 1).

3.2. Effect of MeHg exposure on L-[³H] glutamate release from synaptosomal preparation

Lactate dehydrogenase (LDH) assay indicated that the synaptosomal integrity was not affected by MeHg and/or ebselen treatment (data not shown).

MeHg exposure during suckling caused a significant increase (28%) in basal L-[³H] glutamate release from brain synaptosomes only at PND 24, when compared to the control group (Table 1). Moreover, a tendency for an increasing in basal L-[³H] glutamate release from synaptosomes (P = 0.088) was observed at PND 17, which was not observed at PND 10 (P = 0.252) (Table 1). K⁺-stimulated L-[³H] glutamate release was not affected by MeHg treatment (Table 1). The total amount of loaded L-[³H] glutamate by
synaptosomal preparation before release assay was not affected by MeHg and/or Ebselen exposure (data not shown).

3.3. L-[3H] glutamate uptake by brain cortical slices

Previous studies have reported that an increase in extracellular glutamate concentrations has been shown to cause a compensatory increase in glutamate uptake by cultured neural cells (Duan et al., 1999; Gegelashvili et al., 1996; Munir et al., 2000). Taking into account that, in our study, MeHg increased glutamate release from synaptosomal preparations, which could elevate extracellular glutamate levels, we assayed the effect of MeHg on glutamate uptake by cortical slices (Table 1). The treatment caused a significant increase (56%) in the uptake, only at PND 24, when compared to the age-matched control by Student’s t-Test.

3.4. Relationship between the effects of MeHg on glutamate release and glutamate uptake

The statistical significance of the MeHg effects on both glutamate release and uptake was observed only at PND 24. In order to verify if the significant increase in the uptake could be due to a preceding tendency of increase in the release observed at PND 17, a linear regression × analysis of
variance for the effect of MeHg on both processes was performed. This analysis showed a significant correlation between the MeHg effect on glutamate release and the time of MeHg treatment \((F(1,7)=6.43; \beta=-0.493; P=0.020)\), but not on glutamate uptake \((F(1,7)=1.76; \beta=-0.267; P=0.20)\). This could indicate that MeHg formerly increased the amount of extracellular glutamate and this increase further exerted a stimulatory effect on the uptake. As the MeHg effect was calculated as the difference (delta) between the treated and the control group, matched by age, the age effect was eliminated of analysis.

### 3.5. Effect of ebselen and MeHg interaction on glutamate release and uptake

Table 2 shows the effect of ebselen and/or MeHg on glutamate release from synaptosomal preparations and glutamate uptake by cortical slices, at PND 24. Ebselen, which had no significant effect *per se*, abolished both stimulatory MeHg effects on basal glutamate release and uptake.

### 4. Discussion

Although an ontogenetic evaluation of glutamatergic system was not a major aim of our study, the observed effect of age on glutamate release and uptake is noteworthy. The increase of glutamate release (basal and K⁺-
stimulated) and the decrease of glutamate uptake with age at the postnatal period could represent an ontogenetic physiological modulation of the excitatory tonus at early life. Moreover, considering that the period under study is one of intense gliogenesis (particularly for astroglia, the major site for glutamate), the management of glutamate is likely to show both qualitative and quantitative adjustment with age. Accordingly, some works (Erdo and Wolff, 1990; Haberecht and Redburn, 1996) have reported an increase in the extracellular glutamate levels in different structures during neonatal development.

There is evidence suggesting that MeHg-induced neurotoxicity could be related to over stimulation of the glutamatergic system. In fact, MeHg seems to directly impair astrocytic glutamate transport (Aschner et al., 2000), leading to an increase in extracellular glutamate levels. Accordingly, a recent *in vivo* study showed that MeHg increases glutamate levels in frontal cortex of rats (Juárez et al., 2002).

Our results show that MeHg exposure increased basal glutamate release from synaptosomal preparations of suckling rat pups. This effect was not due to a damage of cell membrane, as observed with LDH assay.

Comparison of basal glutamate release between control and MeHg-treated rats by Student's *t*–Test showed decreasing *P* values (increasing
significance) with time of treatment ($P = 0.252$, $0.088$ and $0.034$ for 7, 14 and 21 days of treatment, respectively) (Table 1). Moreover, linear regression × analysis of variance showed that the MeHg effect on glutamate release was dependent on the length of treatment ($F (1.7)=6.43$; $\beta=-0.493$; $P=0.020$). The absence of significant effect of MeHg on $K^+$-stimulated glutamate release may indicate that the MeHg effect was predominantly on non vesicular glutamate release.

The toxic effects of MeHg exposure are higher in developing than in mature organisms (Kostial, 1983; Sakamoto et al., 1993). In this context, many works present a relationship between MeHg-exposure during phases of rapid brain development and neurological deficits in children (Aschner, 2000; Grandjean et al., 1997; McKeown-Eyssen et al., 1983). Taking into account that glutamate homeostasis is central in developmental events, such as synaptogenesis, dendritic pruning and neurite sprouting, the stimulatory effect of MeHg exposure to suckling rats on glutamate release could be related, at least in part, to neurological deficits in children exposed to MeHg at prenatal or early postnatal stages.

It has been shown that glutamate and glutamatergic ligands may increase glutamate uptake by neuronal and astrocytic cell cultures (Duan et al., 1999; Gegelashvili et al., 1996; Munir et al., 2000). Our results show that
MeHg treatment increased glutamate uptake by brain cortical slices at PND 24. Considering that the MeHg effect on glutamate release was observed earlier, the increase of glutamate uptake could represent a pathophysiological response to the previous MeHg-induced glutamate release.

As pointed above, there are many studies reporting the inhibitory effect of MeHg on glutamate uptake (Allen et al., 2001; Aschner et al., 2000; Brookes and Kristt, 1989). Additionally, there are studies showing the stimulatory effect of MeHg on glutamate release from mouse cerebellar slices (Reynolds and Racz, 1987) and D-aspartate (a glutamate analog) release from neonatal rat primary astrocyte cultures (Aschner et al., 1995). It is noteworthy that these studies were performed under acute and in vitro experimental conditions, and both parameters (glutamate release and uptake) were not measured simultaneously. In contrast, in our study, a relative long-term in vivo MeHg exposure was performed. We realize that the discrepancy among our findings compared to the myriad of studies establishing an inhibitory effect of MeHg on glutamate uptake is related to the long-term in vivo experimental conditions, where increased glutamate release leads to increased extracellular glutamate levels that, in turn, stimulate glutamate uptake.

The neuroprotective role of Ebselen has been extensively reported. Toward the glutamatergic system, the production of thiobarbituric acid
reactive species after quinolinic acid administration in rat brain was blocked by ebselen (Rossato et al., 2002). Moreover, glutamate neurotoxicity in primary cultures of cerebellar neurons, which is believed to be mediated by NMDA receptor activation (Moussaoui et al., 2000; Tsuzuki et al., 1989), is significantly reduced by ebselen (Porciúncula et al., 2001). Furthermore, ebselen is the only therapeutic agent that has been reported to present borderline efficacy against excitotoxicity on Phase III status of acute stroke trials (Lee et al., 1999). Here, we show an in vivo inhibitory effect of ebselen on MeHg-induced glutamate release. Interestingly, an increased glutamate uptake, supposed to be a putative outcome of increased glutamate release from presynaptic terminals, was also prevented by ebselen.

Although our results did not provide substantial mechanistic accounting for the ability of ebselen to counteract MeHg’s effects and reactive oxygen species (ROS) generation was not measured, we believe that the antioxidant properties of ebselen are related to its protective effects against MeHg-induced alterations on glutamate homeostasis. Since the overproduction of H$_2$O$_2$ seems to represent a likely mechanism involved in impairment of glutamate homeostasis (Allen et al., 2001), and ebselen has been found to possess glutathione peroxidase like activity (Müller et al., 1984), it is plausible to suppose that the protective effect of ebselen against MeHg-induced alterations
on glutamate homeostasis could be related to its ability to detoxify hydroperoxides. Accordingly, a recent data from our laboratory shows that ebselen acts protecting against MeHg-induced inhibition of brain glutathione peroxidase activity in mice brain, as well as MeHg-induced alterations on glutamate transport in brain cortical slices (data not published).

Concluding, the present data suggest that the effect of MeHg on glutamate release from presynaptic terminals could be involved in its neurotoxicity in suckling rats and that the increase in the glutamate uptake could correspond to a pathophysiological response to this MeHg effect. Moreover, the observed inhibitory effect of ebselen on MeHg-induced glutamate release could be related to its reported neuroprotective effects. Extrapolating to humans, it is possible that the neurological deficits observed in children exposed to MeHg at perinatal stages could be relate, at least in part, to the disturbance of glutamate homeostasis, reinforcing the perspective of the use of ebselen as an additional therapeutic strategy.

Acknowledgements

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References


Involvement of enhanced sensitivity of N-methyl-D-aspartate receptors in vulnerability of developing cortical neurons to methylmercury neurotoxicity. *Brain Res.* **901**, 252-258.


Legends
Table 1: From the postnatal day (PND) 3, rats were daily injected with MeHg (2 mg/kg body weight; subcutaneously) until PND 10, 17 and 24. Data are expressed as mean ± SE from 8 animals.

a Significantly different from the others groups at the same column (effect of age); $P<0.05$, by one-way ANOVA, followed by the Duncan multiple range test when $F$ was significant.

* Significantly different from the respective control group (rats not injected with MeHg); $P<0.05$, by Student’s t-Test.

Table 2: From the postnatal day (PND) 3, rats were daily injected with MeHg (2 mg/kg body weight; subcutaneously) and/or with ebselen (10 mg/ kg body weight; subcutaneously) for 21 days. Data are expressed as mean ± SE from 8 animals.

a Significantly different from the control group; $P<0.05$, by one-way ANOVA, followed by the Duncan multiple range test when $F$ was significant.
Table 1: Effect of age and methylmercury (MeHg) exposure on basal and K⁺-stimulated L-[³H]glutamate release from brain synaptosomal preparations and on L-[³H] glutamate uptake by brain cortical slices of suckling rat pups.

<table>
<thead>
<tr>
<th>Glutamate release (basal) % of total loaded</th>
<th>Glutamate release (stimulated) % of total loaded</th>
<th>Glutamate uptake pmol /mg protein/min</th>
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<tbody>
<tr>
<td>Control</td>
<td>MeHg</td>
<td>Control</td>
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<tr>
<td>PND = 10</td>
<td>13.4 ± 0.4</td>
<td>14.0 ± 0.3</td>
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<tr>
<td>PND = 17</td>
<td>14.1 ± 1.1</td>
<td>16.8 ± 0.7</td>
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<td>PND = 24</td>
<td>30.7 ± 1.3ᵃ</td>
<td>39.3 ± 3.2ᵃ,⁎</td>
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</table>
Table 2: Effect of methylmercury (MeHg) and ebselen exposure on basal and K⁺-stimulated L-[³H]glutamate release from brain synaptosomal preparations and on L-[³H] glutamate uptake by brain cortical slices of 24 days suckling rat pups.

<table>
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<th>Glutamate release</th>
<th>Glutamate release</th>
<th>Glutamate uptake</th>
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<tr>
<td></td>
<td>(basal)</td>
<td>(K⁺-stimulated)</td>
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<td></td>
<td>% of total loaded</td>
<td>% of total loaded</td>
<td>pmol /mg protein/min</td>
</tr>
<tr>
<td>Control</td>
<td>30.7 ± 1.35</td>
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<tr>
<td>MeHg</td>
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<td>44.2 ± 2.43</td>
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<td>43.3 ± 2.43</td>
<td>181 ± 10</td>
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<tr>
<td>MeHg plus Ebselen</td>
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<td>42.7 ± 3.16</td>
<td>145 ± 25</td>
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