Original Article

Demethylation of methylmercury and the enhanced production of formaldehyde in mouse liver

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ABSTRACT — Methylmercury (MeHg) is gradually changed to inorganic Hg after demethylation in animal tissues, and a selective quantification of inorganic Hg in the tissues is necessary to detect the reaction. We detected inorganic Hg formation in liver and kidney of mouse as early as 24 hr after MeHg injection. As an example of biological demethylation, the cytochrome P450 (P450)-mediated N-demethylation of drugs has been well documented, and formaldehyde was detected as a reaction product. Here we incubated mouse liver homogenate with added MeHg and observed a dose-dependent production of formaldehyde, as well as inorganic Hg formation. Since the amount of formaldehyde was approx. 500 times higher than that of the inorganic Hg that formed, the formaldehyde production would be stimulated by inorganic Hg formed from MeHg. We observed that inorganic Hg caused formaldehyde production, and it was enhanced by L-methionine and sarcosine. Thus, some biomolecules with S-methyl and N-methyl groups may function as methyl donors in the reaction. Using subcellular fractions of mouse liver, we observed that microsomal P450 did not participate in the demethylation of MeHg, but the greatest activity was located in the mitochondria-rich fraction. The addition of superoxide anion in the reaction mixture significantly enhanced the formaldehyde production, whereas Mn-superoxide dismutase depressed the reaction. Our present findings demonstrated that inorganic Hg formed by MeHg demethylation in mouse liver stimulated the endogenous formaldehyde production, and we observed that MeHg demethylation could be estimated by a formaldehyde analysis. Our results also suggested that superoxide anion is involved in the reaction.

Key words: Methylmercury, Demethylation, Mitochondria, Nash method, Formaldehyde

INTRODUCTION

Methylmercury (MeHg) is easily absorbed from the gastrointestinal tract of mammals, and it easily passes through the blood-brain barrier and placental barrier via the transport system for neutral amino acids, to be distributed to nervous tissues and a fetus if one is present (Hirayama, 1980, 1985; Aschner and Clarkson, 1988; Kajiwaru *et al.*, 1996). The neuronal toxicity and fetal toxicity of MeHg are well documented in experimental animals and humans (Choi, 1989; Clarkson, 1997; Clarkson

et al., 2003). Human exposure to MeHg occurs mostly via the consumption of seafood, since it is accumulated in marine animals via a food chain in aquatic environments (ATSDR, 1992). However, ordinary seafood consumption would rarely lead to adverse effects, because in the human body there are metabolism pathways to detoxify MeHg.

The major detoxification pathway for MeHg in humans and terrestrial animals is urinary and fecal excretion. Due to the high affinity of MeHg to anionic sulfhydryl groups (Simpson, 1961), a significant portion of MeHg forms a

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complex with glutathione (GSH), the major low-molecular-weight sulfhydryl compound in the cells, in animal tissues (Yasutake *et al.*, 1989). MeHg-GSH in the cells is eliminated to the extracellular space via the secretion system for GSH, and then finally appears in the urine and feces as a complex with GSH metabolite(s), such as L-cysteine-MeHg, through the bile ducts and renal tubules.

The biological half-life of MeHg in humans is 50-70 days (ATSDR, 1992), and the daily excretion rate has been calculated as 1%-1.4% of the body burden. With this elimination rate, the detoxification capacity may be sufficient, because the intake rate through ordinary fish consumption and the normal excretion rate easily achieve equilibrium, and the amount of MeHg in the body is considered to be kept stable.

An alternative detoxification pathway for MeHg may be a conversion to inorganic Hg.

MeHg gradually changes to inorganic Hg in animal tissues (Norseth and Clarkson, 1970; Mehra and Choi, 1981). In fact, most Hg was found in its inorganic form in tissues including the brain of MeHg-exposed animals over a long period (Lind *et al.*, 1988; Hansen *et al.*, 1989). Unlike MeHg, it is very difficult for inorganic Hg to enter nervous tissue and a fetus due to the rigid blood-brain barrier and placental barrier. The formation of inorganic Hg (i.e., demethylation of MeHg) in the peripheral tissues would contribute to the reduction of a fraction of MeHg that would be taken up by nervous and fetal tissues.

In addition, the reactivity of inorganic Hg can be reduced by forming Hg-metallothionein and insoluble mercuric selenide (Hg-Se) in animal tissues. The contribution of inorganic Hg formation to MeHg detoxification would be more prominent in fish and marine mammals than in terrestrial animals. Since fish and marine mammals have a long biological half-life of MeHg, i.e., up to several hundred days (Van Walleghem *et al.*, 2007; Amlund *et al.*, 2007), the accumulation would easily attain a sufficient level to cause adverse effects, if MeHg is accumulated as it is. Endo *et al.* (2008) reported a typical MeHg demethylation case in a melon-headed whale. The total Hg concentration in the whale liver showed a positive correlation with the inorganic Hg ratio; more than 90% was found as an inorganic form in the liver with a total Hg accumulation above 100 µg/g. It may be speculated that in the whale, hazardous MeHg was changed to inorganic Hg, and the inorganic Hg was stored in the liver as its inert form.

The liver was also suggested as one of the major sites of MeHg demethylation reaction in rats (Hirayama and

Yasutake, 1999; Yasutake and Hirayama, 2001). Although the authors of those studies proposed the involvement of reactive oxygen species in the reaction, the detailed mechanism of the reaction has not been clarified. As a biological demethylation, the cytochrome P450 (P450)-mediated N-demethylation of drugs is well documented (Murray *et al.*, 2000), but the involvement of P450 in MeHg demethylation described to date has been superficial. In the present study we conducted experiments using mouse liver to obtain further information on MeHg demethylation, and we observed enhanced formaldehyde formation without the participation of microsomal P450.

MATERIALS AND METHODS

Animals and sample preparation for *in vivo* experiments

Female C57BL/6N mice (aged 9 weeks) were obtained from CLEA Japan (Osaka, Japan), housed in polycarbonate cages at $23 \pm 2^\circ\text{C}$. They were allowed free access to laboratory chow (CE-2, CLEA Japan) and water for 1 week. MeHg chloride (Strem Chemicals, Newburyport, MA, USA) was dissolved in water in the presence of an equimolar amount of glutathione (GSH) and given orally to six mice at the dose level of 20 µmol/kg body weight, and the mice were kept in metabolism cage (1 mouse/cage) to obtain urine and feces. Twenty-four hours later, the mice were sacrificed under pentobarbital anesthesia to obtain liver and kidney samples. The experimental protocol was approved by the Ethics Committee for Research on Animals at Chlorella Industry Co., Ltd. The animals were cared for according to the U.S. National Institutes of Health guidelines.

Mercury analysis

The liver and kidney samples obtained as described above were subjected to total and inorganic Hg analyses. For the total Hg analysis, an aliquot of the sample was digested in a mixture of nitric acid, sulfuric acid, and perchloric acid (Ministry of Environment of Japanese Government, 2004), and analyzed using an atomic absorption Hg analyzer (RA-3320, Nippon Instruments, Osaka, Japan). Samples for the inorganic Hg analysis were prepared principally according to the method of Yasutake *et al.* (2005) with a slight modification.

Prior to the preparation, liver, the kidney and feces samples were homogenized in distilled water (10% w/v). Briefly, a 250-µL aliquot of the sample solution was acidified by 6N HCl (100 µL) in a 2-mL plastic tube, and toluene (1 mL) was then added. The mixture was shaken vigorously for 2 min to resolve MeHg chloride into the

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organic phase, and centrifuged at 12,000 rpm for 2 min to separate the organic and aqueous phases. The upper toluene layer was removed using an aspirator, and the extraction was repeated five times using fresh toluene (1 mL) to remove MeHg in the sample solution as much as possible. Since single extraction could remove approx. 90% of the MeHg, the present procedure was calculated to remove > 99.99% of MeHg from the sample solution. We analyzed the Hg concentrations in the prepared aqueous sample solutions as described above after digestion.

Demethylation assay of MeHg in the liver homogenate by formaldehyde and inorganic Hg quantification

The fresh liver sample obtained from a female C57BL/6N mouse was immediately homogenized in ice-cold phosphate-buffered saline (PBS) (10% w/v) using a glass homogenizer. The homogenate was then incubated in a 1-mL reaction mixture for 3 min at 37°C with a series of concentrations of MeHg (0 to 2 μ M) in HEPES (50 mM, pH 7.4) containing EDTA (0.1 mM), NADPH (0.33 mM) and $MgCl_2$ (6 mM). The protein content in the reaction mixture was adjusted to 0.075 mg. The formaldehyde that had formed was quantified by the Nash method (Nash, 1953) using an external standard solution (Wako, Osaka, Japan). The content of inorganic Hg formed in the mixture was also determined by the above-described procedure.

The effect of inorganic mercury on formaldehyde production in mouse liver

The liver homogenate prepared as described above was incubated in a 1-mL reaction mixture for 3 min at 37°C with a series of concentrations of $HgCl_2$ (0 to 10 nM) in HEPES (50 mM, pH 7.4) containing EDTA (0.1 mM), NADPH (0.33 mM) and $MgCl_2$ (6 mM). The protein content in the reaction mixture was adjusted to 0.075 mg. The formaldehyde formed was quantified by the Nash method (Nash, 1953) using an external standard solution (Wako).

The effect of L-methionine and that of sarcosine on inorganic Hg-mediated formaldehyde production

The liver homogenate prepared as described above was incubated in a 1-mL reaction mixture for 3 min at 37°C with $HgCl_2$ (10 nM) in the presence of L-methionine (1 mM) or sarcosine (1 mM) in HEPES (50 mM, pH 7.4) containing EDTA (0.1 mM), NADPH (0.33 mM) and $MgCl_2$ (6 mM). The protein content in the reaction mixture was adjusted to 0.075 mg. The formaldehyde formed was quantified by the Nash method (Nash, 1953) using an

external standard solution (Wako).

N-demethylation of benzphetamine and MeHg demethylation in the cytochrome P450-rich fraction of mouse liver

A fresh liver sample obtained from a female C57BL/6N mouse was immediately homogenized in ice-cold PBS (10% w/v) using a glass homogenizer. The homogenate was centrifuged four times (9,000 g, 20 min) to obtain the supernatant as a crude cytochrome P450 fraction. The precipitate was collected as a residual fraction. The whole homogenate, the P450-rich fraction and the residual fraction containing 0.075 mg protein were each incubated as described above with the addition of benzphetamine (1 mM) or MeHg (10 μ M), and we measured the formaldehyde that had formed.

MeHg demethylation activities in the subcellular fractions of mouse liver

Using the liver homogenate prepared as described above, we prepared the subcellular fractions (membrane plus nuclear, mitochondria, microsome and cytosol) according to the method of Lash and Sall (1993). Each fraction of the liver prepared containing 0.075 mg of protein was incubated with MeHg (0.2 μ M) as described above for 3 min at 37°C, and the formaldehyde that had formed was measured.

The effect of superoxide anion and Mn-superoxide dismutase (SOD) on MeHg demethylation

The whole homogenate of mouse liver containing 0.075 mg protein was incubated with MeHg (10 μ M) as described above in the presence of hypoxanthine (1 mM)/xanthine oxidase (1 mU/mL) and/or Mn-SOD (100 U/mL), and the formaldehyde that had formed was measured.

Statistical analysis

The significance of differences was calculated using Student's t-test and Microsoft Office Excel 2010 for Windows (Microsoft Japan, Tokyo, Japan) and a one-way analysis of variance (ANOVA) using Excel-Toukei 2012 software ver. 1.00 (Social Survey Research Information, Tokyo). A *p*-value < 0.05 was considered significant.

RESULTS AND DISCUSSION

MeHg gradually changes to inorganic Hg in animal tissues (Norseth and Clarkson, 1970; Mehra and Choi, 1981). Hirayama and Yasutake (1999) reported that the liver is one of the main organs where inorganic Hg was formed from MeHg, and part of the inorganic Hg formed

is moved to the kidney through the blood circulation. In the present study, to determine the demethylation status of MeHg, we quantified the tissue distribution and excretion and the levels of total and inorganic Hg in mice 24 hr after an injection of a nontoxic dose (20 $\mu\text{mol/kg}$) of MeHg.

Figure 1 shows the Hg distribution in the liver, kidney, urine and feces in female C57BL/6N mice 24 hr after the MeHg administration. A high distribution (12% of the dosed amount) was observed in the liver, and 4.5% of the tissue Hg was found as an inorganic form. A somewhat lower Hg distribution (8% of the dosed amount) was found in the kidney, and 3.5% was detected as inorganic Hg. The mercury contents in the feces and urine collected for 24 hr after the injection were 4% and 1% of the MeHg dose, respectively.

Interestingly, the percent of inorganic Hg in the feces was much higher than in that in the liver. Part of the MeHg in the liver is secreted into the intestinal tube via the bile duct, and then the gut flora would convert MeHg to inorganic Hg (Seko *et al.*, 1981, 2001). Most of the inorganic Hg thus formed would appear in the feces without the intestinal reabsorption. However, the percent of inorganic Hg in the urine was similar to that in the kidney. Both MeHg and inorganic Hg in the kidney would be excreted at similar rates. Since the liver showed greater accumulation and a higher rate of demethylation of MeHg compared to the kidney, we conducted a study on MeHg demethylation in mouse liver.

The demethylation of MeHg in animal tissues could be detected for the first time by a selective quantification of the inorganic Hg that had formed (Yasutake *et al.*, 2005),

but the fate of the methyl group released from MeHg has not been clarified. P450 in the liver is well known as one of the major drug-metabolizing enzymes, and it catalyzes the demethylation of various drugs with N-methyl group (Murray *et al.*, 2000). The demethylase activity can be detected by the quantification of formaldehyde formed from the methyl group (Nash, 1953). If the methyl group in MeHg is changed to formaldehyde after demethylation, the reaction could be detected by formaldehyde measurement (i.e., by the Nash method).

To examine the possibility of estimating MeHg demethylation by the Nash method, we incubated the liver homogenate in the presence of MeHg at a series of concentrations (0.1 to 1.0 μM), and we determined the amounts of inorganic Hg and formaldehyde formed during the incubation. As shown in Fig. 2, both inorganic Hg and formaldehyde increased in a MeHg concentration-dependent fashion (Fig. 2A, B), and a strong correlation was observed between the amounts of inorganic Hg and formaldehyde (Fig. 2C, $R^2 = 0.9482$).

However, the formaldehyde formed would not have originated from MeHg, because the amount of formaldehyde was approx. 500 times higher than the amount of inorganic Hg that had formed (Fig. 2C). We suspect that the inorganic Hg formed from MeHg in the liver homogenate, although its concentration was as low as nanomolar, would interact with some biomolecule(s) to stimulate formaldehyde production. To examine a possibility of the inorganic Hg-stimulated formaldehyde production, the liver homogenate was incubated in the presence of inorganic Hg of up to 10 nM concentrations, and formaldehyde formed was determined.

Fig. 1. Mercury distribution and inorganic Hg contents in mouse tissues 24 hr after MeHg administration. Twenty-four hours after the oral administration of MeHg (20 $\mu\text{mol/kg}$) to female C57BL/6N mice, the total Hg (A) and inorganic Hg (B) contents in liver, kidney, feces and urine were determined.

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Fig. 2. The formation of inorganic Hg (A) and formaldehyde (B), and the correlation between them (C) in the liver homogenate in the presence of MeHg. MeHg (0.1, 0.25, 0.40, 0.50, 0.75, 1 μ M) was incubated in HEPES buffer 50 mM (pH 7.4) containing EDTA (0.1 mM), NADPH (0.33 mM), and $MgCl_2$ (6 mM) with the liver homogenate (0.075 mg protein/mL) for 3 min at 37°C. Inorganic Hg was selectively quantified (22), and the formaldehyde was measured by the Nash method (23).

As shown in Fig. 3, the amount of formaldehyde that had formed increased with the inorganic Hg in a dose-dependent manner up to 10 nM. This result indicated that inorganic Hg that originated from MeHg in the liver homogenate could stimulate endogenous formaldehyde production. It seems that the enzyme-catalyzed reaction is involved in the process because the reaction fits to a Michaelis-Menten equation (Fig. 3). Although the methyl group in MeHg might contribute as a source of formaldehyde formation, its rate would be at most 0.2% of the entire amount of formaldehyde as mentioned above (Fig. 2C). Inorganic Hg-induced alterations of formaldehyde production in pig tissues were reported by Rétfalvi *et al.*

(1998). Those authors suggested that inorganic Hg affected the formaldehyde metabolism in the tissues and that highly accumulated Hg in the liver suppressed the tissue formaldehyde level, while a low accumulation of Hg in the muscle increased the level. Since in the present study formaldehyde production was stimulated, the concentration of inorganic Hg formed from MeHg in the liver homogenate would be low enough for the stimulation. Our findings thus demonstrate, for the first time, the enhanced formation of formaldehyde through the demethylation of MeHg in liver homogenate.

As a source of formaldehyde formed by inorganic Hg, some biomolecules would function as methyl donors. To

Fig. 3. Effect of inorganic Hg on formaldehyde production in the liver homogenate. Inorganic Hg with a series of concentrations up to 10 nM was incubated in HEPES buffer 50 mM (pH 7.4) containing EDTA (0.1 mM), NADPH (0.33 mM), and MgCl_2 (6 mM) with the liver homogenates (0.075 mg protein/mL) for 3 min at 37°C, and the formaldehyde that had formed was determined by the Nash method. Data were fitted to a Michaelis-Menten equation using GraphPad Prism 5.04 software (GraphPad Software, La Jolla, CA, USA). The apparent K_m and V_{max} were 4.02 ± 0.97 nM and 2.57 ± 0.22 nmol/3 min/mL (11.4 ± 1.0 nmol/min/mg protein), respectively.

Fig. 4. Effects of L-methionine and sarcosine on inorganic Hg-mediated formaldehyde production in the liver homogenate. The liver homogenate was incubated with HgCl_2 (10 nM) in the presence of L-methionine (1 mM) or sarcosine (1 mM) in HEPES buffer 50 mM (pH 7.4) containing EDTA (0.1 mM), NADPH (0.33 mM), and MgCl_2 (6 mM) with the liver homogenates (0.075 mg protein/mL) for 3 min at 37°C, and the formaldehyde formed was determined by the Nash method. Significant differences from control group (inorganic Hg) are indicated by * $p < 0.05$ and ** $p < 0.01$.

obtain information concerning possible methyl donors, we incubated L-methionine (S-methyl) and sarcosine (N-methyl) with the liver homogenate in the presence of inorganic Hg. We observed that the inorganic Hg-stimulated formaldehyde production was significantly enhanced by L-methionine and sarcosine (Fig. 4). These results indicated that biomolecules with S-methyl and/or N-methyl groups may function as a part of the methyl donors in the reaction.

It was reported that formaldehyde is an essential intermediate metabolite, and that S-, N-, and O-methyl compounds function as methyl donors (ATSDR, 1999). Thus, a trace amount of inorganic Hg formed from MeHg in the liver homogenate in the present study enhanced the endogenous formaldehyde production, and the MeHg demethylation could be estimated by measuring the formaldehyde by the Nash method without a selective quantification of inorganic Hg.

To examine whether P450s are involved in the demethylation of MeHg, we examined the Hg-mediated formaldehyde production using liver subcellular fractions. Tissue homogenate obtained from mouse liver was separated into the P450-rich fraction and residual fraction

and then incubated with MeHg or benzphetamine, a substrate for P450-mediated N-demethylation. The activity in each fraction was measured by formaldehyde formation. Although high N-demethylation activity of benzphetamine was found in the P450-rich fraction (Fig. 5A), the MeHg demethylation activity was localized in the residual fraction (Fig. 5B).

This result agrees with the finding that the hepatic P450s derived from endoplasmic reticulum catalyze the N-demethylation of various drugs (Murray *et al.*, 2000). However, microsomal P450s are unlikely to contribute to the demethylation of MeHg. Since the MeHg demethylation activity is thought to be located in a fraction other than the P450-rich S9 fraction, we incubated the subcellular fractions that we prepared from the mouse liver with MeHg to identify a fraction with high activity. As shown in Fig. 6, the high formaldehyde formation rate was observed to be localized in the mitochondrial fraction.

Figure 7 shows that Mn-SOD significantly diminished the MeHg-elicited enhanced production of formaldehyde in the mitochondria. Yasutake and Hirayama (2001) demonstrated that the demethylation of MeHg in rat liver slic-

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Fig. 5. Formaldehyde production in cytochrome P450 and residual fractions of the mouse liver in the presence of benzphetamine (A) or MeHg (B). Whole homogenate, cytochrome P450-rich fraction and the residual fraction prepared from the mouse liver were incubated with benzphetamine (1 mM) or MeHg (10 μ M) in 50 mM HEPES buffer (pH 7.4) containing EDTA (0.1 mM), NADPH (0.33 mM), $MgCl_2$ (6 mM) at 37°C for 3 min, and the formaldehyde formed was determined. The protein contents in the reaction mixture were adjusted to 0.075 mg/mL. Significant differences from whole homogenate are indicated by * $p < 0.05$, ** $p < 0.01$.

Fig. 6. MeHg-mediated formaldehyde production in the subcellular fractions of mouse liver. MeHg (10 μ M) was incubated with the nuclear/membrane fraction, mitochondria fraction, microsome fraction and cytosole fraction prepared from the mouse liver in 50 mM HEPES buffer (pH 7.4) containing EDTA (0.1 mM), NADPH (0.33 mM), $MgCl_2$ (6 mM) at 37°C for 3 min, and the formaldehyde formed was determined. The protein contents in the reaction mixture were adjusted to 0.075 mg/mL. Significant differences from whole homogenate: * $p < 0.05$, ** $p < 0.01$.

es was closely related to reactive oxygen production. They further suggested that the use of modulators of the mitochondrial respiration and the reactive oxygen production that superoxide anion produced through the respiration chain in the mitochondrial inner membrane would be involved in the demethylation. In the present study, to confirm the involvement of superoxide anion in the reac-

tion, the liver homogenate was incubated with hypoxanthine-xanthine oxidase, a superoxide producing system (McCord and Fridovich, 1969), and Mn-SOD. The reaction was significantly enhanced by threefold in the presence of additional superoxide, and it was suppressed by Mn-SOD (Fig. 7).

These results suggest that the formation of superox-

ide is necessary for the MeHg-elicited enhancement of formaldehyde production that occurred at the mitochondrial fraction as a conversion of MeHg to inorganic Hg (Yasutake and Hirayama, 2001). Since the high rate of formaldehyde formation was observed in the mitochondrial fraction with MeHg, we speculate that the target site of MeHg and inorganic Hg might be present in the mitochondria. These results agree with the finding that dysfunction of mitochondria caused by MeHg resulted in oxidative stress (Shenker *et al.*, 1999).

Although inorganic Hg caused the enhanced formaldehyde formation (Fig. 3), the contribution of MeHg itself might also be involved in the reaction. However, our finding that the formaldehyde formation was correlated with inorganic Hg formation (Fig. 2C) and the superoxide level (Fig. 7) strongly suggest the involvement of inorganic Hg as the major component to promote the reaction.

Our present findings demonstrated that inorganic Hg formed through MeHg demethylation in the mouse liver homogenate stimulated endogenous formaldehyde production. Formaldehyde is highly toxic and is considered a carcinogen, but there is a pathway to eliminate it after several steps of metabolism (ATSDR, 1999). In conclu-

sion, our results demonstrate that P450s in microsomes do not participate in the demethylation of MeHg in the liver. Further studies are necessary to elucidate the toxicological significance of the formaldehyde production in the mitochondrial dysfunction induced by MeHg.

Conflict of interest---- The authors declare that there is no conflict of interest.

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Fig. 7. The effect of superoxide anion and Mn-SOD on MeHg-mediated formaldehyde production in the mitochondria fraction of mouse liver. MeHg (10 μ M) was incubated with the mouse liver mitochondria fraction (0.075 mg protein) in the presence of hypoxanthine (1 mM)/xanthine oxidase (1 mU/mL) and/or Mn-SOD (100 U/mL). The reaction was carried out in 50 mM HEPES buffer (pH 7.4) containing EDTA (0.1 mM), NADPH (0.33 mM), $MgCl_2$ (6 mM) at 37°C for 3 min, and the formaldehyde formed was determined. * $p < 0.05$, ** $p < 0.01$.

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