ASSOCIATION OF HEAVY METALS WITH METALLOTHIONEIN AND OTHER PROTEINS IN HEPATIC CYTOSOL OF MARINE MAMMALS AND SEABIRDS

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Abstract—Distribution of Cu, Zn, Cd, Ag, Hg, and Se were determined in hepatocytosol of northern fur seals (Callorhinus ursinus), black-footed albatrosses (Diomedea nigripes), and Dall’s porpoises (Phocoenoides dalli). Copper, Zn, and Cd were accumulated preferentially in metallothionein (MT) fraction and their contents in MT fraction increased with the amounts in the hepatocytosol. Silver was bound to both high-molecular-weight substances (HMWS) and MT in the hepatocytosol for all three species, whereas the distribution of Ag in the cytosol was different among the three species. In northern fur seals, Ag mainly was bound to MT, whereas it mainly was associated with HMWS in Dall’s porpoises. In contrast, Ag was distributed almost equally in both HMWS and MT for black-footed albatrosses. Mercury content in HMWS and Se content in HMWS and low-molecular-weight substances (LMWS) increased with their contents in hepatocytosol for all the three species. A significant positive correlation was found between Se and Hg contents in high-molecular-weight (HMW) fraction in cytosol. The molar ratio of Hg and Se was close to unity in HMW fraction of the specimens with high Hg concentration in cytosol, implying that the Hg–Se complex was bound to the HMWS. Analysis of metals in the hepatocytosol by high-performance liquid chromatography/inductively coupled plasma–mass spectrometry (HPLC/ICP-MS) suggests that multiple isoforms of MT are present in hepatocytosol of the three species and that the metal profiles in hepatocytosol are different among the species. To our knowledge, this is the first report on the association of Ag with HMWS and MT in hepatocytosol of marine mammals and seabirds. Also, distribution and interaction of Hg and Se were investigated for the first time in hepatocytosol of the higher trophic marine animals.

Keywords—Marine mammals Metallothionein Heavy metals High-performance liquid chromatography Inductively coupled plasma–mass spectrometry

INTRODUCTION

High accumulation of heavy metals such as Cu, Zn, Cd, Ag, and Hg and Se were determined in hepatocytosol of northern fur seals (Callorhinus ursinus), black-footed albatrosses (Diomedea nigripes), and Dall’s porpoises (Phocoenoides dalli). Copper, Zn, and Cd were accumulated preferentially in metallothionein (MT) in these higher trophic marine animals. Metallothionein, a low-molecular-weight, cystein-rich, metal-binding protein, is ubiquitous in a wide variety of organisms [10,11]. Since the discovery of MT in horse renal cortex [12], their function, structure, and regulation have been studied extensively [13–16]. Generally it is accepted that the primary functions of MT are homeostasis of essential metals such as Zn and Cu and detoxification of heavy metals. Metallothionein can be induced by and bind heavy metals such as Cd, Cu, Hg, Ag, and Zn [17]. Also, MT has been found in some isoforms that have slightly different amino acid compositions and different functions [18–22].

It has been suggested that MT plays a role in accumulation and detoxification of Cu, Zn, and Cd in marine mammals [23], seabirds [24–28], and sea turtles [29]. However, detailed data on the interaction between these metals and MT, and the MT isoforms in the tissues of marine mammals and seabirds still is limited [30–34]. Furthermore, no investigation has been conducted on the association of MT with Ag in the tissues of these animals.

Like MT, Se also seems to participate in detoxification of heavy metals in liver of higher trophic marine animals. It has been suggested that Hg is detoxified by Se via the formation of an equimolar Hg–Se complex in liver of marine mammals and seabirds [35–37]. Indeed, crystalline mercuric selenide (HgSe) has been observed in tissues of some marine mammals and seabirds [38–40]. Selenium also reduces the toxicity of Hg through mechanisms other than the formation of crystalline HgSe. Presence of Hg-selenoproteins compounds was suggested in the liver of striped dolphins [41]. In experimental animals dosed with Hg and Se, equimolar amounts of Hg and Se have been observed in high-molecular-weight substances (HMWS) of hepatic cytosol and in plasma protein [42–46]. Similarly, Se also detoxifies Cd, Ag, and Cu through their interaction [47–49]. Nevertheless, the interactions between Se and heavy metals such as Hg, Ag, Cu, and Cd in hepatocytosol of higher trophic marine animals having high levels of the heavy metals have been poorly characterized.

The purpose of this work is to understand the detoxification mechanisms of heavy metals by MT and Se in greater detail, and also their species-specific differences among the higher trophic marine animals. The interaction of MT and Se with heavy metals (Cu, Zn, Cd, Ag, and Hg) was examined in the hepatic cytosol of northern fur seals, black-footed albatrosses, and Dall’s porpoises. Furthermore, a hyphenated technique, high-performance liquid chromatography/inductively coupled plasma–mass spectrometry, was employed for characterization of MT isoforms in the hepatic cytosol of these marine animals.
**MATERIALS AND METHODS**

**Samples**

Liver tissue of northern fur seals (*Callorhinus ursinus*), Dall’s porpoises (*Phocoenoides dalli*), and black-footed albatrosses (*Diomedea nigripes*) were used in this study. Ten specimens of northern fur seals (male, $n = 1$; female, $n = 9$) and six Dall’s porpoises (male, $n = 5$; female, $n = 1$) were collected from off Sanriku, Japan in 1997 and 1998. These animals were caught for commercial and scientific purposes under appropriate permits. Five black-footed albatrosses (male, $n = 5$) were killed by catch in longline fishing activities in the North Pacific in 1998. All the liver samples were frozen in liquid nitrogen within 1 h after death. These samples were then transported to our laboratory and stored at $-80^\circ$C until analysis. Mean body length and body weight were 124 cm (range, 108–135 cm) and 36.4 kg (range, 24.6–44.0 kg) for northern fur seals, 165 cm (range, 152–176 cm) for Dall’s porpoises, and 79.1 cm (range, 61.1–85.6 cm) and 3.79 kg (range, 3.10–4.50 kg) for black-footed albatrosses, respectively. The age of northern fur seals was determined by counting the growth layers in canine tooth as described by Kasuya [50] and ranged from two to 22 years. For black-footed albatrosses, one specimen was juvenile and four were adult. Data on body weight and age of the Dall’s porpoises were not available.

**Distribution of metals in hepatic cytosol**

Hepatic cytosol was obtained by the method of Anan et al. [29]. A liver sample was homogenized in 100 mM Tris-HCl buffer (1:10 w/v) containing 250 mM glucose (pH 7.4, 4°C). After centrifugation at 106,000 $\times g$ for 90 min, an aliquot of the cytosol fraction was subjected to gel filtration chromatography using a Sephadex G-75 column (3.0 × 90 cm) at 4°C. The column was eluted with 10 mM Tris-HCl buffer (pH 8.6) at a flow rate of 40 ml/h, and fractions of 6.4 ml were collected. The buffer was degassed and then saturated with nitrogen before use.

**Anion exchange chromatography**

To characterize the MT isoforms, an aliquot of MT fraction isolated on the Sephadex G-75 column was applied to an anion-exchange column, TSK gel DEAE-5PW (7.5 mm i.d. × 7.5 cm, Tosoh, Tokyo, Japan), with a precolumn, TSK guardgel DEWE-5PW (6 mm i.d. × 1 cm, Tosoh), using high-performance liquid chromatography (Shimadzu, Kyoto, Japan, LC-10A Series) system [51]. The anion-exchange column was eluted with a linear concentration gradient of Tris-HCl mobile phase (buffer A, 10 mM Tris-HCl, pH 7.4; buffer B, 200 mM Tris-HCl, pH 7.4) from 0 to 40% buffer B in 12 min. The eluate was introduced into the nebulizer (flow rate, 1.0 ml/min) of ICP-MS (Hewlett-Packard, [HP]-4500, Avondale, PA, USA), and the intensity of Cu, Zn, Ag, Cd, and Hg was monitored continuously. The eluate also was monitored by absorptions 254 nm (distinctive absorption of metal-thiolate structure) and 280 nm (distinctive absorption of aromatic amino acids), continuously.

**Chemical analysis**

Each fraction was analyzed for metals by the method described previously [9]. Samples were dried for 12 h at 80°C and then digested by microwave with nitric acid in a Teflon® polytetrafluoroethylene tube. Analysis of Cu, Zn, Ag, and Cd was performed with an ICP-MS using Y as internal standard. Concentrations of Hg and Se were determined with a cold vapor atomic absorption spectrometer (Sanso, Model HG-3000, Tsukuba, Japan) and hydride generation atomic absorption spectrometer (Shimadzu, HVG-1 hydride system), respectively. Accuracy of the methods was assessed using standard reference materials DORM2 (National Research Council Canada) and SRM1577b (National Institute of Standards and Technology, Gaithersburg, MD, USA). Recoveries of the metals ranged from 95.8 to 105%. In the present study, all the metal concentrations were expressed on a wet wt basis (µg/g). In order to compare our values to published values reported on a dry weight basis, we have converted the latter to a wet weight basis assuming the moisture content as 70%.

**Statistical analysis**

Mann–Whitney $U$ test was employed to detect species differences in metal concentration. Simple regression analysis was conducted between the metal concentrations in hepatic cytosol and those of HMW, MT, and low-molecular-weight (LMW) fractions in the cytosol. $p$-value of less than 0.05 was considered to indicate statistical significance. These statistical analyses were executed by the program StatView (Ver 5.0, SAS Institute, Cary, NC, USA).

**RESULTS AND DISCUSSION**

**Metal levels in the liver of northern fur seals, black-footed albatrosses, and Dall’s porpoises**

The concentrations of Hg, Se, Zn, and Cd in whole liver tissue were significantly higher in northern fur seals and black-footed albatrosses than Dall’s porpoises (Mann–Whitney $U$ test, $p < 0.01$), and Cu was highest in northern fur seals among the three species ($p < 0.01$) (Table 1). These values were within the range in the livers of higher trophic marine animals reported in earlier studies [52,53]. Concentrations of Hg and Se in the liver of Dall’s porpoises were an order of magnitude lower than those of the other two species, whereas Ag concentration in Dall’s porpoises (1.3 ± 1.1 µg/g) was five- to 10-fold higher than those in the other two species ($p < 0.01$). These results might reflect the difference in composition of their diets. It is known that Dall’s porpoises prefer to feed on squids and benthic invertebrates [54] with high Ag levels [55,56]. There have been a few investigations on the concentrations of Ag in higher trophic marine animals. An extremely high concentration of Ag was detected in the liver of belugas from North Atlantic (10.1–107.4 µg/g; [57]) and from Alaska (USA) (mean, 15.9 µg/g [58]). However, hepatic Ag concentration usually was below 1 µg/g for marine mammals [2,57,59]. Hence, Ag concentration in the liver of Dall’s porpoises seems to be relatively high compared to those of other marine mammals.

**Comparison of subcellular distribution of metals in liver of marine animals**

Distribution of Cu, Zn, Cd, Ag, Hg, and Se in hepatic cytosol (soluble fraction) of northern fur seals, black-footed albatrosses, and Dall’s porpoises is shown in Table 1. In black-footed albatrosses and Dall’s porpoises with low Cu levels (5.1 µg/g and 5.0 µg/g, respectively), distribution of Cu in cytosol in the liver was relatively low (31.7 and 40.6%, respectively). In contrast, distribution of Cu in cytosol was relatively high (66.7%) in the liver of northern fur seals accumulating high Cu (23 µg/g). For all three species, more than 70% of Zn and...
Cd were present in cytosol (Table 1). Both Hg and Se mainly were distributed in insoluble fraction in the liver of northern fur seals and black-footed albatrosses with high accumulation of Hg in their livers (59 μg/g and 94 μg/g, respectively), whereas distribution of Hg and Se was relatively large (44.4 and 43.7%) in cytosol for Dall’s porpoises with low Hg levels (3.8 μg/g) (Table 1). Silver showed large distribution in insoluble fraction for all the three species (Table 1).

Distribution of Cu, Zn, Cd, Ag, Hg, and Se in the cytosol and insoluble fraction in the liver of these three species were compared to those of other marine animals (Fig. 1). Copper mainly was accumulated in the cytosol in animals accumulating large amounts of Cu, such as northern fur seal, California sea lion [60], and green turtles [29]. However, although all the other animals accumulate relatively low Cu in liver, distribution of Cu in the cytosol was different between the species: Hawksbill turtles contained Cu mainly in cytosol in spite of its low Cu level (Fig. 1). For all the species except the stranded

### Table 1. Concentrations of metals (mean ± standard deviation and range on μg/g liver wet wt) in the liver and the distribution in cytosol in northern fur seals, black-footed albatrosses, and Dall’s porpoises

<table>
<thead>
<tr>
<th>Species</th>
<th>Cu</th>
<th>Zn</th>
<th>Cd</th>
<th>Ag</th>
<th>Hg</th>
<th>Se</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Northern fur seals (n = 10)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole tissue concentration</td>
<td>23±8</td>
<td>82±13</td>
<td>14±8</td>
<td>0.22±0.08</td>
<td>59±43</td>
<td>25±14</td>
</tr>
<tr>
<td>Cytosol</td>
<td>14–37</td>
<td>62–100</td>
<td>5.3–29</td>
<td>0.12–0.35</td>
<td>7.6–121</td>
<td>7.5–43</td>
</tr>
<tr>
<td></td>
<td>66.7±5.4</td>
<td>70.5±7.4</td>
<td>78.1±5.9</td>
<td>29.0±14.3</td>
<td>8.8±6.6</td>
<td>14.7±9.6</td>
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<tr>
<td></td>
<td>56.9–74.2</td>
<td>52.2–77.5</td>
<td>64.4–83.7</td>
<td>13.5–52.2</td>
<td>1.4–23.1</td>
<td>5.0–30.6</td>
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<tr>
<td><strong>Black-footed albatrosses (n = 5)</strong></td>
<td></td>
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</tr>
<tr>
<td>Whole tissue concentration</td>
<td>5.1±1.3</td>
<td>69±13</td>
<td>22±8</td>
<td>0.083±0.043</td>
<td>94±50</td>
<td>30±18</td>
</tr>
<tr>
<td>Cytosol</td>
<td>3.6–6.5</td>
<td>56–86</td>
<td>12–33</td>
<td>0.038–0.14</td>
<td>36–150</td>
<td>11–47</td>
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<tr>
<td></td>
<td>31.7±3.6</td>
<td>75.8±2.8</td>
<td>83.2±2.24</td>
<td>16.4±2.9</td>
<td>6.7±3.6</td>
<td>9.9±5.3</td>
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<tr>
<td></td>
<td>27.5–37.2</td>
<td>72.1–79.7</td>
<td>81.1–86.3</td>
<td>12.3–19.4</td>
<td>4.3–13.0</td>
<td>4.5–18.6</td>
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<tr>
<td><strong>Dalls’ porpoises (n = 6)</strong></td>
<td></td>
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</tr>
<tr>
<td>Whole tissue concentration</td>
<td>5.0±1.1</td>
<td>32±6</td>
<td>2.9±1.8</td>
<td>1.3±1.1</td>
<td>3.8±2.4</td>
<td>3.4±2.3</td>
</tr>
<tr>
<td>Cytosol</td>
<td>3.7–6.1</td>
<td>22–37</td>
<td>0.71–6.0</td>
<td>0.23–3.3</td>
<td>0.58–7.1</td>
<td>1.0–6.4</td>
</tr>
<tr>
<td></td>
<td>40.6±5.9</td>
<td>71.0±1.5</td>
<td>80.2±1.5</td>
<td>24.4±7.7</td>
<td>44.4±10.1</td>
<td>43.7±11.0</td>
</tr>
<tr>
<td></td>
<td>30.4–46.9</td>
<td>69.9–73.1</td>
<td>77.9–82.5</td>
<td>13.1–33.8</td>
<td>30.0–53.9</td>
<td>26.3–57.6</td>
</tr>
</tbody>
</table>

Fig. 1. Comparison of subcellular distribution of metals in liver of marine animals. The concentrations of metals in whole liver tissue are 0.83 μg/g for Cd and 73.2 μg/g for Hg in California (USA) sea lion [62]; Cu 40.5 and Zn 62.9 μg/g for California sea lion [60]; Hg 183 and Se 57.2 μg/g for harbor seals [35]; Cu 3.0, Zn 39.7, and Cd 12.4 μg/g for sperm whale [60]; Cu 2.53, Zn 31.3, Cd 28.6, and Hg 21.7 μg/g for sperm whale [61]; Cu 5.5, Zn 45, Cd 44, and Hg 8.80 μg/g for narwhal [64]; Cu 49.9, Zn 33.3, Cd 8.96, Ag 0.89, and Se 3.3 μg/g for green turtles [29]; Cu 8.62, Zn 25.5, Cd 1.23, Ag 0.38, and Se 19 μg/g for hawksbill turtles [29]. The metal concentrations were expressed on a wet weight basis (μg/g wet wt); NA = not available.
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Fig. 2. Sephadex G-75 (Ameriham Biosciences, Piscataway, NJ, USA) elution profiles of metals in hepatocytosol of northern fur seals (a), black-footed albatrosses (b), and Dall’s porpoises (c). The concentrations of metals in whole liver tissue are 6.5 µg/g wet weight for Cu, 84 µg/g for Zn, 17 µg/g for Cd, 0.24 µg/g for Ag, 53 µg/g for Hg, and 23 µg/g for Se in northern fur seal; Cu 6.5, Zn 78, Cd 33, Ag 0.14, Hg 150, and Se 47 µg/g for black-footed albatross; Cu 4.2, Zn 22, Cd 3.3, Ag 1.9, Hg 5.9, and Se 6.0 µg/g for Dall’s porpoise.

...sperm whale (49.3%) [61], more than 50% of Zn was present in cytosol in the liver (Fig. 1). Cadmium also mainly was contained in the cytosol for most of the species (Fig. 1). Only sperm whales stranded in the southern North Sea [61] and California sea lions accumulating low Cd (0.83 µg/g) [62] showed relatively low distribution of Cd in the cytosol (49.0 and 51.4%, respectively). These results suggest that cytosol has an important role in accumulation of Cu, Zn, and Cd in the liver of these higher trophic marine animals. Alternatively, high accumulation of Cu and Zn may be due to the presence of Cu- and Zn-metalloenzymes in cytosol.

Mercury largely was present in the insoluble fraction of the liver of all the animals, with the exception of Dall’s porpoises with a low-Hg level (Fig. 1). Also, more than 85% of Se was present in insoluble fraction in liver of northern fur seals, black-footed albatrosses, and harbor seals (Fig. 1). Caurant et al. [63] reported that the portion of Hg in insoluble fraction was more than 90% when Hg concentration exceeded 50 µg/g wet weight in the liver of pilot whales. According to Saeki et al. [59], Ag, Hg, and Se mainly were present in insoluble fraction of the liver, and its proportion increased with an increase in hepatic concentration for northern fur seals. Hence, it is assumed that Ag as well as Hg mainly is detoxified by Se in insoluble fraction in the liver of marine mammals and seabirds as suggested by Ikemoto et al. (unpublished data). In contrast, sea turtles showed different patterns of Se and Ag distributions from those of marine mammals and seabirds. More than 65% of Se was present in cytosol in liver of both green turtles with a low Se level (3.3 µg/g) and hawksbill turtles with a relatively high Se level (19 µg/g) (Fig. 1). Silver also mainly was present in cytosol in the sea turtles (Fig. 1), despite the fact that hepatic Ag levels of the sea turtles (green turtle: 0.89 µg/g; hawksbill turtle: 0.38 µg/g) were comparable to those of the other three species used in the present study (northern fur seals, 0.22 µg/g; black-footed albatrosses, 0.083 µg/g; Dall’s porpoise, 1.3 µg/g). These results suggest that significance of the role of Se in detoxification of heavy metals and/or Se metabolism were different between sea turtles, and marine mammals and seabirds.

Sephadex G-75 chromatography of hepatic cytosol fraction in the liver of northern fur seals, black-footed albatrosses, and Dall’s porpoises

To investigate the association of metals with MT in the hepatic cytosols of northern fur seals, black-footed albatrosses, and Dall’s porpoises, gel filtration was performed using a Sephadex G-75 column (Amersham Biosciences, Piscataway, NJ, USA). The typical elution profiles of metals for the three species are shown in Figure 2. The fractions from numbers 35 to 55 corresponded to MT fraction as reported previously [29]. The fractions that were eluted earlier and later than MT were...
defined as HMW substances and LMW substances, respectively.

Copper and Zn were found in both MT fraction and HMWS, while Cd was only in MT fraction of the three species (Fig. 2). These results were consistent with the results of narwhal [64], California sea lions [62], and sea turtles [29]. For all three species, the HMW, MT, and LMW fractions contained Hg, with the highest peak in the HMW fraction (Fig. 2). It has been reported that Hg showed the highest peak in HMWS and the lower peak in MT in hepatocysosol of narwhal [64] and California sea lions [62]. Although it is known that Ag has a high binding affinity for MT [14], very few studies have dealt with the association of Ag with MT in wildlife because of a low concentration of Ag in their tissues. Interestingly, Ag was detected both in MT and HMW fractions in all three species (Fig. 2). To our knowledge, this is the first study demonstrating the existing state of Ag in the hepatic cytosol of marine mammals and seabirds. In contrast, Se was not detected in MT fraction and was present in HMW and LMW fractions in these animals (Fig. 2). The metal composition in MT fraction on a molar basis varied between the species. The composition sequence was Zn (60.2 ± 2.9%) > Cu (21.3 ± 5.2%) > Cd (17.9 ± 3.2%) > Hg (0.5 ± 0.3%) > Ag (0.05 ± 0.01%) in northern fur seals, Zn (71.3 ± 6.9%) > Cd (24.3 ± 6.3%) > Cu (3.7 ± 3.2%) > Hg (0.6 ± 0.3%) > Ag (0.04 ± 0.02%) in black-footed albatrosses, and Zn (72.9 ± 8.1%) > Cu (13.2 ± 4.4%) > Cd (13.1 ± 7.2%) > Hg (0.6 ± 0.6%) > Ag (0.2 ± 0.1%) in Dall’s porpoises. The composition sequence in MT2, which was the major isomer in liver, was reported as Cd (48.9%) > Zn (47.7%) > Cu (2.7%) > Hg (0.7%) for striped dolphin [31] and Zn (54.7%) > Cd (42.2%) > Hg (3.1%) > Cu (not detected) for white-sided dolphin [34]. Notably, high percentage of Cu in MT was observed for northern fur seals (21.3%) and Dall’s porpoises (13.2%), Ag for Dall’s porpoises (0.2%), and Cd for striped dolphin (48.9%) and white-sided dolphin (42.2%). It should be noted that only Cu, Zn, Cd, Hg, and Ag were detected in MT fraction of the liver of the marine animals used in the present study, although MT can ligate various metals such as Co, Pb, and Sb [65].

To characterize the accumulation and detoxification of heavy metals in the hepatic cytosol in detail, the relationship between cytosolic metal concentration and their distribution in cytosol (MT, HMWS, and LMWS) was determined (Fig. 3). Because species difference was not observed in our data except for Ag (Fig. 4), we combined the data from all three species and carried out liner regression analysis. Distributions of Cu, Zn, and Cd in MT fraction markedly increased with their concentrations in cytosol (Cu: Y = 0.80X − 1.1, r = 0.989, n = 21, p < 0.0001; Zn: Y = 0.75X − 10, r = 0.911, n = 16, p < 0.0001; Cd: Y = 0.90X + 0.22, r = 0.989, n = 21, p < 0.0001) (Fig. 3). Similarly, significant positive correlations between concentrations of MT and heavy metals such as Cu, Zn, and Cd were reported for the liver of marine mammals and seabirds [27,66–68]. Hence, it seems likely that MT plays a pivotal role in the detoxification and/or metabolism of Cd, Zn, and Cu in the liver of marine mammals and seabirds. In contrast, Hg content increased markedly in HMW fraction with the concentration in the hepatic cytosol (Y = 0.51X + 0.18, r = 0.917, n = 21, p < 0.0001; Fig. 3). Also, the amount of Hg in MT fraction increased slightly with the cytosol Hg level (Y = 0.15X + 0.039, r = 0.708, n = 21, p = 0.0003). These findings demonstrate that the role of MT in detoxification of Hg is much lower than those of Cd, Zn, and Cu in liver of these higher trophic marine animals. For Se, the content in both HMW fraction (Y = 0.22X + 0.36, r = 0.533, n = 21, p = 0.0128) and LMW fraction (Y = 0.44X − 0.054, r = 0.714, n = 21, p = 0.0003) increased significantly with the concentration in cytosol.

Although the amount of Ag in HMW fraction increased markedly with the concentration in hepatic cytosol of northern fur seals, black-footed albatrosses, and Dall’s porpoises (Y = 0.92X − 0.016, r = 0.942, n = 21, p < 0.0001) (Fig. 3), species difference was observed in its relationship (Fig. 4). The amount of Ag mainly increased in MT fraction for northern fur seals (Fig. 4a), in HMW and MT fractions for black-footed albatrosses (Fig. 4b), and in HMW fraction for Dall’s porpoises (Fig. 4c) with increasing Ag concentration in cytosol. Anan et al. [29] reported that most of Ag in hepatic cytosol was present in MT fraction in sea turtles. Silver might be detoxified by Se via formation of (Ag±Se)±HMWS complex in the hepatic cytosol of black-footed albatrosses and Dall’s porpoises. Sasakura and Suzuki [49] reported that, like Hg, Ag also forms a complex with Se. However, because the concentration of Ag was much lower than that of Hg, the inter-

Fig. 3. Distribution of metals among high-molecular-weight (HMW), metallothionein (MT) and low-molecular-weight (LMW) fractions in hepatic cytosol of northern fur seals, black-footed albatrosses, and Dall’s porpoises.

Fig. 4. Distribution of Ag among high-molecular-weight (HMW), metallothionein (MT) and low-molecular-weight (LMW) fractions in hepatic cytosol of northern fur seals (a), black-footed albatrosses (b), and Dall’s porpoises (c).
action between Ag and Se in HMW fraction was not clear in the present study. Considering this, it may be assumed that detoxification of Ag in liver cytosol is different among the higher trophic marine animals; the northern fur seals and sea turtles detoxify Ag by MT, whereas Dall’s porpoises do it by HMWS.

Naganuma and Imura [43] reported that Hg and Se mainly were present in HMW fraction at a molar ratio of 1:1 in the hepatic cytosol of rabbit administered with Hg and Se. Also in the liver of northern fur seals, black-footed albatrosses, and Dall’s porpoises, both Hg and Se coexist in the HMW fraction of the cytosol (Fig. 2). Hence, we examined the interaction between Hg and Se in HMW fraction of the three species. Both Hg and Se tended to increase simultaneously in HMW fraction (\(Y = 0.17X + 0.52, r = 0.484, n = 21, p = 0.0261\)) (Fig. 5a). Furthermore, the molar ratio of Hg and Se approached around unity with an increase in Hg concentration (Fig. 5b). These results would indicate that Se interacts with Hg and then bind to HMWS in hepatic cytosol, reducing the toxicity of Hg in the liver of these three species. An excess of Hg with respect to Se in HMW fraction was found for black-footed albatrosses: The molar ratio of Se to Hg was 0.24 to 0.89 for black-footed albatrosses (Fig. 5b). According to Sasakiura and Suzuki [49], both Se and S can form a complex with Hg and then bind to a specific plasma protein in bloodstream. Hence, both Se and S might associate with Hg in HMW fraction for black-footed albatrosses.

Ping et al. [69] isolated a LMW compound containing Hg and Se at an equimolar ratio in the liver of striped dolphin. Although both Hg and Se existed in LMW fraction in hepatic cytosol of the three species used in the present study (Fig. 2), the amount of Hg in LMW fraction was too small to obtain the exact quantitative relationship between Hg and Se in the LMW fraction. To our knowledge, this is the first report providing detailed information on the interaction between Hg and Se in hepatic cytosol of higher trophic marine animals.

Characterization of MT of northern fur seals, black-footed albatrosses, and Dall’s porpoises using HPLC/ICP-MS

Isoforms of MT have been identified in a wide variety of species [11,70]. It has been suggested that each MT isoform has a different biological function or detoxification potential for some animals [18–22]. To reveal the species differences in composition of MT isoforms and interaction of metals with these MT isoforms in the hepatic cytosol, the MT fraction on a Sephadex column was applied on a DEAE anion exchange column, and ion intensity of Cu, Zn, Cd, Ag, and Hg was determined by ICP-MS for northern fur seals, black-footed albatrosses, and Dall’s porpoises (Fig. 6). Five metal peaks (2.9, 4.0, 4.3, 7.9, and 8.1 min) for northern fur seal, six (3.3, 4.1, 5.1, 7.3, 7.8, and 9.0 min) for black-footed albatrosses, and five (4.3, 4.7, 6.4, 8.1, and 8.3 min) for Dall’s porpoises were observed (Fig. 6) with absorptions at 254 nm (distinctive absorption of metal-thiolate structure) but not at 280 nm (distinctive absorption of amino acids; data not shown). The result of ultraviolet absorption suggests the presence of mercaptide bond and absence of amino acids in these metalloproteins, which is consistent with the properties of MT [71].

Several Ag and Cu peaks were eluted slightly slower than those of the other metals (Fig. 6). For example, Zn, Cd, and Hg peaks appeared at retention times of 4.0 and 7.9 min, whereas Cu and Ag peaks were eluted at 4.3 and 8.1 min for northern fur seals. Because mammalian MT can bind seven monovalent ions (Ag and Cu) or 12 divalent ions (Zn, Cd, and Hg) [65], it is assumed that differences in metal composition in the same MT isoform might cause the conformational changes in this protein, leading to the slight difference in retention time on the anion-exchange column. If so, northern fur seal, black-footed albatrosses, and Dall’s porpoises have 3, 5, and 3 isoforms of MT, respectively (Fig. 6). However, caution is necessary in the interpretation of the data, because different metal peak does not necessarily mean the different isoform of MT. It was reported that acetylated and nonacetylated forms of the same MT isoform were able to be separated by the tandem size-exclusion and anion-exchange columns [72]. Also, oxidized and dimer of MT would occur during the analysis [32]. Although difference in analytical procedure for characterization of MT isoforms does not allow direct comparison of the results among studies, at least two or three isoforms of MT have been found in the tissues of marine mammals [30–34,73]. Recent studies revealed the presence of at least ten functional MT genes for humans, ten for pigs, and four for sheep [70]. However, primary structure of MT isoform has been identified for only two species of cetaceans, MT2 of striped dolphin [74], and an isoform of bowhead whale [75]. Hence, the exact number of MT isoforms in marine mammals still is unclear.

In Dall’s porpoises, Zn, Cd, Ag, and Hg peaks eluted at 8 min apparently were higher than those obtained at 4 min, and this also was true for northern fur seals to a lesser extent (Fig. 6), suggesting that MT isoform at 8 min is more important for detoxification of the heavy metals except for Cu in these two species. Similar results also were reported for striped dolphin [31] and white-sided dolphin [34]. Also, a high Cu peak was observed at 2.9 min for northern fur seals (Fig. 6). Hence, this isoform of MT also might play a pivotal role in Cu metabolism in northern fur seals.

Although several studies have been conducted on interaction between MT and heavy metals in seabirds [24–28,76,77], no information is available concerning their MT isoforms. In black-footed albatrosses, all the metals exhibited a peak at similar retention time (Zn, Cd, and Hg: 7.3 min; Cu and Ag: 7.8 min) (Fig. 6). Minor metal peaks also were observed at 3.3 min (Cu, Zn, and Cd), 4.1 min (Cu), 5.1 min (Cu), and...
Fig. 6. Elution profiles of Cu, Zn, Cd, Ag, and Hg obtained by applying hepatic metallothionein fraction isolated on a Sephadex column (Amersham Biosciences, Piscataway, NJ, USA) into high-performance liquid chromatography/inductively coupled plasma–mass spectrometry system with a DEAE (Tosoh, Tokyo, Japan) anion exchange column. (a) northern fur seal, (b) black-footed albatross, and (c) Dall’s porpoise.

9.0 min (Cu and Ag) (Fig. 6), suggesting the existence of multiple isoforms of MT in liver of black-footed albatrosses. However, birds generally possess only one isoform of MT [78,79] and only quail [80] and pigeon [81] are reported to have two isoforms of MT in their tissue. Thus, further studies using molecular biology technique should validate the existence of multiple isoforms of MT in black-footed albatrosses. It was remarkable that nearly the entire amounts of Zn, Cd, and Hg in MT fraction were eluted at 7.5 min. This result implies that this MT isoform mainly detoxifies these metals in the liver of black-footed albatrosses.

The present study reveals the interaction of Cu, Zn, Ag, and Hg with MT in the liver of northern fur seals, black-footed albatrosses, and Dall’s porpoises. Copper, Zn, and Cd were accumulated preferentially in MT fraction, whereas Hg mainly was bound to HMWS probably via formation of (Hg-Se)-protein complex in the hepatic cytosol of these higher trophic marine animals. Also, Ag was detected both in MT and HMW fractions in all three species. The detoxification mechanism of Ag in hepatic cytosol might be different among the higher trophic marine animals; the northern fur seals detoxify Ag by MT, whereas Dall’s porpoises do it by HMWS. The HPLC/ICP–MS analysis suggests the existence of multiple isoforms of MT and difference in the number of MT isoforms and metal composition in MT between the species of marine mammals and seabirds. The results described here would help to understand the mechanism of heavy metal tolerance and species difference in the susceptibility to metals in marine mammals and seabirds.

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