Striped dolphin detoxificates mercury as insoluble Hg(S, Se) in the liver

By Poh-Sing NG,*) Hui LI,**) Kazuko MATSUMOTO,**) Sunao YAMAZAKI,*) Toshihiro KOGURE,***) Tokuhei TAGAI,****) and Hiromichi NAGASAWA*),†)

(Communicated by Saburo TAMURA, M.J.A., Nov. 12, 2001)

Abstract: Sea mammals are known to contain high concentrations of mercury and selenium especially in the liver with no apparent symptoms of poisoning. We examined the chemical form of mercury and selenium in the liver of striped dolphin, *Stenella coeruleoalba*. Neither mercury nor selenium could be extracted with acetone, 80% ethanol or 0.2 M ammonium acetate. The residue after extraction was digested most effectively with alkaline protease, resulting in solubilization of almost all the material (99% by weight) but still leaving a small amount of insoluble material (1%), which contained 80% of mercury and 66% of selenium of the original amount. This insoluble material was subjected to transmission electron microscopic analysis, X-ray microanalysis, X-ray diffraction analysis and elemental analysis, which revealed that the material contained spherical crystals of $Hg(S_{0.34}, Se_{0.66})$ with 5-10 nm in diameter as a major component. These crystals may be the final detoxification product of mercury.

Key words: Striped dolphin; *Stenella coeruleoalba*; marine mammal; mercury; selenium; detoxification; biomineralization; bioaccumulation; tiemannite.

Introduction. Marine mammals are typical endpoints of mercury bioaccumulation in the marine food web, and may accumulate mercury at concentrations of more than 100 μg/g wet weight in the liver without any apparent symptoms of poisoning.[1)-5)] In the marine ecosystem, the marine food web is mainly contaminated by methylmercury due to the action of anaerobes. Mercury accumulated in the liver of toothed cetaceans, where mercury is preferentially accumulated, is mostly found in inorganic form,[2),6),7)] though sardines and squids which they consume most are contaminated mostly by methylmercury.[6)] These works support the idea that mercury is not simply transferred from prey to predator tissues but is bioaccumulated by a complex mechanism which remains largely unknown.

In 1973, Koeman first demonstrated a high correlation between mercury and selenium in marine mammals in the North Sea, in which an approximate 1:1 molar ratio of these two elements was detected.[1)] A similar phenomenon was observed in two marine mammals in the Mediterranean Sea,[8)] in the striped dolphin caught off the coast of Japan in the Pacific Ocean,[2),3)] and in Arctic whales and ringed seals in the Canadian Arctic.[9)] Tiemannite (HgSe) granules were identified in the connective tissues of the livers from two marine mammals[8)] and from ringed seals,[10)] and also in the intercellular space in the liver of the striped dolphin[7)] and bottlenosed dolphin,[11)] which well accounted for the equimolar ratio of the two elements. Since marine mammals may consume methyl mercury in the food,[6)] this chemical form of mercury is likely transformed into inorganic or other organic compounds.

In order to understand the detoxification mechanism of mercury, we have been trying to purify and chracterize a water-soluble compound containing mercury and selenium at 1:1 molar ratio from the dolphin liver[3)] and to develop a new highly sensitive method for quantification of mercury and selenium.[3),12)] Here, we describe the presence of biomineral, Hg(S, Se), as a major chemical form in the liver of striped dolphin.

Materials and methods. *Animals.* Livers were obtained from three adult striped dolphin individuals,

*) Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1, Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan.
**) Department of Chemistry, Waseda University, 3-4-1, Okubo, Shinjuku-ku, Tokyo 169-8555, Japan.
***) Department of Earth and Planetary Science, Graduate School of Science, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
****) University Museum, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
†) Correspondence to: H. Nagasawa.

Stenella coeruleoalba, caught off the coast of Taichi in Wakayama Prefecture, Japan, in 1994.

Reagents. Mercury selenite (HgSe) and mercury sulfide (HgS) were purchased from Sigma.

Determination of mercury and selenium. Mercury and selenium in solutions were measured by graphite-furnace atomic absorption spectrometry on a Hitachi Z-9000 spectrometer with a Zeeman background using palladium as a matrix modifier as described previously.[3),12)] Mercury and selenium in the alkaline protease-digested insoluble materials were determined on an inductively coupled plasma emission spectrometer.

Determination of carbon and nitrogen. Elemental analyses for carbon and nitrogen were performed on an NC-Analyzer Sumigraph NC-90A (Sumitomo Chemical Analysis Service, Osaka, Japan).

Purification of mercury-containing materials from the dolphin liver. Livers (6.9 kg wet weight, combined equally from three individuals, sexes and ages unknown) were homogenized successively in cold acetone (60 L) and cold 80% aqueous ethanol (45 L), and the resulting residue was extracted with 0.2 M ammonium acetate (40 L). Each extract was recovered by filtration with No. 2 filter paper (Advantec, Tokyo, Japan).

Proteolytic enzyme digestion of insoluble materials. The insoluble materials (1 g, wet weight; 66% water content) after extraction with 0.2 M ammonium acetate were digested separately with trypsin (Sigma), chymotrypsin (Sigma), collagenase (Nacalai Tesque, Kyoto, Japan), thermolysin, alkaline protease (Seikagaku Co., Tokyo, Japan), Pronase (Nacalai Tesque) in 0.1 M Tris-HCl buffer (pH 8.0) except for alkaline protease digestion in which 50 mM sodium bicarbonate buffer (pH 11.0) with or without 1.0% sodium dodecyl sulfate (SDS). Each digest was determined for mercury and selenium, and the undigested materials were weighed after lyophilization.

Large-scale digestion of insoluble materials with alkaline protease. The insoluble materials (60 g, wet weight) after extraction with 0.2 M ammonium acetate were suspended in 300 ml of 50 mM sodium bicarbonate buffer (pH 11.0) containing 1% SDS, to which alkaline protease in the same buffer (10 mg/ml) was added. The mixture was incubated at 50 °C for 3 h with occasional stirring. The digest was centrifuged at 5000 rpm for 20 min, and the supernatant was removed. The residue was digested twice more with the same enzyme in the same manner as above except for longer incubation period of 14 h, and combined supernatants and precipitated residue were obtained. They were measured for mercury and selenium.

Transmission electron microscopy (TEM) and X-ray microanalysis. A small portion of the powder obtained after digestion with alkaline protease was suspended in ethanol, and the suspension was dropped on a copper grid and dried. This sample was observed under an electron microscope (JEOL, JEM-2010) at 200 kV. X-ray microanalysis was performed with a detector, S-UTW-EDS.

X-ray diffraction analysis. The powdered sample after digestion with alkaline protease, and authentic HgSe and HgS were analyzed on a RAD IC X-ray diffractometer (Rigakudenki Co., Tokyo, Japan) or a RINT X-ray diffractometer (Rigakudenki Co.) at 40 kV and 30 or 20 mA with Cu K_α ray. Scanning was performed at 4 or 1 degree/min from 5 to 120°.

Results and discussion. The average concentrations of mercury and selenium in the liver from three individuals of striped dolphin were 395 and 111 μg/g (wet weight), respectively, as determined by fluorescent X-ray analyses. The molar ratio was calculated to be about 1:0.7 (Hg:Se). These concentrations were almost comparable to those of sea mammals captured at other sea areas in the world.[1),4),5)] Equal amount of livers from three individuals were combined and they were homogenized successively in cold acetone and cold 80% aqueous ethanol, and the resulting residue was extracted with cold 0.2 M ammonium acetate. The quantities of mercury and selenium in the acetone, 80% ethanol and ammonium acetate fractions were measured, and the extraction rates in the three solvents were 0.05, 0.12 and 0.07%, respectively, for mercury and 0.11, 0.16 and 0.10%, respectively, for selenium. The very low extraction ratios of both mercury and selenium in 0.2 M ammonium acetate was in contrast to our previous experiment in which about 14% of mercury was recovered,[3)] but reasons are unclear. These results indicated that almost all mercury and selenium occurred as insoluble materials.

It was expected that mercury and selenium might be contained in protein components, because selenium is known to be involved in selenocysteine, which constitutes an active site in some enzymes,[13)] and a rat serum selenoprotein adsorbs mercury *in vivo*,[14),15)] and because mercury is known to have an ability to bind to selenium easily *in vitro*.[16)] Given the above, the residue after successive extraction with the three types of solvents was digested with various proteolytic enzymes in order to obtain amino acids or small peptides containing mercury and selenium. We tested trypsin, chymo-

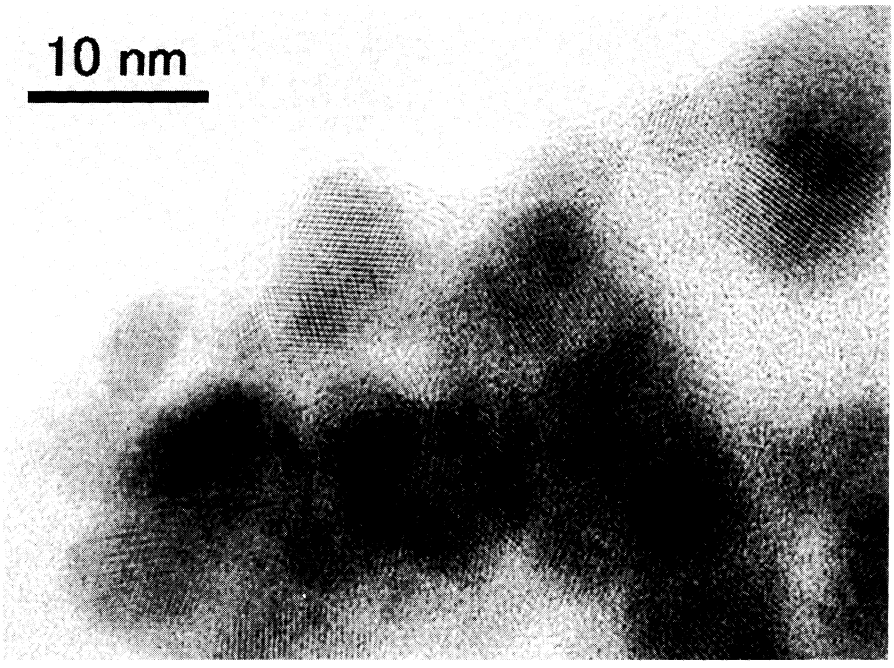

Fig. 1. Transmission electron microscope (TEM) image of the insoluble material obtained after enzymatic digestion. The dried sample was observed under an electron microscope operated at 200 kV.

Table I. Extraction rate of mercury and selenium after digestion with various proteolytic enzymes

Enzyme	SDS	Extraction rate (%) Hg	Extraction rate (%) Se	Molar ratio (Hg : Se)
Trypsin	–*)	0.30	0.40	1 : 1.09
Chymotrypsin	–	0.50	0.50	1 : 1.03
Collagenase	–	0.04	0.03	1 : 0.85
Thermolysin	+**)	4.40	39.2	1 : 6.40
Pronase	+	0.02	0.02	1 : 1.11
Alkaline protease	+	21.0	34.3	1 : 1.52

*)Without SDS. **)With SDS.

trypsin, thermolysin, collagenase, pronase and alkaline protease with or without SDS (Table I). Among those, digestion with alkaline protease in the presence of 1% SDS was most efficient. Digestion of the residue with this enzyme was repeated three times, which resulted in the solubilization of almost 99% of the starting material, with only 1% by weight still remaining insoluble. Unexpectedly, the digested solubilized fraction contained only 21.0% mercury and 34.3% selenium of the original amount, while the insoluble fraction contained more (79.0% mercury and 65.7% selenium) despite the presence of much less total material. It should be noted that each fraction did not contain mercury and selenium at a 1:1 molar ratio. The colour of the starting material was brown, but that of the residue after digestion was grayish black. From 60 g (wet weight, water content; 66%) of the residue, only 0.204 g of dried insoluble material was obtained after digestion.

Since the insoluble fraction was the major one containing mercury and selenium, it was analyzed further after washing repeatedly with distilled water and acetone followed by drying and powdering with an agate mortar and pestle set. Elemental analyses of this powdered material were performed on an NC-Analyzer and on an inductively coupled plasma emission spectrometer for mercury and selenium, revealing that the contents of the four elements, C, N, Hg and Se, were 38.0, 5.6, 1.8 and 0.56%, respectively, on a dry weight basis. These results revealed that this material still contained much organic materials unsolubilized even after proteolytic enzyme digestion. Subsequently the material was subjected to transmission electron microscopy (TEM) observations, which revealed the presence of many spherical crystals measuring 5-10 nm in diameter dispersed throughout an organic matrix (Fig. 1). Electron diffraction patterns and lattice images indicated that these crystals have the sphalerite structure, implying that they are tiemannite (HgSe). However, X-ray

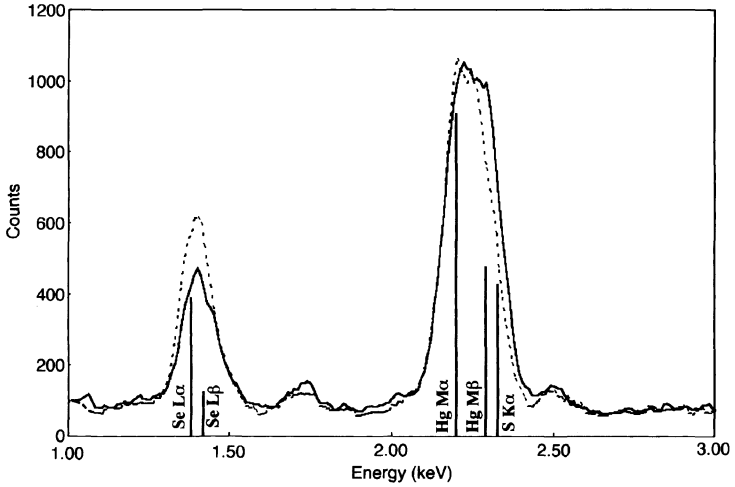

Fig. 2. Energy dispersive X-ray spectra from the crystal in the liver and synthetic HgSe. Data were collected with a Sigma (Kevex Inc.) spectrum analyzer equipped to a Hitachi HF-2000 electron microscope operated at 200 kV. Solid and dotted lines represent spectra for the natural crystal and synthetic HgSe, respectively.

microanalysis for the crystals in the TEM showed that they were deficient in selenium, compared with synthetic HgSe (Fig. 2). If we assume that the synthetic HgSe is stoichiometric, the ratio of mercury and selenium for the crystals in the livers was estimated to be about 1 : 0.7, which was consistent to bulk X-ray analyses as described above. Furthermore, the shoulder in the right of the Hg $M_{\alpha,\beta}$ peak for the crystals in the livers was higher than that for synthetic HgSe (Fig. 2). Since the energy around this position corresponds to sulfur K_α peak (Fig. 2), it was proposed that the crystals consist of not only mercury and selenium but also sulfur, forming $Hg(S_{0.3}, Se_{0.7})$.

Next, X-ray diffraction analysis of the powdered material was performed. Fig. 3 shows the diffraction pattern of the powdered material together with those of the synthetic HgSe and HgS with the sphalerite structure (metacinnabar) for comparison. Four major, rather broad peaks were observed in the pattern for the powdered material. Three of the four peaks were present in between the angles of diffraction (2θ) for HgSe and HgS, indicating that these peaks belong to a solid solution of the two compounds. The d values (lattice spacing) of the three peaks (2θ= 25.70, 42.61 and 50.58°) were calculated to be 3.456, 2.108 and 1.819 Å, respectively, which lead to their indices, 111, 220 and 311, respectively. These parameters were applied in order to determine the lattice constant (a_0 = 6.005 Å) of the solid solution. According to Vegard's rule, a linear correlation exists between the lattice constant and the chemical composition (molar fraction) of a solid solution. Thus, the ratio of HgS and HgSe in this solid solution was found to be 0.34 : 0.66. These values are roughly in agreement with the above results (about 0.3 : 0.7). The strong residual peak at 2θ = 22.53° in the X-ray diffraction could not be assigned thus far.

Tiemannite (HgSe) was identified from liver of a whale species, *Ziphius cavirostris*, captured in the Mediterranean sea, a very polluted area.[8] In this study, we have identified Hg(S, Se) as a major chemical form of mercury in the liver of striped dolphin captured near the coast of Japan. This is the first to demonstrate the existence of Hg(S, Se) as a biomineral in the living organism. These results indicate that not only selenium but also sulfur play an important role in mercury detoxification and it is likely that both elements are incorporated into Hg(S, Se) via a similar mechanism. The particle size of the solid solution was a little smaller (5-10 nm) than the above-mentioned tiemannite (15 nm).[8] These biominerals may be the final product produced in the liver possibly from methyl mercury through an unknown complex biochemical process of mercury detoxification.[17),18)]

In the striped dolphin, total mercury concentration increases with age and reaches a constant level at 20-25 years of age, while the ratio of methyl mercury to total mercury in the liver decreases after about 10 years of age.[4] Therefore, this type of detoxification ability seems to be acquired in the later life stages. Marine

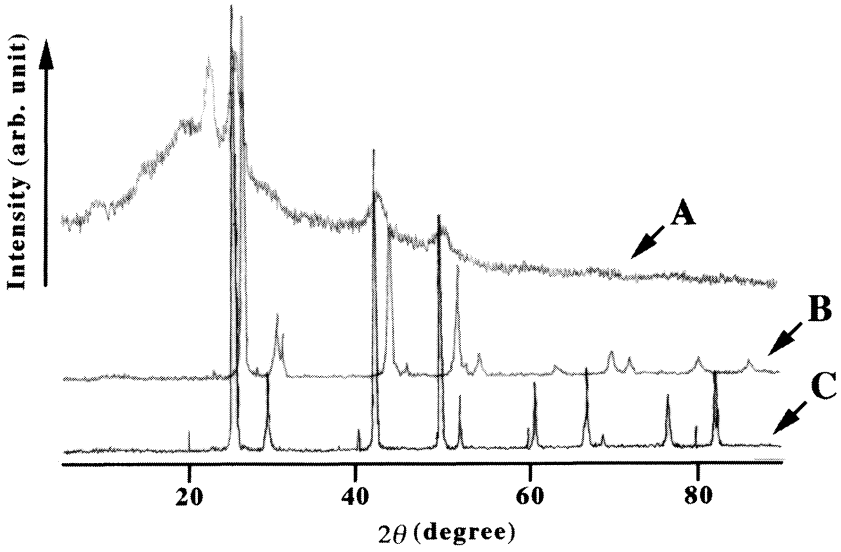

Fig. 3. X-ray diffraction pattern of powdered material obtained after enzymatic digestion. Data were collected with an X-ray diffractometer using CuK_α radiation. A: powdered material from dolphin liver. B: mercuric sulfide (HgS). C: mercuric selenide (HgSe).

mammals may have developed this means of detoxification by utilizing sulfur and selenium, which are richly present in seawater environments, differing greatly from the environment which land mammals inhabit. However, considering that the administration of selenite (H_2SeO_3) to rats which have undergone mercury poisoning is known to be very effective[19] and that mercury and selenium were present at 1:1 molar ratio in human kidney cortex,[20] a similar detoxification mechanism may occur also in land mammals. But their chemical forms are unclear. It was reported that mercuric chloride toxicity was overcome by administration of sodium selenite in rabbit, and the resulting detoxification product was found to be similar to a Hg-Se-S-containing soluble compound synthesized *in vitro* by addition of equimolar mercuric chloride and sodium selenite to aqueous, buffered glutathione.[21] In a previous paper, we reported the presence of a water-soluble, low molecular weight compound containing mercury and selenium with a 1:1 molar ratio in the liver extracts of striped dolphin and purified it to almost homogeneity, although we could not obtain any structural information.[3] We are currently analyzing enzymatically solubilized materials containing mercury and selenium, which might be protein-derived compounds. Preliminary results showed that the apparent molecular weights of the compounds containing mercury and selenium were larger than 1×10^6 as determined by gel-filtration on Sephacryl S-300 even after the treatment of enzymatic digestion. These water-soluble mercury-containing compounds would provide further information clarifying how methyl mercury is converted to Hg(S, Se). The mechanism of detoxification of mercury by biomineralization to form a solid solution, Hg(S, Se), observed in the liver of striped dolphin in this experiment or crystals, HgSe, may be widespread in marine mammals.

Acknowledgments. We are grateful to Prof. N. Miyazaki of the Ocean Research Institute, The University of Tokyo, for providing striped dolphin livers, and to Dr. T. Kunito of Department of Applied Biological Chemistry, The University of Tokyo for the analyses of carbon and nitrogen. We also thank Dr. M. N. Wilder for critical reading of this manuscript. This work was supported by a Grant-in-Aid for Creative Research (#12NP0201) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1) Koeman, J. H., Peeters, W. H. M., Koudstaal-Hol, C. M., Tjoe, P. S., and De Goeij, J. J. M. (1973) Nature **245**, 385-386.
2) Itano, K., Kawai, S., Miyazaki, N., Tatsukawa, R., and Fujiyama, T. (1984) Agric. Biol. Chem. **48**, 1109-1116.
3) Liu, P., Nagasawa, H., Matsumoto, K., Suzuki, A., and Fuwa, K. (1986) Biol. Trace Elem. Res. **11**, 185-199.
4) Skaare, J. U., Degre, E., Aspholm, P. E., and Ugland, K. I. (1994) Pollut. **85**, 153-160.

5) Capelli, R., Drava, G., De Pellegrini, R., Minganti, Y., and Poggi, R. (2000) Adv. Environ. Res. **4**, 31-33.
6) Pelletier, E. (1985) Mar. Environ. Res. **18**, 111-132.
7) Nigro, M. (1994) J. Mar. Biol. Ass. U. K. **74**, 975-978.
8) Martoja, R., and Berry, J.-P. (1980) Vie Milieu **30**, 7-10.
9) Wagemann, R., Innes, S., and Richard, P. R. (1996) Sci. Total Environ. **186**, 41-66.
10) Wagemann, R., Trebecz, E., Boila, G., and Lockhart, W. L. (2000) Sci. Total Environ. **261**, 21-32.
11) Nigro, M., and Leonzio, C. (1996) Mar. Eco-Prog. Ser. **135**, 137-143.
12) Li, H., Nagasawa, H., and Matsumoto, K. (1996) Anal. Sci. **12**, 215-218.
13) Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., and Hafemen, D. G. (1973) Science **179**, 588-590.
14) Yoneda, S., Ohmichi, M., and Suzuki, K. T. (1997) Jpn. Tox. Env. Health **43**, 26.
15) Yoneda, S., and Suzuki, K. T. (1997) Toxicol. Appl. Pharmacol. **143**, 274-280.
16) Sugiura, Y., Tamai, Y., and Tanaka, H. (1978) Bioinorg. Chem. **9**, 167-180.
17) Caurant, F., Navarro, M., and Amiard, J.-C. (1996) Sci. Total Environ. **186**, 95-104.
18) Palmisano, F., Cardellicchio, N., and Zambonin, P. G. (1995) Mar. Environ. Res. **40**, 109-121.
19) Yamane, Y., Fukino, H., Aida, Y., and Imagawa, M. (1978) Chem. Pharm. Bull. (Tokyo) **26**, 703-708.
20) Drasch, G., Wanghoefer, E., Roider, G., and Strobach, S. (1996) J. Trace Elem. Med. Biol. **10**, 251-254.
21) Gailer, J., George, G. N., Pickering, I. J., Madden, S., Prince, R. C., Yu, E. Y., Denton, M. B., Younis, H. S., and Aposhian, H. V. (2000) Chem. Res. Toxicol. **13**, 1135-1142.