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CHARACTERIZATION OF MERCURY AND SELENIUM COMPLEX IN RINGED SEAL LIVER

School of Dietetics and Human Nutrition

McGill University, Montreal

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ABSTRACT

Ringed seal (*Phoca hispida*) is a major component and a major source of mercury (Hg) in the Inuit traditional diet. A high correlation between Hg and selenium (Se), as different forms of Hg-Se complex, has been reported in many species of marine mammals. The chemical form of the Hg-Se complex in ringed seals has never be characterised. In this study, Hg and Se concentrations in different seal tissues: liver, kidney, muscle and brain, were measured. The highest Hg and Se concentrations were found in the liver and a strong linear correlation was also observed between Hg and Se concentration in the seal liver. Extensive chromatographic and mass spectrometry techniques have been used to isolate and characterize the Hg and Se ligands in seal liver. Extraction of Hg and Se showed that Hg and Se were bound to ligands within the cell membranes of ringed seal liver. The Hg/Se binding protein has a MW range of about 65 kDa and Hg and Se had a 1:1 molecular ratio. The Hg/Se binding protein may contain 3 major polypeptides with MW of 6510.8, 14305.1 and 14353.1 Da. The toxicology of this Hg/Se binding protein will be studied using an animal feeding experiment.

Résumé

Le phoque est un élément essentiel de l'alimentation traditionnelle des Inuits, tout en étant une source importante de contamination au mecrure. De nombreuses espèces de mammifères marins ont montré qu'il y avait une forte relation entre le mercure (Hg) et le sélénium (Se) et les différents complexes de mercure-sélénuim trouvés dans ces animaux marins. Chez les phoques, ces différents complexes de Hg-Se n'ont jamais été indentifiés. Lors de cette étude, les différentes concentrations de Se et Hg ont été déterminées dans le foie, les reins, les muscles et le cerveau des phoques; le foie était l'organe où fut trouvée la plus grande concentration de Hg et Se. La chromatographie et la spectrophotométrie de masse furent les techniques utilisées pour charactériser les complexes de Hg et Se du foie de phoque. L'extraction a montré que ces éléments étaient liés à des molécules de la membrane des cellules du foie. Les protéines liant Hg et Se avaient un poids moléculaire d'environ 65 kDa et le rapport moléculaire avec Hg et Se était 1:1. Les protéines liantes de Hg/Se étaient constituées de trois polypeptides ayant des poids moléculaires de 6510.8, 14305.1 et 14353.1 Da. La toxicité de ces protéines serat prochainement étudiée sur un modèle animal.

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Chapter 1. Literature Review

1.1 Introduction

Heavy metals are found in the Arctic environment as a result of long-range atmospheric and oceanic transport and local mining activities (Barrie et al., 1992). In the Canadian Arctic, mercury, cadmium, arsenic, and lead are the toxic metals recognised as pollutants of major concern, partly because of the bioaccumulation in the food chain. Elevated levels of these metals have been reported in terrestrial, fresh water, and marine biota (Lockhart et al., 1992) and have also been identified in food species and human tissues of northern indigenous peoples (Kuhnlein and Chan, 2000). An extensive literature review on levels of environmental contaminants in Northern Canada (Chan 1998), including the ranges of mercury in 79 species of marine mammals, terrestrial mammals, birds, fish and plants, suggested that mercury levels in 32 % of marine mammal meat exceeded the guideline level of 0.5 μg/g. Potential health effects of these metals on indigenous peoples are a concern because human are at the top of the food chain and some of these pollutants are known to accumulate (Lockhart et al., 1992).

Oral exposure of higher levels of these metals can injury several body tissues or target organs. Long-term exposure to mercury can permanently damage the brain, kidney, and developing fetus. Chronic exposure to arsenic can be carcinogenic and may also lead to neurotoxicity, vascular disease and liver injury. Lead toxicity is especially well known for children, with effects detected in the nervous system, in blood cells as anaemia, and in

damage to the kidneys. The kidney is the target organ for cadmium toxicity, with bone disorders as a possible consequence of kidney malfunction (Kuhnlein and Chan, 2000). Bioavailability of metals is a significant factor in toxicity. Organic forms of mercury (methylmercury-MeHg) are much more toxic than the elemental form, whereas inorganic arsenic is much more toxic than the organic form (Kuhnlein and Chan, 2000). Other nutrients in the diet will also have some effects on the toxicity of these metals. For example, selenium, Vitamin C, E and protein has demonstrated protective effects against mercury toxicity (Chapman and Chan, 2000). This literature review will only focus on the interactions between mercury and selenium in traditional food and the potential health effects of mercury on the indigenous peoples.

1.2 Mercury

1.2.1 The Major Physical and Chemical Forms of Mercury

Mercury (Hg) exists in a number of physical and chemical forms in the environment and as a result of chemical syntheses for a variety of medical, agriculture, industrial and other purposes. Hg has three stable oxidation states (Fig. 1). In the ground, Hg exists as the metallic element (Jackson, 1998). The loss of one electron gives the mercurous ion. This oxidation state is commonly found as calomel or mercurous chloride, Hg_2Cl_2. It is still used in electrolytic reference cells. The loss of two electrons results in the formation of the mercuric ion. In this oxidation state, mercuric mercury forms a large number stable chemical compounds, the best known of which is mercuric chloride, $HgCl_2$. In the past it

was commonly used as a disinfectant (WHO, 1990).

INORGANIC

Hg^0	Hg_2^{++}	Hg^{++}
Metalic	Mercurous	Mercuric

ORGANIC

CH_3HgCH_3	CH_3Hg^+
Dimethylmercury	Monomethylmercury

Fig. 1 The major physical and chemical forms of mercury

The mercuric ion can form a number of organomercurial compounds in which the mercury atom is covalently linked to at least one carbon atom. Dimethylmercury is an uncharged, lipophilic compound that is highly volatile. Monomethyl mercury is a cation that forms a variety of compounds.

Overall, the cations of mercury, for example inorganic mercuric mercury (Hg^{++}) and monomethylmercury (CH_3Hg+), readily form stable complexes and chelate with organic ligands. However, the mercury cations have by far the highest chemical affinities for the sulfdryl anions and for selenium in the selenide oxidation state, Se^{2-} (Suzuki et al., 1991).

Hg exists in different forms with different metabolism and toxicological properties. Methylmercury and Hg^0 pass blood-brain barrier into the brain, which is the target organ for these forms of Hg. Inorganic Hg passes into the brain to an very limited extent and the

target organ is the kidney (Bjorkman, et al., 1995).

1.2.2 Environmental Mercury:

Hg undergoes a global distribution. It is emitted to the atmosphere from both natural and anthropogenic sources in the form of elemental vapour (Hg^0). An estimated 10,000 tones of mercury (Hg) are released every year into our environment as a consequence of human activity (Wiken and Hintelmann, 1990). The major "human" source is the burning of fossil fuels, especially coal. Other sources include metal smelter industries, cement manufacture and crematorium. An increasingly important source is the incineration of municipal waste. Anthropogenic sources may contribute more than half of the total emissions. The major natural sources of Hg are degassing of the earth's crust, emissions from volcanoes, and evaporation from nature bodies of water (Suzuki et al. 1991). Hg^0 is converted to a soluble form assumed to be Hg^{++}. The latter is returned to the surface of the earth in rain water and may be reduced to Hg^0 and re-emitted to the atmosphere. Ocean sediment is believed to be the final sink where Hg is deposited.

It has been suggested that anthropogenic releases of Hg to the atmosphere have caused a 3 fold increase in its concentration in marine surface water and in air (WHO, 1990). Current rates of atmospheric Hg deposition in eastern North America exceed preindustrial levels by about 2-4 fold (Lockhart, 1995). Furthermore, elevated levels of Hg have been reported in the terrestrial, freshwater and marine biota in the Canadian North (Jensen et al., 1997). Mercury levels are higher than the guideline level of 0.5 μg/g. in lake trout

throughout the NorthWest Territories (NWT) and northern Quebec. Polar bears, ringed seals and beluga all have higher (than the guideline level of 0.5 μg/g) mercury contamination in the west Arctic (Jensen et al., 1997).

1.2.3 Hg Biotransformation and Bioaccumulation

Two basic concepts, bioaccumulation and biomagnification, will be defined as: bioaccumulation is the transfer of a chemical from water and/or diet into an organism and biomagnification is the successive increase in concentration of a chemical with increasing trophic levels.

Once Hg^{++} has entered bodies of fresh or open water, it undergoes a variety of biotic and abiotic reaction (Suzuki et al., 1991). The change in speciation from inorganic to methylated forms is the first crucial step in the aquatic bioaccumulation process. The mechanism of synthesis of methylmercury compounds (both $CH_3 Hg^+$ and $(CH_3)_2 Hg$) is now well understood (Wood and Wang, 1983). Methylation of inorganic mecury involves the non-enzymic methylation of Hg^{++} by methyl cobalamine compounds (analogues of vitamine B12) that are produced as a result bacterial synthesis. However, other pathways, both enzymic and non-enzymic, may play a role (Beijer and Jernelov, 1979).

Microorganisms have also been isolated that carry out the demethylation process:

$$CH_3 Hg^+ \rightarrow Hg^{++} \rightarrow Hg^0$$

The enzymology of $CH_3 Hg^+$ hydrolysis and mercuric ion reduction is now understood in

some detail, as is the oxidation of mercury vapour to Hg^{++} by an enzyme that is critical to the oxygen cycle (catalase). These oxidation-reduction and methylation-demethylation reactions are assumed to be widespread in the environment, and each ecosystem attains its own steady state with respect to the individual species of mercury. However, owing to the bioaccumulation of MeHg, methylation is more prevalent in the aquatic environment than demethylation (WHO, 1990).

Methylmercury is rapidly accumulated by most aquatic biota and attains its highest concentration in the tissues of fish at the top of the aquatic food chain. Thus, large predatory species, such as trout, pike, walleye, bass, ocean tuna, swordfish, and shark. The bioconcentration factor, i.e., the ratio of the concentration of MeHg in fish tissue to that in water, is usually between 10,000 and 100,000 (WHO, 1990). However, it should be noted that these bioconcentration ratios are not the result of partition between water and tissue but of biomagnification through the food chain. In addition to the influence of trophic level or species, factors such as the age of the fish, microbial activity and mercury in sediment, dissolved organic content (humic content), salinity, pH, and redox potential all affect the level of MeHg in fish (WHO, 1989).

1.2.4 Toxic Effects of Mercury

Methylmercury in the diet is almost completely absorbed into the blood stream and from there is distributed to all tissues, with kidney and brain being most affected. Distribution is completed within 4 days in humans (Kershaw et al., 1980). Toxic effects of mercury are

expressed in different ways according to the chemical form of mercury, the dose and the route of exposure in various species of animals. However, there are the two major forms of toxic effects of mercury, i.e., nephrotoxic effects and neurotoxic effects. Hg^{++} causes renal injury, while the chemical forms of mercury most relevant to the neurotoxic effect are elemental mercury vapour (Hg^0) or MeHg (Suzuki et al., 1991).

1.2.5 Clinical Manifestations of MeHg Toxicity:

Since the manufacture of methylmercury compounds for use as fungicides in agriculture has ceased, human exposure to this form of mercury is now exclusively from consumption of fish and marine mammals (Suzuki et al., 1991). Fetuses are particularly at risk and can suffer damage to the central nervous system, mental retardation, and lack of physical development as a result of mercury exposure. Effects of mercury on adults can also be severe and included sensory disturbance, concentric constriction of the visual field and cerebellar ataxia (Reuther 1996). The clinico-pathology is as follows: visual field disturbances are correlated with neuronal changes in the occipital lobes; sensory disturbances are correlated with loss of peripheral nerve fibres; cerebellar ataxia is correlated with loss of granule cells in the cerebellar cortex (Sato and Nakamura, 1991).

1.2.6 Traditional Food and Its Mercury Content

In the absence of occupational exposure, food is considered to be the primary environmental pathway of Hg for humans. MeHg in the human diet is almost completely

absorbed into the blood stream (WHO 1990). Foods containing higher levels of MeHg, such as certain fish and marine mammals, can be a very significant source of exposure for humans. In Canada, First nation peoples and the Inuit eat more fish or sea mammals than most other Canadian residents and, therefore, are more exposed to the risk of environmental contaminants, such as Hg, than the general population of Canada (Chan et al., 1995).

An assessment report showed that traditional foods are widely consumed in Canadian Northern communities (Jensen et al., 1997). The key food resources being large ungulates (caribou and moose) and fish. Marine mammals are important food sources in Inuit regions. Consequently, potential exposure of aboriginal northerners to contaminants in traditional food is widespread and typical across the Canadian Arctic, including Northern Quebec. A Canada-wide review of 93 community and 10 regional Aboriginal domestic fish catch estimates (Berkes 1990) suggest a median volume of 45 kg edible fish/capita/year and fish is a seasonal staple throughout the north.

Ringed seal (*Phoca hispida*), the most abundant marine mammal species in the Canada Arctic, is a major food component and the major source of Hg in the Inuit traditional diet (Chan et al., 1995). Ringed seal meat constitutes approximately one third by weight of all traditional foods eaten by both adults and children of age 2-12 years old. It also contributes almost 40 % of total Hg intake (Chan et al., 1995). Elevated levels of Hg, i.e. exceeding the Health Canada Consumer guideline (for fish flesh) of 0.5 μg/g wet weight, are commonly found in ringed seal meat (Chan et al., 1997). The average Hg

concentration in liver of ringed seal residing in East Arctic of Canada was reported as 32.9 μg/g wet weight, while the Western counterpart was 8.34 μg/g wet weight (Wheatley, 1996). Mercury in the liver of belugas was found to have increased in both western and eastern Arctic over 10-12 years. Belugas collected during 1993-1994 had a liver Hg approximately twice as that in belugas collected during 1981-1984. In ringed seals, the rate was three times higher in samples collected in 1993 compared to 15-20 years ago (Wagemann, et al, 1996).

Health Canada has established blood Hg guideline level for risk assessment purpose; <20 ppb (normal range), 20-100 ppb (increasing risk), and >100 ppb (at risk). Elevated blood Hg level was reported in Northern Quebec aboriginal communities (Wheatley, 1995). Blood Hg level of 70 % Inuit and 44 % Cree was 20-90 ppb, which means increasing risk according to Health Canada Guideline. Approximately 8 % Inuit and 8 % Cree had a blood Hg level of > 100 ppb. The mercury level of an individual living in one of the native communities has been reported as high as 600 ppb (Health and Welfare Canada, 1979).

1.3 Selenium (Se)

1.3.1 The Major Physical and Chemical Forms of Se

Se is one of the metalloids among the essential elements, which shows both metallic and non-metallic character and is capable of forming both cationic and anionic salts. Some compounds containing this element are highly reactive to thiol (SH) groups, and to some

heavy metals, such as Hg. So far, 14 selenium containing compounds have been reported in living organisms. Limited information available indicates that Se in food occurs in proteins primarily as Se analogs of amino acids. In plant derived foods, the major form is thought to be selenomethionine. In animal products, it appears to be selenocysteine (Shibata, 1992).

1.3.2 Selenoproteins:

Since the report by Schwarz (Schwarz et al., 1957) that selenium (Se) was essential for the prevention of liver necrosis, much work has been done to elucidate the metabolism and the role of Se in living organisms. Especially interesting is the finding of several enzymes having the having the seleno-analog of cysteine residues as the indispensable residue for activity.

They include the family of glutathione peroxidases (GPX), which are the classical GPX, a plasma GPX, a GPX present predominantly in the gastrointestinal tract, and the monomeric phospholipid hydroperoxide GPX (Rotruck et al., 1973; Takahashi et al., 1987; Chu, et al., 1993; Schuckelt et al., 1991). Se-containing GPX catalyzes the decomposition of various peroxides produced in the body, and thus protects living organisms from oxidative damage. A second important enzymatic function of Se was identified when type I, II and III iodothyronine deiodinase were identified as selenoenzyme (Behne et al., 1990; Davey et al., 1995; Croteau et al., 1995). These deiodinases provide a metabolic link between Se and iodine. The most recent selenoenzyme identified was

thioredoxin reductase, which was isolated from human adenocarcinoma (Tumura et al., 1996). A few other selenoproteins have been identified but their biological functions have not yet been identified. They include selenoprotein P, the main selenocompound in plasma the function of which was suspected to be involved in Se transportation in the blood, (Read et al., 1990) and selenoprotein W originally isolated from muscle (Vendeland et al., 1993).

1.3.3 Effects of Selenium on Health

Excessive Se in the diet is associated with two classical diseases described in livestock, one is commonly known as "alkali disease" and the other is "blind staggers". "Alkali disease" usually results from animals grazing on the seleniferous plants that contain between 5-40 ppm Se. Its symptoms are brittleness and even sloughing of the hooves, lameness, rough hair. "Blind stagger" is caused by animals grazing on plants that contain several thousand ppm selenium, its symptoms include impaired vision, loss of appetite, hepatic necrosis, et al.(Combs and Combs, 1986; Rosenfield and Beath, 1964). Both acute and chronic selenosis have been reported in humans (Combs and Combs, 1986).

In humans, a number of diseases have also been associated with a deficiency of selenium in diet. These include Keshan Disease, a severe cardiomyopathy occurring primarily in children(Combs and Combs, 1986), Kashin-Beck or "big-joint" disease, an osteoarthropathy occurring primarily in China, north Korea and eastern Siberia (Combs and Combs, 1986). Because of its antioxidant effect, it has been reported that Se may

reduce the risk of cancer and delay the ageing process(Lee, et al, 1996). Se, in addition, is assumed to act as an antagonist to heavy metals, such as, Hg, cadmium and arsenic (Shibata, et al., 1992).

1.4 Hg and Se Interactions

Since Parizek and Ostadalova(1967) described the protective effect of selenite against mercuric chloride intoxication, the interaction between mercury and selenium has become the object of intensive research.

1.4.1 Co-Existence of Hg and Se

Co-existence of Hg and Se have been reported in shark, seal, dolphin, and whale which feed on fish and occupied the highest position in the marine food chain (Koeman et al. 1973 & 1975; Ganther et al. 1974; Freemen et al. 1978). In 1973, Koeman et al. reported that mercury and selenium are accumulated in seal, porpoise, and dolphin livers in a molar ratio of 1:1 (Koeman et. al., 1973). Concentrations of the elements varied considerably among samples, but the data showed a linear relationship with a molar ratio of 1:1, indicating a strong interaction between these two elements. Similar phenomena were also reported in other marine animals, such as seal and whale, and in some large sized or deep-sea fish (Koeman et al. 1975; Ganther et al. 1974; Freeman et al., 1978). This 1:1 ratio has also been reported in humans. The correlation coefficient between mercury and selenium in the organs of mercury miners at Idrija, Yugoslavia, was 0.99, on a molar basis,

the ratio of mercury to selenium is 0.96 (Whanger 1992). Also, an accumulation of Se together with Hg at a 1:1 stoichiometric ratio in several organs was found in dental stuff and the general population in Sweden (Nylander et al., 1991).

1.4.2 Potential Protective Effects of Se Against Hg

It has been demonstrated that the chemical forms of mercury and selenium are important in the toxicology of both elements. Likewise, the interactions between them are also relied, to a large extent, on the chemical state in which the elements exist (Cuvin-Aralar 1991). Several studies showed that selenite could protect against Hg toxicity. Parizek and Ostadalova (1967) first reported that selenite decreased the renal toxicity caused by mercuric chloride in rats. Chen et al (1974) showed that pre-treatment with selenite followed by injections of mercuric chloride markedly decreased the mercury in the kidneys to one-tenth of control. Kasuya et al (1976) indicated that selenite prevent neurotoxicity caused by MeHg in rats.

More recent studies demonstrated when the level of Hg increases above certain threshold, demethylation starts. Molar ratio of 1:1 between total-Hg (T-Hg) and Se was found in the liver of 10 species of seabirds which contain over 100 μg Hg/g (Kim et al, 1996). However, such a relationship was unclear in other individuals which had relatively low Hg levels. This suggests that Se may play a role in Hg detoxification for those individuals with high Hg (Kim et al, 1996). Cavalli's (1995) also repeated that once T-Hg level is over 100 μg /g wet weight in dolphin live that a Hg-Se complex with a 1:1 Hg/Se molar ratio forms.

Although the interaction between Se and Hg has been the subject of intensive research. The role of Se, particularly the biological Se in the protection against MeHg remained unquantified or even uncertain (Magos, 1991). In support of the protective role of biological Se, diet supplemented with seafood high in Se delayed the onset of MeHg intoxication (Ganther et al., 1972; Ohi et al., 1976; Friedman et al., 1978). However, these experiments did not define the contribution of other dietary constituents present in the selected seafood. Ohi et al. (1976) found a tuna diet was half as effective in preventing the neurological manifestations of methylmercury as a selenite supplemented casein diet which contained as much Se as the tuna diet. The bioavailability of biological Se for Hg-Se formation is less than 20% of the bioavailability of selenite (Magos, 1991). Moreover, Ohi et al. (1976) also found that Se is not the only constituent that offered protection; the increase of casein in the diet without Se supplementation also delayed neurological manifestation. Stillings et al.(1974) also reported increased growth and survival of MeHg exposed rats with increase in casein or fish protein in the diet.

There are other problems regarding Se protection. For example, Magos and Webb (1977) reported that selenite could temporarily increases the concentration of methylmercury in the target brain. They studied the effect of the selenite (5 μmol/kg) on the brain concentration of mercury in rats treated with 5 μmol/kg methylmercury, selenite was administered at day 2, 3 and 7 after methylmercury, and 24 h change in brain mercury concentration was +134%, +103% and +88% respectively. Nishikido et al. (1988) demonstrated that selenium deficiency did not influence the teratogenicity of

methylmercury and at a high level of methylmercury exposure selenite decreased resorption only at a dose which increased the incidence of cleft palate. Even worse, methylmercury can increase the toxicity of selenite (Yonemoto et al., 1985). As the developing fetus is a sensitive target for methylmercury, these observation cast doubt on the effectiveness of Se protection.

1.4.3 Possible Mechanisms of Protection

The exact mechanism if interaction between Hg and Se are not well understood. The following are some of the possible mechanisms for the protective effects of Se against Hg toxicity: (1) redistribution of Hg in the presence of Se, (2) competition for binding sites between Hg and Se, (3) conversion of toxic forms of Hg to other forms, (4) prevention of oxidative damage, (5) formation of a Hg-Se complex.

1.4.3.1 Redistribution

One of the observed effects of the Se treatment of Hg-intoxicated animals was an apparent modification of the distribution pattern of Hg in the different organs and tissues. Several early studies showed that Se promoted the redistribution of Hg from highly sensitive organs and tissues (eg. the kidney) to less sensitive ones (eg. muscle) (Chen et al., 1974; Sheline and Schmidt-Nielsen, 1977). Potter and Matrone (1974) also demonstrated a decreased percentage of Hg in the kidneys of rats fed with selenite. It appeared that Se caused a reduction in the rate at which Hg was taken up by the kidney. In rats, pre-

treatment with selenite followed by injections of mercuric chloride markedly decreased the Hg in the kidneys to one-tenth of control (Chen et al., 1974). In minnows, *phoxinus phoxinus*, a slight reduction in renal Hg was also observed with Se treatment (Cuvin-Aralar and Furness, 1990).

It has also been showed that selenite not only affects Hg uptake by the kidney but also its retention. Se treatment in the killifish *poecilia reticulata* also decreased Hg retention in the kidney. Four and one half hours after Hg injection, the hg concentration in the kidney of Se pre-treated fish was one-half the concentration in controls (Sheline and Schmidt-Nielsen, 1977). From these works, it is reasonable to conclude that Se, whether administered prior to Hg treatment or simultaneously, resulted in the lowing of Hg levels in the kidney. However, contradictory findings were reported by Groth et al. (1972) that the presence of Se increased the concentration of Hg, fed as mercuric chloride, in kidneys. Despite this increase in kidney Hg, Se levels in the kidney increased with simultaneous administration of Hg (Komsta-Szumska and Chmielnicka, 1977).

Hg diverted away from the kidney was believed to be redistributed in the muscle. In rats, there was as much as three times more Hg in the muscle of the Se-treated group compared with the group receiving mercuric chloride only (Fang, 1977). Increased Hg retention in the muscles also had been showed in killifish upon Se treatment. Se was also increased by both methylmercury and inorganic mercury in this tissue (Sheline and Schmidt-Nielsen, 1977).

In the liver, higher Hg levels were found in rats fed with selenite, regardless of the form of Hg administered (Potter and matron, 1974). The same results were observed by Fang (1977) at equimolar doses of Hg and Se. On the other hand, in killifish liver, Hg concentrations were slightly, but not significantly, lowered after Se treatment (Sheline and Schmidt-Nielsen, 1977). The levels of Se were also increased when Hg was administered simultaneously (Komsta-Szumska and Chmielnicka, 1977).

In the subcellular soluble fraction in organs, Hg is bound mainly to metallothionein, a low molecular weight protein. The formation of metallothionein was induced by the presence of certain metals, including Hg (Cuvin-Aralar et al., 1991). Aside from decreases in Hg levels in the soluble fraction, the presence of Se also resulted in the division of the remaining Hg from metallothionein to high molecular proteins (Chen et al., 1974; Komsta-Szumska and Chmielnicka, 1977). This suggests that Se, in one way or another, blocks the binding of Hg to metallothionein or it may even inhibit the induction of metallothionein by Hg. Other data support the view that Se induces the release of Hg bound to cystein (Sumino et al., 1977). Since cystein is a major component of metallothionein and Hg is known to interact with the sulfhydryl group of this amino acid (Winge et al., 1975), the blocking of the induction of metallothionein by Se would thus leave Hg free to bind with other proteins, possibly to those with sulfhydryl groups. The higher molecular weight proteins to which Hg is diverted have not yet been characterized.

The redistribution of Hg from more sensitive targets to less sensitive sites cannot fully explain results of a number of other studies. For instance, the brain is highly sensitive to

Hg and the presence of Se enhances Hg accumulation in this organ (Cuvin-Aralar et al., 1991). It is apparent that redistribution of Hg cannot satisfactorily explain the reduction of neurological damage induced by Se treatment and that more complex mechanisms are involved in the interaction between these two elements.

1.4.3.2 Competition for Binding sites

The variability of Hg-to-Se ratios in fish compared with the concentration of these two elements in the environment led to the assumption that Hg and Se compete for the same receptors located in the animal tissues. This could also explain their toxicological antagonism. It is believed that these binding sites are Se receptors which increase in numbers with age. It is likely that these receptors can be occupied by Hg in proportion to its bioavailability in the environment (Leonzio et al., 1982). The idea of competition for binding sites has also been used not only to explain the varying accumulation rates of Hg and Se but also to explain the rates of elimination of these two elements.

1.4. 3.3 Prevention of Oxidative Damage

Se is an intrinsic component of GPx which is an antioxidative enzyme. Hg is known to have an inhibitory effect on the activity of this enzyme (Hirota, et al., 1980). This explained part of the damaging effect of Hg, particularly in liver and nervous tissue. GPx failed to protect these tissues from oxidative changes. Ganther (1978) has proposed that the possible role of the free radicals formed from the homolytic breakdown of

methylmercury in inducing neurotoxic effects. Methylmercury could be taken up by membranes in target tissues, such as the brain, in close proximity to lipids and then initiate a chain reactionperoxidation. Of various lipid constituents as a result of methylmercury's tendency to undergo homolytic fission. Without Se treatment, methylmercury will thus inhibit GPx activity, making it unable to decompose peroxides that may initiate methylmercury breakdown into methyl and mercury free radicals, and consequently this will result in tissue damage. Treatment with Se will totally alleviate the inhibitory effect of methylmercury on GPx, as shown by Chang and Suber (1982), by securing the integrity of the biological components of cells and tissues via antioxidation. This also could explain why vitamin E, also an antioxidative agent, showed protective effects against methylmercury toxicity (Ganther, 1978).

A more recent study conducted on mallard ducks by Hoffman et al (1998) showed that Se partially or totally alleviated effects of Hg on GSH peroxidase, glucose-6-phosphate dehydrogenase (G-6-PDH) (liver and brain), and hepatic oxidized glutathione (GSSG). It is concluded that since both Hg and excess Se can affect thiol status, measurement of associated enzymes in conjunction with thiol status may be a useful bioindicator to discriminate between Hg and Se effects. The ability of Se to restore the activities of G-6-PDH, GSH peroxidase, and glutathione status involved in antioxidative defense mechanisms may be crucial to biological protection from the toxic effects of methylmercury.

1.4.3.4 Conversion of Toxic Forms of Hg to Other Forms

Different forms of Hg have different toxicity. Methylmercury is known to be more toxic than most other forms. The conversion of methylmercury to less toxic forms may be one of the possible mechanisms of detoxification. Norseth and Clarkson (1970) showed that a small amount of methylmercury can be converted to inorganic Hg. Inorganic Hg was less toxic than methylmercury and had s shorter biological half-life due to its preferential excretion in the feces (Norseth and Clarkson, 1971). It would therefore be an advantage to the organism if methylmercury could be converted into inorganic Hg. Stillings et al. (1974) suggested that the protective effect of Se and cystein against methylmercury may be due to an increased rate of conversion of methylmercury to inorganic Hg. Sheline reported that this did not occur in the killifish (Sheline and Schmidt-Nielsen (1977). They tested for indications of whether demethylation and conversion to inorganic mercury occur by determining whether a breakage of carbon-mercury bond of methylmercury occurs. They used 14C and 203 Hg to label methylmercury and determined the tissue distribution of the two isotopes. Results showed that there was no difference in the distribution of the two isotopes in the tissues leading to the conclusion that no breakage of the carbon-mercury bond of methylmercury had occurred.

Earlier study by Fang (1974) on the effect of the dietary selenite on the activity of C-Hg cleavage enzymes in rat liver and kidney showed that the activity of the methyl mercuric chloride cleavage enzyme was unchanged. No measurable cleavage of the methyl mercuric chloride either with or without selenium was observed. There was also no evidence that methylmercury was converted to dimethylmercury or to inorganic Hg (Sumino et al.,

1977).

1.4.3.5 Formation of a Hg-Se complex

Simultaneous administration of mercuric chloride and selenite to rats radically altered plasma protein binding of Se and Hg compared with those which were given each element alone. After simultaneous administration, both Hg and Se were present in the plasma in much greater quantities due to their binding to a single plasma protein. Despite variations in Hg and Se dose, the molar ratio of Hg to Se in the protein remained close to unity. Further analyses showed that Se was attached to sulfhydryl groups and that Hg was attached to Se. This Hg-Se-protein complex was presumed to play a role in preventing acute inorganic Hg toxicity by binding the Hg and, thus preventing it from reaching target tissues (Burk et al., 1974). This principle might also explain the consistent 1:1 molar ratio between Hg and Se found in tissues of organisms such as seals and other marine mammals (Koeman et al., 1973; 1975).

1.4.4 The Chemical Forms of Hg-Se Complex

The chemical forms of Hg-Se complexes in marine organisms still remain to be clarified. Several different kinds of complexes may be present (Shibata, 1992). A stable Hg-selenoprotein was reported in dolphin livers (Palmisano et al., 1995; Cavalli et al., 1995). Similarly, Caurant et al. (1996) suggested that in pilot whale liver, Se is involved in promoting the binding of Hg with less critical proteins after the formation of Se-trisulphide

groups. Magos (1991) described an unstable adult of bio-methylmercury selenite in rodents. Yoneda et al suggested this complex may be a heparin-bounding protein in human serum (Yoneda and Suzuki 1997a &1997b).But the chemical form of the Hg-Se complex in ringed seals has never be characterised. The Tiemannite granules were identified in the liver of whales (Martoja et al., 1980). This Se-Hg complex, though never characterised, was suggested to be the last stage of the detoxification process through the demethylation of mercury, leading to the fossilisation of Hg and Se form of non-biodegradable compound.

1.5 Summary and Rationale

Elevated levels Hg have been reported in the terrestrial, freshwater and marine biota in the Canadian North (Jensen et al., 1997). Ringed seal (Phoca hispida), the most abundant marine mammal species in the Canadian Arctic, is a major component and the major source of Hg in the Inuit traditional diet (Chan et al., 1995). Since a high correlation between Hg and Se has been reported in many species of marine mammals, and Se was proposed to offer protection against Hg toxicity, the interaction between Hg and Se becomes an important research subject.

Objective

The overall objective of this project is to study the interaction between Hg and Se in ringed seal tissues and elucidate the potential protection of methylmercury toxicity by Se

in the traditional diet.

Specific objectives of this project are:

(a) To isolate and characterise the different species of Hg and Se in livers of ringed seals.

Hypotheses:

(a) Hg:Se molar ratios are close to 1 in ringed seal tissues.

(b) Hg and Se coexist as a complex in ringed seal liver.

Chapter 2. Methods and Materials

2.1 Sample Collections

Seal tissues were supplied by Dr Michael Kwan of the Kuujjuaq Research Centre and Dr. Lyle Lockhart of the Department of Fisheries and Oceans. Seal liver, kidney, muscle, cerebrum and cerebellum from four seals collected from Kuujjuaq were received from Dr. Michael Kwan and the second shipment of 38 seal livers, 20 from Arviat and 18 from Holman, were received from Dr. Lyle Lockhart.

2.2 Study Design

The following experiments were conducted to characterize and/or isolate the Hg/Se complex:

1. Measure Hg and Se in different seal tissues-liver, kidney, muscle and brain, using Atomic Absorption Spectrometry (AAS).
2. Determine the percentage of Hg and Se in dissolved form (cytosol) versus membrane bound form.
3. Dissolve the membrane bound Hg/Se using various buffers.
4. Isolate membrane proteins and determine their molecular weights using gel filtration chromatography (Sephadex-G75)
5. Measure Hg and Se concentration in the fractions from Sepadex G-75 column (FPLC) by ICP-MS.
6. Confirm the Hg-Se complex by size exclusion HPLC (SEC) coupled with ICP-MS.
7. Characterize the seal liver extract using reverse-phase (RP)-HPLC
8. Characterize Hg/Se complex by electron-spray ionization mass spectroscopy (MS).
9. Isolate the Hg-Se complex from seal liver extract in kg quantities using a pressurized dialysis system (Millipore Minitan System) and measure its methylmercury concentration.

2.2.1 Measure Hg and Se in different seal tissues (AAS)

Acid Digestion:

Approximately 2 g of wet tissues was weighed into pre-labeled 20-cm boiling tubes. Two

replicates per sample were prepared. concentrated nitric acid 8 ml (J.T. Baker, Baxter, Missassauga, Ontario) was added to each tube. The tubes were then covered with glass reflux bulbs and the contents were permitted to soak thoroughly in the acid at room temperature overnight. The tubes were then placed on the Thermolyn Dri-Bath and the temperature was increased to 120°C over a period of 2-3 hours. The temperature was hold at 120°C for 5 hours. The tubes were permitted to cool to room temperature. The contents of each tube were then made up to 25.0 ml with Nanopure water. The contents were transferred to a pre-labeled 25ml snap-cap polypropylene vial and stored at room temperature. The nitric acid concentration in the digest was 22% w/v.

Cold Vapour AAS Parameters for Hg Analysis:

The acid-digested sample and the reactant (10%$SnCl_2$-20% HCl) were mixed. Hg vapour was generated and transported by the Argon carrier gas into the quartz cell of a Hitachi HFS-2 hydride formation system and determined with a Hitachi Polarized Atomic Absorption Spectrometer Z-8200 (Nissei Sangyo Canada Inc., Missassauga, Ontario). The Hg hollow cathod lamp was operated at 6.0 mA, the slit width was 1.3 nm and the absorbance was measured at 253.7 nm with background correction. A Hitachi SSC-110 autosampler was used to inject samples.

Graphite Furnace AAS Parameters for Se Analysis:

Se was determined by a Hitachi Polarized Atomic Absorption Spectrometer Z-8200

equipped with a Se electrodeless discharge lamp (EDL) lamp operated at 8.0 mA with a slit width of 1.3 nm. The absorbance was measured at 196.0 nm with background correction. A Hitachi SSC-300 autosampler was used to inject samples.

Quality Assurance/Quality Control

Working standard solutions of Hg or Se were prepared immediately before use by serial dilution of atomic absorption standard containing 1000 ppm of Hg or Se (ACP Chemicals, St. Leonard, Quebec). The instrument was re-calibrated every ten samples.

Two sample blanks were analyzed together with each batch of samples. A spiked blank was analyzed during each analysis to ensure day-to-day reproducibility. Each standard and sample was measured in duplicate and the sample was reanalyzed if the relative standard deviation of the two measurements was greater than ± 5%. Coefficients of variations of the two replicates of the samples were generally less than 10%, and the mean value was considered to be representative of the sample. Standard reference materials, dogfish liver-DOLT 2 and dogfish muscle-DORM 2, from the National Research Council of Canada (Ottawa, Ontario) were digested and analyzed with each batch of samples. Results of the Hg and Se concentrations always fell within 1 SD of the certified values.

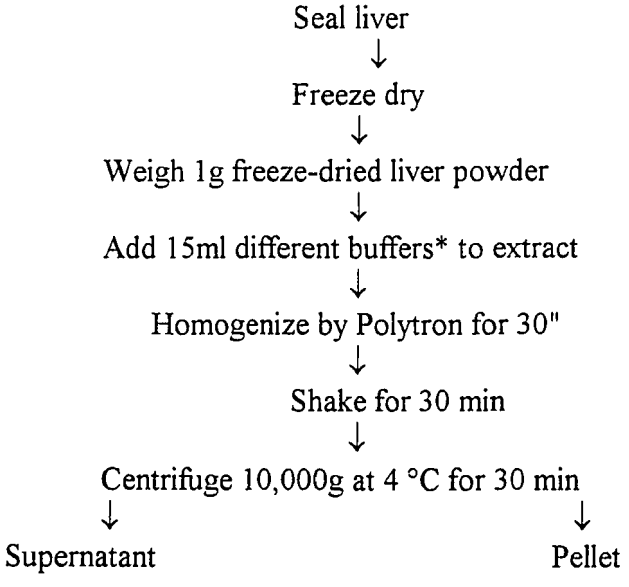
2.2.2 Determine the percentage of Hg and Se in cytosol

Approximately 2 g of seal tissues was homogenized in 1 volume of 100 mM Tris-HCl

buffer (pH 7.4) using a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The homogenates were centrifuged at 10,000g for 30 min at 4 °C in a Sorval RC 5C centrifuge (Dupont, Newtown, Connecticut). Hg and Se concentrations in the supernatant fraction (cytosol) and in the pellet were measured by AAS.

2.2.3 extraction of the membrane bound Hg/Se with various buffers

Seal livers were freeze dried and grounded with a commercial blender. Approximately 1 g of freeze-dried liver powder was weighed accurately then diluted with 15 ml of one of eight different buffers to release membrane proteins (Hg/Se complex). A Polytron homogenizer (Kinematica, Lucerne, Switzerland) was used to homogenize the tissues for 30 seconds. The homogenates were then shaken for 30 min at room temperature and centrifuged at 10,000g for 30 min at 4 °C. Hg and Se concentrations in the supernatant and pellet were measured according to the method described in section 2.2.1.



↓ ↓

Acid digestion and AAS (Hg/Se) | Acid digestion and AAS (Hg/Se)

*Buffers:
1% NaCl
0.2M Phosphate pH7.0
0.2M Ammonium acetate
0.2M Phosphate pH7.0+1% Sodium dodecylsulfate (SDS)
0.2M Ammonium acetate+2%SDS
0.2M Phosphate pH7.0+2% Triton X-100
0.2M Ammonium acetate+1%SDS
0.2M Phosphate pH7.0+2%SDS

2.2.4 Isolation of membrane-bound proteins and the estimation of their molecular weights using gel filtration chromatography (Sephadex-G75)

Seal liver extract, 1 ml (extracted with 0.2 M phosphate pH 7.0-2%SDS), was resolved on Sephadex G-75 with 50 mM Tris-HCl buffer, pH 7.0 at eluted 0.8ml/min. Separate 3 ml fractions were collected. The protein peaks were monitored with an UV detector at 280 nm. Molecular weight markers, including Aporotinin (MW=6,500), lysozyme (MW=14,300), carbonic anhydrase (MW=29,000) and albumin (MW=66,000), were used as reference standards to determine the molecular range of the protein fractions of seal livers.

2.2.5 Characterization of the Hg/Se complex by size exclusion HPLC (SEC)-ICP-MS.

Hg-Se Complex was characterised by HPLC-ICP-MS. Liver extract, 50-100 µl, was applied to the head of a SEC Progel^{TM} –TSK G4000 PWXL column (Supelco, particle

size 10 μm, sample molecular weight 2-300 x 10^3, 30cm x 7.8mm ID) and the column was eluted with 10 mM Tris-HCl pH 8.6 at a flow rate of 0.4 ml/min. The elute was introduced directly into the nebulizer tube of an ICP-MS instrument (Elan 5000 PE-SCIEX), operated under the following conditions: forward power 1000 W, plasma gas (Ar) flow rate 15 L/min, auxilliary gas (Ar) flow rate 0.8 L/min, nebulizer gas (Ar) flow rate 1.0 L/min. Isotopes Se78 and Hg199 were monitored.

2.2.6 Analyse the seal liver extract using reverse-phase (RP)-HPLC

A LC_{18} column (Supelco, 25cm x 4.6mm, particle size 5μm) was used. The mobile phase was a mixture of solvent A (1% trifluoroacetic acid) and solvent B (acetonitrile: H_2O 60:40 plus 0.08% trifluoroacetic acid) and a gradient program at 0.5ml/min. The gradient composition was as follows. The protein peaks were monitored with an UV detector both at 280 and 254 nm.

Time (min)	Rate (ml/min)	Solution A	Solution B
0.10	0.5	100	0
5		0	100
36		100	0
55		100	0

Separate fractions were collected according to the UV response of the eluate. Hg and Se levels associated with the peaks were measured by ICP-MS.

2.2.7 Determinations of Hg and Se concentration in the fractions (isolated by RP-HPLC or Sepadex G-75 chromatography) by ICP-MS.

Hg and Se concentrations in the fractions were measured by ICP-MS (Elan 5000 PE-SCIEX) operated under the following conditions: forward power 1000 W, plasma gas (Ar) flow rate 15 l/min, auxilliary gas (Ar) flow rate 0.8 l/min, nebulizer gas (Ar) flow rate 1.0 l/min, sample uptake rate 1.0 ml/min. Isotopes ^{82}Se and ^{202}Hg were monitored.

2.2.8 Characterization of the Hg/Se complex by electron spray ionization mass spectroscopy (MS).

Two fractions containing Hg and Se were lyophilized and further characterized by mass spectroscopy (MS). Lyophilized RP-HPLC fractions were reconstituted in 70% aqueous acetic acid then analyzed by Electrospray Ionization/MS using a triple quadrupole mass spectrometer (API III MS/MS system, SCIEX, Thornhill, Ontario, Canada). The solutions were infused into the electrospray ion source (fused silica capillary, 100 μm id.) at a rate of 1 μl/min from a low-pressure infusion pump (Model 22, Harvard Apparatus, South Natick, MA).

2.2.9 Isolation of the Hg-Se complex from seal liver extract in kg quantities using a pressurized dialysis system (Millipore Minitan System) and measurement of its methylmercury concentration.

Millipore Minitan System

A pressurized dialysis system, Millipore Minitan System (Millipore, Nepean, Ontario), was used to isolated the Hg-Se complex from the seal liver extract. For each batch, seal liver extract was circulated in the system at 8 psi for about 12 hours. The isolated protein was freeze-dried and the purity assayed by FPLC.

Methylmercury concentrations in the extract were determined by the method of Schintu et al. (1992):

Extraction of Methylmercury

1. Approximately 0.5 g wet tissue of the samples or 0.15 g dried seal liver powder were homogenize in 9 volumes of 50 mM Tris-HCl in 30 ml Sorvall polypropylene centrifuge tubes for 45 seconds at high speed. The homogenizer shaft was rinsed with Nano-pure water in between samples.

2. 0.25 ml of 2mg/ml protease was added to all tubes. The tubes were capped, vortexed for 10 seconds and incubated at 50 °C in a water bath for 1 hour.

3. After removal from the bath, 1.25 ml 40% NaOH and 0.5 ml 1% cysteine were added immediately. The tubes were capped, vortexed for a second and shaken in a shaker for 5 minutes at 200.

4. 0.5 ml of 0.5 M cupric sulfate was added and then 5 mls acidic NaBr. The tubes were capped, vortexed for a second and shaken in a shaker for 5 minutes at 200.

5. 2.5 ml toluene was added and the tubes were capped, vortexed for a second and shaken for 2 minutes at 200. The tubes were then centrifuged in the Sorvall RC5C for 10 minutes

at 6,800 RPM in the SLA-1000 rotor (6,600 g).

6. The toluene (top) layer was removed with a disposable glass pipette and was placed in another pre-labeled plastic test tube. Step 5 was repeated. The second removed toluene layer was added to the first.

7. 4 mls of the toluene layer was transferred into a 15 ml disposable glass centrifuge tube. (If any problems and a different volume is used, be sure to record the volume).

8. 1 ml 5 mM sodium thiosulfate was added and vortexed for 10 seconds. Then it was centrifuged at 4,000 g for 4 minutes.

9. The aqueous (bottom) layer was removed with a pipette and placed in a glass acid-washed pre-labeled test tube (Be sure to remove any toluene transferred along with it by mistake).

10. Steps 8 and 9 were repeated. The aqueous layers from both steps were combined.

11. 1.5 ml of the aqueous layer was transferred into a 50 ml glass acid-washed digestion tube (again if different than 1.5 ml, be sure to record the volume).

Acid digestion:

Nano-pure H_2O 0.5 ml and nitric acid 0.75 ml were added to each sample which was subsequently capped loosely. The samples were heated to 70°C in a dri-bath for 1-2 hours (with periodic monitoring for foaming). The samples were removed from heat and permitted to cool a few minutes. When sufficient cooled, 0.75 ml sulfuric acid was added. Tubes were shaken gently to enhance mixing. hydrochloric acid 0.375 ml was added to each tube. Each tube was shaken again very gently to mix (but be careful of violent

reaction). Samples were heated again at 70°C in a dri-bath for 2-3 hours. Samples were cooled at room temperature overnight. The digestion products were transferred to a 10 ml acid-washed glass graduated cylinder. The test tube and lid was rinsed with 2-mM potassium dichromate-3% HCl using a glass pipette and the contents were added to the same graduated cylinder. The total volume was diluted to 10 mls. After mixing, the acid digested sample was decanted into a 40 ml vial. Then 9.9 ml 1.5% HCl and 100 µl octanol were added respectively (the volume of the digest was then 20 mls). The samples were capped tightly and set aside until Hg determination.

2.3 Statistical Analysis

Correlation analyses were performed using SAS Vs 6.11 (SAS Institute Inc., Cary, NC). A p-value of <0.05 was considered to be significant in all statistical tests.

Chapter 3 Results

3.1 Hg and Se Concentrations in Seal Tissues

Hg and Se concentrations were measured in five different seal tissues from four seals (Tables 1 and 2). Among these tissues, liver, kidney, muscle, cerebrum and cerebellum, the liver was burdened with concentrations of Hg (5.99 ppm) and Se (3.41 ppm), of which, approximately 80% were associated with cell membranes. Around 80% of total Hg were also associated with membranes of seal muscle, cerebrum and cerebellum, while more Se

was present in the cytosol of the same tissue. Hg and Se distributions in kidney were different from other tissues. An approximately half of the Hg and Se were in the membrane.

Hg and Se levels were measured in 37 seal livers. The highest Hg and Se concentration was approximately 58 and 20 (ppm, wet weight), with mean ± SD being 18.5 ± 19.53 and 8.1 ± 6.8 respectively. Hg and Se concentrations in seal livers were significantly correlated (R^2=0.95, $p<0.05$). There was also a significant correlation between molar ratio of Hg and Se (R^2=0.95, $p<0.05$, Figs. 2a and 2b). The length of the seals showed correlation with the Hg/Se molar ratio. Once the length of the seals was greater than 100cm, the Hg/Se molar ratio approached unity (Fig. 3). This figure indicates that when Hg levels reached 20 ppm or Se levels reached 10 ppm, the Hg/Se molar ratios were approaching 1 (Figs. 4 & 5). The ages of the seals were not statistically correlated to either Hg or Se concentrations in the seal livers (Figs. 6 and 7).

3.2 Extraction of Hg and Se from the Biological Membranes

Eight different buffers were used to increase the recovery of Hg and Se from the biological membranes of seal livers. With 0.2 M Na_2HPO_4-NaH_2PO_4, pH7.0 + 2% SDS, a maximum extraction of 60% was obtained for both Hg and Se (Table 3).

3.3 Size Exclusion Gel Filtration Chromatography (FPLC)

Two protein peaks were detected in the seal liver extract, one big peak was eluted at approximately 21 min, whereas the small one was eluted at approximately 101min (Fig. 8). The molecular weight markers were also chromatographed by FPLC. The protein peaks of Aprotinin (MW=6,500), lysozyme (MW=14,300), carbonic anhydrase (MW=29,000) and albumin (MW=66,000) were eluted at 22.5, 40.2, 58 and 82.5 minutes respectively. Retention time and molecular weight (logMW) showed a linear relationship with R^2 =0.9923 (Fig. 9). Regression analyses indicated that the molecular weights for both peaks were 65,000 and 3,000 Daltons (Da) respectively.

3.4 Hg and Se Concentration among FPLC Fractions (ICP-MS)

Hg and Se concentration were measured within the different FPLC fractions by ICP-MS (Fig. 11). Fractions # 8 had the highest Hg level, whereas fraction # 7 had the highest Se level. Hg:Se molar ratios for fraction # 8, 9 and 10 were close to 1. A second Se peak was observed that corresponded to the second protein peak from FPLC (Figs 8 and 11). Hg and Se recoveries were 68% and 110% respectively.

3.5 Corroboration that Hg and Se are co-eluted from the Size Exclusion HPLC (SEC), by ICP-MS

The co-elution of Hg and Se in the fraction corresponding to the first protein peak of FPLC was corroborated by applying a sub-aliquot of the fraction to size exclusion HPLC-ICP-MS. Only one protein peak was detected by both Hg and Se at 17 min that

correspond to MW 65,000 Da (Fig. 10).

3.6 Reverse Phase HPLC (RP-HPLC) with MS Detection

The liver extract from size exclusion HPLC was applied to an RP-HPLC and 17 peaks were detected (Fig 12). Hg and Se were detected in 2 peaks labeled Peak A and B by ICP-MS. The Hg/Se molar ratio for both peaks was approximately 1. The chemical composition of these two fractions were analyzed by ESI-MS. Peak A contained 3 polypeptides (labeled ♦, ✶, and ●) that had MW of 6510.8, 6608.7, and 6706.1 respectively (Fig. 13). The MW of the polypeptides differed from each other by about 97.4 Da (dalton), which is consistent with ionicly bound phosphate group. This suggested that the peaks labeled ✶, and ● are mono- and di-phosphorylated analogues of the peak labeled ♦. Peak B contained 2 polypeptides labeled ● and ✻ had molecular weights of 14305.1 and 14353.1 Da respectively (Fig. 14). The 2 polypeptides differed from each other by 48 Da. The peaks labeled ● ● and ✻✻ represent non-covalently bound phosphorylated analogues of the peaks labeled ● and ✻, with molecular weights 14402.5 and 14450.2 Da respectively. The differences in MW between ● and ● ●, or between ✻ and ✻✻ is 97.4 which is consistent with ionicly bound phosphate group.

3.7 Isolation of the Hg-Se complex from seal liver extract in kg quantities using a pressurized dialysis system (Millipore Minitan System) and measurement its methylmercury concentration.

A total of 10 kg of liver extract were added to the pressurized dialysis system (Millipore Minitan System) fitted with a 30,000 Da cutoff membrane. The isolated protein was freeze-dried and the purity evaluated by FPLC (Fig. 15).

Methylmercury concentrations in seal liver extract (post Millipore Minitan system) was measured. Approximately 11% of total Hg burden was in the form of methylmercury. The recovery, an index of accuracy of the method, of Hg from certified reference material (CRM), DORM 2, was 108 %.

Table 1. Hg Concentration and Its Distribution in Seal Tissues (mean ± SD)

Seal Tissues	Hg (μg/g wet weight)	Distribution (%)	
		Cytosol	Membrane Bound
Liver	5.9917 ± 2.0514	19.7 ± 7.67	80.3 ± 7.67
Kidney	0.7021 ± 0.3091	50.9 ± 7.01	49.1 ± 7.01
Muscle	0.2392 ± 0.1469	11.0 ± 2.44	89.0 ± 2.44
Cerebrum	0.0747 ± 0.0368	18.6 ± 3.59	81.4 ± 3.59
Cerebellum	0.0551 ± 0.0193	20.1 ± 4.31	79.9 ± 4.31

Note: 100mM Tris-HCl pH7.6, 1:1 (tissue:buffer,w/v) homogenized, centrifuged at 10,000g for 30min. n=4.

Table 2. Se Concentration and Its Distribution in Seal Tissues (mean ± SD)

Seal Tissues	Se (μg/g wet weight)	Distribution (%)	
		Cytosol	Membrane Bound
Liver	3.4148 ± 0.6071	19.6 ± 6.36	80.4 ± 6.36
Kidney	0.5748 ± 0.1188	42.6 ± 6.46	57.4 ± 6.46
Muscle	0.0201 ± 0.0040	53.0 ± 1.05	47.0 ± 1.05
Cerebrum	0.0297 ± 0.0062	68.7 ± 5.64	31.3 ± 5.64
Cerebellum	0.0472 ± 0.0118	65.3 ± 2.28	34.7 ± 2.28

Note: 100mM Tris-HCl pH7.6, 1:1 (tissue:buffer,w/v) homogenized, centrifuged at 10,000g for 30min. n=4.

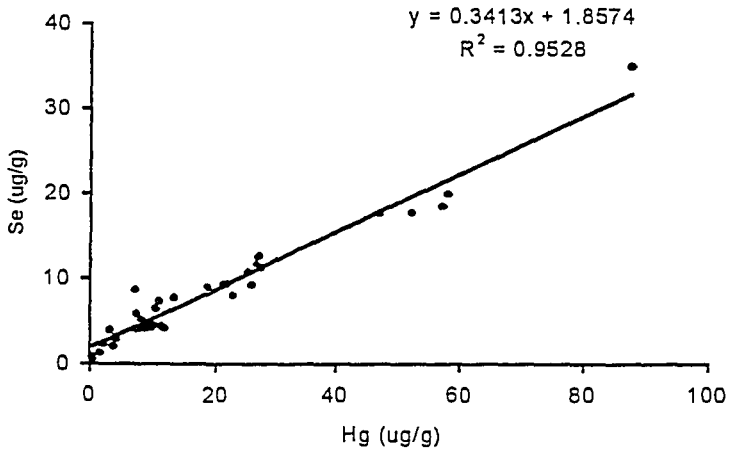


Fig.2a Correlation between Hg and Se in Seal Livers

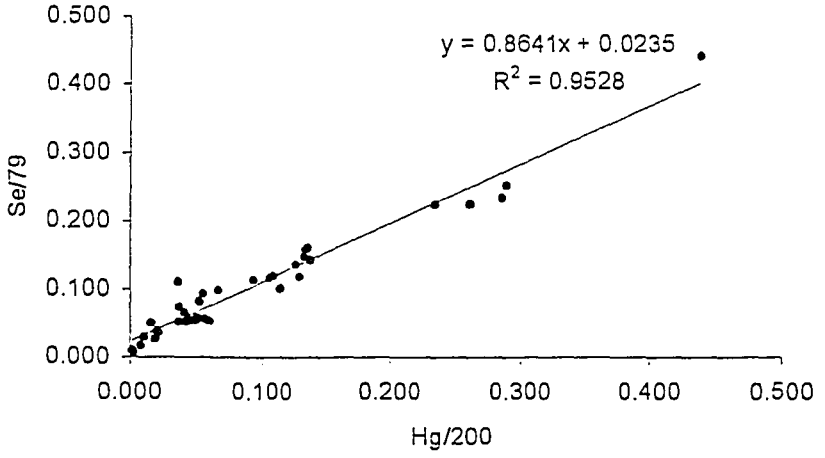


Fig. 2b Relationship between Hg and Se in Seal livers (concentration expressed as mole)

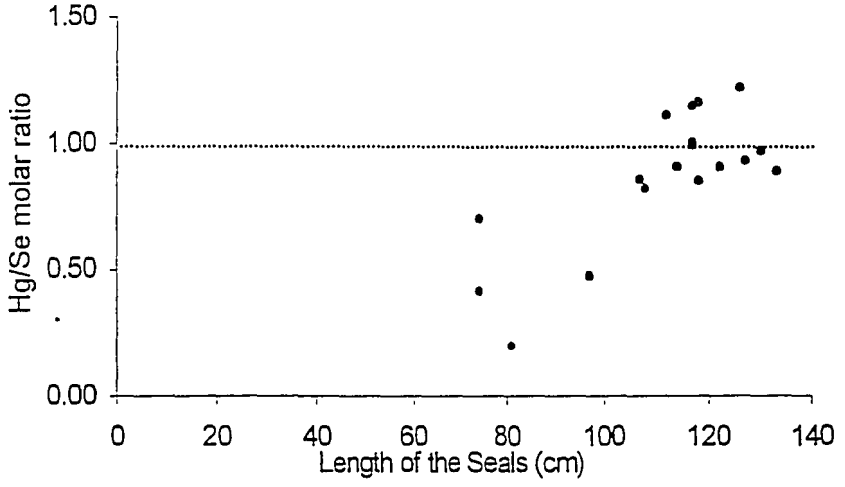


Fig. 3. Relationship between the Length of the Seals and the Hg/Se Molar Ratio

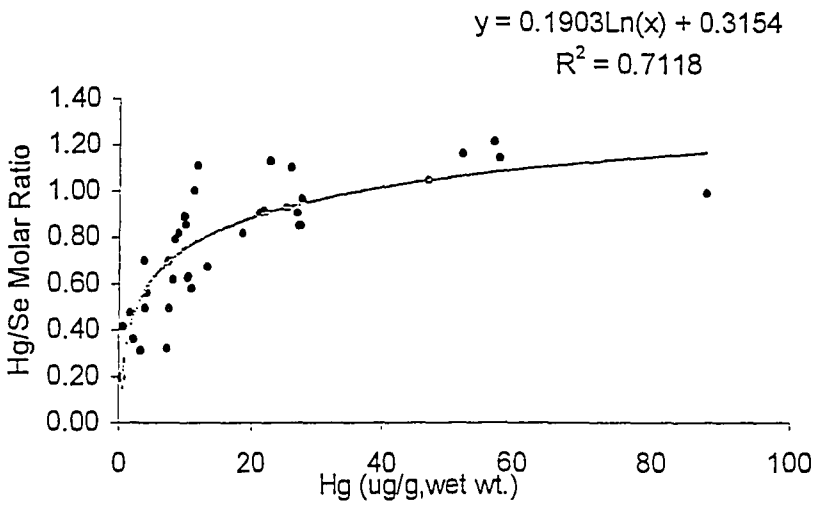


Fig. 4 Relationship between Hg Concentrations in the Seal Livers and Hg/Se Molar Ratio

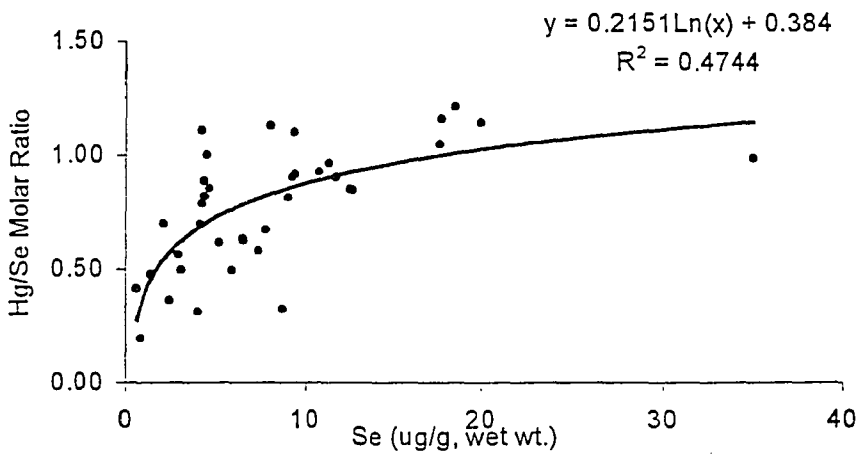


Fig. 5 Relationship between Se Concentrations in the Seal Livers and the Hg/Se Molar Ratio

Fig. 6 Relationship between Age of the Seals and Hg levels in Seal Livers

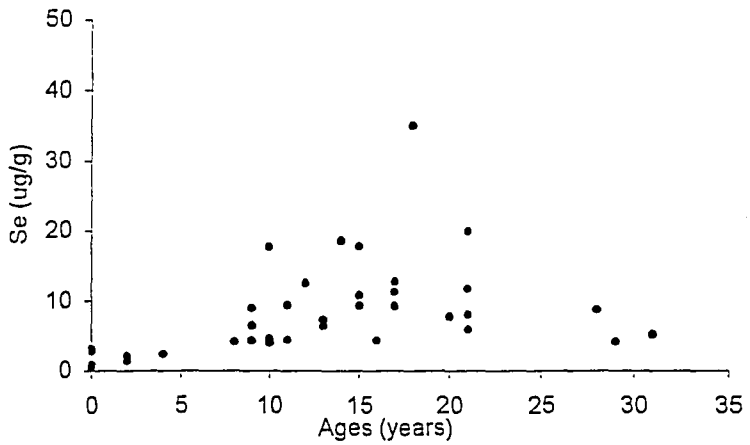


Fig. 7 Relationship between Age of the Seals and Se levels in Seal Livers

Table 3. Hg and Se Extraction Rate Using 8 Different Buffers (%)

	Hg	Se
1% NaCl	10.8	3.6
0.2 M Na_2HPO_4-NaH_2PO_4 pH 7.0	12.4	2.6
0.2 M Ammonium Acetate	8.0	18.4
0.2 M Na_2HPO_4-NaH_2PO_4 pH 7.0 +1% SDS	25.5	30.0
0.2 M Ammonium Acetate +2% SDS	40.0	44.0
0.2 M Na_2HPO_4-NaH_2PO_4 pH 7.0 + 2% Triton X-100	16.3	4.4
0.2 M Ammonium Acetate + 1% SDS	9.0	21.0
0.2 M Na_2HPO_4-NaH_2PO_4 pH 7.0 + 2% SDS	**60.0**	**60.0**

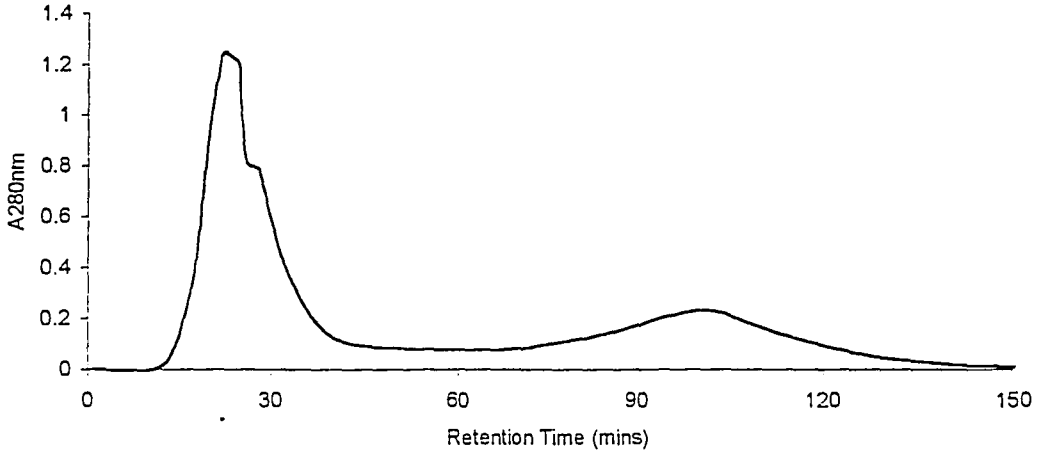
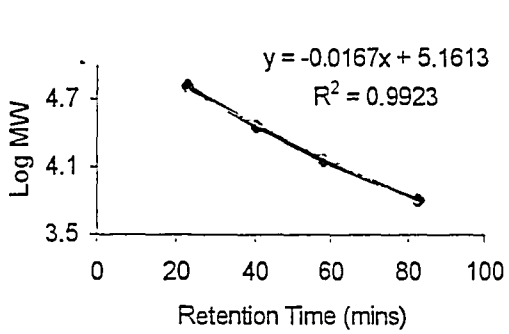


Fig. 8 Gel Filtration (Sephadex G-75) Chromatogram of Seal Liver Extract.



-Aprotinin:
MW 6,500
-Lysozyme:
MW 14,300
-Carbonic
anhydrase
MW 29,000
-BSA
MW 66,000

Fig. 9 Relationship between MW and Retention Time on Gel Filtration Column-Sephadex G75

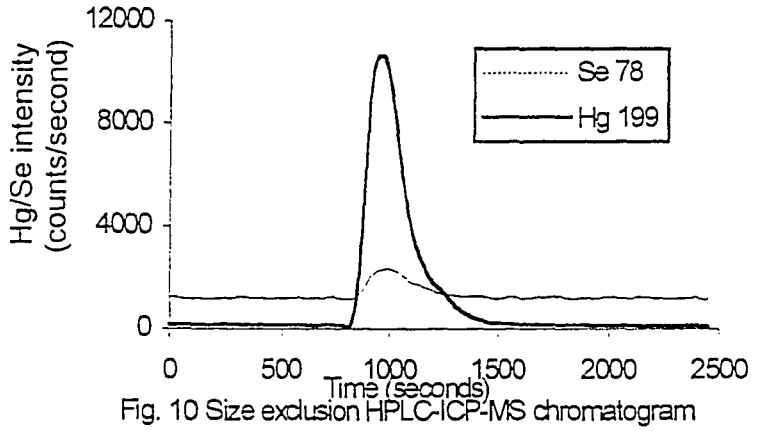


Fig. 10 Size exclusion HPLC-ICP-MS chromatogram of the seal liver extract

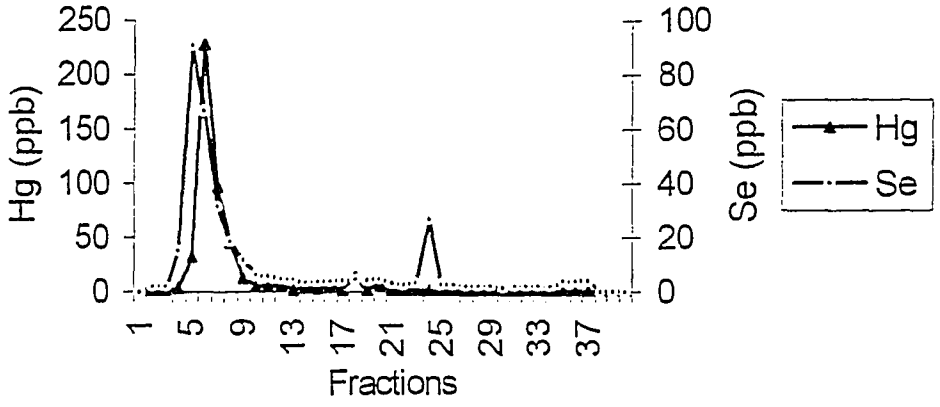


Fig. 11 Hg and Se distributions among fractions from gel filtration column

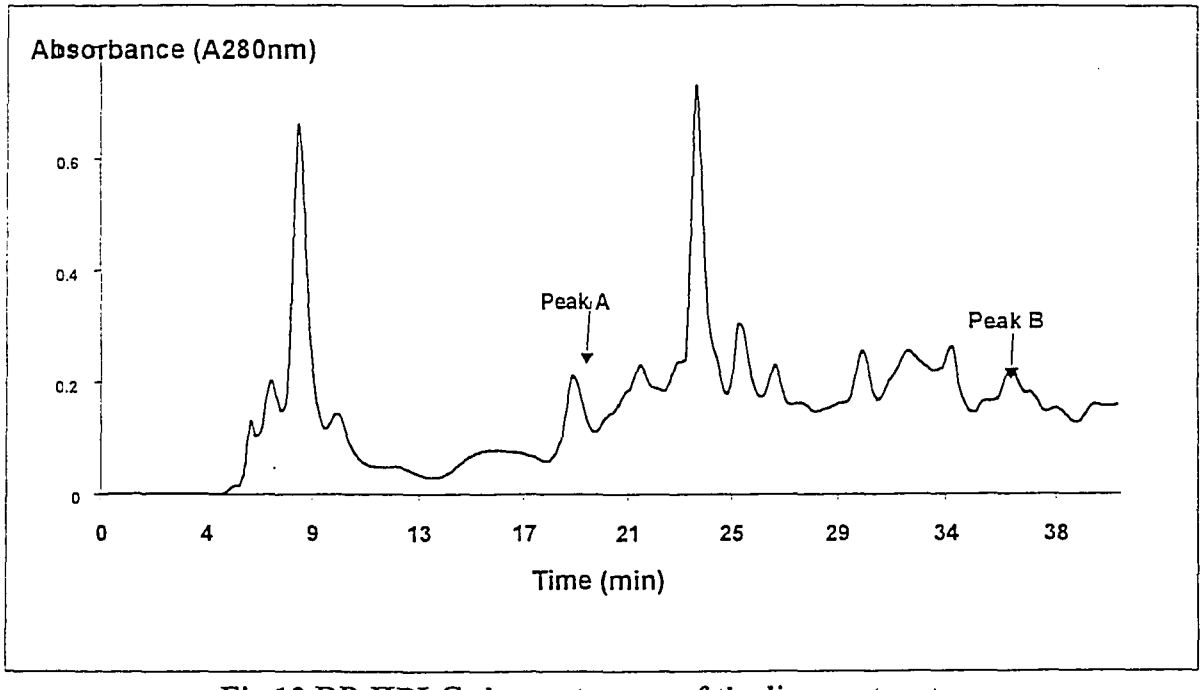


Fig.12 RP-HPLC chromatogram of the liver extract.

Fig. 13. ESI-MS chromatogram of Peak A isolated from the RP-HPLC. 3 polypeptides labeled ♦, ✶, and ● had MW of 6510.8, 6608.7, and 6706.1 respectively. Peaks labeled ✶, and ● are mono- and di-phosphorylated analogues of the peak labeled ♦.

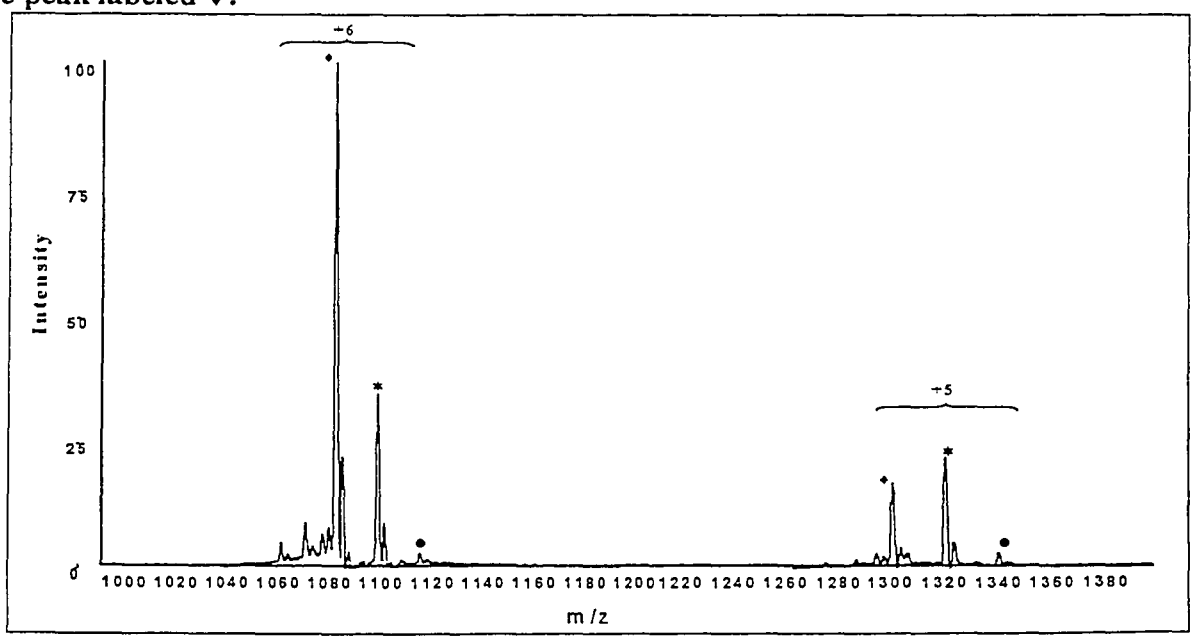
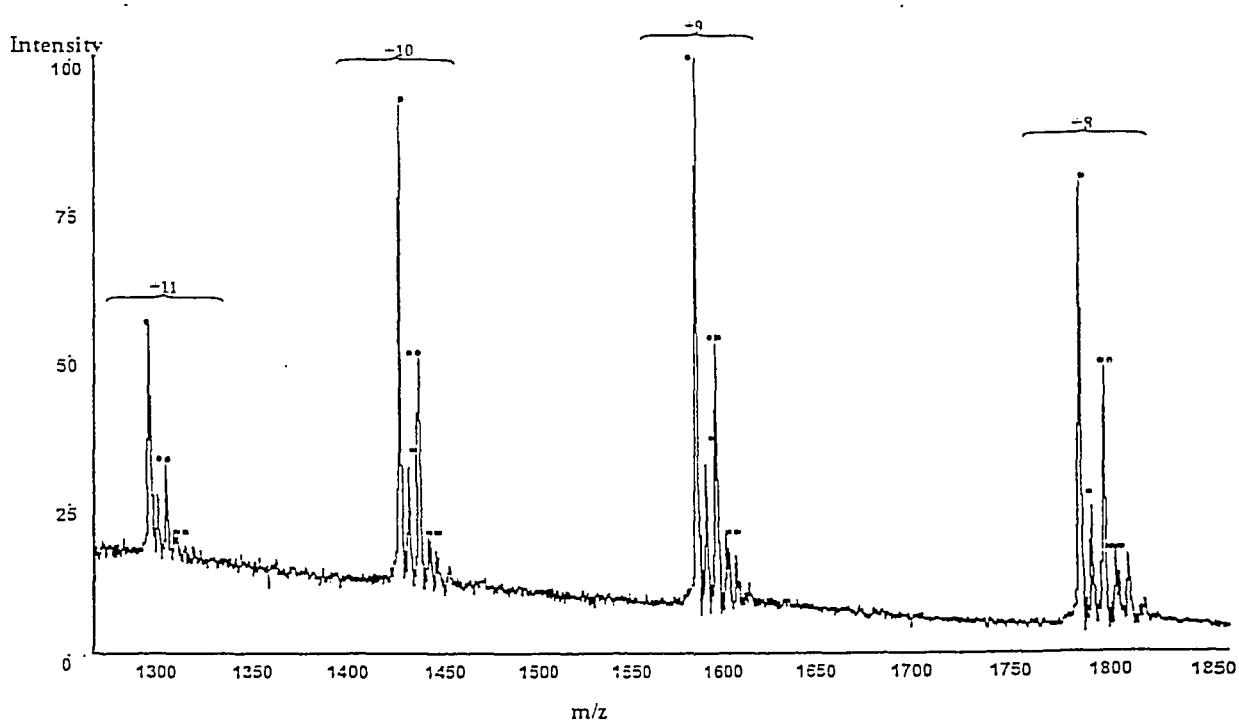


Fig. 14. ESI-MS chromatogram of Peak B isolated from the RP-HPLC. 2 polypeptides labeled with ● and * had molecular weights of 14305.1 and 14353.1 Da respectively. The peaks labeled ● ● and ** represent non-covalently bound phosphorylated analogues of the peaks labeled ● and *



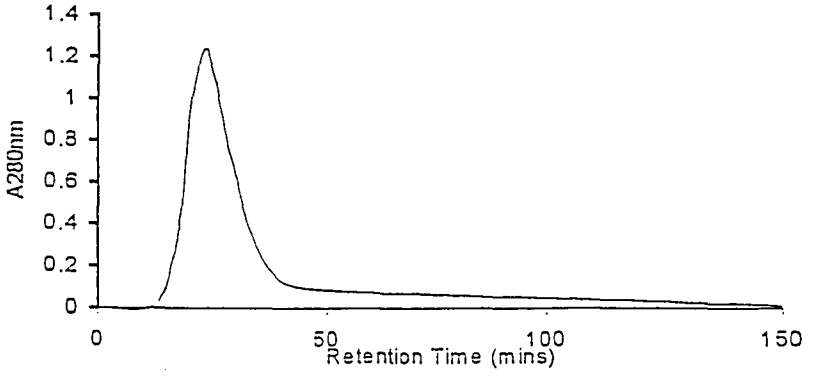


Fig. 15 Gel Filtration Chromatogram of Seal Liver Extract after Millipore Minitan System

Chapter 4. DISCUSSION AND CONCLUSION

Hg and Se concentrations in various seal tissues were characterized by significant variation. The highest Hg and Se concentrations were detected in the livers, averaging 18.5 ppm for Hg and 8.5 ppm for Se (Results section 3.1). These concentrations are comparable to the results reported for ringed seal (Hg: 10.2 ± 8.0 and Se: 15.2 ± 12.9 μg/g, wet weight) by Wagemann et al. (1996). The highest levels of Hg and Se that have been reported in marine mammals have values ranging up to 510 ppm wet weight Hg and 270 ppm wet weight Se in livers of ringed seal *Phoca Hispida* collected in the waters off the Northwest Territory of Canada (Wagemann and Muir, 1984a). Concentrations observed in this study remain an order of magnitude higher than the guideline suggested by Health Canada for consumption of fish flesh (0.5 μg/g wet weight). The effects of such high Hg burden on the animals have not been documented. The exposure guideline for oral Hg ingestion or the provisional tolerable weekly intake (PTWI) recommended by the World Health Organization is 5 μg/kg BW/week (WHO, 1990). Using this guideline, a person weighing 65 kg can only consume 18 g of seal liver per week. There are no comprehensive dietary data available for the Inuit. However, it is not uncommon for the people in the Baffin region to consume a substantial amount of seal tissues (Kuhnlein and Soueida, 1992). Assuming a typical serving of 200 g, a person will exceed the PTWI by 11-fold, if he or she consumes seal liver once a week.

The data also demonstrated that Hg and Se in ranged seal liver were mainly bound to cell membranes (Tables 1 and 2). A similar result was reported by Caurant et al (1996) who

observed that 88 % of total Hg was bound to membranes of pilot whale livers. Studies in the subcellular binding of Hg in rat livers demonstrated that Hg content in the crude nuclear, mitochondrial and microsomal fractions was increased by Se, whereas the Hg content in the soluble fraction was decreased (Fang et al., 1977). It is well recognized that in the subcellular soluble fraction, Hg is bound mainly to metallothionein (MT) and formation of MT is induced by the presence of certain metals, including Hg (Cuvin-Aralar et al., 1991). One study reported that the presence of Se resulted in the diversion of the remaining Hg from MT to high molecular weight proteins and was accompanied by decreases in Hg levels in the soluble fraction (Chen et al., 1974). These results suggest that ringed seals have a mechanism of Hg sub-cellular distribution that is similar to other animals. Formation of Hg/Se complex in the cell membranes may be a protective effect against Hg toxicity.

Almost 50 % of Hg or Se were bound to membranes (Tables 1 and 2) in this study. But different percentages were reported for different species. 73 % of mercury in pilot whales were bound to cell membranes (Wagemann et al., 1984b). For California sea lions, the results varied between 59% and 81% in kidney (Lee et al., 1977). The significance of high percentages of mercury bound to insoluble fraction, as suggested by Caurant (1996), is related to the detoxification of mercury by formation of tiemannite which leads to the fossilization of mercury in tissues. But different detoxification mechanisms may be involved (liver vs kidney).

It was also observed that Hg and Se molar relationship only approached unity when either

Hg or Se had reached a relatively high level, corresponding to 20 ppm for Hg and 10 ppm for Se (Figs 4 and 5). A study conducted on dolphin liver also demonstrated that once Hg or Se concentration reached 10 μg/g wet weight the Hg:Se relative concentration approached 1:1 molar ratio, suggesting that the accumulation of Hg and Se in the dolphin livers was caused by the formation of a compound containing the two elements at a 1:1 molar ratio (Shibata et al., 1992). Co-existence of Hg and Se have been reported in shark, seal, dolphin, and whale which feed on fish that occupy the highest position in the marine food chain (Koeman et al. 1973 & 1975; Ganther et al. 1974; Freemen et al. 1978). The result from size exclusion HPLC separation confirmed that Hg and Se had a 1:1 (molar) association. The significance of formation of Hg-Se complexes in marine mammals has been suggested as the last stage of the detoxification process through the demethylation of Hg, leading to the mineralization of Hg and Se in the form of non-biodegradable compound (Caurant et al, 1996).

The chemical form of the Hg-Se complex in ringed seals has yet to be characterized. In this study, a variety of chromatographic techniques have been used to isolate and characterize the Hg and Se ligands in ringed seal liver. The result from size exclusion HPLC analysis corroborated that Hg and Se were present in a 1:1 (molar) association and that the Hg/Se binding protein had a MW range of about 65 kDa (Figs 8, 9, 10 and 11). RP-HPLC analysis demonstrated that there are 3 major polypeptides that are associated with Hg and Se (Fig. 12). The polypeptides had MW of 6510.8, 14305.1 and 14353.1 Da. It is possible these polypeptides are subunits of the Hg/Se binding protein. For example, a combination of 3 units of the polypeptide in Peak A (3*6510.8, Fig. 13) and 3 units of the

polypeptide in Peak B (3*14402.5, Fig. 14) will result in a protein of MW at about 62000. Further characterization of the complex will require the use of amino acid sequencing and x-ray crytallography to determine the primary and tertiary structure of this chemical. Several different types of complexes can be present in rats, pilot whale liver, dolphin liver and human serum (Shibata et al., 1992, Palmisano et al., 1995; Cavalli et al., 1995, Magos 1991, Yoneda and Suzuki, 1997a &1997b). A stable Hg-selenoprotein was reported in dolphin livers (Palmisano et al., 1995; Cavalli et al., 1995). Similarly, Caurant et al. (1996) suggested that in pilot whale liver, Se be involved in promoting the binding of Hg with less critical proteins after the formation of Se-trisulphide groups. Magos (1991) described an unstable adduct of bio-methylmercury selenite in rodents. Yoneda et al (1997a &1997b) suggested this complex might be a heparin-bounding protein in human serum.

The toxicity of this Hg/Se complex is not known. Since the major form of Hg in ringed seal liver is in this form, it can be assumed to be one of the major sources of Hg in the Inuit diet. Therefore, it is important to characterize the relative toxicity of this compound. We have isolated sufficient quantities of this compound for this study (~600g). It is possible to perform an animal feeding experiment using rodent as a model. The toxicological division of the Food Directorate of Health Canada will perform a comprehensive toxicity study using this compound. Results of such study will be very useful in characterizing the risk of Hg exposure as a result of consumption of ringed seal liver.

General Conclusion

Hg and Se concentrations in different seal tissues: liver, kidney, muscle and brain, were measured; the highest Hg and Se concentrations were observed in the liver. A strong linear correlation between Hg and Se concentration in the seal liver was also noted. Extensive chromatographic separations were used to isolate and characterize the Hg and Se ligands in seal liver. Extractions of Hg and Se demonstrated that Hg and Se were bound mainly to the cell membranes. The result from size exclusion HPLC analysis corroborated that Hg and Se were present in a 1:1 (molar) association and the Hg/Se binding protein had a MW of about 65 kDa. RP-HPLC analysis demonstrated that there are 3 major polypeptides that are associated with Hg and Se. The polypeptides had MWs of 6510.8, 14305.1 and 14353.1 Da. It is possible these polypeptides are subunits of the Hg/Se binding protein. For example, a combination of 3 units of the polypeptide in Peak A (3*6510.8) and 3 units of the polypeptide in Peak B (3*14402.5) will result in a protein of MW at about 62000. The 1:1 molar ratio between Hg and Se, leading to the formation of a Hg-Se complex, suggests that Se has involved. Further research, such as protein sequencing, is needed to confirm the amino acid sequences of the Hg/Se binding protein.

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