Bioaccumulation and Speciation of Mercury and Selenium in tissues of Beluga Whales

Michael K. H. Kwan, Ph.D.

Nunavik Research Centre, Kuujjuaq, Quebec.

Objectives:

1. To elucidate the effect of age on accumulation and tissue distribution of mercury and selenium in beluga whales.
2. To investigate the accumulated forms of mercury as a function of age.
3. To assess the potential risk to human due to the levels of mercury accumulated in the beluga whales from the east coast of Hudson Bay.

Fig. 1a Total mercury concentration in tissues as a function of age

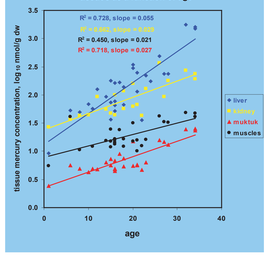


Fig. 1b Selenium concentration in tissues as a function of age

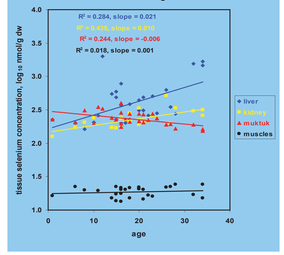


Fig.2 Relationships between mercury levels in the liver and those in other tissues

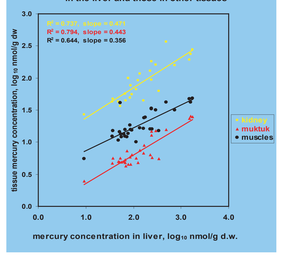


Fig. 3a Ratios of mercury concentration in tissues as a function of age

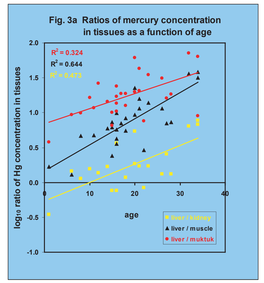


Fig. 3b Ratios of selenium concentration in tissues as a function of age

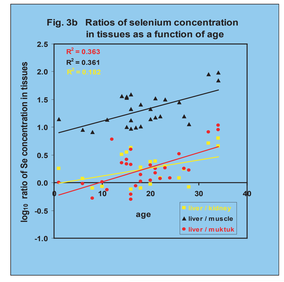


Fig. 3c Ratios of selenium concentration in tissues as a function of age

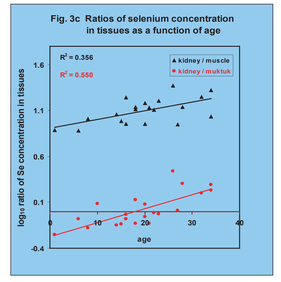
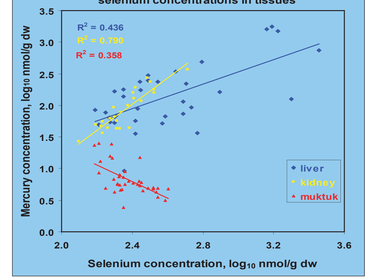


Fig. 4 Relationships between total mercury and selenium concentrations in tissues



Figs. 5a to d. Mercury 9/23/2005 selenium molar ratios in tissues as a function of age

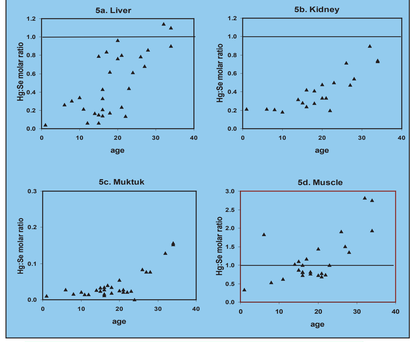


Table 2. Correlation coefficients (r), slope of the best-fit line (m) and significant levels (SL) of correlations between tissue metal concentration ratios and age.
*** p < 0.001, ** 0.001 ≤ p < 0.01, * 0.01 ≤ p < 0.05, ns p > 0.05

log_{10}Tissue Ratio	Mercury r / m / SL	Selenium r / m / SL
liver / kidney	0.688 / 0.027 / ***	0.427 / 0.014 / ns
liver / muscle	0.802 / 0.038 / ***	0.601 / 0.023 / ***
liver / muktuk	0.569 / 0.022 / **	0.602 / 0.027 / ***
kidney / muktuk	0.079 / 0.002 / ns	0.741 / 0.015 / ***
kidney / muscle	0.384 / 0.010 / ns	0.597 / 0.009 / **
muscle / muktuk	0.415 / -0.006 / ns	0.461 / 0.008 / *

Both age and gender were tested for their influence on mercury and selenium accumulation in the four tissues. Only age was found to be an important covariate on mercury accumulation in all four tissues and only for selenium in liver. Comparisons of age-adjusted means between tissues for mercury and geometric means between tissues for selenium can be summarised as follow:

Mercury		Selenium		
liver	> kidney **	liver	>	kidney *
liver	> muscle ***	liver	>	muscle ***
liver	> muktuk ***	liver	>	muktuk **
kidney	> muscle ***	kidney	>	muscle ***
kidney	> muktuk ***	kidney	>	muktuk ns
muscle	> muktuk ***	muscle	<	muktuk ***

(significant levels: *** p ≤ 0.001, ** 0.01 < p ≤ 0.05, * 0.05 < p ≤ 0.1, ns p > 0.1)

(I) Total Mercury and Selenium Determination

Tissues were initially digested in 70% w/v nitric acid in Teflon digestion bombs at 120°C overnight. The primary digests were diluted with Type I water prior to selenium determination. Aliquots of primary digests were further subjected to a more vigorous acid oxidation prior to total mercury determination. The aliquots were digested for 4 hours in a mixture of nitric, sulfuric and hydrochloric acids at 75°C in an open system of boiling tubes on aluminum heating blocks. The secondary digests were diluted with acidic potassium dichromate solution and measured for mercury. Each sample was digested in duplicate.

Cold-vapour atomic absorption spectrometry (CVAAS) was used to determine total mercury concentrations in the secondary digests. A model 4110ZL Zeeman atomic absorption spectrometer (Perkin Elmer) equipped with an electrodeless discharge (EDL) mercury lamp and a FIAS-100 flow injection system (Perkin Elmer) were used. A 10% w/v tin (II) chloride in 30% v/v hydrochloric acid was used as reductant, and a 10% v/v hydrochloric acid as the carrier. Normal calibration method with a standard curves was used in quantitation of absorbance signals. Measurements were made in duplicate for each digest. The typical detection limit achieved was about 0.05 ng/mL.

Graphite furnace atomic absorption spectrometry (GFAAS) was used to determine selenium concentrations in the primary digests. A 4110ZL Zeeman atomic absorption spectrometer, equipped with a transversely-heated graphite atomizer (THGA) and EDL selenium lamp was used. Chemical interference was suppressed by using a palladium / magnesium nitrate matrix modifier. Background absorption was corrected using the longitudinal inverse a.c. Zeeman effect background corrector of the spectrometer. Atomization and ashing temperatures used were 2200°C and 1200°C respectively. The typical characteristic mass achieved was 57-63 pg / 0.0004 abs-sec.

Internal QA/QC: For each batch of samples, two certified reference materials (dogfish muscles DORM-2 and dogfish livers DOLT-2 from the National Research Council of Canada) were digested and analyzed in duplicate. A recovery of the analyte to within 10% of its certified value was used as a criterion for validation of the batch. Digestion blanks in duplicate were included in each batch of samples digested.
External QA/QC: NCP QA/QC exercise and the QUASIMEME Laboratory Performance Studies.

(II) Total Organic Mercury

Tissues were homogenized and digested in protease (2mg/ml) in Tris-HCl buffer (50mM, pH 8.5). An alkaline cysteine reagent (1% w/v cysteine in 40% w/v sodium hydroxide) was added to chelate all mercury from the tissue digest. Cupric sulfate and acidic sodium bromide reagent (28mM sodium bromide in 8% v/v sulfuric acid) were added to liberate alkylmercury from their cysteine complexes. The alkylmercury bromides formed were then quantitatively extracted into toluene and subsequently back-extracted into sodium thiosulphate (5mM). Phase separation was effected by centrifugation (6200 x g, 20 min.). The thiosulphate extrasts were digested with a mixture of nitric, sulfuric and hydrochloric acids at 75°C in test tubes on aluminum heating blocks. Mercury in the acid digests was determined by CVAAS. Certified Reference Materials (DORM2, DOLT2 and Mussel 2976 from the National Research Council of Canada) and methylmercury chloride standards were routinely used in quality control.

(III) Age Determination

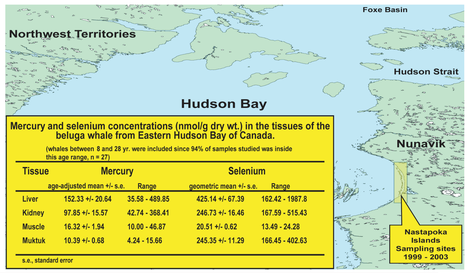
Thin longitudinal sections of the canine (about 1/50 mm thick) were cut using a low-speed jewelry saw. Age was estimated by counting annual layers in the dentine under a stereo-microscope. For the beluga whale, two layers are deposited every year.

(IV) Size Exclusion Chromatography

Liver samples were homogenized in 2 volumes of a buffered saline solution (50mM Tris-HCl, 140mM NaCl, 2.5mM dithiothreitol, 0.5mM PMSP, pH 7.8) at 4°C under nitrogen for 3 min. using a polytron at 30,000 rpm. Supernatants were obtained by centrifuging the homogenate (13,000G, 20 min., 4°C) and filtered through 0.22um PVDF membrane just prior to applying to a Sephacryl S-100HR, (1kD-100kD, 26/100) column (Pharmacia-Biotech Ltd.). The column was then eluted with the buffered saline solution (minus the 0.5mM PMSF) at a flowrate of 0.85 mL/min. Pooled fractions from the S-100HR run were pooled and concentrated by ultrafiltration (MWCO = 50 kD) prior to applying to a Sephacryl S-300HR column.

(V) Anion Exchange Chromatography

The pooled fractions from the Sephacryl S-300 HR run containing the Hg-Se-protein peak were desalted with Sephadex G-25 and exchanged for the starting buffer (5mM Tris-HCl, pH8.0), then filtered through 0.22um PVDF membrane filters before applying to a HiPrep 16/10 DEAE Sepharose column (Pharmacia Biotech). A linear gradient of 5 to 500 mM Tris-HCl, pH 8.0 at a flowrate of 2.4 mlmin. was used in elution. A total eleution volume of 500mL was used.



Mercury and selenium concentrations (nmol/g dry wt.) in the tissues of the beluga whale from Eastern Hudson Bay of Canada.
(whales between 8 and 28 yr. were included since 94% of samples studied was inside this age range, n = 27)

Tissue	Mercury		Selenium	
	age-adjusted mean +/- s.e.	Range	geometric mean +/- s.e.	Range
Liver	152.33 +/- 20.64	35.58 - 489.85	425.14 +/- 67.39	162.42 - 1987.8
Kidney	97.85 +/- 15.57	42.74 - 368.41	246.73 +/- 16.46	167.59 - 515.43
Muscle	16.32 +/- 1.94	10.00 - 46.87	20.51 +/- 0.62	13.49 - 24.28
Muktuk	10.39 +/- 0.68	4.24 - 15.66	245.35 +/- 11.29	166.45 - 402.63

s.e., standard error

Effect of Age on Mercury and Selenium Bioaccumulation - A Summary of Findings:

1. Strong positive correlations ($p < 0.001$) were found between age and mercury concentrations for all four tissues studied (Fig. 1a). The liver showed the highest rate of mercury accumulation as a function of age (slope = 0.051), and muscles the lowest (slope = 0.021). The kidney and muktuk exhibited a similar rate of increase in Hg accumulation with age (similar in slopes of their best-fit lines). Mercury accumulated throughout the entire lifetime of the beluga in all four tissues examined.

2. Selenium concentrations in the liver and kidney increased with age ($0.001 \leq p < 0.01$, Fig.1b). The rate of the increase of selenium accumulation with age in the liver (slope = 0.021) was about twice of that in the kidney (slope = 0.01). A negative correlation was found between selenium concentrations in the muktuk and age ($0.001 \leq p < 0.01$). Selenium concentrations in muscles remained largely unchanged as the beluga grows.

3. Strong positive correlations ($p < 0.001$) between Hg levels in the liver versus that in the kidney, muscle and muktuk (Fig. 2). Kidney and muktuk had a similar rate of increase of their Hg concentrations in relation to the Hg concentrations in the liver, whereas the muscle showed a lower rate.

4. The ratios of Hg concentrations of the liver to that of the other three tissues continued to increase throughout the lifetime of the beluga (Fig. 3a). This indicates that the liver is the most active tissue among the four in accumulating mercury. Kidney/muscle, kidney/muktuk and muscle/muktuk ratios remained relatively constant throughout the animal's lifetime (Table 2), a rough extrapolation of mercury levels between kidney, muscle and muktuk can be made independent of age.

5. Mercury concentrations correlated positively with selenium concentrations in the liver and kidney ($p < 0.001$, Fig. 4), whereas a strongly negative correlation ($0.001 \leq p < 0.01$) between the two metals was observed in the muktuk. This can be explained by the opposing trends these two metals have in the muktuk as a function of age (Fig. 1a and 1b). In the case of muscles (not shown in fig. 4), a marginally positive correlation ($0.05 \leq p < 0.01$) was found between Hg and Se which can be explained by the fact that although Hg concentrations in muscles increased with age (Fig 1a), Se concentrations remained largely constant (Fig. 1b).

6. Mercury: selenium molar ratios for the liver only approached unity when the beluga entered well into maturity of around mid 20s and older (Fig 5a) or when the Hg concentrations in the liver tissues reached about 300 nmol/g d.w.

7. For the kidney, the Hg :Se molar ratios were also found to increase with age and kidney Hg concentrations (Fig. 5b), even though none of the kidney samples examined in this study actually reached or exceeded unit Hg : Se molar ratio. However, the Hg : Se molar ratios came very close to unity at a kidney Hg concentration very similar to that in the case of the liver – about 300 nmol/g d.w. Unlike the liver, the age profile (Fig. 5b) seems to suggest that the Hg : Se molar ratios only came close to unity even for the very old beluga.

8. Despite the Hg : Se molar ratios in the muktuk increased with age in this tissue (Fig. 5c), the ratios remained very low throughout the lifespan of the beluga. The overall low Hg : Se molar ratios is attributed to the opposing trends of these two metals in the muktuk as a function of age (Fig. 1a and 1b).

Health Risk to Consumers due to Mercury

The beluga whale has always been an important part of the diet of Inuit from the Canadian North. Both the muktuk (skin plus a thin layer of blubber under it) and the meat are consumed on a subsistence basis. Beluga hunting is not simply a mean of food harvesting, more importantly it is a way of life and an important part of the Inuit's identity. Like many other marine mammals, beluga provides a highly nutritious diet to the Inuit. Both the meat and the muktuk are high in protein. The blubber is rich in essential fatty acids including omega-3-fatty acids. The muktuk is also a good source of selenium. In recent decades, the discovery of high levels of contaminants (mercury and organochlorines) accumulated in the beluga has posed a health threat to the Inuit.

In this study, high levels of mercury were found in all the four tissues examined. The liver accumulated the highest levels of mercury, followed by kidneys, muscles and muktuk. Selenium followed the same trend as mercury except for muktuk in which selenium is present in high concentrations despite mercury levels are lowest relative to other tissues. The strong age-dependency of mercury levels in tissues (especially the liver and kidneys) is reflected by the wide range of mercury concentrations detected. Although no consumption guideline for mercury in marine mammals has been established, Health Canada has issued a guideline of 0.2 ug/g wet wt. (approximately 3.5 nmol/g dry wt.) for mercury in fish consumed on a subsistence basis. All the samples examined in this study exceeded the guideline. The Health Canada's guideline is based on the assumption that over 90% of mercury in fish muscles are methylmercury. Albeit on the conservative side, the guideline can be applied to the beluga muktuk and the muscle in which an average of 82.5 and 73.4% of mercury are organic mercury respectively (Fig. 6). In view of the high levels of mercury found, recommendations on some degrees of restriction in beluga consumption for certain high-risk groups in a population may be justifiable. High risk groups include pregnant women, young children, infants and women of childbearing age. However, it is an oversimplification to rigidly apply any contaminant guideline in decision-making regarding traditional diets without taking into account a whole myriad of factors. Nutritional benefits of beluga needed to be taken into consideration. Any restriction puts on a traditional food may have social, economic and cultural implications. Another important consideration is the effect of dietary selenium on mercury toxicity. The protective role of selenium against mercury toxicity to marine mammals has long been accepted. The Inuit diet is enriched with selenium from consuming marine mammals and fish. The extent to which dietary selenium in offsetting the mercury toxicity to consumers is still being investigated. Muktuk is probably the most important source of dietary intake of mercury from consuming beluga since it is considered by the Inuit as a delicacy. At the same time, muktuk is an excellent source of selenium, which may protect the consumer from mercury toxicity.

Fig. 6 Percentage organic mercury as a function of age

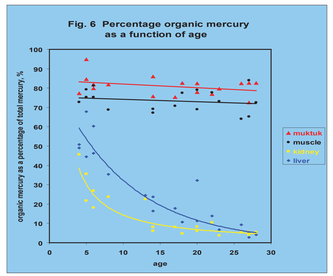


Fig. 7a Percentage of mercury released following sequential buffer extraction of liver tissues

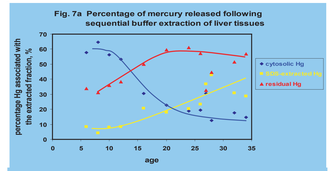
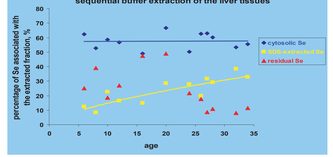
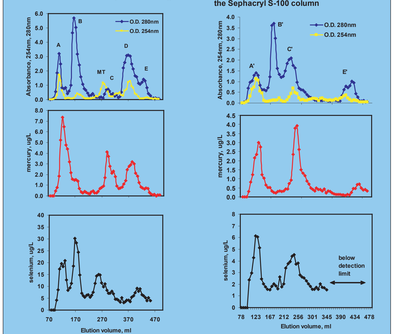


Fig. 7b Percentage of selenium released following sequential buffer extraction of the liver tissues



Sephacryl S-100 elution profile of cytosolic extract of the liver of a 24 years old beluga.

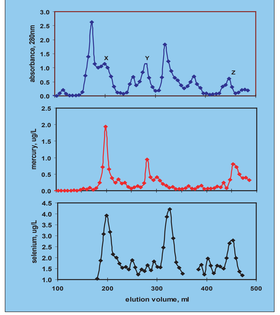
Sephacryl S-300 elution profile of the pooled fractions that contain peak A fractionated by the Sephacryl S-100 column



Size Exclusion Chromatography of Liver Cytosolic Extracts.

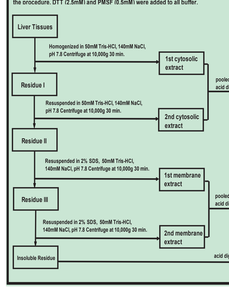
To compare the Hg-Se-protein interactions in the liver between young and old individuals, eight beluga whales were selected for study: the four 'young' individuals were between 4 and 8 years old, and the four 'old' ones were between 24 and 27 years old. Liver tissues were extracted with an aqueous buffer and the cytosolic extracts were fractionated by size exclusion chromatography using a Sephacryl HR S-100 column (fractionation range: 1 to 100 kDa) followed by a Sephacryl HR S-300 column (fractionation range: 10 to 1500 kDa). See Methodology section for details.

DEAE Sepharose elution profiles of peak C' separated by the Sephacryl S-300 column



(VI) Sequential Buffer Extraction Procedure

Tissues were extracted quantitatively first with a Tris-HCl / NaCl buffered saline to dissolve metal species present in the cytosol, then followed by a SDS (sodium dodecylsulfate)-containing buffer to release membrane-bound metal species according to the scheme below. A 4°C condition was maintained throughout the procedure. DTT (2.5mM) and PMSF (0.5mM) were added to all buffer.



Percentage organic mercury in tissues as a function of age, Fig. 6

Tissues of 20 beluga encompassing the age range from 4 to 28 years old were analyzed for total organic mercury (see methodology section). By using standard reference materials certified for methylmercury, it has been demonstrated that the organic mercury extracted by the method used in the study comprised almost entirely of methylmercury.

Both livers and kidneys displayed an essentially continuous decline in percentage organic mercury as the beluga aged. Whereas the percentage organic mercury in muscles and muktuk remained largely unchanged.

At a young age when total mercury in the liver and kidney is relatively low, a substantial percentage of that mercury is present as organic mercury, and as the animal grows and the total mercury increases, the percentage that is organic mercury declines significantly due to demethylation. It was found in this study that the liver exhibited a sharp decline which is best described by an exponential function, $y = 89.27e^{-0.102x}$, $r^2 = 0.863$. A power function was a best fit for the kidneys, $y = 174.78x^{-1.09}$, $r^2 = 0.826$, where y is the percentage organic mercury, x is the total mercury. Since mercury in the liver and kidney accumulated to very high levels with age, the sharp decline in percentage organic mercury observed suggests that as the beluga aged, mechanisms converting the accumulated mercury into less toxic forms in these tissues became dominant.

For both muscles and muktuk, the majority of mercury accumulated remained as organic mercury throughout the lifespan of the beluga. The slopes of the best-fit lines were not significantly different from zero ($p > 0.1$). The mean percentage organic mercury for muscles and muktuk are 73.4% and 82.5% respectively.

Investigation of the accumulated forms of mercury and selenium in the liver tissues by sequential buffer extraction

Liver tissues of 14 beluga encompassing the age range between 6 and 34 years old were subjected to sequential extraction (see methodology section for details). The percentage of metal associated with each extracted fraction was determined.

For mercury (Fig. 7a), there seems to be some form of threshold in the region of between 10 and 12 years old above which the percentage of mercury associated with the cytosol declined steeply, whereas the fraction of the SDS(sodium dodecylsulfate)-extracted mercury started to increase with age. The percentage of mercury bound to the insoluble residue increased with age up to between 15 and 20 years old and then leveled off.

In the case of selenium (Fig 7b), the cytosolic fraction remained largely unchanged with age, whereas a positive correlation was discernible between age and the SDS-extracted selenium. No identifiable trend was found for the percentage of selenium attached to the insoluble residue.

The increase in the percentage of both mercury and selenium present in the SDS-extracted fraction with age and the steep decline in the cytosolic total Hg and organic Hg (Fig. 6) with age seems to support the hypothesis that a Se-mediated mechanism involving membrane-proteins was becoming increasingly important in mercury metabolism as the beluga aged.

Size Exclusion Chromatography: A Summary of Findings

Sephacryl S-100HR Chromatograms:

1. A number of protein peaks were identified. The protein profile remains basically the same for the 'young' and the 'old' individuals.
2. Protein peak A, eluted close to the void volume (V_o) of the column, represents high molecular weight proteins of 100 kDa or larger. The majority of cytosolic Hg and a significant amount of Se were associated with this peak. The relative size of both the Hg and Se peaks associated with peak A were larger for the 'old' beluga.
3. Peak B was the most dominant protein peak (approx. 60-70 kDa) . It coincident with a very small Hg peak and a substantial Se peak. The size of the Se peak (relative to other Se peaks in the Se profile) was larger for the 'young' beluga than that for the 'old' beluga.
4. Peak 'MT' has a high 254nm : 280nm absorbance ratio and a size of about 8 – 10 kDa. Significant amount of Hg was found to associate with this peak but no discernible Se peak was present. The size of the 'MT' protein peak and the Hg peak coincident with it was found to be larger for the 'old' beluga. Peak 'MT' is likely to represent a fraction that contains the metal-sequestering protein – metallothionein. This was supported by the extremely high cadmium levels found in the fractions.
5. Peak C is a small 280nm peak coincided with the shoulder of the Hg peak that associated with the peak 'MT'.
6. Protein peak D eluted close to the void volume (V_o) of the column contained small proteinaceous molecules and peptides of about 1 kDa and smaller. A substantial amount of Hg was eluted with this peak. A broad Se peak was also present. The relative size of the Hg peak associated with peak D was found to be smaller for the 'old' beluga.
7. The small 280nm peak E that came out well after the bed volume is likely to contain small highly charged peptides attached to the gel.
8. Although the majority of Se was found to associate with protein peak B, a significant amount of Se was eluted as a peak (approx. 10 – 20 kDa) that did not coincide with a protein peak or a Hg peak.
9. Overall, There seems to be a shift in the distribution of Hg and Se from the low molecular weight fractions (peak D for Hg and peak B for Se) to the high molecular weight fraction (peak A) as the beluga grow older.

Sephacryl S-300HR Chromatograms:

The fractions obtained from the S-100HR column that contain the protein peak A were pooled, concentrated by ultrafiltration (MWCO = 50 kDa) and further fractionated using a Sephacryl S-300HR column.

1. A small protein peak A' eluted at the void volume (Vo) of the S-300 column (>1500 kDa) associated with a substantial amount of Hg and Se. The extremely high molecular weight of this peak suggested that it might represent some forms of coagulation of large protein molecules. The size of this peak was larger for the 'old' beluga.
2. No Hg or Se peak was found to coincide with the protein peak B'.
3. A broad protein peak C' of about 600 to 800 kDa was identified. The shoulder of this peak was coincided with both a Hg and a Se peaks. The relative size of the Hg peak increased with age whereas the size of both the Se and protein peaks remained unchanged with age.
4. A protein peak D' eluted at the bed volume (< 10 kDa). A small Hg peak was also eluted at this position but no Se was detected.

Anion exchange chromatography of a Hg-Se-protein peak (peak C') separated by the Sephacryl S-300 gel filtration column

The fractions obtained from the Sephacryl S-300HR separation containing peak C' were pooled. The pooled fractions were desalted and applied to a DEAE-Sepharose column. Elution was carried with a linear gradient of 5 to 500mM Tris-HCl pH 8.0 buffer.

A Summary of Findings:

1. A number of protein peaks were observed in the anion exchange chromatogram. Only protein peaks X, Y and Z were associated with mercury peaks. The overall protein profiles were the same between the 'young' and the 'old' beluga whales.
2. The majority of mercury was associated with the protein peak X. A selenium peak was also detected. The size of the mercury peak was significantly larger for the 'old' beluga than for the 'young' beluga. However, the size of both the selenium peak and the protein peak was only slightly increased as the beluga aged.
3. The protein peak Y coincided with a mercury peak but not a selenium peak. The size of both the protein peak and the mercury peak did not change with age.
4. A small protein peak Z eluted near the end of the chromatogram was coincided with a mercury peak and a selenium peak. No differences in size between the 'young' and the 'old' beluga were observed for all three peaks.
5. A significant amount of selenium was eluted with a protein peak at a elution volume of about 320 ml. No mercury peak was present.

Acknowledgements

This project would not have been possible without the help from the Hunting, Fishing and Trapping Association of Nunavik (Northern Quebec) and local Inuit hunters who allowed researchers from Nunavik Research Centre to accompany them in their annual hunts. We are indebted to Dr. D. W. Doidge and Dr. Daniel Leclair who collected the tissue samples. We extend our thanks to the following individuals at the Nunavik Research Centre for technical assistance: Mr. Peter May for age determination, Mr. Chesley Mesher and Mr. Alix Gordon for processing and acid digestion of samples. Part of the funding was provided by Environment Canada and Health Canada through the Toxic Substances Research Initiative (1999-2000) and the Northern Ecosystem Initiative (2001 -2002). Makivik Corporation also provided financial support.