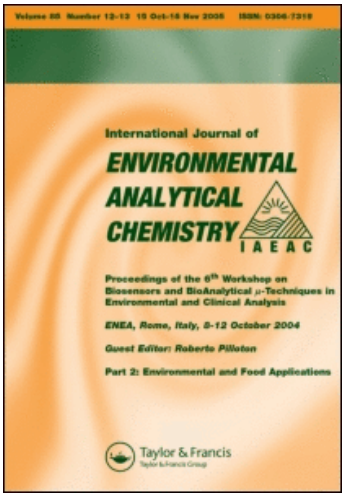

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METALLOTHIONEIN ESTIMATES IN MARINE MAMMAL AND FISH TISSUES BY THREE METHODS: ^{203}Hg DISPLACEMENT, POLAROGRAPHY AND METAL-SUMMATION

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An ^{203}Hg displacement, polarography and the metal-summation method were used to determine metalloproteins, including metallothionein, in narwhal (*Monodon monoceros*) liver and kidney, sucker (*Catostomus commersonii*) and lake trout (*Salvelinus namaycush*) liver after sample fractionation by gel chromatography and ion-exchange chromatography. Metallothionein in marine mammal tissues was high in cadmium and low in copper, and in fish liver it was high in copper and non-detectable in cadmium. Two methods, polarography and metal-summation were in good agreement with each other for mammalian tissues. The mercury displacement results were significantly higher relative to the other two methods. After ion-exchange chromatography of the tissue cytosols, metallothioneins were determined by three different methods, and were subjected to linear regression analysis. The three methods were highly correlated, with a linear slope of 1 between polarography and metal summation, but less than 1 between the mercury displacement and the other methods. Neither of the methods distinguished metallothionein from other thiolic metalloproteins. Any of the three methods were capable of measuring a relative change in tissue metallothionein with only a modest degree of prior separation, but the determination of absolute levels of metallothionein in mammalian tissues requires taking into account the positive bias of the mercury displacement. For fish-liver metallothionein there was good agreement between the mercury displacement and the metal summation method.

KEY WORDS: Metallothionein, fish, ^{203}Hg displacement, polarography, metal-summation.

INTRODUCTION

Metallothionein belongs to a family of low-molecular-weight, cytoplasmic metalloproteins (6000–6500 Da) occurring in practically all animals and in some plants and microorganisms. The physical and chemical characteristics of metallothionein are well known[1,2]. Outstanding features of this protein are its naturally high metal content (6–11% by weight) containing one or more of the metals Zn, Cu and Cd, depending on their availability to the organism

and an amino acid composition characterized by a high systeinyl residue content (30–35%). The biological function of metallothionein is less well understood[3,4]. It is thought to play a role in the metabolism of essential trace metals, the homeostatic control of Cu and Zn, and the detoxification of Cd. Synthesis of this substance in organisms is induced by exposure to heavy metals primarily Zn, Cd, Cu and Hg, as well as by some patho-physiological stimuli[5–8]. The induction of this protein by heavy metals has been observed not only in laboratory animals but also in wild populations of fish[6–12], making it a potential monitoring tool for trace metal pollution or "stress" indication in wild populations[13].

The estimation of metallothionein by a number of different methods has been well reviewed[14,15]. Metal displacement methods are available for measuring metallothionein using Cd, Hg or Ag as the displacing metal. All three metals have radio-isotopes that have been utilized for such measurements. The silver ion, although forming a very stable thiolate complex, has the disadvantage that its salts are photosensitive, and it has a strong affinity for the chloride ion when present in biological fluids, posing a potential interference problem. Additionally, the radio-isotope ^{110m}Ag can be a greater hazard than ^{203}Hg since the former has a longer half-life (253 days) and is a stronger γ-emitter. Three other methods have also been used: radioimmunoassay[16–18], the total metal content of the isolated protein i.e. the metal-summation method[19–23], and polarography[24,25]. The radioimmunoassay method is limited by complex analytical procedures and the metal-summation method relies on the now well established metal stoichiometry of metallothionein[4,26–29], and therefore requires some degree of separation prior to measurement. Metallothionein has been quantified most frequently by one of three methods: ^{203}Hg displacement [30–32], polarography[24–25], cadmium displacement[33–36]. The cadmium displacement method is limited by cadmium's inability to displace Cu and Hg from metallothionein. As with other radio-isotopes precautionary measures must be observed when working with ^{203}Hg. Like silver, mercury displaces other metals and forms strong thiolate complexes, but the radioisotope ^{203} Hg is a comparatively lower-energy γ-emitter and has a shorter half-life (47 days) than the silver radio-isotope. Unlike cadmium, mercury can effectively displace copper from metallothionein.

Valuable comparisons between the different methods of measuring metallothionein have been made[2,37,38], but a comparison of the ^{203}Hg displacement with the polarographic method was not included. Measurements of metallothionein by metal displacement methods are now used increasingly in physiological and environmental studies though the agreement between different methods is still not good [39–41], particularly for crude samples. There is a need for additional work and inter-method comparisons involving the ^{203}Hg displacement, which is potentially a very useful and efficient method because of its simplicity, its relative safety compared to some other radio-isotope methods, and the high affinity of this metal for the thiol group.

We compare here the ^{203}Hg displacement, the polarographic, and metal summation methods for determining metallothionein in mammalian and fish tissues (narwhal liver and kidney, and white sucker and trout liver). The mammalian tissues were chosen because of their high metal content, particularly cadmium[42], and consequently their naturally high metallothionein content, and their previous characterization in terms of metalloproteins[22,43]. The fish tissues were chosen because of their comparatively low metal content, particularly cadmium, and consequently their relatively low metallothionein concentration. The analyses were performed in three different laboratories, each having had experience with the particular method used.

MATERIALS AND METHODS

The narwhal *Monodon monoceros* liver and kidney, and fish liver (white sucker *Catostomus commersonii* and lake trout *Salvelinus namaycush*) were kept frozen at -60°C until used. The tissues were thawed, homogenized with a Polytron and the cytosol was separated and fractionated according to the scheme shown (Figure 1). The characterization of metallothionein in narwhal organs in terms of molecular weight, characteristic light absorbency, metal and sulphydryl content, and electrophoretic mobility has been described in detail elsewhere[22,43]. Exactly the same fractionation procedure was used here as previously described.

The fractionated cytosols were analyzed for metallothionein by the metal-summation and the ^{203}Hg displacement i.e. the mercury displacement method shortly after fractionation. For analysis by polarography they were purged with nitrogen, sealed under nitrogen, and shipped to the Methodologies Consultations Limited laboratory, where they were stored unopened at 5°C until analyzed. Trout liver cytosols were used to test the response of the polarographic

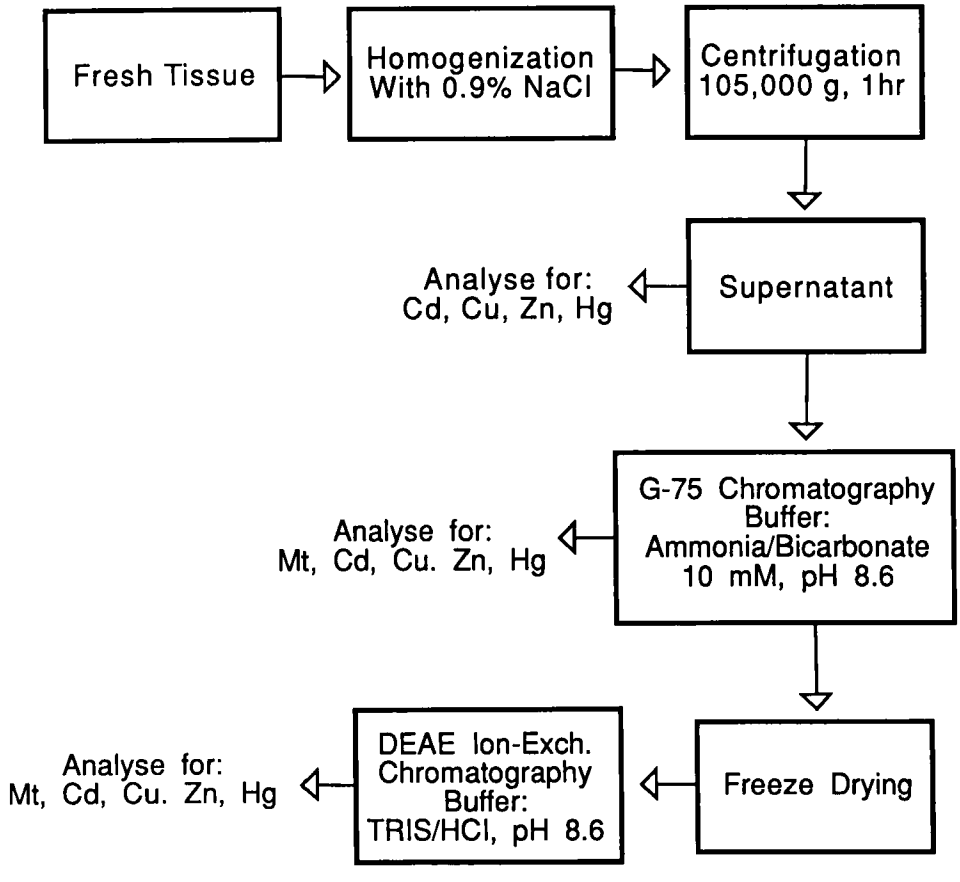

Figure 1 Fractionation scheme used to separate metallothionein from high molecular weight proteins.

method to the degradation of metallothionein by not purging them with nitrogen and storing them for approximately 6 weeks at 5°C prior to analysis. An antioxidant was not added to the buffer so as not to negate this test. Addition of antioxidant was found beneficial for fish-metallothionein (high Cu), but much less so for marine mammalian-metallothionein (high Cd).

Fractionation consisted of gel and ion-exchange chromatography. Gel chromatography (G-75 Sephadex) was performed with 10 mM ammonium bicarbonate buffer adjusted with ammonia to pH 8.6. Approximately 100, 5 ml fractions were collected during each run and analyzed for Cu, Cd, Zn, Hg, and Fe. Fractions #57–73, were under the metallothionein peak; they were pooled and analyzed for metallothionein by the mercury displacement method, by polarography, and by summing the metals under the metallothionein peak. Ion-exchange chromatography (DEAE Sephadex A25) was performed on the pooled fractions (#57–73) from G-75 gel chromatography, by stepwise elution with Tris-HCl buffers of increasing ionic strength (0.002, 0.04, 0.08, 0.18, 0.40, 1 M Tris, and 2 M in NaCl) adjusted with HCl to pH 8.6. Up to 70, 5.0 ml fractions were collected and analyzed for Cu, Cd, Zn, Hg. Metal concentrations in blanks (Tris-HCl buffer) were insignificant relative to metal concentrations in fractions under the metallothionein peaks. Fractions under each ion-exchange chromatography peak were pooled and analyzed for metallothionein by the three methods.

Metals were determined by DC-plasma emission spectrometry as described previously[22]. The metallothionein concentration under a chromatographic peak was calculated (i.e. by the metal summation-method) from the total metal content under the peak (Cu, Cd, Zn, Hg), using a total of 7 metal atoms per metallothionein molecule[27,28,44,45] and an apparent molecular weight we obtained by chromatography, 11,500 Daltons, within the range of molecular weights generally obtained by this method[4,43,46].

The Piotrowski method[47], modified by Dutton *et al.*[48] was used to determine metallothionein by mercury displacement. Piotrowski's method consists essentially of the addition of a mixture of cold and radioactive mercury (^{203}Hg) to a cytosol in excess of that required to displace all metals from metallothionein, and the separation of metallothionein from other proteins by precipitation with trichloroacetic acid followed by centrifugation. The remaining activity of the radiomercury in solution is then counted and used to estimate metallothionein. Pure metallothionein reference samples are treated in the same way, and provide the necessary calibration of the method. The modification consisted essentially of a prior separation of metallothionein from other proteins by heat treatment and centrifugation, followed by acidification of the solution with trichloroacetic acid to a pH of 1.5, prior to the addition of excess hot/cold mercury, and removal from solution of the excess mercuric ion not associated with metallothionein by precipitation with egg white albumin and centrifugation, prior to counting[49]. Rabbit liver metallothionein-II (Sigma Chemical Co., P.O. Box 14508, St. Louis. Mo., USA) served as the reference standard to convert specific activities to metallothionein concentrations.

The methods of Olafson and Sim[24] and Thompson and Cosson[25] were followed generally in the polarographic determination of metallothionein. A Metrohm 626 Polarecord was used, with the 663 Multimode Mercury Electrode operating in the static mercury drop, differential pulse mode (SMDE/DP). The scan range was -1.30 V to -1.75 V versus Ag/AgCl (3 M KCl).

Table 1 Metal concentrations, in supernatant[§] of narwhal and fish tissues (μg/g of fresh tissue) and metal composition (gm-atoms %) of G-75 metallothionein (MT)[†].

Tissue	*Cu*	*Cd*	*Zn*	*Hg*
Narwhal Liver	2.5	37.7	22.8	0.68
MT Composition %	8.2	64.6	27.2	—.-[1]
Narwhal Kidney	1.9	89.9	25.3	0.27
MT Composition %	4.1	80.4	15.4	—.-
Sucker Liver	8.3	<1.	8.5	0.29
MT Composition %	79.7	<2.	19.6	—.-
Trout Liver	5.9	<1.	9.2	0.26
MT Composition %	69.2	<7.	26.1	—.-

§After homogenization of fresh tissue and centrifugation.
†gm-atoms of metal under the MT-peak / (Σgm-atoms of all metals under MT-peak)•100.
[1]Not significant.

The peak potential was about -1.50 V. Modified Brdicka's buffer[50], 15.0 mL, was used for each sample, plus 75 μL of 0.025 % (v/v) Triton X100. This buffer solution was deoxygenated for 5 minutes with oxygen-free nitrogen passed first through a solution of NH_3/NH_4Cl (1 M each). A volume of sample, varying between 75 to 600 μL, depending on the nature of the sample, was then added, and the solution was deoxygenated for another minute. The standard additions or "spiking" method was used, the known-value solutions being prepared from rabbit liver metallothionein II (Sigma Chemical Co.). These additions varied according to the concentration in the sample, being typically 16 to 40 mg/L. For each run the spiking volume was constant, 20 to 80 μL, depending on sample type.

The errors given in Table 2, for the mercury displacement and polarographic methods, represent average deviations from the mean metallothionein concentrations, obtained from 2–3 analyses of the same sample. For the metal summation method the given errors for G-75 cytosols are standard deviations from the mean, obtained from 11 separate fractionations, starting with a fresh narwhal kidney subsample (from the same animal) each time. The coefficient of variation obtained (24%) was then used to calculate standard deviations for all the other tissues, and includes the total methodological errors; not just instrumental errors. Errors for the metal summation method of ion-exchange cytosols were similarly estimated.

RESULTS

Narwhal and fish tissues were characterized by elemental metal analyses of the tissues and their cytosols (prior to chromatographic separations) and metal compositions of metallothionein. Cadmium was relatively high in narwhal tissue and cytosol, but was <1 μg/g in fish tissue and supernatant, which had 2 to 4 times more copper than narwhal supernatant. Zinc was approximately 3 times higher in narwhal than fish supernatant (Table 1). Differences in tissue metal content were reflected in the metal composition of metallothionein.

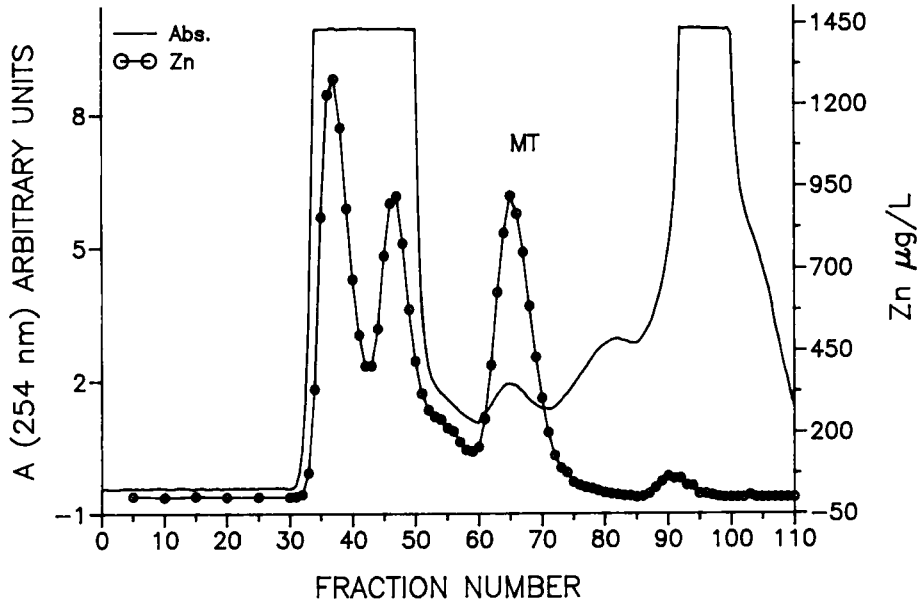

Figure 2 Sephadex G-75 gel permeation chromatographic profile of zinc and UV-absorbency of narwhal liver cytosol.

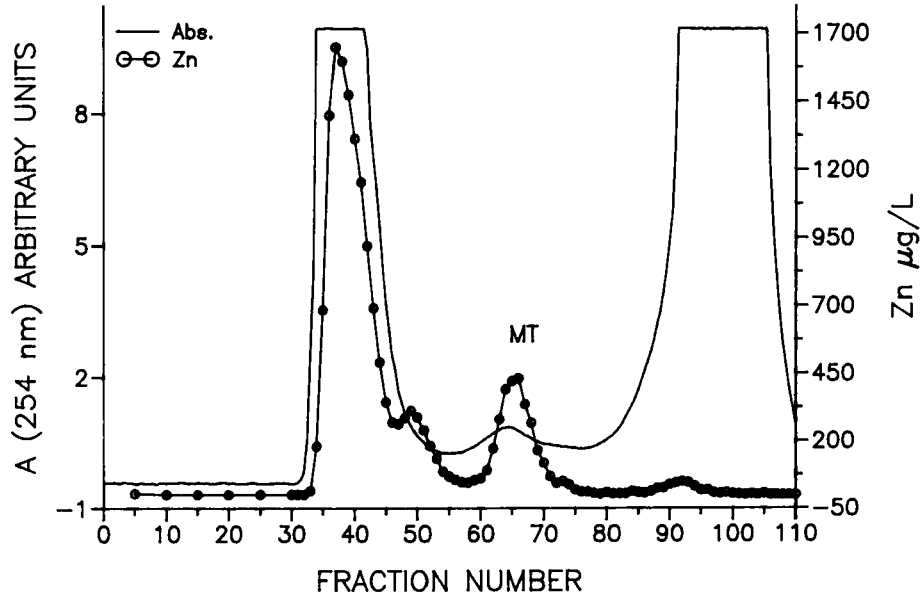

Figure 3 Sephadex G-75 gel permeation chromatographic profile of zinc and UV-absorbency of sucker liver cytosol.

Metallothionein concentrations and metal compositions were derived from metal analyses of the pooled G-75 gel chromatographic fractions under the metallothionein peak, between fractions #59–73, as shown for zinc (Figure 2 and 3). The peaks for Cu and Cd (not shown) were at the same position in the chromatogram as for Zn, for all tissues examined. Metal peaks at fractions #30–55, represented higher molecular weight proteins, including haemoglobin as evidenced by Fe-profiles (data not shown).

Narwhal metallothionein contained 60 to 80 atomic % cadmium, and 4 to 8 atomic % copper. Fish metallothionein contained <7 % cadmium, and 69 to 80 atomic % copper (Table 1). Although zinc was significantly higher in narwhal tissue than in fish tissue, metallothionein contained approximately the same percentage in narwhal and fish; 15 to 27 atomic % in narwhal liver and kidney, and 20 to 26 atomic % in fish liver. Mercury contributed insignificantly to the total metal content of metallothionein.

The pooled fractions under the G-75 chromatogram peak (Figures 2 and 3; #59–73) were analyzed for metallothionein by three different methods: metal-summation, polarography and the ^{203}Hg displacement method. Metal-summation and polarography were in good agreement, differing by <5% for narwhal liver (Table 2, G-75); both methods produced significantly lower estimates (62–75 %) than the ^{203}Hg displacement method. For narwhal kidney, the polarographic and metal-summation methods agreed to within 19%, and for sucker liver the metal summation method and the ^{203}Hg displacement method agreed to within 13%.

The eluent from the G-75 column under the metallothionein peak was further fractionated by ion-exchange chromatography, and the fractions were analyzed for Cu, Cd, Hg, Zn. Only the zinc profiles for narwhal and sucker liver are shown (Figures 4 and 5). Ion-exchange chromatography produced, depending on the type of tissue, 6 to 9 peaks which had been previously characterized [43] by electrophoresis, SH/metals ratio, UV absorbency and molecular weight as non-metallothioneins (peaks #1, #1a), as metallothioneins (peaks #3 and higher), and dimerized metallothionein (peak #2). We used a subsample from the same liver used originally[43].

Table 2 Metallothionein concentrations (μg/g tissue) in narwhal and fish tissues of G-75 and ion-exchange chromatography cytosols (ΣIE) by (^{203}Hg) displacement, polarography and metal-summation (ΣMetals).

Tissue	*Chromatography*	*203-Hg*	*Polargraph.*	*ΣMetals*
Narwhal Liver	G-75	1.70±0.03	0.62±0.01	0.65±0.16
	Total ΣIE[1]	1.06±0.05	0.39±0.01	0.40±0.10[3]
Narwhal Kidney	G-75	1.02[2]±0.33	1.41±0.32	1.14±0.27
	Total ΣIE[1]	1.40±0.48	0.35±0.01	0.58±0.14[3]
Sucker Liver	G-75	0.47±0.09	0.17±0.002	0.41±0.10
	Total ΣIE[1]	0.15±0.01	0.06±0.001	0.13±0.03[3]
Trout Liver	G-75	0.41±0.10	<0.04	0.29±0.07[3]
	Total ΣIE[1]	0.17±0.16	<0.04	0.08±0.02[3]

[1]The sum of metallothionein under all the ion-exchange peaks.
[2]Is < ΣIE (1.40), and does not follow the pattern of other G-75 values.
[3]sd estimated to be the same as in G-75 metal summation.

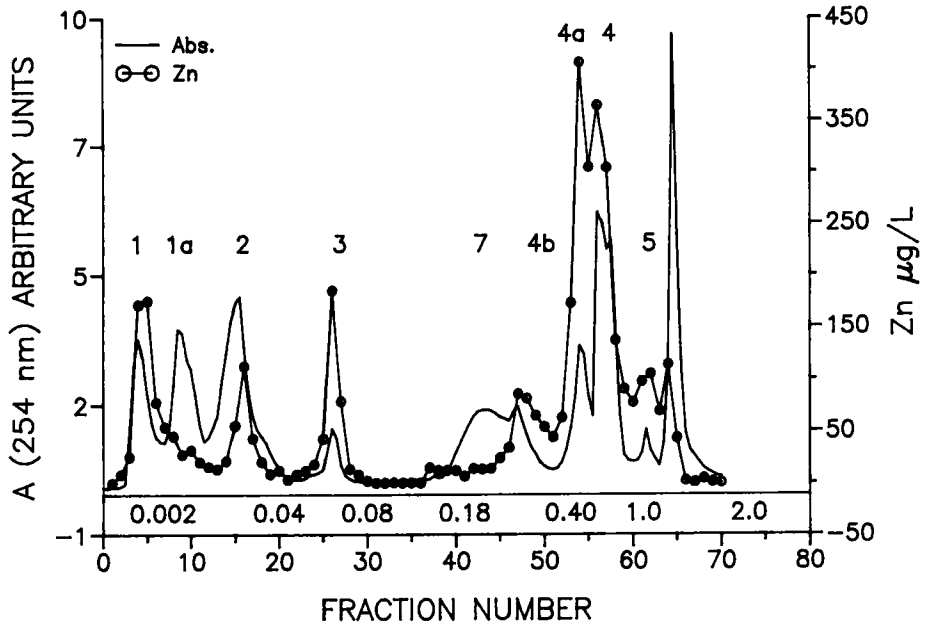

Figure 4 DEAE-Sephadex A25 ion-exchange chromatographic profile of zinc and UV-absorbency of narwhal liver cytosol. Numbers above the x-axis indicate increasing ionic strength (M) of elution buffer.

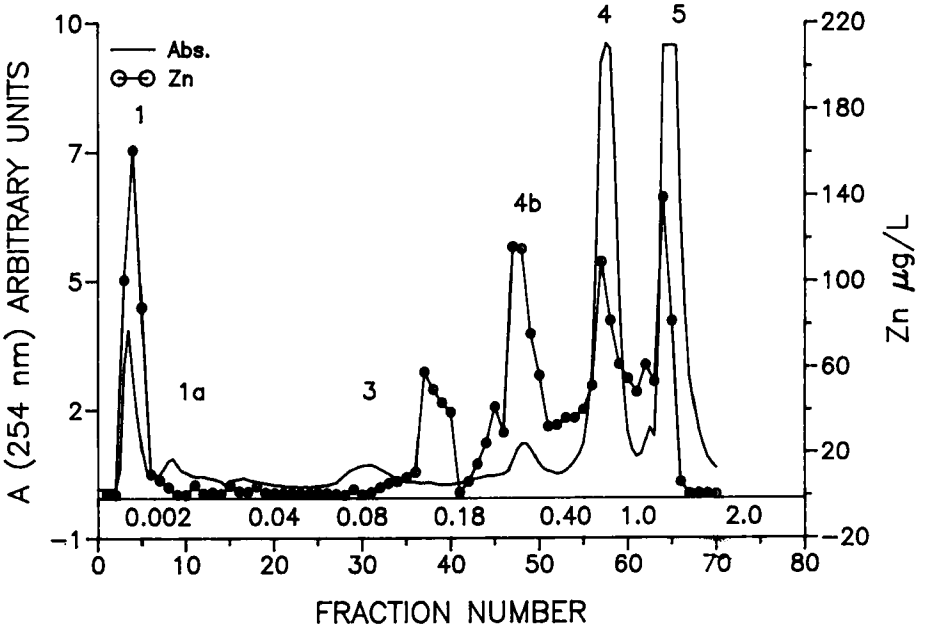

Figure 5 DEAE-Sephadex A25 ion-exchange chromatographic profile of zinc and UV-absorbency of sucker liver cytosol. Numbers above x-axis indicate increasing ionic strength (M) of elution buffer.

The sum of metalloproteins under all ion-exchange peaks was compared (per gm of tissue) by the three methods (Table 2, Total ΣIE). For narwhal liver good agreement (within 2.5%) between the metal-summation and polarographic method was also obtained after ion-exchange fractionation. The ^{203}Hg displacement method gave again higher values (2 to 4 times) than the other two methods for both narwhal tissues. For sucker liver, the ^{203}Hg displacement method and the metal summation method agreed within 13%, while polarographic results were relatively low.

The generally higher concentration of metallothionein in the G-75 cytosols, by all three methods relative to the summed ion-exchange values (Table 2, Total ΣIE) reflected, on the one hand, loss of proteins and metals following the further fractionation of the G-75 cytosol, and on the other hand, the presence of "impurities" in the G-75 cytosol which were measured as "metallothionein" by all three methods. The recovery of Mt under ΣIE relative to that under the G-25 Mt peak was 62 %.

Concentrations of metallothionein under the individual ion-exchange peaks (Figures 4 and 5), obtained by the different methods, were compared by linear regression. For narwhal liver, polarographic and metal summation values were well correlated ($P < 0.01$) and the slope was essentially one (Figure 6). Polarography and the ^{203}Hg displacement method were also well correlated (Figures 7 and 8), but the slope was much less than 1 for both narwhal tissues (liver 0.28, kidney 0.24), indicating that polarographic and metal summation estimates were significantly lower (65 % for liver, 72 % for kidney) than the ^{203}Hg displacement values, in accordance with the mean values obtained after G-75 and ion-exchange chromatography.

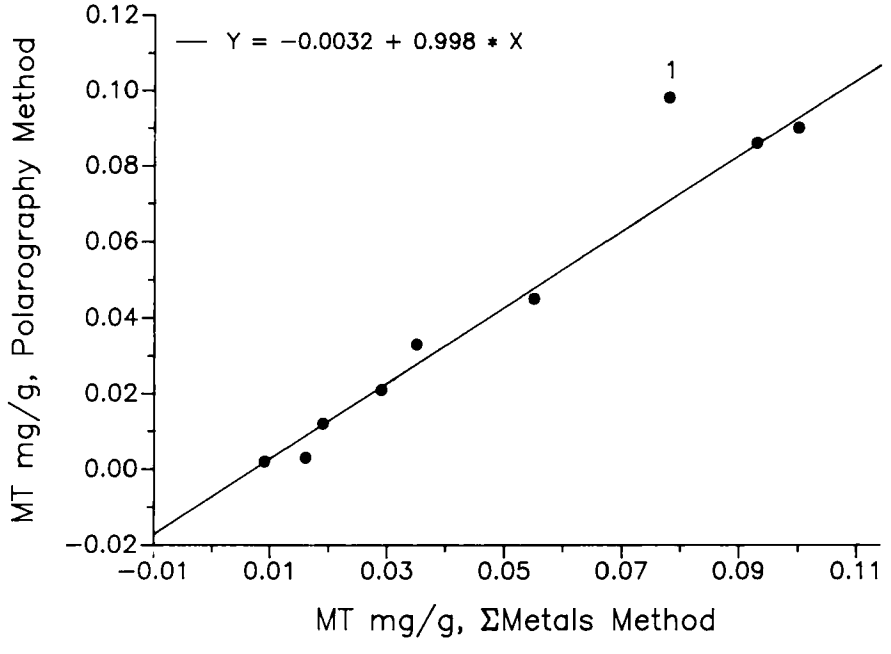

Figure 6 Metallothionein under individual ion-exchange peaks of narwhal liver cytosol, by metal-summation and polarography. Point #1 was excluded from the regression, $R^2 = 0.999$.

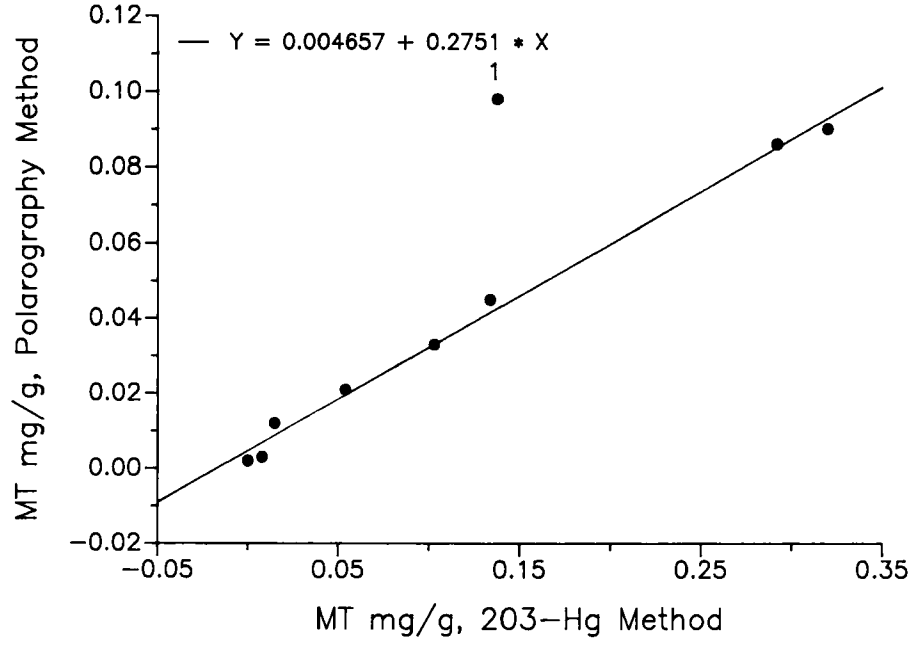

Figure 7 Metallothionein under individual ion-exchange peaks of narwhal liver cytosol, by ^{203}Hg displacement and polarography. Point #1 was excluded from the regression, $R^2 = 0.994$.

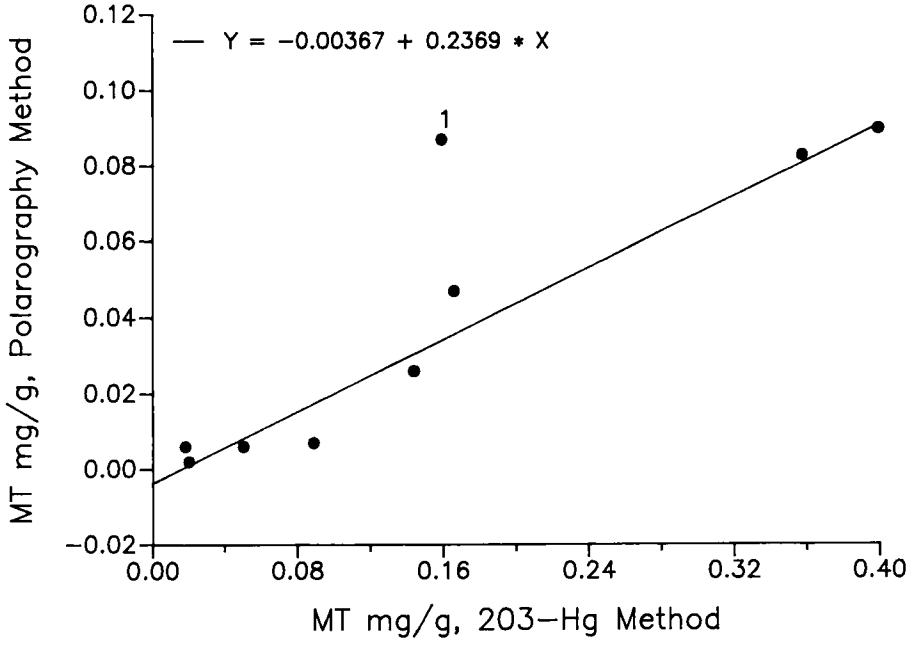

Figure 8 Metallothionein under individual ion-exchange peaks of narwhal kidney cytosol, by ^{203}Hg displacement and polarography. Point #1 was excluded from the regression, $R^2 = 0.952$.

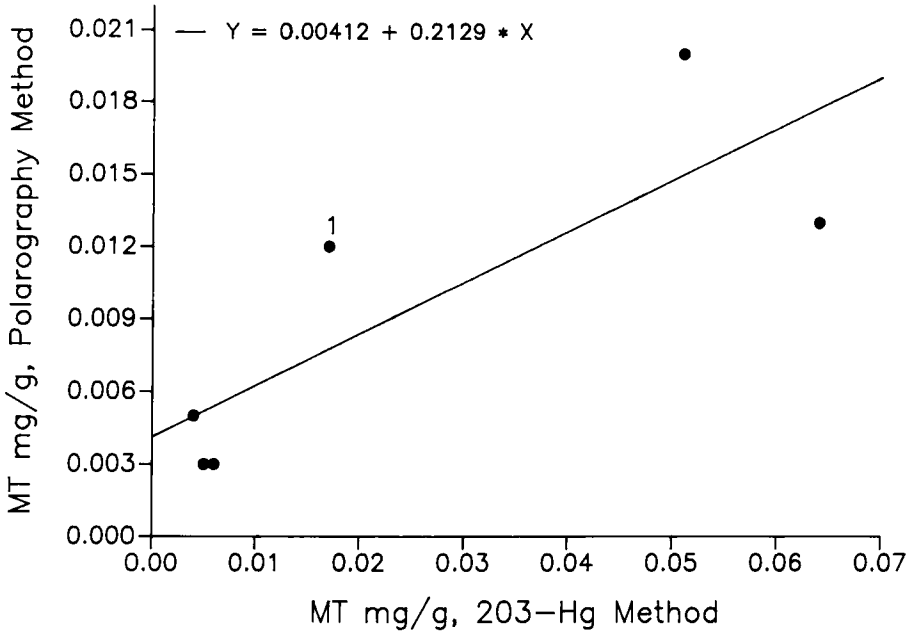

Figure 9 Metallothionein under individual ion-exchange peaks of sucker liver cytosol, by ^{203}Hg displacement and polarography. Point #1 was excluded from the regression, $R^2 = 0.591$.

For sucker liver, the slope between polarography and ^{203}Hg displacement was also much less than one, (Figure 9), but the linear relationship was not highly significant, most likely because of the relatively low metallothionein concentration in this tissue.

The metallothionein concentration was considerably lower, by all three methods, in fish liver than in narwhal liver or kidney, making the analysis of fish tissues more demanding. Additionally, the polarographic response in the case of sucker liver cytosol was erratic, and may have been influenced by unknown factors as evidenced by the low correlation of the two methods (Figure 9).

DISCUSSION

Individually, the methods investigated here have been commonly used in the past to determine metallothionein. However, a bias in a measurement can only be revealed if more than one reliable method, based on different measuring principles, can be utilized, particularly if no certified reference material is available for the analyte in question. In the past, the ^{203}Hg displacement method has been compared with the cadmium displacement (saturation) method and found to overestimate, by a factor of 2^2, somewhat underestimate (10–12 %)[40], and agree (< 10 % difference)[39], with the cadmium-saturation method for mammalian metallothionein. Similarly, polarography has been compared with the cadmium-saturation

method[38], and although the correlation between the two methods was good, differences ranged from +56 to -6% in estimating metallothionein in the liver and kidney cytosol of Cd or Zn-treated rats, the relative difference depending on the type of tissue, the metallothionein concentration (0.1 to 2.0 mg/g), and apparently also on the metal used to treat the animals. Clearly, the comparative agreements reported vary, and probably reflect operational contingencies of individual laboratories.

In this investigation we found that two methods, polarography and metal-summation gave metalloprotein values in good agreement with each other for G-75 cytosol of narwhal liver and kidney, and for narwhal liver ion-exchange cytosol (Table 2). The "Total ΣIE" represented a recovery of 62% relative to the quantity under the G-75 metallothionein peak for narwhal liver, and less for sucker liver. A 56% recovery of Zn has been reported for reversed-phase, high-performance liquid chromatography[51]. Since these methods are based on different measuring principles this provides some confidence that either method can be used with a minimum of prior separation. On the other hand, the ^{203}Hg assay gave higher results for the mammalian cytosols than the other two methods, but was in good agreement with the metal summation method for sucker liver cytosol. The polarographic method behaved more erratically when analyzing fish tissue cytosol which contained largely Cu, Zn-metallothionein than when analyzing mammalian tissue cytosol which contained largely Cd, Zn-metallothioneins. For sucker liver, polarography gave low values possibly as a result of some degradation of metallothionein, precautionary measures against oxidation notwithstanding. For mammalian tissues the ^{203}Hg assay has been reported to be similarly biased relative to the cadmium saturation method[2].

Trout liver cytosol was used to test the polarographic response to degradation of metallothionein by storing without rigorous exclusion of atmospheric oxygen at 5° C for six weeks prior to measurement; there was no significant polarographic response after this time (Table 2). Although the cytosol had been fractionated prior to storage removing most of the high molecular weight proteins (proteases), nevertheless some autolysis of metallothionein may have ocurred and most of this high-Cu(I) metallothionein was probably oxidized because atmospheric oxygen was not rigorously excluded during storage. Cu, Zn-metallothionein is the predominant type of metallothionein in fish tissues[10,49] and is more easily oxidized than Cd- or Zn-metallothionein[52,53]. Antioxidant was not used so as not to negate this test. In contrast, Thompson and Cosson[38] had noted only a small degradation in polarographic response (10–12 %) with crab (*Scylla serrata*) metallothionein after storing it for approximately 10 months. Degradation of mammalian Cd, Zn-metallothionein during normal processing of the cytosol was insignificant. A change in metallothionein prior or during fractionation can also occur, apparently by way of transfer of metals from metallothionein to higher molecular weight proteins, manifesting itself in a changed appearance of the gel permeation chromatographic metal profiles[35,54]. No such shift of Cd or Zn from the metallothionein peak to the higher molecular weight proteins was noticed; more than 80% of the total Cd in G-75 cytosol was found consistently under the metallothionein peak.

After further fractionation by ion-exchange chromatography the metallothionein concentrations were determined under each ion-exchange peak (Figures 4 and 5) by the three different methods. Linear regression analysis of the results showed that the methods were highly correlated ($P<0.01$) for mammalian tissues (Figures 6–8), except for the substance

under ion-exchange chromatographic peak #1 (a non-metallothionein metalloprotein, as determined largely by electrophoresis, and SH/metals ratio[43]) which measured anomalously high by polarography. The nature of this protein (peak #1) was not further identified. While the slope between polarography and metal-summation was essentially one for narwhal liver cytosol (Figure 6), the slope between polarography and the ^{203}Hg displacement method was < 1 for both narwhal tissues (Figures 7 and 8), and was also < 1 between metal-summation and the ^{203}Hg displacement method, indicating that the ^{203}Hg assay produced, notwithstanding further fractionation, higher results than the other two methods. It is noteworthy that the immunological method (not tested here), which is the most specific for metallothionein, in a comparison with metal substitution methods was reported to produce lower values than the ^{203}Hg displacement method and the cadmium-saturation method[2,40,41]. Although the specific cause of the bias in the ^{203}Hg displacement method is unknown, interference by copper, mercury, selenium and silver in the determination of mussel metallothionein has been reported[55].

Nevertheless, since the ^{203}Hg displacement method was linearly related to the polarographic and metal-summation methods, we think that it can be used for mammalian metallothionein determination where a relative change of metallothionein in a tissue, rather than an absolute value, is sought. Until the positive bias of the ^{203}Hg assay relative to other methods is elucidated and rectified, it must be taken into account when absolute metallothionein values are sought.

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