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# $^{210}$Po binding to metallothioneins and ferritin in the liver of teleost marine fish

**J. P. Durand[1,*], F. P. Carvalho[2], F. Goudard[1], J. Pieri[1], S. W. Fowler[2], O. Cotret[2]**

[1]Université de Nantes, Faculté des Sciences, GERMETRAD, Laboratoire de Biochimie et Radiobiochimie, 2 rue de la Houssinière, BP 92208, F-44322 Nantes Cedex 3, France

[2]International Atomic Energy Agency, Marine Environment Laboratory, BP 800, MC-98012 Monaco Cedex

ABSTRACT: The subcellular distribution of the naturally occurring radionuclide $^{210}$Po was investigated in the liver of the Atlantic mackerel *Scomber scombrus*. The majority of the $^{210}$Po was found in the cytosol of the liver cells. Fractionation of the cytosol proteins by high performance size-exclusion and ion-exchange chromatography indicated that about 30% of $^{210}$Po was bound to ferritin and approximately 28% to metallothioneins. The affinity of $^{210}$Po for these proteins was confirmed by a similar binding of the artificial $^{208}$Po isotope incubated *in vitro* with the cytosolic proteins. Two other proteins, likely selenium- and zinc-containing enzymes, may also bind smaller amounts of $^{210}$Po, about 8% each. The extensive binding of $^{210}$Po to ferritin and metallothioneins is not accompanied by a similar strong binding of $^{210}$Pb, the radioactive grandparent of $^{210}$Po, which explains the generally very high $^{210}$Po:$^{210}$Pb ratio observed in fish tissues and, in particular, fish liver

KEY WORDS: Polonium · Fish · Metallothionein · Ferritin

## INTRODUCTION

Polonium-210, a high energy α-particle emitter in the uranium decay chain, is the major source of internal radiation dose to marine organisms (Cherry & Heyraud 1982, Carvalho 1988). This naturally occurring radionuclide is also responsible for a significant proportion of the radiation exposure of humans to background radiation, in particular, through consumption of seafood (Parfenov 1974, Hunt & Allington 1993, Yamamoto et al. 1994, Carvalho 1995a). $^{210}$Po is naturally occurring and ubiquitous in our environment, but, due to its radiotoxic properties, special attention has been paid to the possible enhancement of $^{210}$Po environmental concentrations which may occur from the inputs of effluents of phosphate-ore processing industries (Germain et al. 1992, Pennders et al. 1992, Carvalho 1995b, Swift et al. 1995).

It is well known that $^{210}$Po is accumulated particularly by marine organisms and transferred through marine food chains (Heyraud & Cherry 1979, Carvalho 1988), with ingestion being the major route of entry (Carvalho & Fowler 1994). The tissues of some organisms, such as the hepatopancreas of crustaceans and fish liver may contain particularly high concentrations of $^{210}$Po. Furthermore, they display $^{210}$Po:$^{210}$Pb ratios of $10^2$ to $10^3$ (Carvalho 1988), which correspond to an enhanced ratio in the food chain compared to the $^{210}$Po:$^{210}$Pb ratio in sea water, which is around unity (Heyraud & Cherry 1979). The reason for an elevated $^{210}$Po:$^{210}$Pb ratio in marine food chains is not known. Furthermore, several studies have suggested that the subcellular localization of $^{210}$Po, in particular in the cell nucleus, may have important implications with respect to the genetic radiotoxic effects of the $^{210}$Po α particles (Howell et al. 1990).

Some attempts have been made previously to establish the subcellular localization of $^{210}$Po in tissues of marine invertebrates and small mammals. The aim of this study was to investigate the subcellular distribution of $^{210}$Po and to identify the molecular components that play a role in $^{210}$Po accumulation in marine fish.

*E-mail: jpdurand@nat.svt.sciences.univ-nantes.fr

## MATERIAL AND METHODS

Twenty specimens of mackerel *Scomber scombrus*, 27 cm average length and 140 g body weight, were collected during the spring of 1996 off the northeastern Atlantic coast of France. Livers were removed, weighed and minced with scissors in 6 volumes of ice-cold 0.25 M sucrose, buffered at pH 7.5 with 20 mM Tris HCl buffer and containing 0.1 mM phenyl methyl sulphonyl fluoride (PMSF). The tissue was homogenized in a Potter-Elvejhem type homogenizer using 13 up and down strokes of a Teflon pestle at 1700 rpm. The homogenate obtained was filtered through 4 layers of cheese cloth. The filtrate was then centrifuged at 400 × *g*, at 4°C for 20 min, to sediment nuclei and cell debris. From this centrifugation the supernatant was then centrifuged at 12000 × *g* for 150 min to sediment a heavy mitochondrial and lysosomal fraction. The supernatant was further centrifuged at 45000 × *g* for 30 min to sediment a light mitochondrial/lysosomal fraction and, once separated from the pellet, it was centrifuged again at 115000 × *g* for 70 min to separate the microsomal fraction (pellet) from the soluble components (cytosol) (supernatant). The nuclear fraction was purified by centrifugation through 2.2 M sucrose at 30000 × *g* for 45 min to evaluate more accurately the radioactivity present in the nuclei.

The different fractions were assayed for protein by the method of Lowry et al. (1951) and by spectrophotometric procedures for marker enzymes (cytochrome oxidase for mitochondria, acid phosphatase for lysosomes) as previously described by Goudard et al. (1985).

The cytosol fraction was fractionated by size-exclusion chromatography on a HPLC TSK G3000 SW column (21.5 × 600 mm), equilibrated and eluted with 0.1 M NaCl, 5 mM 2-mercaptoethanol, 20 mM Tris-HCl, pH 7.5. In some experiments, 12.5 µl of $^{208}$Po ($6 \times 10^3$ Bq) in 0.5 M $HNO_3$, or 15 µl of $^{109}$Cd ($6 \times 10^6$ Bq) in 0.9% NaCl, or $^{59}$Fe ($3 \times 10^4$ Bq) in 0.1 M HCl was added to 4 ml of cytosol. After incubation at 20°C (60 min) the cytosolic fractions were analysed by chromatography. Fractions of 2 ml were collected and analysed for absorbance at 280 nm and $^{210}$Po, $^{208}$Po , $^{109}$Cd, or $^{59}$Fe radioactivity. $^{109}$Cd and $^{59}$Fe were measured by gamma spectrometry on a NaI (Tl) well-type detector (Cobra 2, Packard) (Goudard et al. 1991). Polonium isotopes were determined using $^{209}$Po as an internal tracer for radiochemical yield determination, and measured on low background silicon surface barrier detectors (EG&G ORTEC) (Carvalho & Fowler 1994). Stable metals (Cu, Zn) were determined after appropriate dilution by atomic absorption spectrophotometry using a Varian AA 800 apparatus.

The metal-binding protein fractions from the different chromatographic experiments were pooled on the basis of the elution volume of the $^{109}$Cd and $^{210}$Po. The pooled fractions were concentrated with a Savant SpeedVac concentrator system and submitted to a desalting process on a Sephadex G10 column eluted with 1 mM Tris-HCl, pH 7.5. The obtained fractions were applied to a HPLC TSK DEAE 3 SW column (7.5 × 150 mm) equilibrated with 1 mM Tris-HCl, pH 7.5. In some cases, $^{109}$Cd was added to the sample as previously described. Elution was performed at a flow rate of 1 ml $min^{-1}$, using a linear gradient of 1 to 200 mM Tris-HCl, pH 7.5. Fractions of 0.7 ml were collected and analyzed for absorbance at 250 nm and $^{210}$Po, $^{208}$Po and $^{109}$Cd radioactivity.

## RESULTS

The subcellular distribution of $^{210}$Po in the liver of *Scomber scombrus* and the partitioning of marker enzymes are shown in Table 1. Most of $^{210}$Po (80%) was found in the soluble fraction of the cells of the liver (cytosol) and the remainder of the radionuclide was distributed among the other fractions, principally in heavy lysosomes and mitochondria (11.7%) and in light lysosomes and mitochondria (4%). The cytosol contained 39.6% of the acid phosphatase activity which can be attributed to the rupture of lysosomes during homogenisation. Furthermore, the cytosolic fraction obtained was totally free of mitochondria, and soluble proteins accounted for the majority of the protein content of the cell homogenate.

Table 1. *Scomber scombrus*. Distribution of $^{210}$Po and marker enzymes in the subcellular fractions from liver of mackerel. Values are percentages of recovered $^{210}$Po radioactivity, activities of marker enzymes for lysosomes (acid phosphatase, AP) and for mitochondria (cytochrome oxidase, COx), and protein contents

| | $^{210}$Po | AP | COx | Protein |
|---|---|---|---|---|
| Nuclear fraction | 0.8 | 0.4 | 22 | 4 |
| Heavy lysosomal and mitochondrial fraction | 11.7 | 41.8 | 73 | 16.5 |
| Light lysosomal and mitochondrial fraction | 4 | 12 | 5 | 6.5 |
| Microsomal fraction | 3.5 | 6.2 | 0 | 10.5 |
| Cytosolic fraction | 80 | 39.6 | 0 | 62.5 |

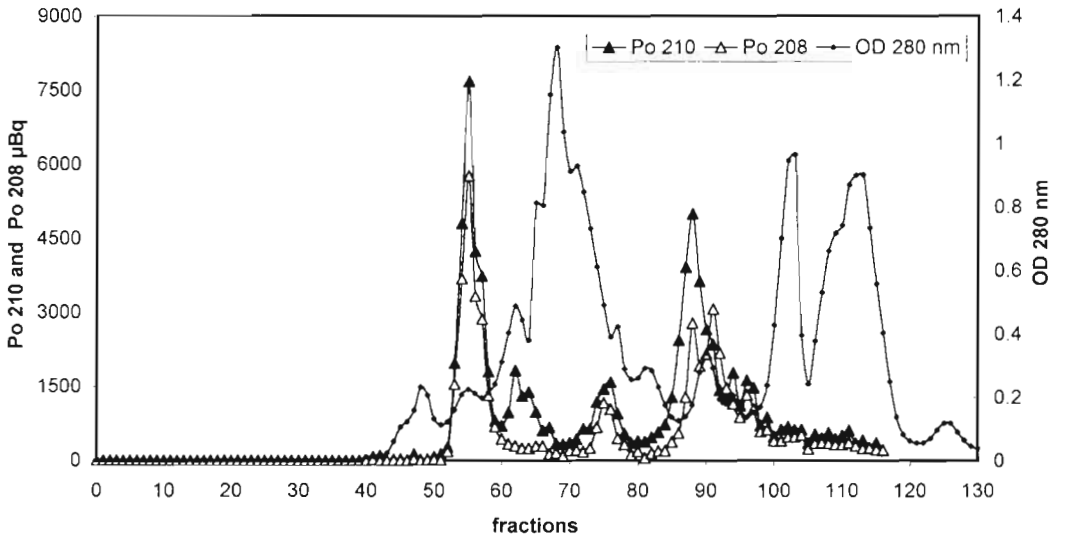


Fig. 1. *Scomber scombrus*. Fractionation of cytosol from liver of mackerel on a TSK G3000 SW column. Distributions of $^{210}$Po, $^{208}$Po and proteins by absorbance at 280 nm (OD, optical density) are shown

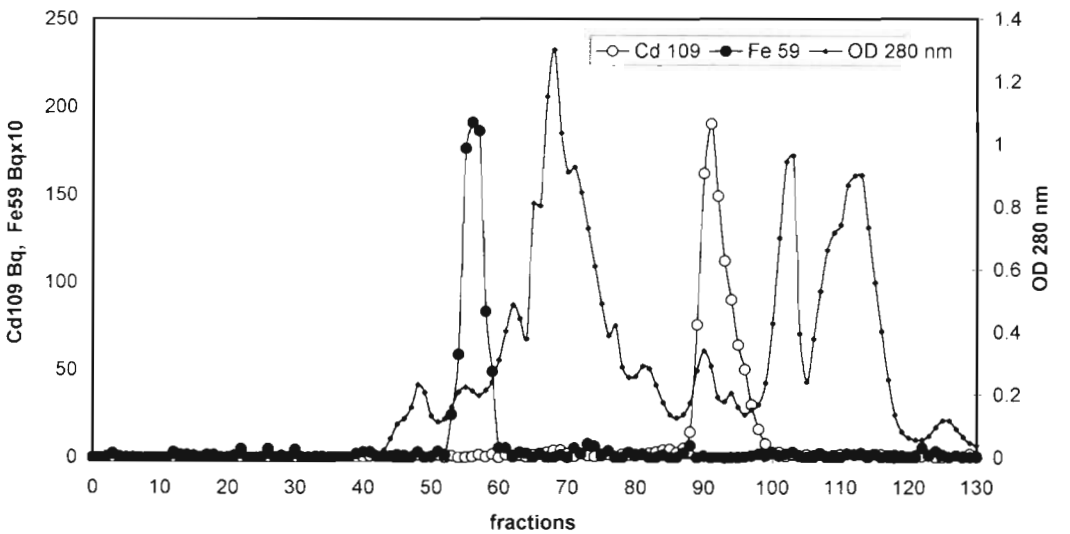


Fig. 2. *Scomber scombrus*. Fractionation of cytosol from liver of mackerel on a TSK G3000 SW column. Distributions of $^{109}$Cd, $^{59}$Fe and proteins by absorbance at 280 nm (OD) are shown

Analysis of the cytosol by size-exclusion chromatography showed that $^{210}$Po is distributed between 4 distinct peaks (Fig. 1). The first peak, containing 30% of the $^{210}$Po activity, was eluted at 110 ml, which is consistent with a molecular weight of approximately 400 kDa and corresponds to the molecular weight of ferritin. In the chromatogram of a cytosol sample incubated *in vitro* with $^{59}$Fe, $^{59}$Fe eluted at the same volume (Fig. 2). A horse spleen ferritin used as calibration standard also eluted at the same volume.

In the second peak, 8% of $^{210}$Po was eluted with proteins of an apparent molecular weight of approximately 200 kDa. The third peak, containing 9% of $^{210}$Po, was eluted between 142 and 158 ml, which is consistent with molecular weights of 40 to 50 kDa. Comparison with the elution profiles of the stable metals present in the cytosol and measured by atomic absorption spectrophotometry shows that this $^{210}$Po peak coincides with a protein peak containing some Zn. It is therefore possible that these proteins may be zinc-containing enzymes.

The fourth peak, containing 28% of the $^{210}$Po activity, was eluted at a volume consistent with a molecular weight of about 10 kDa. The cytosol of mackerel liver

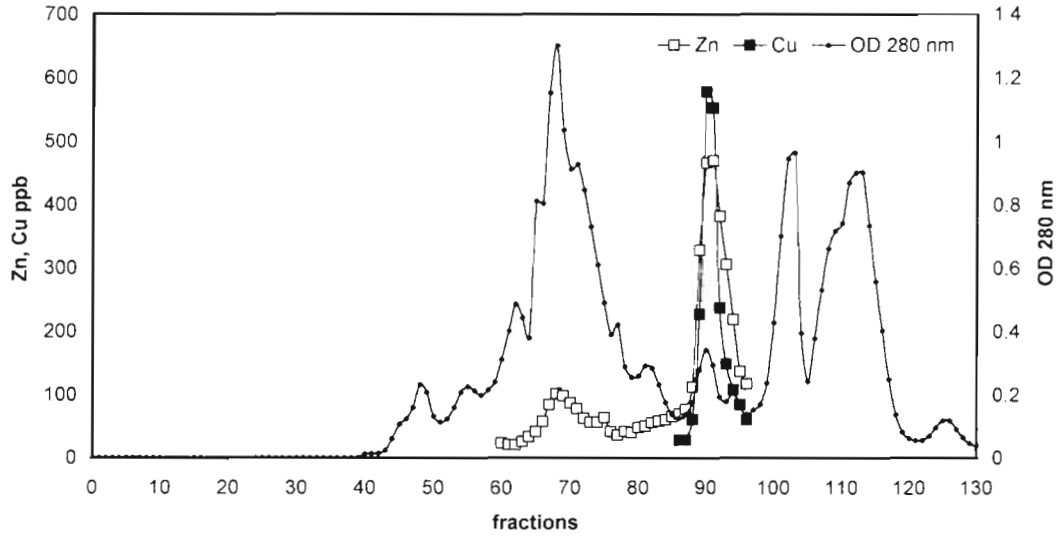


Fig. 3. *Scomber scombrus*. Fractionation of cytosol from liver of mackerel on a TSK G3000 SW column. Distributions of Zn, Cu (atomic absorption spectrometry) and proteins by absorbance at 280 nm (OD) are shown

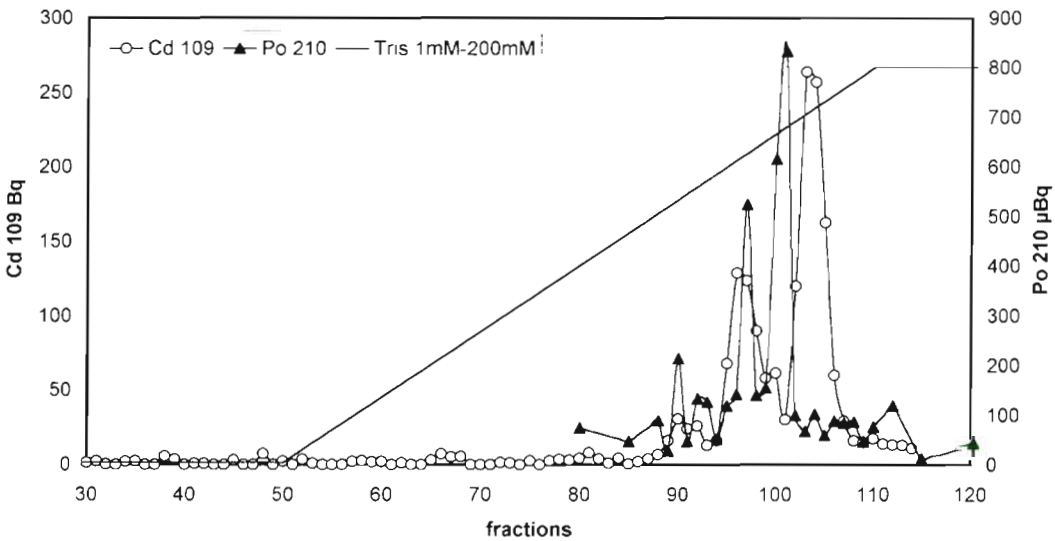


Fig. 4. *Scomber scombrus*. Ion exchange chromatography (TSK DEAE 3 SW) of $^{210}$Po-binding fractions (metallothionein, molecular weight ≈ 10 kDa) from the TSK G3000 SW chromatography. Distributions of $^{210}$Po and $^{109}$Cd and the Tris-HCl linear gradient are shown

was incubated with $^{109}$Cd and then analysed by gel permeation chromatography. Cd-binding proteins were eluted at the same volume as the $^{210}$Po (Figs. 1 & 2). Furthermore, analysis of this fraction for stable metal composition indicated the occurrence of a Cu-binding protein and a Zn-binding protein of 10 kDa molecular weight which is also indicative of the presence of Cu and Zn metallothioneins (Fig 3).

The distribution of $^{208}$Po in the chromatographic fractions of the cytosol incubated *in vitro* with $^{208}$Po was very similar to the distribution of the natural $^{210}$Po. In particular, ferritin and metallothioneins also seem to be very important $^{208}$Po-binding proteins. However, it is noteworthy that $^{208}$Po did not bind *in vitro* with the proteins of the second peak.

The determination of the $^{210}$Pb present in the subcellular fractions and in the chromatographic fractions was performed through a second plating of the $^{210}$Po 1 yr after the first $^{210}$Po analysis. The highest $^{210}$Po:$^{210}$Pb ratio in the subcellular fractions was obtained in the

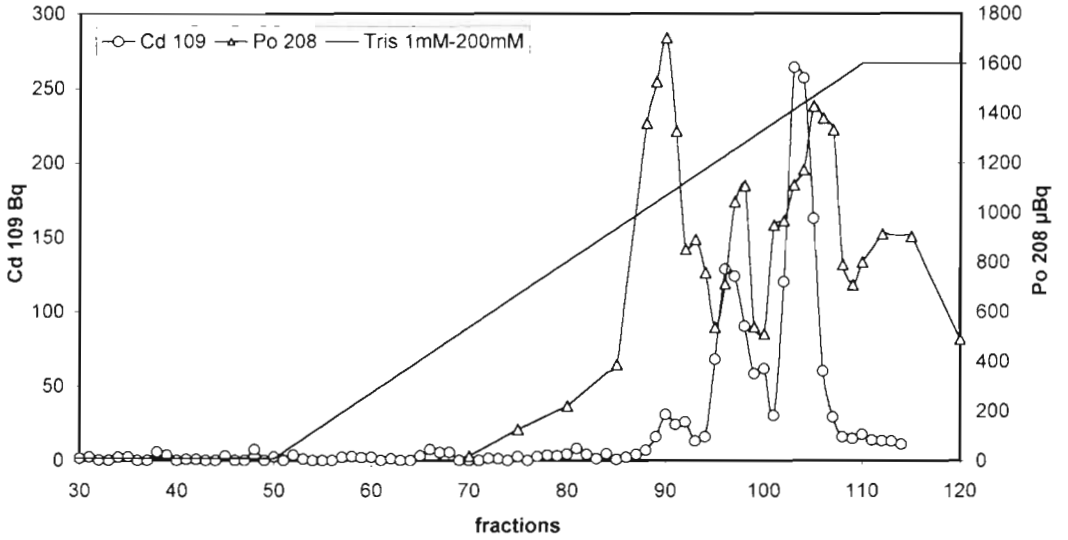


Fig. 5. *Scomber scombrus*. Ion exchange chromatography (TSK DEAE 3 SW) of $^{210}$Po-binding fractions (metallothionein, molecular weight ≈ 10 kDa) from the TSK G3000 SW chromatography. Distributions of $^{208}$Po and $^{109}$Cd and the Tris-HCl linear gradient are shown

cytosol, $^{210}$Po:$^{210}$Pb = 245. This indicates an accumulation in this fraction of unsupported $^{210}$Po which does not originate from $^{210}$Pb decay. Furthermore, in the chromatographic fraction analysis, a very small amount of $^{210}$Pb (traces) was detected in the elution volume of 40 to 50 kDa proteins but no $^{210}$Pb was present in the metallothionein fractions at 10 kDa molecular weight. This result confirms that metallothioneins do not bind Pb *in vivo* (George & Viarengo 1985), and thus the high $^{210}$Po in the metallothionein fraction does not arise from radioactive decay of accumulated $^{210}$Pb. Thus all $^{210}$Po bound to metallothioneins is unsupported by the grandparent radionuclide $^{210}$Pb.

The last peak of size-exclusion chromatography containing $^{210}$Po and metals corresponds to metallothionein molecules. The fractions of this peak were chromatographed on DEAE, and 3 peaks of Cd-binding proteins and 3 major peaks of $^{210}$Po were obtained (Fig. 4). A good correlation in elution volume of $^{109}$Cd and $^{210}$Po was obtained except for the last peak but these slight differences in elution columns have also been observed in previous studies of liver metallothioneins (Aposhian & Bruce 1991). This different elution behaviour is attributed to different molecular conformations of the metallothionein molecule with different metals.

In addition to these observations, the *in vitro* incubation of the metallothionein fraction with added $^{208}$Po resulted, in the case of DEAE chromatography, in 4 peaks of radioactivity (Fig. 5). The first 3 peaks with some slight volume shift correspond closely to the $^{109}$Cd and $^{210}$Po distribution. A fourth peak of $^{208}$Po appeared at the end of the ionic-strength gradient. This lower peak may be due to *in vitro* non-specific binding to some proteins which eluted in these fractions (Nos. 110 to 115) as can be seen by the optical densities (Fig. 6).

## DISCUSSION

Data on the subcellular distribution of $^{210}$Po in marine fish are sparse and published results are mostly related to terrestrial mammals and marine invertebrates. In rat tissues, 50 to 80% of $^{210}$Po was found in the cytosol and minor amounts in other subcellular fractions (Lanzola et al. 1973). This localization of $^{210}$Po is in accordance with the results of our investigation on mackerel which show that $^{210}$Po predominantly resides in the soluble fraction of the liver cells (80%). The subcellular distribution of $^{210}$Po contrasts with the distribution of other metals, such as copper, lead, mercury, plutonium, americium, californium and neptunium, which associate with the lysosomes (George 1982, Galey et al. 1983, George & Viarengo 1985, Seidel et al. 1986, Taylor et al. 1987). On the other hand, a comparison could be made with the distribution of technetium, which is also accumulated in a greater proportion in the cytosolic fraction (Goudard et al. 1985, 1991).

The chromatographic analysis of the cytosolic fraction from the liver cells of mackerel also demonstrates the existence of a specific association between $^{210}$Po and several proteins. $^{210}$Po seems to bind with ferritin,

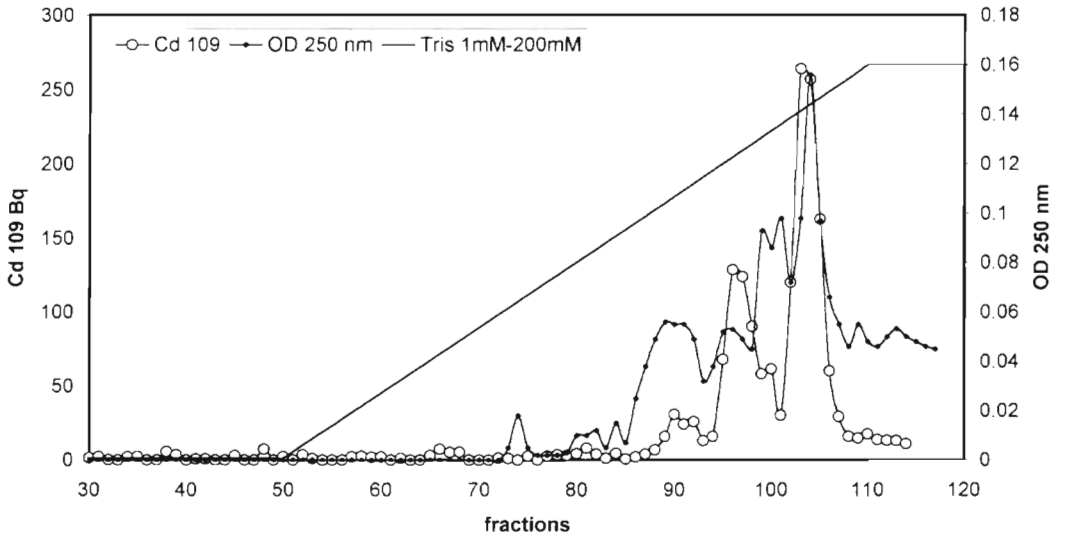


Fig. 6. *Scomber scombrus*. Ion exchange chromatography (TSK DEAE 3 SW) of $^{210}$Po-binding fractions (metallothionein, molecular weight ≈ 10 kDa) from the TSK G3000 SW chromatography. Distributions of $^{109}$Cd and proteins by absorbance at 250 nm (OD) and the Tris-HCl linear gradient are shown

metallothionein and with 2 other proteins of intermediate molecular weights. These findings are in contrast with the observations made by Lanzola et al. (1973), who, despite identifying the site of most of $^{210}$Po in the cytosol, found no specific binding of the $^{210}$Po with the cytosolic proteins. However, recent studies of Aposhian & Bruce (1991) have actually demonstrated the fixation of $^{210}$Po to rat liver metallothioneins.

Metallothioneins have received their designation from their extremely high metal and sulfur content. These proteins are present throughout the animal kingdom including fish, and are most abundant in the liver, kidney, pancreas and intestines (Kägi & Schäffer 1988, Duquesne & Richard 1995, Hogstrand et al. 1996). The role of metallothioneins in protecting aquatic species against the toxic effects of water-borne metal pollutants has been extensively investigated (George 1989, Gagné et al. 1990, Kille et al. 1992, Olsson & Kille 1997).

The results obtained in our chromatographic experiments also clearly show a binding of the naturally occurring $^{210}$Po to ferritin. This association was confirmed by the fixation of $^{208}$Po on ferritin in the *in vitro* incubation of this isotope with the cytosol proteins. The association of polonium with ferritin has not been previously demonstrated in marine vertebrates, although some of the observations recorded in previous studies might support this interpretation. For example, Lanzola et al. (1973) noted that $^{210}$Po in rats was bound to substances of high molecular weight (330 kDa), and, in extracts of marine invertebrates, other authors obtained a peak of $^{210}$Po activity at the void volume of their chromatographic experiments, which suggests that $^{210}$Po was bound to a component of high molecular mass (Finger & Smith 1987, Heyraud et al. 1987). In the cell, ferritin is the major (soluble) iron storage protein and consists of an apoprotein shell (480 kDa) around a core of Fe in the form of ferric-hydroxy-phosphate, which may contain up to 4500 iron atoms (Crichton & Charloteaux-Wauters 1987, Theil 1987). However, ferritin can also combine with other metals such as Cd, Zn, Cu, Ni, Co, Mn, Mg, Pb, Be, Tl and Al (Treffy & Harrison 1984, Wardeska et al. 1986, Fleming & Joshi 1991, Pead et al. 1995), and it has also been shown that transuranium elements such as Am, Cm, Cf and Pu are associated with the cytosolic ferritin in mammals and in marine invertebrates (Stover et al. 1970, Bruenger et al. 1976, Galey et al. 1983, Goudard et al. 1991, Milcent et al. 1996). Thus, some authors have suggested that ferritin may be a general metal detoxicant protein carrier (Joshi & Zimmerman 1988, Joshi et al. 1989, Muller et al. 1991). Taking into consideration these observations, the binding of $^{210}$Po by ferritin in fish liver is, therefore, not surprising.

In our study, $^{210}$Po was shown to bind also to 2 other proteins of intermediate molecular weights. These intermediate molecular weight proteins were not identified; however, they may be zinc metalloproteins or selenoproteins. The configuration of the outer electron shell of polonium is similar to that of selenium and sulfur and the replacement of these elements by $^{210}$Po may occur during metabolism (Aposhian & Bruce 1991).

The localization of $^{210}$Po in mammals and fish indicates that the soluble proteins of the cytosol are the

main subcellular pool of this radionuclide, whereas results for invertebrates show a different distribution of $^{210}$Po. For example, in studies carried out on the hepatopancreas of marine invertebrates, $^{210}$Po was found mainly associated with the microsomal pellet and with an unidentified high molecular weight fraction (Cherry et al. 1979, Finger & Smith 1987, Heyraud et al. 1987). We noted that some authors have shown that ferritin, which may reach a molecular weight of about 850 kDa, is able to precipitate with the microsomal fraction during centrifugation (Stover et al. 1970, Bruenger et al. 1971), whereas other authors have reported that ferritin may also be an integral component of the microsomal fraction (Minotti 1989). Whichever the case, our chromatographic results clearly show that there is an extensive binding of $^{210}$Po by ferritin, which might explain earlier reports on the high $^{210}$Po content of the microsomal fraction in invertebrates.

Many authors have proposed that –SH groups are involved in protein-$^{210}$Po association (Hill 1965, Lanzola et al. 1973, Heyraud et al. 1987, Bulman et al. 1995). In support of this, studies on the elimination of polonium from rats have shown that chelating agents containing thiol groups are the most effective (Rencova et al. 1994, Volf et al. 1995). As metallothioneins have a very large thiol content, different authors suspect these proteins bind $^{210}$Po in aquatic species (Bulman et al. 1995, Swift et al. 1995). Our results effectively demonstrate the binding of $^{210}$Po to metallothioneins in the liver of marine fish. In mackerel liver, we found 3 isoforms of Cd metallothioneins and, clearly, these 3 isoforms have an affinity for natural $^{210}$Po and added $^{208}$Po. This can be compared with mammalian metallothioneins, which usually contain 2 major isoforms, MT-1 and MT-2, differing at neutral pH by a single negative charge. In fish, 1 or 2 isoforms have been observed. The multiple forms of metallothionein may have different functions. For example, in the lobster, 1 form of MT is involved in Cu donation reactions while 2 others are inducible and linked to metal detoxification (Brouwer et al. 1989). Very little information is available on the functional significance of multiple forms of metallothioneins, and their differential roles in the regulation of metals have yet to be studied in detail (Roesijadi 1992).

Despite the significant binding of $^{210}$Po to metallothioneins, this accounts for only 28% of the cytosolic $^{210}$Po. Other soluble proteins, especially ferritin which accounts for 30% of the cytosolic $^{210}$Po, compete for the fixation of this nuclide. Enzyme-like proteins, such as selenoproteins and zinc metalloproteins are also likely to bind $^{210}$Po. Together, the protein-bound cytosolic $^{210}$Po accounts for 80% of the $^{210}$Po in the cells of mackerel liver.

The ability of the cytosolic proteins to selectively bind $^{210}$Po relatively to $^{210}$Pb is probably achieved by $^{210}$Po exchange with sulfur and selenium. Whatever the exact mechanism involved in the intracellular binding of $^{210}$Po to the soluble proteins of the cytosol may be, it actually enhances the accumulation of this radionuclide and increases the ratio of $^{210}$Po:$^{210}$Pb in fish liver relative to the $^{210}$Po:$^{210}$Pb ratio in the food. Hence, this selectivity in binding will clearly affect the transfer of these 2 important radionuclides through marine food chains leading to man.

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