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## Subcellular distribution and binding of heavy metals in the untreated liver of the squid; comparison with data from the livers of cadmium and silver-exposed rats[1]

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*Summary.* In natural squid liver, about 30% of the total Cd present was found in the cytosolic fraction. A large portion of this Cd was bound to high molecular weight species (mol. wt > 70,000). In contrast to Cd, about 60% of the total Ag occurred in the cytosolic fraction; Ag was bound mainly to low molecular weight species (mol. wt < 20,000).

It is well known that in mammals a unique Cd-binding protein of low molecular weight, named 'metallothionein'[2,3], occurs in the cytosolic fractions of the livers or kidneys of animals exposed to high concentrations of Cd. Recent studies have indicated that metallothionein-like proteins are also found in marine invertebrates, such as the mussel, *Mytilus edulis*[4,5], and the crab, *Scyllus serratus*[6], exposed to Cd. In our laboratory, high concentrations of heavy metals including Cd have been observed in the liver of the normal squid, *Todarodes pacificus*[7]. Similar observations have been reported by Martin et al.[8] on 3 other species of squid, *Loligo opalescens, Ommastrephes bartrami* and *Symplectoteuthis oualaniensis.* However, to our knowledge, there are no reports on the subcellular distribution and the molecular association of heavy metals in squid liver. In the present study we determined first the natural subcellular distribution of heavy metals in livers from the squid, *Todarodes pacificus.* Subsequently we compared the gel filtration behavior of the heavy metal-containing components of the liver cytosolic fraction of the squid and of the livers of rats exposed to Cd and Ag. The data on heavy metal-binding substances in squid liver are of practical significance from the view point of food sanitation because it is used in preparing 'Ika-no-shiokara', a food which is very popular in Japan.

*Materials and methods. Samples.* Mature squids, *Todarodes pacificus,* were collected in the Sea of Japan near the Oki Islands. They were frozen immediately after capture and stored at −20 °C or below until used. Female Wistar rats (2–3 months old) weighing 180–220 g were used. The rats were maintained on a commercial laboratory diet but were given a solution of $Cd(NO_3)_2$ and $AgNO_3$ (about 100 µg/ml each of Cd and Ag) as their sole source of drinking water for 7 months prior to killing. Livers of these Cd and Ag-exposed rats were removed immediately after killing and stored frozen at −20 °C or below until used.

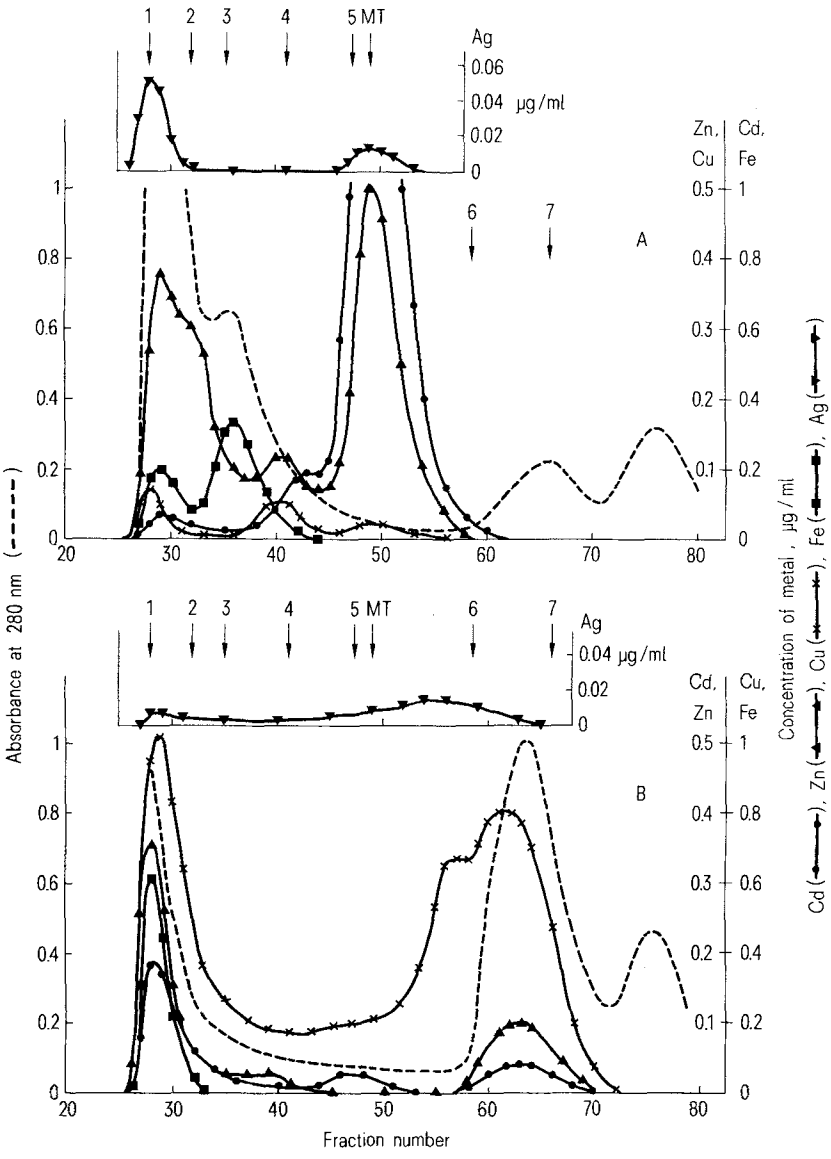
*Subcellular fractionation of squid liver.* After the frozen squid sample was thawed, the liver was removed and immediately homogenized in 4 vols of ice-cold 0.02 M Tris-HCl, pH 8.6, 0.25 M sucrose. In order to isolate subcellular fractions, the liver homogenate was subjected to differential centrifugation at 600×g for 10 min, 10,000×g for 10 min, and 100,000×g for 60 min at 0–4 °C[9]. Each fraction was digested with concentrated $HNO_3$ and monitored for Cd, Zn, Cu, Fe and Ag in a Nippon Jarrel-Ash Model AA-8500 atomic absorption spectrophotometer, using an air-acetylene flame or a heated graphite furnace.

*Gel filtration analysis of the cytosolic fractions of the liver of squid and of Cd and Ag-exposed rats.* The cytosolic fractions were prepared from squid liver and Cd and Ag-exposed rat livers by centrifuging at 100,000×g for 60 min as mentioned above. A sample of the cytosolic fraction (3 ml) was applied to a column of Sephadex G-75 (2.6×62 cm), equilibrated with 0.02 M Tris-HCl buffer, pH 8.6, contain-

ing 0.05 M NaCl and 0.003 M $NaN_3$. The column was eluted with the same buffer at a flow rate of 20 ml/h at 0–4 °C. The elution was monitored at 280 nm using an ALTEX Model 150B Biochemical UV monitor. The effluent fractions (5 ml) were assayed for Cd, Zn, Cu, Fe and Ag by atomic absorption spectrophotometry, as described above, but without prior acid digestion.

*Results and discussion. Subcellular distribution of heavy metals in squid liver.* The subcellular distribution of Cd, Zn, Cu, Fe and Ag in untreated squid liver is shown in the table. Approximately 30% of Cd in the liver homogenates was found in the nuclear, mitochondrial and cytosolic fractions, respectively. In mammals, such as rats and mice, exposed to Cd, it has been observed in several laboratories that more than 60% of Cd in the livers or kidneys occurs in the cytosolic fraction in 24 h or more after a dose of Cd[10,11]. We have also obtained similar results on Cd-exposed rats (unpublished). The experiments described here show that the subcellular distribution of Cd in untreated squid liver is notably different from that in Cd-exposed mammalian liver. The subcellular distribution of Zn was similar to that of Cd. In marked contrast to Cd or Zn, most of the Cu or Ag (about 60% of either) was found in the cytosolic fraction. Approximately 40% of Fe was localized in the microsomal fraction. The data show that there is a close resemblance between the subcellular distributions of Cu and Ag on the one hand, and of Cd and Zn on the other. This is probably related to the fact that Cd and Zn belong to group IIB and Cu and Ag to group IB of the periodic table.

*Comparison of hepatic heavy metal-binding species in untreated squid and Cd and Ag-exposed rat.* Typical Sephadex G-75 gel filtration profiles of the liver supernatants from squid and from Cd and Ag-exposed rats are shown in the figure. In Cd and Ag-exposed rats (graph A), the major portion of Cd (about 90%) in the liver supernatant was associated with the metallothionein fraction, which was shown to have an apparent molecular weight of 11,000–12,000, as estimated from its elution volume from the calibrated column. These results are consistent with those obtained for Cd-exposed rat livers in the gel filtration experiments in several other laboratories[11–13]. A large proportion of the Zn in the liver supernatant was also found in the metallothionein fraction, while Ag was mainly bound to high molecular weight species (mol. wt > 70,000) eluting with the void volume ($V_o$, indicated by arrow 1). By




Sephadex G-75 gel filtration of the liver supernatants prepared from Cd and Ag-exposed rat (A) and from untreated squid, *Todarodes pacificus* (B). The column (2.6×62 cm) was eluted with 0.02 M Tris-HCl buffer, pH 8.6, containing 0.05 M NaCl and 0.003 M $NaN_3$. Fractions of 5 ml were collected. Numbered arrows designate the positions of molecular weight standards: 1, Blue Dextran 2000 ($V_0$); 2, bovine serum albumin; 3, ovalbumin; 4, chymotrypsinogen; 5, ribonuclease; 6, glucagon; 7, $K_2CrO_4$. MT, rat liver metallothionein (as shown clearly in A).

contrast, in untreated squid (graph B), a large portion of Cd in the liver supernatant was bound to high molecular weight species (mol. wt > 70,000). Only a small Cd peak was observed in the 11,000–16,000 molecular weight region close to the position of rat liver metallothionein. In addition, a small portion of the Cd was found to be associated with lower molecular weight species (mol. wt < 3000). The chromatographic distribution of Zn was very similar to that of Cd. However, only very little Zn was detected in the region close to the metallothionein position. The distribution of Ag differed appreciably from that of Cd. A major portion of the Ag was bound to low molecular weight species (mol. wt < 20,000). To sum up, it has been shown in this study that the subcellular and Sephadex G-75 chromatographic distributions of the heavy metals in normal squid liver are strikingly different from those in the livers of Cd and Ag-exposed rats. These results could indicate that the physiological and nutritional significance of the heavy metals in squid are quite different from those in rat.

Subcellular distribution of cadmium, zinc, copper, iron and silver in normal squid *(Todarodes pacificus)* liver homogenates

| Subcellular fraction | Metal contents (% of reconstituted total) | | | | |
|---|---|---|---|---|---|
| | Cd | Zn | Cu | Fe | Ag |
| Nuclei and cell debris | 28±2.7 | 31±4.3 | 20±4.1 | 23±2.8 | 15±2.4 |
| Mitochondria | 33±5.1 | 24±3.3 | 12±1.5 | 20±2.3 | 14±2.4 |
| Microsomes | 14±1.1 | 9.0±0.9 | 4.8±1.0 | 42±2.3 | 6.6±1.6 |
| Cytosol | 26±2.7 | 35±2.1 | 63±4.9 | 14±0.9 | 64±4.3 |

Values represent means ± SEM for 5 liver samples. Ranges of the concentrations of the heavy metals in the liver samples are as follows: Cd, 15–33; Zn, 31–89; Cu, 111–267; Fe, 81–126; Ag, 1.3–2.2 (expressed as µg/g wet wt liver tissue).

Accepting this point of view, and knowing that Cd and Ag are either toxic or nonessential for mammals, further investigations on the Cd or Ag-binding species in untreated squid liver are needed.

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## Free cholesterol not carried by lipoproteins in human serum

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*Summary.* Considerable amounts of nonesterified cholesterol were found in human serum freed from lipoproteins. This cholesterol, when incubated with synaptosomal plasma membranes of dog brain, evokes changes of the ouabain-sensitive ATPase activity, as the exogenously added cholesterol does.

The ability of exogenous cholesterol to become incorporated into the phospholipid bilayer of biomembranes in vitro, causing marked functional changes in integral proteins, has been reported previously[2–5]. Such phenomena have recently been verified in in vivo experiments as well[6]. Monomeric cholesterol, in very dilute aqueous solutions (up to $5 \times 10^{-6}$ M) is a very active compound, affecting integral proteins of biomembranes. For example, the specific activity of adenylate cyclase decreases to 50% after preincubation of aqueous cholesterol solutions with synaptosomal plasma membranes (SPM); the ouabain-sensitive ATPase shows a definite increase in activity after 1–3 h preincubation.

In subsequent studies, we used the ouabain-sensitive ATPase as a measure of the functional changes resulting from the binding of cholesterol into biomembranes. The availability of approximately ideal conditions for kinetic work, and the introduction of the water soluble glucoside of cholesterol, synthesized on purpose in our laboratory, permitted the discovery of the synergistic nature of this binding[2]. Cholesterol glucoside preincubated in monomeric aqueous solutions at concentrations higher than $5 \times 10^{-6}$ M, which was employed in our studies so far, evokes a biphasic curve of changes in the ouabain-sensitive ATPase activity. Up to the concentration of $15 \times 10^{-6}$ M, the glucoside behaves in an identical manner to cholesterol, i.e., it evokes an equally intense increase in the specific activity of the ouabain-sensitive ATPase. Above this concentration, however, the specific activity of the enzyme starts dropping exponentially[5].

These in vitro phemonema suggested a search for an in vivo free fraction of cholesterol, hitherto unsuspected, which would act in a similar manner to aqueous solutions of exogenous cholesterol. The detection of such a fraction was performed and the results are presented and discussed in the present communication.

*Materials and methods.* Serum (2 ml) from freshly drawn human blood was made up to 12 ml with a saturated solution of KBr to achieve a density of d = 1.25 g/ml. This mixture was exposed to 48-h ultracentrifugation, at 321,500 × g (60,000 rpm) in the L5-75 Spinco Ultracentri-