Interaction of Mercury with Human and Bovine Milk Proteins

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The interaction of inorganic mercury with human and bovine milk proteins was studied. Gel filtration chromatography of skimmed milk and whey incubated with mercury showed that, in human milk, mercury was mainly bound to caseins, while a low proportion was bound to albumin. In bovine milk, mercury was associated with two protein fractions, caseins and β-lactoglobulin. Furthermore, it was shown by electrophoresis that mercury induced the formation of dimers of β-lactoglobulin. Thus, in both human and bovine milk, mercury possessed greater ability to interact with milk proteins than to the low-molecular-weight substances. However, the pattern of mercury distribution was different between the milk of these two species.

Key words: mercury; human milk; bovine milk; milk proteins

Mercury is a cumulative poisonous metal whose compounds found in the environment mainly originate from industrial sources. It is well-known that exposure to this metal during lactation may cause adverse effects in the development of infants due to the transfer that occurs from the mother to child through breast milk, especially in populations with a diet based on fish products. This has been supported by the fact that high levels of mercury have been found in the blood and hair of infants exposed to this metal only through breast milk. In ruminants, it has been found that mercury present in milk represented only 10% of that present in blood. Despite this relatively low transfer of mercury from blood to milk, it must be taken into account that bovine milk and dairy products are the most important food in the diet for children.

Most of the mercury poisoning outbreaks reported in the literature have been caused by organomercurial compounds. However, a high proportion of mercury transferred to breast milk has been in the inorganic form, since most organic mercury in a mother's blood is associated with erythrocytes and only the mercury in plasma is transferred into milk. When acute intoxication by mercury takes place, the level of organic mercury in the plasma is higher, and so, therefore, is its level in milk. The demethylation of methylmercury that takes place in vivo also probably plays an important role in the transfer of mercury to milk.

In both human and animals, only a small fraction of the ingested inorganic mercury is absorbed through the gastrointestinal tract, this, however, being more efficiently absorbed in suckling animals than in adults. On the other hand, milk has also been shown to increase the intestinal absorption of mercury in experimental animals, suggesting that its association with some milk proteins, which are readily absorbed. This effect has been observed in suckling and adult rat; therefore, the increased permeability of the intestinal barrier during the first days of life is not the only cause of the enhanced absorption of mercury when milk is administered.

The distribution of such metals as iron, zinc, copper, lead, and cadmium among the fractions of human and bovine milk has been studied. However, there is a lack of information on the distribution of mercury in secreted milk. The aim of this work, therefore, has been to study the interaction of inorganic mercury ions with the different protein fractions obtained from human and bovine milk.

Materials and Methods

Materials. Samples of milk from Holstein cows were supplied by a local farm from the pool of milk and collected immediately after milking. Human milk from the fourth month of lactation was obtained from healthy donors. Human and bovine milk was skimmed by centrifugation at 2000 × g for 30 min at 4°C, the fat layer was removed, and the skimmed milk remaining was filtered through glass wool to obtain fat-free milk. Human and bovine whey were prepared from the skimmed milk by acid precipitation at pH 4.5 and centrifugation at 90,000 × g for 1 h at 20°C. The whey was neutralised to pH 6.5 before being incubated with mercury.

Bovine serum albumin, β-lactoglobulin and α-lactalbumin were provided by Sigma (Poole, Dorset, England) and Sephadex G-100 (medium) by Pharmacia (Uppsala, Sweden).

Fractionation of human and bovine milk or whey by gel filtration. Samples of skimmed milk (0.75 ml) and whey (1 ml) were incubated overnight with mercury (as mercuric chloride) at a final concentration of 3 mg/liter. Afterwards, each sample was subjected to gel filtration chromatography in a Sephadex G-100 column (1 × 100 cm) at room temperature. Elution was carried out with 25 mM sodium acetate at pH 6.5 at a flow rate of 20 ml/h, and fractions of 2 ml were collected. The protein concentration in the eluate was measured as the absorbance at 280 nm, and mercury in each fraction was analysed by cold vapor atomic absorption spectrometry with a model 2100 instrument combined with an MHS-10 system Perkin Elmer (Norwalk, CT, U.S.A.). The chromatographic gel (Sephadex G-100) had been previously modified as described by Lönnrdal and Hoffman to overcome non-specific binding of metals. The recovery of mercury from the chromatographic process was over 80% of that added. Bovine serum albumin, β-lactoglobulin, α-lactalbumin, and mercure chloride were separately chromatographed under the same conditions to determine their elution volumes.

Electrophoresis. To identify which proteins were present in the chromatographic fractions, these were analysed by polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE), using gels that contained a gradient of acrylamide (8-25%). The samples used for SDS-PAGE were heated at 100°C for 5 min with 1% SDS, but without the addition of 2-mercaptoethanol. Electrophoresis was carried out with...
Phat System apparatus (Pharmacia, Uppsala, Sweden), and the proteins separated on the gel were stained with Coomassie Brilliant Blue R-250.

**Study of the aggregation of milk proteins with mercury.** SDS-PAGE and polyacrylamide gel electrophoresis without SDS (native-PAGE) were also used to study the presence of aggregates of β-lactoglobulin or bovine serum albumin induced by mercury. Human and bovine whey, β-lactoglobulin and bovine serum albumin were each incubated with 3 mg/liter of mercury for 12 h, the same samples without added metal being used as controls. The size of the aggregates of β-lactoglobulin was also studied by chromatography in a column of Supersose 12, with an FPLC system (Pharmacia, Uppsala, Sweden). The protein was dissolved in a buffer (Tris-HCl, 50 mM; NaCl, 50 mM; pH 8.0) in which β-lactoglobulin was found as a monomer and was incubated with mercury in molar proportions of 1:1 and 1:4 (mercury:protein) for 12 h. The samples were eluted in the same buffer at a rate flow of 0.4 ml/min, and the absorbance at 280 nm was monitored. A sample of β-lactoglobulin without added metal was chromatographed as a control.

**Effect of the addition of mercury and other metals on the solubility of β-lactoglobulin.** Aliquots of 1 ml of β-lactoglobulin (2.5 mg/ml) dissolved in 25 mM sodium acetate (pH 6.5) were incubated with increasing amounts of mercury, cadmium or lead up to 500 mg/liter. Each sample was held at room temperature for one hour and centrifugated at 15,000 × g for 30 min. The concentration of protein in the supernatant was determined by Bradford’s method using β-lactoglobulin as the standard.

**Results**

The distribution of mercury was studied by the gel filtration chromatography of skimmed milk and whey that had previously been incubated with an inorganic salt of mercury. The composition of each peak was determined, taking into account the molecular weight of the eluted proteins and their electrophoretic pattern by SDS-PAGE.

In the chromatogram with Sephadex G-100 of human skimmed milk (Fig. 1a), measurement of the absorbance at 280 nm showed four peaks corresponding to the casein micelles, lactoferrin, and serum albumin, to α-lactalbumin and to low-molecular-weight (LMW) substances, respectively. The profile obtained for mercury showed a major peak eluted in the void volume of the column together with the casein micelles. Gel filtration chromatography of human whey with Sephadex G-100 (Fig. 1b) resulted in four peaks of absorbance at 280 nm corresponding, in order of elution, to the void volume of the column (in which the remaining caseins and immunoglobulins were eluted), lactoferrin and serum albumin, α-lactalbumin and the LMW substances. The elution pattern for mercury presented two peaks, the first one eluted in the void volume of the column and the second peak eluted in the volume corresponding to lactoferrin and serum albumin.

Gel filtration chromatography on Sephadex G-100 of bovine skimmed milk incubated with mercury (Fig. 2a) showed three protein peaks. The first peak corresponded

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**Fig. 1.** Distribution of Inorganic Mercury in Human Skimmed Milk (A) and Whey (B) Fractionated by Gel Filtration Chromatography. Skimmed milk (0.75 ml) or whey (1 ml) incubated overnight with mercury (as the chloride) at a final concentration of 3 mg/liter was applied to a Sephadex G-100 column and eluted with 25 mM sodium acetate (pH 6.5). Absorbance was measured at 280 nm (○). Concentration of mercury is in µg/ml (●).

**Fig. 2.** Distribution of Inorganic Mercury in Bovine Skimmed Milk (A) and Whey (B) Fractionated by Gel Filtration Chromatography. Skimmed milk (0.75 ml) or whey (1 ml) incubated overnight with mercury (as the chloride) at a final concentration of 3 mg/liter was applied to a Sephadex G-100 column and eluted with 25 mM sodium acetate (pH 6.5). Absorbance was measured at 280 nm (○). Concentration of mercury is in µg/ml (●).
to the void volume of the column, and the following two peaks to β-lactoglobulin and α-lactalbumin, and to LMW substances, respectively. The profile found for mercury showed two major peaks eluted, the first one in the void volume of the column together with the casein micelles, and the second one in the volume corresponding to β-lactoglobulin and α-lactalbumin.

The chromatogram with Sephadex G-100 of bovine whey obtained by acid precipitation is shown in Fig. 2b. Measurement of the absorbance at 280 nm showed four peaks corresponding to the void volume of the column, β-lactoglobulin, α-lactalbumin and, finally, the LMW substances. The profile of mercury contained only one peak that was eluted in the volume corresponding to β-lactoglobulin.

The presence of aggregates of milk proteins induced by mercury was analysed by SDS-PAGE and native-PAGE as shown in Fig. 3. β-Lactoglobulin incubated with mercury revealed by SDS–PAGE two bands with apparent molecular weights of approximately 18,000 and 36,000 while β-lactoglobulin run without added mercury showed only one band corresponding to an apparent molecular weight of 18,000. Likewise, in the electrophoretic run of bovine whey incubated with mercury, a protein band appeared with a molecular weight of 36,000 that was not present in the bovine whey without added mercury. Non-denaturing electrophoresis of β-lactoglobulin or bovine whey also revealed the presence of aggregates.

To confirm the foregoing results, β-lactoglobulin was also dissolved in a buffer of pH 8, in which it should be present as a monomer, and then was incubated with mercury. The protein solution was chromatographed by gel filtration in a column of Superose 12, and the elution volume of β-lactoglobulin was compared with that of the protein without added mercury. As is shown in Fig. 4, β-lactoglobulin incubated with mercury was eluted in a volume less than that of the control without added mercury. When β-lactoglobulin was incubated with a molar proportion of mercury of 1:4 (metal:protein), half of the protein was eluted in the same volume as the complex of β-lactoglobulin-mercury, and the other half as β-lactoglobulin without added mercury. This result indicates that mercury interacted with β-lactoglobulin, causing its aggregation as dimers. On the other hand, mercury did not seem to cause aggregation of albumin or any human whey protein as had previously been shown by SDS–PAGE or native-PAGE.

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**Fig. 3.** SDS-Polyacrylamide Gel Electrophoresis (A) and Polyacrylamide Gel Electrophoresis without SDS (B) of Human and Bovine Whey, β-Lactoglobulin and Serum Albumin with or without Added Mercury. Electrophoresis was carried out in gels containing a gradient of acrylamide (8-25%). Samples 1–8 were incubated overnight with 3 mg/liter of mercury (as the chloride), the same samples without added mercury being used as controls (samples 1–4). 1) human whey; 2) bovine whey; 3) β-lactoglobulin; 4) albumin; 5) human whey; 6) bovine whey; 7) β-lactoglobulin; 8) albumin.

**Fig. 4.** Gel Filtration Chromatography on Superose 12 of β-Lactoglobulin Incubated with Mercury. β-Lactoglobulin (2.5 mg/ml) in 50 mM Tris-HCl and 50 mM NaCl at pH 8.0 without added mercury (A), incubated with an excess of mercury (B), and incubated in a molar proportion 1:4 (mercury:protein) (C). All samples were eluted in the same buffer at a rate flow of 0.4 ml/min, and the absorbance at 280 nm was monitored.
Despite the fact that mercury provoked the aggregation of β-lactoglobulin as it has just been shown, this metal did not cause the precipitation of β-lactoglobulin even at concentrations of the metal as high as 500 mg/liter (Fig. 5), unlike other heavy metals such as lead and cadmium. Under the same conditions, no precipitation of albumin was observed when it was incubated with mercury, lead or cadmium (data not shown).

Discussion

Gel filtration chromatography of human milk with added mercury shows that, in the milk of this species, the casein micelles had high ability to bind inorganic mercury, as it has been also reported for other metals such as lead and zinc. In the chromatography of human whey, mercury was eluted in the void volume of the column, probably bound to the casein micelles which remained in the whey even after centrifugation at 90,000 × g. Another possible explanation might be that mercury would cause the aggregation of some milk proteins such as albumin; however, this phenomenon was not observed in our work. Furthermore, the chromatographic profile of human whey showed a peak of mercury eluted in the volume corresponding to that of albumin, which indicates that part of the mercury in human whey was associated with this protein. Although it has been reported that mercury is able to form dimers of human or bovine serum albumin, the strong bond between the metal and the first albumin molecule caused an effect on the overall conformation that made the formation of the dimer rather difficult. Despite the high affinity of albumin to bind mercury, this interaction is not quantitatively important due to the low concentration of this protein in milk.

In bovine milk, mercury is associated with two protein fractions. The first one is composed of proteins with a high molecular weight, being mainly casein micelles. The interaction of caseins with mercury is probably due to a non-specific mechanism that involves the binding to phosphoserine and carboxyl groups in a way similar to that with other metals. The ability of caseins from bovine milk to bind mercury has been previously reported by Roh et al., who found that about a third of the mercury added to milk was separated with the acid casein, indicating that the interaction between mercury and caseins was retained despite the low pH value.

The other protein that binds an important proportion of mercury in bovine milk is β-lactoglobulin. This protein contains in its structure one free sulphydryl group that is susceptible to interact with mercury, since this metal is known to bind strongly to the sulphur atom of the sulphydryl groups. In our work, it was observed that mercury interacted with β-lactoglobulin to reduce the formation of dimers of protein, probably through the binding of mercury to the free sulphydryl group in each of the two molecules of β-lactoglobulin. This dimer could not be separated by denaturation with SDS, indicating covalent interaction. Furthermore, the formation of dimers was also confirmed by gel filtration with a buffer at pH 8, in which β-lactoglobulin would have been present as a monomer. Since, at the normal pH of milk, this protein is naturally found as a dimer, this could have caused a misinterpretation of the results.

This work identified a different distribution of mercury among the compounds of human and bovine milk, as it had been previously found with other metals such as cadmium, zinc and copper; therefore, the bioavailability of mercury in human or bovine milk may also be different. Although mercury binds to caseins in the milk of both species, in bovine milk, an important proportion of the mercury was bound to β-lactoglobulin, a protein which has been reported to cross the intestinal mucosa intact. More investigations will be necessary to know whether β-lactoglobulin could enhance the intestinal absorption of mercury. No previous report, it has been found comparing the effect of human, bovine milk, or isolated milk compounds on the bioavailability of mercury.

In conclusion, mercury possessed strong ability to interact with proteins from human and bovine milk, rather than with low-molecular-weight substances. Mercury showed high affinity for the sulphydryl groups present in β-lactoglobulin and albumin; however, when such groups were not available, other groups present in caseins such as phosphoserine and carboxyl may efficiently compete for this metal. Furthermore, it must be taken into account that the high concentration of caseins in milk in relation to whey proteins could favor that competition.

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