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# PREGNANCY-ASSOCIATED CHANGES IN RENAL METALLOTHIONEIN CONCENTRATION AND PLASMA DISTRIBUTIONS OF METALS

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**Abstract**—Pregnancy-associated changes in concentrations and distributions of selected essential elements were examined in the blood and tissues of rats. Concentrations of copper (Cu) and zinc (Zn) in the kidneys of dams significantly decreased with gestational age and recovered after delivery. Distribution profiles of multi-elements in the supernatant of the kidneys indicated that Cu and Zn bound to metallothionein decreased with gestational age without affecting their distributions to other components. Although concentrations of Cu and Zn in the liver did not show significant changes during gestational period, Zn bound to metallothionein decreased with gestational age. Plasma concentrations of Cu, iron, phosphorus, sulfur, Zn and other elements were altered by the physiological change, some of those chemical forms being assigned.

Women in pregnancy and lactation can be regarded as a high risk group on two viewpoints. For the mother, pregnancy or gestation and lactation are a kind of load test and cause various significant changes in the maternal body with gestation to adapt to the physiological change. On the other hand, for embryo, fetus and child, the period of gestation and lactation is most susceptible to their environment including the maternal body [1].

Trace elements in the human body are known to be altered by various physiological changes. Pregnancy-associated changes in plasma concentrations of copper (Cu) and zinc (Zn) are well known physiological changes [2–5]. Developmental changes in hepatic Cu and Zn concentrations are now explained in relation to their induction and binding to metallothionein [6–10].

Changes in concentrations of trace elements are relatively well documented for plasma during the gestational period, especially of humans [2–5]. However, those in other tissues are not well known in spite of several toxic effects of contaminant metals during gestational period [11, 12]. In our effort to correlate the changes of trace elements in plasma to those in tissues, we observed a significant decrease of Cu and Zn concentrations in the kidneys of dams with the gestational age of rats.

The present communication reports the changes with gestational age in concentrations of essential elements of tissues and plasma, especially of Cu and Zn in the kidneys in relation to their binding to metallothionein. Concentrations of multi-elements were determined simultaneously to understand the interaction among elements. Distributions of multi-elements in the soluble fraction were also determined simultaneously using an inductively coupled argon plasma-atomic emission spectrometer (ICP) as a multi-elements specific detector for a high performance liquid chromatograph (HPLC–ICP method) [13]. The application of the HPLC–ICP method was shown to be effective for the determination of distributions of multi-elements in biological samples and the participation of renal metallothionein was demonstrated as a source of Cu for fetus during gestation.

## MATERIALS AND METHODS

*Animals*. Female Wistar strain rats were purchased at 4 weeks old from Clea Japan Co. (Tokyo) and given a commercial diet (CE-2, Clea Japan Co.) and distilled water *ad lib*. The rats were mated at 12 weeks old with male rats of the same strain of the same age. Day 1 of gestation was considered when sperms were found in vaginal smears. Pregnant rats were killed at gestation days 6, 13 and 20, and also at the 8th day after delivery. Each time a group of five dams were killed by exsanguination under light ether anesthesia.

*Element concentrations*. Livers and kidneys were homogenized in 3 vol. of 0.1 M Tris–HCl buffer (pH 7.4, containing 0.25 M glucose; bubbled with nitrogen gas before use) using a Potter-Elvehjem glass–Teflon homogenizer in an atmosphere of nitrogen gas under ice-water cooling. The homogenates were centrifuged at 170,000 *g* for 70 min at 4°. A 0.5 ml portion of tissue homogenates and supernatants and plasma in each rat was acid-digested with 1.0 ml of mixed acid ($HNO_3/HClO_4 = 5/1$). Concentrations of multi-elements were determined on an ICP (JY48 PVH, Seiko Instruments & Electronics Ltd., Tokyo).

*Distribution profiles by the HPLC–ICP method*. A 0.1 ml portion of tissue supernatants or plasma was

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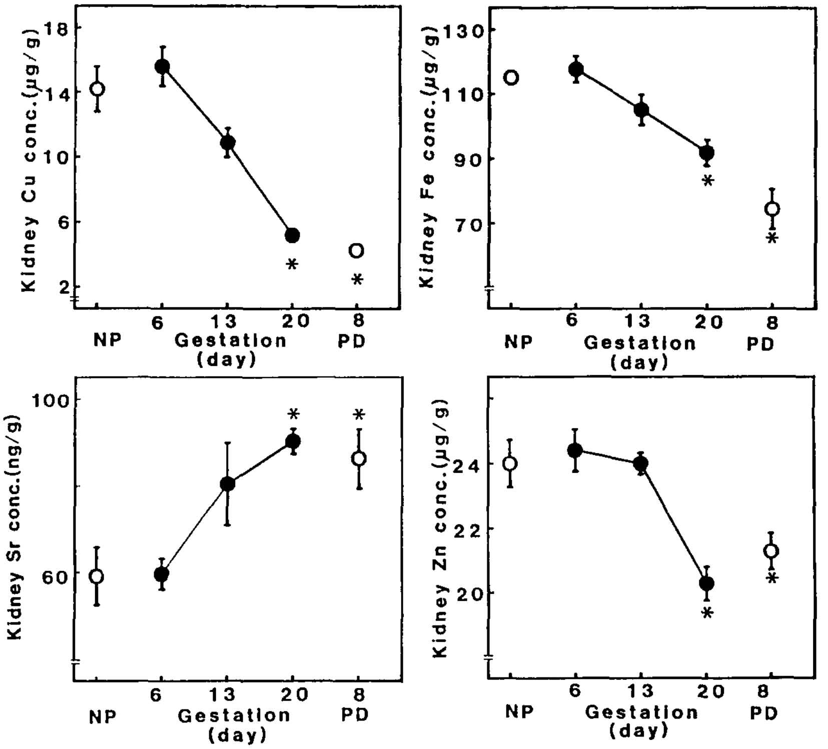


Fig. 1. Changes in concentrations of four elements in the kidneys of rats during gestation and lactation. NP stands for non-pregnant (age-matched non-pregnant rats were used as control), while PD stands for post-delivery and rats nursing 10 offspring were killed at 8th day post-delivery. Values with asterisk are significantly different from control.

applied to gel filtration columns (Asahipak GST-520, 7.6 × 500 mm, Asahi Chemical Industry Co., Kawasaki; or TSK gel G3000SW-XL, 7.8 × 300 mm with a guard column of 6.0 × 40 mm, Tosoh Co. Ltd., Tokyo). A GST-520 column was a modified HPLC column from a GS-520 column, made of vinyl alcohol copolymers. The residual carboxyl groups in the resin of the original GS column were methylated to reduce the ionic interactions between the column materials and metals in substrates [14]. An SW column was made of a chemically bonded silica gel and the ionic interactions with silanol and residual carboxyl groups in the resin were utilized as functional groups for ion exchange during gel filtration chromatography using an alkaline buffer solution [15]. A GST column was eluted with 0.1 M Tris–HCl buffer (pH 7.4, containing 0.9% NaCl and 0.05% $NaN_3$), while an SW column was eluted with 0.1 M Tris–HCl buffer (pH 8.0, containing 0.1% $NaN_3$) at a flow rate of 1.0 ml/min. Absorbances at 254 and 280 nm in the eluate were continuously monitored using a flow cell. Concentrations of multi-elements including sulfur (S) were detected every 2 sec for 26 min (800 data points) for a GST column or every 1 sec for 12 min (700 data points) for an SW column using an ICP as a multi-elements specific detector (the HPLC–ICP method) [13]. Most of the major peaks in each element profile have been already identified on the same GST and SW columns under the same conditions and reported elsewhere [13–16].

*Statistics*. Data were presented as means ± SD. Student's *t*-test was used to compare mean values for statistical significance, and probability values less than 0.05 indicated significant differences.

## RESULTS

### *Changes in concentrations of elements in the kidneys of rats during gestation and lactation*

Concentrations of respective essential elements showed characteristic changes in the kidneys of dams during gestation and lactation. Cooper, ion (Fe) and Zn concentrations decreased with gestational age in the kidneys as shown in Fig. 1. The three metals remained at significantly lower levels even after 8 days post-delivery. On the other hand, calcium (Ca) (78.9 ± 20.8 μg/g, mean ± SD for control), magnesium (Mg) (212 ± 19.3 μg/g), phosphorus (P) (3.15 ± 0.27 mg/g) and S (2.52 ± 0.20 mg/g) concentrations were not altered during this physiological change. Strontium (Sr) is the only one exception that increased with gestational age among elements determined in the present study.

### *Distribution profiles of essential elements in the kidney supernatants of rats during gestation and lactation as determined on an SW column by the HPLC–ICP method*

Distribution profiles of five elements in the kidney supernatant of non-pregnant rats are shown on the left panel in Fig. 2 along with absorbance peaks at 254 and 280 nm. Although distribution profiles of S and P did not change with gestational age (data not shown), those of the other three elements changed.

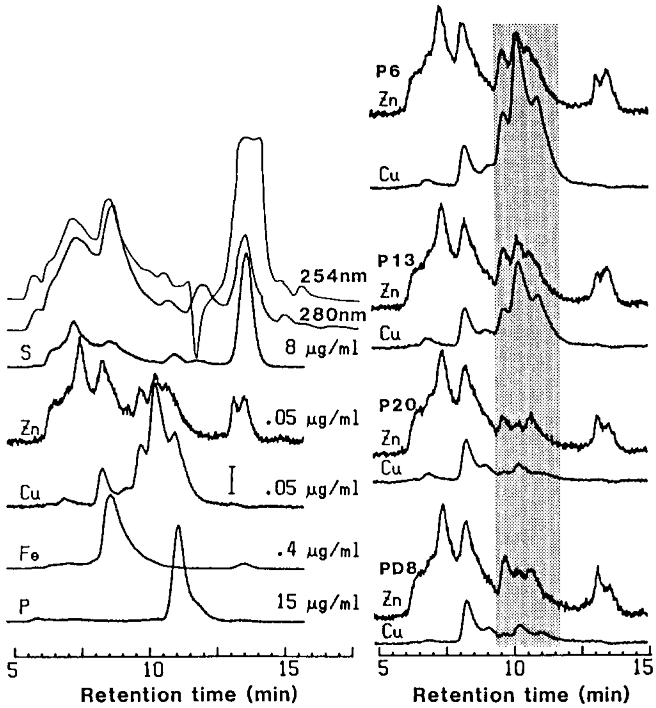


Fig. 2. Distribution profiles of essential elements in the kidney supernatants of non-pregnant rats and those of Cu and Zn during gestation and lactation as determined on an SW column by the HPLC–ICP method. The left panel shows the distribution profiles of five elements and absorbance peaks at 254 and 280 nm for the control rats, while the right panel represents the change in distribution profiles of Cu and Zn during gestation and lactation. The vertical bars indicate the detector levels. Copper and Zn bound to renal metallothionein of rats were eluted between 9.2 and 11.5 min.

In the Fe profiles, the major Fe peak associated with hemoglobin decreased in height with gestational age (data not shown). Copper and Zn profiles changed with gestational age as shown on the right panel in Fig. 2. Although Cu and Zn profiles in the high and low molecular weight fractions remained unchanged, those between retention times of 9.2 and 11.5 min changed dramatically with gestational age. The Cu and Zn peaks in this fraction have been identified earlier as isoforms of Cu-containing rat kidney metallothionein [16]. The present results indicate that the change in Cu and Zn concentrations in the kidneys can be attributed to decreases of Cu and Zn bound to metallothionein with gestational age and their recovery after delivery.

*Changes in concentrations of elements in the liver of rats during gestation and lactation*

Contrary to the changes in the kidneys, several elements in liver increased with gestational age along with Sr as shown in Fig. 3; Ca (30.9 ± 4.1 µg/g before conception, while 40.2 ± 3.6 µg/g at day 20), Mg (232 ± 25.9 µg/g before conception, while 262 ± 13 µg/g at day 20). However, S (2.56 ± 0.25 mg/g) and P (3.57 ± 0.42 mg/g) remained within the control levels. Hepatic Cu and Fe decreased as also observed in the kidneys, while Zn was not altered in the liver.

*Distribution profiles of essential elements in the liver supernatants of rats during gestation and lactation as determined on an SW column by the HPLC–ICP method*

Distribution profiles of five elements in the supernatant of non-pregnant rat liver are shown on the left panel in Fig. 4. Distribution profiles of alkali earth metals were not found to be reliable on an SW column (see Materials and Methods) and are not shown in the present figures. The decrease of Cu in Fig. 3 is attributable to the major Cu peak at a retention time of 8.2 min (PD8 in Fig. 4) that was assigned as Cu and Zn-containing superoxide dismutase (SOD) [13]. The distribution profile of Fe in Fig. 4 indicated that ferritin at the void volume of 6.6 min and hemoglobin at 8.6 min were responsible for the changes. Although Zn in the liver did not show a significant change in Fig. 3, the metal in the metallothionein fraction between 10.0 and 12.0 min decreased with gestational age and then recovered after delivery as shown on the right panel in Fig. 4.

*Changes in concentrations of elements in the plasma of rats during gestation and lactation*

The concentration of P in plasma increased at the late gestational period and then decreased to the control level after delivery (Fig. 5). The concentration of Sr in plasma also increased at the late

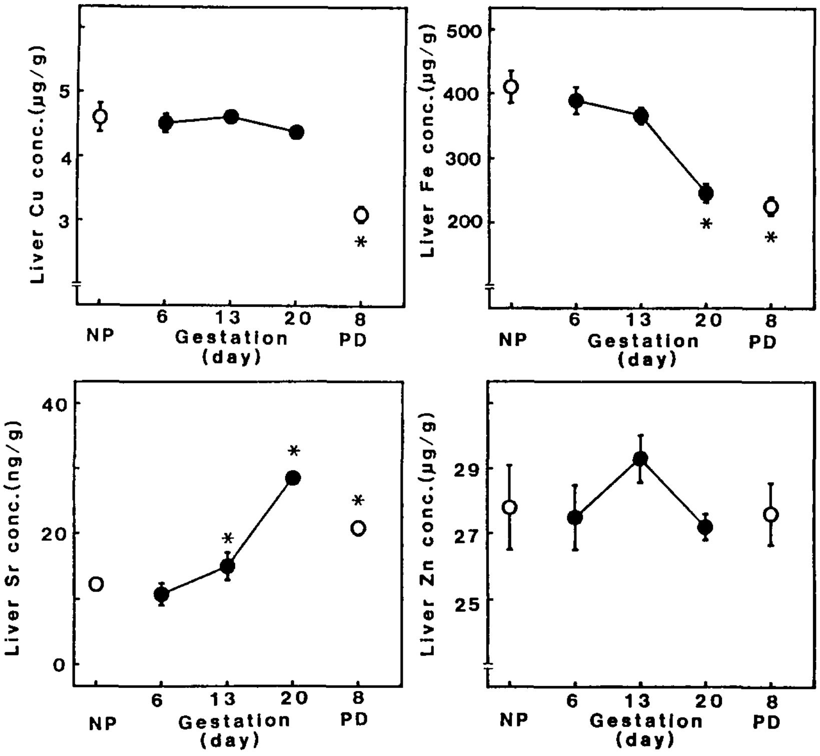


Fig. 3. Changes in concentrations of four elements in the livers of rats during gestation and lactation. See the legend to Fig. 1.

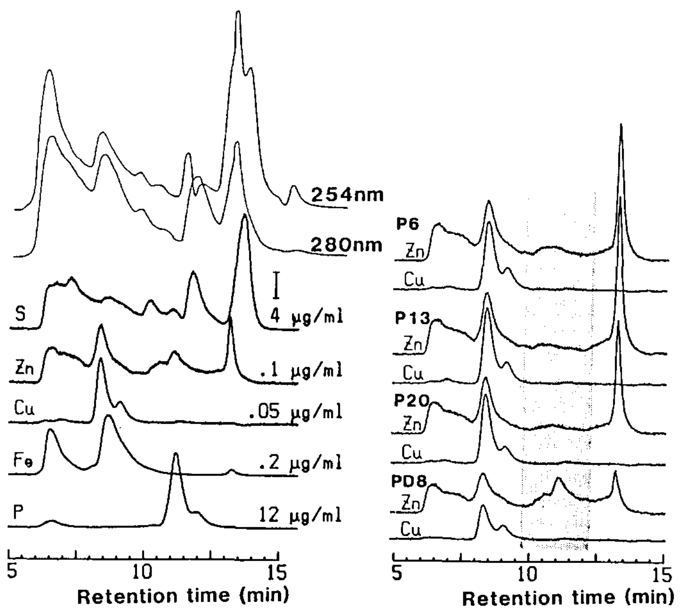


Fig. 4. Distribution profiles of essential elements in the liver supernatants of non-pregnant rats and those of Cu and Zn during gestation and lactation as determined on an SW column by the HPLC–ICP method. Zinc bound to hepatic metallothionein was eluated between 10.0 and 12.0 min under the present condition. See the legend to Fig. 2.

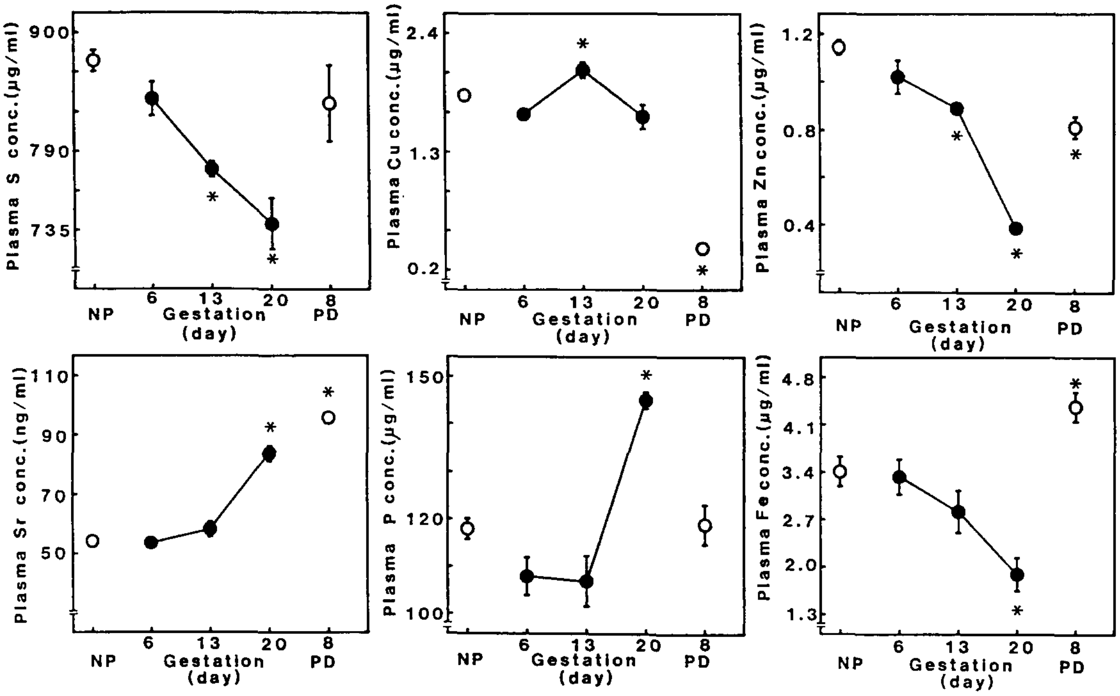


Fig. 5. Changes in concentrations of six elements in the plasma of rats during gestation and lactation. See the legend to Fig. 1.

gestational period and remained at the elevated level even after delivery, indicating that this element was elevated not only in kidneys and liver, but also in plasma with gestational age (Fig. 5). The concentrations of Fe, S and Zn in plasma were decreased with gestational age and then recovered after delivery, while Ca was decreased during gestation ($92.0 \pm 2.1$ before conception, while $88.9 \pm 3.1$, $89.1 \pm 4.0$ and $87.4 \pm 2.7$ at days 6, 13 and 20, respectively).

*Distribution profiles of elements in the plasma of non-pregnant and late gestational rats*

Distribution profiles of elements in plasma were determined on a GST column as shown in Fig. 6. Alkali earth metals were eluted reproducibly at different retention times on this column under the present conditions. The peak height of Sr at 19.2 min became bigger with gestational age, which coincided with the change in total Sr concentration (Fig. 5).

The distribution profile of S in Fig. 6 indicated that albumin was separated into mercaptalbumin at 15.8 min and non-mercaptalbumin at 20.3 min in the non-pregnant rat plasma as already reported elsewhere [14]. The S peak at 16.2 min and the shoulder peak at 17.4 min correspond to sulfate and glutathione, respectively [13]. Globulins were eluted before 15.0 min and separated well from albumins. Although each globulin peak was not assigned in the present study, the S peak at 10.2 min increased characteristically with gestational age, suggesting that this peak was attributable to pregnancy zone protein [17].

The major Cu peak of ceruloplasmin in plasma at 12.3 min [14] was decreased significantly after delivery (profile not shown). Although the major P peak at 16.3 min stayed at the same height throughout, the P peak at the void volume of 8.5 min increased with gestational age and then decreased to the control level after delivery (data not shown). The decrease of Fe concentration in Fig. 5 can be explained by the decreased peak height of transferrin at 8.0 min.

Zinc in plasma distributed to globulins and albumin. In the former fraction Zn was eluted as at least three peaks at 6.8, 7.7 and 8.5 min, while in the latter fraction Zn was eluted as mercaptalbumin [14]. The distribution profiles in Fig. 6 indicate that Zn decreased with gestational age both in the globulin and albumin fractions.

Figure 6 also shows the longitudinal changes in the distribution profiles of S and Zn. The S and Zn profiles in Fig. 6 clearly point out that mercaptalbumin was selectively decreased at the late gestational period. The S peak of mercaptalbumin became the smallest in the profile corresponding to dams just before delivery and it was hidden under the S peak of sulfate ions. Zinc in the albumin fraction was shown to be eluted slower in the profile corresponding to dams just before delivery than in the other gestational periods. Since mercaptalbumin was shown to be eluted slower depending on an applied amount of the protein (unpublished data), the Zn peak in the albumin fraction at gestation day 20 was assigned to the metal bound to mercaptalbumin despite its slower elution than the other Zn bound to mercaptalbumin.

*Concentrations and distributions of elements in the supernatants of red blood cells (RBC)*

Concentrations of S ($2.50 \pm 0.78$ mg/g), P

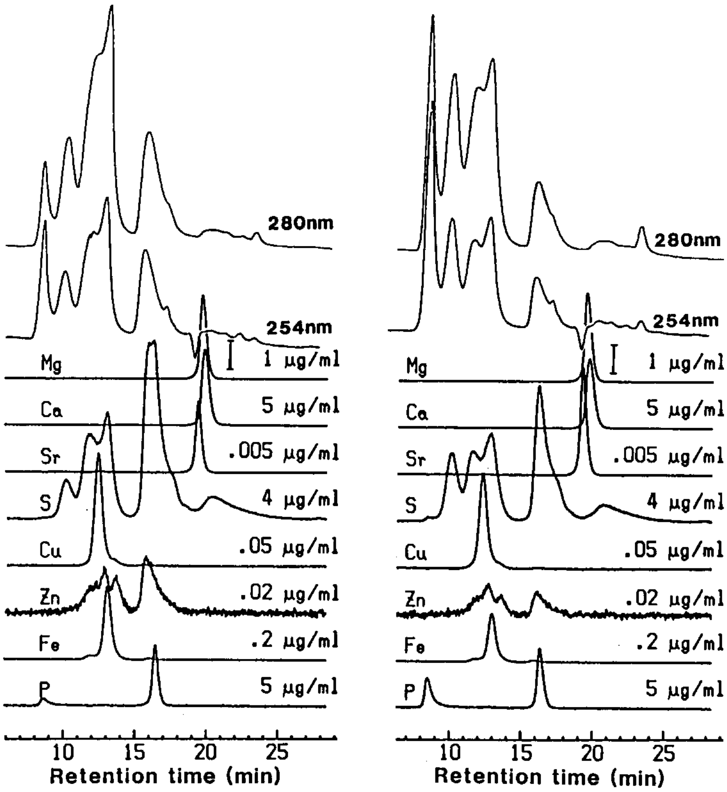


Fig. 6. Distribution profiles of eight elements in the plasma of rats at gestation day 0 and 20 as determined on a GST column by the HPLC–ICP method. The left panel shows the distribution profiles of eight elements in the plasma of the control (before conception), while the right panel represents those of pregnant rats just before delivery (gestation day 20). The vertical bars indicated the detector levels.

(666 ± 32 μg/g), Mg (22.9 ± 1.0 μg/g), Zn (8.21 ± 0.35 μg/g), Cu (0.84 ± 0.04 μg/g) and Fe (919 ± 30 μg/g) in control RBC stayed mostly at the same levels throughout except for some significantly decreased levels after delivery such as for Zn (7.56 ± 0.38 μg/g). The distribution profiles of those elements and others (Mn, Ca and Sr) in the supernatants of RBC were also analysed on an SW column. Figure 7 demonstrates a typical profile. The following peaks were tentatively assigned from their retention times and element specificity; Cu and Zn peaks at 8.2 min (SOD), Fe and S peaks at 8.5 min (hemoglobin), Zn peak at 10.5 min (carbonic anhydrase), S peak at 10.6 min (oxidized glutathione) and S peak at 11.6 min (glutathione). Calcium, Mn, Sr and P were eluted at 9.9 min as main peaks. However, this peak has not been assigned yet.

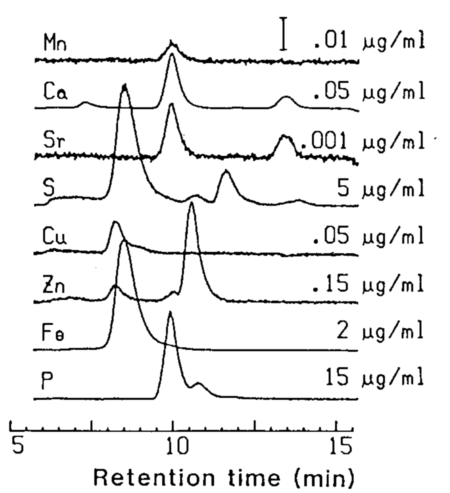


Fig. 7. Distribution profiles of eight elements in the supernatant of control RBC as determined on an SW column by the HPLC–ICP method. The supernatant was prepared by homogenizing RBC in the same manner as employed for tissue supernatants and analysed on an SW column. The vertical bar indicated the detector level.

## DISCUSSION

Copper and Zn concentrations are known to increase with development in the liver of fetus and those increases are well correlated with the increase of hepatic metallothionein [6–10]. The developmental requirement of Cu and Zn in the fetus has to be provided by the maternal supply. The present observation as to the decreases in Cu and Zn concentrations in plasma, kidneys and livers of dams with gestational age may be related to the supply of the metals from the maternal body.

Metallothionein is inducible in liver not only by various kinds of metals but also by a wide range of stresses [18–20]. Although it is known that metallothionein can be induced in liver by a kind of physiological stress such as starvation [21], the present observation is the first that suggests the participation of renal metallothionein in the physiological change associated with pregnancy.

Metallothionein present in kidneys of rats increases with age after weaning [22] and it has characteristic properties compared to metallothionein in the liver of the same animal or renal metallothionein in other mammals [16]; renal metallothionein contains Cu at a higher ratio than Zn in normal rats. Further, renal metallothionein present in normal rats or induced by Cd was shown to be separated into more than the usual two isometallothionein peaks and it was explained by the intramolecular oxidative disulfide bond formation when cupric ions were reduced to cuprous ions by sulfhydryl groups in metallothionein [16]. As an SW column shows not only gel filtration property but also cation exchange property by elution with alkaline buffer solution [15], the Cu-containing metallothionein in the kidneys of rats was separated into at least three peaks as shown in Fig. 2. Therefore, the participation of renal metallothionein in the physiological changes can be most effectively observed in rats from the two properties characteristic of rats; high concentration of metallothionein in the kidneys of normal rats and binding of Cu to metallothionein along with Zn.

Although Zn concentration in the liver did not show a significant change during gestation and lactation, the HPLC–ICP profile suggested that Zn-binding hepatic metallothionein was also decreased with gestational age. However, this change was not observed as changes in hepatic Zn concentration because Zn bound to hepatic metallothionein was negligibly low compared to the total Zn concentration in the liver. This observation is different from that reported for the liver of mouse; maternal metallothionein-I mRNA in the liver increased during the second half of gestation [23].

Plasma Cu concentration is known to increase in humans with gestational age and it is correlated with the increase of ceruloplasmin [24]. On the other hand, plasma Cu in rats was increased only slightly during the gestational period and was decreased significantly after delivery in the present study though it was caused similarly by the decrease of ceruloplasmin. This difference is probably explained by the difference in gestational period between humans and rats. Similarly, as the half-life of RBC is longer than the gestational period in rats, changes in concentrations of elements can not be observed in RBC within the gestational period of rats.

Hemodilution during gestation is a well known physiological process [25, 26]. However, all plasma proteins are not diluted homogeneously as already known, for example, the pregnancy zone protein increases with gestational age. Pregnancy zone protein is called by other names such as pregnancy-associated $\alpha_2$-glycoprotein, pregnancy-associated globulin, $\alpha_2$-pregnoglobulin, and its similarity to $\alpha_2$-macroglobulin has been pointed out [17]. One of globulin peaks was observed to increase characteristically with gestational age in the present study. Although it was not confirmed in the present study, this globulin peak at 10.2 min in Fig. 6 may be assignable to pregnancy zone protein from its chromatographic and gestation-associated properties. Other plasma proteins including albumin decreased with gestational age.

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