Possible Involvement of Metallothionein in the Manifestation of Cadmium Toxicity in Rats

ラットにおけるカドミウムの毒性発現とメタロチオネインの関与に関する研究

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1. Introduction

1.1. Cadmium and its health effects

Cadmium (Cd) is produced as a by-product in the refining process of ores containing zinc (Zn), lead and copper in smelters and is widely used in industries dealing with plating, alloys, catalysts, pigments, plastic stabilizers and batteries. Release of Cd to work and general environments could be potentially harmful to man's health. Indeed, long-term Cd exposure may cause renal damage, from minor tubular dysfunction to severe impairment in tubules as well as glomeruli. Inhalation of Cd may also give rise to obstructive lung disease. However, the kidney is considered to be a target organ, in which earliest adverse effects occur (as reviewed by Friberg et al., 1974; Tsuchiya, 1978; Webb, 1979). Thus, it may be possible to prevent aggravation of Cd-poisoning if one can detect an early sign of the kidney dysfunction (Friberg, 1984).

Investigations on Cd health effects on general populations were started after the occurrence of "itai-itai" disease, a combination of severe renal damage and osteomalacia, that was reported to be endemic in Toyama Prefecture since World War II (Tsuchiya, 1978; Nomiyama, 1979). Regarding the cause of the disease, Cd is considered to be an essential causative factor although other factors such as malnutrition and many pregnancies are suggested to be involved to some extents (Friberg et al., 1974; Tsuchiya, 1978).

Presence of cadmium health effects in work environments was suspected and documented by Friberg as early as 1950.

He pointed out pulmonary emphysema and proteinuria as typical clinical manifestations of Cd-poisoning observed in industrial workers who had inhaled Cd oxide dust. Since then, extensive studies to characterize health effects of Cd have been carried out in epidemiologic and experimental studies. These studies have revealed that clinical features common to man and experimental animals are renal dysfunction (as reviewed by Friberg et al., 1974; Tsuchiya, 1978; Webb, 1979).

1.2. Cadmium and its renal toxicity

Renal dysfunction is classified into two categories, i.e. glomerular type and tubular type. Since the main component of the urinary proteins had a considerably lower molecular weight than albumin and, elevated excretion of low molecular weight proteins (β_2-microglobulin, retinol-binding protein and lyzozyme) were often observed in Cd-poisoned man and experimental animals, it is considered that Cd exposure mainly causes tubular type of renal dysfunction (Kazantzis 1979a, b; Kazantzis et al., 1963; Nogawa et al., 1977, 1978, 1979), whereas Lauwerys and coworkers claimed, from the data on the elevated excretion not only of low-molecular weight protein but also larger molecular weight proteins such as albumin, the presence of mixed (tubular plus glomerular) type of renal damage in Cd-exposed industrial workers and experimental animals (Bernard et al., 1976, 1979, 1980; Buchet et al., 1980; Lauwerys et al., 1974). However, it should be pointed out that normal urine contains larger amounts of albumin than that of low-molecular weight proteins and that even in tubular proteinuria due to Cd the

absolute increase will be highest for albumin but that the relative increase will be highest for low-molecular weight proteins (Friberg, 1984).

To evaluate proteinuria caused by Cd, quantitative methods to determine the low molecular weight proteins, i.e. β_2-microglobulin, retinol-binding protein and α_1-microglobulin are now available. However, in the search of specific indicators of heavy metal exposure, MT, an inducible low-molecular-weight protein that also binds the heavy metals was thus considered to be most appropriate. Development of a radioimmunoassay for MT by Vander Mallie and Garvey (1978, 1979) and Tohyama and Shaikh (1978, 1981) has made it possible to specifically determine MT in biological fluids and tissues and to investigate the physiological and toxicological roles of the protein.

To study whether MT in serum and urine reflects the extent of Cd exposure and/or the severity of renal damage several studies have been carried out in experimental animals and man. In environmentally and occupationally exposed human populations, urinary excretion of MT appears to reflect the extent of Cd accumulation in the liver and the kidney. This was demonstrated in Cd-exposed industrial workers when their urinary MT excretion and hepatic and renal Cd levels were determined by a radioimmunoassay and an in vivo neutron activation analysis (Tohyama et al., 1981a), respectively. A similar relationship was observed between the urinary MT concentration and the Cd levels in the liver and the kidney of rats chronically exposed to Cd. An only major difference between the two species is a drastic in-

crease in the urinary MT excretion in the rat when kidney Cd exceeded approximately 200 μg/g tissue (Tohyama et al., 1981a), a proposed critical concentration in man and animals (Friberg et al, 1974).

The urinary MT level is closely related to urinary excreted levels of Cd in Cd-exposed industrial workers and the general population (Chang et al., 1980a, 1980b; Tohyama et al., 1981b, 1982). Serum MT concentration is also increased upon repeated exposure to Cd in experimental animals (Tohyama and Shaikh, 1981). Even a single injection of Cd causes the serum MT elevation as several-fold high as control (Garvey and Chang, 1981). These observations suggest that serum and urinary MT levels may be used as indicators of the Cd exposure level.

The validity of the urinary MT excretion as an indicator of renal dysfunction was examined in Cd-exposed people. Nordberg et al. suggested that the information derived from these parameters is different from each other because the urinary β_2-microglobulin and MT levels are not always correlated. Shaikh and Smith (1984) summarized that this is in agreement with the view that β_2-MG is a better indicator of general non-specific renal status, and MT a better indicator of Cd body burden. According to Chang et al. (1980b), the urinary MT level is more related to Cd exposure than renal function because no difference was found in subjects with and without renal dysfunction.

In contrast, in the Japanese women living in a Cd-polluted area, the urinary MT level was closely associated levels of various parameters, such as total protein,

glucose, retinol-binding protein, α-amino acid and proline. When the women were classified into two groups based on cutoff values for each of the above parameters, the urinary MT levels were higher in all the groups, the level of the parameter of which is higher than the cutoff value, suggesting that the urinary MT may reflect renal function altered by Cd-exposure (Tohyama et al., 1982). Despite these studies it is neither yet clear as to renal handling of MT in the kidney nor the association of serum and urinary MT with various substances that are used as markers of functions of these organs.

The metabolism of Cd is closely associated with MT that is unique in several physicochemical and biological characteristics (as reviewed by Kägi and Nordberg, 1979): (1) low-molecular weight protein, approximately 7,000 daltons, (2) high cystein contents (20 out of total 61 amino acids), (3) inducible by metal ions such as Cd, Cu, Hg and Zn, (4) high affinity for Cu, Hg, Cd, Zn in the descending order, (5) at least two iso-proteins in mammals, (6) ubiquitous distribution in various living organisms ranging from blue-green algae to primates.

When experimental animals are pretreated with a sub-lethal dose of these metals and MT is preinduced, otherwise lethal dose of either of metals fails to show its effects. It is thus considered that MT plays a protective role in metal toxicity by binding metal ions to the protein moiety (thionein). In contrast, intravenous administration of Cd-MT is selectively taken up by the kidney and the Cd-MT exerts highly toxic effects unless thionein is preinduced.

This occurs at a renal concentration at which no toxic effects are observed if Cd in stead of Cd-MT is administered (Cherian and Shaikh, 1975; Nordberg et al., 1975; Tanaka et al., 1975; Maitani et al., 1986).

1.3. Specific aims of the present study

1.3.1. Fate of metallothionein and Cd in the body with special reference to pathogenesis of the renal damage

Administration of Cadmium (Cd) results in the retention of Cd mainly in liver and kidney and the induction of metallothionein (MT) in these organs (Kägi and Nordberg, 1979). The transfer of Cd from the liver to the kidney was proved when Cd-accumulated liver was intentionally damaged by the administration of hepatotoxicants (Bernard and Lauwerys, 1981; Tanaka et al., 1981). In chronically Cd-poisoned rats the MT level in the circulation was found to be elevated (Tohyama and Shaikh 1981; Dudley et al., 1985). When Cd-MT was intravenously injected to the rat, most of Cd was found in the kidney (Tanaka et al., 1975, Cherian and Shaikh 1975). Thus, it has been speculated that MT, which contains predominant amounts of Cd (abbreviated as Cd-MT), that is released from the liver will be taken up by the kidney and that the Cd ions detached from the protein moiety (thionein) will exert toxic effects (Suzuki, 1982). In contrast, there is an argument that MT in the circulation may be also originated from the kidney of Cd-poisoned rats (Goyer et al., 1984). One of the aims of the present study is to examine the origin of MT in the circulation.

In terms of manifestation of renal toxicity by Cd, several studies argued the saturation of Cd in the kidney

and the occurrence of renal dysfunction at a Cd concentration of approximately 100-200 μg/g tissue. However, when the renal Cd concentration is saturated, the saturation of the liver with this metal is often observed (Suzuki 1980; Bernard et al., 1980; Tohyama and Shaikh, 1981). Recently Dudley et al., (1985) has reported that the occurrence of hepatic injury precedes the renal damage in rats with long-term exposure to Cd. In the present study, the relationship of the manifestation of hepatic and renal damage is further studied under experimental conditions in which the Cd exposure is suspended and thus the hepatic Cd level is decreased below the saturation level.

1.3.2. Significance of serum and urine metallothionein levels as indicators of Cd exposure and/or toxicity

Metallothionein levels in the serum and the urine are increased by continued exposure to Cd. To clarify the significance of the increased levels of MT in these body fluids as an indicator of Cd exposure and/or Cd toxicity, the relationship of MT with other serum and urine substances that are relevant to hepatic or renal functions is studied. The renal handling of MT is also studied in the present study.

1.3.3. Sex difference in the manifestation of Cd-toxicity

In Cd-polluted areas Cd toxicity is more conspicuous in women than in man. In male rats hepatic damage is more clearly observed than in female rats. However, precise comparison between both sexes with regard to Cd behavior and its toxicity has not been carried out. Thus, possible difference between both sexes will be studied on the matters

described in the above sections 1.3.1 and 1.3.2.

1.3.4. Experimental design

The main feature of the present experimental protocol resides in the dosing schedule in which rats are loaded with $CdCl_2$ to allow liver and kidney to accumulate Cd to their maximum concentrations and then left for observation without Cd loading thereafter. To study possible differences in Cd effects between male and female rats the same protocol is applied to both sexes of the rats.

2. Materials and Methods

2.1. Reagents and glass apparatus

Sephadex G-25, G-75 and DEAE A-25 were obtained from Pharmacia Fine Chemicals (Upsala, Sweden); acrylamide, N,N'-methylene bisacrylamide and cadmium chloride from Wako Pure Chemical Industries Co. (Osaka). Bolton-Hunter reagent (N-succinimidyl 3-(4-hydroxy-5-[^{125}I]-iodophenyl propionate; 2200 Ci/mmol) was purchased from New England Nuclear (Boston). Gelatin (Type II) and bovine serum albumin (A4503) were from Sigma Chemical Co. (St. Louis).

Assay kits for alkaline phosphatase (ALP, EC:3.1.3.1), alanine aminotransferase (ALT, EC:2.6.1.2), aspartate aminotransferase (AST, EC:2.6.1.1), lactate dehydrogenase (LDH, EC:1.1.1.27), creatinine (Denka-seiken Co., Tokyo), N-acetyl-β-D-glucosaminidase (NAG, EC:3.2.1.30, Shionogi Pharmaceutical Co., Osaka) and Tonein-TP (Otsuka Pharmaceutical Co., Tokushima) were obtained from manufacturers described in parentheses. Nitric, sulfuric and perchloric acids were metal-determination grade. All glassware including metabolism cages (Sugiyama-gen Co., Tokyo) were used after being soaked in 10% nitric acid solution and rinsed with deionized water.

2.2. Animal treatment

Wistar rats (4 weeks old) of each sex were obtained from Clea Japan Co. (Tokyo) and maintained in stainless steel cages with distilled water and diet (MF diet, Oriental Yeast Co., Tokyo) ad libitum. After quarantine and acclimatization, 130 male and 130 female animals were equally divided into experimental and control groups for

each sex; i.e. 65 rats per group. At 7 weeks old experimental groups of male and female rats were subcutaneously injected with $CdCl_2$ dissolved in physiological saline at a dose of 1.5 mg Cd/kg body weight, 4 days a week (Thursday, Friday, Monday and Tuesday) for up to 6 weeks. This dosing schedule was carefully chosen on the basis of our earlier studies (Tohyama and Shaikh, 1981; Sugihira et al., 1986) to maximize Cd concentrations in liver and kidney by the end of Cd treatment. The rats were left for observation for further 15 weeks. At specified intervals 5 rats from each group were individually transferred to glass metabolism cages and urine was collected from each rat in a glass vessel kept at approximately 6 °C for 24 hrs. During the urine collection only water was provided in order to avoid the contamination of urine with metals from diet. After the urine collection rats were lightly anesthetized with pentobarbital (Nembutal^R, Abbott Lab., North Chicago) and blood was collected from abdominal artery using a 20-G needle connected with polyethylene tubing to minimize hemolysis, and then, liver and kidney were also collected.

Aliquots of urine and serum specimens were kept frozen at -80 °C until MT analysis. The rest of the urine specimen was centrifuged at 2,000 g for 5 minutes and the supernatant was used for determination of enzyme activities and total protein. The rest of the serum was subjected to biochemical analysis. A portion of liver and an entire left kidney were for metal analysis.

2.3. Analytical methods

2.3.1. Metallothionein radioimmunoassay

Metallothionein in serum and urine was assay by a radioimmunoassay as described earlier (Tohyama and Shaikh, 1981). Rat MT-1 and -2 were isolated from the liver of Cd-injected rats as described earlier (Tohyama and Shaikh, 1978). Purity of the isolated protein was proved by disc electrophoresis (Davis, 1964). Rat hepatic MT-2 was iodinated with Bolton-Hunter reagent, according to the manufacturer's instructions (Bolton and Hunter, 1973; Bolton, 1977). The protein (5 µg) in 5 µl of 0.1 M potassium phosphate, pH 8.5, was added to the dried ^{125}I-labeled ester (1 mCi), and the solution was incubated in an ice-bath for an hour and then at 4 °C overnight. The reaction tube was washed three times with 100 µl each of 0.05 M potassium phosphate, pH 7.5, containing 0.25% gelatin. The solution was applied to a Sephadex G-25 column (0.9 x 20.5 cm), equilibrated with 0.05 M potassium phosphate, pH 7.5, containing 0.25% gelatin. Half-milliliter fractions were collected in plastic tubes containing 0.4 ml of 2% bovine serum albumin, the latter being used to improve recovery of MT from the tubes. The radioactivity of ^{125}I in the fractions was determined in a gamma-counter. The test tubes containing ^{125}I-labeled MT were washed three times each with 50 µl of the phosphate buffer, containing 0.25% gelatin in order to recover as much of the iodinated protein as possible.

After optimization of the RIA conditions, the assay was carried out at 4 °C as follows: 100 µl of unlabeled MT solution or sample solution diluted in 10-fold diluted human plasma was mixed with 50 µl of ^{125}I-labeled rat hepatic MT-2 solution (20,000 cpm) and 100 µl of either 400-fold diluted

rabbit antiserum or sheep antiserum and allowed to react for 18 to 24 hours. The antigen-antibody complex was precipitated by the addition of 250 µl of 80% saturated ammonium sulfate (Farr, 1958). Thirty minutes later, the reaction mixture was centrifuged at 2,000 x g for 20 minutes. The supernatant was aspirated, and the precipitate was washed with 500 µl of 40% saturated ammonium sulfate. After centrifugation the radioactivity in the precipitate was measured in a well-type gamma counter. For normalization of the data, the net percent binding value obtained in the presence of the unlabeled antigen was divided by the value obtained in the absence of the unlabeled antigen. The normalized percent binding (B/B_o) was used for the RIA. Computation of analytical results was carried out using logit-log transformation. As shown in Figure 1, a typical result on complete cross-reactivity of rat MT-1 and -2 against sheep anti rat MT-1 antiserum was obtained. For determination of MT in the liver and kidney, portions of the organs were homogenized in 9 to 14 volume of ice-chilled buffer (125 mM borate, pH 8.3) using a Polytron homogenizer under flushing of nitrogen gas. The homogenate was centrifuged at 230,000 g for 40 minutes with an ultracentrifuge (Beckman model L8-55) and the supernatant was appropriately diluted for the assay.

2.3.2. Other analyses

Serum substances (ALP, AST, ALT, creatinine and LDH) and urinary substances (ALP, creatinine, glucose, LDH and NAG) were analyzed by a biochemical analyzer (Toshiba model: TBA-360, Toshiba Medical Co., Tokyo) according to

manufacturer's instructions. Urinary total protein was determined with Tonein-TP assay kit based on a dye (Coomassie Brilliant Blue) binding method.

For metal analysis serum (0.5 ml), urine (2.0 ml) and portions of liver and kidney (0.7-1.0 g) were digested with 2.0 ml of concentrated acid mixture (HNO_3:$HClO_4$:H_2SO_4 = 50:48:2). After being appropriately diluted with 1 N HNO_3, the digested samples were subjected to metal determination. Cadmium, Cu, Zn and iron were determined by inductively-coupled plasma atomic emission spectrometry (ICP; Jarrel Ash model: 975 Plasma Atom Comp). When serum and urinary Cd levels were below detection limit of ICP, it was determined by polarized Zeeman-effect flameless atomic absorption spectrometry (Hitachi model: 180-70). Since no significant difference was observed between Cd values when some urine specimens containing appropriate amounts of Cd were analyzed by ICP and the flameless atomic absorption spectrometry, we regarded it appropriate to use the data obtained by the two methods in a series of Cd analysis.

To evaluate renal handling of MT, the relative clearance of MT to creatinine clearance (C_{MT}(%)) was calculated by the equation described as follows:

$$C_{MT} = \frac{\frac{U_{MT} \times V}{S_{MT}}}{\frac{U_{CR} \times V}{S_{CR}}} \times 100$$

$$= \frac{U_{MT} \times S_{CR}}{S_{MT} \times U_{CR}} \times 100$$

where U_{MT}, S_{MT}, U_{CR}, S_{CR} and V denote concentrations of MT in urine (μg/l) and serum (μg/l), those of creatinine in urine (g/l) and serum (g/l), and urine volume for a known period of time (l/min), respectively. However, since in the present study blood was drawn after the 24 hr urine collection and was not collected during the urine formation, the relative clearance values were used only in estimating time course of the parameter.

2.4. Statistical analysis

Difference in the mean between groups was examined by Welch's *t*-test, that is described in Biomedical Program (Biomedical Computer Programs-P, 1975).

3. Results

Since most of the observations were similar between male and female rats, this section mainly describes the results on female rats (3.1 - 3.9) in order to minimize redundancy of description. Some observations on male rats different from those on female rats are depicted in Section 3.10. Data on the female and male rats are compiled in the first and second half parts, respectively, in "Supplement" to this thesis.

3.1. Body and organ weight and ratio of organ weight to body weight

Table 1 summarizes changes in body and organ weight and in ratio of organ weight to body weight of control and Cd-exposed female rats. In the Cd-exposed female rats that were loaded with Cd for the first 6 weeks and were left for observation thereafter the loss of body weight tends to occur as early as week 2 and continued throughout the observation period.

Liver weight of Cd-exposed female rats was larger than that of control at as early as week 1 and approximately 1.43 fold larger at week 6 and became similar to control level at week 16 (Table 1) When ratio of liver weight to body weight was calculated, the ratio in Cd-exposed female rats was significantly higher than that in control rats throughout the observation period and a transient increase of the ratio from week 4 to 6 was observed (Table 1).

Kidney weight in Cd-exposed female rats was also significantly larger than that in control rats from week 4 and

thereafter (Table 1). Ratio of kidney weight to body weight in control rats was found to gradually decrease with body growth whereas the ratio in Cd-exposed female rats was relatively constant throughout the experiment and significantly higher than control at week 4 and thereafter (Table 1).

Changes in body and organ weight and in ratio of organ weight to body weight of control and Cd-exposed male rats are also summarized in Table 3. Result on the male and female rats was found to be very similar to each other.

These data indicate that body weight loss due to Cd exposure did not recover throughout the observation period and that enlargement occurred in both liver and kidney as an early sign of Cd exposure and persists after cessation of Cd exposure.

3.2. Tissue heavy metal levels

Concentrations and contents of Cd, Cu and Zn in the liver and the kidney of control and Cd-exposed female rats are shown in Figures 2 and 3. The Cd concentration in liver and kidney of control female rats were both below the detection limit (approximately 1.0 μg/g tissue). In Cd-exposed female rats, hepatic Cd concentration was increased during Cd exposure of first 6 weeks and reached a maximum Cd concentration of 584 μg/g tissue at week 6 with a gradual decrease after cessation of Cd exposure. The hepatic Cd concentration remained nearly half the maximum concentration (315 μg/g tissue) at week 21 or the end of observation period. In Cd-exposed male rats time course of the reten-

tion of Cd in the liver was similar to that of Cd-exposed female rats (Figure 11(a)). The maximum hepatic Cd concentration was 597 μg/g tissue at week 6. Time course of Cd content in the liver was similar to that of Cd concentration in both male and female rats (Figure 2(a) and 11(a)).

As to renal Cd concentration of the Cd-exposed female rats (Figure 3(a)), it was also increased during Cd exposure as observed in the liver, but leveled off at a Cd concentration of 194 μg/g tissue at week 7 and thereafter. The Cd content in the the kidney increased until week 12 and leveled off (Figure 2(a)). This apparent increase may be explained by the relatively steady Cd concentration and the increase in the kidney weight during this period (Table 1). An observation on the Cd-exposed male rats (Figure 12(a)) was found similar to that on the Cd-exposed female rats.

Hepatic Cu concentration of control female rats varied between 4.98 and 6.01 μg/g tissue during the observation period (Figure 2(b)). The Cd exposure caused significant increase in the hepatic Cu concentration throughout the observation period (range of means : 5.90 - 7.69 μg/g tissue). Renal Cu concentration of control female rats gradually increased from 6.55 (week 1) to 15.8 (week 21) μg/g tissue with body growth (Figure 3(b)). In Cd-exposed female rats renal Cu metabolism was affected and the renal Cu concentration was markedly elevated at week 2 (29.9 μg Cu/g tissue) and significantly higher than that in the control rats throughout the observation period (range of means : 12.1 - 29.9 μg/g tissue) (Figure 3(b)). The hepatic and renal Cu contents in Cd-exposed female rats were also changed in a

manner similar to Cu concentrations in the respective organs. As to hepatic and renal Cu levels in control and Cd-exposed male rats patterns almost identical with those in the female rats were observed (Figures 11(b) and 12(b)).

As to hepatic and renal Zn concentrations, they varied between 29.3 - 39.1 μg/g tissue for liver and 23.5 - 28.5 μg/g tissue for kidney, respectively, in control rats during body growth (Figures 2(c) and 3(c)). On the other hand, in Cd-exposed female rats hepatic and renal Zn metabolism were markedly affected (Figures 2(c) and 3(c)). The hepatic Zn concentration was approximately 3 fold higher at week 2 and continued to be significantly higher than control level throughout the experiment (Figure 2(c)). Similarly, the renal Zn concentration was remarkably increased for the first 4 weeks and also significantly higher than the control levels thereafter (Figure 3(c)). Changes in hepatic and renal Zn levels in control and Cd-exposed male rats were found very similar to those in the female rats.

3.3. Tissue metallothionein levels

Metallothionein levels in liver and kidney of control and Cd-exposed female rats are shown in Figure 4. The control levels in these tissues ranged from 0.055 to 0.077 mg/g liver and from 0.041 to 0.144 mg/g kidney. In Cd-exposed female rats, the hepatic MT level was increased to 3.91 mg/g tissue at week 6 with duration of Cd exposure and gradually decreased after the cessation of the exposure. On the other hand, the renal MT level was elevated upon Cd exposure and leveled off at 1.4 mg/g tissue at week 5. In control

male rats, hepatic and renal MT concentrations ranged from 0.025 to 0.041 mg/g liver and 0.037 to 0.092 mg/g kidney, respectively, which tends to be lower than the MT levels found in the control female rats. On MT concentrations in the Cd-exposed male rats time course of MT concentrations in both organs were found to be similar to that in the Cd-exposed female rats (Figure 13).

3.4. Serum metal levels

Figure 5 shows serum Cd, Cu and Zn levels in control and Cd-exposed female rats. In the control rats serum Cd, Cu and Zn levels fell in a range of 0.84 and 5.85 ng/ml, 1.55 and 1.91 μg/ml and 0.88 and 1.34 μg/ml, respectively. As to serum Cd concentration in control animals sufficient data have not been reported. Although we took extremely careful precautions to avoid contamination of Cd in the process of glassware handling and acid digestion of tissues, there may be some degree of overestimation of the Cd level due to some degrees of contamination.

Exposure to Cd elevated the serum Cd level as early as week 1 (19.8 ng/ml) and the level reached maximum of 151 ng/ml serum at week 6, followed by a gradual decrease. As to serum Cu level, Cd exposure caused elevation in its level throughout the observation period and the level ranged from 2.07 to 2.97 μg/ml. In contrast, serum Zn level of Cd-exposed female rats ranged from 0.46 to 0.82 μg/ml and was significantly lower than that of control rats throughout the observation period.

Serum Cd, Cu and Zn levels in control male rats were

found to be similar to those in the female rats (Figure 14). In Cd-exposed male rats serum Cd appeared to be cleared from the circulation slower than that in the Cd-exposed female rats after the cessation of Cd exposure. Furthermore, second rise of the serum Cd concentration was observed at week 10 in the male rats. Time course of serum Cu and Zn levels was similar to that in the female rats (Figure 14).

3.5. Serum metallothionein level

Serum MT level of Cd-exposed female rats increased as early as week 1 (96.7 ng/ml), reached a maximum of 485 ng/ml at week 6 and decreased after the cessation of Cd treatment (Figure 6). In the Cd-exposed male rat serum MT level changed in a manner almost identical with that in the female rats (Figure 15). The level was significantly higher than that of control rats throughout the observation period. Since most of the control MT level was close to, or below the assay limit (10-20 ng/ml), actual MT values in control male and female rats are probably smaller than the MT level in the present study.

3.6. Urinary excretion of metals

Excreted amounts of Cd, Cu and Zn during 24 hr urine collection in female rats are shown in Figure 7. The Cd level in control rats could not be detected with ICP and thus determined by flameless atomic absorption spectrometry. The level varied between 0.001 and 0.03 μg/24 hr (Figure 7(a)). In Cd-exposed female rats, the elevated excretion of Cd was first detected at week 4 and reached maximum of

30.7 μg/24 hr at week 6 with a transient decrease until week 11. As to Cu and Zn, elevated excretion of both metals was observed as early as week 2 and increased during Cd loading until week 6 (Figure 7(b) and (c)). The levels of these three metals were increased at week 12 and the Cu and Cd levels decreased with time while the Zn level remained at a steady level thereafter. These levels were significantly higher than control levels throughout the observation period.

Urinary excretion of Cd, Cu and Zn in male rats is illustrated in Figure 16. The time course of these three metals in control and Cd-exposed male rats was very similar to that in the female rats except that the urinary Zn level in the Cd-exposed male rats reached a plateau at week 6 and leveled off thereafter.

3.7. Urinary excretion of metallothionein

Urinary MT level in control female rats was no higher than 1.7 μg/24 hr (Figure 8). In the Cd-exposed female rats the urinary MT output reached maximum at week 6 (1210 μg/24 hr), followed by a transient increase between weeks 7 and 12 and a gradual decrease. Urinary MT levels in control and Cd-exposed male rats presented marked similarity to those in the female counterparts, respectively. (Figure 17).

3.8. Effects on the liver

To examine toxic effects of Cd on the liver several enzymes (ALT, AST, ALP and CHE) were analyzed in female and male rats (Figures 9 and 17). Upon Cd treatment serum AST

level in the Cd-exposed female rats elevated and was significantly higher than control level at week 2 and thereafter (Figure 9(a)). It gradually increased until week 10 and then decreased thereafter although the increased level did not return to the control level. A trend of serum ALT level in Cd-exposed female rats was almost identical with that of the AST level (Figure 9(b)). The elevation of both serum ALT and AST levels in the Cd-exposed male rats at week 6 appeared to be more drastic than that in the female rats and its level was significantly higher than that in the control rats at week 7 and thereafter (Figure 18(a) and (b)).

As to serum ALP level, it became markedly higher in Cd-exposed female rats during weeks 4 and 6 than in control rats and gradually decreased after the cessation of Cd exposure (Figure 9(c)). However, it did not return to control level until the end of the observation period. This observation was consistent with that in Cd-exposed male rats (Figure 18(c)).

Analysis of another enzyme, CHE, that was recently proposed to be a very sensitive indicator of Cd exposure (Uehara et al., 1985), showed that the level is considerably depressed during Cd exposure (weeks 1 and 6) and recovers to control level after the cessation of Cd exposure in Cd-exposed female rats (Figure 9(d)). As described below (Section 3.10), serum CHE activity in the Cd-exposed male rats behaved in a different fashion from that in the female rats (Figure 18(d)). Nevertheless, the data of well-established enzyme markers such as ALT, AST and ALP show the occurrence and persistence of hepatic injury caused by Cd

exposure in both male and female rats.

3.9. Effects on the kidney

Changes in daily output of urinary substances in female and male rats due to Cd treatment are shown in Figures 10 and 19, respectively. In the control female rats urinary levels of LDH, NAG, total protein and glucose remained at low levels during the observation period; i.e., LDH; 0.09 - 0.27 U/24 hr, NAG; 0.035 - 0.29 IU/24 hr, total protein; 1.27 - 1.85 mg/24 hr, and glucose; 0.08 - 0.58 μg/24 hr. Urinary ALP level of the control rats appeared to be constant (1.16 - 2.30 U/24 hr) except some variation at weeks 2 and 4. In Cd-exposed female rats excreted amounts of LDH, NAG and ALP became larger with duration of Cd treatment and the LDH and NAG levels were significantly higher than respective control levels as early as week 4 (Figure 10(a) and (b)). The urinary glucose and total protein levels in Cd-exposed female rats tended to increase at weeks 5 and 6 but decreased from weeks 6 to 7 and remained at relatively low levels thereafter (Figure 10(d) and (e)). The urinary LDH level in the Cd-exposed male rats was similar in its time course to that in the female rats (Figure 19(a)). Because of large fluctuation in the urinary NAG and ALP levels in the control male rats the time course of these substances in the Cd-exposed male rats was not as marked as that in the Cd-exposed female rats (Figure 19(b) and (c)). Furthermore, the urinary glucose level was rather low in the Cd-exposed male rats (Figure 19(d)). Observation on urinary excretion of total protein is described in Sec-

tion 3.10.

Interestingly, the second peak of the elevated excretion of LDH, NAG and ALP was observed at week 12, which is similar to the urinary excretion pattern of MT, Cd and Cu (Figures 7 and 8). Although patterns of the second rise in Cd-exposed male rats were not as evident as those in the Cd-exposed female rats, the second rise was also observed for the urinary excretion of LDH, MT, Cd and Cu in the Cd-exposed male rats, supporting the fact that this second peak is attributable to Cd exposure (Figures 16,17 and 19). No marked difference in urine volume was observed between control and Cd-exposed female rats during the observation period (Figure 10(f)).

Table 2 summarizes time course of the relative clearance of MT to creatinine clearance in control and Cd-exposed female rats. In the control rats most of MT filtered through glomerulus appears to be reabsorbed. On the other hand, in the Cd-exposed rats, the relative clearance of MT to creatinine clearance showed marked increase and the values exceeded 100% between weeks 4 and 18, suggesting the possibility that some portions of MT in urine are of renal origin. The same was true for the observation in the control and Cd-exposed male rats summarized in Table 4.

3.10. Some observations limited to male rats

As described in the beginning of the Results section, most of the effects caused by Cd treatment are commonly observed in both male and female rats. In this section two major differences between both sexes are described.

As to serum cholinesterase activity of Cd-exposed male rats, the level was not depressed but rather elevated throughout the observation period (Figure 18(d)) whereas the enzyme activity in Cd-exposed female rats was remarkably reduced during Cd loading (Figure 9(d)).

The other difference between male and female rats is the excretion of protein in urine. The urinary total protein level was considerably increased after week 7 in Cd-exposed male rats (Figure 19(e)). Furthermore, spontaneous proteinuria was observed in control male rats at as early as week 2.

4. Discussion

4.1. Toxicological significance of serum and urine metal lothionein

The present study clearly demonstrates metabolic fate and behavior of Cd and MT in Cd-exposed male and female rats. This was made possible by determining MT with an RIA in tissues and body fluids of rats that are treated under a specially designed dosing schedule. As shown in Figures 2, 3, 11 and 12 Cd was accumulated in the liver and the kidney during Cd exposure. However, after the cessation of Cd treatment, hepatic Cd level was decreased whereas renal Cd level was continuously increased (Figures 2(a), 3(a),11(a) and 12(a)). Metallothionein levels in these organs behave in a fashion similar to Cd levels in the respective organs. Furthermore, serum MT was found to be closely associated with the serum Cd (Figures 6 and 13), as has been suggested by Nordberg and coworkers (1971). Earlier studies showed that a pattern of time course of serum MT resembles those of hepatic MT and renal MT. For instance, when rats were continuously administered with Cd for 10 to 26 weeks in those studies, serum MT as well as the hepatic and renal MT levels reached a plateau and did not decrease (Tohyama and Shaikh, 1981; Dudley et al.,1985; Sugihira et al., 1986). In contrast, the present study shows that time course of serum MT level is very similar to that of hepatic rather than renal MT level (Figures 2(a), 3(a) and 6), strongly suggesting that serum MT comes from the liver. This supposition is further supported by the concomitant increased levels of serum enzymes such as ALT, AST and ALP that are known to be

released from the liver at the onset of hepatic injury (Figures 9 and 18). It is thus conceivable that the increased level of serum MT reflects not only the extent of Cd exposure but also the presence of hepatic damage.

The renal handling of MT is not completely understood. As to the significance of MT excretion in urine, it has been considered that the increased MT excretion in urine reflects the extent of Cd exposure until renal dysfunction occurs (Tohyama et al., 1981a). This supposition was made because in this earlier study the urinary MT level in Cd-exposed rats appears to be proportional to liver and kidney Cd levels that are considered to reflect Cd body burden. In control male and female rats of the present study almost entire amount of MT filtered through glomerulus into tubular lumen appeared to be reabsorbed (Tables 2 and 4). In contrast, increased excretion of MT in urine of Cd-exposed male and female rats could be partly explained by overflow theory in that filtered amount of MT exceeds the maximum capacity of reabsorption. In addition, as a toxic sign of Cd the reabsorption is depressed in rabbits whose renal Cd concentration exceeds 200 μg/g tissue (Foulkes, 1978). Furthermore, the present study provides some evidence that at least portion of urinary MT may come from damaged tubular epithelial cells. This idea is supported by the fact that the relative clearance of MT to creatinine clearance exceeded 100% and that urinary excretion of LDH, NAG and ALP increased (Tables 2 and 4, Figures 10 and 19). The possibility that MT is secreted through tubular cells to lumen is very limited (Foulkes, 1978). Taken together, the in-

creased level of urinary MT in the Cd-exposed rats during the Cd loading period indicates not only the extent of Cd exposure but also the occurrence of renal damage.

Selenke and Foulkes (1981) found specific binding sites for Cd-MT in brush border membrane in the kidney of rabbits and a decreased ability in the brush border membrane isolated from rabbits chronically exposed to Cd. Foulkes (1982) stated, based on studies on saturation of the binding sites in vitro and in vivo, that "Cd-MT excreted in urine of Cd-poisoned rabbits, and perhaps other animals, would presumably originate in damaged renal parenchyma, rather than resulting from tubular rejection following filtration". The present study not only support his speculation but showed that upon the saturation of the specific binding sites with Cd-MT "tubular rejection following filtration" may occur.

4.2. Reappraisal of critical concentration of Cd for renal toxicity

It has been believed that renal dysfunction occurs at an approximate Cd concentration of 200 μg/g tissue in experimental animals and man (Friberg et al., 1974). In the present study no severe renal damage was observed in Cd exposed female rats. Rather, the extent of renal damage as well as hepatic injury appeared to attenuate by the end of observation period. The present result is the first to show possible recovery of renal damage in rats whose renal Cd concentration is similar to the proposed critical concentration. However, in Cd-exposed male rats no such apparent recovery was observed althogh the difference be-

tween male and female rats cannot explained in the present study.

It should be pointed out that urinary excretion of other enzyme markers such as LDH, NAG and ALP was remarkably increased by week 6 in Cd-exposed female rats (Figure 10) and that this may be due to the earlier sign of renal damage before the renal Cd level reached a maximum level. Since so-called critical concentration defined by the Subcommittee on the Toxicity of Metals of the Permanent Commission and International Association of Occupational Health (1976) includes both reversible and irreversible adverse functional changes, the critical concentration value could be set at lower than 150 μg/g tissue based on these elevated excretion of LDH, NAG and ALP. This observation is consistent with the supposition of Nomiyama (1974) that in Cd-exposed rabbits urinary enzymes are earlier indices of Cd toxicity than total protein and that critical concentration may differ among parameters.

4.3. Involvement of Cd-metallothionein in the manifestation of renal toxicity

When Cd-MT is experimentally infused into the circulation, the Cd-MT is selectively taken up by the kidney and exerts renal toxicity characterized by proteinuria, glucosuria and increased excretion of RNAse as well as degeneration of tubule cells (Tanaka et al., 1975; Nordberg 1975; Cherian and Shaikh, 1975; Suzuki et al., 1979a; Squibb and Fowler, 1984). As to underlying mechanisms, two hypotheses were under consideration. One is that Cd-MT

damages the proximal tubule cell membrane during pinocytosis of the MT molecule (Cherian et al., 1976). The other one is that Cd ions released from the protein moiety (thionein) due to degradation of Cd-MT in proximal tubule cells give rise to damages in the kidney cells (Webb and Etienne, 1977; Squibb et al., 1982; Cain and Holt, 1983).

Several lines of study favor the second hypothesis. Administration of Zn-MT itself is not harmful to rats (Webb and Etienne, 1977). Furthermore, pretreatment of Zn-MT protects rats against lethal dose of Cd-MT (Webb and Etienne, 1977) and pretreatment of Zn also prevents rats from developing renal functional changes (Squibb and Fowler, 1984). In contrast, Cd-MT administration is highly toxic and Cd ions are found in high-molecular weight fractions from the kidney of rats administered with Cd-MT before newly-synthesized MT appears (Squibb and Fowler, 1984).

For elucidation of the Cd toxicity in the liver and the kidney, it may be appropriate to use data from Cd-exposed female rats as a typical example and to divide the entire observation period into two phases which correspond to the Cd loading period (first 6 weeks) and the rest. During the first phase, both hepatic damage and renal injury were observed. The former was characterized by increased levels of ALT, AST and ALP (Figure 9) and the latter by significant increase in urinary excretion of LDH, NAG, ALP and total protein as well as apparent elevated excretion of glucose (Figure 10). It should be emphasized that the increase in serum AST level was observed as early as week 2 and that the elevation in urinary LDH, NAG and total

protein levels was statistically significant at week 4. Although detection of tissue damage depends on the assay sensitivity of a given substance, it seems that the present result seems to be consistent with the study of Dudley et al. (1985), who reported that hepatic injury precedes renal dysfunction and that Cd liberated from the liver is responsible for the renal damage. This idea is substantiated by the facts that the elevation of Cd and MT in the circulation was observed as early as week 1 (Figures 5(a) and 6) and that Cd-MT experimentally infused into the circulation is known to selectively accumulate in the kidney (Cherian and Shaikh, 1975; Tanaka et al., 1975).

An interesting observation may reside in the second phase of the observation period. After the cessation of Cd loading at week 6, levels of urinary substances such as LDH, NAG, ALP, Cd, Cu and MT appeared to decrease until week 7 and to increase again until week 12, followed by a gradual decrease (Figures 7, 8 and 10). On the other hand, urinary glucose and total protein levels were found to decrease after the cessation of Cd loading (Figure 10). It must be stressed that even after the cessation of Cd loading renal Cd level (Figure 3) remained to be similar to the critical organ concentration of Cd for renal toxicity proposed by Friberg and associate (1974). In earlier studies (Suzuki, 1980; Dudley et al., 1985; Sugihira, 1986) it was shown that proteinuria, for example, approximately 30 mg protein /day, persists as long as Cd loading continues and the kidney harbors the critical organ concentration of Cd. Thus, the present study may provide new suggestive evidence on the

recovery from renal damage in Cd-exposed female rats. It may be important to point out that proteinuria was observed throughout the observation period in Cd-exposed male rats that were treated with $CdCl_2$ by the same protocol as the present study, suggesting the possibility that there may be some difference in vulnerability between both sexes of rats. Furthermore, the second rise, at week 12, of the several urinary substances that are more sensitive to Cd exposure than glucose and total protein may reflect the presence of rebound of renal damage to a certain extent. Although data from Cd-exposed male rats were not as conspicuous as those from Cd-exposed female rats, an observation similar to the second rise was also obtained in the study using the male rats (Figures 16, 17 and 19), suggesting that this phenomenon could be reproducible under given experimental conditions. Taken together, it is reasonable to assume that when the influx of Cd ions or those from Cd-MT surpasses the detoxification system for Cd toxicity (Squibb et al., 1982) or the capacity of MT synthesis in the kidney, renal damage may be detected by parameters that are sensitive to Cd exposure. In order to clarify this hypothesis the relationship of maximum capacity of MT synthesis with Cd toxicity remains to be studied.

In the experimental studies where parental administration of Cd and/or relatively high dosage of Cd are used, hepatic damage has been observed (Axelsson and Piscator, 1966; Stowe et al., 1972; Faeder et al., 1977; Dudley et al., 1985), which is not the case in human populations at risk in the general and work environments. Although the

present study has limited relevance to Cd toxicity in man, it provides an elaborate model that may be useful in elucidating the mechanism of Cd toxicity in the liver and the kidney.

4.4. Difference in Cd metabolism between male and female rats

As has been described in the result section, most of effects of Cd exposure were commonly observed in both male and female rats. No marked sex difference in accumulation and elimination of Cd in tissues and body fluids was observed.

Two distinct differences by sex observed in the present study were serum cholinesterase activity and urinary protein excretion. In the present study serum cholinesterase level in Cd-exposed male rats was similar or higher than that in control male rats. This observation is not only different from data on serum cholinesterase activity in female rats in the present study but also inconsistent with the study of Suzuki and coworkers (Uehara et al.,1985), who revealed that serum cholinesterase activity is depressed by a subcutaneous injection of $CdCl_2$ in male and female rats. Raab (1969) reported that serum cholinesterase activity is rather increased in rats afflicted with nephrosis. Since as described below proteinuria was more evident in Cd-exposed male rats than female rats, there may be some association of the kidney damage with the elevation of serum cholinesterase activity in the Cd-exposed male rats.

Excretion of total protein in urine differs markedly between male and female rats. In Cd-exposed male rats the

urinary protein level was significantly elevated after the cessation of Cd exposure, suggesting that male rats are more susceptible than female rats in terms of renal damage. Relatively high urinary protein level observed even in control male rats may be due to spontaneous proteinuria, the occurrence of which is occasionally documented in male rats.

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Supplement

Tables and Figures on control and Cd-exposed female and male rats

(4 Tables and 19 Figures)

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Table 1 Body, liver and kidney weights and percentage of the organ weight to body weight in control and Cd-exposed female rats[a]

Group	Week	Body weight (g) (A)	Liver weight (g) (B)	(B)/(A) x100 (%)	Kidney weight (C)	(C)/(A) x100 (%)
Control	1	139±1.1	4.84±0.13	3.48±0.08	1.16±0.02	0.83±0.013
Cd-exposed		141±2.3	5.93±0.15[d]	4.21±0.11[d]	1.20±0.04	0.85±0.018
Control	2	158±2.4	5.02±0.08	3.17±0.04	1.25±0.03	0.79±0.009
Cd-exposed		148±6.4	6.07±0.30[b]	4.09±0.07[d]	1.23±0.03	0.83±0.018
Control	4	184±3.3	5.15±0.15	2.80±0.09	1.25±0.05	0.68±0.018
Cd-exposed		165±2.9[c]	6.14±0.11[d]	3.72±0.06[d]	1.47±0.05[b]	0.89±0.018[d]
Control	5	182±4.4	4.96±0.11	2.72±0.06	1.22±0.04	0.67±0.013
Cd-exposed		171±3.0	7.06±0.14[d]	4.14±0.04[d]	1.44±0.02[c]	0.84±0.022[d]
Control	6	195±3.3	5.38±0.13	2.77±0.04	1.33±0.03	0.68±0.013
Cd-exposed		173±4.5[c]	7.70±0.11[d]	4.46±0.08[d]	1.58±0.02[d]	0.91±0.018[d]
Control	7	203±4.8	5.46±0.13	2.70±0.04	1.31±0.06	0.65±0.018
Cd-exposed		175±6.9[b]	7.60±0.20[d]	4.36±0.12[d]	1.58±0.09[b]	0.90±0.040[c]
Control	9	197±3.3	5.25±0.14	2.67±0.04	1.24±0.02	0.63±0.004
Cd-exposed		183±5.4	7.12±0.25[d]	3.90±0.09[d]	1.69±0.06[d]	0.93±0.040[d]
Control	10	207±1.5	5.23±0.19	2.52±0.08	1.29±0.04	0.62±0.013
Cd-exposed		177±4.2[d]	6.80±0.19[d]	3.85±0.08[d]	1.68±0.05[d]	0.95±0.022[d]
Control	12	209±4.7	5.08±0.17	2.43±0.04	1.27±0.05	0.61±0.009
Cd-exposed		195±3.0[b]	6.89±0.18[d]	3.53±0.05[d]	1.84±0.05[d]	0.95±0.036[d]
Control	14	225±6.2	5.61±0.07	2.50±0.08	1.36±0.02	0.61±0.018
Cd-exposed		191±1.3[b]	6.87±0.08[d]	3.59±0.06[d]	1.84±0.06[c]	0.96±0.031[d]
Control	16	235±4.2	5.94±0.11	2.53±0.03	1.43±0.03	0.61±0.013
Cd-exposed		185±4.6[d]	6.04±0.21	3.27±0.07[d]	1.70±0.06[c]	0.92±0.018[d]
Control	18	228±2.3	5.71±0.11	2.51±0.06	1.37±0.02	0.60±0.013
Cd-exposed		204±9.6	6.64±0.43	3.26±0.10[d]	1.89±0.07[c]	0.93±0.022[d]
Control	21	234±3.1	5.87±0.18	2.51±0.08	1.45±0.03	0.62±0.013
Cd-exposed		202±3.2[d]	6.06±0.09	3.00±0.05[d]	1.70±0.03[d]	0.84±0.013[d]

a. Values are expressed as arithmetic mean ± SE for 5 rats.
b. Significantly different from matched control ($P < 0.05$).
c. Significantly different from matched control ($P < 0.01$).
d. Significantly different from matched control ($P < 0.001$).

Table 2 **Relative clearance of metallothionein to creatinine clearance in Cd-exposed female rats**

Week	Control (% C_{cr})	Cd-exposed (% C_{cr})
1	0.0 ± 0.0 (4)[a]	68.1 ± 26.9
2	12.4 ± 4.7	56.9 ± 13.0[b]
4	3.4 ± 2.1	174.0 ± 38.0[b]
5	3.4 ± 1.4	158.0 ± 25.9[c]
6	2.7 ± 1.6 (3)	440.7 ± 116.8[b]
7	7.2 ± 4.6	81.0 ± 12.1[c]
9	2.2 ± 0.9	222.0 ± 57.0[b]
10	2.3 ± 1.0	382.0 ± 36.0[d]
12	4.4 (1)	472.4 ± 109.0
14	4.4 ± 0.8	219.9 ± 47.6[b]
16	3.8 ± 0.5	139.8 ± 12.0[d]
18	2.8 ± 1.2	111.6 ± 29.1[b]
21	7.8 ± 1.4	77.3 ± 18.2[b]

a. The number in parentheses denotes the number of rats used for calculation of the relative clearance of metallothionein to creatinine clearance. Unless specified, data from 5 rats were used.
b. Statistically significant at $P < 0.05$.
c. Statistically significant at $P < 0.01$.
d. Statistically significant at $P < 0.001$.

Table 3 **Body, liver and kidney weights and percentage of the organ weight to body weight in control and Cd-exposed male rats[a]**

Group	Week	Body weight (g) (A)	Liver weight (g) (B)	(B)/(A) x100 (%)	Kidney Weight (g) (C)	(C)/(A) x100 (%)
Control	1	212± 6.1	7.08±0.23	3.34±0.08	1.66±0.05	0.79±0.022
Cd-exposed		199± 3.0	7.82±0.11[b]	3.94±0.06[c]	1.57±0.02	0.79±0.004
Control	2	238± 4.1	7.89±0.18	3.32±0.07	1.79±0.06	0.75±0.013
Cd-exposed		208± 4.6[d]	8.35±0.11	4.02±0.05[d]	1.60±0.04[b]	0.77±0.009
Control	4	278± 8.7	8.13±0.26	2.93±0.02	1.90±0.06	0.68±0.004
Cd-exposed		237± 4.1[c]	8.87±0.14[b]	3.74±0.03[d]	1.89±0.05	0.80±0.018[c]
Control	5	300± 8.1	8.73±0.17	2.92±0.05	1.88±0.04	0.63±0.008
Cd-exposed		244± 7.5[d]	9.88±0.28[b]	4.05±0.06[d]	1.95±0.08	0.80±0.013[d]
Control	6	309± 5.4	8.89±0.24	2.87±0.04	1.99±0.08	0.64±0.018
Cd-exposed		244± 8.9[c]	10.86±0.26[c]	4.47±0.06[d]	1.95±0.04	0.80±0.013[d]
Control	7	311± 3.2	8.86±0.21	2.86±0.06	1.93±0.03	0.62±0.008
Cd-exposed		252±11.1[c]	11.56±0.53[c]	4.59±0.11[d]	1.98±0.08	0.78±0.009[d]
Control	9	340±11.2	9.52±0.34	2.80±0.01	2.13±0.05	0.63±0.018
Cd-exposed		277± 9.7[c]	11.74±0.43[c]	4.24±0.06[d]	2.04±0.07	0.74±0.018[c]
Control	10	333±10.5	8.91±0.37	2.67±0.03	1.97±0.06	0.59±0.008
Cd-exposed		285± 3.4[c]	12.09±0.34[d]	4.24±0.08[d]	2.16±0.12	0.76±0.040[b]
Control	12	387± 7.9	10.26±0.23	2.65±0.03	2.17±0.07	0.56±0.013
Cd-exposed		293± 9.5[d]	11.53±0.31[b]	3.94±0.06[d]	2.16±0.06	0.74±0.013[d]
Control	14	362± 7.0	9.31±0.12	2.58±0.04	1.96±0.04	0.54±0.013
Cd-exposed		302± 7.2[d]	10.88±0.37[b]	3.60±0.06[d]	2.12±0.03[b]	0.70±0.013[d]
Control	16	387± 6.4	9.81±0.31	2.54±0.06	2.12±0.05	0.55±0.008
Cd-exposed		292±10.0[d]	9.63±0.11	3.30±0.08[d]	2.18±0.06	0.75±0.022[d]
Control	18	377±12.5	9.64±0.30	2.56±0.05	2.19±0.10	0.58±0.013
Cd-exposed		306± 7.5[c]	10.32±0.20	3.37±0.07[d]	2.32±0.06	0.76±0.022[d]
Control	21	409± 6.3	10.35±0.31	2.53±0.05	2.15±0.04	0.53±0.008
Cd-exposed		344± 8.6[d]	10.79±0.26	3.14±0.02[d]	2.41±0.06[b]	0.70±0.013[d]

a. Values are expressed as arithmetic mean ± SE for 5 rats.
b. Significantly different from matched control ($P < 0.05$).
c. Significantly different from matched control ($P < 0.01$).
d. Significantly different from matched control ($P < 0.001$).

Table 4 **Relative clearance of metallothionein to creatinine clearance in Cd-exposed male rats**

Week	Control (% C_{cr})	Cd-exposed (% C_{cr})
1	4.9 (2)[a]	63.4 ± 9.0[c]
2	1.3 ± 0.8 (4)	43.7 ± 8.3[c]
4	1.8 ± 2.8 (5)	212.1 ± 38.8[c]
5	4.8 ± 2.8 (4)	292.2 ± 58.4
6	8.4 ± 1.1 (4)	348.1 ± 49.6[c]
7	4.3 ± 1.9	269.6 ± 57.7[c]
9	11.9 ± 3.5	153.5 ± 18.5[d]
10	12.8 ± 6.0	360.5 ± 92.7[b]
12	0.1 ± 0.1	462.3 ± 149.3[b]
14	2.2 ± 2.1	434.8 ± 101.8[b]
16	0.5 ± 0.4	150.2 ± 21.6[c]
18	1.3 ± 0.7	110.1 ± 16.6[c]
21	2.3 ± 0.7 (4)	58.6 ± 6.3[d]

a. The number in parentheses denotes the number of rats used for calculation of the relative clearance of metallothionein to creatinine clearance. Unless specified, data from 5 rats were used.
b. Statistically significant at $P < 0.05$.
c. Statistically significant at $P < 0.01$.
d. Statistically significant at $P < 0.001$.

Figure 1. Cross-reactivity of rat metallothionein-1 (open circle) and -2 (closed circle) with sheep antiserum against rat metallothionein-2.

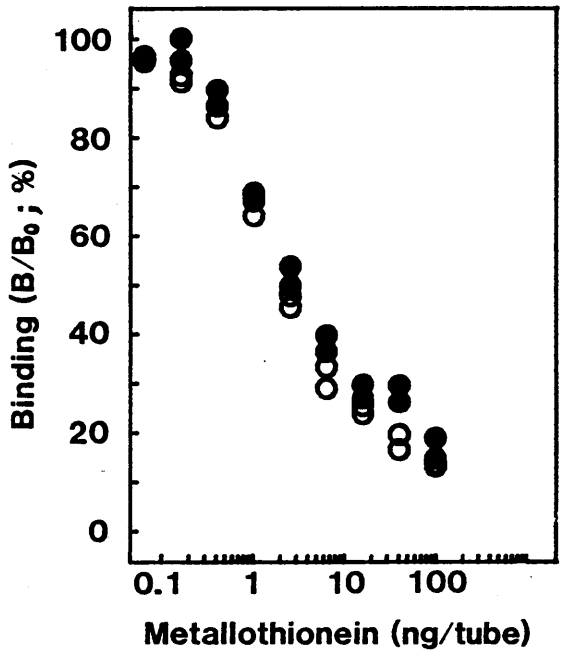

100
80
60
40
20
0
Binding (B/B0 ; %)
0.1
1
10
100
Metallothionein (ng/tube)

Figure 2. (a) Cadmium, (b) copper and (c) zinc concentration and content in the liver of control and Cd-exposed female rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open circle and square denote concentration and content in the control rats, respectively, whereas closed circle and square represent concentration and content in the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

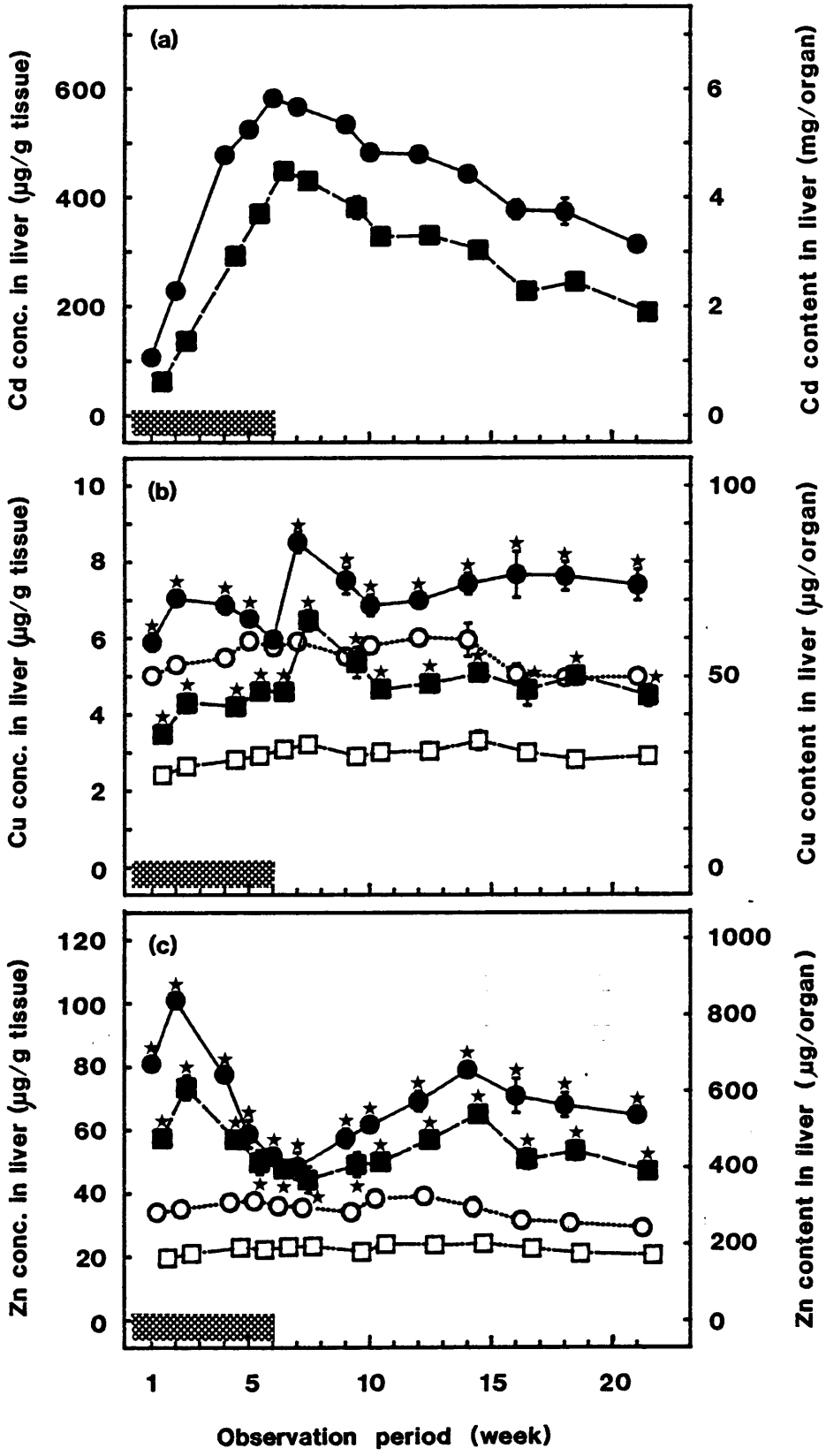

(a)
Cd conc. in liver (μg/g tissue)
600
400
200
0
6
4
2
0
Cd content in liver (mg/organ)
(b)
Cu conc. in liver (μg/g tissue)
10
8
6
4
2
0
100
50
0
Cu content in liver (μg/organ)
(c)
Zn conc. in liver (μg/g tissue)
120
100
80
60
40
20
0
1000
800
600
400
200
0
Zn content in liver (μg/organ)
1
5
10
15
20
Observation period (week)

Figure 3. (a) Cadmium, (b) copper and (c) zinc concentration and content in the kidney of control and Cd-exposed female rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open circle and square denote concentration and content in the control rats, respectively, whereas closed circle and square represent concentration and content in the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

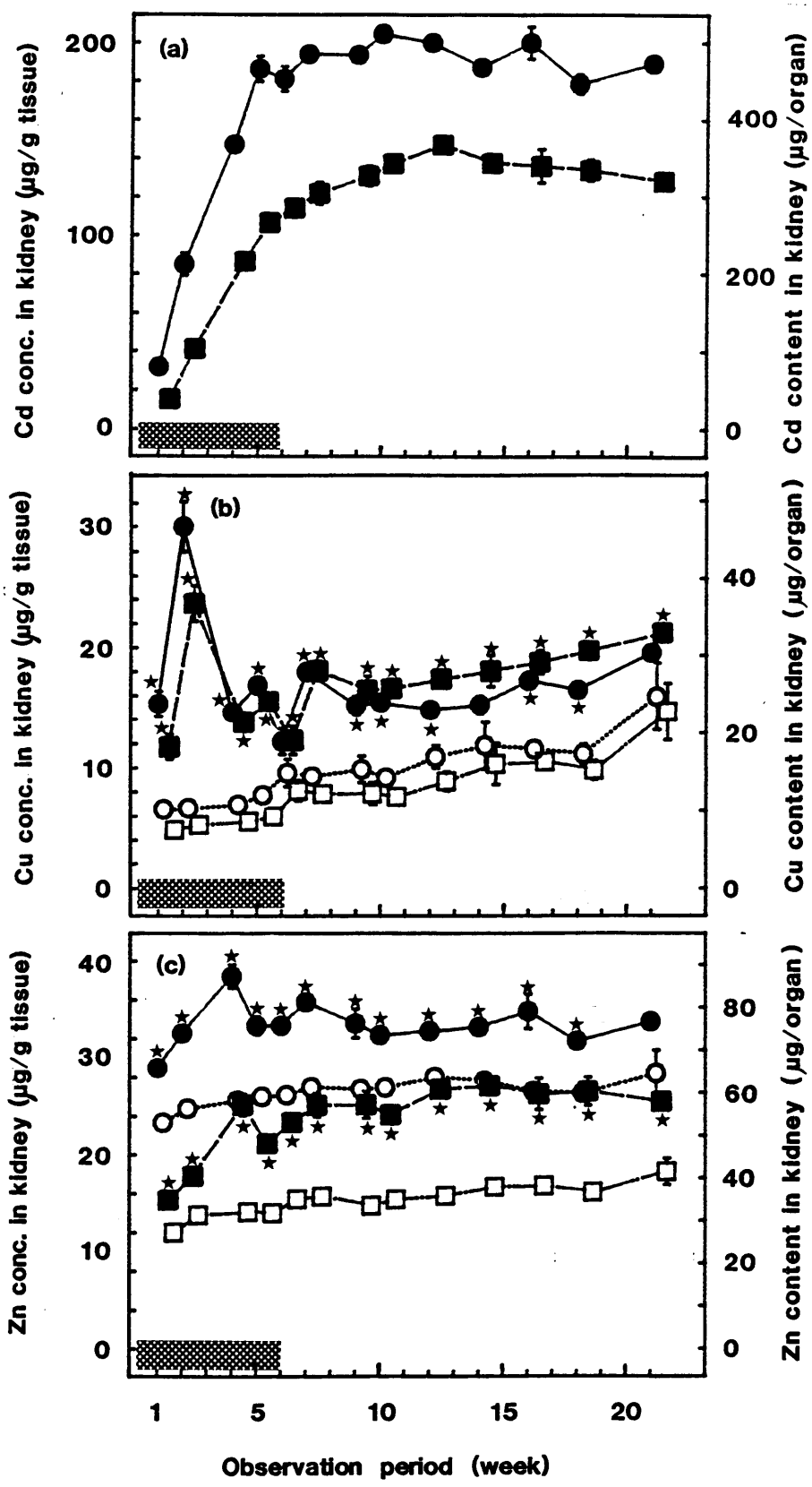

(a)
(b)
(c)
Cd conc. in kidney (μg/g tissue)
Cd content in kidney (μg/organ)
Cu conc. in kidney (μg/g tissue)
Cu content in kidney (μg/organ)
Zn conc. in kidney (μg/g tissue)
Zn content in kidney (μg/organ)
Observation period (week)

Figure 4. Metallothionein concentration and content in the liver (a) and kidney (b) of control and Cd-exposed female rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open circle and square denote concentration and content in the control rats, respectively, whereas closed circle and square represent concentration and content in the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size.

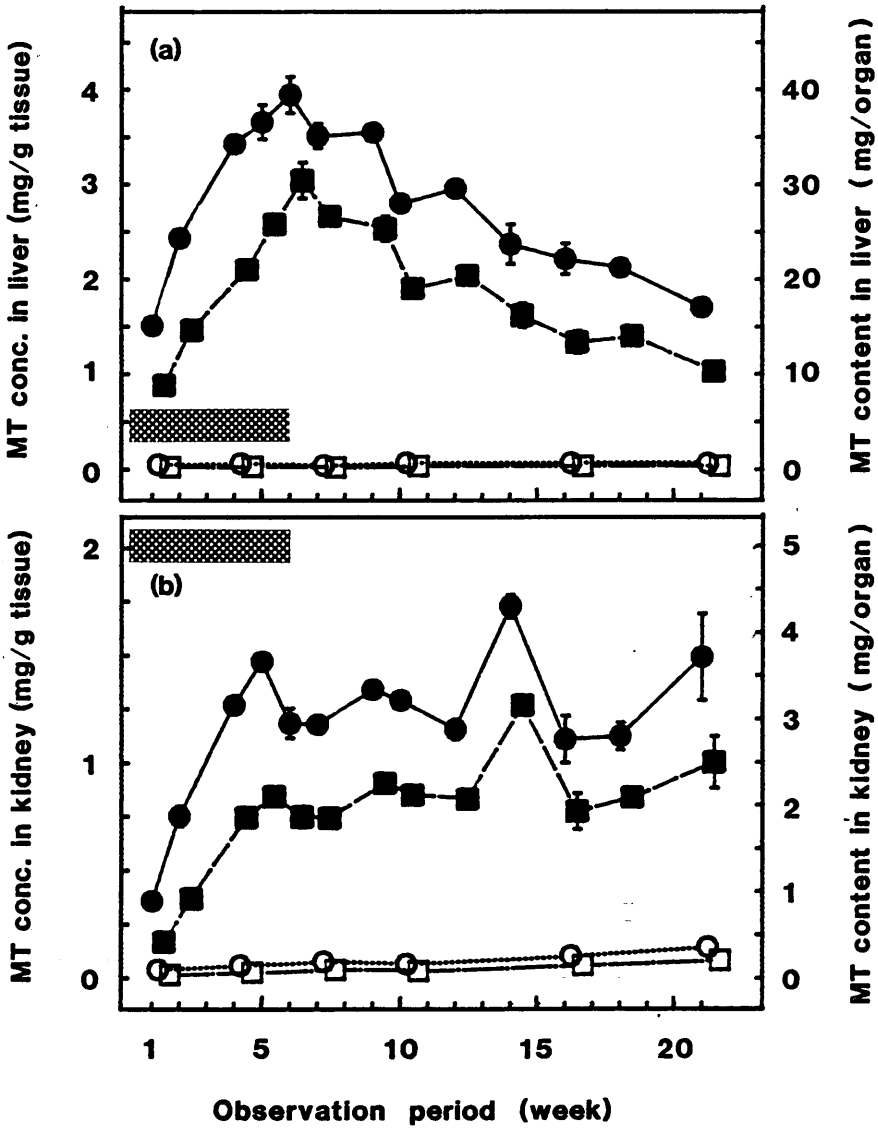

(a)
(b)
MT conc. in liver (mg/g tissue)
MT content in liver (mg/organ)
MT conc. in kidney (mg/g tissue)
MT content in kidney (mg/organ)
Observation period (week)
0
1
2
3
4
10
20
30
40
5
15

Figure 5. (a) Cadmium, (b) copper and (c) zinc concentration in the serum of control and Cd-exposed female rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open and closed circles denote concentrations in the control and the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

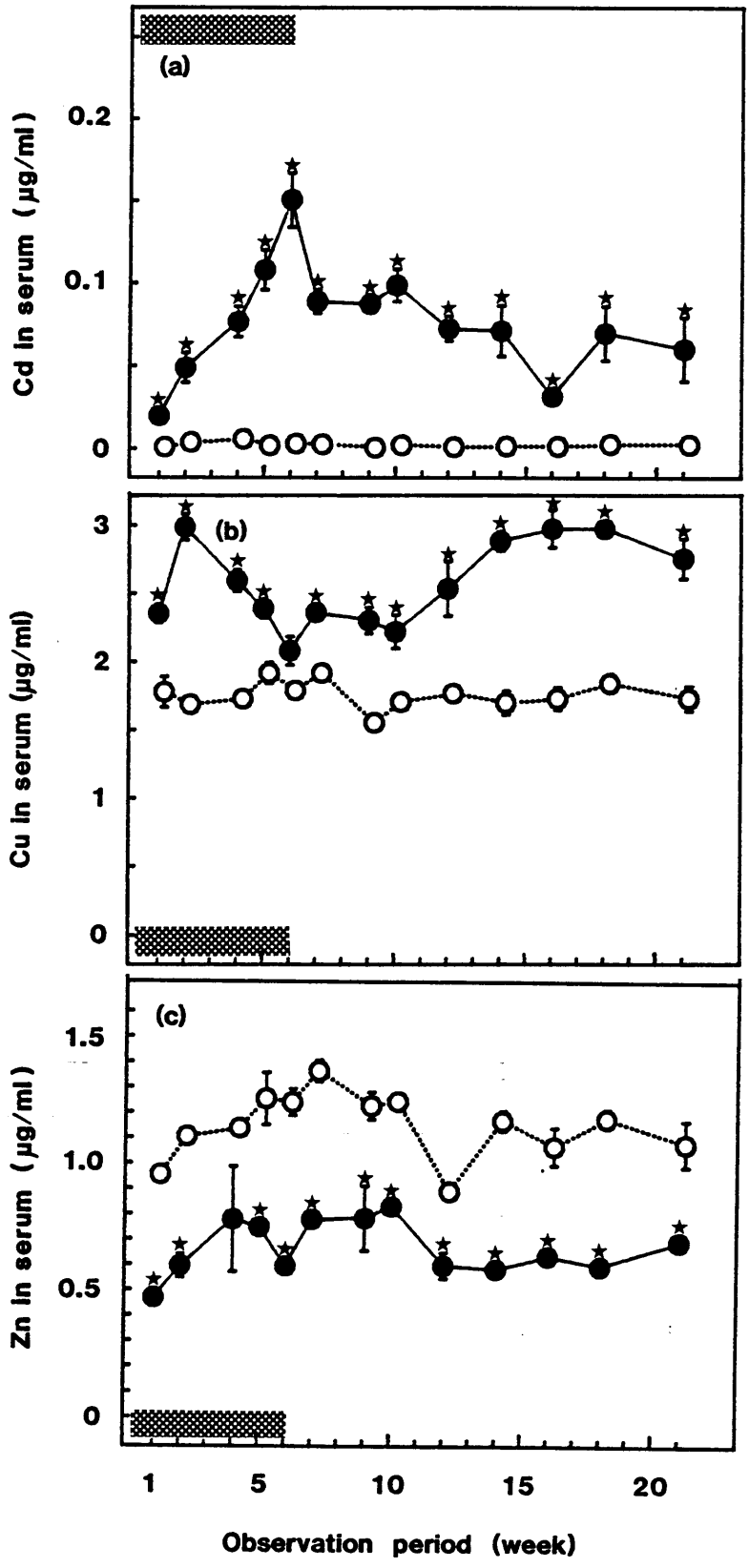

(a)
Cd in serum (μg/ml)
0.2
0.1
0
(b)
Cu in serum (μg/ml)
3
2
1
0
(c)
Zn in serum (μg/ml)
1.5
1.0
0.5
0
1
5
10
15
20
Observation period (week)

Figure 6. Metallothionein concentration in the serum of control and Cd-exposed female rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open and closed circles denote concentrations in the control and the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

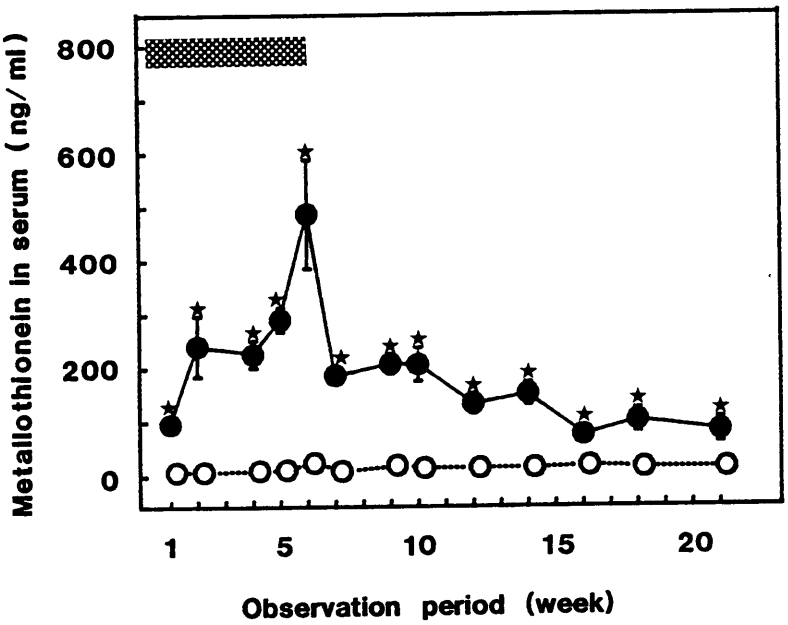

Metallothionein in serum (ng/ ml)
800
600
400
200
0
1
5
10
15
20
Observation period (week)

Figure 7. (a) Cadmium, (b) copper and (c) zinc amounts in the urine of control and Cd-exposed female rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open and closed circles denote excreted amounts in the control and the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

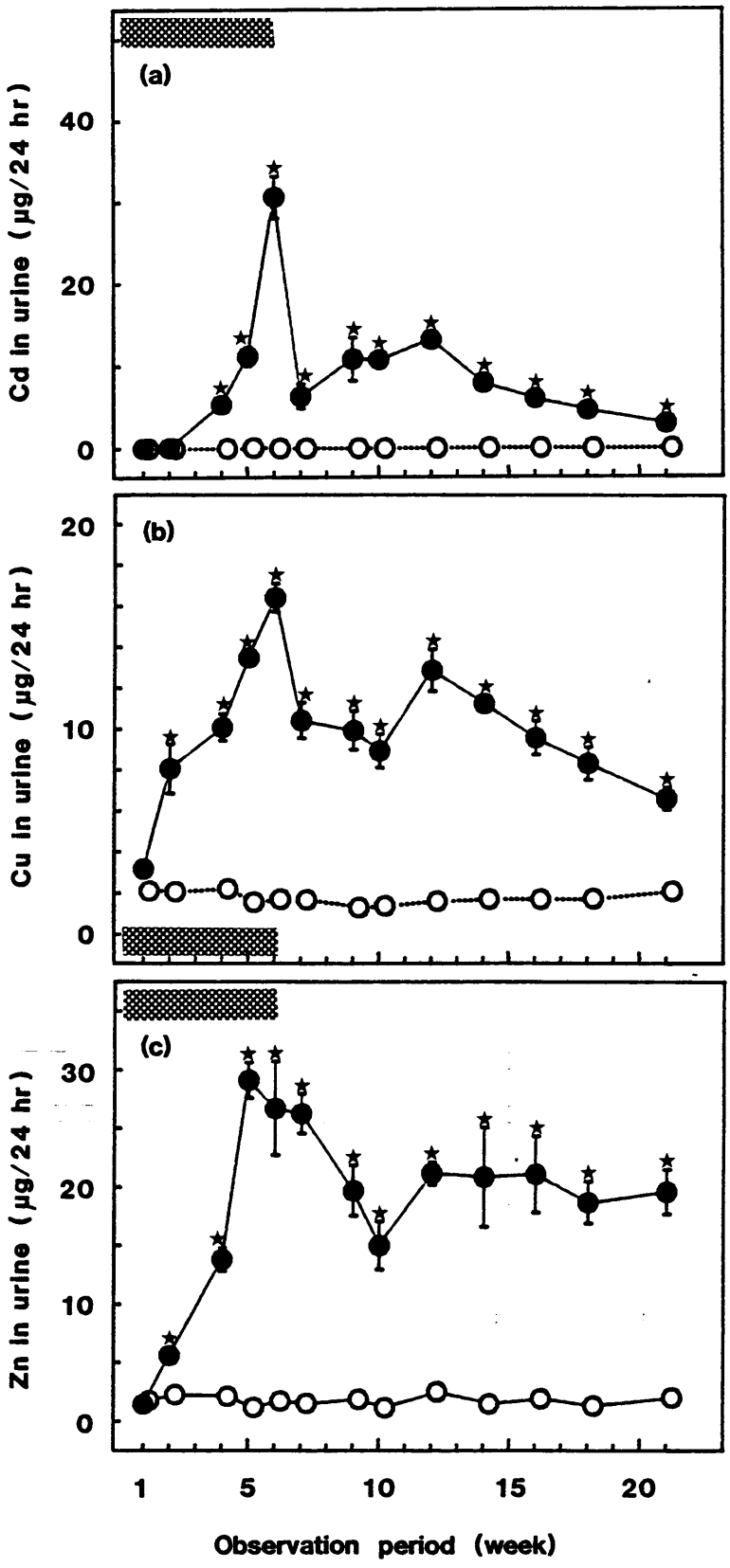

(a)
Cd in urine (μg/24 hr)
40
20
0
(b)
Cu in urine (μg/24 hr)
20
10
0
(c)
Zn in urine (μg/24 hr)
30
20
10
0
1
5
10
15
20
Observation period (week)

Figure 8. Metallothionein excretion in the urine of control and Cd-exposed female rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open and closed circles denote the excreted amounts in the control and the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

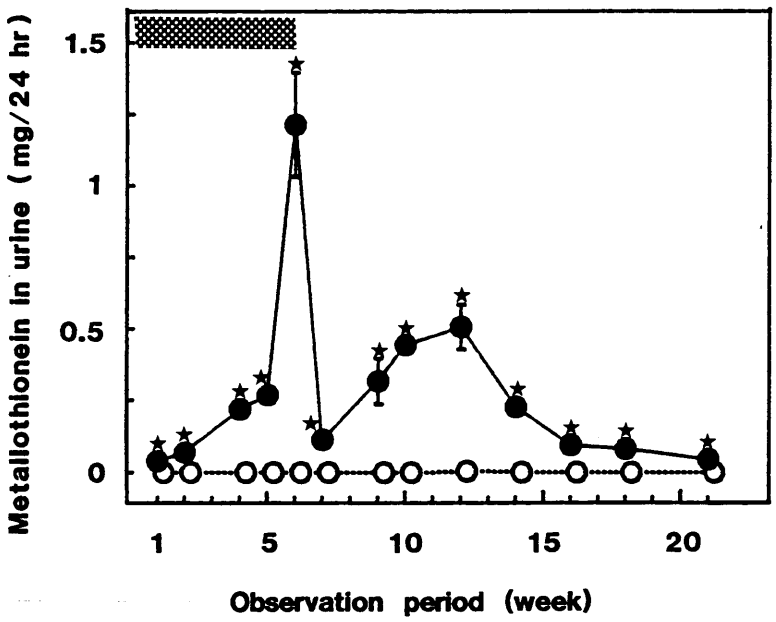

Metallothionein in urine (mg/24 hr)
1.5
1
0.5
0
1
5
10
15
20
Observation period (week)

Figure 9. Changes in activities of (a) serum alanine aminotransferase, (b) aspartate aminotransferase, (c) alkaline phosphatase and (d) cholinesterase in control and Cd-exposed female rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open and closed circles denote the activities in the control and the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

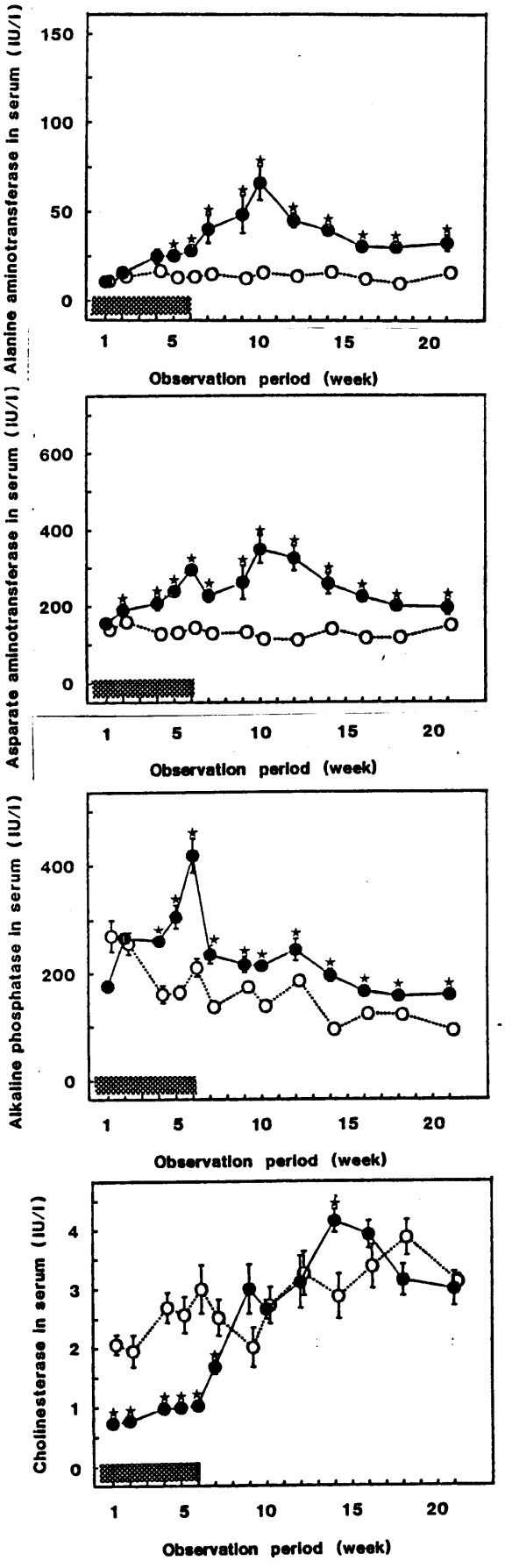

Alanine aminotransferase in serum (IU/l)
150
100
50
0
1
5
10
15
20
Observation period (week)
Asparate aminotransferase in serum (IU/l)
600
400
200
0
1
5
10
15
20
Observation period (week)
Alkaline phosphatase in serum (IU/l)
400
200
0
1
5
10
15
20
Observation period (week)
Cholinesterase in serum (IU/l)
4
3
2
1
0
1
5
10
15
20
Observation period (week)

Figure 10. Changes in the urinary excretion of (a) lactate dehydrogenase, (b) N-acetyl-β-D-glucosaminidase, (c) alkaline phosphatase, (d) total protein and (e) glucose and in (f) urine volume in control and Cd-exposed female rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open and closed circles denote the unit of a given parameter in the control and the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

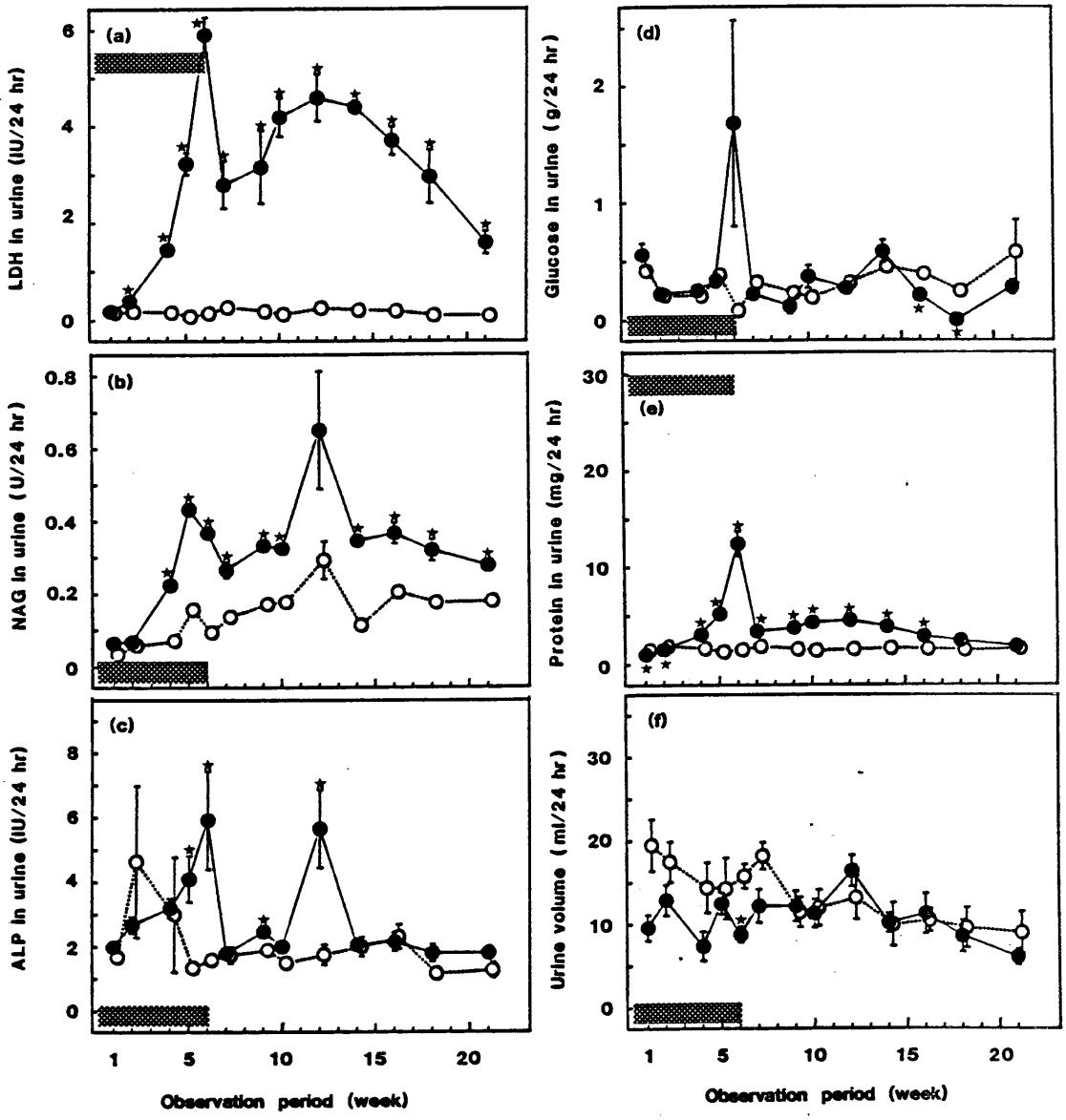

(a)
LDH in urine (IU/24 hr)
(b)
NAG in urine (U/24 hr)
(c)
ALP in urine (IU/24 hr)
(d)
Glucose in urine (g/24 hr)
(e)
Protein in urine (mg/24 hr)
(f)
Urine volume (ml/24 hr)
Observation period (week)
Observation period (week)

Figure 11. (a) Cadmium, (b) copper and (c) zinc concentration and content in the liver of control and Cd-exposed male rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open circle and square denote concentration and content in the control rats, respectively, whereas closed circle and square represent concentration and content in the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

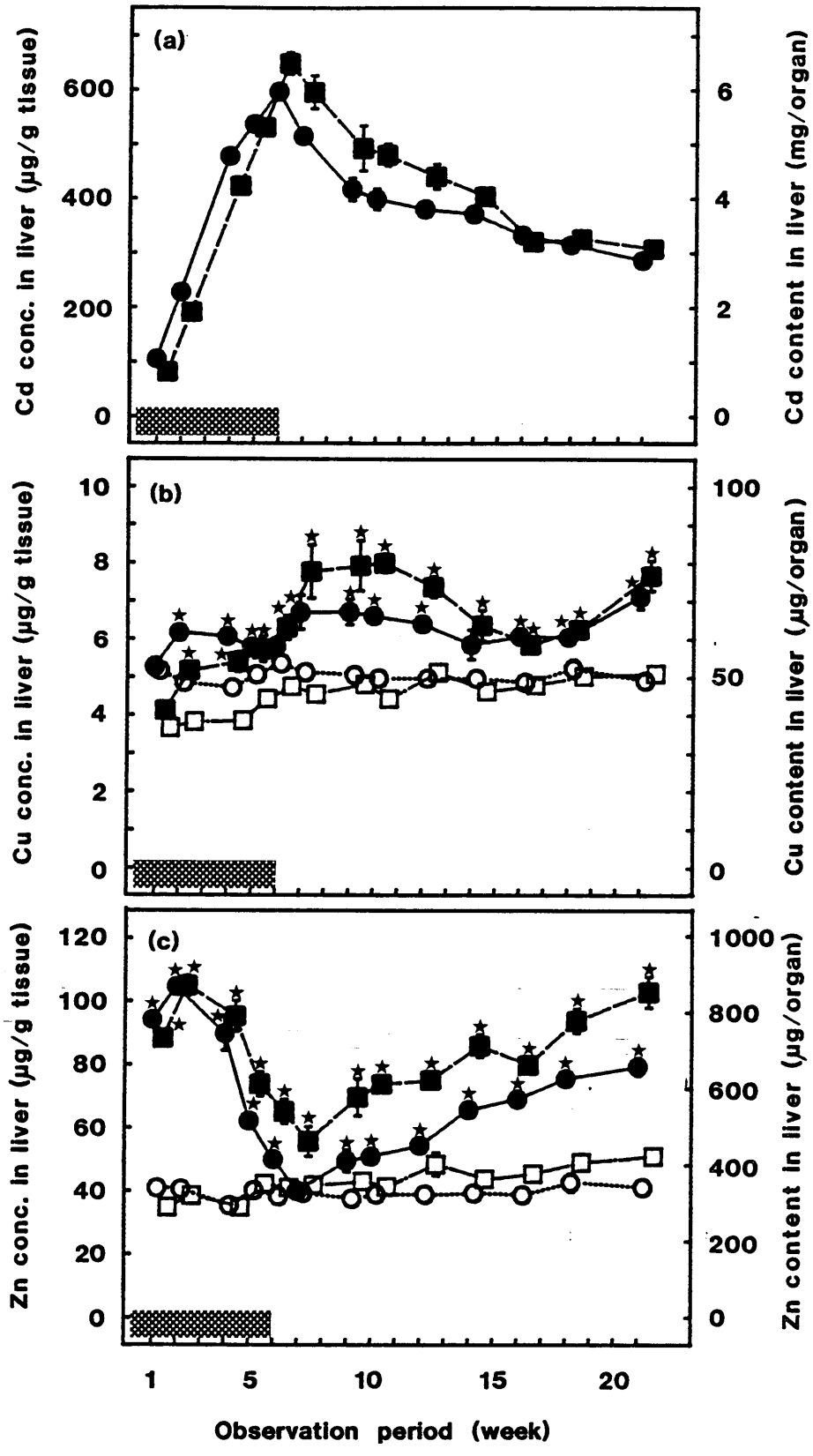

(a)
Cd conc. in liver (μg/g tissue)
Cd content in liver (mg/organ)
600
400
200
0
6
4
2
0
(b)
Cu conc. in liver (μg/g tissue)
Cu content in liver (μg/organ)
10
8
6
4
2
0
100
50
0
(c)
Zn conc. in liver (μg/g tissue)
Zn content in liver (μg/organ)
120
100
80
60
40
20
0
1000
800
600
400
200
0
1
5
10
15
20
Observation period (week)

Figure 12. (a) Cadmium, (b) copper and (c) zinc concentration and content in the kidney of control and Cd-exposed male rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open circle and square denote concentration and content in the control rats, respectively, whereas closed circle and square represent concentration and content in the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

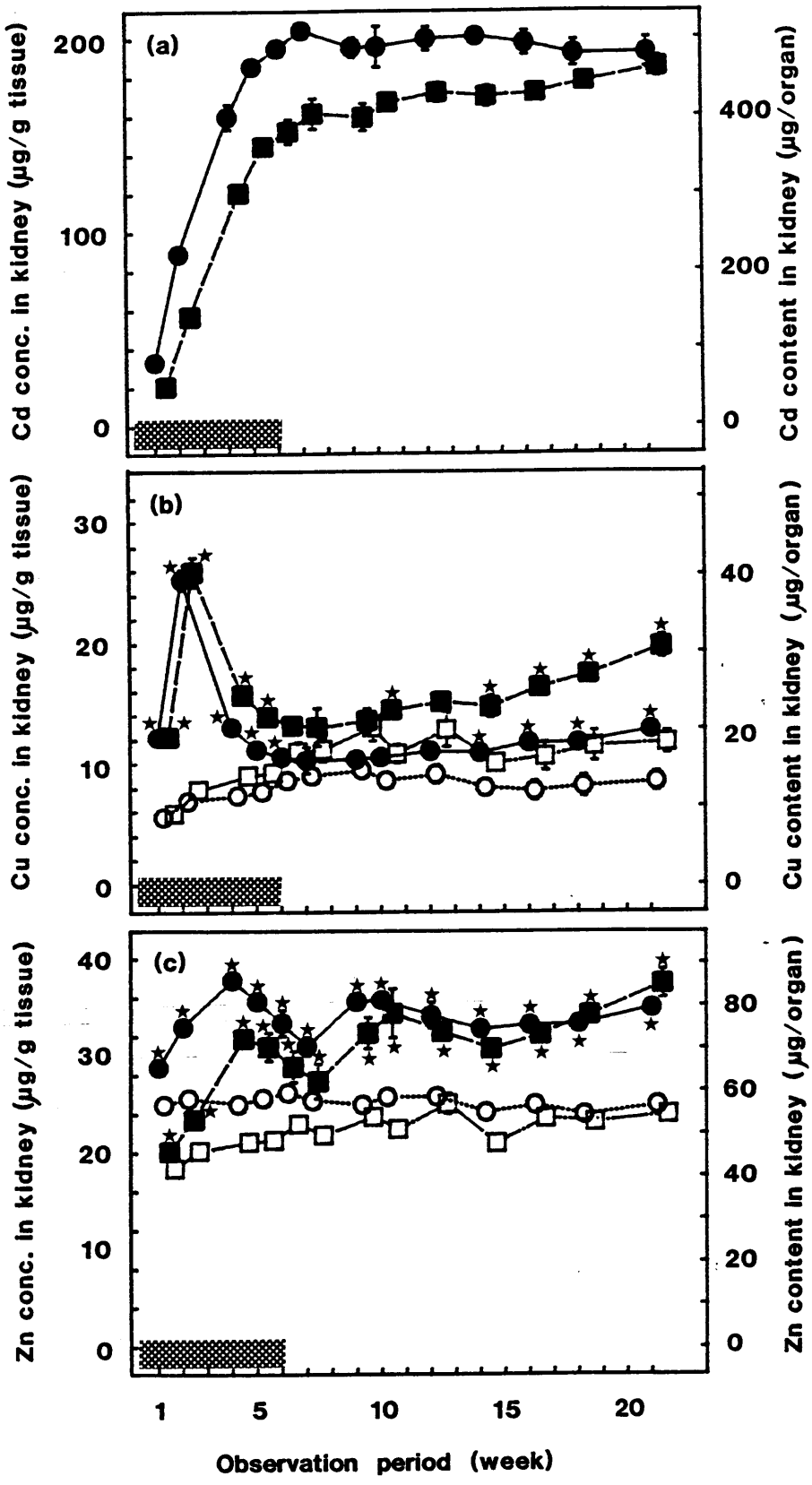

(a)
(b)
(c)
Cd conc. in kidney (μg/g tissue)
Cd content in kidney (μg/organ)
Cu conc. in kidney (μg/g tissue)
Cu content in kidney (μg/organ)
Zn conc. in kidney (μg/g tissue)
Zn content in kidney (μg/organ)
200
100
0
400
200
0
30
20
10
0
40
20
0
40
30
20
10
0
80
60
40
20
0
1
5
10
15
20
Observation period (week)

Figure 13. Metallothionein concentration and content in the liver (a) and kidney (b) of control and Cd-exposed male rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open circle and square denote concentration and content in the control rats, respectively, whereas closed circle and square represent concentration and content in the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

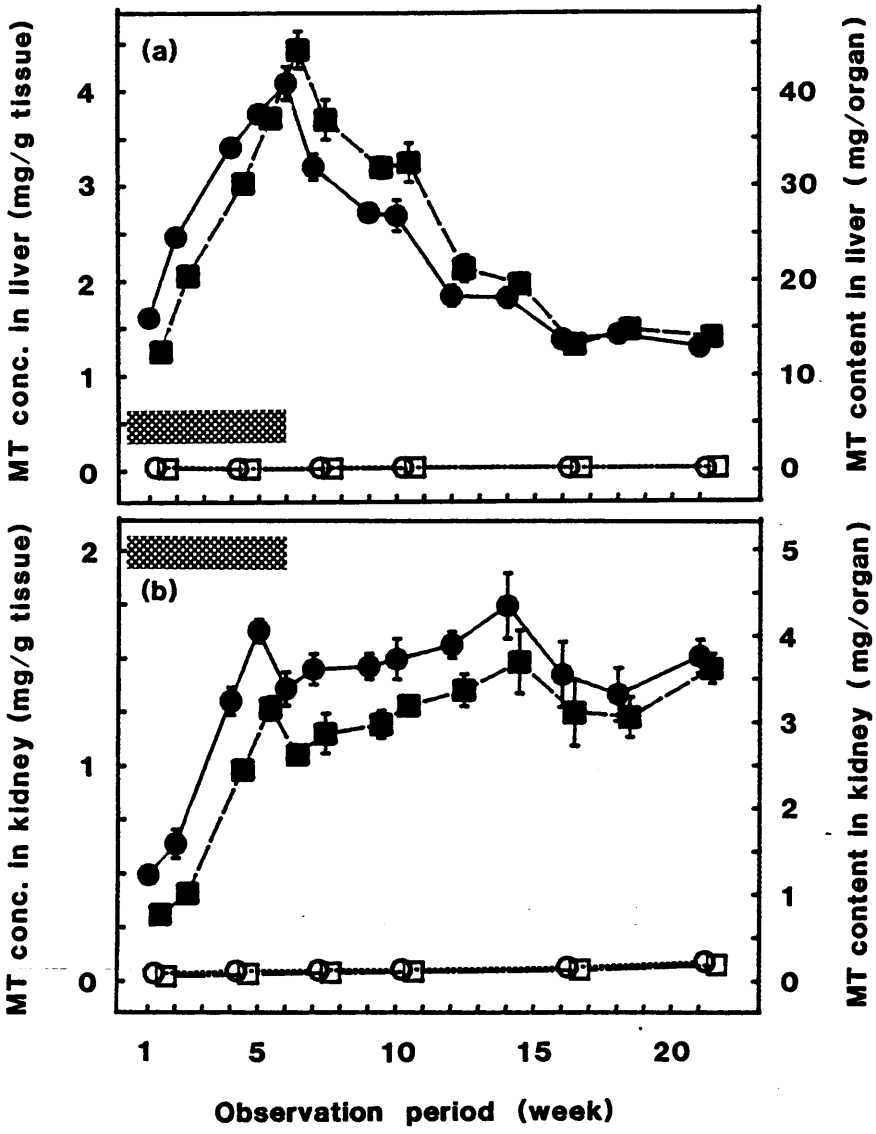

(a)
(b)
MT conc. in liver (mg/g tissue)
MT content in liver (mg/organ)
MT conc. in kidney (mg/g tissue)
MT content in kidney (mg/organ)
Observation period (week)
0
1
2
3
4
10
20
30
40
5
15

Figure 14. (a) Cadmium, (b) copper and (c) zinc concentration in the serum of control and Cd-exposed male rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open and closed circles denote concentrations in the control and the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

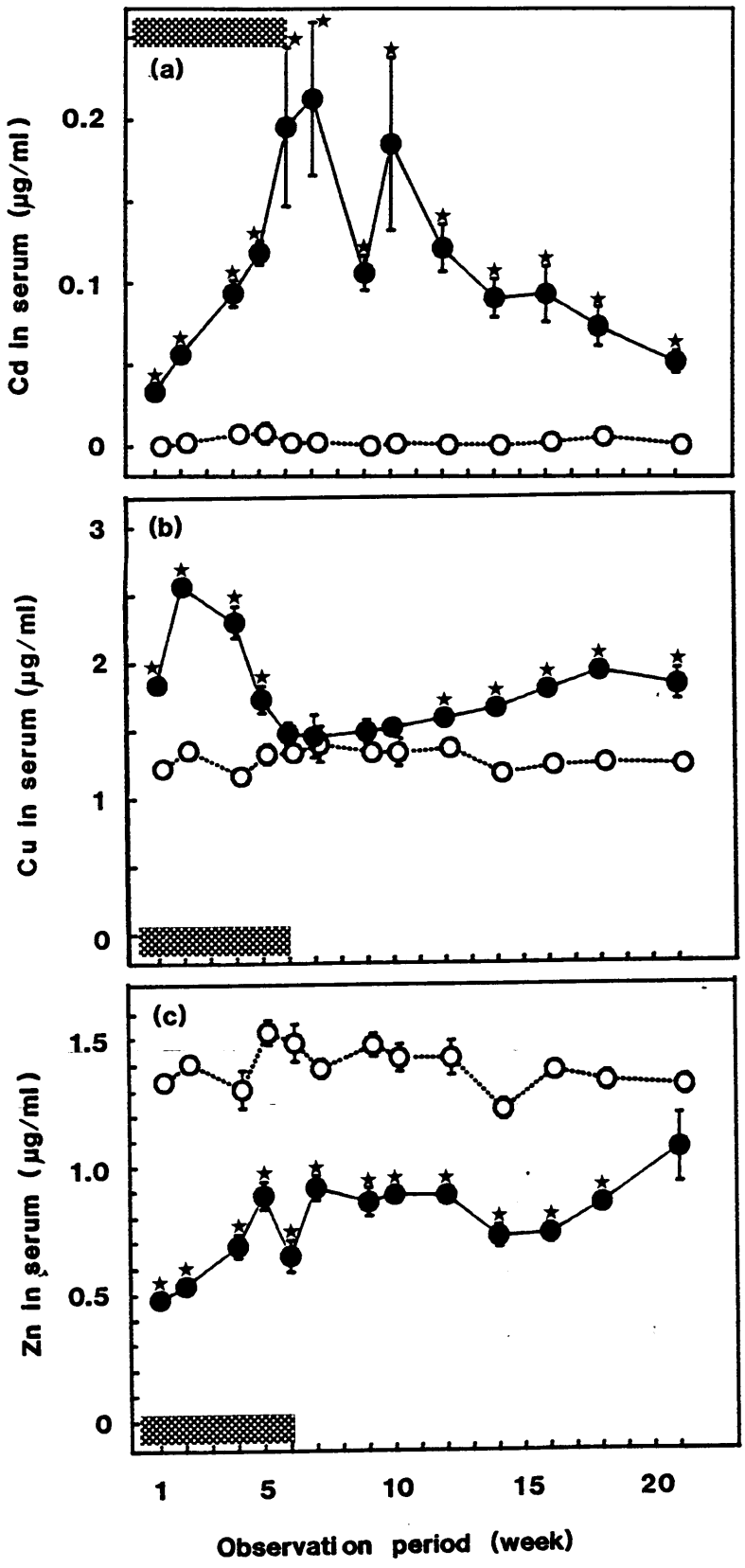

(a)
Cd in serum (μg/ml)
0.2
0.1
0
(b)
Cu in serum (μg/ml)
3
2
1
0
(c)
Zn in serum (μg/ml)
1.5
1.0
0.5
0
1
5
10
15
20
Observation period (week)

Figure 15. Metallothionein concentration in the serum of control and Cd-exposed male rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open and closed circles denote concentrations in the control and the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

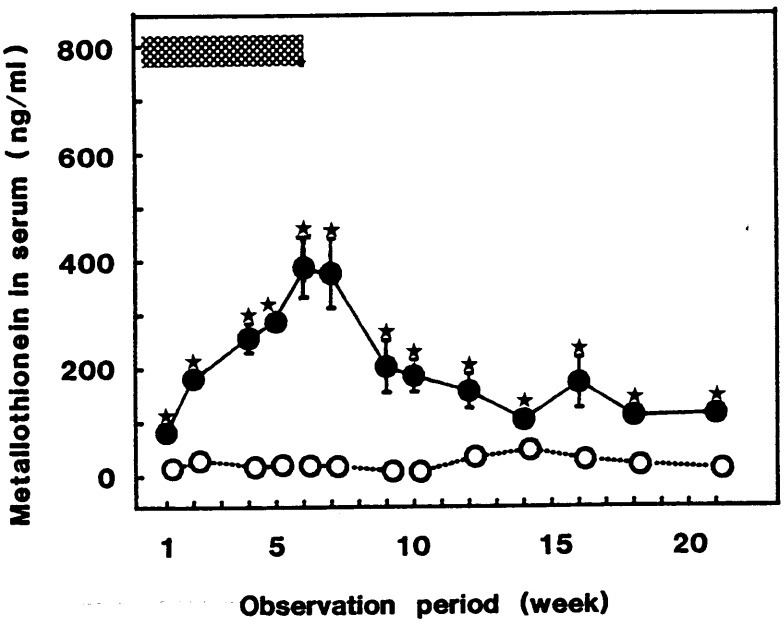

Metallothionein in serum (ng/ml)
800
600
400
200
0
1
5
10
15
20
Observation period (week)

Figure 16. (a) Cadmium, (b) copper and (c) zinc amounts in the urine of control and Cd-exposed male rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open and closed circles denote excreted amounts in the control and the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

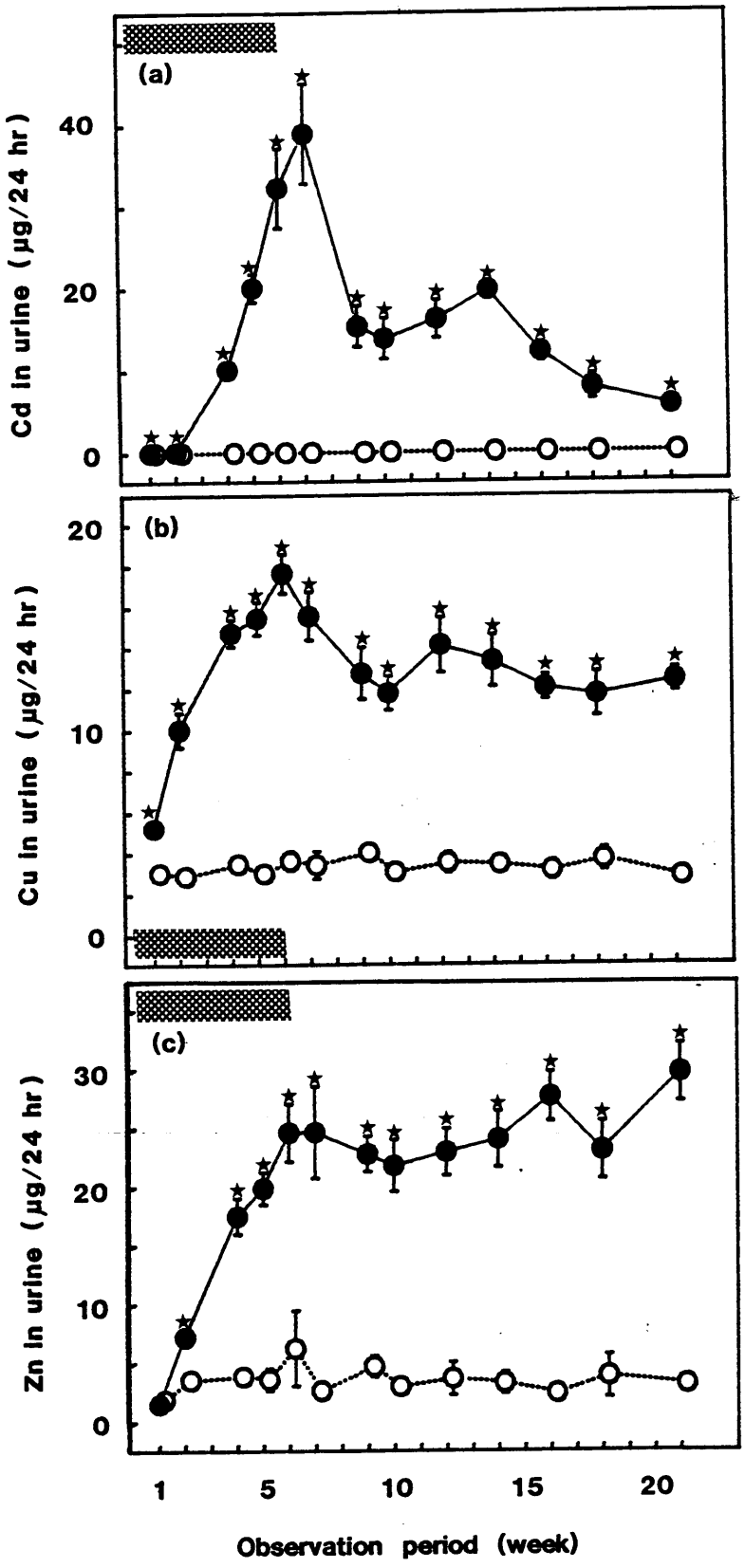

(a)
Cd in urine (μg/24 hr)
40
20
0
(b)
Cu in urine (μg/24 hr)
20
10
0
(c)
Zn in urine (μg/24 hr)
30
20
10
0
1
5
10
15
20
Observation period (week)

Figure 17. Metallothionein excretion in the urine of control and Cd-exposed male rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open and closed circles denote excreted amounts in the control and the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

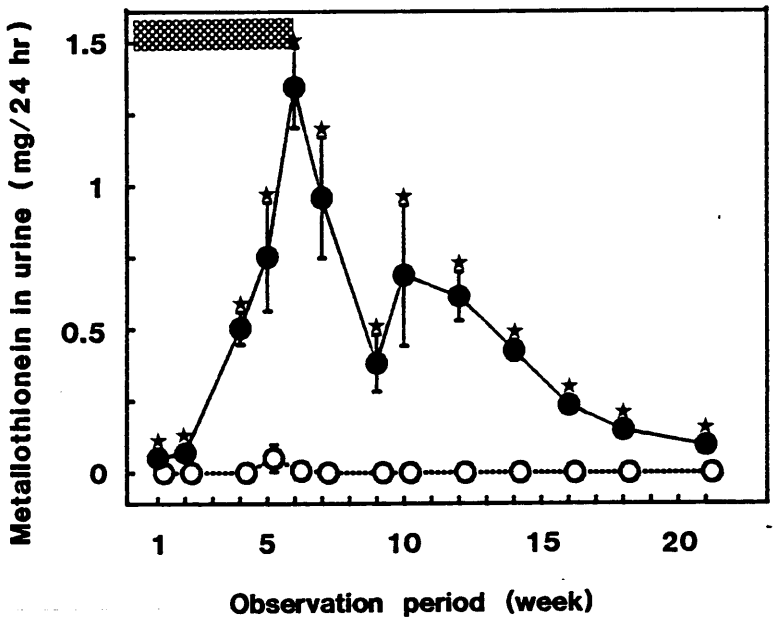

Metallothionein in urine (mg/24 hr)
1.5
1
0.5
0
1
5
10
15
20
Observation period (week)

Figure 18. Changes in activities of (a)alanine aminotransferase, (b) aspartate aminotransferase, (c) alkaline phosphatase and (d) cholinesterase in the serum of control and Cd-exposed male rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open and closed circles denote the enzyme activities in the control and the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).

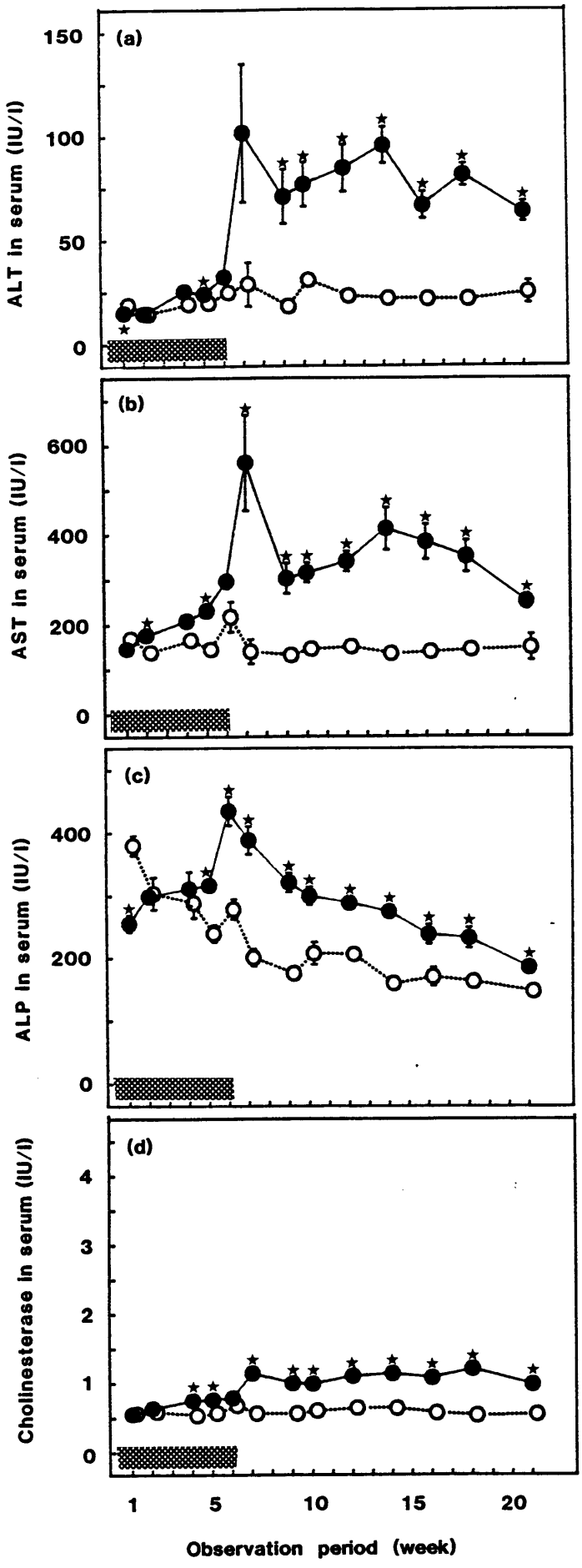
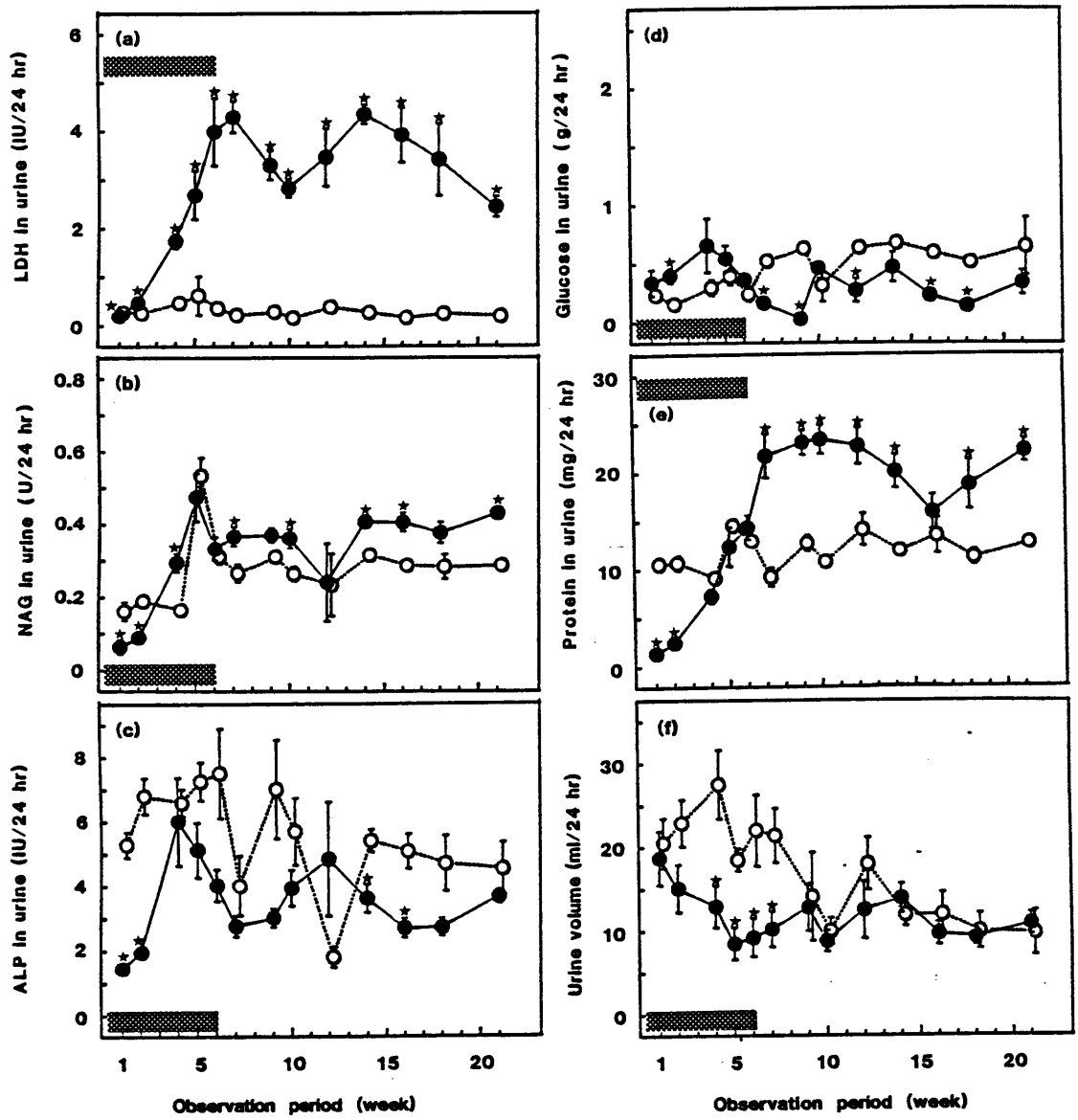

(a)
ALT in serum (IU/l)
150
100
50
0
(b)
AST in serum (IU/l)
600
400
200
0
(c)
ALP in serum (IU/l)
400
200
0
(d)
Cholinesterase in serum (IU/l)
4
3
2
1
0
1
5
10
15
20
Observation period (week)

Figure 19. Changes in the urinary excretion of (a) lactate dehydrogenase, (b) N-acetyl-β-D-glucosaminidase, (c) alkaline phosphatase, (d) total protein and (e) glucose and in (f) urine volume in control and Cd-exposed male rats. A period of Cd injection (week 0 to 6) is indicated with a shaded bar. Open and closed circles denote the unit of a given parameter in the control and the Cd-exposed rats, respectively. Values represent mean ± SE for 5 rats that were sacrificed at corresponding time points. A point without a bar means that the SE is smaller than symbol size. Asterisk indicates statistically significant difference between means of Cd-exposed and its matched control rats ($P<0.05$).


(a)
LDH in urine (IU/24 hr)
(b)
NAG in urine (U/24 hr)
(c)
ALP in urine (IU/24 hr)
(d)
Glucose in urine (g/24 hr)
(e)
Protein in urine (mg/24 hr)
(f)
Urine volume (ml/24 hr)
Observation period (week)
Observation period (week)