The Effect of Dietary Zinc Status on Biliary Metal Excretion of Rats

SARAH JAW AND ELIZABETH H. JEFFERY

Division of Nutritional Sciences and Institute for Environmental Studies, University of Illinois at Urbana-Champaign, Urbana, IL 61801

ABSTRACT The effect of dietary zinc status on biliary excretion of zinc, cadmium and mercury administered as a bolus of metal chloride (1 mg metal/kg body weight i.v.) was studied. Female rats were fed a purified diet containing either 9 μg/g (low), 45 μg/g (adequate) or 1150 μg/g (high) zinc for 8 d. Hepatic metallothionein (MT) was similar in low- and adequate-zinc groups, but was 18-fold higher in the high-zinc group than in the other two groups. Liver zinc content varied in relation to dietary zinc level. Biliary excretion of all metals studied was significantly lower in the high-zinc group than in the low-zinc group. The cumulative excretion of zinc, cadmium and mercury over 2.5 h in rats fed these two diets was 6.2 ± 1.4 vs. 33.5 ± 7.7, 0.006 ± 0.02 vs. 22.8 ± 8.4 and 1.6 ± 0.6 vs. 14.9 ± 5.3 μg/kg body weight, respectively. A relationship was found between the disposition of metal in liver and the extent of biliary metal excretion. The cumulative excretion of zinc, cadmium and mercury over 2.5 h in rats fed these two diets was 6.2 ± 1.4 vs. 33.5 ± 7.7, 0.006 ± 0.02 vs. 22.8 ± 8.4 and 1.6 ± 0.6 vs. 14.9 ± 5.3 μg/kg body weight, respectively. A relationship was found between the disposition of metal in liver and the extent of biliary metal excretion. Biliary metal excretion was highly correlated with liver cytosolic non-MT-bound metal; r = 0.999, 0.998 and 0.993 for endogenous + exogenous zinc, cadmium and mercury, respectively. J. Nutr. 118: 1385-1390, 1988.

INDEXING KEY WORDS:
- biliary metal
- dietary zinc
- metallothionein

Metallothionein (MT) is a low molecular weight, cysteine-rich cytoplasmic protein that binds metals. Metallothionein synthesis can be induced many-fold by administration of metals such as cadmium [1] or zinc [2]. This inducible nature of MT may be intimately involved in the biological function of MT, possibly by effecting a control over the free metal-content of tissues. The functions of MT are not yet clearly determined but are thought to involve binding of endogenous metals, copper [3] and zinc [4], and consequent homeostatic control of these endogenous metals. Metallothionein is thought to perform a similar role in the metabolism of several xenobiotic metals, complexing with them to produce a lower free-metal concentration. This process has been described as a detoxification of heavy metals [5, 6, 7], since the toxicity of xenobiotic metals appears to be decreased by prior MT induction [2]. The reduced toxicity results from enhanced binding to MT and therefore less free metal available to interact with critical cellular components [8]. Induction of hepatic MT by pretreating with cadmium dramatically decreases the biliary excretion of a bolus of cadmium suggesting that once cadmium is bound to hepatic MT, it is no longer available for biliary excretion [9, 10]. Biliary excretion of copper, mercury, zinc and silver is similarly decreased by cadmium pretreatment [10]. Klaassen proposed that the reduced excretion of these metals was also due to their incorporation into MT [10].

Cadmium is known to bind MT far more avidly than does zinc, and the turnover of MT is greatly influenced by the composition of the metal complex of the MT [11-13]. The half-life for cadmium-MT is 84 h, while that for zinc-MT is 18-20 h [12, 13]. Thus the composition of the complex may also influence the metabolic fate of bound metal ions. As dietary zinc varies, so does hepatic zinc and hepatic MT content. For this reason, the present studies were designed to determine whether induction of hepatic MT by feeding increasing concentrations of zinc would modify biliary metal excretion in the same manner as induction by cadmium. Further, we wanted to extend the study to determine whether biliary metal excretion is directly related to non-MT-bound cytosolic metal, a relationship that would be consistent with the findings of both Cherian [9] and Klaassen [10].

MATERIALS AND METHODS

Female Sprague-Dawley rats (Harlan Industries, Indianapolis, IN), housed individually in stainless steel cages, were acclimated to a nonpurified diet (Wayne Rat Chow, Continental Grain Co., Chicago, IL), analyzed to contain 70 μg/g zinc, until they reached a body weight of 180-220 g. All rats were provided tap water.
TABLE 1
Composition of purified diet¹

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>20.0</td>
</tr>
<tr>
<td>Corn starch</td>
<td>15.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>50.0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.0</td>
</tr>
<tr>
<td>Fiber</td>
<td>5.0</td>
</tr>
<tr>
<td>Vitamin mix²</td>
<td>1.0</td>
</tr>
<tr>
<td>Mineral mix³,⁴</td>
<td>3.5</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.2</td>
</tr>
<tr>
<td>dl-Methionine</td>
<td>0.3</td>
</tr>
</tbody>
</table>

¹This diet contained by analysis 9 µg/g zinc.
²Composition (per kg diet): thiamin HCl, 6.0 mg; riboflavin, 6.0 mg; pyridoxine HCl, 7.0 mg; nicotinic acid, 30.0 mg; d-Ca pantothenate, 16.0 mg; folic acid, 2.0 mg; d-biotin, 0.2 mg; cyanocobalamin (B-12), 10 µg; retinyl acetate, 40.0 IU; dl-α-tocopheryl acetate, 50.0 IU; cholecalciferol, 25 µg; menaquinone, 50 µg.
³Composition (per kg diet): calcium carbonate, 4.38 g; sodium chloride, 2.59 g; potassium citrate, monohydrate, 7.70 g; potassium sulfate, 1.82 g; magnesium oxide, 0.84 g; manganous carbonate, 122 mg; ferrous sulfate, 210 mg; cupric carbonate, 10.5 mg; potassium iodate, 0.35 mg; sodium selenite, 0.35 mg; chromium potassium sulfate, 19.3 mg.
⁴The zinc-supplemented diets (45 µg/g or 1150 µg/g) were obtained by supplementing the above mineral mix with an appropriate quantity of ZnCl₂.

ad libitum. Rats were then randomly assigned to one of three dietary treatments (Table 1). The purified diets varied only in the content of zinc and are referred to as low-zinc (9 µg/g), adequate-zinc (45 µg/g), and high-zinc (1150 µg/g). After 8 d, the rats were anesthetized with a single intraperitoneal injection of sodium pentobarbital [50 mg/kg body weight]. The bile duct was cannulated with PE-10 tubing (13–14 cm length), and the femoral vein was cannulated with PE-30 tubing for the administration of metal chlorides in saline (zinc, cadmium, or mercury, 1 mg/kg body weight). Alteration in biliary flow and excretion due to hypothermia was prevented by maintaining rectal temperature at 37°C with a single intraperitoneal injection of sodium pentobarbital [50 mg/kg body weight]. The bile duct was cannulated with PE-10 tubing (13–14 cm length), and the femoral vein was cannulated with PE-30 tubing for the administration of metal chlorides in saline (zinc, cadmium, or mercury, 1 mg/kg body weight). Alteration in biliary flow and excretion due to hypothermia was prevented by maintaining rectal temperature at 37°C with a temperature-regulated heating pad.

Before administration of any metal via the femoral vein, 0.6 ml blood was collected for plasma zinc estimation, and a 30-min control bile sample was collected into an ice-cold polyethylene tube. Following intravenous injection of metal, bile was continuously collected over 3 h and stored in 30-min fractions. When saline, rather than a bolus of metal, was administered, zinc continued to be excreted at the control rate throughout the experimental period. At the completion of bile collection, rats were killed by cervical dislocation and livers were excised. Liver homogenates [50%, wt/vol] were prepared in ice-cold isotonic KCl and centrifuged at 10,000 × g for 10 min at 4°C. The resulting postmitochondrial supernatant was centrifuged at 104,000 × g for 60 min and the resulting supernatant was used as the cytosol fraction. The concentrations of metal in bile and of metal and MT in liver were measured as described below. Liver cytosol was chromatographed on a Sephadex G-75 [Pharmacia Inc., Piscataway, NJ] gel filtration column (1.4 × 21.5 cm), and the fractions were analyzed for metal and MT.

Metal analysis. Metals were estimated by flame atomic absorption spectrometry (Varian, SpectrAA-20, Varian, Palo Alto, CA). Metal chloride dissolved in triple-distilled water was used as standards. Samples were prepared as follows: plasma was diluted 1:4 to 0.1 N HNO₃, liver was digested in concentrated HNO₃ then diluted in triple-distilled water. Bile samples, diluted in water as necessary, and the fractions eluted from the G-75 column were aspirated directly into the atomic absorption spectrometer.

Metallothionein. Metallothionein was determined by the cadmium-saturation method of Onosaka and Cherian [15], which depends on saturation of the sample with CdCl₂ (4 mM), then estimation of cadmium by atomic absorption spectrometry after non-MT-bound cadmium has been removed by binding to excess rat hemoglobin.

Metal ligand analysis. Liver cytosol [0.7 ml] was fractionated on a Sephadex G-75 column (1.4 × 21.5 cm) previously equilibrated with 0.01 M Tris-Cl buffer, pH 8.0. The elution was carried out at 4°C with the same buffer and 1-ml fractions were collected. Fractions were analyzed for MT, and the amount of metal in each fraction was determined by atomic absorption spectrometry.

Statistical analysis. Because multiple bile samples were taken from each rat over a period of time, a repeated measures analysis was carried out. Where multivariate analysis revealed a significant treatment effect by the Wilk’s Lambda criterion, Tukey’s test was used to determine significant differences (P < 0.05) between pairs of treatments at each time point.

The cumulative data were analyzed using analysis of covariance. After demonstrating the homogeneity of the covariate time (i.e., equality of covariate slopes across treatments) we examined the overall treatment effect. The method of linear contrasts was used to determine significant differences (P < 0.05) between pairs of treatments. The Student-Newman-Keuls test was used to compare data between groups in Tables 2 and 3.

RESULTS

Hepatic metallothionein content. The effect of feeding rats purified diets with different zinc contents on hepatic MT content is shown in Table 2. No difference in hepatic MT content between rats fed the low-zinc and adequate-zinc diets was observed. However, the concentration of hepatic MT in rats fed a high-zinc diet for 8 d was 18-fold higher than that in rats fed a low- or adequate-zinc diet.
### TABLE 2
Effect of dietary zinc on hepatic metallothionein level

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MT content1 (µg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-zinc diet (9 µg/g)</td>
<td>34.2 ± 6.2*</td>
</tr>
<tr>
<td>Adequate-zinc diet (45 µg/g)</td>
<td>46.5 ± 5.8*</td>
</tr>
<tr>
<td>High-zinc diet (1150 µg/g)</td>
<td>732.0 ± 51.2*</td>
</tr>
</tbody>
</table>

1Values are means ± SEM of 5 rats. Values not sharing a common superscript differ significantly (P < 0.05).

### FIGURE 1
(a) Biliary zinc excretion in rats fed a low- (9 µg/g; open bars), adequate- (45 µg/g; hatched bars) or high-zinc (1150 µg/g, closed bars) diet for 8 d, and administered zinc (1 mg/kg, as zinc chloride) intravenously. Endogenous metal excretion, shown on the left, has been subtracted from total excretion to report exogenous excretion. Values are means ± SEM of 5 rats per group. Data not sharing the same superscript are significantly different from each other (α < 0.05). b) The data from Figure 1a are plotted as cumulative biliary metal excretion for rats fed low- (—o—), adequate- (—•—), or high-zinc (—A—) diets for 8 d. Excretion from the high-zinc rats was significantly less than from the adequate-zinc or low zinc rats (P < 0.001).

### TABLE 3
Zinc content of plasma and liver from rats fed various levels of dietary zinc

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Plasma (µg/dl)</th>
<th>Liver (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-zinc diet (9 µg/g)</td>
<td>98.0 ± 2.8*</td>
<td>15.1 ± 2.4*</td>
</tr>
<tr>
<td>Adequate-zinc diet (45 µg/g)</td>
<td>92.7 ± 2.8*</td>
<td>24.1 ± 1.1*</td>
</tr>
<tr>
<td>High-zinc diet (1150 µg/g)</td>
<td>546.5 ± 99.1b</td>
<td>120.1 ± 18.7b</td>
</tr>
</tbody>
</table>

1Values are means ± SEM of 5 rats. Values not sharing a common superscript differ significantly (P < 0.05).

### Zinc content in plasma and liver.
The effect of feeding rats different dietary zinc levels for 8 d on the zinc content of plasma and of liver is shown in Table 3. There was no significant difference in plasma zinc between rats fed 9 µg/g or 45 µg/g. However, plasma zinc in rats fed a 1150 µg/g zinc diet was 6-fold higher than that in rats fed a 9 µg/g or 45 µg/g zinc diet. While mean hepatic zinc content increased with increasing dietary zinc, the difference between the low-zinc dietary group and the adequate-zinc dietary group was not significant (P > 0.05). Hepatic zinc content did vary significantly between adequate- and high-zinc dietary groups (P < 0.05).

### Biliary metal excretion.
Biliary excretion of endogenous zinc and of a bolus of zinc as zinc chloride (1 mg/kg, i.v.) is shown in Figure 1a. Rats fed 1150 µg/g dietary zinc excreted significantly more endogenous zinc than rats fed 9 µg/g or 45 µg/g zinc, 0.080 ± 0.012 µg·kg⁻¹·min⁻¹ vs. 0.040 ± 0.015 µg·kg⁻¹·min⁻¹ and 0.040 ± 0.012 µg·kg⁻¹·min⁻¹, respectively. Conversely, the excretory rate of exogenous zinc from rats fed the high zinc diet was significantly less than that excreted by low-zinc rats for all collection times after the first 60 min, and significantly less than that excreted by adequate-zinc rats at one time point only (120 min). The cumulative biliary excretion (Figure 1b) was therefore significantly less in the high-zinc animals than in the adequate- or low-zinc animals whether reported as exogenous zinc only (6.16 ± 1.45 µg/kg vs. 22.89 ± 11.56 µg/kg or 33.52 ± 7.70 µg/kg) or total zinc excretion (21.58 ± 3.78 µg/kg vs. 35.38 ± 13.99 µg/kg or 41.68 ± 9.7 µg/kg). Although there was a trend toward a greater excretion of a bolus of zinc in low-zinc rats when compared to adequate-zinc rats, this trend was not significant.

The biliary excretory rate for cadmium (Figure 2a) was significantly lower in the rats fed 1150 µg/g zinc than in rats fed either 9 µg/g or 45 µg/g zinc for most of the collection intervals. For individual collection periods, cadmium excretion did not vary significantly between low and adequate groups. However, the cumulative data revealed that the low-zinc group excreted significantly less cadmium than the adequate-zinc group (Figure 2b). In 2.5 h, the rats fed the high zinc diet actually excreted only 0.2% of that excreted by rats fed the adequate-zinc diet.

Biliary excretion of mercury in rats fed the high-zinc diet was significantly lower than in rats fed the low-zinc diet at all time points (Figure 3a). The excretion rate of mercury reached maximum 1 h after i.v. admin-
istration in all groups of rats. Although mercury excretion in rats fed the low-zinc diet tended to be greater than in rats fed the adequate-zinc diet throughout the experimental period, there was no significant difference in excretion between the low-zinc and adequate-zinc groups (Figure 3a). This was due to the large variation in excretion rate that occurred in the low-zinc group. When the data were replotted to display the cumulative excretion of mercury, rats fed the high-zinc diet excreted significantly less than rats fed either the low-zinc or adequate-zinc diets (Figure 3b). Also, rats fed the adequate-zinc diet excreted significantly less mercury than rats fed the low-zinc diet (Figure 3b).

**Metal binding in liver cytosol.** Sephadex G-75 gel filtration of liver cytosol samples is shown in Figure 4. Analysis of fractions revealed that MT eluted at approximately $V_e/V_0 = 2$, the position typically reported for MT [10]. Zinc was mainly bound to the MT fraction in rats fed the high-zinc diet. In rats fed low- or adequate-zinc diets, zinc was mainly associated with the high molecular weight (HMW) fraction. In rats administered cadmium, most cadmium was associated with the MT fraction in rats fed the low-zinc and high-zinc diets. In rats fed the adequate-zinc diet, most cadmium was found to associate with the HMW fraction. When mercury was administered, it bound mainly to the HMW fraction in rats fed the low-zinc diet. However, most of the mercury bound to the MT fraction of liver cytosol from rats fed the adequate- or high-zinc diets.

**DISCUSSION**

Biliary excretion of exogenous metals was lowest in rats fed the high-zinc diet. Previous work has shown a decrease in biliary excretion of cadmium [9] and several other metals [10] after cadmium pretreatment. In this study there was significantly more metal bound to MT in the high-zinc group than in the adequate- or low-zinc groups. Therefore, it is quite possible that the decreased biliary metal excretion in rats fed the high-zinc diet is due to the trapping of metal in MT, leaving less metal free for biliary excretion. Fractionation of hepatic cytosol on a Sephadex G-75 column allows determination of MT-bound and non-MT-bound metal. When the amount of metal found in the MT or non-MT fraction of liver cytosol is plotted against biliary metal excretion, a correlation is found between the form of bound metal in liver and biliary metal excretion (Figure 5). Biliary mercury excretion is inversely proportional to MT-bound mercury ($r = 0.944$) and directly proportional to non-MT-bound mercury ($r = 0.993$). However,
FIGURE 4 Sephadex G-75 chromatogram of rat liver cytosol. A 0.7-ml aliquot of 104,000 × g supernatant fraction from rats fed various dietary zinc levels was separated on a 1.4 × 21.5 cm Sephadex G-75 column. One-ml fractions were collected and the fraction containing MT was identified by analysis.
Figure 5 The amount of metal found in the MT a) or non-MT b) fraction of liver cytosol after fractionation on a Sephadex G-75 column is plotted against biliary metal excretion. Biliary metal excretion is reported as percentage of that excreted by rats fed adequate-zinc diet.

Literature Cited