

## Effects of $\text{Ca}^{2+}$ , $\text{Zn}^{2+}$ and $\text{Cd}^{2+}$ on Uridine Diphosphate-Glucuronyltransferase and $\beta$ -Glucuronidase Activities in Rat Liver Microsomes

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The effect of various metals on uridine diphosphate (UDP)-glucuronyltransferase and  $\beta$ -glucuronidase activities in rat liver microsomes was investigated. The presence of  $\text{Mn}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{V}^{5+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cu}^{+}$  or  $\text{Ca}^{2+}$  ( $20\text{ }\mu\text{M}$ ) in the enzyme reaction mixture did not cause a significant alteration of UDP-glucuronyltransferase activity in hepatic microsomes. Of these metals,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  ( $20\text{ }\mu\text{M}$ ) caused a remarkable increase in hepatic microsomal  $\beta$ -glucuronidase activity. Appreciable effects of  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  on  $\beta$ -glucuronidase activity were seen at  $5.0\text{ }\mu\text{M}$ , and the effects were saturated at  $50\text{ }\mu\text{M}$ .  $\text{Ca}^{2+}$  ( $5.0\text{--}50\text{ }\mu\text{M}$ ) and/or the  $\text{Ca}^{2+}$ -binding protein regucalcin ( $2.0\text{ }\mu\text{M}$ ) did not have an appreciable effect on UDP-glucuronyltransferase and  $\beta$ -glucuronidase activities in hepatic microsomes. Thus,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  uniquely increased  $\beta$ -glucuronidase activity. The  $\text{Zn}^{2+}$ - and  $\text{Cd}^{2+}$ -induced increase in  $\beta$ -glucuronidase activity was completely reversed by the presence of an SH group-protecting reagent (dithiothreitol). The response of the microsomal enzyme to  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  ( $20\text{ }\mu\text{M}$ ) was no longer seen after treatment with 0.2% Triton X-100 [polyoxyethylene(10) octylphenyl ether], indicating that the stimulation by these metals is dependent on membrane association. The present study suggests that, of various metals tested,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  can uniquely increase hepatic microsomal  $\beta$ -glucuronidase activity, and that their effect is based on binding to membranous SH groups, beside the enzyme protein.

**Keywords** zinc; cadmium; calcium; regucalcin; uridine diphosphate-glucuronyltransferase;  $\beta$ -glucuronidase; rat liver microsome

Liver microsomal uridine diphosphate (UDP)-glucuronyltransferase catalyzes the glucuronidation of a wide variety of endogenous and xenobiotic compounds.<sup>1)</sup> A significant fraction of  $\beta$ -glucuronidase in liver is associated with the hepatic microsomal fraction,<sup>2,3)</sup> although the function of the microsomal enzyme remains poorly defined.<sup>2)</sup> Since both UDP-glucuronyltransferase and  $\beta$ -glucuronidase are components of the endoplasmic reticulum, a conjugation-deconjugation cycle has been proposed as a determinant of net glucuronide production from a substrate generated *via* mixed-function oxidation or supplied from other sources.<sup>4)</sup> Thus, liver microsomal UDP-glucuronyltransferase and  $\beta$ -glucuronidase may play a cell physiological role in glucuronidation, which is a major pathway for non-oxygenative metabolism of many drugs, steroids, and toxic and carcinogenic chemicals.<sup>5)</sup>

In recent years, it has been demonstrated that  $\text{Ca}^{2+}$  plays an important role in liver metabolism.<sup>6,7)</sup>  $\text{Ca}^{2+}$  and other metal ions may modulate glucuronidation in hepatic microsomes. This, however, has not been confirmed thus far. The present investigation was undertaken, therefore, to clarify the effects of various metal ions on UDP-glucuronyltransferase and  $\beta$ -glucuronidase activities in rat liver microsomes. It was found that  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  increase  $\beta$ -glucuronidase activity, but not UDP-glucuronyltransferase activity in the hepatic microsomes, and that  $\text{Ca}^{2+}$  does not influence either of the enzyme activities.

### Materials and Methods

**Animals** Male Wistar rats, weighing 100–120 g, were used. They were obtained commercially from Japan SLC Inc. (Hamamatsu, Japan). The animals were fed commercial laboratory chow (solid) containing 57.5% carbohydrate, 1.1% Ca, and 1.1% P at a room temperature of  $25^{\circ}\text{C}$ , and were allowed distilled water freely.

**Reagents** All reagents were purchased from Wako Pure Chemical Co. (Osaka, Japan) and Sigma Chemical Co. (St. Louis, Mo., U.S.A.), and were dissolved in distilled water then passed through an ion-exchange resin to remove metal ions.

**Isolation of Regucalcin**  $\text{Ca}^{2+}$ -binding protein regucalcin in the cytosol fraction of rat liver was purified to electrophoretic homogeneity by gel filtration on Sephadex G-75 and G-50 followed by ion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose, as reported previously.<sup>8)</sup>

**Preparation of Hepatic Microsomes** Rats were sacrificed by cardiac puncture, and the liver was perfused with ice-cold 250 mM sucrose solution, immediately cut into small pieces, suspended 1:9 in the homogenization medium containing 250 mM sucrose, 20 mM Hepes 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1.0 mM EGTA ethylene glycol bis(2-aminoethylether) *N,N,N',N'*-tetraacetic acid and 1.0 mM dithiothreitol, pH 7.2 and homogenized in Potter-Elvehjem homogenizer with a Teflon pestle.<sup>9)</sup>

The homogenate was centrifuged at  $1000\times g$  for 10 min to remove nuclei, unbroken cells and cell debris. The resultant supernate was centrifuged at  $7700\times g$  for 20 min to remove the mitochondrial fraction. The postmitochondrial supernate was then centrifuged at  $110000\times g$  for 60 min to sediment the microsomal fraction. The microsomal fraction was resuspended in 120 mM KCl, 10 mM Hepes, pH 6.8, to a final protein concentration of 20–30 mg/ml. In a separate experiment, the microsomal fraction was resuspended in 5.0 mM 2,6-pyridinedicarboxylic acid (dipicolinate) solution for 10 min at  $4^{\circ}\text{C}$ , or in 0.2% polyoxyethylene(10) octylphenyl ether (Triton X-100) solution for 60 min at  $4^{\circ}\text{C}$ . The suspensions were then centrifuged at  $110000\times g$  for 60 min to obtain the microsomes. The microsomal fraction was resuspended in 120 mM KCl, 10 mM Hepes, pH 6.8.

**Analytical Methods** The glucuronidation of 4-nitrophenol by liver microsomes was measured as described by Burchell and Weatherill.<sup>10)</sup> A 20 mM solution of UDP-glucuronic acid triammonium salt, adjusted to pH 7.4 with KOH, was used in assays. UDP-glucuronyltransferase activity was measured by incubation for 10 min at  $37^{\circ}\text{C}$  in the reaction mixture (final volume 0.25 ml) containing 0.5 M Tris-HCl, 1.0 mM 4-nitrophenol, 20 mM UDP-glucuronic acid, 10 mM  $\text{MgCl}_2$ , other metal ions, and the microsomes (0.7–0.8 mg as protein). The reaction was initiated by the addition of 20 mM UDP-glucuronic acid. After incubation, ice-cold 0.5 M trichloroacetic acid was added and the mixture was rapidly vortexed and kept in ice before removal of the protein pellet by centrifugation at  $2000\times g$  for 10 min. The supernatant solution was mixed with 2.0 M NaOH solution and then diluted by addition of distilled water. Absorbance was measured at 405 nm to assess the reduction in color caused by the formation of 4-nitrophenyl glucuronide. The enzyme activity was expressed as nmol of 4-nitrophenol glucuronidated per min per mg protein.

$\beta$ -Glucuronidase activity was measured by incubation for 45 min at  $37^{\circ}\text{C}$  in a final volume of 0.4 ml containing 75 mM Hepes buffer (pH 7.3), 5.0 mM  $\text{MgCl}_2$ , 125 mM KCl, 1.5 mM phenolphthalein glucuronide, metal ions, and the microsomes (0.7–0.8 mg as protein).<sup>11)</sup> The reaction was

stopped by the addition of 50 mM  $\text{NaHCO}_3\text{--Na}_2\text{CO}_3$  buffer (4.0 ml), pH 10.3. Absorbance was measured at 555 nm to assess the increase in color caused by the formation of phenolphthalein. The enzyme activity was expressed as nmol of phenolphthalein liberated per min per mg protein.

Protein concentration was determined by the method of Lowry *et al.*<sup>12)</sup>

**Statistical Methods** The significance of differences between values was estimated by using Student's *t* test; *p* values of less than 0.05 were considered to indicate statistically significant differences.

## Results

**Effect of Metal Ions on UDP-Glucuronyltransferase Activity in Hepatic Microsomes** The effect of metal ions on UDP-glucuronyltransferase activity in rat liver microsomes is shown in Fig. 1. Various metal ions were present at 20  $\mu\text{M}$  (final concentration) in the enzyme reaction mixture. UDP-glucuronyltransferase activity was not significantly altered by addition of  $\text{MnCl}_2$ ,  $\text{CdCl}_2$ ,  $\text{ZnCl}_2$ ,  $\text{V}_2\text{O}_5$ ,  $\text{NiCl}_2$ ,  $\text{CoCl}_2$ ,  $\text{CuCl}$  or  $\text{CaCl}_2$ . A previous investigation showed that addition of  $\text{Ca}^{2+}$  causes a significant increase of glucose-6-phosphatase activity in rat liver microsomes, and that hepatic  $\text{Ca}^{2+}$ -binding protein regucalcin reverses the  $\text{Ca}^{2+}$  effect.<sup>13)</sup> Then, the effect of increasing concentrations of  $\text{Ca}^{2+}$  on UDP-glucuronyltransferase activity was exam-

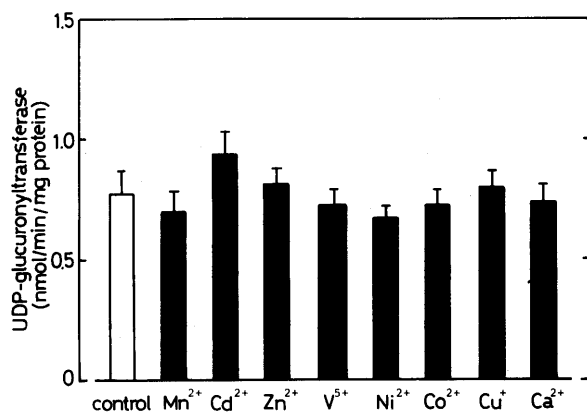


Fig. 1. Effect of Various Metals on UDP-Glucuronyltransferase Activity in the Microsomes of Rat Liver

The enzyme activity was measured in the reaction mixture containing 20  $\mu\text{M}$  metal (final concentration). Each value represents the mean  $\pm$  S.E.M. of 5 experiments. The differences were not significant.

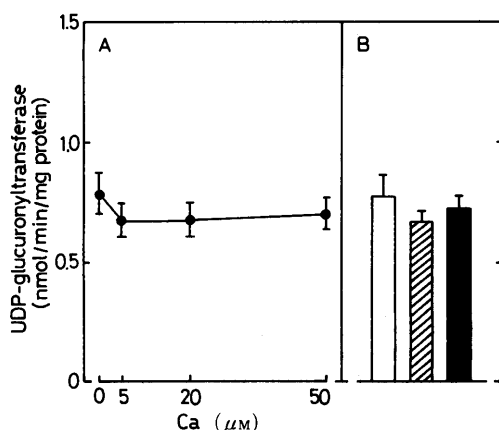


Fig. 2. Effect of  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$ -Binding Protein Regucalcin on UDP-Glucuronyltransferase Activity in the Microsomes of Rat Liver

Figure A shows the effect of increasing concentrations of  $\text{Ca}^{2+}$  (5.0, 20 and 50  $\mu\text{M}$ ). Figure B shows the effect of regucalcin (2.0  $\mu\text{M}$ ) on UDP-glucuronyltransferase activity in the presence of 20  $\mu\text{M}$   $\text{Ca}^{2+}$ . Each value represents the mean  $\pm$  S.E.M. of 5 experiments. The differences were not significant.  $\square$ , control;  $\square$ , 2.0  $\mu\text{M}$  regucalcin;  $\blacksquare$ , 20  $\mu\text{M}$   $\text{Ca}^{2+}$  and 2.0  $\mu\text{M}$  regucalcin.

ined (Fig. 2).  $\text{Ca}^{2+}$  (5.0–50  $\mu\text{M}$ ) did not cause a significant alteration of UDP-glucuronyltransferase activity, and the  $\text{Ca}^{2+}$  effect was not modulated by regucalcin (2.0  $\mu\text{M}$ ). Thus, it seemed that hepatic microsomal UDP-glucuronyltransferase activity was not altered by a comparatively low concentration of metal ion.

**Effect of Metal Ions on  $\beta$ -Glucuronidase Activity in Hepatic Microsomes** The effect of metal ions on  $\beta$ -glucuronidase activity in rat liver microsomes is shown in Fig. 3. When metal ion at 20  $\mu\text{M}$  (final concentration) was contained in the enzyme reaction mixture,  $\beta$ -glucuronidase activity was markedly increased by the presence of  $\text{Cd}^{2+}$  or  $\text{Zn}^{2+}$ . The enzyme activity was not significantly altered by the presence of  $\text{Mn}^{2+}$ ,  $\text{V}^{5+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Cu}^+$  or  $\text{Ca}^{2+}$ . As shown in Fig. 4, the effects of increasing concentrations of  $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{V}^{5+}$  and  $\text{Ca}^{2+}$  were examined. None of the metals at 1.0  $\mu\text{M}$  had an appreciable effect on  $\beta$ -glucuronidase activity. The presence of 5.0  $\mu\text{M}$   $\text{Cd}^{2+}$  or  $\text{Zn}^{2+}$  caused a significant increase in the enzyme activity. The effect of  $\text{Cd}^{2+}$  or  $\text{Zn}^{2+}$  was saturated at 50  $\mu\text{M}$ . Meanwhile,  $\text{V}^{5+}$  or  $\text{Ca}^{2+}$  (1.0–100  $\mu\text{M}$ ) did not cause a significant alteration of  $\beta$ -glucuronidase activity.

It has recently been reported that  $\text{Zn}^{2+}$  can modulate the

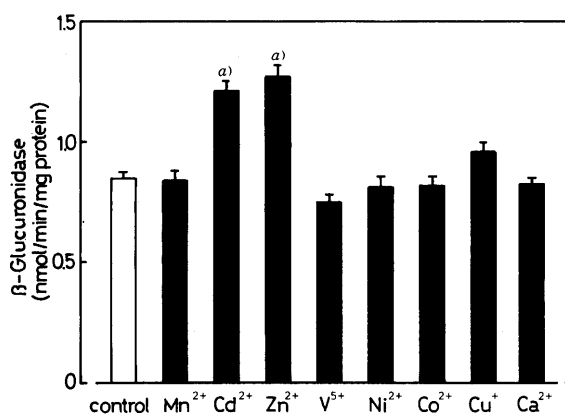


Fig. 3. Effect of Various Metals on  $\beta$ -Glucuronidase Activity in the Microsomes of Rat Liver

The enzyme activity was measured in the reaction mixture containing 20  $\mu\text{M}$  metal (final concentration). Each value represents the mean  $\pm$  S.E.M. of 5 experiments. *a)*  $p < 0.01$ , as compared with the control value.

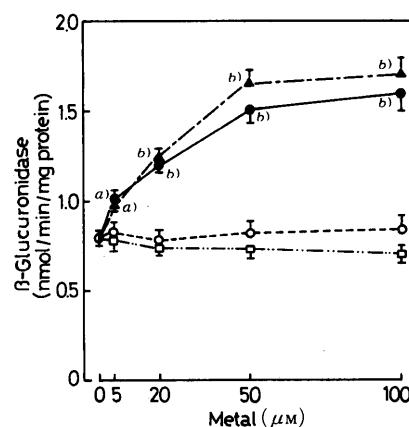


Fig. 4. Effect of Increasing Concentrations of  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{V}^{5+}$  on  $\beta$ -Glucuronidase Activity in the Microsomes of Rat Liver

The enzyme activity was measured in the reaction mixture containing 1.0, 5.0, 20, 50 or 100  $\mu\text{M}$  metal. Addition of 1.0  $\mu\text{M}$  metal did not alter the enzyme activity. Each value represents the mean  $\pm$  S.E.M. of 5 experiments. *a)*  $p < 0.05$ , and *b)*  $p < 0.01$ , as compared with the value without metal.  $\circ$ ,  $\text{Ca}^{2+}$ ;  $\blacktriangle$ ,  $\text{Zn}^{2+}$ ;  $\bullet$ ,  $\text{Cd}^{2+}$ ;  $\square$ ,  $\text{V}^{5+}$ .

$\text{Ca}^{2+}$  effect on protein kinase  $\text{C}^{14}$ ) and  $\text{Ca}^{2+}$ -binding protein.<sup>15)</sup> Thus, the effect of  $\text{Zn}^{2+}$  on hepatic microsomal  $\beta$ -glucuronidase activity was examined in the presence of  $\text{Ca}^{2+}$ . The effect of  $\text{Zn}^{2+}$  (5.0 and 20  $\mu\text{M}$ ) to increase  $\beta$ -glucuronidase activity was not enhanced by the presence of 5.0 or 20  $\mu\text{M}$   $\text{Ca}^{2+}$  (Fig. 5). Moreover, hepatic  $\text{Ca}^{2+}$ -binding protein regucalcin (2.0  $\mu\text{M}$ ) did not modulate the effects of  $\text{Cd}^{2+}$  (20  $\mu\text{M}$ ) and  $\text{Zn}^{2+}$  (20  $\mu\text{M}$ ) to increase hepatic microsomal  $\beta$ -glucuronidase activity (Fig. 6). The presence of both  $\text{Ca}^{2+}$  (20  $\mu\text{M}$ ) and regucalcin (2.0  $\mu\text{M}$ ) had no effect on the enzyme activity. Thus, hepatic microsomal  $\beta$ -glucuronidase activity was uniquely increased by  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$ , among the various metal ions tested.

**Effect of SH Group-Protecting Reagent on  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$ -Increased  $\beta$ -Glucuronidase Activity in Hepatic Microsomes** The effect of dithiothreitol, an SH group-protecting reagent, on the  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$ -induced increase of  $\beta$ -glucuronidase activity in rat liver microsomes is shown in Fig. 7. Dithiothreitol (5.0–100  $\mu\text{M}$ ) was contained in the enzyme reaction mixture. The presence of 20  $\mu\text{M}$  dithio-

threitol clearly prevented the effect of  $\text{Cd}^{2+}$  (20  $\mu\text{M}$ ) to increase  $\beta$ -glucuronidase activity. At 100  $\mu\text{M}$  dithiothreitol, the effects of  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$  were completely blocked. Thus, the effects of  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  to increase hepatic microsomal  $\beta$ -glucuronidase activity were related to SH groups. If the binding sites of  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  on the microsomal membranes were identical,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  at sub-maximum concentration (20  $\mu\text{M}$ ) would be expected to cause an additive increase of  $\beta$ -glucuronidase activity. This was examined, and the result is shown in Fig. 8.  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  did cause an additive increase in the enzyme activity.

**Effect of Dipicolinate, a  $\text{Zn}^{2+}$ -Chelating Reagent, on Hepatic Microsomal  $\beta$ -Glucuronidase Activity**  $\text{Zn}^{2+}$ , but not  $\text{Cd}^{2+}$ , is an essential trace metal in mammals. The role of endogenous  $\text{Zn}^{2+}$  on  $\beta$ -glucuronidase activity in rat liver microsomes was examined by using dipicolinate, a  $\text{Zn}^{2+}$ -chelating reagent (Fig. 9). The hepatic microsomes were suspended in 5.0 mM dipicolinate solution at 4°C for 10 min, and recentrifuged at 110000  $\times g$  for 60 min. The hepatic microsomal  $\beta$ -glucuronidase activity was decreased (about 10%,  $p < 0.01$ ) by washing with dipicolinate in comparison with that of control (not washed). The presence

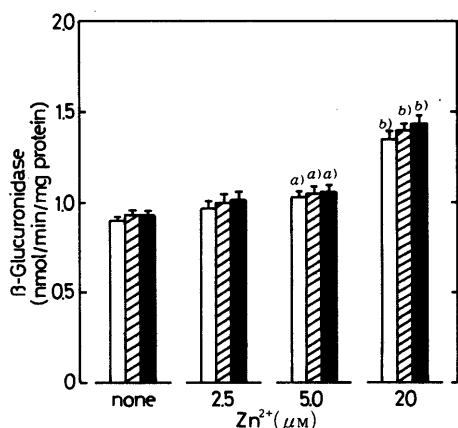


Fig. 5. Effect of  $\text{Ca}^{2+}$  on the  $\text{Zn}^{2+}$ -Induced Increase of  $\beta$ -Glucuronidase Activity in the Microsomes of Rat Liver

The enzyme activity was measured in the reaction mixture containing 5.0 or 20  $\mu\text{M}$   $\text{Ca}^{2+}$  (final concentration) in the presence of 2.5, 5.0 and 20  $\mu\text{M}$   $\text{Zn}^{2+}$ . Each value represents the mean  $\pm$  S.E.M. of 5 experiments. a)  $p < 0.05$ , and b)  $p < 0.01$ , as compared with the control value without metal.  $\square$ , none;  $\square$ , 5.0  $\mu\text{M}$   $\text{Ca}^{2+}$ ;  $\blacksquare$ , 20  $\mu\text{M}$   $\text{Ca}^{2+}$ .

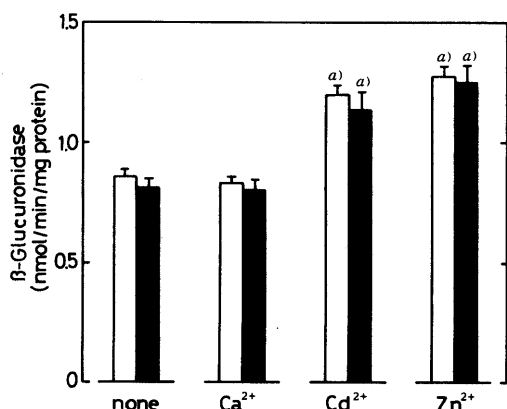


Fig. 6. Effect of the  $\text{Ca}^{2+}$ -Binding Protein Regucalcin on the Metal-Induced Increase of  $\beta$ -Glucuronidase Activity in the Microsomes of Rat Liver

The enzyme activity was measured in the reaction mixture containing  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$  or  $\text{Cd}^{2+}$  (20  $\mu\text{M}$ ) in the presence or absence of 2.0  $\mu\text{M}$  regucalcin. Each value represents the mean  $\pm$  S.E.M. of 5 experiments. a)  $p < 0.01$ , as compared with the control (none) value.  $\square$ , control;  $\blacksquare$ , regucalcin.

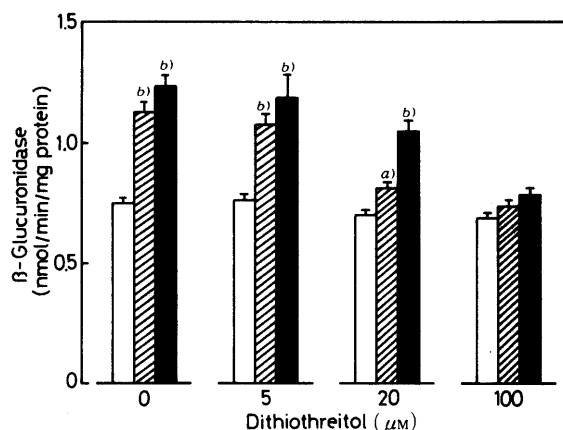


Fig. 7. Effect of Dithiothreitol on the  $\text{Zn}^{2+}$  or  $\text{Cd}^{2+}$ -Induced Increase of  $\beta$ -Glucuronidase Activity in the Microsomes of Rat Liver

The enzyme activity was measured in the reaction mixture containing  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  (20  $\mu\text{M}$ ) in the presence of 5.0, 20 and 100  $\mu\text{M}$  dithiothreitol. Each value represents the mean  $\pm$  S.E.M. of 5 experiments. a)  $p < 0.05$ , and b)  $p < 0.01$ , as compared with the value without metal.  $\square$ , control;  $\square$ ,  $\text{Cd}^{2+}$ ;  $\blacksquare$ ,  $\text{Zn}^{2+}$ .

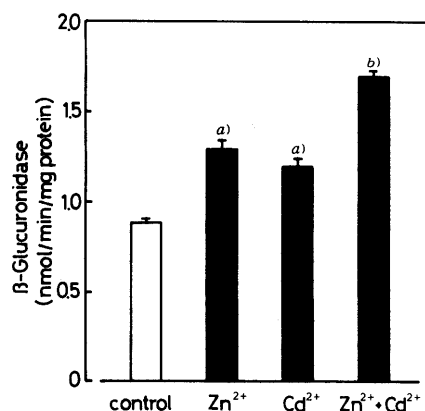


Fig. 8. Effect of  $\text{Cd}^{2+}$  on the  $\text{Zn}^{2+}$ -Induced Increase of  $\beta$ -Glucuronidase Activity in the Microsomes of Rat Liver

The enzyme activity was measured in the reaction mixture containing either  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ , or  $\text{Zn}^{2+}$  plus  $\text{Cd}^{2+}$  (20  $\mu\text{M}$ ). Each value represents the mean  $\pm$  S.E.M. of 5 experiments. a)  $p < 0.01$ , as compared with the control value. b)  $p < 0.01$ , as compared with the value of  $\text{Zn}^{2+}$  or  $\text{Cd}^{2+}$  alone.

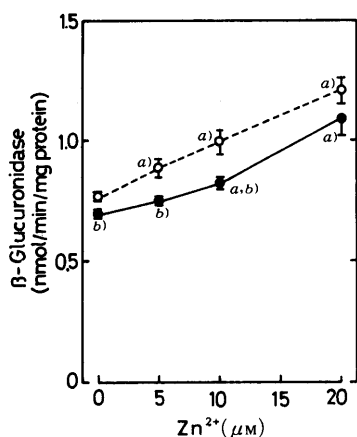


Fig. 9. Effect of Dipicolinate Treatment of  $\beta$ -Glucuronidase Activity in the Microsomes of Rat Liver

The microsomes were suspended in 5.0 mM dipicolinate solution at 4°C for 10 min, and then re-centrifuged at  $110000 \times g$  for 60 min. The enzyme activity was measured in the reaction mixture containing  $Zn^{2+}$  (5.0, 10 and 20  $\mu M$ ) and the microsomes washed with or without dipicolinate. Each value represents the mean  $\pm$  S.E.M. of 5 experiments. a)  $p < 0.01$ , as compared with the value without  $Zn^{2+}$ . b)  $p < 0.01$ , as compared with the value without dipicolinate treatment.  $\circ$ , control;  $\bullet$ , dipicolinate treatment.

TABLE I. Effects of  $Ca^{2+}$ ,  $Zn^{2+}$  and  $Cd^{2+}$  on the Activity of  $\beta$ -Glucuronidase Solubilized from Rat Liver Microsomes by Treatment with Triton X-100

| Treatment <sup>a)</sup> | $\beta$ -Glucuronidase <sup>b)</sup><br>(nmol/min/mg protein) |                 |                 |
|-------------------------|---------------------------------------------------------------|-----------------|-----------------|
|                         | None                                                          | 10 $\mu M$      | 20 $\mu M$      |
| Supernatant             |                                                               |                 |                 |
| Control                 | 3.18 $\pm$ 0.58                                               |                 |                 |
| CaCl <sub>2</sub>       |                                                               | 3.00 $\pm$ 0.54 | 2.99 $\pm$ 0.53 |
| ZnCl <sub>2</sub>       |                                                               | 2.99 $\pm$ 0.53 | 2.95 $\pm$ 0.54 |
| CdCl <sub>2</sub>       |                                                               | 3.05 $\pm$ 0.55 | 3.01 $\pm$ 0.54 |
| Pellet                  |                                                               |                 |                 |
| Control                 | 0.42 $\pm$ 0.10                                               |                 |                 |
| CaCl <sub>2</sub>       |                                                               | 0.40 $\pm$ 0.10 | 0.39 $\pm$ 0.11 |
| ZnCl <sub>2</sub>       |                                                               | 0.49 $\pm$ 0.11 | 0.59 $\pm$ 0.14 |
| CdCl <sub>2</sub>       |                                                               | 0.45 $\pm$ 0.12 | 0.49 $\pm$ 0.12 |

a) Samples of a microsomal suspension containing 10–15 mg/ml were treated with 0.2% Triton X-100 at 4°C for 60 min. After treatment, aliquots were removed for assay and the samples were centrifuged at  $110000 \times g$  for 60 min. The resultant pellets and supernatants were assayed for  $\beta$ -glucuronidase. b) Values are mean  $\pm$  S.E.M. of the samples from the liver microsomes of 5 rats. The differences were not significant.

of 5.0  $\mu M$   $Zn^{2+}$  in the reaction mixture with dipicolinate-washed microsomes did not cause a significant increase in  $\beta$ -glucuronidase activity, although the metal ions significantly raised the enzyme activity in unwashed microsomes. With greater concentrations of  $Zn^{2+}$  (10 and 20  $\mu M$ ), the enzyme activity was increased significantly. Thus, endogenous  $Zn^{2+}$  has a role in the preservation of hepatic microsomal  $\beta$ -glucuronidase activity.

**Effect of Triton X-100 Treatment on  $Zn^{2+}$  or  $Cd^{2+}$ -Increased Hepatic Microsomal  $\beta$ -Glucuronidase Activity**  
The  $\beta$ -glucuronidase is membrane-bound in microsomes. About 90% of the enzyme associated with the microsomes from rat liver was solubilized by treatment with 0.2% Triton X-100 at 4°C for 60 min (Table I). To test whether  $Zn^{2+}$  and  $Cd^{2+}$  stimulation is dependent on membrane association, we examined the response of the microsomal enzyme to metal ions after treatment with 0.2% Triton X-

100 (Table I). The solubilized form of  $\beta$ -glucuronidase showed a greatly reduced sensitivity to  $Zn^{2+}$  or  $Cd^{2+}$  (20  $\mu M$ ). After treatment with 0.2% Triton X-100, the enzyme activity remaining associated with microsomal membranes was not significantly increased by  $Zn^{2+}$  or  $Cd^{2+}$  (20  $\mu M$ ). Addition of  $Ca^{2+}$  (20  $\mu M$ ) also did not cause a significant increase in  $\beta$ -glucuronidase activity in either the supernatant or pellet fraction after treatment with 0.2% Triton X-100.

## Discussion

The effect of various metals on the enzymes which catalyze glucuronidation was investigated in microsomes isolated from rat liver. Glucuronidation is a major pathway for nonoxygenative metabolism of many drugs, steroids, and toxic and carcinogenic chemicals.<sup>5)</sup> Liver microsomal UDP-glucuronyltransferase is a major enzyme related to glucuronidation. It has been reported that liver microsomal UDP-glucuronyltransferase activity is increased by addition of  $MnCl_2$  (1.0 and 2.0 mM).<sup>16,17)</sup> The effect of other metals, however, was not examined. We used a comparatively low concentration of metals. Liver microsomal UDP-glucuronyltransferase activity was not significantly altered by addition of  $Mn^{2+}$ ,  $Cd^{2+}$ ,  $Zn^{2+}$ ,  $V^{5+}$ ,  $Ni^{2+}$ ,  $Co^{2+}$ ,  $Cu^{+}$  or  $Ca^{2+}$  (20  $\mu M$ ) to the enzyme reaction mixture containing 10 mM  $MgCl_2$  (optimum condition). UDP-glucuronyltransferase and  $\beta$ -glucuronidase in hepatic microsomes both catalyze a conjugation-deconjugation cycle which was proposed to be a determinant of net glucuronide production from substrates generated *via* mixed-function oxidation or supplied from other sources, although the enzymes are present in multiple forms.<sup>4)</sup> Liver microsomal  $\beta$ -glucuronidase activity was markedly increased by addition of  $Zn^{2+}$  and  $Cd^{2+}$  (20  $\mu M$ ) to enzyme reaction mixture containing 5 mM  $MgCl_2$  (optimum condition), while  $Mn^{2+}$ ,  $V^{5+}$ ,  $Ni^{2+}$ ,  $Co^{2+}$ ,  $Cu^{+}$  and  $Ca^{2+}$  (20  $\mu M$ ) did not have an appreciable effect. The present finding, that  $Zn^{2+}$  and  $Cd^{2+}$  can uniquely increase hepatic microsomal  $\beta$ -glucuronidase activity, suggests that the metals influence glucuronide production from substrates.

Recently, it has been established that  $Ca^{2+}$  plays an important role in the regulation of liver metabolism.<sup>6,7)</sup> Hepatic microsomal UDP-glucuronyltransferase and  $\beta$ -glucuronidase activities were not significantly altered by addition of  $Ca^{2+}$  (5.0–50  $\mu M$ ) to the enzyme reaction mixture. Moreover, the hepatic  $Ca^{2+}$ -binding protein regucalcin, which can regulate the  $Ca^{2+}$  effect on enzymes,<sup>18–20)</sup> did not have an appreciable effect on either of the enzyme activities. Calmodulin also had no effect on hepatic microsomal UDP-glucuronyltransferase and  $\beta$ -glucuronidase activities (data not shown). Thus,  $Ca^{2+}$  and  $Ca^{2+}$ -binding protein may not regulate hepatic microsomal glucuronidation.

The effect of  $Zn^{2+}$  and  $Cd^{2+}$  to increase hepatic microsomal  $\beta$ -glucuronidase activity was seen at 5.0  $\mu M$  metals, and was saturated at 50  $\mu M$ .  $\beta$ -Glucuronidase activity was additively increased by addition of both  $Zn^{2+}$  and  $Cd^{2+}$  (20  $\mu M$ ) to enzyme reaction mixture, while  $Ca^{2+}$  (20  $\mu M$ ) did not enhance the effect of  $Zn^{2+}$  (20  $\mu M$ ) on the enzyme activity. It is assumed that  $Zn^{2+}$  and  $Cd^{2+}$  bind to identical sites on hepatic microsomes. Dithiothreitol is an SH group-protecting reagent. The presence of dithiothreitol (20  $\mu M$ )

significantly prevented the  $\text{Cd}^{2+}$  ( $20\ \mu\text{M}$ )-induced increase in the microsomal  $\beta$ -glucuronidase activity, while the  $\text{Zn}^{2+}$  ( $20\ \mu\text{M}$ ) effect was not blocked significantly. At  $100\ \mu\text{M}$  dithiothreitol, however, the effects of  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  ( $20\ \mu\text{M}$ ) were completely blocked. Thus,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  may bind to SH groups of microsomal membranes.

Dipicolinate can bind  $\text{Zn}^{2+}$  in biological systems.<sup>21)</sup> Rat liver microsomes contain endogenous  $\text{Zn}^{2+}$ .<sup>22)</sup> When hepatic microsomes were washed with  $5.0\ \text{mM}$  dipicolinate solution, the microsomal  $\beta$ -glucuronidase activity was significantly decreased in comparison with that obtained from unwashed microsomes. This finding suggests that the microsomal endogenous  $\text{Zn}^{2+}$  can preserve  $\beta$ -glucuronidase activity.

About 90% of the enzyme associated with the microsomes from rat liver was solubilized by treatment with 0.2% Triton X-100. The solubilized form of  $\beta$ -glucuronidase showed a greatly reduced sensitivity to  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  ( $20\ \mu\text{M}$ ). This indicates that  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  stimulation is dependent on membrane association, and that the metals do not activate the enzyme directly. As mentioned above,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  may bind to SH groups of hepatic microsomal membranes to stimulate microsomal  $\beta$ -glucuronidase activity.  $\text{Cd}^{2+}$  is a toxic metal and may bind to the binding sites (SH groups) for  $\text{Zn}^{2+}$ , since the  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  effects on the enzyme activity were both completely blocked by dithiothreitol. Although the liver cytosolic free concentration of  $\text{Zn}^{2+}$  is unknown, the total cytosolic concentration of  $\text{Zn}^{2+}$  has been estimated at about  $200\ \mu\text{M}$ .<sup>23,24)</sup> Addition of more than  $5.0\ \mu\text{M}$   $\text{Zn}^{2+}$  to the enzyme reaction mixture caused a remarkable increase in hepatic microsomal  $\beta$ -glucuronidase activity.  $\text{Zn}^{2+}$  may have a physiological significance to preserve and to stimulate hepatic microsomal  $\beta$ -glucuronidase activity.

In conclusion, it has been demonstrated that, of various metals tested,  $\text{Zn}^{2+}$  and  $\text{Cd}^{2+}$  could uniquely increase hepatic microsomal  $\beta$ -glucuronidase activity, and that the

metals stimulation is dependent on membrane association.

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