Biochemical Pharmacology, Vol. 44, No. 11, pp. 2139–2147, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

MECHANISM OF CADMIUM-DECREASED GLUCURONIDATION IN THE RAT

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(*Received* 24 *January* 1992; *accepted* 1 *September* 1992)

Abstract—In isolated rat hepatocytes, cadmium (0–200 μM) decreased the overall glucuronidation of both isopropyl *N*-(3-chloro-4 hydroxyphenyl)carbamate (4-hydroxychlorpropham, 4-OHCIPC) and 4-nitrophenol in a concentration-dependent manner. In contrast, in native rat liver microsomes, glucuronidation of 4-OHCIPC was increased by cadmium through activation of microsomal 4-OHCIPC glucuronosyl transferase. In addition, in rat microsome incubations, the net amount of 4-OHCIPC glucuronide was also indirectly increased by cadmium through a reduction in the activity of β-glucuronidase.

As the effect of cadmium on the activity of 4-OHCIPC glucuronosyl transferase could not account for the decrease in glucuronide formation in intact hepatocytes, the influence of cadmium on the availability of UDP-glucuronic acid (UDPGA) was investigated further. In isolated rat hepatocytes, cadmium depleted the UDPGA content in a dose-dependent manner without a change in the UDP glucose (UDPG) content. Cadmium did not increase the breakdown of UDPGA by microsomal UDPGA pyrophosphatase but strongly decreased (30–66%) the synthesis of the cofactor in the cytosol by inhibiting UDP-glucose dehydrogenase (UDPGDH). Cadmium (10–50 μM) was found to inhibit the purified enzyme from bovine liver (EC 1.1.1.22) non-competitively. *In vivo* in the absence of a substrate undergoing glucuronidation, cadmium administration, 1.5 and 2.5 mg Cd/kg i.v., to normally fed rats resulted in a 15 and 30% decrease of hepatic UDPGA, respectively. However, in the liver, neither the NAD^+/NADH ratio nor the UDPG content was significantly changed following cadmium treatment. Both *in vitro* and *in vivo* results support the conclusion that in intact cells the reduction in overall 4-OHCIPC glucuronidation caused by cadmium was due to a decrease in UDPGA availability which results from the inhibiting effect of cadmium on UDPGDH.

In a recent paper [1] concerning the effects of cadmium (Cd) on the metabolism of the widely used herbicide isopropyl *N*-(3-chlorophenyl)carbamate (chlorpropham, CIPC‡) by isolated rat hepatocytes, we showed that both sulphation and glucuronidation of isopropyl *N*-(3-chloro-4 hydroxyphenyl)carbamate (4-hydroxychlorpropham, 4-OHCIPC), the major metabolite of CIPC, were reduced by Cd to a similar extent (60% at 200 μM Cd). Moreover, in isolated rat hepatocytes the combined effects of Cd and free 4-OHCIPC resulted in a marked reduction in intracellular ATP which, in turn, is known to affect strongly glucuronidation *in vitro* [2, 3] and to a lesser extent *in vivo* [4, 5]. Also, the degree of decrease in glucuronidation was surprisingly great since this conjugation reaction is known to be much less dependent on energy supply than sulphation [6, 7]. In support of this lower energy requirement it has been shown that in strongly ATP-depleted hepatocytes, the decrease in sulphation was about twice that in glucuronidation [2]. Consequently, our results suggest that another superimposed mechanism is involved to account for the dramatic decrease in glucuronidation. The present study was undertaken to investigate the effects of Cd on the rate-controlling steps involved in 4-OHCIPC glucuronidation.

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‡ Abbreviations: CIPC, isopropyl *N*-(3-chlorophenyl)-carbamate, chlorpropham; 4-OHCIPC, isopropyl *N*-(3-chloro-4 hydroxyphenyl)carbamate, 4-hydroxychlorpropham; UDPG, UDP-glucose; UDPGA, UDP-glucuronic acid; UDPGDH, UDP-glucose dehydrogenase; DTT, dithiothreitol.

MATERIAL AND METHODS

Chemicals. NAD^+, UDP-glucose (UDPG), UDP-glucuromic acid (UDPGA), Triton X-100, D-saccharic acid-1,4-lactone and UDP-glucose dehydrogenase (UDPGDH) (EC 1.1.1.22) from bovine liver were purchased from Sigma (La Verpillière, France). Arylsulphatase (EC 3.1.6.1), β-glucuronidase (EC 3.2.1.31), glutathione, dithiothreitol (DTT) and NADH were from Boehringer Mannheim (Meyland, France). Tetrabutylammonium hydrogen sulphate and 4-nitrophenol were from Merck (Nogent-sur Marne, France). 4-OHCIPC which is not commercially available was synthesized as described previously [8].

Animal treatment. Male Sprague–Dawley rats (180–220 g) purchased from Iffa-Credo (L'Arbresle, France) were used as liver donors. The animals were maintained under a 12 hr light/dark cycle and were allowed a standard laboratory diet and water *ad lib*.

Treated rats were injected i.v. with 1.5 or 2.5 mg Cd/kg ($CdCl_2$ in saline) and controls received saline only.

Hepatocyte isolation and incubation. Hepatocytes were isolated by the collagenase perfusion method according to Seglen [9]. The final washing was performed with a modified Hank's incubation

medium (137 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 0.35 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.5 mM glucose, 4.2 mM $NaHCO_3$, and 15 mM Hepes brought to pH 7.4 with 1 M NaOH). The cells were pelleted by centrifugation and the cell pellet resuspended in 50 mL modified Hank's medium. Initial viability was determined by Trypan blue exclusion. The final hepatocyte suspension consisted of at least 85% viable cells. Without further separation of viable cells from dead cells, the hepatocyte suspension was diluted with modified Hank's medium to a cell density of 1.5×10^6 cells/mL. To 10 mL of the cell suspension, Cd ($CdCl_2$) was added at final concentrations of 0–200 μM. Then, 4-OHCIPC dissolved in dimethyl sulphoxide was added giving final concentrations of 0.1 mM 4-OHCICP and 35 mM dimethyl sulphoxide respectively. Incubations were commenced within 15 min of obtaining the final cell suspension and carried out in air for 1 hr at 37° in a shaking water bath with 25 oscillations/min. The reaction was stopped by addition of 10 mL ice-cold methanol. After removal of protein by centrifugation, a 20 μL aliquot of the supernatant was directly chromatographed. Incubations with 100 μM 4-nitrophenol as substrate were carried out under the same conditions.

Microsome preparation. Livers from rats were homogenized in 150 mM KCl–10 mM KH_2PO_4 buffer (pH 7.5). After centrifugation at 11,000 *g* for 15 min (4°), the ensuing supernatant was spun at 105,000 *g* for 60 min (4°). The resulting microsomal pellet was resuspended in the same buffer (20 mg protein/mL). Before use the microsomal suspensions were stored at −80° for 1 month. Protein was determined according to Lowry *et al.* [10] using bovine serum albumin as standard.

Analytical methods. Separation of the metabolites of 4-OHCICP was achieved on a Resolve 10 μm C18 Radial Compression column (0.8 × 10 cm) (Waters) operating in an 8 × 10 Radial Compression Module (Waters). The column was protected with a 20 × 4 mm guard column packed with 10 μm Nucleosil C18. The eluent consisted of methanol–water (50:50, v/v) containing 10 mM KH_2PO_4 and 5 mM tetrabutylammonium hydrogen sulphate. The final pH was adjusted to 7.0 with dilute KOH.

Flow rates of 0.7 and 1.0 mL/min were used for metabolite separation in hepatocyte and microsomal incubations, respectively. Detection was accomplished by UV absorption at 240 nm. Glucuronide of 4-OHCIPC was characterized from guinea pig microsomal incubations (fortified with 5 mM UDPGA and 5 mM $MgCl_2$) after hydrolysis by β-glucuronidase (16 hr, 37°, pH 4.5). The expected glucuronide peak completely disappeared yielding a corresponding peak of free 4-OHCICP which was quantified using an authentic standard. Under the same conditions, the formation of free 4-OHCIPC was completely suppressed by the addition of D-saccharic acid-1,4-lactone (20 mM), a specific inhibitor of β-glucuronidase. Further evidence that the peak corresponded to the glucuronide was given by the increase in the peak area with increasing UDPGA levels in the microsomal incubation medium.

Sulphate of 4-OHCIPC was characterized from hepatocyte incubations after hydrolysis with arylsulfatase in the presence of D-saccharic acid-1,4-lactone.

Separation of the metabolites of 4-nitrophenol in hepatocyte incubations was performed with the same chromatographic system at a flow rate of 1 mL/min using the procedure described by Motohashi *et al.* [11] for microsomal incubations. Under these conditions 4-nitrophenol glucuronide and free 4-nitrophenol gave well-resolved peaks with retention times of 5.5 and 16 min, respectively.

Glucuronidation of 4-OH*CIPC in microsomal incubations.* Incubations with native microsomes were carried out for 30 min at 37° in a final volume of 0.5 mL 50 mM Tris–HCl buffer (pH 7.4). The reaction mixture contained 1 mg microsomal protein, 400 μM 4-OHCIPC (in 10 μL methanol), 5 nM $MgCl_2$, 5 mM UDPGA and 0–200 μM Cd. When, Tritron X-100 was used, it was added at a final concentration of 0.25 mg/mg protein. The reaction was stopped by addition of 2 mL ice-cold methanol and protein was removed by centrifugation. A 50 μL aliquot of the supernatant was chromatographed.

Biological synthesis of 4-OH*CIPC glucuronide.* Glucuronide of 4-OHCIPC was biologically synthesized in 1 mL Tris–HCl buffer (pH 7.4) containing 5 mM UDPGA, 5 mM Mg^{2+}, 5 mM 4-OHCIPC and guinea pig microsomes (4 mg protein) which have a low UDPGA pyrophosphatase activity [12]. After 2 hr incubation at 37°, the sample was extracted three times with 20 mL ether to remove unconjugated 4-OHCIPC.

The remaining aqueous phase containing 4-OHCIPC glucuronide, excess UDPGA and UDP as a by-product of the forward reaction was transferred to a Sep Pak C18 column (Waters) which was washed twice with 10 mL distilled water to remove UDPGA and UDP since the reverse glucuronidation is known to be UDP dependent [13, 14]. Then, 4-OHCIPC glucuronide was eluted with 10 mL methanol. The solvent was evaporated to dryness in vacuum and the residue dissolved in Tris–HCl buffer (pH 7.4). Then, a 1 mL aliquot of the solution was added to 1 mL methanol and a 100 μL aliquot of the mixture was chromatographed to ascertain that the final solution was free from unconjugated 4-OHCIPC.

Effect of Cd *on the activity of rat microsomal β-glucuronidase.* Incubations which contained 90 μM 4-OHCIPC glucuronide, 0–200 μM Cd and 2 mg microsomal protein were carried out at 37° for 1 hr in a final volume of 2 mL Tris–HCl buffer (pH 7.4). Incubations were stopped by adding 2 mL methanol and protein was removed by centrifugation. Free 4-OHCIPC and the remaining glucuronide were assayed in the supernatant by HPLC.

Effect of Cd *on UDPGA levels in isolated hepatocytes.* Hepatocytes (40×10^6) were preincubated for 30 min in 10 mL modified Hank's buffer (pH 7.4) at 37°. Then, Cd (0–200 μM) was added and the incubation was continued for 30 min. The sample was boiled for 2 min and centrifuged. UDPGA was assayed in the supernatant by HPLC according to Aw and Jones [15].

Effect of Cd *on the activity of rat microsomal UDPGA pyrophosphatase.* The reaction mixture

contained rat microsomes (2 mg protein), 0.1–1 mM UDPGA and 0–200 μM Cd in a final volume of 0.5 mL Tris–HCl buffer (pH 7.4). After 30 min at 37° the reaction was stopped by boiling (2 min) and the sample centrifuged (5000 *g*, 10 min). UDPGA was assayed in the supernatant by HPLC.

Effect of Cd *on the activity of cytosolic UDPGDH.* The supernatant from the 105,000 *g* centrifugation of rat liver homogenate was immediately used as UDPGDH source. Incubations were carried out for 30 min at 37° and contained 130 μg cytosolic protein, 1 mM UDPG, 1.5 mM NAD^+, and 0–200 μM Cd in a final volume of 0.5 mL Tris–HCl buffer (pH 7.4). The reaction was stopped by boiling (2 min) and protein was removed by centrifugation. UDPGA was determined in the supernatant by HPLC.

Effect of Cd *on purified UDPGDH from bovine liver.* Incubations were carried out for 1 hr at 37° in 5 mL 50 mM Tris–HCl buffer (pH 7.4) containing 1.5 mM NAD^+, 0.5 mM UDPG, 0–200 μM Cd and 0.036 enzyme units. At the end of the incubation UDPGA was directly determined in the medium by HPLC. In order to determine the type of inhibition caused by Cd, incubations were carried out for 15 min at 37° in a final volume of 0.7 mL Tris–HCl buffer (pH 7.4) containing 2 mM NAD^+, 25–200 μM UDPG, 0–50 μM Cd and 0.01 enzyme units. NADH production was monitored at 340 nm. Concerning the reversal of inhibition, 0.01 enzyme units were preincubated with Cd (30, 60 μM) for different times (15–240 min) in Tris–HCl buffer (pH 7.4). Then 2 mM DTT was added and the incubation continued for 1 hr. NAD^+ (2 mM) and UDPG (200 μM) were added and NADH formation was monitored at 340 nm for 15 min.

Effect of Cd *treatment on hepatic glycogen, glucose, NAD^+, NADH and UDPGA levels.* The rats were killed by decapitation 6 hr after Cd treatment. Livers were quickly excised and placed in liquid nitrogen. For the different determinations, a piece of frozen liver was finely ground in a mortar containing liquid nitrogen. A weighed 1 g aliquot of liver powder was transferred to a Potter–Elvehjem apparatus containing different ice-cold extraction solutions and homogenized for 30 sec. For glycogen and glucose the apparatus contained 20 mL of 150 mM KCl–10 mM KH_2PO_4 buffer (pH 7.5). Glycogen was determined as described by Hinson *et al.* [16] and was estimated as the difference between total hepatic anthrone-positive sugars and liver free glucose. Glucose was determined after deproteinization of the homogenate with perchloric acid (0.6 M final concentration). The resulting supernatant was cooled on ice and neutralized with 8 M KOH and $KHCO_3$ powder. After removal of insoluble material by centrifugation, glucose was assayed in the supernatant by the glucose hexokinase method using a kit from Boehringer Mannheim. For the determination of NAD^+, 1 g of frozen liver was homogenized in 20 mL of ice-cold 1 M $HClO_4$. After deproteinization the supernatant was neutralized (8 M KOH, $KHCO_3$) and the precipitate removed. NAD^+ was assayed in the supernatant by HPLC as described by Litt *et al.* [17] with UV absorption at 260 nm. For NADH analysis, the extraction procedure described by Kalhorn *et al.* [18] was used. HPLC determination was performed under the same chromatographic conditions as for NAD^+ with UV absorption at 340 nm. For UDPG analysis 1 g of frozen liver powder was homogenized in 5 mL of ice-cold 0.6 M $HClO_4$. After protein removal, the supernatant was neutralized (8 M KOH, $KHCO_3$) and centrifuged. UDPG was enzymatically determined in the supernatant as described previously [19]. UDPGA was determined in a 1 g aliquot of frozen liver homogenized in 20 mL of precooled 150 mM KCl–10 mM KH_2PO_4 buffer (pH 7). The homogenate was placed in a boiling water bath for 5 min and centrifuged (5000 *g*, 10 min). The supernatant was filtered (0.2 μm) to remove glycogen and a 20 μL aliquot of the filtrate was immediately chromatographed. The chromatographic system was that used for the separation of 4-OHCIPC metabolites. The flow rate was 1 mL/min. UDPGA was monitored by UV absorption at 260 nm. Under these conditions, UDPGA gave a well-resolved peak (T_r = 40 min) which was characterized by retention time, coelution with authentic standard of UDPGA and formation of uridine monophosphate after hydrolysis in 1 M HCl medium (24 hr, 25°).

RESULTS

An isocratic ion-pair reversed-phase HPLC method was developed for the direct determination of 4-OHCIPC metabolites in hepatocyte and microsomal incubations.

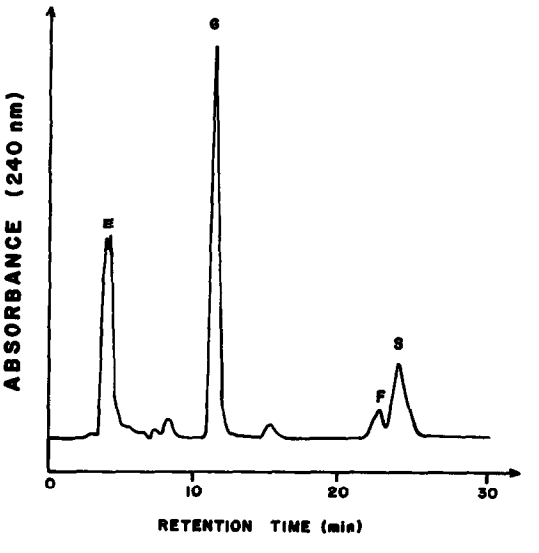

Fig. 1. Representative HPLC chromatogram of 4-OHCIPC metabolites in isolated rat hepatocyte incubations. Endogenous peak from disrupted hepatocytes (E), free 4-OHCIPC (F), glucuronide (G) and sulphate (S). Hepatocytes (15×10^6) were incubated in 10 mL of modified Hank's medium (pH 7.4) with 0.1 mM 4-OHCIPC for 2 hr. After deproteinization with methanol and centrifugation, a 20 μL aliquot of supernatant was directly injected. Chromatographic conditions are described in Materials and Methods.

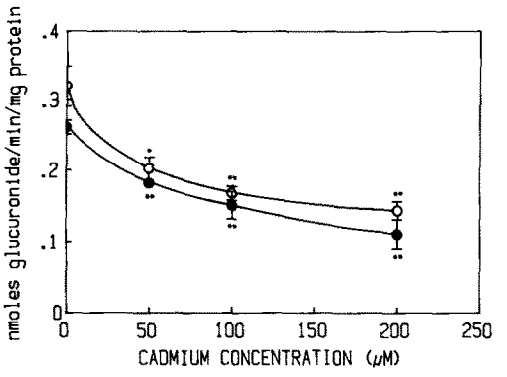

Fig. 2. Effect of Cd on 4-OHCIPC and 4-nitrophenol glucuronidation in isolated rat hepatocyte incubations. Hepatocytes (15×10^6) were incubated for 1 hr at 37° in 10 mL modified Hank's buffer (pH 7.4) with 0.1 mM 4-OHCIPC (O) or 4-nitrophenol (●) as substrate. After deproteinization glucuronides were determined in the supernatant by HPLC. Values are the means ± SD for four to five separate incubations. Key: significantly different from control * $P < 0.05$; ** $P < 0.01$.

The described method is simple, rapid and enables the chromatographic separation of free 4-OHCIPC, glucuronide and sulfate in hepatocyte incubations (Fig. 1). Since preliminary experiments carried out with isolated rat hepatocyte suspensions showed that Cd reduced cell viability in a concentration- and time-dependent manner, in the present study the incubation time was limited to 1 hr.

Under these experimental conditions with 100 μM 4-OHCIPC as substrate and without Cd, glucuronide, sulphate and unconjugated 4-OHCIPC represented typically 50, 15 and 35% of the dose, respectively.

Effect of Cd *on* 4-OH*CICP and 4-nitrophenol glucuronidation in isolated hepatocytes*

Addition of Cd to the incubation medium resulted in a significant and dose-dependent decrease in the rates of 4-OHCIPC glucuronidation (Fig. 2). As this effect of Cd on glucuronidation was inferred from an unusual substrate for this type of study, further experiments were carried out under the same experimental conditions with 4-nitrophenol, a widely used substrate for glucuronidation studies. Figure 2 shows that the reduction in the glucuronidation rates of 4-nitrophenol was similar to that observed for 4-OHCIPC, reaching 30, 45 and 56% at 50, 100 and 200 μM Cd, respectively. Since Cd-induced cytotoxicity could lead to an overestimation of the specific effect of Cd on glucuronidation, the respective reductions in cell viability and in glucuronide formation were determined simultaneously. When compared with control (without Cd) the results showed that at each Cd dose, the percentage of reduction in glucuronidation was always 2-fold higher than that of cell viability.

Effect of Cd *on* 4-OH*CIPC glucuronidation in microsomal incubations*

Table 1 shows that Cd increased the activity of 4-OHCIPC glucuronosyl transferase in native microsomes both in the absence and presence of Mg^{2+}. In the two cases, maximal activation caused by Cd was reached at a concentration of 100 μM; above this, a slight decrease in activation occurred. To gain further insight into the effect of Cd on UDP glucuronosyl transferase activity, experiments were carried out with microsomes activated by prior

Table 1. Effect of Cd on glucuronidation of 4-OHCIPC in rat microsomal incubations

	4-OHCIPC glucuronide formed (nmol/min/mg protein)			
	Native microsomes		Triton-X-100-treated microsomes	
Cd (μM)	Without Mg^{2+}*	5 mM Mg^{2+}†	Cd treatment with substrates‡	Cd treatment without substrates§
0	0.970 ± 0.026	2.272 ± 0.194	16.700 ± 1.957	16.700 ± 1.957
50	1.353 ± 0.110¶	3.050 ± 0.265¶	15.533 ± 0.681	14.733 ± 0.462
100	2.410 ± 0.130¶	3.207 ± 0.189¶	13.333 ± 1.222‖	11.667 ± 2.082‖
200	2.133 ± 0.119¶	2.950 ± 0.311¶	8.667 ± 0.577¶	6.333 ± 0.306¶

Incubations with native microsomes (1 mg protein) were carried out for 30 min at 37° in a final volume of 0.5 mL Tris–HCl buffer (pH 7.4) and contained 400 μM 4-OHCIPC, 5 mM UDPGA and no Mg^{2+}* or 5 mM Mg^{2+}†.

Incubation with Triton X-100-treated microsomes: ‡ In a volume of 0.48 mL 50 mM Tris–HCl buffer (pH 7.4), 1 mg of microsomal protein was preincubated for 25 min at 37° in the presence of 5 mM $MgCl_2$ and 0.25 mg Triton/mg protein. UDPGA, 4-OHCIPC and Cd were added giving final concentrations of 5 mM 400 μM and 0–200 μM, respectively, in a final volume of 0.5 mL. The incubation was continued for 5 min and stopped by addition of methanol.

§ In a volume of 0.48 mL 50 mM Tris–HCl buffer (pH 7.4), 1 mg microsomal protein was preincubated for 25 min at 37° in the presence of 5 mM $MgCl_2$, 0.25 mg Triton/mg protein and Cd (0–200 μM). UDPGA and 4-OHCIPC were added giving final concentrations of 5 mM and 400 μM, respectively, in a final volume of 0.5 mL. The incubation was continued for 5 min and stopped by addition of methanol.

Values represent the means ± SD for three to four different incubations.

Key: significantly different from the corresponding control ‖ $P < 0.05$; ¶ $P < 0.01$.

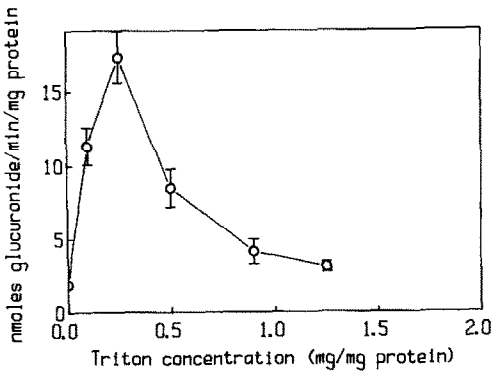

Fig. 3. Effect of Triton X-100 concentration on the activities of 4-OHCIPC glucuronosyl transferase in rat microsomes. Microsomal protein (1 mg) was preincubated for 25 min at 37° with 5 mM $MgCl_2$ and Triton in 490 μL of 50 mM Tris–HCl buffer (pH 7.4). UDPGA and 4-OHCIPC were added to give 5 mM and 400 μM final concentrations, respectively, in a final volume of 500 μL 50 mM Tris–HCl buffer (pH 7.4), The incubation was continued for 5 min, and stopped by addition of methanol. 4-OHCIPC glucuronide was detected by HPLC. Values are the means ± SD for three separate incubations.

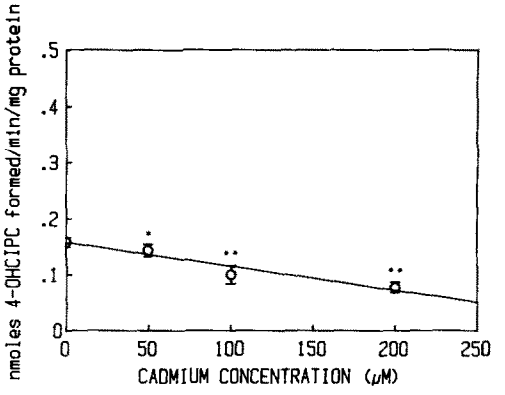

Fig. 4. Effect of Cd on β-glucuronidase activity in rat microsomal incubations. Incubations were carried out for 60 min at 37° and contained 90 μM 4-OHCIPC glucuronide, 0–200 μM Cd and 2 mg protein in a final volume of 2 mL Tris–HCl buffer (pH 7.4). After deproteinization with methanol, free 4-OHCIPC formed from the hydrolysis of the glucuronide was determined by HPLC. Values are the means ± SD of three separate incubations. Key: significantly different from control * $P < 0.05$; ** $P < 0.01$.

treatment with Triton X-100. As shown in Fig. 3, maximum activation (8-fold) of 4-OHCIPC glucuronosyl transferase was reached with 0.25 mg Triton X-100/mg protein. After preincubation of rat microsomes with Triton X-100 (0.25 mg Triton/mg protein), addition of Cd (100–200 μM) resulted in a significant decrease in the rates of glucuronidation (Table 1). When preincubation with Triton X-100 was carried out in the presence of Cd the decrease in glucuronidation rate was greater only with the highest dose of Cd.

Effect of Cd *on rat microsomal β-glucuronidase activity*

The possibility that the increase in glucuronide formation observed in Cd-treated rat native microsomes could be due partially to Cd-induced changes in β-glucuronidase activities was investigated using 4-OHCIPC glucuronide as substrate. Since in such incubations an additional formation of free aglycone could be related to a non-enzymatic degradation of the substrate, the stability of the glucuronide was investigated previously in Tris–HCl buffer (pH 7.4) in the presence of Cd (0–200 μM) and in the absence of microsomal protein. Under these experimental conditions, no spontaneous degradation of the glucuronide occurred (not shown).

As depicted (Fig. 4), addition of Cd to rat microsomal incubations resulted in a dose-dependent decrease in β-glucuronidase activity (I_{50} = 200 μM). Similar experiments carried out in the presence of 20 mM saccharic acid-1,4-lactone, a specific inhibitor of β-glucuronidase, resulted in a complete recovery of intact 4-OHCIPC glucuronide (not shown).

This result demonstrated that under our experimental conditions, the formation of free 4-OHCIPC resulted only from the deconjugation of 4-OHCIPC glucuronide by β-glucuronidase.

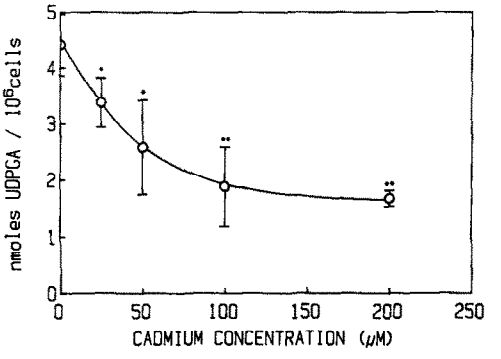

Fig. 5. Effect of Cd on UDPGA levels in isolated rat hepatocytes. After preincubation of 40 × 10^6 cells in 10 mL modified Hank's buffer (pH 7.4) for 30 min, Cd (0–200 μM) was added and the incubation continued for 30 min. Values are the means ± SD for four separate incubations. Key: significantly different from control * $P < 0.05$; ** $P < 0.01$.

Effect of Cd *on UDPGA levels in isolated hepatocytes*

As shown in Fig. 5, addition of Cd to rat hepatocyte incubations caused a dose-dependent decrease in the cofactor. The marked depletion in UDPGA content was accompanied by only a slight decrease in cell viability (−15% at 200 μM Cd).

Interestingly, UDPG levels in Cd-treated hepatocytes were not changed when compared with untreated hepatocytes (3 ± 0.3 μmol/16^6 cells) (not shown).

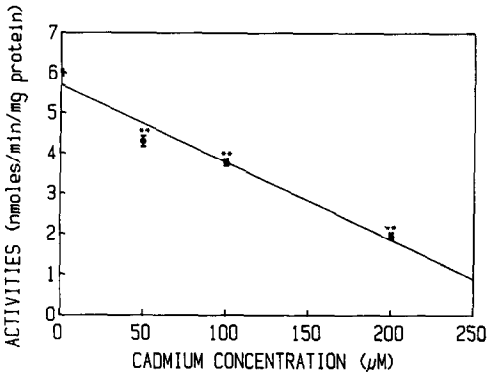

Fig. 6. Effect of cadmium on UDPGDH activities in cytosol. Incubations contained 130 μg cytosolic protein, 1 mM UDPG, 1.5 mM NAD^+ and 0–200 μM Cd in a final volume of 0.5 mL Tris–HCl buffer (pH 7.5). The amounts of UDPGA were determined as described in Materials and Methods. Values are the means ± SD of four separate incubations. Key: significantly different from control ** $P < 0.01$.

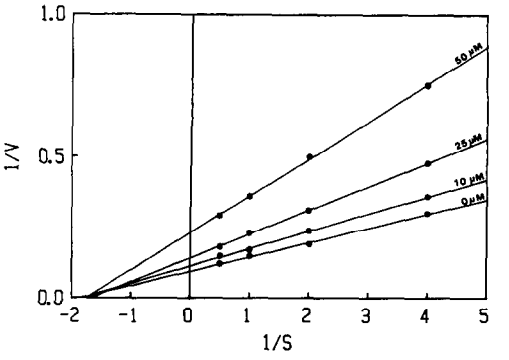

Fig. 7. Lineweaver–Burk plot for inhibition of purified UDPGDH from bovine liver by Cd. In a final volume of 720 μL Tris–HCl buffer (pH 7.5), the reaction mixture contained 0.01 U of purified UDPGDH from bovine liver (EC 1.1.1.22), 2 mM NAD^+, and variable concentrations of UDPG and of Cd (as noted on the curves). The amounts of NADH formed were determined at 340 nm for 15 min. The velocity (v) is given as nmol/min/0.01 enzyme units. S = 25, 50, 100 and 200 μM UDPG.

Effect of Cd *on UDPGA synthesis in rat cytosolic fractions*

In the presence of an excess of both cofactor (NAD^+) and substrate (UDPG), addition of Cd to freshly prepared cytosolic fractions resulted in a marked decrease in cytosolic UDPGDH activity (Fig. 6) ranging from 30 to 66% at 50 and 200 μM Cd, respectively (IC_{50} = 150 μM). In order to confirm the effect of Cd on UDPGDH activity, additional experiments were carried out with purified UDPGDH from bovine liver (EC 1.1.1.22). Here, the reduction of UDPGDH activity was greater than that observed in rat cytosolic fractions, reaching 30 and 97% at 10 and 200 μM Cd, respectively. This difference in Cd susceptibility may be attributed to a reduction of Cd availability for the enzyme in the rat cytosol due to the high protein content of the cytosol and to the strong Cd binding capacity of proteins [20]. On the other hand, the high sensitivity of the enzyme to Cd suggests that the metal interacts with the SH-group(s) of UDPGDH [21]. A strong increase in absorbance at 250 nm occurred when Cd interacted with purified UDPGDH providing additional evidence that SH-group(s) are involved in the direct interaction of Cd with UDPGDH.

Lineweaver–Burk plots constructed using the direct spectrophotometric assay for NADH showed that the inhibition was of a non-competitive type (Fig. 7) suggesting that Cd interaction with UDPGDH is not concerned with the active center of the enzyme.

In view of the effect of Cd on the activity of UDPGDH, it was important to ascertain whether exogenous thiols reversed the inactivation caused by Cd. Reactivation of UDPGDH activity was studied with GSH and DTT as reversers. The inhibitory effect of Cd was only partially reversed by GSH (4 mM) added at the start of the incubation. At Cd doses of 100 and 200 μM we obtained 50 and 30% reversal, respectively. On the other hand, when Cd and UDPGDH were preincubated for 1 hr before GSH addition, the exogenous thiol did not significantly reverse the inhibition. DTT (2 mM) was a more effective reverser than GSH since complete reversal of inhibition was obtained at all Cd doses when DTT was added from the onset of incubation.

When UDPGDH and Cd were preincubated (1 hr) before adding DTT the results show that the inhibition was only partially reversed. The percentages of reversal were 55 and 30% with 30 and 60 μM Cd, respectively. In addition, for a given Cd dose (60 μM) the percentage of reversal decreased from 30 to 10% with increasing preincubation times from 1 to 2 hr.

The results of reversal of Cd inhibition showed that partially reversible inhibition was further accompanied by an irreversible inactivation which was both dose and time dependent. The progressive increase in irreversibility probably resulted from slower structural alterations of the protein caused by Cd.

As a reduction in UDPGA availability could be due to an increase in cofactor breakdown, the effect of Cd on the activity of microsomal UDPGA pyrophosphatase was investigated. Under our experimental conditions, in the absence of both Cd and the substrate undergoing glucuronidation with UDPGA concentrations lower than 0.5 mM, more than 90% of the UDPGA was degraded. With UDPGA in the range 0.1–1 mM addition of Cd (50, 100, 200 μM) caused no significant change in the activity of microsomal phyrophosphatase (not shown).

Effect of Cd *on hepatic levels of glycogen, glucose, NAD^+, NADH, UDPG and UDPGA*

Table 2 shows that administration of Cd resulted in a dramatic decrease in liver glycogen in agreement with a previous qualitative study [22], and that the glycogen depletion was accompanied by a slight

Table 2. Biochemical changes in the liver of Cd-treated rats

Treatment group	Control	Cd 1.5 mg/kg	Cd 2.5 mg/kg
Glycogen (mg/g)	56.6 ± 5.6	15.15 ± 5.50*	2.03 ± 0.64†
Glucose (mg/g)	1.55 ± 0.91	1.37 ± 0.20	0.80 ± 0.29†
UDPG (μmol/g)	0.70 ± 0.03	0.67 ± 0.03	0.66 ± 0.10
UDPGA (μmol/g)	0.41 ± 0.04	0.35 ± 0.03†	0.28 ± 0.05†
NAD^+ (μmol/g)	0.73 ± 0.10	—	0.70 ± 0.04
NADH (μmol/g)	0.20 ± 0.03	—	0.20 ± 0.03

Six hours after Cd administration the metabolic intermediates were measured in whole liver as described in Materials and Methods and expressed per gram weight.
Key: different from value in control rat * $P < 0.05$, † $P < 0.01$.

decrease in intracellular free glucose levels, while the UDPG content remained unchanged.

In contrast, hepatic levels of UDPGA were significantly depleted by Cd in a dose-dependent manner. This result is in agreement with a previous *in vivo* study concerned with the effect of various chemicals on liver UDPGA levels [23]. Table 2 shows that both the NAD^+ and NADH contents of liver were not significantly different in controls and Cd-treated rats. When considering the activity of cytosolic UDPGDH it can be expected that the cytosolic content of NAD^+ and NADH is more relevant than the whole liver content. However, it has been shown that total NAD^+ and NADH in the cytosol accounts for 65% of that in the whole liver [24]. Accordingly, it can be considered that our data are representative of the cytosolic NAD^+ and NADH levels.

DISCUSSION

The present data show that the addition of Cd (0–200 μM) to isolated rat hepatocyte suspensions results in a dose-dependent reduction in the overall glucuronidation of two phenolic substrates. As in intact hepatocytes, glucuronidation is regulated by both the activity of glucuronosyl transferase and the availability of UDPGA [25], the effect of Cd on the activity of microsomal 4-OHCIPC glucuronosyl transferase was further investigated. In incubations carried out with rat native microsomes, Cd activates 4-OHCIPC glucuronosyl transferase even in the absence of Mg^{2+}, a known activator of many UDP glucuronosyl transferases. As already observed with bilirubin and 4-nitrophenol [26], Cd in the presence of Mg^{2+} causes an additional increase in 4-OHCIPC glucuronosyl transferase activity.

This activating effect of Cd probably results from a direct effect of the metal on the enzyme protein. However, with Cd as activator an indirect effect through membrane perturbation cannot be ruled out since Cd is known to cause dilation of the rough endoplasmic reticulum [22] and to perturb membrane phospholipids [27].

Recent studies dealing with both the amino acid sequence and the localization of UDP-glucuronosyl transferases have shown that the majority of the enzyme protein is oriented in the luminal side of the endoplasmic reticulum and that the protein is embedded within the membrane with cystein residues involved in the stabilization of the enzyme–phospholipid linkage [28, 29]. This model appears to be consistent with the observed modulation of catalytic activities by many membrane perturbants such as detergents [30] and also by thiol reagents [31, 32]. Therefore, to gain further insight into the effect of Cd on 4-OHCIPC glucuronosyl transferase, experiments were carried out with rat microsomes fully activated by prior treatment with Triton X-100. Under these conditions, the results show that the activating effects of Triton and Cd were non-additive. The activating effects of detergents on UDP-glucuronosyl transferases have been generally attributed to membrane perturbations, which in turn increase the accessibility of the active centre to substrates [33] or cause phospholipid alterations in enzyme conformation [13]. Whatever the mechanism postulated, it may be expected that after Triton pretreatment some −SH group(s) in the enzyme, which were masked in native microsomes, become more accessible and thus more susceptible to Cd interaction leading in turn to modification of the enzyme structure incompatible with full activity. Moreover, the absence of a protective effect afforded by the substrates up to 100 μM Cd and the weak protective effect observed at 200 μM Cd suggest that the active centre of the enzyme is not involved in Cd interaction.

The significant inhibition of β-glucuronidase caused by Cd resulted indirectly in a net increase in the amount of glucuronide, although this effect can be expected to be of no great significance in intact hepatocytes inasmuch as the glucuronide is quickly excreted from the cell. Considered together, the activation of 4-OHCIPC glucuronosyl transferase in native microsomes and the inhibition of β-glucuronidase, both caused by Cd, were in complete contrast with the decreased overall glucuronidation occurring in Cd-treated hepatocytes. Therefore, we were led to investigate the effect of Cd on the availability of UPDGA, the essential cofactor regulating glucuronidation in intact cells [34, 35]. In isolated rat hepatocyte incubations, Cd caused a large decrease in the UDPGA level which can conceivably account for the decrease in overall glucuronidation. Since in intact cells the UDPGA

level is dependent on both synthesis and breakdown, the influence of Cd on these two pathways was further investigated. The results show that Cd is without effect on UDPGA pyrophosphatase activities suggesting that it is responsible for a decrease in the synthesis of the cofactor. The *in vitro* results are consistent with this hypothesis since Cd strongly inhibits rat cytosolic UDPGDH. This fact was further confirmed by additional experiments carried out with purified UDPGDH from bovine liver (EC 1.1.1.22) since the purified rat enzyme is not available.

However, *in vivo* and in isolated hepatocytes a decrease in UDPGA availability may also be caused by alterations in the $NAD^+/NADH$ redox state since UDPGDH is a NAD^+-requiring enzyme and is inhibited by NADH [36]. On the other hand, UDPGA is derived from glycogen and glucose, and a reduction in carbohydrate reserves has been reported to reduce glucuronide formation [37]. *In vivo* results excluded the possibility that a change in $NAD^+/NADH$ ratio is responsible for a decrease in UDPGA synthesis since Cd was without effect on the pyridine nucleotide levels in whole liver. On the other hand, the *in vivo* results show that the glycogen level was still dramatically reduced 8 hr after Cd treatment. This finding is consistent with both the stimulation of glycogenolysis and the reduction in insulin secretion following Cd treatment [38]. Moreover, the present *in vivo* results show that although the level of glycogen was essentially depleted, that of UDPGA was only one third decreased. This finding is in agreement with results found earlier in fasted rats [39] and suggests that UDPGA availability is not directly related to the hepatic glycogen level *per se*.

After stimulation of glycogenolysis by Cd, the intracellular levels of free glucose would be expected to increase but the results show that this is not the case since free glucose was also depleted. Both the decrease in free glucose and the maintenance of UDPG levels can be explained by the shunt of glucose to the UDPG pathway. However, the maintenance of UDPG levels may also be due to the absence of glycogen synthesis after treatment at the highest Cd dose since UDPG is the precursor of glycogen synthesis. As *in vivo* experiments showed that Cd is without effect on either the redox state or UDPG content, the most reasonable interpretation to explain the decrease in UDPGA is that Cd reduces synthesis of the cofactor through a reduction in UDPGDH activity. This opinion is strongly supported by the *in vitro* results.

Although glucuronosyl transferases represent a family of isoenzymes with different substrate specificity induction [40] and developmental profiles [41, 42], the cofactor for the glucuronidation of xenobiotics and endogenous compounds is always UDPGA. Consequently, as Cd decreases glucuronidation through a reduction in UDPGA synthesis, it appears that the metal must be considered as a non-specific inhibitor of glucuronidation.

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