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BILIVERDIN REDUCTASE: CHARACTERIZATION IN THE RAT KIDNEY AND THE INHIBITION OF ACTIVITY BY MERCURIC CHLORIDE

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Abstract—The effects of metal ions on the activities of biliverdin reductase in the rat kidney and liver were examined; the pH optimum and the cofactor requirement for the enzyme activity in the kidney were also studied. The reduction of biliverdin IXα by biliverdin reductase in the rat kidney cytosol fraction could be supported by NADPH and NADH. The activity was optimal around pH 8.7 when NADPH was the cofactor. The activity with NADH was undetectable at this pH. NADH-dependent biliverdin reductase was optimal at pH 7.0, where the NADPH-dependent activity was negligible. Biliverdin reductase activity was not inducible in the kidney or liver in response to treatment of rats with metal ions—Co^{2-}, Ni^{2-}, Pb^{2+}, Sn^{2-}, Zn^{2-}, Cd^{2+}, and Cu^{2-} or sodium selenite. Rather, both NADPH- and NADH-dependent activities in the kidney were decreased markedly in a time- and dose-related manner following the adminstration of $HgCl_2$ (10–30 μmoles/kg, 24 hr). The pretreatment of rats (30 min) with sodium selenite (5 μmoles/kg) effectively blocked the Hg^{2+} (20 μmoles/kg, 24 hr) inhibition of the kidney cytosol biliverdin reductase activity. Similarly, *in vitro* Hg^{2+} was an effective inhibitor of the kidney biliverdin reductase. In addition, highly purified biliverdin reductase also was extremely sensitive to Hg^{2+} and the thiol reagent, 5, 5′-dithiobis-(2-nitrobenzoic acid). The inhibition of purified reductase by 5,5′-dithiobis-(2-nitrobenzoic acid), but not by Hg^{2-}, could be reversed by dithiothreitol.

Biliverdin constitutes the end product of heme (Fe-protoporphyrin-IX or heme *b*) degradation. In mammalian systems, the catalytic reduction of biliverdin to bilirubin is mediated by the cytosolic enzyme, biliverdin reductase [1–5]. The enzyme also catalyzes the reduction of hematobiliverdin, the oxidation product of hematoheme (heme *c*), to hematobilirubin [6]. Recently, biliverdin reductase was purified to homogeneity from the rat liver [1] and was shown to exhibit enzymatic criteria which were markedly different from those previously ascribed to the enzyme [2–5]. Moreover, the liver enzyme exhibited certain unique characteristics; namely, the presence of two distinct pH optima, 8.7 and 7.0, for the enzyme activity, and an absolute specificity for pyridine nucleotides, NADPH and NADH, at the pH optima, respectively. To date, however, information pertaining to the kidney biliverdin reductase is not available. Furthermore, with the exception of one isolated report on the induction by Co^{2+} of biliverdin reductase activity in the rat liver [7], the effect of metal ions on the enzyme activity is not known.

The activity of biliverdin reductase does not constitute the rate-limiting step in the degradation of heme. Rather, the microsomal heme oxygenase functions in this capacity [8, 9]. This enzyme catalyzes the oxidative cleavage of heme b molecule at the α-methene bridge to form biliverdin; and that of hematoheme at α-, β-, γ-, and δ-methene bridges to form corresponding hematobiliverdins [6]. The activity of heme oxygenase in various organs including the kidney and the liver is induced by a host of chemicals including heavy and transition elements such as Co^{2+}, Ni^{2+}, Pb^{2+}, Sn^{2+}, Zn^{2+}, Cd^{2+}, and Cu^{2+} [10–16]. Mercury is among the metal ions known for their potencies in increasing the activity of heme oxygenase in the kidney [11]. In addition, the kidney is known as the target tissue for Hg^{2+} toxicity [17–21].

The present study was undertaken to characterize the kidney biliverdin reductase with respect to pH optima and cofactor requirements, and to examine the effect of various metal ions on the activity of the reductase in the kidney and the liver.

MATERIALS AND METHODS

Materials and treatment of animals. Sprague–Dawley rats (180–220 g) were used throughout the study. Bilirubin and biliverdin hydrochloride were obtained from Porphyrin Products, Logan, UT. NADH and NADPH were obtained from the Sigma Chemical Co., St. Louis, MO. All other chemicals were of analytical reagent grade available commercially.

The animals were allowed access to food and water *ad lib.* and were maintained on a 12 hr light (7:00 a.m. to 7:00 p.m.) and dark cycle. Rats were injected subcutaneously, 24 hr before killing, with a single dose of 200 μmoles/kg of $NiCl_2$, $CoCl_2$ or $SnCl_2$, 100 μmoles/kg of Pb $(C_2H_3O_2)_2$, 600 μmoles/kg of $ZnSO_4$ or 5 μmoles/kg of Na_2SeO_3. Mercuric chloride ($HgCl_2$) was administered subcutaneously at 5, 10, 20 or 30 μmoles/kg. The listed compounds were dissolved in saline. The control groups received the vehicle. Sodium selenite was injected 30 min

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before the adminstration of Hg^{2+} (20 μmoles/kg). All injections were made between 8:30 and 9:30 a.m.

Tissue preparation. The animals were decapitated, and the liver and kidney were perfused *in situ* with saline. The organs were homogenized in 5 vol. of Tris–HCl buffer (0.01 M, pH 7.5) containing 0.25 M sucrose. The homogenates were centrifuged for 20 min at 10,000 *g*. The cytosol fractions were prepared by centrifuging the 10,000 *g* supernatant fractions at 105,000 *g* for 1 hr.

Purification of biliverdin reductase. The biliverdin reductase was purified to homogeneity from the rat liver with slight modification of the procedure described before [1]. It was observed that the addition of 0.1 mM dithiothreitol (DTT) to the various buffer systems used for the enzyme purification enhanced the stability of the enzyme preparation. Rat livers were homogenized in 0.1 M sodium citrate buffer, pH 5.0, containing 10% glycerol and 0.1 mM DTT. The homogenate was centrifuged at 9000 *g* for 20 min. The resulting supernatant fraction was centrifuged at 105,000 *g* for 1 hr, and the supernatant fraction was subjected to $(NH_4)_2SO_4$ precipitation. The precipitate obtained between 25 and 50% saturation of $(NH_4)_2SO_4$ was dissolved in 0.1 M potassium phosphate buffer, pH 7.4, containing 10% glycerol and 0.1 M DTT. Thereafter, solid KCl was added to a concentration of 2.0 M, the mixture was centrifuged, and the supernatant fraction was applied to an NADP–Agarose column preequilibrated with 0.1 M potassium phosphate buffer, pH 7.4, containing 10% glycerol, 0.1 mM DTT and 2.0 M KCl. The column was washed with the equilibrating buffer containing 1 mM NADH, and the enzyme was eluted with the equilibrating buffer containing 1 mM NADPH. The active fractions were pooled and subjected to gel filtration on a Sephadex G-200 (1.6 × 40 cm) column, equilibrated with 0.1 M potassium phosphate buffer, pH 7.4, containing 10% glycerol. Dithiothreitol was not added to the buffer system at this step of purification, since the presence of DTT was found to interfere with the reactivity of Hg^{2+} and other metal ions with the enzyme. The enzyme preparation showed purity and specific activity in the range reported previously [1].

Enzyme assay. The biliverdin reductase activity was determined by measuring the rate of bilirubin formation at 450 nm as described earlier [1]. For the routine measurements, a reaction mixture (1.0 ml) consisting of an appropriate amount of the cytosol preparation (0.5 to 1 mg protein), 0.1 mM NADPH, 5.0 μM biliverdin and 0.1 M Tris–HCl buffer, pH 8.7, was used. The reaction was carried out at 25° in an Aminco Chance DW-2 spectrophotometer. All measurements and preparations were made in subdued lighting. The amount of each cytosol preparation used in assay mixture was adjusted to obtain a linear rate of activity with respect to enzyme concentration. The enzyme activity was calculated using a millimolar extinction coefficient of 53 mM^{-1} cm^{-1} for bilirubin at 450 nm. Kinetic data, double-reciprocal plots, were analyzed by least-squares linear regression analysis.

The NADH-dependent biliverdin reductase activity was measured in a similar manner. The reaction mixture (1.0 ml) contained the appropriate amount of cytosol, 1.0 mM NADH, 5.0 μM biliverdin, and 0.1 M potassium phosphate buffer, pH 7.0.

In vitro *experiments.* The effects of metal ions or thiol reagents on biliverdin reductase activity were tested as follows. The purified biliverdin reductase or the cytosol preparations were preincubated with the reagents for 3 min at 25° prior to use as the enzyme source in the assay mixtures.

Estimation of mercury in kidney homogenate. The estimation was performed with a Varian AA-475 series atomic absorption spectrophotometer using a model 65 vapor generation accessory. Samples (1 ml of kidney homogenate equivalent to 30 mg of protein) were digested with concentrated HNO_3 (10 ml) until complete dissolution and, thereafter, diluted with distilled water. The estimation of mercury was performed on aliquots according to the flameless procedure described by Hatch and Ott [22] as simplified by Stux and Parker [23].

Other methods. Protein was measured by the method of Lowry *et al.* [24]. The *in vivo* experiments were repeated four to six times, and the data were analyzed using Student's *t*-test. The results of *in vivo* experiments are presented as mean ± S.D. The *in vitro* experiments were repeated three times, and the representative values are presented.

RESULTS

The pH and cofactor requirements for the kidney biliverdin reductase activity.

The rat kidney cytosol biliverdin reductase catalyzed the reduction of biliverdin into bilirubin in the presence of pyridine nucleotides, NADPH and NADH; however, NADPH was the more effective cofactor (Table 1). The kidney biliverdin reductase activity exhibited the complete spectrum of characteristics of the purified rat liver biliverdin reductase [1]. The enzyme exhibited maximal activity at pH 8.7 when NADPH was used as the cofactor. At this pH, the NADH-dependent activity was not detectable. A pH optimum of 7.0 was observed when NADH was used as a cofactor. At this pH, when NADPH was used as the cofactor the reductase activity was negligible. The NADPH-dependent activity measured at optimum pH value was inhibited by concentrations of NADH in excess of 0.5 mM, whereas the NADH-dependent activity was severely inhibited by minute amounts of NADPH (0.001 mM). Both activities were inhibited by concentrations of substrate, biliverdin, in excess of 5.0 μM (data not shown). The apparent K_m value for biliverdin was 1.6 μM and was independent of the nature of the cofactor.

Effect in vivo *of metal ions on biliverdin reductase.* The effects of Co^{2+}, Ni^{2+}, Pb^{2+}, Sn^{2+}, Zn^{2+}, Cd^{2+}, and Cu^{2+} on the kidney and the liver were investigated. These metal ions and their dosages were selected on the basis of previous findings, demonstrating their potent abilities to induce heme oxygenase in the liver and the kidney. The possible effect of Co^{2+} on biliverdin reductase activity was of particular interest in the light of a recent report [7] on the induction of the enzyme in the liver by the metal ion. It was observed that the treatment of rats (24 hr) with large doses (100–600 μmoles/kg) of the

Table 1. Cofactor requirements and the pH optima of rat kidney biliverdin reductase*

Reaction condition	Biliverdin reductase activity [nmoles bilirubin · (mg protein)$^{-1}$ · hr^{-1}]
At pH 8.7 with:	
1.0 mM NADH	0
0 mM NADPH	0
0.01 mM NADPH	64.4
0.10 mM NADPH	92.1
1.00 mM NADPH	92.8
0.01 mM NADPH + 0.5 mM NADH	66.3
0.01 mM NADPH + 5.0 mM NADH	6.4
At pH 7.0 with:	
0.1 mM NADPH	2.3
0 mM NADH	0
0.1 mM NADH	19.9
1.0 mM NADH	59.8
10 mM NADH	71.7
1.0 mM NADH + 0.001 mM NADPH	19.2

* The activity of biliverdin reductase in the kidney cytosol fraction was measured using standard assay conditions described in Materials and Methods. Buffers, 0.1 M Tris–HCl or 0.1 M potassium phosphate, were used to obtain the indicated pH values. In experiments in which both NADPH and NADH were used, NADH was added to the enzyme preparation prior to the addition of NADPH.

metal ions did not alter the activity of the enzyme in the liver and the kidney (data not shown). Moreover, the finding reported by Frydman *et al.* [7] on the induction of the enzyme in the liver could not be substantiated.

Effect in vivo *of mercuric chloride on kidney biliverdin reductase activity.* The effect of Hg^{2+} treatment (20 μmoles/kg, 24 hr) on the rate of NADPH-and NADH-dependent biliverdin reductase activities was determined, using the kidney cytosol preparation as the enzyme source and measuring the increase in absorbance at 450 nm (Fig. 1, a and b). Using the control preparation, as shown, the rate of enzyme activity with NADPH as the cofactor was linear with respect to time for approximately 40–45 sec. and that of NADH-dependent activity was linear for up to 75–80 sec. Hg^{2+} treatment did not alter the pattern of rapid deviation from linearity of enzyme activity. However, this treatment caused a 40% reduction in the initial rate of enzyme activity with both cofactors. As shown, the NADPH- and NADH-dependent activities exhibited similar responses to Hg^{2+} treatment *in vivo*. The similarity of the repsonses plus the fact that NADPH was apparently the preferred cofactor over NADH prompted us to use NADPH as the cofactor for other experiments conducted in this study.

The dose–response and the time-dependent effects of Hg^{2+} treatment on the enzyme activity were measured. Rats were treated with 5, 10 and 30 μmoles/kg of Hg^{2+} and were killed after 24 hr. As shown in Fig. 2, the reductase activity was decreased significantly in a dose-dependent manner following Hg^{2+} treatment. The enzyme activity in animals treated with 10 μmoles/kg was decreased by approximately 30%, and by nearly 50% following administration of the metal ion at a dose of 30 μmoles/kg. The activity was not decreased significantly in response to treatment with 5 μmoles/kg Hg^{2+}. Moreover, a reduction in the

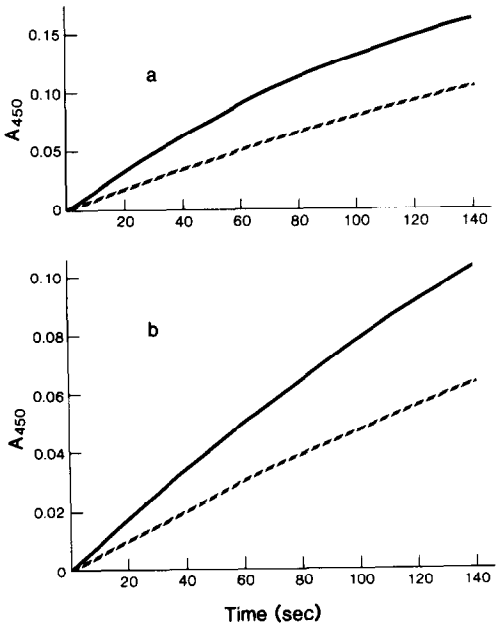

Fig. 1. Effect *in vivo* of mercuric chloride on NADPH- and NADH-dependent biliverdin reductase activities of the rat kidney cytosol. Rats were treated with $HgCl_2$ (20 μmoles/kg, s.c.) and were killed 24 hr later. The control group received saline. The kidney cytosol fraction was prepared as described in Materials and Methods and was used as the enzyme source. The NADPH-dependent biliverdin reductase activity was determined using a reaction mixture (1.0 ml) containing cytosol (0.8 mg protein), 0.1 mM NADPH, 5.0 μM biliverdin and 0.1 M Tris–HCl buffer, pH 8.7, at 25°. The NADH-dependent activity was determined using a reaction mixture containing cytosol (0.8 mg protein), 1.0 mM NADH, 5.0 μM biliverdin, and 0.1 M potassium phosphate buffer, pH 7.0. The experimental details are provided in Materials and Methods. Key: (a) NADPH-dependent biliverdin reductase activity; and (b) NADH-dependent biliverdin reductase acitivity; (——) control; and (---) Hg^{2+}-treated.

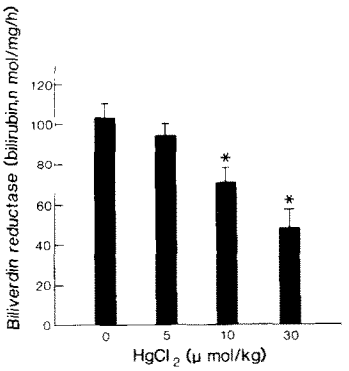

Fig. 2. Dose-dependent *in vivo* effect of mercuric chloride on rat kidney biliverdin reductase activity. Groups of four rats (180–220 g) were treated (s.c.) with 0, 5, 10 or 30 μmoles $HgCl_2$/kg. After 24 hr the animals were killed, the kidney cytosol fractions were prepared, and biliverdin reductase activity was determined using NADPH as the cofactor. Detailed procedure is outlined in Materials and Methods. The data presented are the means ± S.D. of six rats. Key: (*) $P \leq 0.05$, when compared to the control animals.

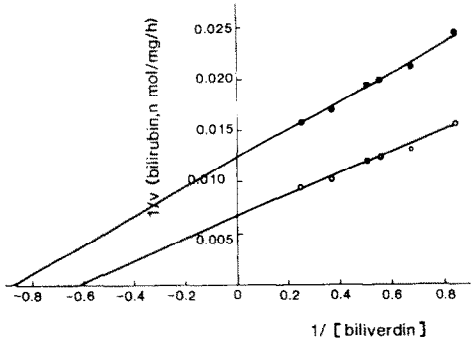

Fig. 3. Lineweaver–Burk plot of the kidney biliverdin reductase activity of control and mercuric chloride-treated rats. The NADPH-dependent biliverdin reductase activities of kidney cytosol preparations obtained from the control and Hg^{2+}-treated (10 μmoles/kg, 24 hr) rats were estimated as described in Materials and Methods. Key: (○——○) control; and (●——●) Hg^{2+}-treated.

biliverdin reductase activity was not observed when the duration of treatment with 5, 10, and 30 μmoles/kg Hg^{2+} was reduced to 3 or 12 hr.

The possibility that the observed difference in the response of biliverdin reductase to Hg^{2+} at 12 and 24 hr was related to the differential levels of the metal ion in the kidney was investigated. Two groups of rats were treated with 10 μmoles/kg of Hg^{2+} and the animals were killed 12 and 24 hr later. The Hg^{2+} content in the kidneys was estimated as described in Materials and Methods. The tissue metal ion levels measured 159.08 ± 22.73 nmoles/g and 148.88 ± 16.38 nmoles/g kidney weight at 12 and 24 hr respectively. Therefore, it appears that the Hg^{2+}-mediated inhibition of biliverdin reductase activity at 24 hr after treatment was not a reflection of the tissue content of the metal ion.

The kinetic parameters of biliverdin reductase activity using the control kidney cytosol fraction and the cytosol preparation obtained from the Hg^{2+}-treated rats (10 μmoles/kg, 24 hr) were investigated. The Lineweaver–Burk plot of the enzyme activity is shown in Fig. 3. As noted, the maximal velocity of the reductase was considerably reduced following Hg^{2+} treatment *in vivo*. However, the apparent K_m of the enzyme for biliverdin was only slightly reduced. The values for the control and the Hg^{2+}-treated animals measured 1.6 and 1.2 μM respectively.

The effect *in vivo* of Hg^{2+} on the liver biliverdin reductase activity was also assessed. The enzyme in this organ was not decreased significantly in comparison to control value (59.2 ± 3.6 nmoles bilirubin per mg protein per hr vs 51.3 ± 4.9 nmoles bilirubin per mg protein per hr) in animals treated with 30 μmoles/kg Hg^{2+} (24 hr). Similarly, the enzyme activity was not altered in response to treatment with 5 and 10 μmoles/kg Hg^{2+} (24 hr).

Effect of selenium on Hg^{2+}-mediated decrease in the kidney biliverdin reductase activity. Selenium compounds, selenite in particular, are known to have an inhibitory effect on the biological activities of Hg^{2+} [25–29]. Accordingly, the effect of selenium on Hg^{2+}-mediated inhibition of the kidney biliverdin reductase was investigated. As shown in Table 2, treatment of animals with 20 μmoles/kg Hg^{2+} caused a 40% reduction in the kidney biliverdin reductase

Table 2. Inhibition *in vivo* of the kidney biliverdin reductase by mercuric chloride and the reversal of inhibition by selenium

Treatment and dose	Biliverdin reductase activity [nmoles · (mg protein)$^{-1}$ · hr^{-1}]
Control	84.6 ± 2.4
Hg^{2+} (20 μmoles/kg)	50.9 ± 6.0†
Selenium (5 μmoles/kg)	84.6 ± 3.0
Hg^{2+} (20 μmoles/kg) + Selenium (5 μmoles/kg)	78.0 ± 1.8

* Groups of four rats were injected (s.c.) with indicated doses of $HgCl_2$ or Na_2SeO_3. When both salts were injected, selenium was administered 30 min prior to Hg^{2+}. The animals were killed 24 hr later, the kidney cytosol fractions were prepared, and biliverdin reductase activity was estimated as described in Materials and Methods. The data presented are means ± S.D.

† $P \leq 0.05$, when compared to the control animals.

Table 3. *In vitro* effect of metal ions and selenium on rat kidney biliverdin reductase activity*

Additions	Concn (μM)	Relative activity (% of control)
Control	0	100
Ni^{2+}	250	59
Pb^{2+}	250	76
Sn^{2+}	125	38
Zn^{2+}	25	54
Cd^{2+}	10	57
Cu^{2+}	10	52
Co^{2+}	10	60
Hg^{2+}	10	20
Hg^{2+}	5	48
Selenium	1000	100
Hg^{2+} + Selenium	5 1000	48

* The kidney cytosol fraction obtained from the control rats was utilized. The metal salts were dissolved in buffer (Tris–HCl, pH 8.7), and 10 μl aliquots of the solutions were added to biliverdin reductase enzyme source in 0.1 M Tris–HCl buffer, pH 8.7. The reaction was started after 3 min by the addition of the substrate and the cofactor (NADPH). The activity of the enzyme was measured as described in Materials and Methods. The control value was designated as 100% and measured 98.2 nmoles bilirubin per mg protein per hr. The data shown are representative of three determinations.

activity. The treatment of rats with selenium (5 μmoles/kg), 30 min prior to the injection of Hg^{2+} (20 μmoles/kg), effectively blocked the inhibitory effect of Hg^{2+} on the enzyme. The kidney biliverdin reductase in this group of rats measured 92% of that of the control group. Treatment of rats with selenium alone did not elicit a significant change in the kidney biliverdin reductase activity.

In vitro *studies.* The effects *in vitro* of Hg^{2+} and those of Ni^{2+}, Pb^{2+}, Sn^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, and Co^{2+} on the activity of the rat kidney biliverdin reductase are shown in Table 3. The kidney cytosol fraction prepared from the control rats was preincubated with various concentrations of metal ions for 3 min at 25° prior to assay for enzyme activity. The NADPH-dependent biliverdin reductase activity was estimated as described in Materials and Methods. As shown, the reductase activity was effectively inhibited by Hg^{2+}, Cd^{2+}, Cu^{2+} and Co^{2+}, at final concentrations of 10 μM. The extent of inhibition of the enzyme activity ranged from 40 to 80%. However, Ni^{2+}, Pb^{2+}, Sn^{2+} and Zn^{2+} required higher concentrations to exert an inhibitory effect on the reductase. Hg^{2+} at a concentration of 10 μM produced 80% inhibition of enzyme activity, and at a 5 μM concentration it produced 52% inhibition of the activity.

In vitro selenium up to a 1 mM concentration was ineffective in altering biliverdin reductase activity when tested in a system containing the kidney cytosol preparations obtained from Hg^{2+}-treated and control animals. Similarly, selenium (1 mM) was ineffective in blocking the *in vitro* inhibitory action of Hg^{2+} on the reductase activity. The *in vitro* effect of Hg^{2+} on the kidney biliverdin reductase activity of Hg^{2+}-treated rats was also investigated (Table 4). It was observed that not only the enzyme activity in this preparation was inhibited by the addition of Hg^{2+}, but the extent of inhibition was somewhat greater than that produced in the control enzyme preparation. The molecular basis for the increased *in vitro* sensitivity of the enzyme to Hg^{2+} is not clear.

The mechanism of *in vitro* inhibitory action of Hg^{2+} on biliverdin reductase activity was further investigated using purified rat liver enzyme. The inhibitory effects of Hg^{2+} and that of the thiol reagent, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), on the enzyme activity were compared with respect to the possibility of the reversal of the inhibition by DTT, a sulfhydryl compound. Previous studies with the purified liver biliverdin reductase had revealed that the reductase is exceedingly sensitive to the inhibitory action of sulfhydryl reagents including DTNB [1]. As shown in Table 5, DTNB and Hg^{2+} at concentrations of 1 and 0.1 μM, respectively, fully inhibited the enzyme activity. However, whereas the treatment of the DTNB-inhibited enzyme with DTT (100 μM) restored the reductase activity by nearly 90%, the inhibitory action of Hg^{2+} was not reversed by treatment with the sulfhydryl compound. Dithiothreitol itself had no effect on the enzyme activity, that is, at the concentration used (100 μM) non-enzymatic reduction of biliverdin was not detected. The results support the conclusion [1] that biliverdin reductase depends on thiol group(s) for its activity, which suggests that the interaction of the thiol group(s) of the enzyme with Hg^{2+} is covalent in nature.

The possibility that the observed *in vivo* inhibitory effect of Hg^{2+} on the reductase is caused by the metal

Table 4. Effect *in vitro* on mercuric chloride on the kidney cytosol biliverdin reductase activity of mercuric chloride-treated rats*

$HgCl_2$ (μM)	Biliverdin reductase activity (% of control)	
	Saline-treated	Hg^{2+}-treated
0	100	100
1	100	84
2	90	74
3	86	58
5	56	10
8	8	0

* The kidney cytosol fractions were obtained from rats treated (s.c.) with saline or $HgCl_2$ (20 μmoles/kg, 24 hr). $HgCl_2$ was dissolved in buffer, and 10-μl aliquots of the solutions were added to the enzyme preparation. Hg^{2+} was allowed to react with the enzyme preparation in the buffer for 3 min prior to the addition of other assay components. The reaction mixture (1.0 ml) contained rat kidney cytosol preparation (0.8 mg), 100 μM NADPH, 5.0 μM biliverdin and indicated concentrations of Hg^{2+} in Tris–HCl buffer (0.1 M, pH 8.7). The activity of the enzyme was measured as described in Materials and Methods. The control value was designated as 100% and measured 86.2 nmoles bilirubin per mg protein per hr for the saline-treated rats and 51.4 nmoles bilirubin per mg protein per hr for the Hg^{2+}-treated animals. The data shown are representative of three determinations.

Table 5. Effect of dithiothreitol on the inhibition of biliverdin reductase by 5,5'-dithiobis-(2-nitrobenzoic acid) and mercuric chloride*

Condition	Biliverdin reductase activity (% of control)
1. Complete reaction mixture	100
2. Reaction mixture with enzyme + DTT (100 μM)	100
3. Reaction mixture with enzyme treated with DTNB (1 μM)	0
4. Reaction mixture with enzyme treated with DTNB (1 μM) + DTT (100 μM)	90
5. Reaction mixture with enzyme treated with Hg^{2+} (100 nM)	0
6. Reaction mixture with enzyme treated with Hg^{2+} (100 nM) + DTT (100 μM)	0
7. Reaction mixture without enzyme	0
8. Reaction mixture without enzyme + DTT (100 μM)	0

* The complete reaction mixture (1.0 ml) contained NADPH (100 μM), biliverdin (5.0 μM), and purified rat liver biliverdin reductase (0.1 μg, activity = 3260.0 nmoles bilirubin per mg per min) and Tris–HCl buffer (0.1 M, pH 8.7). The enzyme was treated with DTNB or Hg^{2+} for 3 min, in the buffer, prior to the addition of NADPH and biliverdin. Reaction was carried out as described in Materials and Methods. The data shown are representitive of three determinations. Abbreviations: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); and DTT, dithiothreitol.

ion present in the cytosol preparation was investigated. The kidney cytosol fraction obtained from rats treated with Hg^{2+} (20 μmoles/kg, 24 hr) was dialyzed for 24 hr at 0–4° against 0.1 M potassium phosphate buffer containing 10 mM EDTA. The biliverdin reductase acitivity in the samples was analyzed before and subsequent to dialysis. The specific activity of biliverdin reductase remained unchanged by dialysis, suggesting that the *in vivo* inhibitory action of Hg^{2+} reflects the interaction of the metal with enzyme protein rather than the reactivity of free Hg^{2+} with components of the assay system.

The activities of certain enzymes are known to be regulated by disulfide exchange. Accordingly, this possibility was tested by examining the effects of several sulfhydryl and disulfide compounds on Hg^{2+}-inhibited biliverdin reductase. In these studies, the kidney cytosol preparation of Hg^{2+}-treated rats and that of control rats were incubated at 0° and 37° for 0–1 hr with the following compounds: cysteine, cystine, cysteamine, cystamine, reduced glutathione, oxidized glutathione and DTT. These compounds were dissolved in dilute solutions of HCl or NaOH, as required, the pH was adjusted to 7.4, and small aliquots were added to cytosol fractions to obtain a final concentration of 1 mM. It was observed that the incubation of kidney cytosol preparations with the listed thiol compounds for various intervals (up to 1 hr) did not reactivate the Hg^{2+}-inhibited enzyme activity. Also, the treatment of the control rat kidney cytosol preparations with the listed agents did not modify the rate of enzyme activity.

DISCUSSION

The rat kidney biliverdin reductase, as the liver enzyme, exhibited unique catalytic properties with respect to the pH optimum and cofactor requirement. The enzyme displayed two distinctly different pH optima for activity with NADPH (pH 8.7) and NADH (pH 7.0). Moreover, the enzyme from both the kidney and the liver was non-inducible by a host of metal ions known for their abilities to induce heme oxygenase activity. Rather, the enzyme activity in the kidney was severely inhibited in response to Hg^{2+} treatment. The previously reported capacity of Co^{2+} to induce the activity of the enzyme [7] could not be substantiated. The presently observed inability of Co^{2+}, in specific, and metal ions, in general, to elicit an induction response in biliverdin reductase activity may reflect intrinsic differences in sex and species of rats used in the present study and that of Frydman *et al.* [7]. Alternatively, it could represent the conditions of assay for enzyme activity. The unusual catalytic requirements of the reductase, and the exceedingly rapid rate of enzyme activity (Fig. 1), could readily lead to equivocal results if not considered. At this time, however, the biochemical basis for the observed discrepancy is not clear, since experimental detail for the reported [7] induction of biliverdin reductase by Co^{2+} is not available.

The present observed inability of metal ions to induce biliverdin reductase activity may represent a major biological safeguard against deleterious actions of bilirubin in the mammalian system. As noted earlier, the activity of heme oxygenase is readily increased in response to exposure to a host of chemicals of diverse structures including the metal ions [10–16]. However, the immediate product of heme oxygenase activity, biliverdin, is rather innocuous in biological systems when compared to bilirubin, the ultimate product of heme degradation. The array of disruptive actions of bilirubin in biological systems, including the neurotoxicity and free radical-mediated destruction of cellular membranes [30], is well established. It follows that the inability of metal ions to enhance the conversion of biliverdin to bilirubin may have a significant protective role against such effects of bilirubin. It is plausible that this property of metal ions may be shared by other

agents, known to enhance heme oxygenase activity.

Mercuric chloride is known for its potent ability to induce heme oxygenase activity in both the liver and the kidney. In the case of biliverdin reductase activity, however, a different response to the metal ion was observed in these organs. As noted, whereas the activity of the reductase in the liver was not altered in response to Hg^{2+} treatment, the activity of the enzyme in the kidney was inhibited markedly by the metal ion. The *in vivo* inhibitory action of Hg^{2+} on the kidney biliverdin reductase for the most part may reflect both the known capacity of the metal ion to accumulate in the kidney and the interaction of the agents with the -SH groups of the enzyme. The latter concept is consistent with the known high affinity of inorganic mercurials for thiol groups and with the presently reported *in vitro* experiments using the purified enzyme (Table 5) that demonstrate binding of Hg^{2+} to the sulfhydryl group(s) of the enzyme and the irreversible inhibition of the enzyme activity. The finding that several other metal ions also known for their high affinity for -SH groups, including Cd^{2+}, Cu^{2+}, and Co^{2+}, inhibited the reductase activity *in vitro* (Table 3) but, at the dose and the regimen of treatment used in the present study, did not exert similar action *in vivo* may reflect the lesser extent of accumulation of these metal ions in the kidney in comparison to Hg^{2+}.

The loss of the enzyme activity in the kidney of rats treated with Hg^{2+} may not solely reflect the direct interaction between the metal ion and the -SH group(s) of the enzyme. Rather, it may involve factors such as the inhibition by the metal ions of the synthesis of the enzyme protein, or the inhibition of enzyme activity by the substrate, and/or the product of reductase activity [1]. The finding (Table 4) that the activity of the kidney biliverdin reductase of the Hg^{2+}-treated rats could be further inhibited *in vitro* by Hg^{2+} suggests that the presently observed (Fig. 2) *in vivo* inhibition of the enzyme activity by Hg^{2+} does not solely represent the binding of the metal ion to the enzyme active site and the direct inhibition of the activity. The possibility of inhibitory actions of the substrate, biliverdin, and the product, bilirubin, on biliverdin reductase activity is consistent with the previous finding [1] that the activity of purified liver biliverdin reductase is remarkably sensitive to both the substrate and the product concentration. In studies with the purified rat liver, enzyme bilirubin and biliverdin in excess of 0.5 and 20 μM, respectively, were shown to inhibit the enzyme activity by nearly 50%. Considering the known ability of Hg^{2+} to induce kidney heme oxygenase activity, the possibility of excess production of biliverdin and, subsequently, bilirubin in the kidney of the treated animals is plausible. Of course, the possibility of loss of enzyme protein subsequent to Hg^{2+} exposure cannot be ruled out since it is known that the exposure of rats to Hg^{2+} leads to the expression of proteinurea [31]. This factor could be responsible for the inhibition of the enzyme activity at 24 hr, but not at 3 and 12 hr, after Hg^{2+} treatment. However, unless a selective loss of biliverdin reductase enzyme protein had occurred in the treated animals in the present experiments, the likelihood of a major contribution by this factor to the decrease observed at 24 hr in enzyme activity subsequent to Hg^{2+} treatment is rather unlikely.

The precise mechanism by which the *in vivo* inhibition of biliverdin reductase activity by Hg^{2+} was blocked by selenium is not clear. The inactivation of Hg^{2+} through direct interaction with selenium is a distinct possibility. The *in vitro* studies (Table 3) indicate that a direct interaction between Hg^{2+} and selenium (as Ne_2SeO_3) did not occur. However, the observed *in vivo* protection by selenium, when administered as Na_2SeO_3, against the inhibitory effects of Hg^{2+} may occur following biotransformation of SeO_3^{2-} to Se^{2-} [32]. It has been postulated that the conversion of SeO_3^{2-} to Se^{2-} is required for interaction of the element with metal ions [32, 33]. However, judging by the finding that the treatment of rats with a 1:4 molar ratio of selenium/Hg^{2+} was fully effective in preventing the inhibitory action of Hg^{2+} on the reductase, it is unlikely that the observation solely represents a direct selenium/Hg^{2+} interaction. In addition, the presently employed regimen of treatment of animals with the agents (Hg^{2+} was injected 30 min after selenium) does not favor the exclusive occurrence of selenium/Hg^{2+} interaction. Moreover, experimental findings [26] indicate the accumulation and the occurrence of selenium and Hg^{2+} in the mammalian systems with an atomic ratio of one. It follows that the recently described [34] effects of selenium to increase the levels of reduced glutathione (GSH) in the liver and kidneys and to induce the activities of glutathione reductase and γ-glutamylcysteine synthetase, the rate-limiting enzyme in glutathione biosynthesis, may also be involved in the blockade by selenium of Hg^{2+} inhibition of biliverdin reductase. Such action of selenium has been postulated to play a role in protection against Hg^{2+}-mediated inhibition of the activities of the enzymes of the glutathione metabolism pathway [35].

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