Phenylhydrazine-mediated induction of haem oxygenase activity in rat liver and kidney and development of hyperbilirubinaemia

Inhibition by zinc-protoporphyrin

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Phenylhydrazine was found to be a potent inducer of microsomal haem oxygenase activity in rat liver and kidney, but not in spleen. The phenylhydrazine-mediated increase in haem oxygenase activity was time-dependent. Maximum activity was attained 12 h after treatment in the liver, and 24 h after treatment in the kidney. The increases in the activity of haem oxygenase in the liver and the kidney could be inhibited by cycloheximide. Furthermore, the increases could not be elicited by the treatment of microsomal preparations *in vitro* with phenylhydrazine. In consonance with the increased haem oxygenase activity, a marked increase (16-fold) was observed in the serum total bilirubin concentration in phenylhydrazine-treated rats. The mechanism of haem degradation promoted by phenylhydrazine *in vivo* appears to differ from that *in vitro*; only in the former case is bilirubin formed as the end-product of haem degradation. When rats were given zinc-protoporphyrin ($40 \mu\text{mol/kg}$) 12 h before and after phenylhydrazine treatment, the phenylhydrazine-mediated increases in haem oxygenase activity in the liver and the kidney were effectively blocked. Treatment of rats *in vivo* with the metalloporphyrin also inhibited the activity of splenic haem oxygenase, and promoted a major decrease in the serum bilirubin levels. In phenylhydrazine-treated animals, the microsomal content of cytochrome *P*-450 was significantly decreased in the absence of a decrease in the microsomal haem concentration. The decrease in cytochrome *P*-450 content was accompanied by an increased absorption in the 420 nm region of the reduced CO-difference spectrum, suggesting the conversion of the cytochrome to an inactive form. The marked depletion of cellular glutathione levels suggests that this conversion may be related to the action of active intermediates and free radicals formed in the course of the interaction of phenylhydrazine with the haem moiety of cytochrome *P*-450.

Hydrazine (-N-N-) derivatives are chemically and biologically highly reactive compounds, and are used in a host of applications, including therapeutics and the defence industry (Juchau & Horita, 1972). Also, hydrazine compounds are recognized for their biological toxicity, which includes methaemoglobinaemia, carcinogenesis, and hyperbilirubinaemia. The haemolytic action of phenylhydrazine has been the subject of much research. However, interest has usually centred

around the nature of the oxygen species generated in the course of the phenylhydrazine reaction, and the identification of haemolytic active intermediates (Harley & Mauer, 1960; Cohen & Hochstein, 1964; Goldberg & Stern, 1975; Augusto *et al.*, 1982). In the presence of haemoglobin, phenylhydrazine causes the increased production of H_2O_2 , an end-product of the dismutation of O_2^- within the erythrocytes. Moreover, the alkylation of haem and the formation of *N*-phenylhaem in the haemolytic reaction of phenylhydrazine has been demonstrated (Ortiz de Montellano & Kunze, 1981). In addition to its oxidative effect on haemoglobin, phenylhydrazine also causes oxidative alterations in the integrity of blood cell mem-

Abbreviations used: GSH and GSSG, reduced and oxidized glutathione.

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branes, thus enhancing its haemolytic action (Jain & Hochstein, 1980). Phenylhydrazine-mediated haemolysis is accompanied by the development of hyperbilirubinaemia. However, the biochemical events underlying this observation remain obscure.

Lemberg & Legge (1942) in their classical studies proved that choleglobin and biliverdin are formed in the circulating erythrocytes of rabbits treated with phenylhydrazine. Since erythrocytes are devoid of endoplasmic reticulum, the source of bile pigments in these cells would appear to be the nonenzymic degradation of haemoglobin haem, in accordance with the model systems previously described (Kondo *et al.*, 1971; O'Carra & Colleran, 1976; Brown *et al.*, 1980). Nonetheless, clearly a potential exists for the stimulatory action of haemoglobin released in the course of the haemolysis of erythrocytes on the activity of microsomal haem oxygenase. This, in turn, could significantly contribute to an increase in the serum levels of bilirubin. These concepts are consistent with the findings of the following investigations: the induction of haem oxygenase activity in the rat kidney by haemoglobin infusion (Pimstone *et al.*, 1971); the promotion of an increase in haem oxygenase activity by methaemalbumin in the rat liver (Tenhunen *et al.*, 1970); and the development of postparturition hyperbilirubinaemia which accompanies increased haem oxygenase activity in the liver and the kidney in rats (Maines & Kappas, 1978). Similarly, the possibility of a direct haem oxygenase-inducing action of phenylhydrazine and/or its metabolites cannot be dismissed.

Haem oxygenase, the rate-limiting enzyme in the degradation of haem (Tenhunen *et al.*, 1968), oxidatively cleaves the haem molecule to form biliverdin. The latter is subsequently reduced to form bilirubin, an activity that is catalysed by the cytosolic enzyme, biliverdin reductase (Colleran & O'Carra, 1977; Kutty & Maines, 1981). The ability of synthetic metalloporphyrins, Zn-protoporphyrin and Sn-protoporphyrin, to inhibit competitively *in vivo* the activity of haem oxygenase in rat liver, kidney and spleen, and to curtail increased production of serum bilirubin, has recently been described (Maines, 1981; Drummond & Kappas, 1982). Moreover, studies *in vivo* with purified biliverdin reductase have revealed the inhibiting action of these metalloporphyrins on the activity of the enzyme (Kutty & Maines, 1981). Therefore, it would appear reasonable to assume that a curtailment of the elevation of the serum bilirubin levels, subsequent to the administration of synthetic metalloporphyrins, would reflect the enzymic mode of haem degradation.

The present study was undertaken to investigate the effect of phenylhydrazine on cellular haem and haemoprotein metabolism, to assess the contribu-

tion of the enzymic mode of haem degradation to phenylhydrazine-mediated hyperbilirubinaemia, and to explore the usefulness of Zn-protoporphyrin in suppressing haem oxygenase activity in experimentally induced hyperbilirubinaemia.

Experimental

Materials

Male Sprague-Dawley rats (180–200g) were used throughout the study. The animals were allowed access to food and water *ad libitum* throughout the experiments. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Porphyrins were purchased from Porphyrin Products, Logan, UT, U.S.A. Phenylhydrazine was a product of Sigma. Solutions of Zn-protoporphyrin, haematin and biliverdin were prepared as described elsewhere (Kutty & Maines, 1982). Rats were injected between 08:00h and 09:00h with phenylhydrazine (100mg/kg body wt. in saline, subcutaneously) and were killed 12 or 24h later. Zn-protoporphyrin was administered (40 μ mol/kg body wt., intraperitoneally) 12h before, as well as 12h after, phenylhydrazine treatment, as indicated in the appropriate Figures and Tables. The control animals received iso-osmotic saline.

Tissue preparations

Blood samples were drawn by cardiac puncture immediately before killing the animals, and were used for the preparation of serum and the estimation of serum total bilirubin. The animals were decapitated, and unless otherwise indicated, the livers were perfused *in situ* with 0.9% NaCl (4°C) until fully blanched. Thereafter, the liver, spleen, and kidneys were removed and homogenized in 0.05M-Tris/HCl buffer (pH 7.4) containing 0.25M-sucrose. For the measurement of reduced glutathione (GSH) levels in unperfused tissues, the tissues were homogenized in the extraction mixture described below. The mitochondrial, microsomal and cytosolic fractions were prepared by the differential centrifugation described before (Kutty & Maines, 1982). The microsomal fraction was 'washed' prior to use for the determination of the contents of haem and cytochromes. Biliverdin reductase was purified from rat liver (Kutty & Maines, 1981) and had a specific activity of approx. 3500nmol of bilirubin/min per mg of protein.

Assay procedures

Freshly prepared subcellular fractions were utilized in all experiments. Haem oxygenase activity was measured using protohaem (haem, Fe-protoporphyrin IX) as the substrate according to the

procedure described previously (Maines & Kappas, 1975). Unless otherwise indicated, a reaction mixture (1.4 ml), consisting of 15 μ M-haematin, microsomal fractions (0.5–1 mg of protein/ml), 0.85 mM glucose 6-phosphate, 3 units of glucose-6-phosphate dehydrogenase and 2 mM-MgCl₂, in a 90 mM-potassium phosphate buffer (pH 7.4), was preincubated for 5 min at 37°C, and thereafter divided into two equal aliquots. A mixture of NADP⁺ and an excess amount (50–100 μ g of protein) of purified rat liver biliverdin reductase in a 100 μ l of potassium phosphate buffer was added to the test incubation mixture. The blank incubation mixture did not receive NADP⁺. The duration of incubation was 10 min at 37°C. The formation of bilirubin was measured from the difference in absorption between 464 and 530 nm of the blank and the test incubation mixtures, using an absorption coefficient of 40 mM⁻¹·cm⁻¹ (Maines & Kappas, 1975).

The haem content was estimated by the pyridine haemochromogen method of Paul *et al.* (1953) measuring the reduced minus oxidized difference spectrum between 557 and 575 nm, and utilizing an absorption coefficient of 31.0 mM⁻¹·cm⁻¹. The contents of cytochromes P-450 and P-420 were measured by the procedure of Omura & Sato (1964). The reduced CO-difference spectrum of the suspension was obtained by using sodium dithionite as the reducing agent. The concentration of cytochrome P-450 was calculated by using a difference absorption coefficient of 91 mM⁻¹·cm⁻¹ between 450 and 490 nm, and that of cytochrome P-420 was determined using a difference absorption coefficient of 111 mM⁻¹·cm⁻¹. Biliverdin reductase activity was determined as described before (Kutty & Maines, 1981) from the increase in absorbance at 450 nm of a reaction mixture (1.0 ml) consisting of 100 μ M-NADPH, 5 μ M-biliverdin and cytosol preparation (300–500 μ g of protein) in a 0.1 M-Tris/HCl buffer (pH 8.7). An absorption coefficient of 53 mM⁻¹·cm⁻¹ was used.

Hepatic GSH levels were measured by a modification of the method of Cohn & Lyle (1966). The tissue was homogenized in 5 vol. of an extraction mixture consisting of 10% (w/v) trichloroacetic acid/1 mM-disodium EDTA/0.01 M-HCl (1:1:1, by vol.), and protein was removed by centrifugation at 500g (10 min). Na₂HPO₄ (1 ml of 0.5 M) and o-phthalaldehyde (100 μ l of 1 mg/ml in methanol) were added to a 50 μ l sample of the supernatant fraction. The concentration of GSH was measured spectrofluorometrically using authentic GSH as the standard. The serum total bilirubin concentration was estimated by the diazo method as described by MacDonald (1965). The samples were measured in triplicate. The activity of glutathione (GSSG) reductase was determined as described by

Massey & Williams (1965) by measuring the disappearance of NADPH at 25°C using the dual beam mode of a spectrophotometer at 340 nm. Protein was measured by the method of Lowry *et al.* (1951).

All spectral studies were conducted using an Aminco-Chance DW-2 spectrophotometer. The fluorometric measurements were carried out using an Aminco-Bowman fluorometer. All experiments *in vivo* were carried out four to six times. The results are expressed as the mean $\pm$ S.D. The data were analysed by using Student's *t* test and a *P* value ≤ 0.05 was used to denote significance.

Results

Induction of microsomal haem oxygenase activity in the liver and the kidney by phenylhydrazine, and the inhibition of the activity by Zn-protoporphyrin

Fig. 1 shows the time-course of response of haem oxygenase activity in the rat liver and kidney to

Fig. 1. Time-course of increase in haem oxygenase activity in the liver (○) and the kidney (●) of rats treated *in vivo* with phenylhydrazine

Male Sprague-Dawley rats (180–200 g body wt.) were treated with phenylhydrazine (100 mg/kg, subcutaneously) and were killed 12 or 24 h after treatment. The control animals received saline. The microsomal fractions were prepared from the perfused organs, and used for the measurement of haem oxygenase activity. The enzyme activity was measured as described earlier (Maines & Kappas, 1975). The procedures are described in detail in the Experimental section. The values shown are means $\pm$ S.D. for four determinations; one rat was used for each determination. **P* ≤ 0.05 when compared with the control animals.

Fig. 2. Effect *in vivo* of phenylhydrazine and Zn-protoporphyrin on the activity of haem oxygenase in liver, spleen and kidney

Male Sprague-Dawley rats (180–200 g body wt.) were treated with (■) phenylhydrazine (100 mg/kg, subcutaneously), (▨) Zn-protoporphyrin (40 μ mol/kg, intraperitoneally), or (□) both. The control animals (□) received saline. When given both agents, Zn-protoporphyrin was injected 12 h before and 12 h after phenylhydrazine administration. The animals were killed 24 h after phenylhydrazine treatment. The livers were perfused, the organs were removed, and the microsomal fractions were prepared as described in the Experimental section. The activity of haem oxygenase was measured as described before (Maines & Kappas, 1975) using haematin as the substrate. The values shown are means $\pm$ S.D. for six determinations; one rat was used for each determination. * $P \leq 0.05$ when compared with the control animals.

phenylhydrazine treatment. In these experiments the enzyme activity was measured as the rate of conversion of haem to bilirubin. This Figure also demonstrates the differential temporal response elicited by phenylhydrazine in the activity of haem oxygenase in these organs. As shown in the liver, the maximum increase in enzyme activity was observed at 12 h, whereas in the kidney the peak haem oxygenase activity was not reached until 24 h after treatment. The splenic enzyme activity was not altered in phenylhydrazine-treated rats (results not shown). This finding is consistent with the previously noted refractory response of the splenic enzyme to certain known inducers of the liver and the kidney enzymes (Maines & Kappas, 1974).

The potent action of Zn-protoporphyrin in blocking the action of phenylhydrazine to increase the activity of haem oxygenase is shown in Fig. 2. As shown, the enzyme activity in the liver and the kidney was dramatically reduced when rats were treated with Zn-protoporphyrin 12 h before and 12 h after phenylhydrazine administration. Indeed,

Table 1. Inhibition by cycloheximide of induction of haem oxygenase by phenylhydrazine

Two groups of four male Sprague-Dawley (180–200 g body wt.) rats were injected subcutaneously with phenylhydrazine (100 mg/kg). After 4 h cycloheximide, dissolved in ethanol (0.18 mg/kg), was injected intraperitoneally into one group. The second group received the same volume of ethanol. A third group of rats served as control and received injections of vehicle only. At 8 h after the last injection the animals were killed, and the microsomal fractions were prepared from the liver and the kidney and used for measuring haem oxygenase activity. The enzyme activity was determined using haematin as the substrate as described in the Experimental section. The data shown represent the mean $\pm$ S.D. for four determinations; one rat was used for each determination. * $P \leq 0.05$ when compared with the phenylhydrazine-treated animals; ‡ $P \leq 0.05$ when compared with the control animals.

Treatment	Haem oxygenase (nmol of bilirubin/h per mg of protein) in:	
	Liver	Kidney
Control	1.73 $\pm$ 0.27	0.99 $\pm$ 0.19
Phenylhydrazine	13.25† $\pm$ 2.03	1.86† $\pm$ 0.49
Phenylhydrazine + cycloheximide	5.07* $\pm$ 1.59	0.81* $\pm$ 0.28

haem oxygenase activity in the liver and the kidneys of animals treated in this manner was less than or equal to, respectively, those of control animals. Similarly, the splenic haem oxygenase activity was significantly decreased in phenylhydrazine-treated rats given Zn-protoporphyrin. As noted for rats treated with Zn-protoporphyrin alone, the basal activity of the enzyme in the liver and the spleen, but not in the kidney, was significantly decreased. Table 1 provides data indicating that the increased haem oxygenase activity in the liver and the kidneys of rats treated with phenylhydrazine was not a stimulation phenomenon due to the activation of a pre-existing enzyme; rather, it was an induction process as suggested by the requirement for protein synthesis. In these studies rats were injected with phenylhydrazine 4 h prior to the administration of cycloheximide, and were killed 8 h after the last injection. As shown, the increase in haem oxygenase activity could be effectively inhibited by treating the animals with the protein synthesis inhibitor.

As expected, a marked increase in the serum levels of total bilirubin, subsequent to phenylhydrazine treatment, was observed. As noted in Fig. 3, a nearly 16-fold increase in the serum total bilirubin concentration was detected 24 h after phenylhydrazine treatment. The extent of the in-

Fig. 3. Elevation of serum total bilirubin levels by phenylhydrazine treatment, and the inhibitory action of Zn-protoporphyrin

Male Sprague-Dawley rats (180–200g body wt.) were treated with (■) phenylhydrazine (100mg/kg, subcutaneously), (▨) Zn-protoporphyrin (40μmol/kg, intraperitoneally) or (▩) both. Controls animals (□) received saline. When both agents were administered, Zn-protoporphyrin was injected 12h before and 12h after phenylhydrazine treatment. Blood samples were obtained by cardiac puncture from control rats, and from animals treated with phenylhydrazine, Zn-protoporphyrin, or both. The serum total bilirubin levels were determined as described in the Experimental section. The data shown are means $\pm$ S.D. for six determinations; one rat was used for each determination. * $P \leq 0.05$ when compared with the control animals.

crease was pronouncedly reduced by concomitant treatment with Zn-protoporphyrin. As shown, the concentration of bilirubin declined to approx. 6–7-fold that of the control animals. This finding strongly suggests the involvement of haem oxygenase in the aetiology of phenylhydrazine-mediated hyperbilirubinaemia. In rats treated with Zn-protoporphyrin alone a statistically significant decrease in the serum total bilirubin levels was not observed. This observation may reflect the limitation of the sensitivity of the diazo method in the measurement of serum bilirubin, as well as the low serum bilirubin concentration in control and in Zn-protoporphyrin-treated rats.

The ultimate formation of bilirubin from haem requires the activity of the cytosolic enzyme, biliverdin reductase. Therefore, the activity of bili-

verdin reductase in the liver and the kidney, in response to treatment with phenylhydrazine, or phenylhydrazine plus Zn-protoporphyrin, was assessed. In phenylhydrazine-treated animals the rate of enzyme activity was unchanged. However, a significant decrease in the activity of biliverdin reductase in the liver and the kidney of rats treated with phenylhydrazine plus Zn-protoporphyrin compared with the control animals was observed. In the treated rats the activity of the enzyme in the liver and the kidney was 44.7 ± 1.2 and 93.6 ± 8.3 nmol of bilirubin/h per mg of protein, respectively. The control values for biliverdin reductase activity in the liver and the kidney were 57.1 ± 0.4 and 120.0 ± 15.3 nmol of biliverdin/h per mg of protein, respectively. These findings suggest the inhibitory action of Zn-protoporphyrin on the enzyme activity. Consistent with this suggestion is the finding that in both organs the magnitude of reduction in biliverdin reductase activity was similar to that noted in animals receiving Zn-protoporphyrin alone. The enzyme activity in the liver and the kidney of Zn-protoporphyrin-treated animals was 44.3 ± 1.2 and 92.5 ± 9.0 nmol of bilirubin/h per mg of protein, respectively. As noted, a nearly 2-fold difference in the reductase activity was detected in the liver and the kidney. The presently observed inhibitory action *in vivo* of Zn-protoporphyrin on the biliverdin reductase activity is in agreement with previous findings on the inhibitory action of the metalloporphyrin on the purified rat liver enzyme (Kutty & Maines, 1981).

Studies *in vitro*

The possibility was examined that the increased serum bilirubin levels reflect the nonenzymic mode of degradation of haem by phenylhydrazine. Also, the possibility of the stimulatory action *in vitro* of this agent on haem oxygenase activity was explored. The latter possibility was examined in a series of experiments in which microsomal fractions obtained from the liver of control rats were treated with phenylhydrazine (10mM, 10min at 25°C), resedimented (105000g, 1h), and 'washed' once with the buffer (0.1M-potassium phosphate, pH7.4) prior to use in the haem oxygenase assay system. Treatment of the microsomal preparation did not produce an increase in the enzyme activity (results not shown). This finding indicates that the phenylhydrazine-mediated increase in haem oxygenase activity *in vivo* does not represent a direct stimulation of the enzyme activity. In a second series of experiments (Table 2) the direct effects *in vitro* of phenylhydrazine on the degradation of haem and the formation of bilirubin were assessed. In these experiments, either phenylhydrazine at a concentration of 10mM was added to the haem oxygenase assay system, with haematin (30μM) as the

Table 2. *Effects in vitro of phenylhydrazine on the oxidation of haem and the formation of bilirubin by the microsomal fractions of rat liver*

'Washed' liver microsomal fractions were used in the standard haem oxygenase assay system described in the Experimental section. The system (3ml) consisted of an NADPH-generating system, microsomal preparation (1.6 mg/ml), rat liver cytosol fraction (0.8 mg/ml), and haematin (30 μ M). The inactivation of the enzyme source was accomplished by heating the microsomal fraction at 60°C for 10 min. The addition of phenylhydrazine was made at the commencement of the incubation time. At the beginning and at the end of the incubation period the formation of bilirubin was assessed as described before (Maines & Kappas, 1975), as was the concentration of haem by the procedure described by Paul *et al.* (1953). The data shown are representative of two series of experiments.

Phenylhydrazine (mM)	Enzyme treatment	Incubation time (min at 37°C)	Concentration (nmol/mg) in assay system of	
			Bilirubin	Haem
0	Control	10	0.59	19.6
0	Control	0	0	25.58
10	Heat-inactivated	10	0	8.92
10	Control	10	0	1.78

Table 3. *Effects in vitro of phenylhydrazine on the concentrations of haem and cytochromes P-450 and P-420 in the microsomal fractions of rat liver*

'Washed' liver microsomal preparation (1.6 mg/ml) was incubated with phenylhydrazine. The addition of phenylhydrazine was made at the commencement of the incubation time. The inactivation of the enzyme source was accomplished by heating the microsomal fraction at 60°C for 10 min. The concentrations of cytochromes P-450, P-420 and haem were measured at the beginning and at the end of the incubation period. The procedure described by Omura & Sato (1964), was used for the measurement of cytochromes, and that of Paul *et al.* (1953) was used for the determination of the haem concentration. The data shown are representative of two series of experiments.

Phenylhydrazine (mM)	Microsomes	Incubation time (min at 37°C)	Concentration (nmol/mg) in assay system of		
			Cytochrome P-450	Haem	Cytochrome P-420
0	Control	10	0.63	1.29	0.07
0	Control	0	0.72	1.57	0
10	Heat-inactivated	10	0.0	0.15	0.03
10	Control	10	0.12	0.53	0

substrate and inactivated (10 min, 60°C) liver microsomal fraction as the enzyme source, or the rat liver microsomal fractions were treated (10 min, 4°C) with 10 mM-phenylhydrazine prior to use in the haem oxygenase assay system. At the commencement of the incubation time and at the termination of the incubation period (10 min, 37°C), the formation of bilirubin was assessed by measuring the increased absorbance at 463 nm (the absorption maximum of bilirubin in such a system). As shown in Table 2, the formation of bilirubin could not be detected in the presence of phenylhydrazine regardless of the regimen of pretreatment of the enzyme source. The pretreatment, however, did not alter the ability of phenylhydrazine to promote the nonenzymic degradation of haem. As shown, in the presence of phenylhydrazine, with an active or heat-inactivated enzyme source, the concentration of haem at the end of the incubation period was decreased to a minor fraction of the zero-time value. The haem concentration at zero time reflects both the endo-

genous, microsomal haem level, as well as that of the exogenously added substrate haem. These findings suggest the degradation of the haem molecule to pigments other than bilirubin in the presence of phenylhydrazine.

The effect *in vitro* of phenylhydrazine on microsomal levels of cytochromes P-450, P-420, and haem were investigated. As shown in Table 3, in the presence of phenylhydrazine, regardless of the pretreatment regimen of the enzyme source, cytochrome P-450 and haem levels were markedly decreased at the end of the incubation period. Moreover, the degradation of cytochrome P-450 did not result in the formation of cytochrome P-420.

Effect in vivo of phenylhydrazine treatment on the activity of GSSG-reductase, the microsomal contents of cytochromes P-450, P-420 and haem, and the concentration of glutathione in the liver

The effects *in vivo* of phenylhydrazine on the microsomal haem and cytochrome P-450 contents were examined. Also, the possibility of the active

Table 4. *Effect in vivo of phenylhydrazine treatment on the activity of glutathione reductase, glutathione content, and the microsomal concentrations of cytochromes P-450, P-420 and haem in rat liver*

Male Sprague-Dawley rats (180–200 g body wt.) were treated with phenylhydrazine (100 mg/kg, subcutaneously) and killed after 24 h. The control animals received saline. The cytosol and the 'washed' microsomal fractions were prepared from perfused livers as described in the Experimental section and used for the measurement of GSSG-reductase activity and the concentrations of cytochrome P-450, P-420 and haem. The tissue homogenate from unperfused liver was used for the measurement of the GSH content. The activity of GSSG-reductase represents nmol of NADPH oxidized/min per mg of protein. The details of the experimental procedures are described in the Experimental section. The data presented are the means $\pm$ S.D. for four determinations; one rat was used for each determination. * $P \leq 0.05$ when compared with the control animals.

Treatment	GSH (mM)	Cytochrome P-450 (nmol/mg)	Cytochrome P-420 (nmol/mg)	Haem (nmol/mg)	GSSG-reductase (nmol/min per mg)
Control	5.20 $\pm$ 0.40	0.76 $\pm$ 0.07	0	1.18 $\pm$ 0.09	91 $\pm$ 9
Phenylhydrazine	3.12* $\pm$ 0.36	0.56* $\pm$ 0.10	0.13 $\pm$ 0.01	1.24 $\pm$ 0.09	86 $\pm$ 4

intermediate and/or free-radical-mediated destruction of microsomal haem and cytochrome P-450 was explored by measuring the cellular level of GSH and the activity of GSSG-reductase. The data shown in Table 4 demonstrate that the microsomal content of cytochrome P-450 was markedly decreased in the phenylhydrazine-treated rats. An unexpected finding, however, was that the microsomal content of haem was not decreased in animals exposed *in vivo* to phenylhydrazine. Moreover, in the microsomal fractions of the treated animals a major increase in absorption in the 420 nm region of the reduced-CO difference spectrum, corresponding to that of cytochrome P-420, was observed. This finding is interpreted as suggesting the conversion of cytochrome P-450 to its inactive form, cytochrome P-420, through the formation of active metabolite species. The increase in the absorption at 420 nm did not appear to be caused by haemoglobin contamination of the microsomal preparation. This assertion is made on the basis of the following observations: the requirement for the presence of dithionite for the appearance of the absorption band at 420 nm of the CO-treated microsomal suspension, and the reasonable agreement of the magnitude of increase in cytochrome P-420 with the loss of cytochrome P-450.

Moreover, since 'washed' microsomal fractions prepared from perfused livers were used for these studies, the possibility of a significant contamination of the microsomal preparations with haemoglobin would appear rather unlikely.

The above interpretation of the data is consistent with the observed marked decrease of nearly 40% in the tissue GSH levels in phenylhydrazine-treated rats. This finding further suggests that phenylhydrazine *in vivo*, as *in vitro*, promotes the formation of reactive molecular species that are inactivated by reaction with GSH. The decrease in

GSH levels did not appear to reflect an inhibition of the production of the tripeptide, as judged by the unaltered activity of GSSG-reductase.

Discussion

Given the wide range of application of hydrazine compounds, the finding reported here that phenylhydrazine exerts inducing effects on the activity of haem oxygenase, the rate-limiting enzyme of the haem metabolic pathway, may have significant biological implications. Phenylhydrazine exhibited a potent ability to increase haem oxygenase activity in a time-dependent manner in the liver and the kidney of treated rats. This finding suggests that, for the most part, the biological basis for the development of hyperbilirubinaemia by phenylhydrazine may be related to the enhanced rate of enzymic conversion of haemoglobin haem to bilirubin. This suggestion is supported by findings that the inhibitions of the activities of haem oxygenase (Fig. 2) and biliverdin reductase by Zn-protoporphyrin were accompanied by markedly reduced serum total bilirubin levels (Fig. 3). However, the finding that the serum total bilirubin levels did not fully return to the control value may be interpreted as suggesting that the increased activity of haem oxygenase was not solely responsible for the increased serum levels of the bile pigment, and that non-enzymic mechanisms contributed to the enhanced degradation of haemoglobin haem to bilirubin in phenylhydrazine-treated animals. This suggestion is consistent with observations reported by Lemberg & Legge (1942). Alternatively, the finding may reflect an incomplete inhibition of haem oxygenase activity in various tissues and organs in response to the Zn-protoporphyrin dose used in the present study.

The present findings suggest that the mechanism of the degradative action of phenylhydrazine on haem and cytochrome *P*-450 *in vitro* may be distinctly different from that of its action *in vivo*. As shown in Tables 2 and 3, the addition *in vitro* of phenylhydrazine to the microsomal preparation caused marked depressions in the haem and the cytochrome *P*-450 levels. However, the degradation of the haem molecule and cytochrome *P*-450 by phenylhydrazine did not lead to the formation of bilirubin or cytochrome *P*-420. On the other hand, the treatment *in vivo* of rats with a high dose of phenylhydrazine did not decrease the microsomal content of haem (Table 4), although a pronounced decrease in the microsomal content of cytochrome *P*-450 was produced along with the formation of cytochrome *P*-420. Based on the observation (Table 3) that the heat-inactivated microsomal fraction *in vitro* was as effective as the control microsomal fraction in supporting the degradation of the haem moiety of cytochrome *P*-450 by phenylhydrazine, it may be suggested that the degradation process was not catalysed by an active enzyme protein.

The differential actions *in vivo* and *in vitro* of phenylhydrazine on microsomal haem and cytochrome *P*-450 may reflect the rapid inactivation by cellular GSH of the reactive molecular species generated in the course of the interaction of phenylhydrazine with cytochrome *P*-450. This suggestion is consistent with the following findings: (a) the cytochrome *P*-450 concentration in the liver of phenylhydrazine-treated rats was decreased without a concomitant reduction in the haem concentration (Table 4); (b) the decrease in the cytochrome *P*-450 content of the liver was accompanied by an increase in the absorption at the 420 nm region of the CO-reduced difference spectrum of the microsomal fractions; and (c) there was a marked decrease in the levels of GSH in the liver of phenylhydrazine-treated animals (Table 4). Moreover, the 'suicide' inactivations of cytochrome *P*-450 and haemoglobin have been amply documented in systems *in vitro* (De Matteis & Cantoni, 1979; Ortiz de Montellano *et al.*, 1981; Cantoni *et al.*, 1981). Also, the interaction of GSH with active intermediates and free radicals is well established (Jollow *et al.*, 1974).

The cellular basis for the phenylhydrazine-mediated increase in haem oxygenase activity is not clear. It may involve any of the following factors: (a) the intermediate action of haemoglobin released in the course of haemolysis; (b) the direct action of the parent compound; (c) the activity of the reactive metabolites of phenylhydrazine; and (d) a secondary response to the altered cellular homeostasis including decreased GSH levels. The first suggestion is consistent with the known induc-

tion of haem oxygenase activity in the liver and the kidney in response to haemolytic conditions (Pimstone *et al.*, 1971; Maines & Senft, 1981). The last possibility is consistent with the reported effect of diethylmaleate on haem oxygenase activity (Burk & Correia, 1979; Maines, 1982). In rats treated with diethylmaleate a severe decrease in the GSH levels is accompanied by an increase in haem oxygenase activity in the liver and the kidney in the absence of a decrease in cellular haem levels (Maines, 1982). The present study, however, does not permit a preference for any of the suggested possibilities.

The finding that Zn-protoporphyrin fully blocked the phenylhydrazine-mediated increase in the activity of haem oxygenase suggests the utility of this metalloporphyrin for the suppression of the enzyme activity in various haemolytic conditions. The effectivity of metalloporphyrins in subsiding the postparturition increase in haem oxygenase activity in the newborn rat has already been demonstrated (Maines, 1981; Drummond & Kappas, 1982).

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