Cobalt Induction of Hepatic Heme Oxygenase; with Evidence That Cytochrome *P*-450 Is Not Essential for This Enzyme Activity

(heme oxidation/enzyme induction/mixed-function oxidation)

M. D. MAINES AND ATTALLAH KAPPAS

The Rockefeller University, New York, N.Y. 10021

ABSTRACT   Treatment of rats *in vivo* with cobalt chloride stimulated heme oxidation by hepatic microsomes to levels up to 800% above controls. This treatment also caused increases in liver weight and in total microsomal protein; in contrast, marked decreases were produced in microsomal oxidation of ethylmorphine (80%), and in cytochrome *P*-450 (60–70%) and heme (30–50%) contents. Cobalt chloride treatment did not affect heme oxidation by the spleen heme oxygenase system.

The rate of heme oxidation by hepatic microsomal enzymes and the microsomal content of cytochrome *P*-450 were found to be unrelated. This conclusion was reached from studies in which microsomal heme oxygenase activity from cobalt-treated animals could be increased by 900% above control levels in the same microsomal preparation in which cytochrome *P*-450 content was decreased to spectrally unmeasurable amounts after incubation with 4 M urea. The same treatment eliminated ethylmorphine demethylation and decreased microsomal NADPH-cytochrome *c* reductase (EC 1.6.2.4) activity by 75%.

It is concluded that (*i*) the hepatic microsomal enzyme system that oxidizes heme compounds is not the same as that which metabolizes drugs, (*ii*) cytochrome *P*-450 is not essential for the oxidation of heme by liver cells, (*iii*) there is no direct relationship between the rate of heme oxidation and the level of NADPH-cytochrome *c* reductase activity, and (*iv*) the oxidation of heme is protein-dependent and the active proteins are inducible, but are different from those involved in drug metabolism.

These studies were undertaken to examine the effects of cobalt on hepatic heme oxygenase activity and to explore the relationship between heme catabolism and cytochrome *P*-450 in liver cells. It has been proposed that this microsomal hemoprotein plays an essential role in the oxidation of heme (1) and that the components of the microsomal mixed-function oxidase system are involved in this process (2).

In recent studies (3), we have shown that several of the heme compounds that are substrates for the heme oxygenase system *in vitro* have potent degradative effects on the components of the microsomal mixed-function oxidase complex, a finding that seemed paradoxical in light of the reported role of these compounds as natural substrates for the complex (4). These observations, together with the reported ability of cobalt to diminish the content of hepatic cytochrome *P*-450 and to depress microsomal drug oxidations (5), prompted the studies reported here in which cobalt effects on heme catabolism have been examined.

The results of these studies indicate that cobalt treatment leads to a marked induction of liver cell capacity to oxidize heme; and that the catabolism of heme by hepatic cells continues at a very high rate in the total absence of detectable levels of cytochrome *P*-450 or drug metabolism by the microsomal mixed-function oxidase system.

MATERIALS AND METHODS

All chemicals used in this study were purchased from Sigma Chemical Co. Male Sprague-Dawley rats, 160–190 g, were used.

Treatment of Animals and Preparation of Enzyme. Rats were injected with cobalt chloride (60 mg/kg, subcutaneously), methemalbumin (40 μmoles/kg, intraperitoneally), or with both cobalt chloride (60 mg/kg, subcutaneously) and methemalbumin (40 μmoles/kg, intraperitoneally) twice at 24-hr intervals. The control animals were injected with saline. After the second injection, the animals were starved for 24 hr and then killed by decapitation. The body weights of cobalt-treated animals did not differ by more than 5% from those of control animals, at the time of killing. Hepatic microsomal fractions were prepared as described (6). Livers were perfused *in situ* with 0.9% NaCl, and homogenized in potassium phosphate buffer (0.1 M, pH 7.4). The spleens were removed and homogenized without perfusion. The homogenates were centrifuged at 9000 × *g* for 20 min, and the supernatant fraction was filtered and centrifuged at 163,000 × *g* for 40 min. The microsomal pellets were resuspended in buffer to a protein concentration of 18 mg/ml and 8–9 mg/ml for liver and spleen, respectively. The liver microsomal supernatant fraction served as the source of biliverdin reductase; the protein concentration of this fraction was adjusted to 10 mg/ml.

Heme Oxygenase Assay. Heme oxygenase activity was assayed as described by Schacter *et al.* (2), and bilirubin formation was calculated from an extinction coefficient of 40 mM⁻¹ cm⁻¹ between 464 and 530 nm. The formation of bilirubin as the end product of heme oxygenase activity was confirmed by extraction of the incubation mixtures with chloroform and crystallization (7) and spectral analysis of the bile pigment. The concentration of hematin routinely used was 17 μM, except where noted.

Hepatic Mixed-Function Oxidase Activity Assay. The activity of this system was monitored by measuring the oxidation of the prototype drug, ethylmorphine. Formaldehyde, formed by *N*-demethylation of ethylmorphine, was determined by the method of Nash (8) with the incubation mixture described earlier (9).

Urea Treatment of Hepatic Microsomal Fraction. In order to further decrease the microsomal content of cytochrome *P*-450

TABLE 1.   *Stimulatory effect of treatment in vivo with cobalt on hepatic microsomal heme oxygenase activity, and the inhibitory effect of cobalt on the mixed-function oxidase system*

Treatment		Heme (μM)	Bilirubin formed (nmol/mg per hr)	Liver wt (g)	Ethylmorphine N-demethylation (nmol/mg per hr)	Cytochrome P-450 (nmol/mg)	NADPH-cytochrome c reductase (nmol/mg per hr)	Microsomal heme (nmol/mg)
Control	1	17	1.11	9.8	315	0.71	91.9	1.49
		34	1.89					
	2	17	1.05	8.9	295	0.69	92.6	1.34
		34	1.59					
	3	17	1.02	9.1	290	0.70	76.0	1.52
		34	1.69					
Cobalt chloride	1	17	6.56	11.4	55	0.27	98.2	0.95
		34	10.16					
	2	17	6.18	11.8	43	0.28	84.1	0.87
		34	9.57					
	3	17	8.39	11.0	64	0.12	77.0	0.93
		34	13.67					

Rats (170–185 g) were injected with cobalt chloride (60 mg/kg, subcutaneously) twice at 24-hr intervals, and were starved for 24 hr subsequent to the second injection. Control animals were injected with saline. Heme oxidation was measured by the method of Schacter *et al.* (2). Ethylmorphine demethylation was assayed as described by Correia and Mannering (9). Formaldehyde formed was determined by Nash's method (8). NADPH-cytochrome c reductase activity was measured by the method of Williams and Kamin (11). Total microsomal heme content was determined by the pyridine hemochromogen method of Paul *et al.* (12).

of the cobalt-treated animals, this cellular fraction was treated with urea. For these experiments the liver microsomal fractions from control and cobalt-treated animals were suspended in buffer to a concentration of 1 mg/ml, and one aliquot of each suspension was incubated for 30 min at 25° with 4 M urea. The fractions were then diluted once in order to decrease the urea concentration and to facilitate the resedimentation of the microsomal membranes in the subsequent centrifugation. Thereafter, the suspensions were centrifuged (163,000 × g, 40 min), and the pellets thus obtained were resuspended in buffer. This preparation was used as the enzyme source for the heme oxygenase activity, ethylmorphine oxidation, and the spectral studies reported in Table 3.

Spectral Studies. Cytochrome P-450 content was measured by the method of Omura and Sato (10), with NADPH or sodium dithionite as the reducing agent. NADPH-cytochrome c reductase (EC 1.6.2.4; NADPH:ferricytochrome oxidoreductase) activity was measured as described by Williams and Kamin (11). The concentration of microsomal heme was determined by the pyridine hemochromogen method of Paul *et al.* (12). All spectral studies were conducted with an Aminco Chance DW2 spectrophotometer.

Protein was determined by the method of Lowry *et al.* (13). The procedure described by Tenhunen *et al.* (14) was used for the preparation of heme solutions. All experiments were conducted at least three times, and the results are expressed as the average of these experiments.

RESULTS

Effect of Cobalt Chloride, Methemalbumin, and Methemalbumin Plus Cobalt Chloride on the Hepatic Enzymes. As Table 1 shows, comparison of hepatic microsomal heme oxygenase activities of the cobalt-treated animals and of the controls indicates a profound stimulatory effect of cobalt treatment on this enzyme activity. In the treated animals, the magnitude

of heme oxygenase activity was increased by 6- to 8-fold over that of untreated animals. This induction of heme oxygenase activity contrasts strikingly with the effects of cobalt on hepatic mixed-function oxidase activity and on contents of cytochrome P-450 and total microsomal heme; these were reduced to levels, respectively, of 20%, 30%, and 60% of control (Table 1). These enzyme changes were accompanied by a significant increase (20%) in liver weight and in total content of liver microsomal protein (20%). No changes in NADPH-cytochrome c reductase activity were observed after cobalt treatment alone. Figs. 1 and 2 show that in the cobalt-treated rats there were no alterations in the substrate dependence of heme oxygenase activity, as reflected in the same apparent K_m value (31 μM) obtained with hepatic microsomes of treated and control animals; however, large differences were observed in the rate of enzymatic activity, the V_{max} value being about eight times higher with liver microsomes from cobalt-treated animals (25 nmoles/mg of protein per hr compared with 3.1 nmoles/mg of protein per hr) than with control microsomes.

In contrast to the stimulatory effects of cobalt treatment *in vivo* on hepatic heme oxygenase activity, there was no significant effect of the metal on heme oxygenase activity of spleen microsomal fractions, with the values for the cobalt-treated and the control spleens being, respectively, 12.51 and 13.89 nmoles of bilirubin formed per mg of protein per hr.

It has been shown (15) and substantiated here (Table 2) that the activity of the heme oxygenase system is stimulated by methemalbumin. Accordingly, the effects of simultaneous administration of cobalt and methemalbumin were investigated on the parameters described above. In these studies, additive stimulatory effects on heme oxygenase activity were noted when the animals were treated with methemalbumin in addition to cobalt chloride. Furthermore, the decreases in the microsomal contents of cytochrome P-450 and total heme produced by the cobalt alone were somewhat magnified in those animals treated with both cobalt and methemalbumin.
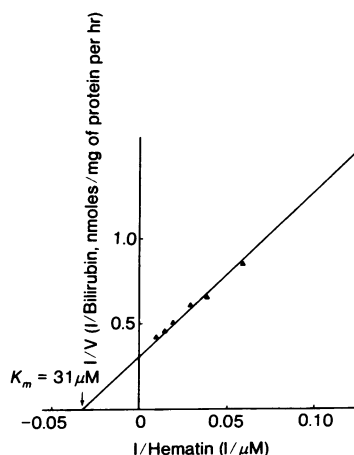
Fig. 1.   Lineweaver–Burk plot demonstrating the rate of the hepatic microsomal heme oxygenase reaction in control rats.
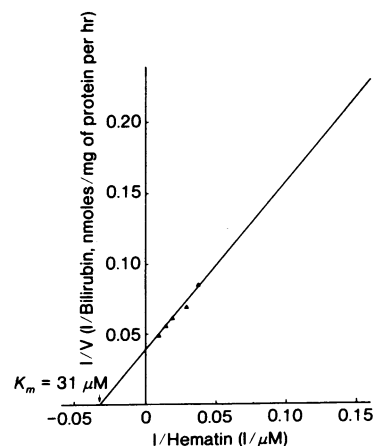

Fig. 2.   Lineweaver–Burk plot demonstrating the rate of the hepatic microsomal heme oxygenase reaction in cobalt chloride-treated rats.

These findings in cobalt-treated animals established that there was no direct correlation between the rate of heme oxidation and the microsomal content of cytochrome P-450 in liver cells. In an attempt to completely disassociate hepatic heme oxygenase activity and cytochrome P-450, further studies were undertaken. It is known that treatment of microsomes with 4 M urea causes destruction of cytochrome P-450 and its conversion to the inactive form, cytochrome, P-420 (16). Therefore, microsomes from cobalt-treated animals in which microsomal content of cytochrome P-450 had already been reduced to about 30% of control levels were treated with 4 M urea (30 min, 25°). Table 3 shows that this treatment reduced the spectrally measurable microsomal content of cytochrome P-450 to insignificant or undetectable amounts; nevertheless, heme oxygenase activity of these microsomes remained highly elevated, at a level about 900% above normal. As expected by the complete loss of cytochrome P-450, the mixed-function oxidative activity of such microsomes was also totally eliminated. In addition, in this study two unexpected observations were made.

These were that (i) the microsómal fraction from cobalt-treated animals, which served as the control for the urea treatment (row 3, Table 3), showed an exaggerated heme oxygenase activity (15 times normal); and (ii) although NAPH-cytochrome c reductase activity of urea-treated microsomes from the cobalt-treated animals was decreased to only a fraction of the control, heme oxygenase activity was still nine times greater than normal. Thus, although the oxidation is NADPH-dependent, NADPH-cytochrome c reductase

activity is not rate-limiting in this process. The sources of the microsomes in which heme oxygenase activity was increased by six to eight times that of the control (Table 1) and the above fraction in which this enzyme activity was increased to 15 times the control level were the same, i.e., cobalt-treated animals. However, the latter preparation was only a very dilute version of the former (0.5 mg/ml compared to 22 mg/ml) and was centrifuged one more time (163,000 X g, 40 min). These results suggest that the proteins induced by cobalt treatment apparently have different hydrophobicity and/or sedimentation properties than those involved in drug oxidation; thus, when the microsomes from cobalt-treated animals (which contain increased amounts of proteins involved in heme oxidation) were centrifuged in a highly dilute form, these proteins were selectively sedimented. This selective sedimentation of the active proteins would account for the further increase in the specific activity of heme oxygenase found in these experiments.

Mechanism of Cobalt Stimulation of the Heme Oxygenase System. In order to investigate the mechanism by which cobalt exerts its stimulatory effect, we conducted several studies. In one series of experiments the role of cell sap constituents in determining heme oxygenase activity was investigated. In these studies the heme oxygenase assay was carried out by exchanging the microsomal and supernatant fractions from the cobalt-treated and the control animals. It was found that full heme oxygenase activity required only the presence of hepatic microsomes from cobalt-treated animals and that no activating components were present in the supernatant frac-

TABLE 2.   *The additive stimulatory effect of treatment of rats with cobalt and methemalbumin in vivo on heme oxygenase activity, and the inhibitory effect of such treatment on the microsomal mixed-function oxidase system*

Treatment	Bilirubin formed (nmol/mg per hr)	Ethylmorphine N-demethylation (nmol/mg per hr)	Cytochrome P-450 (nmol/mg)	Microsomal heme (nmol/mg)
Control	1.31	312	0.68	1.39
Cobalt chloride	7.69	40	0.22	0.69
Cobalt chloride + methemalbumin	9.18	25	0.14	0.58
Methemalbumin	3.24	132	0.40	1.19

Rats (170–185 g) were treated as described in *Materials and Methods*. The concentration of hematin was 17 μM.

TABLE 3. *Effect of urea on heme and drug oxidation activities and on cytochrome P-450 content of cobalt-treated (in vivo) rat hepatic microsomal fractions*

Treatment *in vivo*	Treatment *in vitro*	Bilirubin formed (nmol/mg per hr)	Ethylmorphine N-demethylation (nmol/mg per hr)	Cytochrome P-450 (nmol/mg)	NADPH-cytochrome c reductase activity (nmol/mg per min)
Control	None	1.01	205	0.61	56.6
Control	Urea	0.18	26	0.07	9.4
Cobalt chloride	None	15.66	18	0.22	52.3
Cobalt chloride	Urea	9.71	0	0	12.5

Rats were injected with cobalt chloride or saline, and the microsomal fractions were prepared and treated as described in *Materials and Methods*. Cytochrome P-450 was measured with sodium dithionite as the reducing agent. The concentration of hematin was 17 μM.

tion. In addition, it was of interest to establish whether the stimulatory effect of cobalt *in vivo* on heme oxygenase could be duplicated *in vitro*. In such experiments microsomal fractions from control animals were first incubated (30 min, 37°) with 50–500 μM cobalt chloride, and heme oxygenase and other microsomal enzyme parameters were measured.

As Table 4 indicates, cobalt chloride at concentrations up to 100 μM had little or no effect on heme oxygenase activity; at higher concentrations there was an inhibitory effect on this enzyme activity. In contrast, with all concentrations of cobalt studied, the mixed-function oxidase activity of the microsomal fraction was stimulated, with no differences being noted in the content of cytochrome P-450. Further studies were carried out in which cobalt chloride at concentrations of 1–1000 μM was directly added to the heme oxygenase incubations mixture without preincubation. In these studies, again, there was no change in heme oxygenase activity with cobalt concentrations up to 100 μM. Above this concentration, cobalt progressively inhibited the oxidation of heme, with this activity decreasing to about 20% of control in the presence of 1 mM cobalt chloride.

In studies in which the effect of heat on the ability of the microsomal fraction from the cobalt-treated animals to oxidize heme was investigated, it was found that heating at 60° for 5 min totally abolished heme oxidative activity. Furthermore, replacement of the atmosphere of the incubation mixture with nitrogen completely inhibited the oxidation of heme by the urea-treated microsomal fractions of cobalt-

TABLE 4. *Effects of treatment in vitro of the hepatic microsomal fractions with cobalt on heme oxygenase and mixed-function oxidase system activities and cytochrome P-450*

Cobalt chloride (μM)	Bilirubin formed (nmol/mg per hr)	Ethylmorphine N-demethylation (nmol/mg per hr)	Cytochrome P-450 (nmol/mg)
0	1.03	221	0.70
50	0.98	260	0.72
100	0.96	285	0.70
200	0.68	315	0.78
500	0.46	319	0.67

Hepatic microsomal fractions were prepared as described earlier with a protein concentration of 4–5 mg/ml and incubated with cobalt chloride at the final concentrations indicated for 30 min. Thereafter, microsomal enzymes were assayed and cytochrome P-450 content was determined.

treated animals. NADH was partially effective in replacing NADPH in the incubation mixture, the oxidation of heme by microsomes from both cobalt-treated as well as control animals in the presence of NADH (0.8 mM) being 10–20% of the value obtained with NADPH. However, no significant synergistic effect was observed when both NADPH and NADH were added to the incubation mixture. Ascorbate in equimolar concentrations could not substitute for NADPH in the reaction mixture.

DISCUSSION

The precise mechanism through which heme compounds are degraded in biological systems is not yet resolved and there exist several hypotheses concerning this process (reviewed in ref. 17). The most recent and attractive has been that proposed by Tenhunen *et al.* (4), in which heme compounds serve as substrates for a microsomal enzyme system referred to as "heme oxygenase". Criteria such as inhibition by carbon monoxide and partial reversal of carbon monoxide inhibition of the reaction (18) by light, immunological studies involving NADPH-cytochrome c reductase (2), and the requirements for molecular oxygen and NADPH (4) were used as evidence for the common involvement of components of the microsomal electron transport system in the oxidative metabolism of heme, as well as drug compounds. Cytochrome P-450 has been specifically proposed as being "essential" (1) for the catabolism of heme by the heme oxygenase system.

Certain characteristics of the system for heme oxidation, however, distinguish it from the microsomal system involved in the mixed-function oxidation of drugs and have been noted by various investigators (4, 19, 20). These differences, as well as our own observations that heme substrates for the heme oxygenase system *in vitro* (3) had significant degradative effects on the activity and components of the drug-metabolizing microsomal enzyme complex, led us to investigate the relationship between these two systems.

Tephly and Hibbeln (5) had earlier shown that treatment of rats with cobalt markedly diminished hepatic microsomal drug metabolism and microsomal content of cytochrome P-450. These findings prompted us to investigate the effect of cobalt treatment on the oxidation of heme in the liver. Our findings confirm those made by these investigators, in that short-term cobalt treatment produces a major decrease in microsomal content of cytochrome P-450 and total heme, and of drug metabolism. In striking contrast, however, the oxidative metabolism of heme by microsomes is greatly enhanced, ranging in the experiments reported here (Table 1) from 600

to 800% above control levels. In other unreported studies, increases in heme oxygenase activity after cobalt treatment alone have reached levels as high as 10 times greater than normal.

This dissociation between the rate of heme catabolism and the hepatic content of cytochrome *P-450* prompted the further experiments (Table 3) with urea-treated microsomes, in which we were able to demonstrate that heme oxygenase activity can continue at a very high rate (900% above control levels) in the complete absence of spectrally detectable cytochrome *P-450*. In the same urea-treated microsomes, moreover, drug metabolism by the mixed-function oxidase system was totally abolished, as expected, and there was a marked reduction in NADPH-cytochrome *c* reductase activity as well. These findings thus establish that cytochrome *P-450* is not essential for heme oxidation, and that the microsomal components necessary for the mixed function oxidation of such prototype drugs as ethylmorphine are not identical to those components required for heme catabolism. The data reported in Table 3 also indicate that the relationship between NADPH-cytochrome *c* reductase activity and heme oxygenase activity is apparently not a direct one; or in any case that only low levels of the former are required to sustain very high levels of heme oxygenase activity.

The mechanism by which cobalt treatment leads to an enhancement of heme oxygenase activity in the liver is under further investigation; the phenomenon does however manifest certain criteria associated with drug or other chemically mediated hepatic "enzyme induction", such as an increase in liver weight and in hepatic microsomal protein content. In addition, the lack of cobalt stimulatory effects on heme oxygenase activity *in vitro* and the large increase in the V_{max} of the reaction would also be consistent with an "induction" mechanism. The nature of the protein, or proteins, "induced" by cobalt and the manner in which they are related to heme oxidation are not known. It is evident, nevertheless, that the protein components of the system involved in heme breakdown in the livers of cobalt-treated animals are not identical to those involved in the mixed-function oxidation of drugs despite the fact that both systems may function in an analogous manner.

1. Tenhunen, R., Marver, H. S. & Schmid, R. (1969) *Trans. Ass. Amer. Physicians* **82,** 363–371.
2. Schacter, B. A., Nelson, E. B., Marver, H. S. & Masters, B. S. S. (1972) *J. Biol. Chem.* **247,** 3601–3607.
3. Maines, M. D. & Kappas, A. (1974) *Fed. Proc.* **33,** 588 abstr.
4. Tenhunen, R., Marver, H. S. & Schmid, R. (1969) *J. Biol. Chem.* **244,** 6388–6394.
5. Tephly, T. R. & Hibbeln, P. (1971) *Biochem. Biophys. Res. Commun.* **42,** 589–595.
6. Maines, M. D., Andres, M. W. & Muller-Eberhard, U. (1974) *Mol. Pharmacol.* **10,** 204–213.
7. Ostrow, J. D., Hammaker, L. & Schmid, R. (1961) *J. Clin. Invest.* **40,** 1442–1452.
8. Nash, T. (1953) *Biochem. J.* **55,** 416–421.
9. Correia, M. A. & Mannering, G. J. (1973) *Mol. Pharmacol.* **9,** 455–464.
10. Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239,** 2379–2385.
11. Williams, C. H. & Kamin, H. (1962) *J. Biol. Chem.* **237,** 587–595.
12. Paul, K. G., Theorell, H. & Åkeson, Å. (1953) *Acta Chem. Scand.* **7,** 1284–1287.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
14. Tenhunen, R., Marver, H. S. & Schmid, R. (1968) *Proc. Nat. Acad. Sci. USA* **6,** 748–755.
15. Tenhunen, R., Marver, H. S. & Schmid, R. (1970) *J. Lab. Clin. Med.* **75,** 410–421.
16. Mason, H. S., North, J. C. & Vanneste, M. (1965) *Fed. Proc.* **24,** 1172–1180.
17. Lathe, G. H. (1972) *Essays Biochem.* **8,** 107–148.
18. Tenhunen, R., Marver, H. S., Rimstone, N. R., Truger, W. F., Cooper, D. Y. & Schmid, R. (1972) *Biochemistry* **11,** 1716–1720.
19. Nichol, A. W. (1970) *Biochim. Biophys. Acta* **22,** 28–40.
20. Schacter, B. A. & Mason, J. I. (1974) *Arch. Biochem. Biophys.* **160,** 274–278.