HEME AND METHEMOGLOBIN: NATURALLY OCCURRING REPRESSORS OF MICROSOMAL CYTOCHROME

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The carbon monoxide-binding cytochrome of liver microsomes plays an essential role as the terminal oxidase in the metabolism of xenobiotics and steroids (Cooper et al., 1965). Although this cytochrome in reality may represent more than one protein (Hildebrandt et al., 1968), we shall designate it collectively as cytochrome P-450. Cytochrome P-450 may be induced by a large number of compounds administered individually or in combinations; the activity of microsomal drug oxidation closely parallels this drug-mediated stimulation of cytochrome P-450 (Remmer and Merker, 1965). The mechanisms responsible for the induced synthesis of cytochrome P-450 are unknown but several recent observations suggest that the rate of heme biosynthesis may exert a regulatory effect on the formation of microsomal cytochrome (Granick, 1966; Marver et al., 1966 a).

The biosynthesis of heme*, which probably is identical in all cells, is initiated by the condensation of glycine and succinyl-CoA resulting in formation of δ-aminolevulinic acid (Shemin, 1956). This step is catalyzed by δ-aminolevulinic acid synthetase (ALA synthetase) which in mammalian liver is located within mitochondria (Granick and Urata, 1963) and is rate limiting in heme biosynthesis. Physiologic control of heme formation appears to be exercised primarily at the level of ALA synthetase (Granick and Urata, 1963; Marver et al., 1966 b). Hepatic ALA synthetase is induced in the liver of experimental animals and in cultured embryonic chick liver by a number of compounds (Granick, 1966; Marver, in press), some of which are known to be stimulators of microsomal cytochrome (Remmer and Merker, 1965). Recent evidence suggests that heme interferes with the induction of hepatic ALA synthetase (Granick, 1966; Hayashi et al., 1968), thereby providing a regulatory mechanism for its own biosynthesis.

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*Heme refers to iron protoporphyrin IX complexes collectively. The specific heme compounds employed are identified in the text.

The present study demonstrates that heme, methemoglobin and methemalbumin, administered parenterally, are potent repressors of the drug-mediated induction of hepatic δ-aminolevulinic acid synthetase and of microsomal cytochrome. Moreover, heme prevents the enhancement of microsomal protein synthesis resulting from drug administration.

We used female Sprague-Dawley rats weighing 140-160 g, and except as otherwise noted, all experiments were carried out on groups of 6 rats; the animals were fasted for 40 hours before the beginning and throughout the course of the experiments. After decapitation, the liver was perfused with isotonic saline "in situ", then homogenized in 0.25 M sucrose and finally fractionated by the method of Schneider (1948). ALA synthetase and the rate of aminoacetone formation were assayed in liver homogenate (Marver et al., 1966 b). Tyrosine transaminase was measured by the method of Lin and Knox (1957). Cytochrome P-450 was determined by measuring the CO-difference spectrum of dithionite-reduced suspensions of microsomes (Omura and Sato, 1964). The increment of molar extinction between 450 and 490 mμ was assumed to be 91 cm^{-1} mM^{-1}. Cytochrome b_5 was quantitated from the spectral difference between NADH-reduced and air saturated microsomes based on an extinction coefficient between 424 and 409 mμ of 185 cm^{-1} mM^{-1} (Omura and Sato, 1964).

The continuous intravenous infusion or intermittent intraperitoneal injection of heme prevented induction of ALA synthetase by either the porphyrogenic drug, allylisopropylacetamide, or phenobarbital (Table 1). By contrast, heme failed to affect the production of aminoacetone which resembles ALA in being formed by acylation of glycine in liver mitochondria (Table 1). Moreover, the prednisone-mediated induction of tyrosine transaminase (8.2 to 8.6 x control levels) is not influenced significantly by intermittent, intraperitoneal administration of methemalbumin (8.0 to 8.7 x control levels). This effect of heme on ALA synthetase does not result from enzyme inhibition. In concentrations up to 0.05 mM heme does not significantly inhibit ALA synthetase (ALA synthetase activity was ± ten per cent of control values) (Marver et al., 1966 c). Also, the effect cannot be attributed to the formation of inhibitors "in vivo" because admixture of liver homogenate prepared from rats treated with heme to homogenates of drug treated animals failed to reduce the activity of ALA synthetase. Moreover, it cannot be explained by interference with the capacity of the liver to generate succinyl-CoA. Despite incubation with an excess of succinyl-CoA (Marver et al., 1966 a), mitochondria isolated from the livers of rats treated with allylisopropylacetamide and heme did not produce greater than normal amounts of ALA.

The induction of ALA synthetase either by allylisopropylacetamide or by phenobarbital is followed by enhanced heme synthesis as measured by the incorporation of Fe^{59} or of glycine-2-C^{14} into liver heme (Marver, in press). The following observations suggest that at least a portion of this newly synthesized heme is utilized for the synthesis of microsomal

TABLE I

Conditions	ALA Synthetase (mμmoles ALA)	AA Synthetase (mμmoles AA)
Saline infusion	22 (19-27)	78 (75-84)
Saline infusion + AIA	300 (283-327)	83 (77-86)
Heme-Alb. infusion + AIA	31 (25-33)	79 (74-82)
Heme-Alb. infusion	15 (14-17)	80 (73-84)
Intraperitoneal saline	24 (21-26)	83 (80-89)
Phenobarbital	88 (79-95)	87 (83-91)
Hematin + phenobarbital	28 (23-31)	85 (79-88)
Hematin	15 (14-17)	86 (85-90)

The effect of heme on the allylisopropylacetamide (AIA) or phenobarbital-mediated induction of hepatic δ-aminolevulinic acid synthetase and on aminoacetone synthesis. Methemalbumin (Heme-ALB.) was administered by constant intravenous infusion (0.25 μmoles/100 g/hr.) for two hours prior to administration of AIA (300 mg/kg subcutaneously) and thereafter until sacrifice 16 hours later. Hematin (2.5 μmoles/100 g) was injected intraperitoneally 2 hours before and immediately after administration of phenobarbital (125 mg/kg). The rats were sacrificed 8 hours later. In the infusion experiments, enzyme activities in each condition were determined on the liver homogenate of each of the 5 rats. Enzyme activities following intraperitoneal injections were determined on the pooled liver homogenates of each group. Values given are the mean and the range (per gram liver/hour).

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cytochrome: (a) More than 80 per cent of the labeled heme is localized in the microsomal fraction and is soluble in 1 per cent deoxycholate; (b) Administration of an inducing drug such as phenobarbital, is followed sequentially by an increase in ALA synthetase, in heme synthesis and eventually in microsomal cytochrome and; (c) Carbohydrate, puromycin, actinomycin D and, under certain circumstances, 2-diethylaminoethyl-2,2-diphenylvalerate (SKF-525A) block drug-mediated induction both of ALA synthetase and of microsomal cytochrome (Marver, in press).

Heme also suppresses the drug-mediated induction of microsomal cytochrome (Fig. 1; Table 2). Hematin (2-4 μmoles/100 g) given intraperitoneally every six to twelve hours, or by constant intravenous infusion (0.25-0.5 μmoles/100 g/hour) blocked the phenobarbital-stimulated increase of cytochrome P-450 and of cytochrome b. Methemalbumin (2-4 μmoles/100 g intraperitoneally) and methemoglobin (1.5-2 μmoles/100 g intraperitoneally) had effects similar to that of hematin. At comparable concentrations peroxidized heme, hemoglobin or inorganic iron were ineffective. Administration of heme reduced microsomal cytochromes to levels below those of saline-treated control animals (Fig. 1; Table 2). This may

TABLE 2

Treatment of rats		Microsomal fraction	
Heme-Alb.	Phenobarbital	Protein (mg/g liver)	Cyt. P-450 (mμmoles/mg protein)
—	—	18.0	0.69
—	+	31.2	2.04
+	—	19.8	0.43
+	+	20.2	0.58

The effect of methemalbumin (Heme-Alb.) on the phenobarbital-mediated increase of microsomal protein and cytochrome P-450 (cyt. P-450). Phenobarbital (100 mg/kg subcutaneously) was administered at zero time and again 24 hours later. Methemalbumin (2.5 μmoles/100 g) was injected intraperitoneally 2 hours before and immediately after the first injection of phenobarbital and every 12 hours thereafter until sacrifice 48 hours after zero time. Groups of 6 rats were employed for each set of conditions; protein and cytochrome P-450 were determined in the pooled liver homogenates of each group.

represent interference by heme with physiologically occurring stimulators of microsomal hydroxylation which may include certain sex hormones (Murphy and DuBois, 1958).

In addition to repressing the stimulatory effect of drugs on ALA synthetase and microsomal cytochromes, heme also blocks the phenobarbital-mediated increase of microsomal protein (Table 2). In phenobarbital-treated rats, microsomal protein content was almost double that in control animals or in animals given the drug and heme. It appears unlikely that the reduction in protein could be accounted for only by the heme-induced repression of microsomal cytochromes. For example, if cytochrome P-450 were to account for all of the phenobarbital-mediated increase in the protein content of microsomes (Table 2), this enzyme would have to represent 15 per cent of the total protein in this subcellular fraction. This figure seems unreasonably high in view of the heterogeneity of the microsomal fraction (Siekevitz, 1963). Furthermore, administration of phenobarbital leads to an increase in a number of individual microsomal proteins (Remmer and Merker, 1965). It was ascertained that the determination of cytochrome P-450 and b_5 and of protein content of liver microsomes of non-heme treated rats is not interfered with by the addition "in vitro" of hematin or methemalbumin, or of liver microsomes from heme-treated animals. Finally, it should be noted that on a molar basis heme interferes with drug stimulation at dosage levels well below those of the drug. In our experiments the molar ratio of heme to drug ranged from 1 to 50 (Table 1) to 1 to 4 (Table 2).

Our data provide evidence for repression by heme of mammalian hepatic ALA synthetase, the rate limiting enzyme in heme biosynthesis. In addition, heme interferes with the drug-

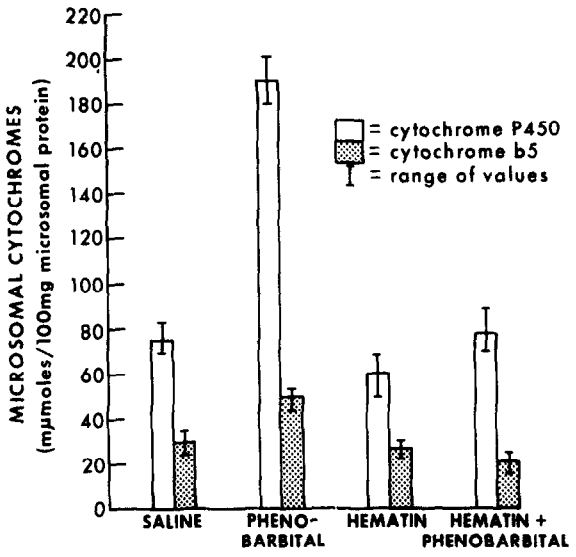

FIGURE 1. Blockade by hematin of the phenobarbital-mediated enhancement of microsomal cytochrome activities. Fasted rats were sacrificed 36 hours after a single subcutaneous injection of phenobarbital (125 mg/kg). Hematin was given intraperitoneally (3 μmoles/100 g) 2 hours before and immediately after phenobarbital and 2 μmoles/100 g at 6, 14 and 26 hours. Enzyme activities for each of the conditions were determined on the pooled liver homogenates from the six rats in each group.

induced synthesis of microsomal cytochromes and protein. The following observations indicate that heme exerts these effects by direct action on the liver: (a) Heme appears to interfere with the induction of ALA synthetase in cultured embryonic chick liver (Granick, 1966); (b) Hematin-C^{14} or hematin-Fe^{59} injected into rats is taken up largely in the liver and can be crystallized from that organ (Snyder and Schmid, 1965; Marver, in press). However, the mechanism and the subcellular site of action accounting for these inhibitory effects of heme are unknown. They do not result from interference by heme with the uptake of inducer by the liver because administration of heme in an effective dosage does not diminish the hepatic concentration of phenobarbital-2-C^{14} (Marver, in press). If the repressive effects of heme on ALA synthetase, microsomal cytochromes and microsomal protein are interrelated, then they could result either from intereference with a single step in an interdependent system or from blockade of a step common to all three mechanisms. For example, since ALA synthetase is induced by drugs to provide heme for microsomal cytochromes, repression of ALA synthetase by heme could result in a diminished responsiveness of microsomal cytochromes to drug administration. However, this hypothesis would require that the drug-stimulated increase in microsomal protein depends upon the induced synthesis of heme or microsomal cytochromes, or both. There is no convincing

evidence in support of this hypothesis. An alternative explanation is that heme interferes with the uptake of inducer at its intracellular sites of action in the liver.

The repressive effect of heme may play an important role in the biologic control of microsomal cytochromes and thereby regulate microsomal hydroxylation. Such regulation will result not only from hepatic heme formed in situ but also from circulating heme delivered to the liver in the forms of methemoglobin and methemalbumin. These hemoproteins may be derived from the physiologic breakdown of senescent red cells; their formation is enhanced in hemolytic states (Bunn and Jandl, 1968).

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