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STUDIES ON THE INDUCTION OF SERUM HEMOPEXIN BY PENTOBARBITAL AND POLYCYCLIC HYDROCARBONS

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Abstract—Induction of the serum glycoprotein hemopexin by sodium pentobarbital and 3-methylcholanthrene is established. Serum hemopexin levels are assayed by a radial immunodiffusion technique. Hemopexin levels were increased up to 200 per cent after repeated injections of pentobarbital, compared with control rabbits injected with saline or distilled water. Induction was confirmed by simultaneous administration of an inhibitor of protein synthesis, cycloheximide, which temporarily interrupted and delayed hemopexin induction. Other aspects of hemopexin synthesis studied are: the effect of starvation, which markedly reduced the serum levels; the combined action of pentobarbital and polycyclic hydrocarbons on induction, which was nonadditive; and the lack of response to repeated doses of 3-methylcholanthrene.

HEMOPEXIN (Hx), the heme-binding serum β-glycoprotein, is induced by polycyclic hydrocarbons and porphyrinogenic agents.[1] However, this induction is not mediated by factors controlling heme* biosynthesis,[2] although injections of small amounts of heme significantly increase serum Hx levels.[3] Hemopexin has an extremely high affinity for heme, which it binds in an equimolar ratio. This protein *in vitro* has been shown to prevent the inhibitory effect of high heme concentrations on δ-aminolevulinic acid synthetase† and to remove heme *in vitro* from the microbial enzyme, cytochrome P-450, resulting in a reduction in activity of this enzyme.[4]

Known inducers of hepatic microsomal enzymes have been shown to increase serum hemopexin in a manner qualitatively similar to that of the drug-metabolizing enzymes.[1] It is of interest to study further the effect on the biosynthesis of hemopexin of certain conditions and agents known to influence hepatic enzyme synthesis. Starvation[5] markedly decreases the content of total hepatic microsomal protein in rats. Protein levels, particularly of albumin, are depleted in states of malnutrition and dietary deficiency.[6–9] Phenobarbital is one of the many drugs that stimulate various pathways of metabolism by liver microsomes, whereas the polycyclic hydrocarbons stimulate a more limited number of these metabolic processes.[10] Both 3-methylcholanthrene and 3,4-benzpyrene increase serum hemopexin levels in rabbits. Simultaneous administration of D,L-ethionine prevented this rise.[1] However, since this agent, an inhibitor of adenosine triphosphatase, is hindering an early step in protein synthesis, interference with later steps by other drugs had to be established.

The present study was undertaken to evaluate the effect of starvation, pentobarbital, an analogue of penobarbital, and 3-methylcholanthrene on hemopexin synthesis.

* Heme is used to denote all iron protoporphyrin IX compounds.

† G. R. WARNICK and B. F. BURNHAM, *J. biol. Chem.* **246**, 6880 (1971).

MATERIALS AND METHODS

Animals. Adult male New Zealand white rabbits, weighing 1·5–2·5 kg, obtained from Rancho de Conejo, Vista, Calif. were used in all experiments. Unless otherwise stated, they were maintained on Harrison–Riedy rabbit pellets and tap water *ad lib.*, and housed in individual cages at 22°. All animals remained in local animal facilities for 3–5 days prior to experimentation and were weighed three times a week.

Drugs. Sodium pentobarbital was purchased from Western Medical Supply, Inc., (65 mg/ml of physiological saline) and Diamond Laboratories (60 mg/ml of water). 3-Methylcholanthrene and 3,4-benzpyrene were obtained from Eastman Organic Chemicals and both were dissolved in corn oil, to a final concentration of 8 mg/ml. Cycloheximide (Acti-dione) was obtained from Nutritional Biochemicals Corp., and chloramphenicol (Chloromycetin) from Parke–Davis. The experimental details are given in Table 1.

TABLE 1. AGENTS INJECTED INTRAPERITONEALLY, WITH DOSAGE AND LENGTH OF TIME GIVEN FOR 15 GROUPS OF RABBITS

Group		No. of rabbits	Pharmacological agents*	Dosage (kg/day)	No. of days given
II		11	0·85% NaCl	1 ml	4
III		5	Distilled H_2O	1 ml	4
V		12	NaPB	40 mg	4
VI	A	4	NaPB + CH	40 mg	4
				2·5 mg	1 on day 2
	B	4	NaPB + CH	40 mg	4
				2·5 mg	1 on day 3
VII		6	CH	2·5 mg	1
VIII		6	3-MC + CH	4 mg	1
				2·5 mg	1
IX		6	3-MC	4 mg	1
X		4	NaPB + CP	40 mg	4
				500 mg	1 on day 3
XI		4	CP	500 mg	1
XII	A	6	NaPB + 3-MC	40 mg	4
				4 mg	1 on day 1
	B	5	BP + 3-MC	20 mg	1
				4 mg	1
XIII	A	2	3-MC	4 mg	1
			3-MC	4 mg	1 on day 15
	B	2	3-MC	4 mg	1
			3-MC	12 mg	1 on day 15
	C	2	3-MC	12 mg	1
			3-MC	12 mg	1 on day 15

* Abbreviations: NaPB, sodium pentobarbital; CH, cycloheximide; 3-MC, 3-methylcholanthrene; CP, chloramphenicol; BP, 3,4-benzpyrene.

Serum hemopexin (Hx) concentrations were determined, as previously described,[11] by radial immunodiffusion. The antibody to rabbit Hx was developed in goats by injecting 10 μg purified rabbit Hx weekly three times in complete and subsequently three times in incomplete Freund's adjuvant. The goats were exsanguinated at the end of 6 weeks, and their sera were used as the source of antibody for the Hx determinations. Two ml of goat serum, diluted 1 : 5 with normal saline, and 6 ml of agar solution

were mixed to a final concentration of 4 and 1·5 per cent respectively. This mixture was layered onto a 7·5 × 5 cm plastic slide (Meloy Laboratories) and, after solidification, 27 wells were cut with a size 14 needle and aspirated. Each well was completely filled, as judged visually, with rabbit serum dilutions using a capillary pipette. The pooled normal rabbit serum Hx standards were not used for more than 3 consecutive days when kept at 4° after being thawed from − 70°. Seven different points were used to obtain the standard curve, each standard being applied three times. Rabbits were bled from the marginal ear vein and the sera stored at 4°. The rabbit Hx was quantitated either the same day or within 48 hr, although a stoppered sample stored at 4° shows no change in Hx concentration for up to 14 days. Each sample was applied in triplicate; the experimental error was 10 per cent. At least three baseline values for serum Hx were obtained, and only those rabbits whose level remained stable within the normal range (200–600 μg/ml) were used. To minimize any error due to diurnal variation in rabbit Hx, samples were collected at 9 a.m. when rabbits were bled only once a day. However, in some experiments it was necessary to bleed them as often as every 4 hr. The hematocrit was monitored throughout all experiments.

Variation in serum hemopexin. The normal variation in serum hemopexin concentration during the day was studied in six untreated rabbits (group I). They were bled at 9 a.m., 1 p.m., 5 p.m. and 9 p.m. for 4 days, and in addition at 9 a.m. and 9 p.m. for another 3 days.

Starvation. Six rabbits (group IV) received only water *ad lib.* for 10 days, whereupon normal diet was resumed. Serum Hx concentration was determined daily throughout the starvation period and for 1 week after diet was continued.

Pentobarbital induction. Twelve rabbits (group V) were injected intraperitoneally with sodium pentobarbital (NaPB), 40 mg/kg daily for 4 days. They were bled every 4 hr for 12 hr after the first injection and then every 12 hr for 36 hr, followed by daily bleedings for 10 days. Control animals (groups II and III) received physiological saline or distilled water, 1 ml/kg i.p. for 4 consecutive days.

Cycloheximide inhibition. Cycloheximide (CH) dissolved in physiological saline, 5 mg/ml, was injected i.p. after the induction of Hx by either NaPB or 3-methylcholanthrene (3-MC). Initially, cycloheximide was given daily with NaPB injections, but this combination proved so toxic that the majority of rabbits died. Therefore, a single dose of cycloheximide, 2·5 mg/kg i.p., was administered with the second (group VI A) or third (group VI B) dose of four consecutive daily doses of NaPB, 40 mg/kg i.p. Since cycloheximide has a shorter duration of action than 3-MC, it was injected daily for 3 days beginning on the day on which 3-MC was given. However, this also was too toxic. Therefore, a single dose of CH, 2·5 mg/kg i.p., was given 18 hr after 3-MC at which time the Hx concentration was already increasing. Six other animals (group VII) received CH, 2·5 mg/kg i.p., alone.

Chloramphenicol. Chloramphenicol, 500 mg/kg i.p., was administered to four rabbits on the third day of a series of four sodium pentobarbital injections (group X). Animals in group XI received the same dose of chloramphenicol alone.

Combination induction. (a) Pentobarbital + 3-methylcholanthrene: six rabbits were given NaPB, 40 mg/kg i.p., daily for 4 days and 3-MC, 4 mg/kg i.p., on day 1 (group XII A). Hemopexin concentrations were determined every 12 hr for 4 days and thereafter daily for 8 days.

(b) Benzpyrene + 3-methylcholanthrene: in addition, five rabbits (group XII B)

were injected with 3-MC, 4 mg/kg i.p., and simultaneously with benzpyrene, 20 mg/kg i.p. Hemopexin concentrations were measured as for group XII A.

Tolerance to 3-methylcholanthrene induction. Three rabbits were given two doses of 3-MC, 4 mg/kg i.p., 7 days apart in one rabbit, and 10 days apart in the two others. All three failed to respond to the second injection with regard to Hx induction. However, in all cases the Hx level had not completely returned to the pre-injection level when the second dose was given. To ascertain whether or not rabbits are tolerant to repeated doses of 3-MC, six rabbits (group XIII) were given two doses of 3-MC, 14 days apart. The first pair were given two doses of 4 mg/kg; in the second pair the first dose was 4 mg/kg and the second 12 mg/kg; the third pair received two doses of 12 mg/kg i.p. Hx concentrations were assayed daily in these six rabbits for 4 weeks.

RESULTS

Variation in serum hemopexin concentration. The percentage change in hemopexin levels during the day for the six untreated rabbits is shown in the first three rows in Table 2. The 1 p.m. (group Ia), the 5 p.m. (group Ib) and the 9 p.m. (group Ic) values were compared to the 9 a.m. value and the differences in serum Hx concentration calculated. For a particular rabbit in groups Ia and Ib, an average of four values was obtained. In observing the variation from 9 a.m. to 9 p.m., two time intervals were

TABLE 2. PERCENTAGE CHANGE IN HEMOPEXIN AFTER INJECTION OF DRUGS IN SEVEN GROUPS OF RABBITS*

Group	No. of rabbits	Pharmacological agents†	%Δ Hx (Mean + 2 S.D.)
I (untreated)			
Ia‡	6		0·9 ± 9
Ib§	6		4·7 ± 14
Ic‖	6		1·6 ± 7
II	11	NS	60 ± 100
III	5	H_2O	55 ± 124
IV	6	ST	−73 ± 70
V	12	PB	110 ± 118
VI	8	CH + PB	99 ± 75
VII	6	CH	142 ± 146

* Each bleeding was related to the 9 a.m. value, the time of daily sampling.

† Abbreviations: NS, normal saline; PB, pentobarbital; CH, cycloheximide; ST, starved.

‡ Represents change in Hx from 9 a.m. to 1 p.m.

§ Represents change in Hx from 9 a.m. to 5 p.m.

‖ Represents change in Hx from 9 a.m. to 9 p.m.

studied where possible: 9 a.m.–9 p.m. and 9 p.m.–9 a.m. the following day (group Ic). Fourteen separate 12-hr intervals were studied for each of the six rabbits. The average change in serum Hx concentration for each rabbit was calculated and, from these values, the mean percentage change for the six rabbits. The results are shown in Table 2, group Ic. All values are related to the 9 a.m. value, the time at which daily bleedings were performed. No significant deviation in serum Hx level was encountered for any

rabbit tested. Eighty-three per cent of the values lay within ± 7 per cent of the 9 a.m. value and the remainder within ± 10 per cent. This variation remains well within the 10 per cent accuracy of the method of radial immunodiffusion. Therefore, the serum Hx concentration in rabbits is not subject to circadian fluctuations.

Starvation. Table 2 shows the pronounced fall in serum Hx in all animals deprived of food for 10 days. The mean drop was 73 per cent. It took between 3 and 7 days after onset of the period of fast for a drop in serum Hx levels to occur. The lowest Hx concentration occurred the day after normal diet was resumed in five animals, but 3 days later in the remaining rabbit. The average weight loss over the 10-day period was 0·4 kg. It took from 3 to 6 days after diet was resumed for Hx levels to return to prestarvation values and in three rabbits the Hx level rose above the pre-starvation concentration. No change in hematocrit was observed in this or following experiments.

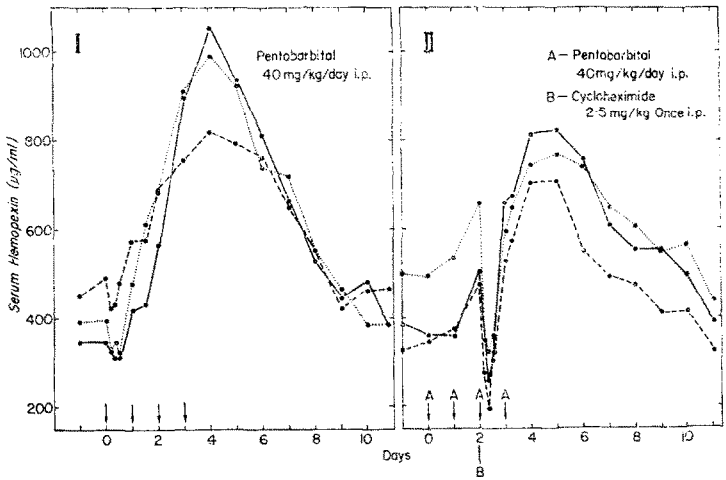

FIG. 1. Pentobarbital induction of serum Hx and its inhibition by cycloheximide. Part I shows the increase in serum Hx concentrations for three representative rabbits after repeated daily injections of sodium pentobarbital. Part II depicts the precipitous decrease in serum Hx caused by one dose of cycloheximide on the third day of pentobarbital injections in three other animals.

Pentobarbital induction. Figure 1, part I, shows for three representative animals the 2- to 3-fold increase in serum Hx levels produced by repeated injections of NaPB. The mean rise for the 12 rabbits was 110 per cent, the range being 27–202 per cent. In 9 of the 12 rabbits, the peak level occurred on the last day of NaPB injections or the following day. Hemopexin levels of the control animals receiving four doses of either physiological saline or distilled water were variably elevated, the mean percentage increase from groups II and III combined was 58. However, a significant increase ($P < 0·01$) in serum hemopexin, compared to that in control groups, occurred after NaPB.

Cycloheximide inhibition. A striking fall in serum Hx concentration followed cycloheximide administration (Fig. 1, part II). Without exception, the fall in hemopexin levels was pronounced, regardless of whether cycloheximide was given with the second

or third injection of NaPB. The mean decrease for the eight rabbits was 50 per cent and occurred 4–8 hr after cycloheximide administration. This drop occurred at a time when the hemopexin level was already rising in response to NaPB. Thus, cycloheximide interrupts the induction of serum Hx by NaPB. Within 24 hr, the induction process resumed. The maximum value attained was almost as high as that produced by NaPB alone. The serum Hx levels then returned to baseline values within 1 week.

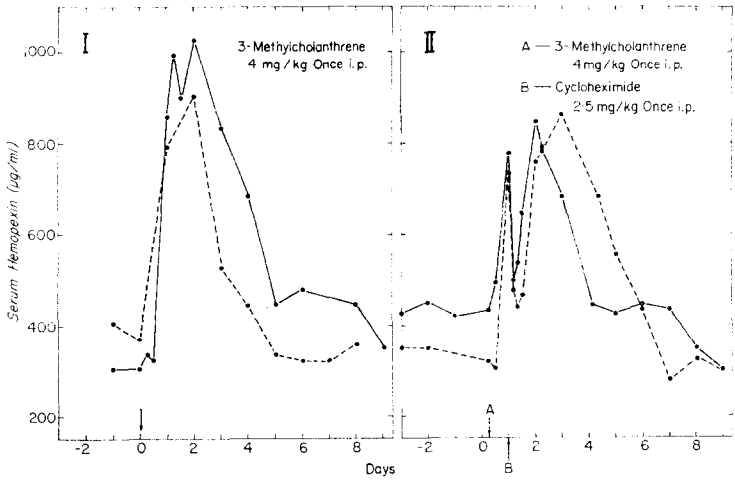

FIG. 2. 3-Methylcholanthrene induction of serum Hx and its inhibition by cycloheximide. Part I shows the steep rise in serum Hx concentration for two representative animals after a single dose of 3-MC. Part II illustrates the fall, delay and reduction in the rise of serum Hx in two other rabbits produced by one dose of cycloheximide 18 hr after 3-MC.

After a single injection of 3-MC, a 2·5– to 3-fold increase in serum Hx was observed as shown in Fig. 2, part I. Part II illustrates the interruption of this induction after a single dose of cycloheximide 18 hr after 3-MC. The Hx levels in the six rabbits fell 36 per cent after CH and started to rise only 24 hr later. The induction process was not only temporarily interrupted and delayed by CH, but also reduced in magnitude. The maximum induction levels occurred 2 days after 3-MC in two rabbits, 3 days later in three rabbits, and on the fifth day, in the remaining rabbit, compared to 24–48 hr in rabbits receiving 3-MC alone. Pre-experimental levels were not reached for a further 7–10 days.

Cycloheximide alone, as shown in Table 2, also produced a significant rise in hemopexin levels; the mean increase in six rabbits was 142 per cent. However, prior to the increase, hemopexin levels fell markedly (36 per cent) below pre-injection levels, with the minimum concentration being reached 8 hr after cycloheximide administration. The final peak in Hx concentration was reached 30–48 hr after the injection of CH, long after CH has ceased to act. This increase is therefore probably a rebound phenomenon.

Chloramphenicol. Chloramphenicol injections had no effect on serum hemopexin concentrations and, in addition, did not interrupt the induction by NaPB when given on the third day of four daily NaPB injections.

Combination therapy. Four daily injections of NaPB in combination with a single dose of 3-MC did not increase serum Hx concentrations above the values seen when either drug was given alone (mean = 91 ± 20*). Similarly, a single injection of two different polycyclic hydrocarbons, benzpyrene and 3-MC, had no additive effect on hemopexin induction. The mean increase in concentration was 114 ± 23* in five rabbits.

Tolerance to 3-methylcholanthrene induction. Two rabbits alone of the six tested for tolerance to 3-MC (group XIII) were induced by both injections. Only one of these rabbits showed a similar response to both injections; the other showed a smaller increase in response to the second. The remaining four rabbits responded to only one injection with respect to hemopexin induction.

DISCUSSION

Circadian fluctuations exist in man and animals for many biochemical and hematological parameters.[12–15] It was suggested that liver protein metabolism shows rhythmic changes throughout the day in response to successive meals containing protein.[16] This was verified for the hepatic enzyme, tyrosine transaminase.[17] Circadian rhythms are found in plasma protein levels of the rat[14] and amino acid levels of man.[13] However, in rabbits, hemopexin, a serum protein synthesized in the liver,[18] did not show this variation. In contrast to previous studies, our animals were fed *ad lib.* and periods of light and dark were not strictly regulated, nor was the amount of external stimuli. Nevertheless, within our experimental framework and the limitation for accurately detecting changes in serum hemopexin concentration of less than 10 per cent, no alteration occurred.

The changes in serum hemopexin levels due to fasting were not unexpected, considering previous observations on plasma protein synthesis in states of fasting and protein deficiency.[19–22] The 73 per cent decrease in serum Hx concentration is greater than that reported for albumin and transferrin levels in rats.[9,21] The effect of starvation on serum hemopexin became apparent after a delay of several days. Albumin synthesis, on the other hand, decreases within 18 hr of commencing a fast, but no significant drop in serum albumin is seen at that time.[19]

Four days elapsed, after diet was commenced, before serum hemopexin levels rose. This correlates well with observations of others[9,21] for albumin and transferrin. Although synthesis rates of these proteins increase within 30 min of refeeding, serum levels only gradually rise after 24 hr. Our results in fasted rabbits compare with the lowered serum Hx seen in marasmus and Kwashiorkor.†

Studies on enzyme induction are copious.[10,23] Induction of the serum protein Hx by agents known to enhance the synthesis of the hepatic microsomal drug-metabolizing enzymes was indicated previously.[1] In the initial studies, D,L-ethionine was used as an inhibitor of protein synthesis. As D,L-ethionine depletes the liver of ATP, its effect on protein synthesis is indirect. In the present investigation, cycloheximide was used as an inhibitor. Its action is believed to be specific for protein synthesis and not involved in that of DNA or RNA. The inhibition interferes with one or more steps in the reaction sequence transferring amino acids from aminoacyl transfer-RNA into nascent peptides on ribosomes.[24]

* Mean ± S.E. (in per cent).

† Results obtained in collaboration with Dr. R. Suskind, Chiang Mai, Thailand.

A marked elevation in serum Hx concentration followed repeated injections of pentobarbital or a single injection of 3-MC. After a single injection of cycloheximide, there was a striking drop in serum Hx levels at a time when hemopexin concentrations were rising in response to NaPB or 3-MC administration. This temporary interruption and delay in the rise of serum hemopexin established induction of hemopexin by NaPB and 3-MC. Had the rise in serum Hx concentration been due to decreased catabolism or mobilization from extravascular sites, cycloheximide would not have had this dramatic effect. Hemopexin concentrations fell within 4 hr of CH administration, and the effect of CH was limited to 12 hr. Others also observed a very rapid and short duration of action for CH.[25] The variable rise in control animals after injections of saline or water was probably a result of peritoneal irritation, but compared to these controls serum hemopexin rose significantly ($P < 0{\cdot}01$) in animals receiving an inducer.

The degree of induction of serum hemopexin was not nearly as great as that observed *in vitro* for membrane-bound microsomal liver enzymes, using liver homogenates. This is explained by the fact that the level of this protein, molecular weight 57,000,* rises concomitantly intra- and extravascularly[26] as other proteins of this size diffuse readily into the extravascular space.[27] The magnitude of induction of a serum protein is therefore only partially reflected in its serum concentration.

Failure of chloramphenicol to interrupt the induction by NaPB or to decrease serum Hx levels on its own was not unexpected, since protein synthesis in mammalian systems is usually markedly resistant to this antibiotic.[24]

The development of tolerance to the repeated administration of certain drugs is well documented. In the majority of studies on the induction of hepatic enzymes, the animals are sacrificed prior to liver fractionation. Hence, tolerance with respect to the ability of an enzyme system to respond to induction cannot be observed. Tolerance to several barbiturates is related to their accelerated metabolism, but not all cases of tolerance can be explained by enzyme induction.[10] For these reasons, we are unable to compare our findings regarding tolerance to the induction of this serum protein by 3-MC to those of others.

Our results regarding induction of hemopexin with a combination of two polycyclic hydrocarbons or one carcinogen and pentobarbital[10,28] suggest that the two types of drugs induce by acting on the same step in the biosynthetic pathway of this serum protein. This finding contrasts with those of others for hepatic enzymes. However, the mechanism of induction[10,28] has yet to be elucidated.

After administration of phenobarbital or a polycyclic hydrocarbon, it has been shown that δ-aminolevulinic acid synthetase activity is stimulated, which results in increased heme synthesis followed by elevated levels of microsomal heme and cytochrome P-450, and subsequently by enhanced activity of certain microsomal mixed-function oxidase reactions.[29,30] Thus, synthesis of cytochrome P-450 and its induction by various agents are presumably dependent on heme synthesis.[29–32] Furthermore, it has also been shown[29] that heme represses the drug-mediated induction of δ-aminolevulinic acid synthetase, and other components of the microsomal oxidative pathway.

An essential role in heme catabolism has been assigned to hemopexin.[18,33] Plasma heme is transported to the liver solely or predominantly by Hx. Indeed, preliminary studies also suggest that hemopexin-bound heme is a suitable substrate for microsomal

* V. L. SEERY, G. HATHAWAY and U. MULLER EBERHARD, *Archs Biochem. Biophys.* (in press).

heme oxygenase, which is thought to catalyse the oxidation of heme at the α-methene bridge to form biliverdin.[34]

The present study shows the induction of a heme-binding serum glycoprotein by the same agents which induce tissue-bound enzymes involved in heme and drug metabolism. At present, no definite conclusions can be drawn as to the significance of these findings. Since Hx has been shown to prevent the inhibitory effects of high concentrations of heme *in vitro*, one may speculate that this protein serves to regulate drug-metabolizing hepatic enzymes by binding any excess heme produced by δ-aminolevulinic acid synthetase induction. This surplus heme, if hemopexin were not present to bind it, would otherwise inhibit the metabolism of drugs,[29] resulting in the accumulation of toxic substances.

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