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Apocytochrome *P*-450: Reconstitution of Functional Cytochrome with Hemin *In Vitro*

(hemoprotein synthesis/organelle interaction/rat liver)

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ABSTRACT **Synthesis of microsomal cytochrome *P*-450 in rat liver requires synthesis of apoprotein in rough endoplasmic reticulum and of heme in mitochondria. Dissociation of apoprotein and heme synthesis by concomitant treatment of rats with inducers of cytochrome *P*-450 (i.e., phenobarbital) and inhibitors of heme synthesis (i.e., cobalt) resulted in a relative excess of apocytochrome *P*-450. Under these circumstances, it was possible to reconstitute the holocytochrome by addition of hemin *in vitro*. The holocytochrome was detected spectrophotometrically by its CO-binding properties and functionally by its increased oxidative activity. Heme-mediated reconstitution was most efficient in cell fractions rich in mitochondria-rough endoplasmic reticulum complexes (640 × *g* fraction), suggesting that the structural association of these two organelles may represent a functional unit essential for the synthesis of holocytochrome *P*-450. These findings indicate that phenobarbital-mediated induction of apocytochrome *P*-450 is independent of heme synthesis. It is suggested that synthesis of the apocytochrome may be the primary and rate-limiting event in the formation of cytochrome *P*-450.**

Liver microsomal oxygenases are multicomponent enzyme systems which metabolize a wide variety of xenobiotics. An important component of the oxygenase system is a carbon monoxide binding hemoprotein or a group of hemoproteins, collectively known as cytochrome *P*-450, which functions as a terminal oxidase. A variety of lipophilic substances induce cytochrome *P*-450 synthesis in the liver, and this is associated with enhanced microsomal oxidation (1–4).

Synthesis of cytochrome *P*-450 requires the synthesis of apoprotein and of heme. Most likely, the former occurs in the rough endoplasmic reticulum, whereas the latter is essentially a mitochondrial function. It is not known whether or how these two synthetic processes are coordinated. Some form of coordination may be inferred from the observation that induction of the cytochrome in the liver is preceded by enhanced hepatic protein and heme synthesis (5–13). Moreover, coordination of heme and protein synthesis has been demonstrated in the formation of other hemoproteins, such as hemoglobin (14–17), tryptophan oxygenase (18), and cytochrome *c* (19, 20). A similar interdependence between heme and apoprotein synthesis may exist in the synthesis of cytochrome *P*-450.

Administration of heme precursors such as δ-aminolevulinic acid failed to increase the synthesis of cytochrome *P*-450, indicating that at least under steady-state conditions heme synthesis apparently is not rate-limiting for the synthesis of this cytochrome (21, 22). We, therefore, considered the possibility that synthesis of cytochrome *P*-450 may be regulated primarily by the synthesis of its apoprotein. If this were true, it may be anticipated that under appropriate experimental conditions, a pool of "free" apocytochrome *P*-450 would be demonstrable in the liver. Recently, a small but significant pool of free apoprotein was demonstrated in rat liver for microsomal cytochrome b_5. In these studies, ^{14}C-labeled apoprotein or ^{59}Fe-labeled hemin were used to reconstitute the microsomal holocytochrome which was then solubilized, purified, and quantitated. On the basis of these studies, it was estimated that a pool of apocytochrome b_5 may be 7.5% of the total microsomal cytochrome content of rat liver (23, 24). This estimate of apocytochrome b_5 was facilitated by the relative ease of solubilization and purification of this cytochrome. Difficulties in the solubilization and isolation of cytochrome *P*-450 of liver microsomes have limited the use of this direct approach for this hemoprotein. To circumvent this methodological problem, we selected an indirect experimental technique for detection of apocytochrome *P*-450 in rat liver. It is based on simultaneous induction of cytochrome *P*-450 by phenobarbital and other inducers, and partial inhibition of heme synthesis by agents such as cobalt. If synthesis of apoprotein *P*-450 should occur independently of heme synthesis, then inhibition of heme formation would result in a relative excess of apocytochrome *P*-450. Indeed, under these experimental conditions, apocytochrome *P*-450 was identified and quantitated in liver homogenates by reconstitution with hemin to the functionally active holocytochrome. Present studies define some of the biochemical and structural requirements for reconstitution of the holocytochrome. Preliminary findings of these studies have been reported in abstract form (25, 26).

MATERIALS AND METHODS

Phosphatidylcholine and phosphatidylethanolamine were obtained from Sigma Chemical Co., ethylmorphine-HCl and *p*-chloro-*N*-methylaniline-HCl from Mallinckrodt Chemical Works and from Calbiochem, respectively; 3-methylcholanthrene and 3,5-diethoxycarbonyl-4,4-dihydro-2,4,6-trimethylpyridine were obtained from Eastman Kodak Co. Pregnenolone 16α-carbonitrile was a gift from Dr. John Babcock, Upjohn Chemical Co., Kalamazoo, Mich.

Male Sprague–Dawley rats (160–210 g) were treated with phenobarbital sodium [50 mg/kg, intraperitoneally (i.p.)] and cobaltous chloride [60 mg/kg, subcutaneously (s.c.)] at 48 and 24 hr before they were killed, except where stated

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otherwise. The animals were fasted overnight, then stunned and decapitated; the livers were excised after perfusion *in situ* with ice-cold isotonic KCl solution. The livers were homogenized in 0.1 M phosphate buffer (pH 7.4) to yield a 50% suspension.

Incubation with Hemin. Hemin (ferriprotoporphyrin IX hydrochloride, Sigma Chemical Co.) was dissolved in 0.1 M NaOH and the pH was adjusted to 7.4 with 0.1 M Na^+-K^+ phosphate buffer. Aliquots (10 ml) of the homogenate were supplemented with phosphatidylcholine (1 mM) and phosphatidylethanolamine (0.25 mM) and incubated in the presence and absence of hemin (40 μM) for 20 min at 37° in a Dubnoff metabolic shaker. At the end of the incubation, the homogenate was centrifuged at 10,000 × *g* for 15 min at 4°, and the supernatant was recentrifuged at 105,000 × *g* for 60 min at 4°. The microsomal pellet was suspended in 1.15% KCl and resedimented. Microsomes were resuspended in 0.1 M phosphate buffer (pH 7.4), and ethylmorphine and *p*-chloro-*N*-methylaniline *N*-demethylase activities were determined as described (27, 28). Protein was quantitated according to the method of Lowry *et al.* (29). Microsomal cytochrome *P*-450 was determined by the [(CO + $Na_2S_2O_4$)-CO]-difference spectrum as described by Schoene *et al.* (30).

Mitochondria were prepared by sedimentation at 9000 × *g* for 20 min as described (27). The 640 × *g* fraction was obtained by sedimenting the homogenate at 640 × *g* for 10 min at 4°. The pellet was suspended in 0.1 M phosphate buffer and resedimented at 10,000 × *g* for 10 min at 4°. It was then resuspended in 0.1 M phosphate buffer (pH 7.4) and used as the "640 × *g*" fraction.

RESULTS

Sequential Appearance of Apocytochrome P-450. After a single injection of phenobarbital and cobalt, the concentration of cytochrome *P*-450 in the liver fell markedly, but recovered after reaching a minimum at 24 hr (Fig. 1). A second injection of phenobarbital and cobalt at 24 hr led to a further decrease in cytochrome *P*-450, which reached a minimum at 48 hr and then recovered. In all instances where cytochrome *P*-450 concentration was reduced, incubation of liver homogenate with hemin increased the cytochrome *P*-450 level, reflecting presence of free apocytochrome. This was most prominent at 48 hr in animals that had received two doses of phenobarbital and cobalt (Fig. 1), and this injection schedule, therefore, was selected for all subsequent experiments. The accumulation of apocytochrome *P*-450 under these conditions could not be explained by a hemin-mediated increase in protein synthesis during the period of incubation. This was verified by the absence of detectable [^{14}C]leucine incorporation into microsomal protein in liver homogenates from phenobarbital and cobalt-treated rats, incubated in the presence or absence of hemin (for 30 min at 37°). Comparable concentrations of [^{14}C]leucine resulted in measurable incorporation of label into microsomes only when liver homogenates were supplemented with ATP, confirming findings by Hoagland *et al.* (31). Moreover, the amount of cytochrome *P*-450 that could be reconstituted with hemin *in vitro* was approximately 10-fold higher than the estimated amount of cytochrome *P*-450 that would have been formed *in vivo* during a 30-min period of incubation.

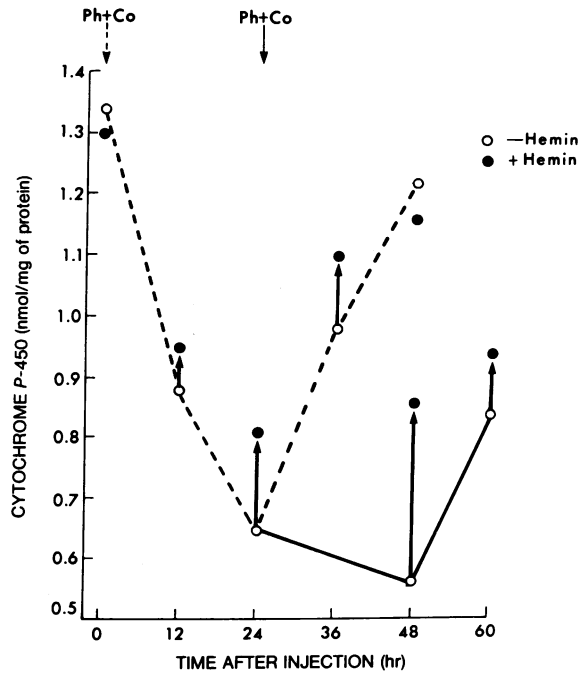

FIG. 1. Effect of hemin *in vitro* on microsomal cytochrome *P*-450 after treatment of rats with phenobarbital (Ph) and cobalt (Co). Rats were given phenobarbital sodium (50 mg/kg intraperitoneally) and cobaltous chloride (60 mg/kg subcutaneously): at 0 hr, and killed after 0, 12, 24, 36, and 48 hr (dashed curve); and at 0 and 24 hr and killed at 24, 36, 48, and 60 hr (solid curve). Liver homogenate (pooled from at least two rats) was incubated with hemin, microsomes were prepared, and cytochrome *P*-450 was determined as described in *Materials and Methods*.

In a separate set of experiments heme was synthesized from δ-aminolevulinic acid (0.32 mM) *in vitro* in a system containing rat liver mitochondria (40–50 mg of protein), cytoplasmic fraction (15–20 mg of protein), and ferrous sulfate (20 nM), as described by Yoda and Israels (32). This preparation was preincubated for 10 min and then added to liver homogenate of rats treated with phenobarbital and cobalt, supplemented with phospholipids, and further incubated for 20 min at 37°. Reconstitution of cytochrome *P*-450 under these conditions was comparable to that achieved with direct addition of hemin to liver homogenate.

Preliminary data also indicated that the addition of sulfhydryl reagents such as cysteine and dithioerythritol to the reconstitution system *in vitro* further enhanced the hemin-mediated increases of cytochrome *P*-450. Yu and Gunsalus (33) recently described an active role of cysteine in the reconversion of cytochrome $P\text{-}420_{cam}$ to cytochrome $P\text{-}450_{cam}$. Thus, formation of the active holocytochrome *P*-450 may involve sulfhydryl groups.

Effect of Various Inhibitors of Heme Synthesis and Inducers of Cytochrome P-450. In addition to cobalt, a number of other agents, including nickel, lead, and 3,5-diethoxycarbonyl-4,4-dihydro-2,4,6-trimethylpyridine, have been shown to inhibit one or several enzymes of heme synthesis. Treatment of rats with phenobarbital in combination with each of these inhibitors yielded reconstitution with hemin comparable to that obtained with cobalt (Table 1). Similarly, reconstitution of apocytochrome *P*-450 to the holocytochrome was demonstrable

TABLE 1. *Reconstitution of microsomal cytochrome P-450 with hemin after concomitant administration of various inducers of cytochrome P-450 and various inhibitors of heme synthesis*

Pretreatment*		Cytochrome P-450† (nmol/mg of microsomal protein)		% Increase	
Inducer	Inhibitor	− Hemin	+ Hemin		
None		1.11 ± 0.04	1.14 ± 0.04	2.7	N.S.
Phenobarbital (Ph)		2.25 ± 0.06	2.35 ± 0.05	4.4	$P < 0.05$
Cobalt ($CoCl_2$)		0.31 ± 0.03	0.25 ± 0.01	—	
Ph	$CoCl_2$	0.71 ± 0.06	0.87 ± 0.07	22.5	$P < 0.001$
Ph	Lead ($PbCl_2$)	1.28 ± 0.16	1.47 ± 0.24	16.4	$P < 0.05$
Ph	DDC‡	0.80 ± 0.00	0.93 ± 0.00	15.8	$P < 0.05$
Ph	Nickel ($NiCl_2$)	0.32 ± 0.02	0.38 ± 0.02	18.8	$P < 0.05$
3-Methylcholanthrene (3-MC)		1.10 ± 0.00	1.15 ± 0.00	4.5	$P < 0.05$
3-MC	$CoCl_2$	0.73 ± 0.06	0.84 ± 0.06	15.1	$P < 0.001$
Pregnenolone 16α-carbonitrile (PCN)		1.69 ± 0.00	1.55 ± 0.00	—	—
PCN	$CoCl_2$	0.44 ± 0.11	0.55 ± 0.11	25.0	$P < 0.005$

* Ph (50 mg/kg; i.p.); $CoCl_2$ (60 mg/kg; s.c.); $PbCl_2$ (10 mg/kg; i.p.); DDC (300 mg/kg; i.p.); $NiCl_2$ (20 mg/kg; s.c.); 3-MC (20 mg/kg; s.c.); PCN (100 mg/kg; p.o.), administered at 0 and 24 hr. Animals were killed at 48 hr.

† All values are expressed as means ± SD of at least three experiments using liver homogenates pooled from at least two rats. N.S., not significant.

‡ DDC, 3,5-diethoxycarbonyl-4,4-dihydro-2,4,6-trimethylpyridine.

with inducers other than phenobarbital, such as 3-methylcholanthrene and pregnenolone 16α-carbonitrile (Table 1).

Reconstitution of Cytochrome P-450 with Hemin in Various Liver Cell Subfractions. Significant reconstitution of cytochrome *P*-450 was demonstrable only in whole liver homogenate, as no positive effect of hemin was found with isolated microsomes or mitochondrial fractions (Table 2). In fact, with microsomal suspensions we consistently observed a decrease in cytochrome *P*-450 concentration, confirming earlier reports (34). Because of this apparent absolute requirement of liver homogenate for reconstitution, we examined the possibility that a factor present in homogenate but lost in the course of cell fractionation may be essential for reconstitution. Indeed, a recent report of Lewis and Tata (35) suggested that during an early step in the usual cell fractionation, complexes of mitochondria with fragments of rough endoplasmic reticulum are discarded with the nuclear and debris fraction. Reconstitution of microsomal cytochrome *P*-450 with hemin in this crude fraction (640 × *g* pellet) was found to be more efficient than in the parent homogenate, suggesting that the apocytochrome *P*-450 is present primarily in this fraction (Table 3). Electron microscopy of the 640 × *g* pellet from livers of rats treated with cobaltous chloride and phenobarbital confirmed the earlier observation of Lewis and Tata in untreated rat liver, that mitochondrial–rough endoplasmic reticulum complexes are a major component of this fraction.

Metabolic Activity of Reconstituted Cytochrome P-450. Reconstitution of cytochrome *P*-450 in liver homogenate with hemin was associated with a parallel increase in oxidative activity. In rats treated with cobalt and either phenobarbital or 3-methylcholanthrene, the *N*-demethylation of ethylmorphine and *p*-chloro-*N*-methylaniline was increased after addition of hemin to the homogenate (Fig. 2). Ethylmorphine and *p*-chloro-*N*-methylaniline were chosen as prototypes for

TABLE 2. *Reconstitution of cytochrome P-450 in cell fractions after concomitant administration of phenobarbital and cobalt**

Cell fraction	Cytochrome P-450 (nmol/mg of microsomal protein) − Hemin	+ Hemin	% Increase	
Homogenate	0.71 ± 0.06	0.87 ± 0.07	22.5	$P < 0.001$
Microsomes	0.71	0.54	—	
Microsomes + mitochondria	0.71	0.73	2.1	
Microsomes + 105,000 × *g* supernatant	0.68	0.73	7.3	

* Phenobarbital and cobalt treatment was as described in Table 1. The value obtained with homogenate is the mean ± SD of 25 separate experiments using pooled livers from at least two rats. For the other cell fractions, the data represent the mean values of at least two experiments, using livers pooled from at least two rats.

TABLE 3. *Reconstitution of cytochrome P-450 in mitochondria–endoplasmic reticulum complexes (640 × g pellet) after concomitant administration of phenobarbital and cobalt**

Cell fraction	Cytochrome P-450 (nmol/mg of microsomal protein) − Hemin	+ Hemin	% Increase	
Homogenate	0.62 ± 0.13	0.73 ± 0.13	17.8	$P < 0.05$
640 × *g* pellet	0.42 ± 0.05	0.52 ± 0.08	23.8	$P < 0.05$
640 × *g* supernatant	0.74 ± 0.12	0.83 ± 0.12	12.2	$P < 0.05$

* Phenobarbital and cobalt treatments were as described in Table 1. Values obtained represent the mean ± SD of three individual experiments using liver homogenate pooled from at least two rats.

Type I and Type II substrates for cytochrome *P*-450-mediated reactions. No apparent specificity for either ethylmorphine or *p*-chloro-*N*-methylaniline was observed, although phenobarbital is known to induce the metabolism of both the substrates, while 3-methylcholanthrene and other polycyclic hydrocarbons stimulate preferentially the metabolism of *p*-chloro-*N*-methylaniline, with little effect on that of ethylmorphine. A possible explanation for this apparent discrepancy may be inferred from the observation that in rats treated with cobalt and phenobarbital, or with cobalt and 3-methylcholanthrene, cytochrome *P*-450 concentrations before reconstitution are lower than in untreated animals. Under these circumstances, the reduced ratio of cytochrome *P*-450 to the flavoprotein, NADPH-cytochrome *P*-450 reductase, may make the cytochrome the rate-limiting component for both ethylmorphine and *p*-chloro-*N*-methylaniline. This is in contrast to the physiological situation in which the reductase appears to be rate-limiting for the oxidation of most substrates (4, 36, 37). The reduced ratio of cytochrome to reductase activity could account for the finding that reconstitution of cytochrome *P*-450 in rats treated with phenobarbital or 3-methylcholanthrene was associated with enhanced *N*-demethylation of both substrates studied.

DISCUSSION

These findings suggest the presence of apoprotein *P*-450 in the liver of rats in which heme and apoprotein synthesis have been dissociated. Under these circumstances, it was possible to reconstitute the holocytochrome by addition of hemin *in vitro*. The holocytochrome was detected spectrophotometrically by its CO-binding properties and functionally by increased substrate oxidation (Table 1 and Fig. 2). The presence of preformed apoprotein was supported by the finding that during the short period of incubation required for reconstitution, no demonstrable *de novo* protein synthesis occurred. Furthermore, it appears unlikely that the observed increase of cytochrome *P*-450 could be accounted for by a protective effect of hemin on hemoprotein degradation. Hemin failed to increase the cytochrome *P*-450 concentration when it was incubated with microsomes alone, in combination with other subcellular fractions, or with liver homogenates of untreated or cobalt-treated rats.

Interaction of mitochondria (heme synthesis) and rough endoplasmic reticulum membranes (protein synthesis) appears to be required for efficient hemoprotein biosynthesis, as reconstitution of cytochrome *P*-450 was most effective with mitochondria–rough endoplasmic reticulum complexes (Table 3; 640 × *g* pellet). Structural association of mitochondria and rough endoplasmic reticulum may be a prerequisite for the transfer of heme to the apoprotein in microsomal hemoprotein synthesis. Indeed, mitochondria–rough endoplasmic reticulum complexes may represent a functional unit involved in regulation of microsomal cytochrome *P*-450 synthesis, as suggested by Jones and Emans (38).

Black and Bresnick (39) and Siekevitz (40) recently demonstrated by sodium dodecyl sulfate–gel electrophoresis the occurrence of apocytochrome *P*-450 in liver microsomes of fetal and immature rats. In the present study, we failed to detect reconstitution of apocytochrome *P*-450 with hemin in untreated adult male rats (Table 1). It is possible that more sensitive techniques are required to detect the small quantity of apoprotein expected under these conditions. Furthermore,

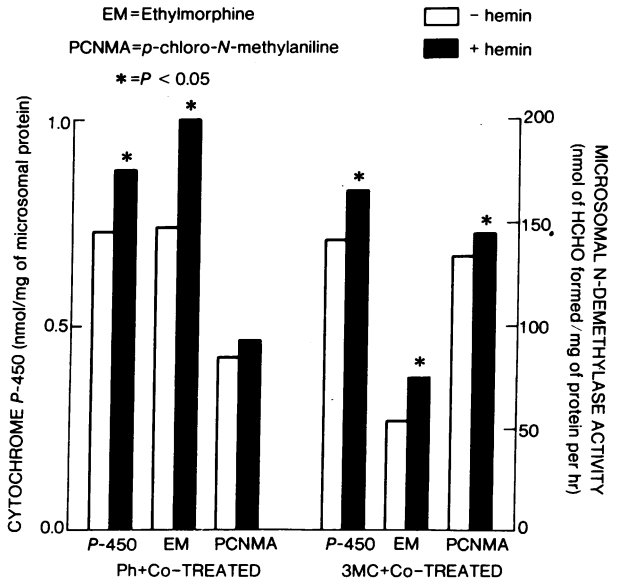

FIG. 2. Functional activity of reconstituted cytochrome *P*-450. Rats were given phenobarbital (Ph) and cobalt (Co), or 3-MC and cobalt (Co) as described in Table 1. Liver homogenates were incubated with hemin, microsomes were prepared, and microsomal cytochrome *P*-450 content and EM and PCNMA *N*-demethylase activities were determined. Values are expressed as the mean ±SD of at least five separate experiments using livers pooled from at least two rats. While the mean PCNMA values (+ or − hemin) from Ph + Co-treated rats were statistically not significant, each pair of values in five individual experiments was significantly different.

partial impairment of heme synthesis by cobalt alone does not result in appreciable accumulation of apoprotein *P*-450 (Table 1). Even in combination with an inducer of cytochrome *P*-450 synthesis, such as phenobarbital, the amount of reconstitutable cytochrome *P*-450 was lower than the expected value. Thus, after incubation with hemin of homogenates from rats treated with cobalt and phenobarbital, the concentration of microsomal cytochrome *P*-450 was 0.87 nmol/mg of protein, as compared to 2.35 nmol/mg of protein in rats treated with phenobarbital alone. Only a minor portion of the induced apoprotein thus appears to be available for reconstitution. This incomplete reconstitution may be due to any of the following factors: (*i*) competition between heme, porphyrins, or cobalt-protoporphyrin for the heme-binding site on the apoprotein; (*ii*) a direct inhibitory effect of cobalt or other heavy metals on protein synthesis or breakdown; (*iii*) inhibition of microsomal protein synthesis by impaired heme synthesis, and (*iv*) suboptimal experimental conditions with only partial reconstitution of the available apoprotein.

Without impairment of heme synthesis and stimulation of cytochrome *P*-450 synthesis, accumulation of apoprotein may be imperceptible because of rapid combination of the synthesized apoproteins with heme generated in mitochondria. This is possible because δ-aminolevulinic acid synthetase, the rate-limiting enzyme in heme synthesis, has a very short half-life (41), which permits it to respond rapidly to stimuli requiring increased heme formation. Nevertheless, when rats were treated with phenobarbital and 3-methylcholanthrene without impairment of heme synthesis, we consistently observed significant, albeit small, reconstitution of cytochrome *P*-450 (Table 1), suggesting that under conditions of maximal

induction of cytochrome *P*-450, heme synthesis may lag slightly behind apoprotein synthesis. This is consistent with the interpretation that apoprotein synthesis may be the primary and rate-limiting event in the control of cytochrome *P*-450 formation. Whether and how free apoprotein regulates the rate of heme synthesis is unknown. In support of this hypothesis, we observed that in rats pretreated with phenobarbital and cobalt, induction of microsomal protein synthesis (maximal incorporation of [^{3}H]leucine into microsomal protein) preceded induction of δ-aminolevulinic acid synthetase (M. A. Correia and U. A. Meyer, manuscript in preparation). Although these observations and interpretations are of a preliminary nature, they warrant reevaluation of the role of heme synthesis in the regulation of cytochrome *P*-450 induction. They suggest that apocytochrome *P*-450, either in the cytosol or in association with mitochondria–rough endoplasmic reticulum complexes (rather than heme synthesis), may be the primary effector in this regulatory process.

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