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Mechanistic Aspects of the Hematin-Mediated Increases in Brain Monooxygenase Activities

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SUMMARY

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Addition of micromolar concentrations of hematin to reaction vessels containing 9000*g* supernatant fractions of rabbit brain homogenates together with cofactors necessary for monooxygenation resulted in up to 70-fold increases in rates of benzo[*a*]pyrene hydroxylation relative to rates observed for incubations performed in the absence of hematin. Time course determinations revealed the hematin-independent activity to be linear with time for at least 2 hr while the hematin-dependent activity was nonlinear, following an upward hyperbolic course of product formation. Preincubation of samples in the presence of substrate and cofactors before the additions of hematin resulted in increased initial rates for the hematin-dependent reaction. However, preincubations of supernatant fractions with hematin alone did not result in increases in initial reaction rates. At equivalent heme concentrations, hemoglobin was approximately one-ninth as effective as hematin in eliciting the stimulatory response, whereas protoporphyrin IX, biliverdin, myoglobin, catalase, and $FeCl_2$ were essentially ineffective in producing enhanced rates of reaction. Hematin did not produce increases in metabolic rates when incubated with hepatic homogenates. Both hematin-dependent and hematin-independent activities were found to be inhibited by carbon monoxide, aniline, cytochrome *c*, 17β-estradiol, 7,8-benzoflavone, butylated hydroxyanisole, and 2-mercaptoethanol. Increasing enzymatic activities by pretreating animals with phenobarbital, 3-methylcholanthrene, or Aroclor 1254 caused a decrease in the stimulatory capabilities of hematin *in vitro*. Hematin also produced increases in rates of oxidative metabolism of 7,12-dimethylbenz[*a*]anthracene and 17β-estradiol, but not of *N*-2-fluorenylacetamide. Metabolic profiles obtained with high-pressure liquid chromatography and benzo[*a*]pyrene or 7,12-dimethylbenz[*a*]anthracene as substrate illustrated that additions of hematin increased the quantity of individual metabolites produced without causing qualitative changes in the metabolic profiles.

INTRODUCTION

Although studies of the biotransformation of polycyclic aromatic hydrocarbons (PAHs)[1] have been extensive, many fundamental questions remain with regard to the bioactivation of PAHs and the nature of their carcinogenic properties. Regulatory and developmental aspects of the biosynthesis of the *P*-450 cytochromes that mediate the epoxidation/hydroxylation of these ubiquitous chemicals, as well as the modulation of cytochrome *P*-450-dependent monooxygenase activities by various effectors—both *in vivo* and *in vitro*—are among the important aspects that remain to be elucidated thoroughly.

Recent reports from our laboratory (1–3) have demonstrated that micromolar additions of hematin *in vitro* produced consistent and oftentimes marked increases in rates of monooxygenation reactions (benzo[*a*]pyrene hydroxylase; EC 1.14.14.2) as catalyzed by enzyme sources derived from extrahepatic tissues of fetal, maternal, and adult rats, rabbits, chickens, and humans. The stimulation produced by hematin appeared specific for extrahepatic tissues (only very slight hematin-mediated effects occurred with hepatic preparations) and tissues of the rabbit responded to hematin additions to the greatest extent.

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[1] The abbreviations used are: PAH, polycylic aromatic hydrocarbons; HPLC, high-pressure liquid chromatography; BaP, benzo[*a*]pyrene; DMBA, 7,12-dimethylbenz[*a*]anthracene; G6P, glucose 6-phosphate; BH, butylated hydroxytoluene; BHA, butylated hydroxyanisole; BF, 7,8-benzoflavone; MC, 3-methylcholanthrene.

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In this investigation, we have attempted to further characterize the hematin-mediated increase in oxidative activities. The phenomenon has been evaluated using subfractions of rabbit brain homogenates as the enzyme source and examined with regard to kinetic parameters, porphyrin derivative and substrate specificity, nature of the metabolites formed utilizing high-pressure liquid chromatography (HPLC) and the effects of inducers and other modifiers of monooxygenase activity.

Our data are consistent with the proposal that cytochrome *P*-450 is the component affected by the additions of hematin and are interpreted to support a suggested model in which free pools of apocytochrome *P*-450 molecules existing in these tissues can complex with added hematin, subsequently yielding functional holocytochrome.

MATERIALS AND METHODS

Materials. *S*-[*methyl*-^{3}H]Adenosyl-1-methionine (specific activity: 10.7 Ci/mmole) was purchased from New England Nuclear, Boston, Massachusetts. 7,12-[G-^{3}H]-benzo[*a*]pyrene (BaP; specific activity: 27 Ci/mmole) and 7,12-dimethylbenz[a]anthracene (DMBA; specific activity: 37 Ci/mmole) were purchased from Amersham/Searle, Arlington Heights, Illinois. [^{3}H]BaP and [^{3}H]-DMBA were further purified using preparative HPLC and TLC in hexane, respectively, to a final purity of >99% when analyzed by HPLC.

BaP and 2-mercaptoethanol were obtained from Eastman Kodak Company, Rochester, New York. BaP was purified by column chromatography on silica gel followed by recrystallization from benzene. DMBA was obtained from Sigma Chemical Company, St. Louis, Missouri, and purified using an aluminum oxide column followed by recrystallization from benzene and ethanol. Glucose 6-phosphate (G6P) was purchased from Boehringer-Mannheim, West Germany. NADPH, NADH, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), hematin, protoporphyrin-IX-dimethyl ester, biliverdin, myoglobin (Type IV), cytochrome *c* (type III), 17β-estradiol, acetylsalicylic acid, indomethacin, L-tryptophan, arachidonic acid, catalase, and reduced glutathione all were obtained from Sigma. 7,8-Benzoflavone (BF) was purchased from Aldrich, Milwaukee, Wisconsin. Metyrapone was obtained through Ciba/Geigy, Summit, New Jersey. Human hemoglobin was purchased from Pentex Biochemicals, Kankakee, Illinois. Cumene hydroperoxide was obtained from MC/B, Norwood Ohio. 3-Methylcholanthrene (MC) and Aroclor 1254 were purchased from Mann Research Labs, New York, and Analabs, Inc., New Haven, Connecticut, respectively. Purified protein kinase (rabbit skeletal muscle) and phosphatase (bovine skeletal muscle) were provided by Dr. E. G. Krebs, University of Washington.

Protoporphyrin IX was prepared by hydrolysis of the dimethyl ester as described by Falk (4) and isolated from ether according to previously reported methods (5). Hematin (purity = 99%) was prepared in 0.01 N NaOH (0.19 mg/ml). All other chemicals utilized were of reagent grade and obtained through standard commercial sources.

Animals and tissue preparation. Male, New Zealand White rabbits weighing approximately 5 lb were purchased from Totem (Lab Assoc.), Kirkland, Washington. Pretreated animals were dosed according to the regimen described by Atlas *et al.* (6). The rabbits were sacrificed by air embolism. The entire brain was rapidly removed and rinsed in ice-cold 1.15% KCl. All subsequent homogenization procedures were performed at 4°. Typically, brains from three to six animals were pooled and homogenized in 3 vol of 1.15% KCl in a Teflon-glass homogenizer. Homogenates of liver and kidney were prepared similarly except 4 vol of KCl were used. The homogenates were then centrifuged at 9000*g* for 5 min (10 min for liver); the supernatants were decanted and frozen at −80°. Enzyme activities were found to be stable for at least 2 months.

BaP assay. A radiometric technique for assaying BaP metabolites (7) was employed with modifications. The procedure [extraction into 1 N NaOH from acetone:hexane (1:4) mixtures] measures the formation of predominantly phenolic and the more polar diol metabolites of BaP (8).

Supernatant (S-9) fractions (9000*g*; 5–10 min) were incubated with shaking at 37° and 100% oxygen tension. Reactions were carried for 2 hr unless otherwise indicated and were initiated by addition of substrates. Reaction vessels typically were assayed in triplicate and contained final concentrations of the following: [G-^{3}H]BaP (2–3 μCi), BaP (80 μM), NADPH (1.1 mM), NADH (0.7 mM), G6P (2.5 mM), tissue supernatant fractions (9000*g*; 1–2.5 mg protein/flask), hematin (18 μM) and sufficient potassium phosphate buffer (0.1 M; pH 7.35) to bring the total volume to 1.0 ml. Blanks contained heat-inactivated (100°; 5–10 min) enzyme. The presence of hematin did not affect the dpm values observed in blanks. The cofactor, substrate, and hematin concentrations employed were determined to yield optimal activities under the conditions utilized in these assays. Maximal rates of metabolism were found to occur at pH 7.35. Reaction rates were found to be linear with protein concentration over the range used. All experiments were performed in darkened conditions under yellow lights. Protein was assayed according to the method of Lowry *et al.* (9).

Activities are expressed as picomoles product per milligram of protein and were calculated (dpm values occurring in blanks were subtracted) according to the formula

$$\frac{\text{dpm recovered in NaOH phase} \times 80\ \text{nmole} \times 1000}{\text{total dpm added} \times \text{mg protein/flask}}.$$

Greater than 97% of the total radioactivity added to each flask was extracted into the organic phase. Samples were counted for a sufficient length of time to insure <3% error with a 95% confidence interval.

Other assays. Analyses of the formation of catecholic estrogens with 17β-estradiol as substrate were performed according to the method of Paul *et al.* (10). Metabolism of *N*-2-fluorenylacetamide was assayed using TLC as described by Juchau *et al.* (11).

High-pressure liquid chromatography. The metabolism of BaP was assayed by HPLC as described (1) except that ethyl acetate:acetone (2:1) was used to extract in-

TABLE 1

Specificity of the hematin effect

Incubation conditions are described under Materials and Methods. Reaction flasks contained 1 mg of rabbit brain S-9 protein. Each compound was added to yield a final heme concentration of 18 μM in reaction flasks. Protoporphyrin IX was prepared as described under Materials and Methods. Activities are means of triplicate determinations with standard deviations <10% of mean values.

Compound	Activity (pmole/mg protein/2 hr)	Ratio
Control	29.5	1.0
Hematin	2111	71.6
Protoporphyrin IX	57.1	1.9
Proto-IX-dimethylester	29.9	1.0
Biliverdin	29.5	1.0
Hemoglobin	237	8.0
Myoglobin	12.0	0.4
Catalase	29.7	1.0
$FeCl_2$	30.0	1.0

cubation media and [G-^{3}H]BaP was employed as substrate. The DMBA metabolic profiles using HPLC were performed according to methods described previously (12).

RESULTS

Specificity of the hematin response. The results of experiments performed to test the specificity for the observed increase in enzymatic activity in rabbit brain tissues are presented in Table 1. These results demonstrated the apparent specificity for hematin as the porphyrin moiety necessary to elicit the stimulatory response. $FeCl_2$ and various porphyrin derivatives tested, including protoporphyrin IX and biliverdin (the immediate biosynthetic precursor and degradation product, respectively) were largely ineffective in enhancing the oxidation of BaP. Additions of hemoglobin to incubation mixtures produced a comparatively moderate increase (8-fold) in the formation of oxidized products relative to the control flasks. In contrast, catalase and myoglobin, the other hemoproteins tested produced no increase in enzyme activity, with myoglobin actually exhibiting an inhibitory effect on measured product formation.

Table 1 indicates that hematin produced a 71-fold increase in the formation of hydroxylated products relative to control flasks. Some variations in enzymatic activities were observed with these tissues. Additions of hematin to incubation vessels containing homogenates of rabbit brain normally produced a 30- to 40-fold enhancement of product formation.

Reaction time course and preincubation experiments. Time course determinations for BaP-hydroxylase activities in rabbit brain subfractions are depicted in Figs. 1a and b. Figure 1a represents monooxygenase activity in these homogenates for a complete incubation system minus hematin. The reaction was linear with time for at least 2 hr. However, metabolite formation did not occur linearly in the presence of hematin (Fig. 1b). After an initial lag period during approximately the first 20 min of the incubation, product formation then dramatically increased and followed an upward hyperbolic course which was still increasing at the 2-hr time point. Due to the nonlinear time plot of the hematin-stimulated reaction, the differences in measured activities between the controls and the hematin experiments were magnified at longer incubation times. A 2-hr time point was arbitrarily chosen as our standard incubation time and activities were thus expressed on a 2-hr, rather than a per minute, basis.

Results presented in Table 2 indicated that, although the initial reaction rate was not increased by preincubation of supernatant fractions with hematin alone or with cofactors, the initial rate of the hematin-dependent reaction could be increased by either preincubation of samples with cofactors and substrate prior to hematin additions, or by adding 3-hydroxy-BaP (1–10 μM) to the reaction mixture immediately before adding BaP to initiate the reaction.

Effects of enzyme induction. The effects of pretreating rabbits with MC, Aroclor 1254, and phenobarbital on the metabolism of BaP in various tissues and the effects of added hematin are shown in Table 3. Phenobarbital pretreatment resulted in only a very slight increase in brain monooxygenase activity. In the kidney and liver, however, phenobarbital pretreatment resulted in the largest increases in BaP monooxygenation—approximately 8-fold and 7-fold increases, respectively, over control activities. Pretreatment with MC or Aroclor 1254 resulted in approximately 2-fold increases in rabbit brain preparations. Hepatic activities for MC- or Aroclor-pretreated animals were also increased only 2- to 3-fold, similar to the findings of other investigators (6). For both

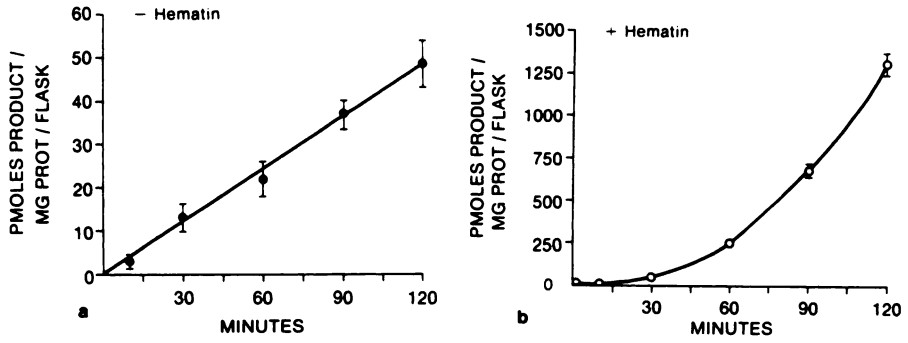

FIG. 1. *Time course determinations of BaP-metabolite formation in the absence (a) and in the presence (b) of hematin*
Details of incubations are given under Materials and Methods. Values represent means of triplicate determinations ± standard deviations.

TABLE 2

Effects of preincubation and additions of 3-hydroxy-BaP on hematin-mediated increases in brain monooxygenase activity

Except as specifically indicated, incubations were performed as described under Materials and Methods. Values in the table are means of specific activities ± SD with *n* values in parentheses.

Experiment	Hematin (18 μM) addition	3-Hydroxy-BaP (5 μM) addition	Preincubation[a] (37°; 15 min)	Activity (pmole product/mg protein/30 min)
1	−	−	−	13.6 ± 6.6 (6)
2	+	−	−	146.1 ± 16.6 (4)
3	−	+	−	12.1 ± 4.8 (4)
4	+	+	−	262.2 ± 22.5 (6)
5	−	−	+	25.3 ± 8.9 (4)
6	−	−	+	13.4 ± 5.7 (4)
7	−	+	+	12.9 ± 6.1 (4)
8	+	−	+	278.6 ± 13.7 (4)

[a] In experiment 5, the preincubated mixture contained only buffer, brain S-9, NADH, and NADPH; all other components were added after preincubation. In experiments 6 and 7, the preincubated mixture contained all components except NADH and NADPH. In experiment 8, the preincubated mixture contained all components except hematin.

the kidney and liver, MC appeared to be a more potent inducer of BaP monooxygenation than was Aroclor 1254.

A striking aspect of these data involves the effect of hematin on these preparations. With each tissue and inducing agent studied, increased levels of enzymatic activity were associated with smaller ratios for the hematin-mediated increases. Similar results were obtained using extrahepatic tissues of MC-pretreated rats (3). Thus, it appeared that higher levels of hydroxylase activity produced by inducer pretreatment resulted in a decreased capacity for hematin to stimulate the reaction.

Cofactor requisites. Investigations performed to examine the cofactor requirements for BaP monooxygenation by rabbit brain subfractions are summarized in Table 4. NADPH and NADH in combination produced the largest observable reaction rates for both basal and hematin-enhanced activities, although NADPH alone was nearly as effective (95%) as both cofactors in combination and values did not differ significantly. Incubation with NADH alone resulted in approximately a 50% reduction in the maximal observable reaction rate in the absence of hematin. For the hematin-mediated hydroxylation, NADH alone was a fairly effective (~80% of controls) reducing agent. These results indicated a more selective requirement for NADPH in the basal activity than for the hematin-mediated reaction. The absence of any added cofactors resulted in large decreases in reaction rates relative to controls. Reduced glutathione was also tested and found to be ineffective as a source of reducing equivalents for the reactions. Interestingly, when cumene hydroperoxide was incubated in the absence of added cofactors (but in the presence of hematin), the hydroperoxide-mediated hydroxylation of BaP was also greatly enhanced. In all cases, no measurable hydroxylation occurred with heat-inactivated (100°; 5 min) supernatant fractions or in the presence of all components (including hematin and reduced nucleotides) except S-9. The hematin-mediated increases also were observed with microsomal fractions prepared by centrifuging the S-9 fractions for 1 hr at 105,000*g*.

Potential modifiers and their effects on enzymatic activities. A number of classical inhibitors of cytochrome *P*-450-mediated monooxygenation reactions also were found to be effective inhibitors of both the control and hematin-stimulated activities, as measured in our system (see Table 5). The substances include carbon monoxide, aniline, cytochrome *c*, 7,8-benzoflavone, and 17β-estradiol. Incubations performed under reduced oxygen tension also resulted in inhibition of the reactions. Of three antioxidants tested, BHT, BHA, and 2-mercaptoethanol were inhibitory to the basal monooxygenase activity at final concentrations of 0.1 mM. BHT exhibited the weak-

TABLE 3

Effects of pretreatment with MC, Aroclor 1254, or phenobarbital on BaP-hydroxylase activities in the presence and absence of hematin

Pretreatment of animals was performed as described under Materials and Methods. Results are expressed as means of duplicate determinations (<10% variation). Activities for brain and kidney are expressed per 2-hr incubation. Activities for liver are expressed per 15-min incubation. Ratios of activities obtained +/− hematin appear in parentheses.

Rabbit tissue	Activity (pmole product/mg protein)			
	Control	MC	Aroclor 1254	Phenobarbital
Brain[a]				
− hematin	31	55	69	37
+ hematin	1040 (34)	993 (18)	989 (15)	963 (26)
Kidney[a]				
− hematin	53	378	174	427
+ hematin	722 (14)	705 (2)	941 (5)	950 (2)
Liver[b]				
− hematin	560	1695	1089	3929
+ hematin	569 (1.0)	1450 (0.9)	1022 (0.9)	3420 (0.9)

[a] 2-hr incubation.

[b] 15-min incubation.

TABLE 4

Cofactor requirements for BaP-hydroxylation catalyzed by rabbit brain supernatant (9000g) fractions in the presence and absence of hematin

Incubations were performed as described under Materials and Methods. Values are expressed as means of activities ± SD ($n = 3$).

Compound added	Concentration (mM)	Activity (pmole product/mg protein)		Ratio[a]	
		− Hematin	+ Hematin	− Hematin	+ Hematin
NADPH, NADH	1.1, 0.7	32.5 ± 1.1	1235.4 ± 66.9	1.00	1.00
NADPH	1.1	31.0 ± 4.8	1175.7 ± 69.2	0.95	0.95
NADH	0.7	17.3 ± 8.1	692.1 ± 30.8	0.54	0.78
−NADPH, −NADH	—	10.8 ± 0.1	6.9 ± 4.1	0.33	0.01
Cumene hydroperoxide[b]	1.0	1.3 ± 0.5	43.9 ± 7.4	—	—

[a] Ratios represent comparisons with values obtained in experiments in which both NADPH and NADH were added to the reaction flasks.

[b] Cumene hydroperoxide was added to incubation flasks in the absence of NADPH and NADH and was incubated for only 1 hr due to the deleterious effects of the hydroperoxides on *P*-450-dependent systems.

TABLE 5

Effects of potential modifiers of BaP-hydroxylase activity assayed using 9000g supernatant fractions of rabbit brain homogenates

All assays were performed as described under Materials and Methods. Modifiers were dissolved in 10 μl of appropriate solvent (H_2O or 100% ethanol) and added to incubation flasks. Values are expressed as ratios of means of activities (experimental/control) obtained for each experiment. Control values in these experiments ranged from 25 to 50 pmole product/mg protein for hematin-independent activities and from 1000 to 2200 pmole product/mg protein for hematin-dependent activities.

Modifier	Concentration (M)	Ratio (experimental[b]/control)	
		−Hematin	+Hematin
CO	—[a]	0.75	0.02
Aniline	10^{-4}	0.43	0.02
Cytochrome *c*	10^{-5}	0.68	0.06
β-Estradiol	10^{-4}	0.09	0.50
Metyrapone	10^{-3}	2.83	1.13
7,8-Benzoflavone	10^{-4}	0.29	0.88
BHT	10^{-4}	0.67	1.00
BHA	10^{-4}	0.29	0.22
2-Mercaptoethanol	10^{-4}	0.52	0.43
Indomethacin	10^{-3}	0.85	0.24
Acetylsalicylate	10^{-2}	0.58	0.62
L-Tryptophan	10^{-3}	0.87	1.26
Arachidonic acid	10^{-4}	1.03	1.10
$MgCl_2$	5×10^{-3}	0.75	1.10
$CaCl_2$	5×10^{-3}	0.96	1.06
EDTA	10^{-4}	1.50	0.83

[a] Incubations with CO were performed in a 50% CO:50% O_2 atmosphere and compared to controls incubated in a 50% N_2:50% O_2 atmosphere.

[b] "Experimental" refers to those flasks to which modifiers were added. Only the corresponding vehicles (buffer or ethanol) were added to control flasks.

est inhibitory effect on basal activity and was devoid of effect on the hematin-enhanced activity at this concentration. Both 2-mercaptoethanol and BHA produced marked inhibitory effects of hematin-mediated reactions as well as the control activity.

The effects of BF and metyrapone (0.1 and 1 mM, respectively) on the formation of oxygenated products of BaP in brain homogenates are also presented in Table 5. The addition of BF resulted in inhibition of metabolism for both the control and hematin-enhanced activities. Additions of metyrapone to incubation flasks led to interesting results. Metyrapone produced a 2.8-fold stimulation of hematin-independent activity as well as a 13% increase in hematin-mediated activity. These results could not be explained, but are somewhat analogous to the observations of Leibman (13) regarding the stimulatory effects of metyrapone on acetanilide hydroxylation.

In light of recent reports (14, 15) that BaP can be cooxygenated by an arachidonic acid-dependent cyclooxygenase (also a hemoprotein), we examined the effect of indomethacin and acetylsalicylate (both are known to be inhibitors of cyclooxygenase) on BaP hydroxylation, as measured in our system. Unexpectedly, at final concentrations of 1 mM or greater, each compound elicited inhibitory effects on both the control levels (15 and 42% inhibition for indomethacin and acetylsalicylate, respectively) and hematin-enhanced levels (76 and 38%, respectively) of enzymatic activity. Similar concentrations were assayed using Aroclor 1254-pretreated rat hepatic homogenates. In these preparations, a slight (~60%) stimulation of AHH activities occurred. These results again illustrated the differences between hepatic and extrahepatic hydroxylation systems.

To further investigate the possible involvement of cyclooxygenase cooxygenation we examined the effect of the addition of arachidonate and L-tryptophan to incubation flasks. Although the addition of arachidonate (0.1 mM) appeared to produce a slight stimulatory effect, the reaction was not dependent on the presence of arachidonate, which appears to be requisite to cooxygenation reactions (16, 17). L-Tryptophan additions did not inhibit the hematin-dependent reaction. Additions of $MgCl_2$, $CaCl_2$ (5 mM) or EDTA (10^{-4} M) to incubation vessels also resulted in only slight effects.

In a sensitive assay designed to measure the formation of catecholic estrogen metabolites, we examined the capacity of hematin to alter rates of 17β-estradiol hydroxylation using homogenates of rabbit brain. We observed an approximate 4-fold increase in rates of metabolism of 17β-estradiol upon the addition of hematin during a 10-min incubation. Attempts to detect increases in hydroxylation of *N*-2-fluorenylacetamide according to described methods (11) proved negative, however, in that hematin produced no detectable increase in rates of metabolism of this substrate when using rabbit brain homogenates as the enzyme source.

High-pressure liquid chromatography. Results of analyses performed with HPLC and BaP or DMBA as substrates are presented in Figs. 2 and 3, respectively. A

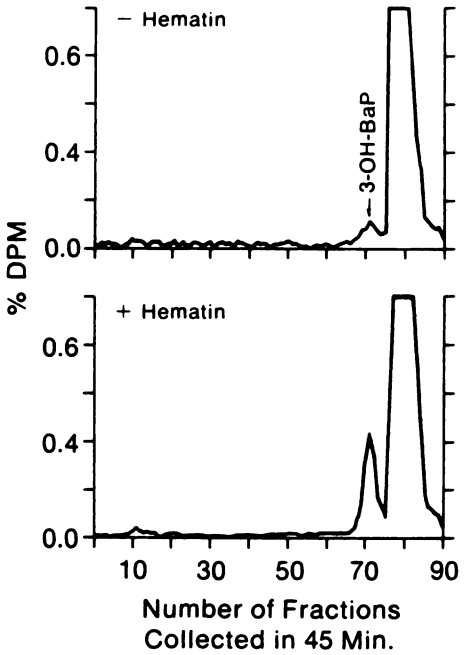

FIG. 2. *HPLC profiles of BaP metabolism in rabbit brain S-9 in the absence (upper profile) and in the presence (lower profile) of hematin*

See Materials and Methods for experimental details.

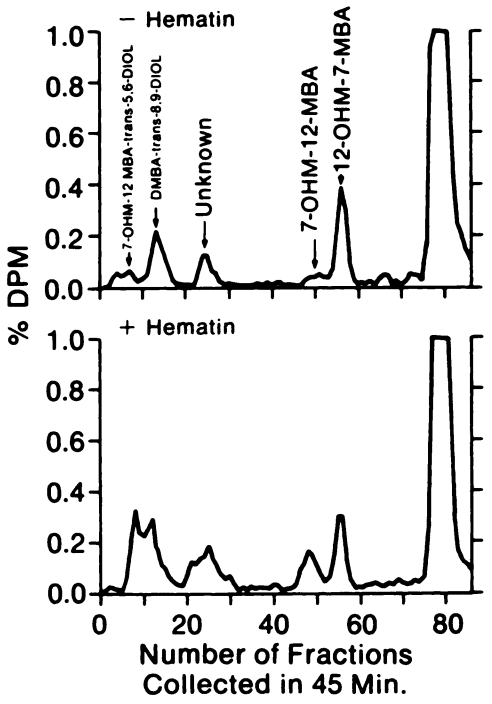

FIG. 3. *HPLC profiles of DMBA metabolism in rabbit brain S-9 in the absence (upper profile) and in the presence (lower profile) of hematin*

See Materials and Methods for experimental details.

metabolite cochromatographing with the 3-hydroxy metabolite of BaP was the only detectable metabolic product formed with BaP as substrate. Addition of hematin to the incubation medium resulted in an increase in the quantity of 3-hydroxy metabolite without any other detectable alterations in metabolite patterns.

When DMBA was used as substrate, a number of metabolites cochromatographing with reference standards of DMBA metabolic products were detected. In the control preparations metabolism occurred at both methyl carbons; the 12-hydroxymethyl metabolite of DMBA appeared to be the major detectable metabolic product in control preparations. Also detected were metabolites cochromatographing with the 7-hydroxymethyl-12-methyl-5,6-diol, the DMBA-*trans*-8,9-diol, and an unknown metabolite of DMBA that did not cochromatograph with any available standard. Additions of hematin to the incubation flasks resulted in apparent increases in all of the above-mentioned metabolites with the exception of 12-hydroxymethyl derivative. As was the case with BaP, no qualitative alteration in metabolite formation was observed upon the addition of hematin to flasks containing DMBA.

DISCUSSION

In this report we have examined a number of biochemical and pharmacological parameters associated with the hematin-mediated increase in oxidative activities as measured with homogenates of rabbit brain. Although rabbit tissues are exceptionally sensitive to the hematin effect, the metabolic stimulation produced by hematin has been found to occur with every extrahepatic tissue preparation in every species examined (2, 3).

A large number of possible mechanisms could be postulated to account for the enhancement of oxidative activities produced by hematin. We have considered some of the more likely possible explanations and, in terms of the available evidence, have ruled out the following as probable causes of the stimulatory effect of hematin:

1. *Nonenzymatic oxidation of substrate by hematin.* Experiments performed using heat-inactivated enzyme or enzyme blanks have demonstrated that, under these conditions, hematin is unable to mediate oxygenation reactions.

2. *Enzyme stabilization.* Time course determinations (see Figs. 1a and b) indicate that the action of hematin is not one of extending the linear period of the product formation curve.

3. *Hematin inhibition of further product transformation.* Analyses performed using HPLC and BaP as substrate (Fig. 2) indicate that increased product formation occurred only in the phenolic fractions and that the effect of hematin was not due to inhibition of product transformation.

4. *Inactivation of endogenous inhibitors, facilitation of electron transport, or conversion of inactive enzyme to active enzyme by phosphorylation or dephosphorylation.* In experiments that monitored initial rates of reaction as a function of varying preincubation times with hematin (reactions in these experiments were initiated by the addition of substrate), we were unable to detect any increases in *initial* reaction rates with preincubation times of up to 2 hr.

It has been reported that heme plays an important role in the regulation of rates of protein synthesis by modulating the phosphorylation of initiation factors present in reticulocyte lysates (18, 19). Preliminary experiments to test for similar mechanisms in the regulation of monooxygenase activity utilized purified preparations of phosphatase and of catalytic and regulatory subunits of protein kinase, together with appropriate cofactors for these phosphorylation/dephosphorylation reactions. However, no stimulation of rates of hydroxylation were detected in these experiments, either in the presence of absence of hematin.

5. *Involvement of cyclooxygenase.* The function of BaP in such a cooxygenation reaction, theoretically, would be to serve as an aromatic source or donor of electrons to the enzyme–substrate complex, in a fashion similar to that suggested for L-tryptophan (see Ref. (14)). L-Tryptophan has been reported to stimulate activities or purified preparations of cyclooxygenase (15), and, in addition, Cavalieri *et al.* (20) have reported that BaP can participate in one-electron oxidation reactions. If cooxygenation of BaP were the mechanism responsible for the hematin stimulation effect, we reasoned that additions of L-tryptophan should effectively compete with BaP as the electron source for the reaction. However, even at a 1 mM final concentration, L-tryptophan additions did not exhibit any inhibitory effect on the hematin-dependent reaction (in fact, a slight stimulation of the reaction was observed).

Also arguing against cooxygenation were the metabolic profiles of BaP obtained in our experiments as compared

to those obtained by researchers who have investigated the cooxygenation reaction (16). Phenolics appeared to be the only metabolites formed by rabbit brain homogenates (Fig. 5) either in the presence or absence of hematin, whereas quinones were reported as the only detectable metabolites in arachidonate-dependent cooxygenase systems. These observations also tend to rule out other non-*P*-450-mediated mechanisms.

6. *Inhibition of lipid peroxidation.* Since the stimulatory effect of hematin on BaP metabolism becomes magnified relative to controls at longer incubation times, the possibility was considered that hematin could be inhibiting lipid peroxidation, thereby preserving increased levels of enzyme at longer incubation periods. Several lines of evidence rule against this hypothesis. First, it has long been known that lipid peroxidation readily occurs in hepatic microsomal preparations and this tissue has been the focus of many studies of this nature. The stimulatory effect of hematin on hepatic oxidative activities, however, is not apparent and appears to be specific for extrahepatic tissues. Second, low rates of lipid peroxidation occur in the rabbit (21), as compared to the rat; yet the enhancing effect of hematin is greatest with tissues from rabbit (3). Third, the antioxidant BHT is known to inhibit lipid peroxidation *in vitro*; yet when tested in our assay system, BHT had no effect on the hematin-dependent hydroxylation of BaP. EDTA, an effective inhibitor of lipid peroxidation, also exhibited minor effects.

The results presented in this study fulfill many of the criteria usually considered necessary to implicate the involvement of a cytochrome *P*-450-dependent monooxygenase system as the enzyme complex responsible for the hematin-dependent metabolism we have observed. Both the control incubation systems and those containing the hematin share a number of similarities with regard to these criteria. Results indicate that each system requires molecular oxygen and NADPH as the preferred source of reducing equivalents. Both enzymatic reactions are inhibited by carbon monoxide, aniline, cytochrome *c*, BF, and 17β-estradiol. Each of these substances inhibit cytochrome *P*-450-dependent monooxygenase reactions. The results of experiments with BF are similar to those reported by Wiebel *et al.* (22) regarding the inhibitory effects of this compound on aryl hydrocarbon hydroxylase activities in extrahepatic tissues and hepatic tissues from inducer-pretreated animals. The differential inhibitory effects by BF on hematin-independent and -dependent reactions may be similar to the differential effects produced by BF on various species of *P*-450s *in vitro* (23).

In addition, other known substrates of *P*-450-dependent monooxygenases (specifically, DMBA and 17β-estradiol) also exhibit increased rates of metabolism in the presence of hematin.

Although the cytochrome *P*-450-dependent monooxygenase system requires NADPH and molecular oxygen for activity, these cofactors can be replaced *in vitro* by organic hydroperoxides, the most common being cumene hydroperoxide (24, 25). Recent evidence that hydroperoxide-dependent aromatic hydroxylations are attended by an "NIH shift" comparable to that occurring in the presence of NADPH and molecular oxygen (26), suggests that hydroxylation in the two systems proceeds via a common intermediate, presumably an arene oxide. We observed that the addition of cumene hydroperoxide to incubation vessels in the absence of cofactors and molecular oxygen resulted in the production of oxygenated metabolites of BaP and that the rate of this metabolism could be markedly enhanced upon the further addition of hematin (Table 4). These data also are consistent with a *P*-450-dependent monooxygenase in the hematin-enhanced reaction.

Furthermore, pretreatment of animals with agents that produced increases in rates of BaP-metabolism was associated with a decrease in the magnitude of the stimulatory effect of hematin when added to these systems (Table 3). At this point, such observations cannot be readily explained. Yet, the fact that classical inducing agents that are known to alter the cytochrome *P*-450 system *in vivo* can also affect the action of hematin *in vitro* also suggests that hematin is acting upon a cytochrome *P*-450-dependent monooxygenase system.

The results presented in this study are, thus, interpreted to indicate not only an apparent specificity for hematin as the moiety necessary in the production of large increases in enzymatic activity, but also to implicate cytochrome *P*-450 as the enzyme on which hematin acts in producing this response. Insofar as it has been studied in the liver, evidence has accumulated that the availability of heme for cytochrome *P*-450 biosynthesis in the normal animal is not rate limiting (27, 28). The rabbit brain has also been found to possess relatively high heme-biosynthetic activity (29).

The following model is proposed as an explanation of the stimulatory effects of hematin in extrahepatic tissues: It is postulated that in these tissues, pools of free apocytochrome exist that have a low affinity for their heme prosthetic group but a comparatively high turnover number when in the holocytochrome state. These unsaturated pools of apoprotein can undergo a change, possibly in conformation, producing an increase in the relative affinity for heme and thereby resulting in heme incorporation, i.e., the formation of a functional holoenzyme molecule. Support for this model has been obtained in the preliminary observations on the time course with which the hematin-mediated reaction proceeds: The initial lag period (see Fig. 2) found to be characteristic of the hematin effect could be partially circumvented by first preincubating the system in the presence of cofactors and substrate before the addition of hematin, or by the addition of 3-hydroxy BaP, the major metabolite, to the incubation system immediately before initiating the reactions (Table 2). These preliminary results suggested that accumulation of some substance in the reaction mixture (apparently a metabolite) was required for, or at least facilitated, the hematin effect. This substance may alter the apoenzyme in some fashion, resulting in a rapid incorporation of heme into the apoprotein. A similar mechanism has been demonstrated to regulate the activity of tryptophan pyrrolase (30), a cytoplasmic hemoenzyme. Experiments with modifiers also suggest that if this model is valid, the reconstituted *P*-450 differs from that mediating basal activity.

In previous experiments, we have been unable to detect spectral increases in holoenzyme as a consequence of adding hematin, even in systems that exhibited very large increases in enzymatic activity (3). However, it is possible that small pools of cytochrome *P*-450 possessing high turnover numbers may not be spectrally detectable.

If such a model proves correct, this would indicate that extrahepatic tissues possess a short-term regulatory mechanism for cytochrome *P*-450-mediated monoxygenase activities—the regulation provided by substrate metabolism, whereby specific species of *P*-450 could be activated (reconstituted) in response to the presence of specific substrates. Further work is required to determine the applicability of this concept and to test the postulated model.

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