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SUBSTRATE AND POSITION SPECIFICITY OF HEMATIN-ACTIVATED MONOOXYGENATION REACTIONS

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Abstract—Rates of hydroxylation of benzo[*a*]pyrene (BaP), benzo[*e*]pyrene (BeP), chrysene, acetanilide (AC), 7,12-dimethylbenz[*a*]anthracene (DMBA), and 17β-estradiol (E_2) *in vitro* could be increased by as much as 70-fold by additions of micromolar quantities of hematin. Such increases were observed primarily when extrahepatic tissues were utilized as the enzyme source; the greatest increases occurred with rabbit brain. Mixed-function oxygenations of aniline, ethylmorphine, benzphetamine, *N*-2-fluorenylacetamide (FAA), dibenz[*a,h*]anthracene (DBA), and benz[*a*]anthracene (BA), were affected minimally or not at all by hematin additions. Analyses of metabolites of BaP, AC and DMBA with high-pressure liquid chromatography revealed a high degree of position specificity for the hematin-mediated reactions. This specificity was dependent upon the enzyme source, e.g. with rabbit kidney as enzyme source and AC as substrate, hematin additions resulted in only minor increases (30–40 per cent) in quantities of 3- and 4-hydroxylated metabolites and decreases (approximately 40–50 per cent; possibly a result of further degradation) in amounts of 2-hydroxylated AC. With hematin additions to rabbit brain homogenates, quantities of the measured 2-hydroxylated AC increased by 10 to 12-fold and of the 3-hydroxylated product by 3 to 4-fold, but no detectable changes in 4-hydroxylated AC were observed. With BaP as substrate, hematin elicited the formation of large quantities of an unidentified and hitherto undetected metabolite. Results of the study were consistent with the concept that hematin additions result in the reconstitution of a number of functionally distinct, tissue-specific cytochrome P-450 apoproteins.

Previous investigations in this laboratory [1–6] have revealed that additions of hematin to reaction vessels containing benzo[*a*]pyrene (BaP) and necessary enzymes and cofactors for cytochrome P-450-dependent monooxygenation resulted in extremely large increases in the formation of hydroxylated metabolites. The most profound effects were observed when extrahepatic homogenates or homogenate subfractions were utilized as the source of enzyme; only slight or no effects were observed with hepatic tissues. Species specificity was demonstrated, inasmuch as rabbit tissues clearly exhibited the largest increases. Data obtained to date are consistent with a model presented previously [4]. The model suggests that pools of apocytochrome(s) P-450 exist in extrahepatic tissues; that the apocytochrome(s) exhibits a relatively low affinity for hematin but may undergo a change (possibly in conformation) resulting in an increased affinity for hematin and subsequent formation of holocytochrome; that additions of hematin *in vitro* can then result in the formation of catalytically active hemoprotein; and that the resulting reconstituted holocytochrome(s) is characterized by a comparatively high substrate turnover number.

The present investigation was designed to provide information pertaining to the substrate and position specificity of hematin-mediated monooxygenation reactions. The results presented here indicate that such specificity is highly dependent on the enzyme source and suggest the possibility that a number of functionally distinct P-450 cytochromes may exist as free apoproteins in the various extrahepatic tissues.

MATERIALS AND METHODS

Chemicals. [G-^{3}H]Benzo[*a*]pyrene (BaP, 27 Ci/mmole) and [7,10-^{14}C]BaP (60.7 mCi/mmole) were obtained from Amersham, Arlington Heights, IL, and from the New England Nuclear Corp., Boston, MA, respectively. [G-^{3}H]-7,12-Dimethylbenz[*a*]anthracene (DMBA, 37 Ci/mmole), [12-^{14}C]benz[*a*]anthracene (BA, 49 mCi/mmole), [9-^{14}C]*N*-2-fluorenylacetamide (FAA, 26 mCi/mmole). *p*-[G-^{3}H]hydroxyacetanilide (1.9 Ci/mmole) and [7-^{14}C]dibenz[*a,h*]anthracene (DBA, 8.8 mCi/mmole) were purchased from Amersham. Manufacturers' estimates of purity for these purchased compounds were approximately 98 per cent; however, all labeled and unlabeled hydrocarbons were repurified as described below.

[G-^{3}H]Chrysene (0.4 Ci/mole) and [G-^{3}H]-benzo[*e*]pyrene (BeP, 0.12 Ci/mole) were prepared from the unlabeled hydrocarbons by catalytic exchange. *S*-[Methyl-^{3}H]adenosyl-1-methionine (SAM, 10.7 Ci/mmole) was purchased from the New England Nuclear Corp.

Unlabeled BaP, BeP and chrysene were purchased from the Aldrich Chemical Co., Milwaukee, WI. Unlabeled DMBA, BA, DBA, 17β-estradiol (E_2), catechol-*O*-methyltransferase (COMT), NADPH, NADH, glucose-6-phosphate dehydrogenase (G6PDH), G6P and hematin all were obtained from the Sigma Chemical Co., St. Louis, MO. FAA, aniline and *p*-aminophenol were obtained from the Eastman Kodak Co., Rochester, NY. Benzpheta-

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mine was a gift from Upjohn Inc., Kalamazoo, MI. Ethylmorphine was a gift from Merck, Sharp & Dohme, Inc., West Point, Pa.

Sources for the metabolite standards used in these studies for BaP, DMBA and FAA were as described previously [2, 7, 8]. Derivatives of AC hydroxylated in positions 2 and 3 were a gift from Dr. Jack Hinson, NIH, Bethesda, MD. The *p*-hydroxylated derivative was obtained from the Eastman Kodak Co.

Reverse-phase preparative high-pressure liquid chromatography (h.p.l.c.) was employed to purify both the unlabeled and radioactive AC derivatives and BaP, BA and DBA. The chemicals were injected onto a Whatman Partisil ODS-2 Mag-9 column (9.4 mm i.d. × 25 cm) and eluted with a methanol: water (75–100% methanol) linear gradient (4 ml/min; 30 min) or a 33% methanol isocratic elution for AC. Greater than 99 per cent purity was achieved with these techniques. DMBA was purified using an alumina column followed by recrystallization from benzene and ethanol. [G-^{3}H]DMBA was purified on thin-layer chromatograms with hexane. The purity was greater than 99 per cent. E_2 was purified by recrystallization from ethanol. AC was prepared by acetylation of redistilled aniline with acetic anhydride in benzene solution, followed by recrystallization from ethanol (2x). *p*-Aminophenol was purified by recrystallization from water and ethanol. Hematin (purity = 99 per cent) was prepared fresh in 0.01 N NaOH (0.19 mg/ml) before each use.

Tissue preparation. Male, New Zealand White rabbits weighing 2.0–2.5 kg were obtained from R & R Laboratories, Inc., Kirkland, WA, and allowed to acclimate for 3–5 days. All animals were fed standard laboratory chow (Purina Co., St. Louis, MO) and water *ad lib*. Rabbits were killed by injecting air intravenously; kidneys, livers and brains were removed and placed in ice-cold 0.15 M KCl/0.05 M potassium phosphate buffer at pH 7.35. Tissues were pooled from three to six animals and homogenized in 3 volumes of the buffer solution with a teflon–glass homogenizer. Homogenates were centrifuged at 9000 *g* for 5 min (10 min for liver); the supernatant fractions were decanted and frozen at −80°. Enzyme activities were stable for at least 2 months.

Male, Sprague–Dawley rats weighing 200–220 g were purchased from Tyler Laboratories, Inc., Bellevue, WA, and allowed to acclimate for 2–3 days. Rats were fed standard laboratory chow (Purina Co.) and water *ad lib*. They were killed by cervical dislocation; tissues were prepared as described above for rabbits. Some rats were pretreated with Aroclor 1254 (500 mg/kg, 5 days before killing them) and S-9 fractions from these animals served as control standards for the enzyme assays.

Enzyme assays. Phenolic, and the more polar non-phenolic, oxidized, metabolites of BaP, BA, DBA, BeP and chrysene were assayed by measurements of base-extractable radioactivity from an organic phase according to a modified [4] method of Brown and Kupfer [9]. Supernatant fractions (S-9) were incubated with shaking at 37° and 100% oxygen tension. Reactions in which rabbit brains were utilized as enzyme source were carried for 2 hr [4] and were initiated by addition of substrate. Reactions with hepatic tissues were carried for 15 min. Reaction vessels were assayed in triplicate and contained final concentrations of the following: radioactive substrate (1–3 μCi), unlabeled substrate (80 μM), NADPH (1.1 mM), NADH (0.7 mM), G6P (2.5 mM), tissue supernatant (1–2.5 mg protein/flask), hematin (10–18 μM), and sufficient potassium phosphate buffer (0.1 M, pH 7.35) to bring the total volume to 1.0 ml. Incubations were terminated by addition of supersaturating quantities of NH_4Cl and 4 ml of ice-cold acetone–hexane (1:4, v/v) except in the case of chrysene in which acetone–hexane–isoamyl alcohol (1:3:1, by vol.) replaced the acetone–hexane mixture. Extraction and scintillation counting were further performed as described [4]. For chrysene, a second extraction with ether–hexane–isoamyl alcohol (1:3:1, by vol.) was performed and the organic phase extracts were pooled. All assays were performed in darkened conditions under yellow lights.

The metabolism of [9-^{14}C]FAA was assayed by t.l.c. as described previously [8]. This technique allows determinations of hydroxylation at carbons 1, 3, 5 and 7 and of *N*-hydroxylation. Analyses of the formation of catechol estrogens with E_2 as substrate were performed with a coupled assay procedure utilizing [^{3}H]SAM and purified COMT as described in detail by Paul *et al.* [10] and by Chao *et al.* [11]. Aniline hydroxylase was measured according to methods described by Imai *et al.* [12]. Assays for benzphetamine and ethylmorphine *N*-demethylation were performed according to methods described by Hewick and Fouts [13] estimating the production of formaldehyde as described by Nash [14]. All reactions were zero order with respect to substrates and cofactors and were carried for varying time intervals as described in Results. Hematin additions (10–20 μM) to the reaction flasks did not interfere with the assay procedures.

High-pressure liquid chromatography. Metabolites of BaP, DMBA and AC were analyzed with h.p.l.c. Biotransformation of [G-^{3}H]DMBA was measured according to procedures described by DiGiovanni *et al.* [7] and Bond *et al.* [2]. Two separation techniques were employed for the analysis of BaP metabolites. The first procedure has been described previously [15]. A second procedure was similar except that a Whatman Partisil 10-ODS-2 column (4.6 mm i.d. × 25 cm) was utilized; 10 μl of the reconstituted sample was injected onto the column and eluted with a linear water–methanol gradient (1 ml/min; 80–100% methanol over a period of 30 min). Fractions were collected at 24-sec intervals for 30 min followed by 1-min fractions for an additional 15 min. This procedure resulted in an approximate 2.5-min separation of the 9-hydroxy-BaP and 3-hydroxy-BaP metabolites as contrasted with a 40-sec separation time with the first procedure.

For measurements of AC hydroxylation, incubation flasks contained 25 mM G6P, 1.1 mM NADPH, 0.7 mM NADH, 10 μM heme (when applicable), 3 mM substrate (2 μCi of [U-^{14}C]AC), various quantities of the enzyme source and suffcient Tris–HCl buffer (0.1 M, pH 7.6) to yield a total volume of 1.0 ml. Reactions were carried for various time periods and were terminated by adding 2.5 ml of ice-cold ethyl acetate to the reaction mixture. After

shaking for 10 min, the samples were centrifuged and the ethyl acetate layer was removed and saved. The aqueous material was reextracted as above, and the organic layer was pooled with the first extract. The pooled extract was evaporated to dryness under a stream of nitrogen and reconstituted in 0.2 ml methanol. The 2-, 3-, and 4-hydroxylated metabolites of AC were separated from the substrate by a slight modification of the procedures described by Guenthner *et al.* [16]. A 10 μl aliquot of methanol was injected onto a Whatman Partisil 10-ODS-3 column and eluted with an isocratic gradient of 33% methanol–water and a flow rate of 2 ml/min. Fractions were collected at 20-sec intervals and counted for radioactivity. Retention times of eluted metabolites were compared with the retention times of unlabeled standards detected with u.v. at 254 nm and with the retention time of a [G-^{3}H]-4-hydroxyacetanilide spike added to the ethyl acetate extracts.

Liquid scintillation counting. Scintillation counting was performed in Beckman LS 8000 and LS 9000 scintillation counters. Digital output from the LS 8000 was recorded on cassette tapes using write-and-read-interface units (Meditek Labs, Seattle, WA), and data reduction was performed on an HP 9830A computer/plotter and programmed in Basic language. Standard quench curves were computed using [^{3}H]toluene and [^{14}C]toluene standards. Aquasol (New England Nuclear) was employed as scintillation mixture, and all samples were counted for a sufficient length of time to ensure less than a 3% error with a 95% confidence interval. All samples were evaluated for the possibility of interference from chemiluminescence. None of the conditions of modifiers utilized posed interference problems. Flasks containing heat-inactivated (100°, 10 min) tissue preparations (otherwise identical to test flasks) were always utilized as sample blanks, and dpm values occurring in these blanks were subtracted from the dpm values of the test samples.

RESULTS

Analyses of oxidized metabolites (primarily phenolic compounds) of various polynuclear aromatic hydrocarbons (Table 1) revealed that hematin additions produced quantitatively different effects on the oxidation reactions, dependent upon the specific substrate under investigation as well as the enzyme source. Increases were greatest for BaP with brain tissue as the source of enzyme. In brain tissue, 20- to 40-fold increases were commonly observed and in some experiments increases were as high as 70-fold. Hematin additions also resulted in 10- to 12-fold increases in oxidation of BeP and 3- to 5-fold increases in oxidation of chrysene. With the method utilized, no increases were observed with BA, DBA, or with DMBA as substrates. However, h.p.l.c. analyses [4] revealed that marked increases in metabolites cochromatographing with 7-hydroxymethyl-12-methylbenz[*a*]anthracene and with 7-hydroxymethyl-12-methylbenz[*a*]anthracene-*trans*-5,6-diol could be observed upon incubating DMBA with rabbit brain preparations.

The observation that hematin additions resulted in decreased quantities of base-extractable, oxidized metabolites of the benzanthracene derivatives DMBA, BA and DBA (Table 1) was unexpected. These experiments were repeated four times with triplicate samples to verify this observation; similar results were obtained in each experiment. Further, in experiments with some of the substrates commonly utilized in drug metabolism studies (Table 2), hematin additions also produced no increases but rather minor and consistent decreases in observed specific activities when rabbit kidneys were employed as enzyme source. With liver or brain, hematin produced no significant changes in observed enzyme activities.

In experiments with FAA, hematin produced only very minor changes (Table 3) which tended toward

Table 1. Effects of heme additions on the conversion of polynuclear aromatic hydrocarbons to base-extractable, oxidized metabolites*

Substrate	Tissue	Incubation time (min)	Specific activity [pmoles · (mg protein)$^{-1}$ · (incubation time)$^{-1}$] −Heme†	+Heme†
Benzo[*a*]pyrene	Brain	120	23 ± 7 (6)	657 ± 83 (6)
	Liver	15	560 ± 61 (5)	569 ± 61 (6)
Benzo[*e*]pyrene	Brain	120	34 ± 19 (6)	408 ± 96 (5)
	Liver	15	1592 ± 463 (3)	976 ± 221 (3)
Chrysene	Brain	120	69 ± 17 (6)	245 ± 19 (6)
	Liver	15	722 ± 103 (3)	1604 ± 292 (3)
Benz[*a*]anthracene	Brain	120	87 ± 23 (12)	28 ± 15 (12)
	Liver	15	684 ± 154 (3)	610 ± 173 (3)
Dibenz[*a*]anthracene	Brain	120	78 ± 14 (12)	21 ± 12 (12)
	Liver	15	2171 ± 562 (3)	2024 ± 399 (3)
7,12-Dimethylbenz[*a*]anthracene	Brain	120	712 ± 113 (6)	68 ± 29 (6)
	Liver	15	2502 ± 186 (6)	3138 ± 374 (6)

* These metabolites were extracted into acetone–hexane (1:4) and back-extracted into 1 N NaOH. In experiments with chryseme, metabolites were extracted once with isoamyl alcohol–hexane–acetone (1:3:1) and a second time with isoamyl alcohol–hexane–ether (1:3:1). These extracts were pooled and back-estracted into 1 N NaOH. Values obtained with heat-inactivated (100°, 10 min) tissue preparations were subtracted as background. Rabbit tissues were utilized in all experiments. Reactions without hematin proceeded linearly during the stipulated incubation times. Values in the table are means ± S.E. Values in parentheses are the number of experiments performed.

† Hematin concentrations were 10 μM in all experiments.

Table 2. Effects of heme additions (10 μM) on the monooxygenation of common drug substrates in hepatic and extrahepatic tissues*

Substrate	Tissue	Incubation time (min)	Specific activity [nmoles · (mg protein)$^{-1}$ · (incubation time)$^{-1}$] −Heme	+Heme
Aniline	Brain	60	11.9 ± 1.3	11.7 ± 3.4
	Kidney	60	2.8 ± 0.6	1.4 ± 1.3
	Liver	10	14.9 ± 1.9	14.4 ± 0.9
Benzphetamine	Brain	60	5.5 ± 1.0	5.4 ± 1.6
	Kidney	60	6.2 ± 0.9	4.0 ± 1.2
	Liver	15	58.5 ± 5.2	61.5 ± 3.5
Ethylmorphine	Brain	60	2.6 ± 1.1	2.6 ± 2.7
	Kidney	60	7.1 ± 1.0	5.9 ± 3.4
	Liver	15	29.3 ± 3.1	28.2 ± 4.0

* Results are means ± S.E. of triplicate determinations with pooled S-9 preparations (see Materials and Methods). Experiments were repeated at least twice with similar results.

Table 3. Metabolism of [^{14}C]FAA in S-9 preparations of brain, lung and liver*

Tissue	Specific activities† 7-OH-FAA	5-OH-FAA	*N*-OH-FAA	3-OH-FAA	1-OH-FAA
Rabbit brain					
−Heme	157.2	10.8	36.6	61.2	22.4
+Heme	93.0	11.4	40.2	37.8	20.6
Rabbit lung					
−Heme	79.5	10.6	51.5	26.2	36.8
+Heme	74.3	14.9	49.6	24.0	31.4
Aroclor 1254-rat liver					
−Heme	2894.3	441.0	359.7	628.3	100.2

* Assayed with t.l.c. as described in Ref. 8.

† Specific activities are expressed as pmoles product · (mg S-9 protein)$^{-1}$ · (incubation time)$^{-1}$. Results are means of duplicate determinations on the same tissue pool (< 10 per cent deviation). Rabbit brain and lung S-9 fractions were incubated for 120 min. Hepatic S-9 fractions from Aroclor 1254-pretreated (500 mg/kg, 5 days prior to killing) rats were utilized as positive control standards and were incubated for 15 min.

inhibition rather than activation when brain or lung was utilized as enzyme source. With brain tissue as enzyme source, hematin produced increased rates of catechol estrogen formation via aromatic ring hydroxylation (Table 4) but, unlike the results obtained with polycyclic hydrocarbons (in which the hematin effect is magnified with increasing incubation times), the magnitude of the stimulatory effect of hematin decreased as incubation times increased [11].

Improvements in h.p.l.c. technology (see Materials and Methods) allowed us to evaluate more precisely the effects of hematin additions on the profile of oxidized metabolites of BaP. The effects of hematin additions on BaP metabolism in incubation flasks containing rabbit brain are illustrated in Fig. 1. Very large increases in quantities of metabolites eluting with 3-hydroxy-BaP, the 6,12-quinone and 9-hydroxy-BaP were noted. In addition, large quantities of radioactivity eluted as a single peak following

Table 4. Catechol estrogen formation in rabbit brain and liver S-9 fractions

Rabbit tissue	Incubation time (min)	Specific activity* −Heme	+Heme
Brain	10	0.28 ± 0.11	0.72 ± 0.18
	30	2.26 ± 0.54	5.60 ± 0.43
	60	4.98 ± 1.26	8.42 ± 1.39
Liver	10	151.72 ± 9.18	157.90 ± 8.62

* Specific activities are expressed as pmoles catechol estrogen formed · (mg S-9 protein)$^{-1}$ · (incubation time)$^{-1}$; values are means ± S.E. of triplicate determinations performed on pooled samples (see Materials and Methods). This experiment was repeated once with similar results.

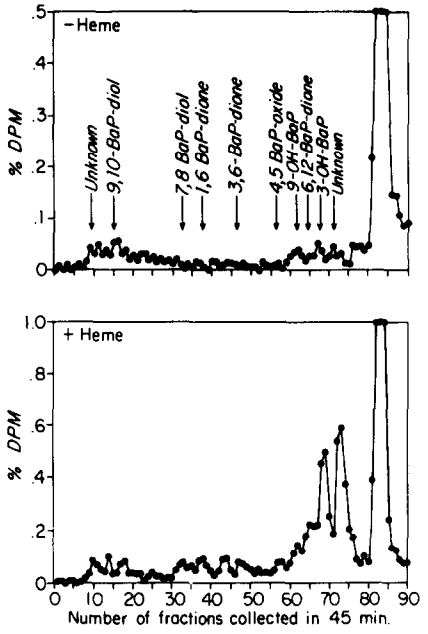

Fig. 1. Metabolite profile of organic solvent [acetone–hexane (1:4, 1x) and ethyl acetate–acetone (2:1, 1x)]-extractable benzo[*a*]pyrene metabolites formed in incubation vessels containing S-9 fractions from rabbit brain homogenates and the effect on the profile of hematin added *in vitro* (10 μM). Incubation conditions and methods of h.p.l.c. analyses are described in Materials and Methods.

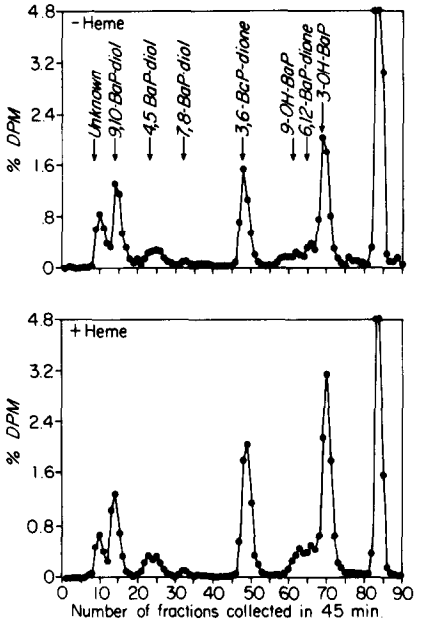

Fig. 3. Metabolite profile of organic solvent-extractable (see legend to Fig. 1) benzo[*a*]pyrene metabolites formed in incubation vessels containing S-9 fractions from rabbit liver homogenates and the effect on the profile of hematin added *in vitro* (10 μM).

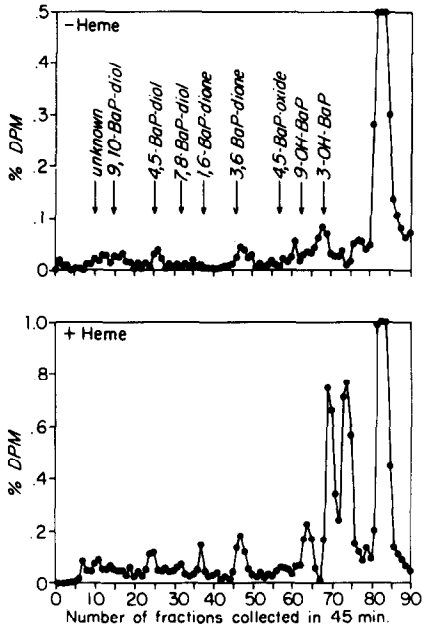

Fig. 2. Metabolite profile of organic solvent-extractable (see legend to Fig. 1) benzo[*a*]pyrene metabolites formed in incubation vessels containing S-9 fractions from rabbit kidney homogenates and the effect on the profile of hematin added *in vitro* (10 μM).

the elution of 3-hydroxy-BaP. This radioactivity cochromatographed with 9,10-dihydro-BaP but did not cochromatograph with 3-, 7- or 9-hydroxy-BaP. Extraction of ethyl acetate extracts with 1 N NaOH effectively eliminated the peak, suggesting that the unknown metabolite(s) was a phenolic compound. Preliminary analyses with fluorescence and u.v. spectroscopy also suggested that the metabolite(s) was a phenol. Further rigorous experiments will be required to firmly identify the compound or compounds eluting in this fraction. The same phenomenon also was observed in experiments with rabbit kidneys (Fig. 2) but not in experiments with rabbit livers (Fig. 3) or in heat-inactivated samples incubated with hematin. No major changes in metabolic profiles were noted upon addition of hematin to hepatic preparations.

The effect of hematin additions on the metabolism of AC by preparations of rabbit brain, kidney and liver are presented in Figs. 4, 5 and 6 respectively. Again, additions of hematin produced marked stimulation of the oxidative metabolism of AC in the brain but elicited only comparatively minor effects in hepatic preparations. In brain tissues, profound effects on 2-hydroxylation were noted, with 10- to 15-fold increases observed consistently. Somewhat lesser increases in quantities of radioactivity eluting with 3-hydroxy-AC resulted from hematin additons. Further, increased radioactivity eluted as a peak prior to the 4-hydroxy-AC standard; the elution characteristics suggested that the peak may have been a dihydroxy compound(s). No significant increases in radioactivity eluting with 4-hydroxy-AC were noted.

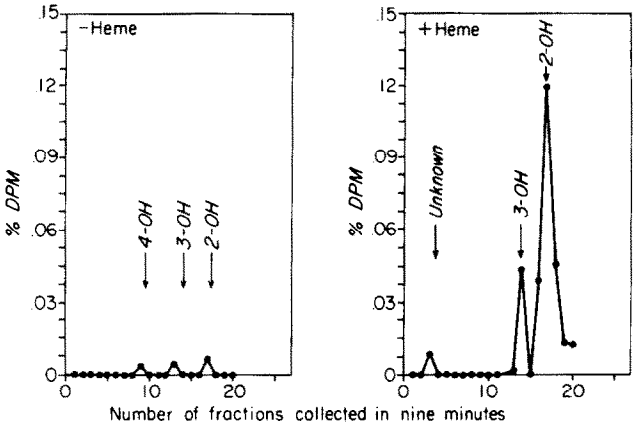

Fig. 4. Metabolite profile of ethyl acetate-extractable (2x) acetanilide metabolites formed in incubation vessels containing S-9 fractions from rabbit brain homogenates and the effect on the profile of hematin added *in vitro* (10 μM). Incubation conditions and methods of h.p.l.c. analyses are described in Materials and Methods.

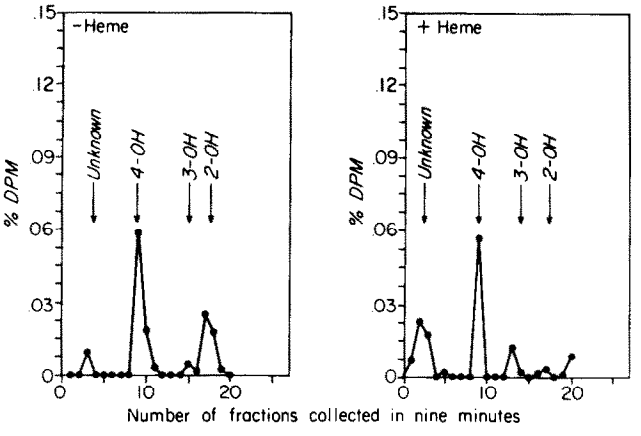

Fig. 5. Metabolite profile of ethyl acetate-extractable (2x) acetanilide metabolites formed in incubation vessels containing S-9 fractions from rabbit kidney homogenates and the effect on the profile of hematin added *in vitro* (10 μM).

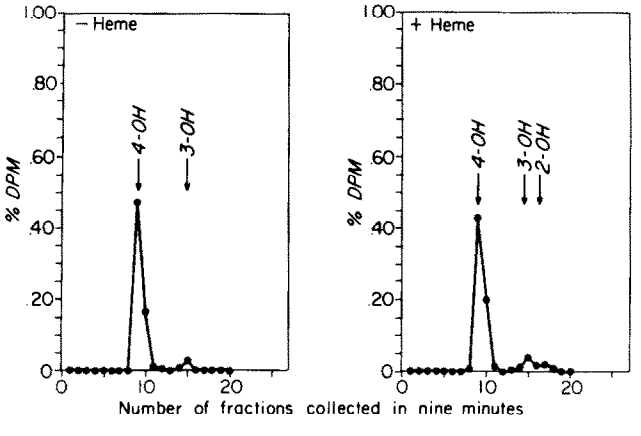

Fig. 6. Metabolite profile of ethyl acetate-extractable (2x) acetanilide metabolites formed in incubation vessels containing S-9 fractions from rabbit liver homogenates and the effect on the profile of hematin added *in vitro* (10 μM).

Table 5. Effect of heme additions on the position-specific monooxygenation of AC as assessed with h.p.l.c.

Tissue	Hematin addition (10 μM)	Specific activity* [pmoles · (mg protein)$^{-1}$ · min^{-1}] ortho(2)	meta(3)	para(4)	Unknown†	Total
Brain	No	2.3 ± 1.3	2.3 ± 1.1	ND‡	ND	4.6 ± 1.3
Brain	Yes	27.6 ± 3.4	7.8 ± 0.8	ND	4.9 ± 3.0	40.3 ± 2.7
Kidney	No	7.9 ± 1.7	2.3 ± 0.2	6.8 ± 2.8	5.1 ± 1.9	22.1 ± 1.8
Kidney	Yes	4.8 ± 2.2	3.4 ± 1.4	9.8 ± 1.8	9.3 ± 2.6	27.3 ± 2.1
Liver	No	1.3 ± 1.2	49.3 ± 2.1	684 ± 29	1.3 ± 0.0	736.6 ± 31
Liver	Yes	58.7 ± 4.3	94.7 ± 9.6	677 ± 34	12.3 ± 2.8	843.0 ± 30
Liver (rat)	No	56.9 ± 4.6	64.8 ± 8.7	1034 ± 93	16.0 ± 4.2	1172 ± 81
Liver (rat)	Yes	82.7 ± 8.9	71.4 ± 3.2	1018 ± 81	18.6 ± 3.0	1191 ± 92
Liver (rat)§ (PCB-pretreated)	No	50.7 ± 9.1	223.3 ± 9.9	2807 ± 79	18.8 ± 2.3	3100 ± 87

* Kidney and brain preparations were incubated for 2 hr; liver preparations were incubated for 15 min. Tissues were from rabbits unless otherwise specified. The same experiment was repeated three times with closely similar results. Numbers in the table are means ± S.D. of the three experiments.

† Radioactivity in this peak did not cochromatograph with any of the available standards; retention times suggested that it was a diphenol.

‡ ND indicates not detectable. Specific activities less than 1.0 were not significantly different from blank values.

§ Rats in this group were pretreated with Aroclor 1254 (500 mg/kg, 5 days prior to being killed).

In the kidney, hematin additions resulted in a quantitatively and qualitatively different profile (Fig. 5) of AC metabolites. A marked decrease in quantities of radioactivity eluting with 2-hydroxy-AC was concomitant with almost equal increases in radioactivity eluting prior to 4-hydroxy-AC. Some increase in radioactivity eluting with 3-hydroxy-AC was observed but virtually no changes in quantities chromatographing with 4-hydroxy-AC. An additional minor peak eluted prior to 4-hydroxy-AC (see Fig. 5) and appeared consistently in the profiles. This peak likewise has not yet been identified. In hepatic preparations, the only striking change in the profile (Fig. 6) was the appearance of increased radioactivity eluting with 2-hydroxy-AC. Results from experiments in which AC was utilized as substrate are tabulated in Table 5. In both rabbits and rats, hepatic preparations exhibited increases in radioactivity eluting with 2- and 3-hydroxy-AC but no increases in 4-hydroxy-AC. Pretreatment of rats with Aroclor 1254, on the other hand, produced increases in 3- and 4-hydroxy-AC but no significant change in 2-hydroxy-AC.

DISCUSSION

The evidence supporting the suggestion that the very profound hematin-mediated increases in extrahepatic oxidation of aromatic substrates occur as the result of incorporation of the added heme into P-450 apocytochrome(s) has been summarized previously [4]. According to the suggested hypothesis, a metabolite-mediated change (allosteric?) appears to increase the affinity of the apocytochrome for heme, thus resulting in the formation of active holocytochrome. The results presented in this study are interpreted to suggest further that functionally distinct free apocytochromes with relatively low affinity for heme may be present in various tissues and cells and that the reconstituted holocytochrome forms each may display a somewhat unique but overlapping substrate specificity. This seems to be best supported by observations with h.p.l.c. of position-specific hydroxylation by hematin-dependent monooxygenases in studies with BaP, AC and DMBA.

Currently, an extensive body of evidence is available to suggest the existence of a large number of distinct P-450 cytochromes with varying functional characteristics existing in different tissues, in different subcellular fractions of the same cells, and even in the same subcellular organelles. Various reports [17, 18] have indicated that some of the P-450s found in extrahepatic tissues do not have detectable hepatic counterparts. Other studies [e.g. Refs. 19 and 20] have demonstrated that P-450 cytochromes are under complex genetic control, with various tissues exhibiting tissue-specific and temporal genetic regulation. In view of such observations, it seems plausible to suggest that differing P-450 apocytochromes may vary in their affinities for heme and that some do not function as monooxygenases *in vitro* in the absence of added heme. Rigorous testing of this hypothesis will require considerable additional research but a body of data already exists to intimate that hepatic P-450(s) can exist both *in vitro* and *in vivo* as the apocytochrome(s) [21–23 and references therein].

It should be appreciated that, until more direct evidence can be obtained, a number of other possible mechanisms for the hematin-mediated increases in monooxygenation reactions must be entertained. Several possible mechanisms have been tentatively ruled out on the basis of experimental data [4]. These include nonenzymatic oxidation of substrate by hematin, enzyme stabilization, inhibition of further product transformation, arachidonate-dependent, cyclooxygenase-mediated cooxygenation, inactivation of endogenous inhibitors, facilitation of electron

transport, conversion of inactive enzyme to active enzyme via phosphorylation or dephosphorylation, and inhibition of lipid peroxidation. Examination of the time course of the hematin-mediated effect showed that no increase in *initial* reaction velocity could be observed. This absence of increase in *initial* reaction velocity would argue strongly against several conceivable mechanisms.

One mechanism that has been suggested as a viable possiblity is that of a hydroperoxide-mediated oxidation reaction, e.g. a flavoprotein reductase plus heme-catalyzed reaction. This suggestion remains a possibility but the experimental data obtained to date tend to argue against it. The data which suggest that it is not a likely mechanism are:

1. If the suggested mechanism were operative, an increase in the initial reaction velocity should be expected. As stated above, this was not observed.
2. If the suggested mechanism were operative, there should be little or no species or organ specificity of the type that has been observed. In particular, tissues with the highest flavoprotein concentrations would be expected to exhibit the greatest hematin-mediated increases. The increases appear to be unrelated to tissue and species differences in flavoprotein activity or concentrations.
3. A hydroperoxide attack on substrates would be expected to result in oxidations at the most reactive positions on the substrate molecule. For benzo[*a*]pyrene, for example, this would be at carbon 6, and for benzo[*a*]pyrene-7,8-diol at carbons 9 and 10. With benzo[*a*]pyrene as substrate one would expect to observe primarily 1,6-, 3,6-, and 6,12-quinones as products of the reaction. With the 7,8-diol, one would expect to observe the 7,8-diol-9,10-oxide and/or subsequent products. The studies of Marnett and coworkers [24 and references therein] support these expectations. Formation of the 3-phenol of benzo[*a*]pyrene is predominant in cytochrome P-450-mediated monooxygenations, and the cytochrome appears to serve as a template for orientation of the molecule for oxidation in that otherwise unlikely 3-position. In view of this, it is highly significant that a major reaction product detected in the hematin-mediated reaction is 3-hydroxybenzo[*a*]pyrene.
4. Lack of inhibition of the hematin-mediated reaction by antioxidants. Butylated hydroxytoluene failed to inhibit the reaction at 10^{-4} M concentrations [4].
5. Specific and sensitive inhibition of the hematin-mediated reaction by P-450 inhibitors. Carbon monoxide, aniline and cytochrome *c* were extremely effective inhibitors. This suggests a P-450-mediated rather than a hydroperoxide-mediated reaction, particularly if considered in conjunction with the specific cofactor requirements [4].

The data presented in this paper demonstrating that hydroxylation of only certain substrates is affected by hematin additions suggest that, if our hypothesis is correct, only a very limited number of P-450 cytochromes are affected by hematin and that these may exhibit a high degree of substrate specificity. The fact that aromatic hydroxylation of some polynuclear aromatic hydrocarbons but not others (as well as specific positions on the hydrocarbons) was increased by hematin additions also tends to indicate a very high degree of substrate specificity of reconstituted apocytochromes. The data indicating that rates of hydroxylation of the aromatic rings of BA, DBA and DMBA were actually decreased following hematin additions (as opposed to marked increases in rates of hydroxylation of aromatic rings of BaP, BeP and chrysene) were unexpected but very consistent. The substrate and position specificities exhibited were reminiscent of those displayed by the P-450 systems involved in the metabolism of steroid hormones. Increases in rates of one position-specific reaction are accompanied frequently by decreases in another hydroxylation reaction. Important also were the observations that rates of oxygenation of a large number of commonly utilized substrates for P-450-dependent monooxygenation reactions were not affected appreciably by hematin additions. Position specificity for the hematin-mediated hydroxylation reactions was well demonstrated in studies of the h.p.l.c.-separated metabolites of BaP, AC and DMBA. Further progress in this area of research will depend upon the successful isolation and purification of hematin-activated components.

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