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ANILINE HYDROXYLATION IN THE HUMAN PLACENTA—MECHANISTIC ASPECTS*†

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Abstract—Attempts to demonstrate aniline *p*-hydroxylating activity in particulate fractions of human or rat placental homogenates yielded consistently negative results, even in subfractions with very high aryl hydrocarbon hydroxylase activity. *p*-Hydroxylation of aniline, however, was readily detected in 104,000 *g* supernatant fractions from both species. Characteristics of the placental reaction differed markedly from the reaction classically observed in hepatic microsomes. Ammonium sulfate fractionation and column chromatography analyses indicated that hemoglobin and/or methemoglobin were strongly associated with placental catalytic activity. Substitution of hemoglobin for placental tissues in reaction flasks resulted in an easily measurable reaction. Cytochrome *c* could substitute for hemoglobin but was a much less efficient catalyst under the reaction conditions utilized. Flavins were not required as components of these model system reactions, but FMN or FAD markedly accelerated the cytochrome *c* catalyzed reactions. Comparisons of tissue-free (model system) reactions with those catalyzed by components present in placental homogenates indicated that hemoglobin was a principal factor responsible for catalysis of *p*-hydroxylation of aniline in these placental preparations, thus suggesting that placental trophoblasts *per se* were either devoid of or extremely low in aniline *p*-hydroxylating activity. The results also indicated separate mechanisms for aniline and 3,4-benzpyrene hydroxylation processes.

IN RECENT years, several investigators have reported oxidations of xenobiotic substrates catalyzed by mixed-function oxidative enzyme systems present in placentas from a variety of species, including humans. A current review of these aspects of drug metabolism has appeared very recently.[1] The most intensively investigated of these xenobiotic biotransformation reactions has been the hydroxylation of 3,4-benzpyrene. Studies on placentas from cigarette-smoking women and in methylcholanthrene-pretreated experimental animals tend to indicate that the placental enzyme system (referred to as aryl hydrocarbon hydroxylase or benzpyrene hydroxylase) exhibits many characteristics similar to those observed in the corresponding hepatic microsomal system.[2–5] One of the principal differences appears to be a much lower apparent affinity of the reduced nucleotide cofactor (NADPH) for the placental system of either species.[2,3] In previous studies on placental benzpyrene hydroxylation catalysis with 3-methylcholanthrene (MC)-pretreated pregnant rats,[6] we discovered that 9000 *g* supernatant fractions of rat placental homogenates would catalyze an easily measurable aniline *p*-hydroxylation reaction. The present study represents an attempt

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to characterize the placental reaction and to present preliminary studies on the catalytic mechanism. Characteristics of aniline *p*-hydroxylation catalysis by hemoglobin and cytochrome *c* also are reported.

METHODS

Animal tissues. Timed-pregnant Sprague–Dawley rats were obtained from Simonsen Laboratories (Gilroy, Calif.) and were 14 days pregnant upon arrival. Test animals were given 20–160 mg/kg of 3-methylcholanthrene (MC) in corn oil as a single dose via intraperitoneal injection on day 15, 16 or 17 of pregnancy. Controls were given an equal volume of corn oil vehicle. Rats were killed by cervical dislocation 48 hr later, and maternal livers and uterine horns were removed. Placentas and fetal livers were dissected from the horns and all tissues were placed on ice. After blotting and weighing, tissues were homogenized in a Potter homogenizer with a teflon pestle in 2 vol. of ice-cold 0·25 M sucrose. Homogenates were then centrifuged at 9000 *g* for 20 min in a refrigerated IEC centrifuge. Incubations were performed within 2 hr after the animals were sacrificed. Supernatants from 9000 *g* centrifugations were employed in most instances; in others, the 9000 *g* supernatants were centrifuged for 1 hr at 104,000 *g* and the 104,000 *g* supernatants (soluble fractions) or pellets (microsomes) were employed. Microsomal pellets were washed twice with isotonic KCl and reconstituted in KCl such that microsomes from 1 g of liver or 10 g of placenta were present in each milliliter of suspension.

Human tissues. Human placentas were obtained from the delivery room of the University Hospital (Seattle) at term within 15 min following normal vaginal deliveries or cesarean sections. Placental cotyledenous tissues were dissected out, placed in approximately 500 ml of ice-cold isotonic KCl solution, minced, and rinsed twice with similar volumes of KCl solution. After blotting and weighing, the placental tissues were homogenized with 2 vol. of ice-cold 0·25 M sucrose or 1·15% KCl for 1 min at high speed in a Waring blender. The homogenates were centrifuged at 760 *g* for 20 min in an IEC refrigerated centrifuge. Subsequent centrifugations were at 9750 *g* for 10 min and 104,000 *g* for 1 hr. Particulate fractions were washed three times with isotonic KCl solution. The 760 *g* pellets were washed by rehomogenization in the Waring blender in the same volume of original solution and were resuspended in isotonic KCl such that sediment from 1 g of placenta was present in 1 ml suspension. The 9750 *g* pellets were washed by hand with the use of a Potter homogenizer and teflon pestle and finally resuspended such that material from 5 g of placenta was present in 1 ml suspension. The 104,000 *g* pellet was handled in a similar fashion except that washed "microsomes" from 10 g of placenta were present in 1 ml of the suspension. Preliminary studies with marker enzymes, electron microscopy, and chemical analyses indicated that the 9750 and 104,000 *g* pellets contained high respective concentrations of mitochondria and endoplasmic reticular components as compared to other intracellular elements.

Chemicals. NADPH, NADH, FAD, FMN, bovine hemoglobin, methemoglobin and hematin, horse heart cytochrome *c* (type III) and highly purified milk xanthine oxidase were obtained from Sigma Chemical Co., St. Louis, Mo. Carbon monoxide (99·5 per cent minimum purity) and aminopyrine were purchased from Matheson Co., Inc., Joliet, Ill. Aniline hydrochloride, *p*-aminophenol, 3,4-benzpyrene, MC,

TABLE 1. HYDROXYLATION OF ANILINE AND 3,4-BENZPYRENE IN HOMOGENATE SUBFRACTIONS OF LIVER AND PLACENTA

Pre-exposure to inducing agents		Aniline hydroxylase* pH 7·0		Aniline hydroxylase* pH 7·4		Aniline hydroxylase* pH 8·1		Benzpyrene hydroxylase* pH 7·4	
		Liver	Placenta	Liver	Placenta	Liver	Placenta	Liver	Placenta
Rats	MC†	5·6	1·1	7·9	0·8	13·4	0·3	8·9	0·4
(9000 *g* sup.)	None	4·6	1·2	3·8	0·8	3·2	0·3	2·8	0
Humans	Cigarettes	‡	0§		0		0		6·5
(104,500 *g* pellet)	None		0		0		0		0

* Activities are expressed as micromoles of substrate hydroxylated per hour per gram of protein. For aniline hydroxylation, incubation vessels contained 1 ml homogenate fraction, 1·1 ml 0·1 M tris buffer, 10 μmoles aniline hydrochloride, 14 μmoles glucose 6-phosphate and 3·6 μmoles NADPH in a total volume of 3·0 ml. Flasks were incubated for 5 hr in an atmosphere of 100% oxygen at 37·5°. For benzpyrene hydroxylation, vessels contained 0·5 ml homogenate fraction, 1·2 ml potassium phosphate buffer (0·1 M, pH 7·35), 2 units of glucose 6-phosphate dehydrogenase, 9 μmoles glucose 6-phosphate, 2·4 μmoles NADPH and 0·2 μmole 3,4-benzpyrene in a total volume of 2·0 ml. Flasks were incubated for 15 min in an atmosphere of 100% oxygen at 37·5°.

† Rats were given 40 mg/kg of MC in corn oil 48 hr prior to sacrifice.

‡ Blanks indicate no determinations were made.

§ Zero indicates no detectable activity.

p-nitrobenzoic acid (PNBA) and *p*-aminobenzoic acid (PABA) were purchased from Eastman Organic Chemicals. All other reagents and solvents were of analytical grade.

Assay procedures. Quantitative determinations of amounts of *p*-aminophenol formed from aniline HCl in reaction vessels were performed by the methods described by Schenkman *et al.*[7] This method is specific for the *para*-hydroxylated metabolite; i.e. *ortho*- or *meta*-aminophenol do not couple with phenol to yield the blue compound which is assayed in the procedure.[8] Determinations of hydroxylated 3,4-benzpyrene metabolites were made by slight modifications of the method of Wattenberg *et al.*[9] as previously described.[3] Incubation conditions, substrate and cofactor concentrations, etc. varied considerably and are described for each experiment in the text. Amounts of 4-aminoantipyrine formed during incubation with aminopyrine were determined according to methods described by La Du *et al.*[10] PABA formation was assayed according to previously described methods.[11] Protein concentrations were analyzed by the methods of Lowry *et al.*[12] using bovine serum albumin as standard. Hemoglobin analyses were performed according to the method of Levere and Granick[13] utilizing 6004 mM^{-1} cm^{-1} as extinction coefficient for the difference in absorbance between 418 and 433 nm.

RESULTS

Previous experience with human placental aryl hydrocarbon hydroxylase[3] indicated that high concentrations of the electron donor (NADPH) might be required to detect a measurable placental aniline hydroxylation reaction *in vitro*. Certain human placentas obtained at term were found to contain particularly high levels of aryl hydrocarbon (benzpyrene) hydroxylase activity in each of the three particulate fractions (see Methods) tested. The highest specific activity was found in a 104,000 *g* pellet fraction (microsomes). Examination of this same microsomal subfraction for aniline *p*-hydroxylation revealed that it was devoid of detectable activity. The results of this initial experiment are outlined in Table 1. Maternal livers and placentas from MC-pretreated pregnant rats also were examined for purposes of comparison and control. In these experiments, however, 9000 *g* supernatant fractions of rat tissues were utilized. As indicated, no aniline hydroxylase activity was detected in the human particulate fractions, but an easily measurable reaction was observed in flasks containing the rat placental 9000 *g* supernatant.

Subsequent experiments then were designed to determine whether these observations represented a species difference or were a function of the subfraction employed. As shown in Table 2, subfractionation experiments demonstrated that the placental aniline *p*-hydroxylating activity was localized almost exclusively in 104,000 *g* supernatant fractions of rat placental homogenates. It was subsequently found that the same subfraction of human placental homogenates also contained catalysts for the reaction. Moreover, specific activities in 104,000 *g* supernatant fractions were slightly higher than in 9000 *g* supernatant fractions, further indicating localization in the soluble portion of the homogenates. Pretreatment of pregnant rats with phenobarbital (80 mg/kg/day for 4 consecutive days, beginning on day 15 of gestation) or MC (40 mg/kg—see Methods) did not produce detectable levels of activity in particulate fractions of placental homogenates, nor did such procedures produce increased activity in 9000 or 104,000 *g* placental supernatant fractions (Table 1). Marked

TABLE 2. ANILINE HYDROXYLASE ACTIVITY IN SUBFRACTIONS OF PLACENTAL HOMOGENATES*

Homogenate subfraction	Human	Rat
Whole homogenate	0·72	0·66
760 *g* pellet	<0·04†	<0·04
9750 *g* pellet	<0·04	<0·04
104,000 *g* pellet	<0·04	<0·04
104,000 *g* supernatant	1·71	1·48
9750 *g* supernatant	1·64	1·40

* Activities are expressed as micromoles of aniline hydroxylated per hour per gram of protein. Reaction conditions are the same as those given in Table 1 at pH 7·0. Pretreatment of rats with MC or phenobarbital (see text) resulted in the same distribution pattern with no increase in activity in any subfraction at 5- or 1-hr incubation periods.

† Values less than 0·04 were not significantly different from corresponding blanks.

increases in aniline and benzpyrene hydroxylase activities, however, were observed in maternal livers from the same animals.

In these preliminary experiments, NADPH appeared to be a more efficient electron donor than NADH in placental soluble fractions, particularly in dialyzed preparations (Table 3). It was also noted that activity diminished with time in unfrozen preparations and that heat inactivation (100°, 5 min) reduced the activity by 60–80 per cent.

Experiments designed to determine optimal conditions for the placental catalyzed reaction were next performed. The reaction appeared to proceed in a linear fashion for approximately 1 hr. Lesser increases in PAP recovery from flasks were observed during the next 5 hr, but at 8 hr the quantity of PAP recovered from flasks was significantly less than that recovered at 1 hr. This observation may have been due to

TABLE 3. ELECTRON DONOR SPECIFICITY IN HUMAN PLACENTAL 104,000 *g* SUPERNATANT FRACTIONS

	Aniline hydroxylase activity*	
Preparation	NADPH	NADH
Undialyzed (−15°, 24 hr)	0·84	0·51
Undialyzed (4°, 24 hr)	0·73	0·49
Dialyzed† (4°, 24 hr)	0·61	0·05

* Same as Table 2 except that no glucose 6-phosphate was added to reaction vessels.

† Ten ml of placental supernatant was dialyzed against 800 ml of 0·1 M phosphate buffer, pH 7·0, for a period of 24 hr with three changes of the dialysate.

further oxidation of PAP. In subsequent experiments, incubations with placental soluble fractions were of 45-min duration.

Utilizing potassium phosphate buffer, optimal pH was observed at approximately 7·0, and this buffer at pH 7·05 (0·1 M) was utilized in all subsequent experiments. With a final substrate concentration of $1{\cdot}4 \times 10^{-2}$ M and an NADPH concentration of $1{\cdot}2 \times 10^{-3}$ M, the reaction rate increased linearly with increasing placental protein concentrations. In contrast to the reaction observed in rat hepatic 9000 *g* supernatant fractions, no substrate inhibition was apparent even at $2{\cdot}3 \times 10^{-2}$ M final concentration of aniline hydrochloride. High concentrations of the NADH or NADPH were required for quantitative observations.

A series of potential activators and inhibitors was tested in an attempt to further characterize the system and to draw comparisons between the placenta and liver (Table 4). Steroids synthesized by the human placenta and cholesterol exhibited no

TABLE 4. EFFECTS OF POTENTIAL INHIBITORS AND ACTIVATORS ON HUMAN PLACENTAL ANILINE HYDROXYLASE ACTIVITY*

Compound	Concentrations tested	Effect
SKF-525 A	10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}	No significant effect
FMN, FAD	10^{-6}, 5×10^{-6}, 10^{-5}, 5×10^{-5}, 10^{-4}, 5×10^{-4}, 10^{-3}, 5×10^{-3}	Biphasic, optimal (20%) Activation at 10^{-5} M, 40% Inhibition at 5×10^{-3} M
β-Estradiol	10^{-5}, 10^{-3}	No significant effect
Estrone	10^{-5}, 10^{-3}	No significant effect
Progesterone	10^{-5}, 10^{-3}	No significant effect
Cholesterol	10^{-5}, 10^{-3}	No significant effect
Nicotinamide†	10^{-5}, 10^{-4}, 10^{-3}	No significant effect
Acetone	10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 10°	No significant effect
Potassium cyanide	10^{-5}, 10^{-4}, 10^{-3}	Total inhibition at 10^{-3} M
p-Chloromercuribenzoate	10^{-5}, 10^{-4}, 10^{-3}	Total inhibition at 10^{-3} M
p-Hydroxymercuribenzoate	10^{-5}, 10^{-4}, 10^{-3}	Total inhibition at 10^{-3} M
EDTA	10^{-5}, 10^{-4}, 10^{-3}	Slight inhibition at 10^{-3} M
Mg^{2+}	10^{-5}, 10^{-4}, 10^{-3}	No significant effect

* Incubation flasks contained 0·7 ml of 104,000 *g* placental supernatant, 3·0 mg aniline hydrochloride, 3·0 mg NADPH, 9 μmoles glucose 6-phosphate and sufficient potassium phosphate buffer (0·1 M, pH 7·05) to yield a total volume of 3·0 ml. Flasks were incubated for 45 min in air at 37°.

† Nicotinamide exhibited significant inhibitory effects when incubations were carried 3 hr or longer.

measurable effects at 10^{-5} or 10^{-3} M concentrations. The same steroids, particularly the estrogens, strongly inhibited the hepatic microsomal aniline *p*-hydroxylation reaction. SKF525-A, a powerful inhibitor of microsomal mixed-function oxidation reactions, also appeared to be ineffectual as an inhibitor in the placental system. Addition of FMN to reaction flasks resulted in a biphasic effect with approximately 20 per cent activation observed at 10^{-5} M and approximately 40 per cent inhibition at 5×10^{-3} M. Acetone, which markedly activates hepatic microsomal aniline hydroxylase, exhibited no significant effects on the placental reactions at the concentrations tested. Nicotinamide which, like aniline, exhibits a type II difference spectrum when added to rat liver microsomes, likewise did not affect that reaction. Potassium

cyanide and sulfhydryl reagents (*p*-chloromercuribenzoate and *p*-hydroxymercuribenzoate) completely inhibited the reaction at 10^{-3} M final concentration. EDTA exhibited a slight inhibitory effect at 10^{-3} M. Additions of magnesium ion produced no observable effects.

Incubations were run also under various atmospheric conditions (Table 5). The reaction was nearly as rapid in an atmosphere of air as in 100% O_2 but was almost

TABLE 5. RATE OF HYDROXYLATION OF ANILINE IN HUMAN PLACENTAL SOLUBLE FRACTIONS UNDER VARIOUS ATMOSPHERES

Gas phase	Aniline hydroxylase activity*
Oxygen 100%	0·55
Nitrogen 100%	0·05
Air	0·51
Oxygen 80%, Carbon monoxide 20%	0·29
Oxygen 60%, Carbon monoxide 40%	0·16

* Activities are expressed as micromoles of aniline hydroxylated per hour per gram of protein. Reaction conditions are the same as for Table 4.

totally inhibited in 100% N_2. A mixture of 80% O_2 and 20% CO inhibited the reaction by approximately 40 per cent. These observations indicated a mixed-function oxidation reaction and suggested the involvement of a heme compound as a catalyst.

Kinetic studies utilizing pooled human placental soluble fractions and Lineweaver–Burke plots yielded a K_m value of $2{\cdot}5 \times 10^{-1}$ M and V_{max} of 33·3 μmoles/g of protein/hr for aniline hydrochloride as substrate. With respect to NADPH as substrate, the K_m value determined was $3{\cdot}4 \times 10^{-4}$ M and the V_{max} was 548 μmoles/g of protein/hr. By comparison, Anders[14] reported a K_m value of $6{\cdot}9 \times 10^{-5}$ and V_{max} of 1·4 μmoles/g of liver/hr with respect to aniline HCl in rat liver microsomes.

TABLE 6. RECOVERY OF HUMAN PLACENTAL ANILINE HYDROXYLASE ACTIVITY AFTER FRACTIONAL PRECIPITATION WITH AMMONIUM SULFATE*

Experiment	Electron donor	Ammonium sulfate saturation (%)						
		30	40	50	60	70	80	90
1	NADPH	0†	0	0	0	97‡	3	0
2	NADPH	0	0	0	25	55	20	0
3	NADH	0	0	15	33	16	37	0

* In Experiment 1 incubations were carried 4 hr in an atmosphere of air at 37·5°. Incubation flasks contained 5·4 mg aniline hydrochloride, 3 mg NADPH, 2 units glucose 6-phosphate dehydrogenase, 9 μmoles glucose 6-phosphate, 0·7 ml reconstituted pellet and sufficient potassium phosphate buffer (0·1 M, pH 7·05) to yield a total volume of 3·0 ml. Conditions for experiment 2 were the same except that flasks were incubated for 45 min. Conditions for experiment 3 were the same as for experiment 1 except that NADH (2·6 mg) replaced NADPH in reaction flasks.

† Zero indicates no detectable activity.

‡ Numbers represent the percentage of total activity recovered in all fractions.

Attempts were made to isolate and purify the placental catalyst(s). Ammonium sulfate fractionation (Table 6) resulted in the recovery of a large percentage of the aniline hydroxylase activity in the pellet which precipitated between 60 and 70 per cent saturation when NADPH was utilized as electron donor. Filtration of this fraction through Sephadex G-200 also indicated that most of the activity on the placental homogenate subfraction was associated with hemoglobin-rich fractions collected from the Sephadex column. These observations, together with the consideration that hemoglobin, methemoglobin and a variety of other heme compounds would catalyze aromatic nitro group reduction,[13] led us to test some of these compounds as catalysts for the aniline *p*-hydroxylation reaction. It was found that a preparation of hemoglobin (twice recrystallized; Sigma) catalyzed a readily measurable reaction. Subsequently we found that purified methemoglobin and oxidized cytochrome *c* (Table 7) also would catalyze the reaction but that hematin, which is an efficient catalyst for aromatic nitro group reduction,[15] did not catalyze the aniline *p*-hydroxylation reaction to a measurable extent.

TABLE 7. COMPARISON OF HEME COMPOUNDS IN THE CATALYSIS OF ANILINE HYDROXYLATION*

Heme compound	Control	+FAD 10^{-5} M	+FAD 10^{-4} M	+FAD 10^{-3} M	+Xanthine oxidase (0·25 unit)
Hemoglobin	7·8	8·1	7·6	7·3	5·6
Methemoglobin	5·6	5·8	5·6	5·4	†
Hematin	<0·04	<0·04	<0·04	<0·04	
Cytochrome *c*	0·08	1·2	1·9	3·7	3·5

* Reaction conditions were the same as listed for Fig. 1. Hemoglobin (1·4 mg) and equal monomer heme concentrations of other heme compounds were present in reaction vessels. Values are expressed as millimicromoles of *p*-aminophenol formed per hour per flask. Values less than 0·04 were not significantly different from corresponding blanks.

† Dashes indicate that determinations were not made.

Assay of the commercial hemoglobin preparation indicated that 85–90 per cent was present in the form of methemoglobin, yet this preparation was significantly more active than a corresponding preparation of purified (twice recrystallized) methemoglobin obtained from the same company, suggesting that the reduced compound (hemoglobin) was the active form. Nevertheless, methemoglobin was considerably more active than cytochrome *c* when compared on the basis of total monomer heme. These results suggest the importance of the nature of the ligands involved in the heme protein-catalyzed reaction mechanism.

Early experiments with human whole blood yielded negative results; however, experiments with hemoglobin indicated a biphasic effect in the reaction system (Fig. 1). A 5-fold dilution of human whole blood then yielded an easily measurable reaction. The biphasic effect observed was apparently responsible for the low activity observed in the earlier experiments with whole blood.

Addition of 0·25 unit of purified milk xanthine oxidase to reaction vessels markedly accelerated the cytochrome *c*-catalyzed reaction but slightly decreased the rate of the hemoglobin-catalyzed *p*-hydroxylation process. In contrast, however, flavins did not

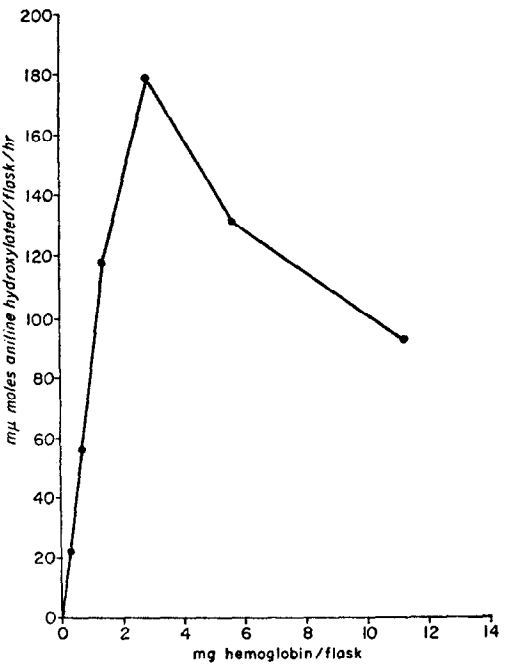

FIG. 1. Effect of increasing hemoglobin concentration on the rate of aniline hydroxylation. Incubations were carried for 10 min. Other conditions were as described in Table 6 for experiments 1 and 2.

appear to accelerate the placental reaction, and exerted little effect on hemoglobin catalysis.

In addition to the similarities between hemoglobin and placental catalysis with respect to the effects of flavins, further comparisons yielded a number of other parallelisms. NADPH was the preferred electron donor in both the purified hemoglobin reaction system and the placental system (Table 8); both reactions were inhibited similarly by carbon monoxide; heat inactivation by boiling reduced catalytic activity

TABLE 8. HEMOGLOBIN CATALYSIS OF ANILINE HYDROXYLATION*

Electron donor	*p*-Aminophenol formation†	
	100% O_2	Air
NADPH	10·2	8·4
NADH	6·3	5·8
Ascorbate	4·9	3·9
GSH	2·8	2·7

* Reaction conditions are the same as described in Table 7. Electron donors were present in equimolar concentrations (10^{-3} M).

† Values in the table indicate millimicromoles of *p*-aminophenol formed per hour per milligram of protein.

to approximately the same level in both cases; sulfhydryl reagents inhibited the reaction to a similar degree in both systems and, when expressed in terms of heme concentration, specific activities were also similar. These correlative data, taken together with the results of attempted isolation, strongly suggest that hemoglobin is a principal factor in the catalysis of aniline *p*-hydroxylation in placental homogenates and soluble fractions Since hemoglobin may be regarded as a contaminant of such preparations, the results also suggest that placental cells *per se* contain little or no catalytic activity with respect to this particular xenobiotic biotransformation reaction.

The hemoglobin model (tissue-free) system also was tested for capacity to hydroxylate 3,4-benzpyrene and *N*-demethylate aminopyrine. The system contained purified hemoglobin (Sigma) ($1{\cdot}3 \times 10^{-5}$ M), NADPH ($1{\cdot}2 \times 10^{-3}$ M), and 3,4-benzpyrene ($1{\cdot}4 \times 10^{-3}$ M) or aminopyrine ($8{\cdot}3 \times 10^{-3}$ M) and sufficient 0·1 M phosphate buffer at pH 7·05 to yield a total volume of 3·0 ml. Although this system catalyzed an easily measurable *p*-hydroxylation of aniline hydrochloride (7×10^{-2} M), it did not convert either of the other two substrates to their oxidized products to a measurable extent.

Significant levels of aryl hydrocarbon hydroxylase or aminopyrine *N*-demethylase also could not be observed in reaction flasks containing placental soluble fractions. The same model systems likewise did not measurably catalyze the conversion of PNBA (8×10^{-3} M) to the corresponding amine but addition of 5×10^{-4} M flavins or 0·25 unit of xanthine oxidase resulted in a rapid reduction reaction.

Addition of 0·4 mg of L-α-cephalin (Sigma, grade II) to reaction flasks significantly increased the rate of the hemoglobin-catalyzed NADPH or NADH-dependent aniline *p*-hydroxylation (20 and 29 per cent respectively) but additions of several concentrations of egg yolk phosphatidylcholine (Sigma, type II-E) produced no apparent effects on the reaction rate.

DISCUSSION

The experiments conducted in this study permit several tentative conclusions regarding the catalytic capabilities of the human placenta at term and also provide insights into mechanisms of mixed-function oxidations of xenobiotics. It is now well established that particulate fractions of placentas at term will catalyze hydroxylation of 3,4-benzpyrene at readily measurable rates,[1–6] particularly if the pregnant female has been exposed to inducing agents such as are present in tobacco smoke. In our experience we have found that in certain instances (frequently those in which the placental donor would admit to a high level of exposure during pregnancy to drugs and foreign compounds) the placental benzpyrene-hydroxylating activity can approach that observed in rat liver preparations. Nevertheless, the same placental subfractions which catalyzed such comparatively rapid hydroxylation of 3,4-benzpyrene were devoid of detectable aniline *p*-hydroxylating activity. The same situation appeared to exist in rat placentas and, in addition, pretreatment of pregnant rats with either class of inducing agents (barbiturates or polycyclic aromatic hydrocarbons) did not result in detectable levels of activity in particulate fractions or increased levels of activity in the soluble fractions. Since evidence was found to indicate that the aniline *p*-hydroxylating activity detected in soluble fractions was due primarily to the catalytic action of contaminating hemoglobin, it would appear that placental cells *per ce* contain little or no capacity for *p*-hydroxylation catalysis with aniline as substrate. This, in

turn, supports the view that *p*-hydroxylation of aniline and 3,4-benzpyrene can occur by two separate and distinct mechanisms. Further evidence for this premise is supplied by the finding that purified heme compounds would catalyze the *p*-hydroxylation of aniline but not 3,4-benzpyrene under the same reaction conditions. Separate reaction mechanisms for aminopyrine *N*-demethylation and aniline *p*-hydroxylation are also indicated on the basis of studies in tissue-free model systems.

Our studies suggest that the only required biological constituents of an aniline *p*-hydroxylating system are an electron donor, such as NADPH or NADH, a suitable hemoprotein and molecular oxygen together with the substrate. These results are supported by results of other investigators who have shown that aniline hydroxylation occurs readily in the presence of dihydroxyfumarate (a non-biological electron donor), peroxidase and molecular oxygen.[16,17] The studies of Lu *et al.*,[18] on the other hand, indicate that the electron donor, a flavo-protein, a suitable phospholipid, cytochrome P-450 and molecular oxygen are required for the optimal mixed-function oxidation of substrates which exhibit type I difference spectra.

By analogy, Kuntzman *et al.*[19] have shown a similar requirement for hydroxylation of 3,4-benzpyrene in reconstituted systems except that cytochrome P-448 was much more efficient than P-450 in the reaction vessels. It is our belief that the study of reactions in such model systems appears to offer great promise for the elucidation of mechanisms of drug hydroxylation.

Although catalysis of the *p*-hydroxylation of aniline was clearly demonstrated in these reaction systems, quantitative studies of rates of formation of dihydroxy compounds (which also appear to be produced in such systems) represent an important area of future investigation. Preliminary semi-quantitative observations (utilizing the methods of Mitoma *et al.*[20]) in our laboratory tend to indicate that rates of *p*-hydroxylation exceed rates of *ortho-* or *meta*-hydroxylation in methemoglobin-catalyzed reaction systems. Quantitative studies with radioactive substrates are in progress.

Finally, it should be noted that considerable confusion exists with respect to the capability of the placenta to catalyze mixed-function oxidations of drug substrates. While there is general positive agreement concerning the placental hydroxylation of 3,4-benzpyrene, conflicting reports in the literature have appeared regarding oxidation of other drug substrates by placental tissues.[1] Although it is evident that considerable effort will be required to resolve such questions, it is hoped that the present studies will provide clarification in this important area of reproductive pharmacology.

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