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Cytochrome P-450-Dependent Biotransformation of a Series of Phenoxazone Ethers in the Rat Conceptus during Early Organogenesis: Evidence for Multiple P-450 Isoenzymes

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SUMMARY

Using highly sensitive probe-substrate analyses, investigations of drug biotransformation in tissues of the rat conceptus during an early stage of organogenesis revealed that three separate tissue components each contained P-450 isozymes capable of catalyzing the monooxygenation of foreign organic chemicals. Tissues of the embryo proper contained constitutive P450(s) that catalyzed readily measurable *O*-dependentylation and *O*-debenzylation of pentoxyphenoxazone and benzyloxyphenoxazone, respectively, but no measurable *O*-demethylation of methoxyphenoxazone and barely detectable *O*-deethylation of ethoxyphenoxazone. Higher specific activities for the *O*-dependentylation and *O*-debenzylation reactions were measured in preparations of the yolk sac and this organ also appeared to contain constitutive P450(s) for the readily detectable *O*-deethylation of ethoxyphenoxazone. The *O*-demethylation of methoxyphenoxazone could not be detected in the yolk sac. Only the *O*-debenzylation reaction could be detected in tissues of the ectoplacental cone. Treatment of conceptuses *in utero* with 3-methycholantherene (MC) resulted in significantly increased rates of *O*-deethylation reactions in preparations of yolk sac and embryo but not ectoplacental cone. Demethylation was not detectable in the same preparations. Treatment with phenobarbital, pregnenolone-16α-carbonitrile, or isosafrole produced no observable effect on any of the reactions studied. Carbon monoxide ($CO:O_2$ = 80:20 versus $N_2:O_2$ = 80:20) markedly inhibited all reaction rates and inhibition could be reversed by replacement of CO with N_2. Deethylation and debenzylation were inhibited by anti-P450IA1 IgG after MC induction but were not affected by the same IgG fraction in untreated conceptuses. Dependentylation reactions were not inhibited by anti-P450IA1 or anti-P450IIB1/2 antibodies under any of the conditions used. Deethylation was strongly inhibited by 1.0 μM 7,8-benzoflavone in tissues from MC-treated but not untreated conceptus. Metyrapone (0.1 mM) failed to significantly inhibit any of the measurable conceptus-catalyzed dependentylation reaction. The results indicated the presence of four (or more) functional P450 isozymes in tissues of the conceptus during organogenesis, a constitutive dependentylase(s) in the yolk sac and embryo, a constitutive deethylase(s) present in the yolk sac, an MC-inducible deethylase(s) in the embryo and yolk sac, and constitutive debenzylase(s) present in all three tissues. No *O*-demethylation was detectable in any of the three tissues, even after *in utero* exposure to inducers. Conversion of testosterone to androstenedione by washed embryonic particulate fractions was readily measurable, but conversion to other oxidized metabolites could not be detected.

The development of a system for the culturing of whole embryos (1, 2) has resulted in a recently renewed interest in the exploration of biochemical/molecular mechanisms whereby drugs and other chemicals produce dysmorphic effects on developing conceptuses during the period of organogenesis (3–5). Utilization of a modification of the culture system (6, 7) has provided evidence that the embryotoxic effects of several chemicals can be profoundly influenced by routes and rates of biotransformation. Certain of these agents are enzymatically converted to reactive intermediary metabolites with much greater embryotoxic/dysmorphogenic potential than the parent chemical (3–8). In addition, evidence has accumulated to indicate that tissues of the conceptus *per se* contain a complement of enzymes that are capable (at least under certain circumstances) of bioactivating various chemical teratogens (9–11). These observations immediately raise questions regarding the specific biotransformational capabilities of embryonic target tissues in terms of xenobiotic bioactivation/inactivation processes. In previous studies (9–12) we provided evidence for the existence in cultured whole conceptuses of P450 cytochromes in quantities amply sufficient to effect the dysmorphogenic bioactivation of the model substrate/teratogen 2-acetylaminofluorene. In the present study, we have used a series of phenoxazone ethers (commonly referred to as resorufin ethers) as sensitive and specific probe substrates for the detection and

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ABBREVIATIONS: PB, phenobarbital; MC, 3-methylcholanthrene; PCN, pregnenolone 16α-carbonitrile; ISF, isosafrole; MTY, metyrapone; AMF, 7,8-benzoflavone; HPLC, high performance liquid chromatography.

characterization of embryonic P450 cytochromes. Use of these ethers as probes has been developed largely by Burke and Mayer and their associates (13–16) and has become an exceptionally useful tool for detection and characterization of P450 isozymes. Lubet *et al.* (16) have recently documented the specificity of pentoxyresorufin for PB-inducible isozymes, and Guengerich *et al.* (17) have documented the specificity of the ethoxy ether for purified MC-inducible forms. In combination with specific antibody probes and selective inhibitors and inducers, our approach has permitted the tentative conclusion that at least four (three constitutive and one inducible) P450 isozymes are enzymatically active in tissues of the rat conceptus on day 11 of gestation and that each of these isozymes is functionally active in the monooxygenation of small foreign organic chemicals. However, only one of the four conceptus isozymes could be related to an identifiable adult hepatic counterpart. All P450 isozymes are designated in accordance with the recently standardized nomenclature provided by Nebert *et al.* (18). P450IA1 has also been designated trivially as P-450$_c$ and BNF-B; P450IA2 as P-450$_d$ and ISF-G; P450IIB1 as P450$_b$, PBB, and PB-4; and P450IIB2 as P-450$_e$, PBD, and PB-5. These represent commonly used designations for the rat hepatic isozymes. In these experiments, the preparation enriched with P450IIB1 may have contained small quantities of the closely homologous (98%) IIB2. Because no attempt was made to further separate P450IIB1 from P450IIB2, and for convenience, both the purified preparation and its corresponding antibody are designated as P450IIB1/2 and anti-P450IIB1/2, respectively.

Materials and Methods

Chemicals. Methoxyphenoxazone, ethoxyphenoxazone, pentoxyphenoxazone, and benzyloxyphenoxazone were synthesized in our laboratory according to methods described by Mayer *et al.* (14) and were purified by methods described by Klotz *et al.* (15) and purity was verified (>99.5%) by analytical HPLC. Melting points of the purified chemicals were in excellent agreement with the published (15) literature values. Resorufin and ANF were purchased from Aldrich Chemical Co. (Milwaukee, WI) and phenoxazone was supplied by Dr. A. E. Rettie, Department of Medical Chemistry, University of Washington. Resorufin and phenoxazone were also purified by published methods (15) and purity was verified (>99.5%) by analytical HPLC. Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, dicoumarol, Protein A-Sepharose CL-4B, NADPH, nonradiolabeled testosterone, and MC were purchased from Sigma Chemical Co. (St. Louis, MO); ISF was purchased from ICN Pharmaceuticals (Plainview, NY); PCN and MTY were received as generous gifts from Searle Laboratories (Chicago, IL) and CIBA Pharmaceutical Co. (Summit, NJ), respectively; PB (as the pure powdered sodium salt) was obtained from Drug Services, University of Washington (Seattle, WA); carbon monoxide (99.4% purity) from the Matheson Co. (St. Louis, MO). [4-^{14}C]Testosterone (59.6 mCi/mmol, 99% pure) was obtained form the Amersham Co. (Arlington Heights, IL). Testosterone metabolites were supplied by Steraloids Inc. (Wilton, NH) (androstenedione, 6β-, 7α-, 11α-, 11β-, 16α-, and 16β-hydroxy), from the Steroids Reference Collection, courtesy of Professor D. N. Kirk (6α-, 2α-, 2β-, 6β-, 7α-, 7β-, 16α-, and 16β-hydroxy) and from Searle Research Division (Skokie, IL) (hydroxy). Purity of these samples was verified with analytical HPLC. All other chemicals used were of the highest purity commercially available.

P450s and P450 antibodies. P450s IA1, IA2, and IIB1/2 were prepared from rat liver microsomes exactly according to methods described by Ryan *et al.* (19, 20) except that the final immunopurification step for IA2 was not carried out and no antibodies were elicited for this isozyme. The purified isozymes had specific contents of 13–17 nmol of P450/mg of protein. Electrophoresis yielded a single protein staining band on sodium dodecyl sulfate-polyacrylamide gels. NADPH-cytochrome P450 reductase was purified from rat liver microsomes from PB-induced rats by methods described by Shepard *et al.* (21). The preparation exhibited a specific activity of 57.2 μmol/min/mg at 24° in a solution containing 0.1 M potassium phosphate buffer (pH 7.4), 10 mM EDTA, 3 mM $MgCl_2$, 0.1 mM KCN, 0.05 mM cytochrome *c*, and 0.1 mM NADPH. Antibodies were raised against P450s IA1 and IIB1/2 by intradermally injecting 0.25 mg of the corresponding purified antigens in Freund's adjuvant into adult female New Zealand White rabbits according to methods described by Thomas *et al.* (22). Polyclonal antibodies raised against P450IA1 and P450IA2 are known to exhibit considerable cross-reactivity. Likewise, antibodies raised against P450IIB1 and P450IIB2 are known to cross-react extensively, as these two isozymes exhibit 97–98% homology. However, polyclonals raised against IA1 or IA2 do not normally cross-react with those raised against IIB1 or IIB2. Analyses performed with enzyme-linked immunosorbent assays according to the methods described by Paye *et al.* (23) and with Western blots according to methods described by Towbin *et al.* (24) indicated that the antibodies raised during the course of these investigations exhibited the same properties. (For Western blots, 5% nonfat dry milk was used to block nonspecific binding and the substrate used for color development was 4-chloro-1-naphthol.) IgG fractions, prepared from the corresponding antisera in accordance with methods described by Ey *et al.* (25), were used as antibody sources. For use as potential enzyme inhibitors, IgGs were preincubated with the enzyme sources at 25° for 15 min with a ratio of 0.5–10 mg of IgG/nmol of P450 as determined in preliminary experiments with a range of concentrations. For embryonic enzyme sources (in which quantities of P450 cannot be assessed with standard methodology), a ratio range of 0.01–0.5 mg IgG/ml of the reaction mixture was used.

Enzyme sources. Sprague-Dawley (Wistar-derived) rats obtained from Tyler Laboratories (Bellevue, WA) were used in all experiments. For comparative purposes, we prepared washed microsomes from the hepatic homogenates of adult (230–260 g) rats in accordance with established methods (26). Primigravida rats were obtained between 4 and 5 days of pregnancy and were kept in plastic cages on crushed corncob bedding materials (Sanicel). The morning after copulation was designated as day 0 of gestation. Animals were housed with a 14-hr light, 10-hr dark lighting cycle and had free access to food (Purina Rat Chow) and water. Embryos were also exposed *in utero* to various inducing agents by injecting the dams intraperitoneally according to the schedule in Table 1. On day 11, the dams were anesthetized with diethyl ether and blood was collected from the abdominal aorta. The uteri were removed and placed in cold Hank's balanced salt solution and the individual implantation sites were removed. A spectroscopic dissecting microscope was then used to surgically remove and discard the decidua, parietal yolk sac, Reichert's membrane, and trophoblastic remnants. The embryos, together with the visceral yolk sac, amnion, and ectoplacental cone remained and are hereafter referred to as the conceptus. For initial experiments and in experiments using testosterone as substrate, the entire conceptus was gently homogenized by hand in a 0.1 M potassium phosphate buffer (pH 7.4) in a Potter homogenizer

TABLE 1

Exposure of rat conceptus in utero to various inducing agents

Conceptuses were exposed by treating the pregnant dams with the indicated intraperitoneally administered doses of inducing agents in accordance with indicated schedules.

Treatment	Dose	Vehicle	Frequency
	mg/kg		
MC	40	Corn oil	Once daily, day 9*
PB	80	Normal saline	Once daily, days 8–10
PCN	40	Corn oil	Twice daily, days 7–10
ISF	150	Corn oil	Once daily, days 7–10
Control		Corn oil	Once daily, days 7–10

* Day of gestation on which injections were given. The morning after copulation was designated as day 0 of gestation. Conceptuses were removed on the morning of day 11.

with a Teflon pestle and the homogenate was centrifuged at 600 × *g* for 5 min. The resulting supernatant fraction was used as an enzyme source. In other experiments, the visceral yolk sac, ectoplacental cone, and embryo were separated and homogenates of each of these tissues were prepared as described above for the entire conceptus. No attempts were made to prepare homogenates of the amnion because of its extremely small size and, in the latter experiments, the amnion remained attached to the embryo. Each preparation represented a pooled homogenate from 30–70 conceptuses from 5–8 dams. Preparations were maintained at 4° and were assayed within 2–5 hr. No loss of activity could be detected during this time period.

Enzyme assays. Hydroxylation of phenoxazone, *O*-dealkylation of the methoxy-, ethoxy-, and pentoxy-ethers, and *O*-debenzylation of benzyloxyphenoxazone each result in the generation of the highly fluorescent resorufin metabolite, which can be monitored continuously as a function of time. Slight modifications of the method described by Burke *et al.* (27) were used in these experiments. Reactions were carried out at 37° with the use of a properly jacketed cuvette holder with a Farrand manual spectrofluorometer. Excitation and emission wavelengths were 530 and 585 nm, respectively. Reaction cuvettes contained dimethyl sulfoxide-dissolved substrate (0.01 mM), enzyme sources (0.02–2 mg of protein), NADPH (1.0 mM), and potassium phosphate buffer (0.1 M, pH 7.4) in a total volume of 1.0 ml. For effects of MTY or ANF, 5 μl of the inhibitors (in dimethyl sulfoxide) were added to the reaction mixture. For reconstituted systems, reaction cuvettes contained 1.0 μmol of NADPH, 20–100 pmol of purified P450 isozyme, 40 nmol of dilauroyl L-phosphatidylcholine, 10 nmol of substrate, 200 pmol of NADPH cytochrome P450 reductase, and 0.1 M potassium phosphate buffer (pH 7.4) in a total volume of 1.0 ml. The temperature of the reaction mixture was first raised to 37° while establishing a baseline and the reactions were started by the addition of NADPH, except in the case of reconstituted systems. For the latter experiments, reactions were started by the addition of substrate. Only those reactions exhibiting linear increases in fluorescence over a period of 2 min were regarded as within the limits of detectability. Additions of glucose 6-phosphate (50 mM), glucose 6-phosphate dehydrogenase (1 unit), or dicoumarol (0.01 mM) produced less than 20% increases in reaction rates and, in routine experiments, were omitted from the reaction cuvettes. Each assay was performed in duplicate or triplicate and standardized by the addition of a known quantity of purified resorufin. Means of duplicate/triplicate determinations were calculated for each assay and means and standard deviations of three or four repeated assays were calculated. Assays for the hydroxylation of testosterone were carried out with HPLC analyses as described by Wood *et al.* (28) except that as much as 2 μCi of radiolabeled testosterone per flask were utilized as substrate with no unlabeled testosterone added, and incubations were carried for 15 min to 2 hr. Fractions were collected (24 sec each) and assayed by scintillation counting. Metabolite standards were run with each assay. Activities were expressed as pmol/min/mg of protein with protein concentrations determined by the method of Lowry *et al.* (29) with bovine serum albumin as the standard. Activities less than 0.1 or 0.05 pmol/min were regarded as below the limits of detectability for assays with phenoxazone ethers and radiolabeled testosterone, respectively.

Results

Assays with entire conceptuses. Initial experiments were designed to determine whether significant dealkylation or debenzylation could be detected in homogenates of whole conceptuses at day 11 of gestation. As shown in Table 2, three reactions were detectable. These were the deethylation of ethoxyphenoxazone, dependentylation of pentoxyphenoxazone, and debenzylation of benzyloxyphenoxazone. Treatment of conceptuses *in utero* with MC resulted in increased rates of deethylation and lesser increases in debenzylation but no significant changes in rates of dependentylation. Rates of these reactions were almost totally blocked when the reaction cuvettes were gassed with a mixture of oxygen and carbon monoxide (O_2:CO = 20:80) as is illustrated for the yolk sac dependentylation reaction in Fig. 1. Rates were partially restored by regassing the cuvettes with a mixture of oxygen and nitrogen (O_2:N_2 = 20:80) as is also shown in Fig. 1. Pretreatment of dams with PCN, PB, or ISF did not result in significant changes in observed enzymic activities. Neither hydroxylation of phenoxazone nor demethylation of methoxyphenoxazone was detected in any of the conceptus preparations used in these experiments although both reactions were readily measurable with adult hepatic microsomes. Preparations containing only the cytosolic (104,000 × *g* supernatant) fractions exhibited no detectable activity.

Assays with various components of the conceptus. Subsequent experiments demonstrated that tissues of the untreated embryo proper contained enzymes that catalyzed the *O*-debenzylation and *O*-dependentylation reactions but not *O*-demethylation or hydroxylation (Table 2). Measured *O*-deethylation of ethoxyphenoxazone was close to the borderline of detectability. By contrast, the untreated yolk sac contained enzymes that catalyzed not only more rapid dependentylation and debenzylation but also easily measured deethylation of the ethoxy ether. Tissues of the untreated ectoplacental cone catalyzed only the debenzylation reaction, demonstrating that this particular reaction was measurable in all three tissues. Treatment of embryos *in utero* with MC resulted in marked increases in rates of deethylation reactions and relatively smaller increases in debenzylation but no changes in rates of any of the other measured reactions or appearance of measurable *O*-demethylation or phenoxazone hydroxylation.

As also reported by previous investigators (14–17, 27), studies with reconstituted P450IA1 demonstrated that this isozyme catalyzed the deethylation and debenzylation reactions at very rapid rates but catalyzed the dependentylation and hydroxylation reactions at minimal rates under the conditions used and also catalyzed much lower rates of demethylation (Table 2). P450IIB1/2 effectively catalyzed only the dependentylation and debenzylation reactions. P450IA2 was highly effective in catalyzing the demethylation reaction but catalyzed *O*-deethylation and hydroxylation at very minimal rates. The effects of various inducing agents on the rates of reactions catalyzed by enzymes present in adult hepatic microsomes were all consistent with these observations.

Effect of isoenzyme-specific inhibitors. The effects of antibodies raised against P450IA1 and P450IIB1/2 are given in Table 3. As expected, anti-P450IA1 markedly inhibited the deethylation reaction in tissues on conceptuses exposed to MC *in utero*. Somewhat unexpectedly, the same IgG fraction had no detectable effect on the same reaction in tissues of untreated animals. Also, anti-P450IIB1/2 failed to inhibit (<10% inhibition) dependentylation in conceptus-catalyzed reactions while exhibiting the expected inhibition of purified reconstituted P450IIB1/2-catalyzed dependentylation (93%) and dependentylation catalyzed by hepatic microsomes from PB-induced adult rats (96%).

Experiments with MTY and ANF corroborated the results obtained with antibodies (Table 4). At 1.0 μM final concentration, the flavone strongly inhibited deethylation after exposure of conceptuses to MC but, at the same concentrations, did not inhibit deethylation in tissues of MC-unexposed conceptuses.

TABLE 2

Monooxygenation of phenoxazone ethers by preparations (see Materials and Methods) of day 11 rat conceptuses, adult rat hepatic microsomes, and reconstituted P450 systems

Numbers in the table are specific activities (pmol/mg of protein/min ± standard deviation), except for reconstituted systems. Specific activities in the reconstituted systems are expressed as pmol/nmol of P450/min. Reconstituted systems for purified P450s IA1, IA2, and IIB1/2 contained 0.02–0.1 nmol of P450, 0.01–0.02 nmol of NADPH cytochrome P450 reductase (specific activity 50–60 μmol/mg/min), 10 nmol of substrate, 1 mmol of NADPH, and 40 nmol of dilauroyl phosphatidyl choline in 0.1 M potassium phosphate buffer, pH 7.4.

Preparation	Treatment	Demethylation	Deethylation	Depentylation	Debenzylation	Hydroxylation
Whole conceptus	Control	ND[a]	0.25 ± 0.03	0.24 ± 0.02	0.46 ± 0.03	ND
Whole conceptus	MC	ND	0.68 ± 0.07	0.21 ± 0.01	0.64 ± 0.06	ND
Whole conceptus	PB	ND	0.27 ± 0.04	0.24 ± 0.03	0.46 ± 0.05	—[b]
Whole conceptus	PCN	ND	0.20 ± 0.05	0.25 ± 0.02	0.42 ± 0.03	—
Whole conceptus	ISF	ND	0.29 ± 0.04	0.25 ± 0.05	0.42 ± 0.04	—
Embryo	Control	ND	0.12 ± 0.07	0.25 ± 0.07	0.40 ± 0.10	ND
Embryo	MC	ND	0.42 ± 0.08	0.30 ± 0.01	0.53 ± 0.09	ND
Yolk sac	Control	ND	0.99 ± 0.16	0.55 ± 0.17	0.61 ± 0.02	ND
Yolk sac	MC	ND	2.56 ± 0.32	0.86 ± 0.08	1.40 ± 0.23	ND
Ectoplacental cone	Control	ND	ND	ND	0.30 ± 0.08	ND
Ectoplacental cone	MC	ND	ND	ND	0.47 ± 0.09	ND
Maternal liver	Control	19.7 ± 1.8	17.7 ± 2.2	0.68 ± 0.09	5.1 ± 1.1	—
Maternal liver	MC	291 ± 27	2868 ± 196	21.1 ± 3.6	155 ± 23	—
P450IA1	MC	117	3371	80	693	ND
P450IA2	ISF	1694	12.6	91	121	ND
P450IIB1/2	PB	ND	6.1	65	323	ND
Adult male liver	Control	8.2 ± 2.3	37.4 ± 4.9	5.9 ± 1.8	18.0 ± 3.2	2.1 ± 0.6

[a] ND indicates that activities were below the level of detectability.
[b] Dashes indicate that no experiment was performed.

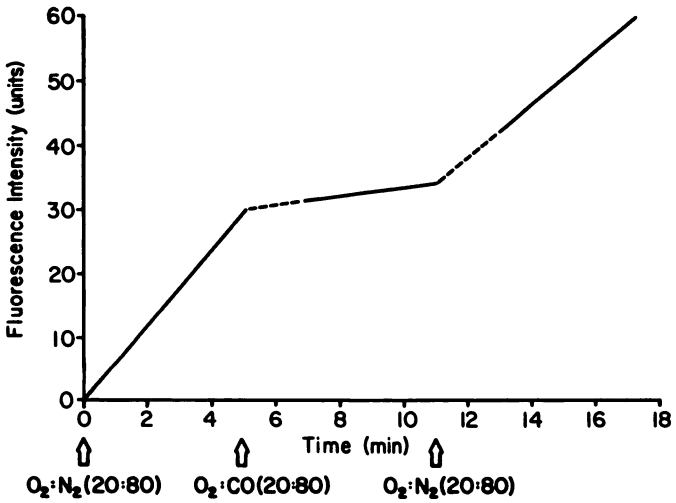

Fig. 1. Inhibition of the conceptus-catalyzed depentylation reaction with carbon monoxide and restoration of activity with molecular oxygen. *Dotted lines* indicate periods in which the reaction mixtures were gassed for 2 min with the indicated gas mixtures.

At the same concentration, ANF also inhibited debenzylation in MC-treated conceptuses by 37% (embryos) and 39% (yolk sacs) but failed to inhibit in reactions containing homogenates from untreated conceptuses. At these concentrations, ANF did not inhibit depentylation reactions in tissues of MC-treated or control conceptuses. MTY, at 1.0 μM final concentrations, strongly inhibited P450IIB1/2-catalyzed depentylation (91%) as well as debenzylation reactions occurring in reaction cuvettes containing hepatic microsomes from PB-induced adult rats (72%). At the same concentrations (as well as at 0.1 mM), MTY failed to inhibit depentylation or debenzylation in any reaction in which tissue of the conceptus served as enzyme source. MTY also was not inhibitory to embryonic or yolk sac deethylation reactions at either concentration, although it produced the expected effect on PB-induced adult rat hepatic microsomes (Table 4).

Because the results suggested that tissues of the rat conceptus contained three or more constitutive P450 isozymes, we attempted to determine whether such isozymes might exhibit similarities to constitutive P450(s) present (and characterized) in the hepatic tissues of adult rats. As a probe, we used radiolabeled testosterone, which is selectively converted to specific oxidized metabolites by constitutive P450 isozymes present in the livers of adult rats (reviewed by Conney; Ref. 30). Washed particulate fractions (104,000 × *g*, for 1 hr, sediment after an initial 600 × *g*, for 10 min, centrifugation) of homogenates of whole conceptuses catalyzed a relatively rapid oxidation of testosterone to androstenedione (14% conversion in 15 min; 61% conversion in 2 hr), but, in spite of the use of large quantities (2 μCi/flask) of ^{14}C-labeled substrate and incubations for as long as 2 hr, no hydroxylated testosterone metabolites appeared at detectable levels. In addition, the conversion of testosterone to androstenedione could not be inhibited by carbon monoxide, suggesting that the reaction catalyzed by washed particulate fractions was due to a dehydrogenase rather than to a P450 isozyme(s).

Discussion

In rodent embryos, the impracticality of investigating P450 isozymes via the classical solublization/isolation/purification/characterization route engenders the necessity of alternate approaches. At present, a "multiple probe" design appears to be the most reasonable approach to the characterization of P450 enzymes in tissues for which isolation and purification procedures are impractical. Substrates, inducers, and inhibitors (including specific antibodies) that exhibit specificity for one or more P450 isozymes seem the most useful probes for detection and characterization of functional P450s in such tissues at present. Hybridization probes for mRNAs of specific isozymes may also prove to be of value but do not measure functional protein. Phenoxazone and a homologous series of its

TABLE 3

Effects of P450 antibodies on monooxygenase activities assayed with preparations (see Materials and Methods) of the rat conceptus and adult rat liver microsomes

Numbers in the table are percentages of activities observed under identical conditions except in the presence of an equal quantity (0.05–0.5 mg/ml) of preimmune IgG fraction. Repeated experiments yielded almost identical results.

Preparation	Treatment	Antibody	Deethylation	Depentylation	Debenzylation
Whole conceptus	Control	anti-IA1	95	100	99
Whole conceptus	MC	anti-IA1	41	—[a]	—
Whole conceptus	Control	anti-IIB1/2	89	90	94
Whole conceptus	MC	anti-IIB1/2	108	—	—
Embryo	Control	anti-IA1	—	99	—
Embryo	MC	anti-IA1	44	100	—
Embryo	Control	anti-IIB1/2	—	96	—
Embryo	MC	anti-IIB1/2	106	100	—
Yolk sac	Control	anti-IA1	96	109	—
Yolk sac	MC	anti-IA1	43	101	—
Yolk sac	Control	anti-IIB1/2	100	99	—
Yolk sac	MC	anti-IIB1/2	110	102	—
Adult female liver	PB	anti-IA1	42	102	—
Adult female liver	PB	anti-IIB1/2	62	4	—
Adult female liver	MC	anti-IA1	2	18	—
Adult female liver	MC	anti-IIB1/2	98	71	—

[a] Dashes indicate no experiment was performed.

TABLE 4

Effect of MTY and ANF on monooxygenase activities in preparations (see Materials and Methods) of the rat embryo and yolk sac and adult rat liver microsomes.

Numbers in the table are percentages of activities observed under identical conditions except in the presence of an equal volume of inhibitor vehicle (dimethyl sulfoxide). Very similar values were obtained in separate repeated experiments.

Preparation	Treatment	Inhibitor (1 μM)	Deethylation	Depentylation
Embryo	Control	MTY	—[a]	103
Embryo	MC	MTY	91	99
Embryo	Control	ANF	—	104
Embryo	MC	ANF	45	100
Yolk sac	Control	MTY	99	102
Yolk sac	MC	MTY	107	101
Yolk sac	Control	ANF	101	96
Yolk sac	MC	ANF	52	99
Maternal liver	Control	MTY	97	131
Maternal liver	MC	MTY	98	84
Maternal liver	Control	ANF	69	87
Maternal liver	MC	ANF	5	49
Maternal liver	PB	MTY	77	12

[a] Blanks indicate that no experiment was performed. Deethylase activities in the embryo proper were close to the borderline of detectability, rendering experiments with inhibitors of doubtful value.

ethers have proven to be particularly useful as substrate probes because of their selectivity for particular P450 isozymes, assay sensitivity, and facility of analysis (13–17, 27, 31–33). For example, the ethoxy ether, commonly referred to as ethoxyresorufin, undergoes *O*-deethylation that is selectively catalyzed by P450IA1. Increases in deethylase activity have been regarded as diagnostic for increases in that isozyme (17) and possibly other MC-inducible forms. In like fashion, changes in depentylation rates have been regarded as directly diagnostic of change in tissue levels of P450IIB1/2 and possibly other PB-inducible isozymes (16).

The results of the presently reported experiments provide suggestive evidence for the presence of three (or more) constitutive P450 isozymes and one inducible form in various tissues of the rat conceptus on day 11 of gestation. The MC-inducible isozyme(s) in the conceptus may also be present in hepatic tissues of adult rats. Inducibility by MC, preference of the ethoxy ether as substrate, and selective inhibition by ANF and anti-P450IA1 all suggest that the induced conceptus isozyme(s) is either identical to or highly homologous with rat hepatic P450IA1. It was of interest that the MC-induced increases in deethylase activity in the conceptus were relatively low (2–3-fold) as compared with those observed in adult tissues (160-fold). This contrasts with earlier observed marked increases in rates of 7-hydroxylation of 2-acetylaminofluorene observed in conceptuses of comparable gestational age after treatment *in utero* with MC (11). Reasons for the differences remain to be explored, but it should be recognized that activity was not detectable in untreated whole conceptuses with 2-acetylaminofluorene as substrate whereas deethylation was readily measured in untreated conceptuses in the experiments reported here. The "fold" induction therefore is expectedly less.

Measurements of significant deethylase and depentylase activities without prior induction suggest that *constitutive* isozymes with some similarities in substrate specificity to P450IA1 and P450IIB1/2 are functionally present in normal inducer-unexposed tissues of the conceptus during organogenesis. That they are probably not the same isozymes, however, is evidenced from several characteristics, lack of induction by MC, PB, or other enzyme inducers, lack of inhibition by the corresponding inhibitory antibodies, and lack of inhibition by MTY and ANF. A constitutive deethylase(s) appears to be definitively present in the yolk sac, marginally present in the embryo proper, and undetectable in the ectoplacental cone. In contrast, the constitutive depentylase(s) exhibited relatively high activity in both the embryo and the yolk sac although it was undetectable in the ectoplacental cone. Although the ectoplacental cone lacked both deethylase and depentylase activities, debenzylase activity was readily detectable in untreated ectoplacental cones, suggesting that a separate constitutive form (or forms) of P450 is present in that tissue. The benzyloxy ether represents a substrate that is less differentially attacked, appears to be a substrate for a large number of P450 isozymes (27), and, as such, may be particularly useful as an *initial* probe to detect the presence of any functional P450 and thus determine the potential for further investigations with other, more specific, probes. Recently, we have used this approach in characterizing P450 enzymes present in human placental tissues (31).

Dependylase and deethylase activities observed in tissue of the untreated conceptuses seem best explained by the presence of two or more constitutive P450 isozymes. If, for example, it were presumed that a single P450 could catalyze these two reactions in the yolk sac and embryo, it would follow that the ratio of dependylation/deethylation should be the same in each tissue. However, observed ratios calculated from the data given in Table 2 indicate that the dependylation/deethylation ratio was 0.56 for the yolk sac as compared with 2.08 for the embryo. Thus the data appear consistent with separate P450s for dependylation and deethylation. Similar calculations can be made to argue for a separate constitutive P450(s) for debenzylation of benzyloxyphenoxazone in tissues of the ectoplacental cone. However, none of the constitutive forms appeared capable of utilizing testosterone as a substrate.

Although the specific activities measured with these substrates were low in tissues of the conceptus, investigations have previously demonstrated that even very low activities can have profound morphologic consequences during this stage of embryogenesis (9–12). In terms of the teratogenic effects of chemicals, the period of organogenesis is the most sensitive, yet information concerning the bioactivation of chemicals by the relevant target tissues (conceptus) during this period is very scarce at present. These experiments demonstrate that P450-dependent biotransformation of foreign organic chemicals proceeds not only in the tissues of conceptuses exposed to various inducing agents but also in conceptuses unexposed to inducers. The implications for investigations in chemical teratogenesis are immediately obvious.

Activities measured in tissues of the conceptus may have been somewhat underestimated due to recognized minor problems with the assay. It is well known that various oxidoreductases [e.g., quinone oxidoreeductase (32) and P450 oxidoreductase (16)] can catalyze not only reduction of the phenoxazone ether substrates but also the reduction of the reaction product (resorufin) to their corresponding nonfluorescent alcohols. The problem is circumvented, at least partially, by measuring only the maximal slope increase that occurs within the first few minutes and, in cases in which cytosolic fractions are included, by adding 0.01 mM dicoumarol, a specific inhibitor of the quinone oxidoreductase (33). In our experiments with tissues of the conceptus, additions of 0.01 mM dicoumarol to the reaction mixtures did not significantly alter the rates of the measured reactions. This suggested that only minor or no amounts of quinone reductase are present in rat embryos at this stage of gestation. Activities measured in these experiments in microsomal fractions of adult male rats (used as comparison standards) were lower than those reported by other investigators (27) for reasons that may be partially explicable in terms of reaction conditions or specific strain of animal used. In addition, we discovered during the early phases of these investigations that the commercially obtained resorufin (used as standard metabolite) contained considerable quantities of impurities. Thus, our use of a highly purified standard also has the potential to contribute to lower calculated specific activities than those reported in the literature.

The period of organogenesis in the rat extends roughly from the appearance of the primitive streak on day 9 to the complete closure of the palate on day 16–17 of gestation (34). Thus, the stage of development chosen for investigation in this study represents a relatively early period in organogenesis. It also represents a stage during which the culturing of whole embryos is commonly carried out. Thus, the results of the study provide valuable information for researchers interested in elucidating mechanisms of chemical teratogenesis. The importance of studies during the earlier stages of organogenesis is well recognized in the science of teratology, as susceptibility to dysmorphogenic insults is highest during this period of development.

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