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# IMMUNODETECTION, IMMUNOINHIBITION, IMMUNOQUANTITATION AND BIOCHEMICAL ANALYSES OF CYTOCHROME P-450IA1 IN TISSUES OF THE RAT CONCEPTUS DURING THE PROGRESSION OF ORGANOGENESIS*

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**Abstract**—Polyclonal antibodies raised against the adult form of rat hepatic cytochrome P-450IA1 were used to immunologically detect, inhibit and quantitate an analogous isozymic form(s) in various tissues of the rat conceptus during the progression of organogenesis. Tissues investigated were the embryo proper, the visceral yolk sac and the ectoplacental cone/chorioallantoic placenta. Studies were performed on conceptuses from day 10 (day of conception = day 0) to day 14 of gestation. Ethoxyphenoxazone deethylation, benzo[*a*]pyrene (BaP) hydroxylation, and ring- and N-hydroxylation of 2-acetylaminofluorene (AAF) were utilized in assessments of cytochrome P-450IA1-dependent monooxygenase activities during the same gestational period. In untreated conceptuses, cytochrome P-450IA1 could not be detected immunologically in any of the three tissues at any stage of gestation investigated. The deethylation reaction was quantifiable in embryos and yolk sacs of untreated conceptuses, but was not inhibited by cytochrome anti-P-450IA1 antibodies, α-naphthoflavone or metyrapone. Treatment of pregnant rats with 40 mg/kg of 3-methylcholanthrene 48 hr prior to removal of the conceptuses resulted in marked increases in measured enzymatic activities as well as in readily immunodetectable cytochrome P-450IA1. Inducibility for the deethylase was greatest in the visceral yolk sac (3–8×), was evident in the embryo proper (2–3×) but was minimal in the ectoplacental cone (1.5–2×). Much greater induction (up to 70×) was observed with BaP and AAF as substrates. Induced activities were inhibited effectively (70–100% inhibition) by cytochrome anti-P-450IA1 antibodies and by α-naphthoflavone but not by metyrapone. Inducibility increased as a function of gestational age in the ectoplacental cone/chorioallantoic placenta but reached maxima on day 12 in the embryo and visceral yolk sac. A good correlation between antibody/α-naphthoflavone-inhibited enzymatic activities and quantities of immunodetectable cytochrome P-450IA1 was also apparent. The results indicate that cytochrome P-450IA1, or a very closely related isoform(s), is both inducible and enzymatically functional in tissues of the conceptus throughout organogenesis and have important implications for the potential effects of bioactivatable proteratogens.

Studies conducted during the past few years have demonstrated that target tissues of the conceptus are capable of converting proteratogens to reactive intermediates in sufficient quantities to elicit grossly observable anatomical anomalies in the selfsame conceptuses (for reviews, see Refs. 1–4). Such studies raise numerous questions with regard to the enzymatic capabilities of the conceptus during the stage of development that is most susceptible to the kinds of chemical insults that result in permanent or semipermanent damage, i.e. during organogenesis. Several studies have shown that preimplantation embryos are capable of converting benzo[*a*]pyrene (BaP‡) to hydroxylated metabolites [5–7] and studies in our own laboratory have documented the capacity of postimplantation embryos to convert 2-acetylaminofluorene (AAF) [8–10] and nitroheterocycles [11–13] to dysmorphogenic metabolites. Most recently, we have provided evidence to suggest that P-450IA1, designated according to recently recommended, standardized nomenclature [14], may be the embryonic isozyme responsible for the dysmorphogenic bioactivation of AAF by the conceptus. This isozyme is also commonly referred to as P-450c and P-450βNF-B and is the major P-450 induced by 3-methylcholanthrene (MC), dioxins, planar polyhalogenated biphenyls and β-naphthoflavone in adult rats. The purpose of this investigation was to utilize a combination of immunologic methods, highly sensitive probe substrates (e.g. ethoxyphenoxazone, BaP and AAF, substrates whose hydroxylation is catalyzed efficiently by P-450IA1) and diagnostic inhibitors to determine the extent to which this or a closely-related P-450 isozyme(s) was present and functional in various tissues of the rat conceptus during the critical period of organogenesis. The tissues selected for investigation were the embryo

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‡ Abbreviations: BaP, benzo[*a*]pyrene; AAF, 2.acetylaminofluorene; MC, 3-methylcholanthrene; ANF, α-naphthoflavone; MTY, metyrapone; P-450IA1, cytochrome P-450IA1—also commonly known as P-450c and P-450βNF-B; and G6PDH, glucose-6-phosphate dehydrogenase.

proper, the visceral yolk sac and the ectoplacental cone/chorioallantoic placenta because they are the predominant tissues associated with the conceptus during organogenesis and are also those that are commonly included in the now popular whole embryo culture preparations. In addition, they are the most probable target sites for the teratogenic effects of chemicals that act directly on the conceptus.

The period of organogenesis is defined frequently as the period extending between the appearance of the neural plate and the closure of the palate. For rats, the period of organogenesis so defined would thus extend from day 9.5 to day 16 [15]. (In humans, the corresponding period is from days 18–20 to days 55–60 of gestation.) The gestational period covered in these investigations on rat conceptuses was from day 10 to day 14. The results demonstrate both constitutive and inducible xenobiotic monooxygenation activities in tissues of the rat conceptus throughout organogenesis and that the inducible activities are attributable, at least in part, to the presence of immunodetectable, functional P-450IA1 or a very closely related isozyme(s).

## MATERIALS AND METHODS

*Chemicals.* Nonradiolabeled BaP, glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDH), MC, Protein A-Sepharose CL-4B, dicoumarol, 4-chloro-1-naphthol and NADPH were purchased from the Sigma Chemical Co. (St. Louis, MO). Methoxyphenoxazone, ethoxyphenoxazone, pentoxyphenoxazone and benzyloxyphenoxazone were synthesized in our laboratory according to methods described by Mayer *et al.* [16] and were purified by methods described by Klotz *et al.* [17]. Purity of the ethers was verified (>99.5%) by analytical HPLC and by determination of melting point values, which were in excellent agreement with published [17] literature data. Resorufin and α-naphthoflavone (ANF) were purchased from the Aldrich Chemical Co. (Milwaukee, WI) and phenoxazone was supplied by Dr. Alan E. Rettie, Department of Medicinal Chemistry, University of Washington. Resorufin and phenoxazone were also purified by published methods [17], and purity was verified (>99.5%) by analytical HPLC. Metyrapone (MTY) was a gift from the CIBA Pharmaceutical Co. (Summit, NJ). 2-Aminofluorene and 9-ketoaminofluorene were synthesized in our laboratory according to methods described by Fletcher and Namkung [18] and by Pan and Fletcher [19] respectively. AAF and 9-keto-AAF were subsequently synthesized by acetylation of 2-aminofluorene and 9-ketoaminofluorene with acetic anhydride as described earlier [18]. The purity of each of these compounds was >99% as assessed with analytical HPLC. All hydroxylated metabolites of AAF and BaP were acquired from the Chemical Repository of the National Cancer Institute. [9-$^{14}$C]AAF (52 mCi/mmol, 98% purity) and [G-$^{3}$H]BaP (17.2 Ci/mmol, 97% purity) were purchased from the Amersham Corp., Arlington Heights, IL. [9-$^{14}$C]AAF was further purified (>99.5%) by HPLC using a Whatman Partisil ODS-2 Magnum (M9 10/25) column and eluting isocratically with methanol:water (80:20), and [G-$^{3}$H]BaP was purified (>99%) with preparative, reverse phase HPLC on a Whatman Partisil ODS-2 Magnum (M9 10/25) column with a linear methanol–water gradient (80–100%, 30 min). All other chemicals utilized were of the highest quality commercially available.

*Enzyme sources.* Timed pregnant, primigravida Sprague–Dawley (Wistar-derived) rats, obtained from Tyler Laboratories (Bellevue, WA), were used in all experiments. The animals 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. Conceptuses were exposed *in utero* to MC by injecting the dams intraperitoneally with 40 mg/kg of MC in corn oil with a single dose 48 hr prior to killing the animals via anesthetization with diethyl ether. The pregnant dams were killed on days 10, 11, 12 or 14 of gestation and, in each case, MC was injected 48 hr prior to sacrifice. These treatments produced no detectable toxic effects on the embryos exposed to MC *in utero*. Controls were injected at the same time with an equal volume of vehicle. After sacrifice, the uteri were removed and placed in cold Hanks' balanced salt solution, and 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 certain experiments, the entire conceptus was gently homogenized by hand in 0.1 M potassium phosphate buffer (pH 7.4) in a Potter homogenizing vessel 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/chorioallantoic placenta, and embryo were separated by dissection, and homogenates of each of these separated 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 the amnion remained attached to the embryo. Each preparation was derived from 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. For comparative purposes, we prepared washed microsomes from hepatic homogenates of adult (230–260 g) female rats in accordance with established methods [20].

*Enzyme assays.* Conversion of AAF to oxidized metabolites was determined by HPLC analyses as previously described by Faustman-Watts *et al.* [10]. Linearity of conversion as a function of time and protein concentration was indicated in preliminary experiments. Reaction vessels contained 1.0 μmol NADPH, 2.0 μmol G6P, 2 units G6PDH, 0.05 to 2.0 mg protein, 40 nmol AAF (2.0 μCi) in ethanol

and potassium phosphate buffer to a final volume of 1.0 ml. Incubations were carried out at 37° for 120 min under 100% atmospheric oxygen. Retention times in minutes were: AAF, 23.9; *N*-hydroxy-AAF, 20.8; 1-hydroxy-AAF, 18.3; 3-hydroxy-AAF, 16.2, 9-keto-AAF, 12.9; 5-hydroxy-AAF, 7.9; 9-hydroxy-AAF, 6.8, and 7-hydroxy-AAF, 5.3.

Conversion of BaP to oxidized metabolites was determined by HPLC as previously described by Dean *et al.* [21]. Preliminary experiments indicated linearity of oxidation as a function of time and protein concentration. Reaction vessels contained 1.0 μmol NADPH, 2.0 μmol G6P, 2 units of G6PDH, 0.1 to 1.0 mg protein, 0.12 nmol BaP (2.0 μCi; the radioactive BaP was not diluted with cold BaP in order to maximize the sensitivity of the assay) in 20 μl acetone, and potassium phosphate buffer (0.1 M, pH 7.4) in a total volume of 1.0 ml. Incubations were carried out at 37° for 120 min under 100% atmospheric oxygen. Retention times in minutes for substrate and metabolites were: BaP, 33.6; 3-hydroxy-BaP, 22.7; 7-hydroxy-BaP, 22.5; 9-hydroxy-BaP, 21.2; BaP-6,12-dione, 18.4; BaP-3,6-dione, 17.1; BaP-1,6-dione, 15.2; BaP-7,8-diol, 10.3; BaP-4,5-diol, 8.7; and BaP-9,10-diol, 6.3. For assays of hydroxylation of both BaP and AAF, activities are expressed as pmol/mg protein/120 min. Activities less than 30 or 60 pmol/120 min were regarded as below the limits of detectability for AAF and BaP respectively.

Hydroxylation of phenoxazone, O-dealkylation of the corresponding methoxy-, ethoxy-, and pentoxy-ethers, and O-debenzylation of benzyloxyphenoxazone each resulted in the generation of the highly fluorescent resorufin metabolite which was monitored continuously as a function of time according to slight modifications [22] of the methods described by Burke *et al.* [23]. Reaction cuvettes contained dimethyl sulfoxide-dissolved substrate (0.01 mM), enzyme sources (0.02 to 2.0 mg protein), 1.0 mM NADPH and 0.1 M potassium phosphate buffer (pH 7.4) in a total volume of 1.0 ml. Reactions were initiated by addition of NADPH. Only those reactions exhibiting linear increases in fluorescence over a period of 2 min were regarded as within the limits of detectability. Additions of 2.0 mM G6P, 2 units of G6PDH or 0.01 mM dicoumarol produced less than 20% increases in reaction rates and were omitted from the reaction flasks in routine experiments. Each assay was performed in duplicate or triplicate and standardized by addition of a known quantity of purified resorufin. Activities are expressed as pmol/mg protein/min. The limit of detectability was 0.1 pmol/min. Protein concentrations were determined by the method of Lowry *et al.* [24] with bovine serum albumin as the standard.

*Immunoassays.* Rat hepatic P-450IA1, also commonly known as P-450c and P-450β-NF-B, was purified from the microsomes of MC-induced adult males according to the methods described by Ryan *et al.* [25, 26]. The purified isozyme exhibited a specific content of 16.7 nmol P-450/mg of protein. Sodium dodecyl sulfate (SDS)-gel electrophoresis yielded a single protein staining band. Polyclonal anti-P-450IA1 antibodies were produced by intradermally injecting 0.25 mg of the purified antigen in Freund's complete adjuvant into adult female New Zealand White rabbits according to the methods described by Thomas *et al.* [27]. Injections in Freund's incomplete adjuvant were given after 1 and 2 months, and blood was withdrawn 1 week after the final injection. An IgG-enriched fraction was prepared [28] by passage of sera through a Protein A-Sepharose CL-4B column. Specificity of the antibody was verified as described previously [22, 29].

For immunoblotting, various quantities of 600 *g* (5 min) supernatant fractions of conceptal homogenates were applied to SDS-polyacrylamide gels and electrophoresed according to the method of Laemmli [30]. Various quantities of P-450IA1, purified from the livers of MC-induced adult rats, were utilized as standards for quantitation and were routinely run on the same gels. After electrophoresis, the samples were transferred to nitrocellulose membranes (0.45 μm) according to slight modifications of the method of Towbin *et al.* [31]. The blocking solution used was 1% bovine serum albumin. The nitrocellulose membrane was incubated with anti-P-450IA1 IgG for 3 hr. The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase. 4-Chloro-1-naphthol was used as the color-developing substrate. Immunoquantitation of conceptal P-450IA1 was effected by scanning of the immunoblots on nitrocellulose membranes with an LKB 2400 Gel-Scan laser densitometer. Quantities of the conceptal hemoprotein were estimated by comparison with a standard curve for purified P-450IA1 run on the same gels and transferred to nitrocellulose membranes and simultaneously immunostained.

## RESULTS

Results of experiments presented in Table 1 demonstrated that ethoxyphenoxazone deethylase activity, regarded as indicative of the presence of functional, MC-inducible (IA1 or IA2) isozymes, was readily detectable in the homogenates of rat conceptuses at day 10 of gestation. In addition, the depentylation of pentoxyphenoxazone and debenzylation of benzyloxphenoxazone were also easily measurable at day 10 in the same conceptuses (Table 1). Pretreatment *in utero* of conceptuses with MC by injection of the corresponding pregnant dams resulted in a 124% increase in deethylase activity, a slight and statistically insignificant decrease (11%) in depentylase activity, and a 52% increase ($P < 0.05$) in debenzylase activity. Importantly, no demethylation of methoxyphenoxazone could be detected in any of the investigated tissues of the day 10–14 conceptus, even after preinduction with MC. The demethylase activity is regarded as indicative of the presence of functional P-450IA2 [29], an immunologically cross-reactive hemoprotein.

For analyses of conceptuses at days 11, 12 and 14 of gestation, conceptal tissues were separated and analyzed individually as the embryo proper, the visceral yolk sac and the ectoplacental cone (days 11 and 12) or chorioallantoic placenta (day 14). Of these three components, the visceral yolk sac (Fig. 1) strikingly exhibited not only the highest specific activities for each of the phenoxazone ethers studied,

Table 1. Dealkylation and debenzylation reactions catalyzed by homogenates of whole conceptuses at day 10 of gestation*

| Substrate | Specific activity (pmol/mg protein/min | | |
|---|---|---|---|
| | Control embryos | MC-treated embryos | Maternal hepatic microsomes |
| Ethoxyphenoxazone | 0.200 ± 0.08 | 0.448 ± 0.07† | 17.7 ± 2.23 |
| Pentoxyphenoxazone | 0.412 ± 0.05 | 0.358 ± 0.05 | 0.68 ± 0.09 |
| Benzyloxyphenoxazone | 0.256 ± 0.04 | 0.390 ± 0.10† | 5.09 ± 1.07 |

* Values are means ± SD of a minimum of four determinations. Conceptal preparations were supernatant fractions of whole homogenates centrifuged at 600 *g* for 5 min. No attempt was made to separate embryos, yolk sacs and ectoplacental cones in these experiments because of the extremely small quantities of tissue. Demethylation of methoxyphenoxazone could not be detected in preparations of control or MC-treated conceptuses. For comparative purposes, activities assayed in maternal hepatic microsomal preparations (non-induced) are presented.

† Significantly different from control embryos ($P < 0.05$).

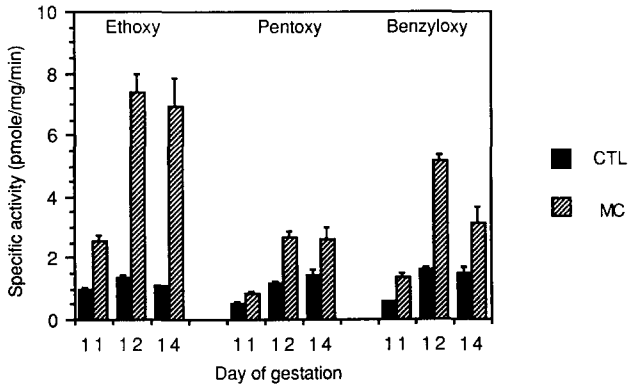


Fig. 1. Rates of dealkylation and debenzylation of phenoxazone ethers in incubated preparations containing yolk sac homogenates prepared at various stages of gestation. Reaction conditions are described in Materials and Methods.

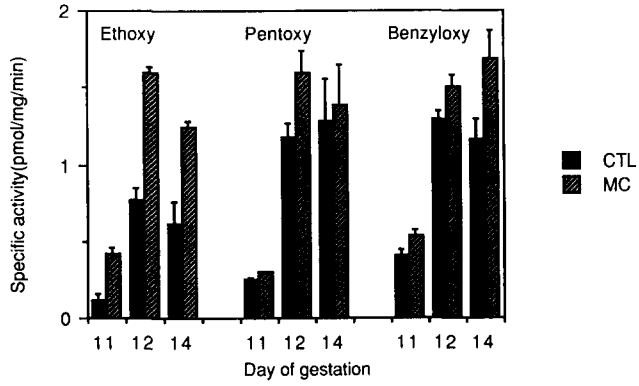


Fig. 2. Rates of dealkylation and debenzylation of phenoxazone ethers in incubated preparations containing embryo homogenates prepared at various stages of gestation. Reaction conditions are described in Materials and Methods.

but also the greatest inducibility (3–8×) after MC pretreatment. The embryo proper (Fig. 2) exhibited the lowest levels of activity, particularly on day 11, but did exhibit significant inducibility of the deethylase by MC. Uninduced activities were much higher at day 12 than at day 11 but were not significantly different on days 12 vs 14. Inducibility was also evident on days 12 and 14 but was not profound (2 to 3-fold). In the ectoplacental cone (Fig. 3), deethylase and depentylase activities at day 11 were below the levels of detectability regardless of MC pretreatment. At days 12 and 14, however, marked increases in each of these activities were observed and inducibility of deethylase activity in the ecto-

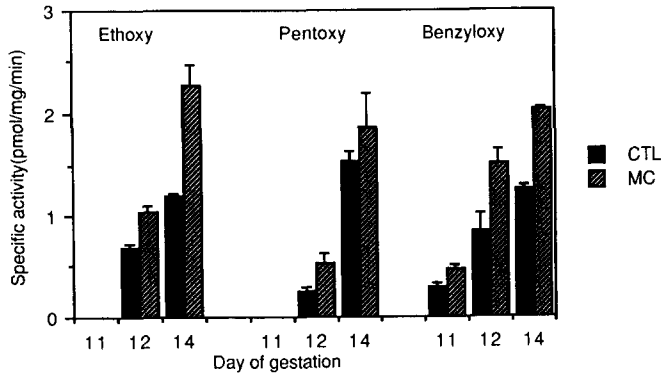


Fig. 3. Rates of dealkylation and debenzylation of phenoxazone ethers in incubated preparations containing homogenates of ectoplacental cones (days 11 and 12) or chorioallantoic placenta (day 14). Reaction conditions are described in Materials and Methods.

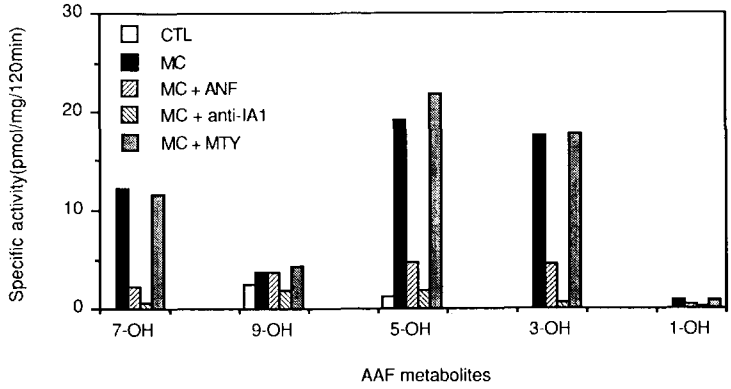


Fig. 4. Hydroxylation of 2-acetylaminofluorene in incubated preparations containing homogenates of conceptuses at day 11 of gestation. Reaction conditions, induction *in utero* with 3-methylcholanthrene (MC), and inhibition *in vitro* with 7,8-benzoflavone (ANF, 1.0 $\mu$M), anti-P-450IA1 IgG (anti-IA1, 0.5 mg/ml) or metyrapone (MTY, 0.1 mM) are described in Materials and Methods.

placental cone (day 12) and chorioallantoic placenta (day 14) was approximately 2-fold. Debenzylase activities in the ectoplacental cone/chorioallantoic placenta were readily measurable on days 11, 12, and 14. They were not affected significantly by MC pretreatment on day 11 but did exhibit marked increases on days 12 and 14. Activities for all three substrates increased progressively as a function of gestational age in the ectoplacental cone/chorioallantoic placenta, as did also the apparent inducibility of the deethylase.

Because each of the dealkylation/debenzylation assays rely on the same fluorimetric measurements, we investigated two additional substrates by HPLC analyses. Results obtained in analyses of the monooxygenation of AAF in whole conceptuses at day 11 are presented in Fig. 4. Except for very low hydroxylation at positions 9 and 5 on the AAF molecule, hydroxylation at other positions on the molecule was below the limits of detectability in experiments with uninduced conceptuses. Preinduction with MC, however, resulted in profound increases in hydroxylation at each of the positions assessed, with the exception of N-hydroxylation, which was below detectability limits under all circumstances. Increases in observed hydroxylation at positions 5, 7, and 3 were especially striking after MC pretreatment. The induced activities were inhibited markedly by anti-P-450IA1 antibodies and by ANF but were largely unaffected by metyrapone.

Similarly, MC pretreatment resulted in profound increases in hydroxylation of BaP in experiments with whole conceptuses at day 11 (Fig. 5). Hydroxylation was below the limits of detectability in untreated conceptuses but was increased markedly in MC-pretreated conceptuses for each of the oxidation products assayed. Particularly impressive were the profound effects on the rates of 3-hydroxylation. Because the 3- and 7-hydroxy-BaP metabolites exhibited very similar retention times, we collected the radioactive material that cochromatographed with these metabolites and subjected it to fluorimetric scanning (Shimadzu RF 5000 U Spectrofluorophotometer with DR-15 controller/printer) of both excitation and emission spectra. All spectra were virtually identical to that of the 3-hydroxy-BaP standard, and no evidence could be found for the presence of a 7-hydroxy-BaP metabolite, although the presence of very small quantities could not be ruled out entirely. BaP-9,10-diol and BaP-6, 12-dione also were not detected in these experiments. Again, anti-P-450IA1 antibody and

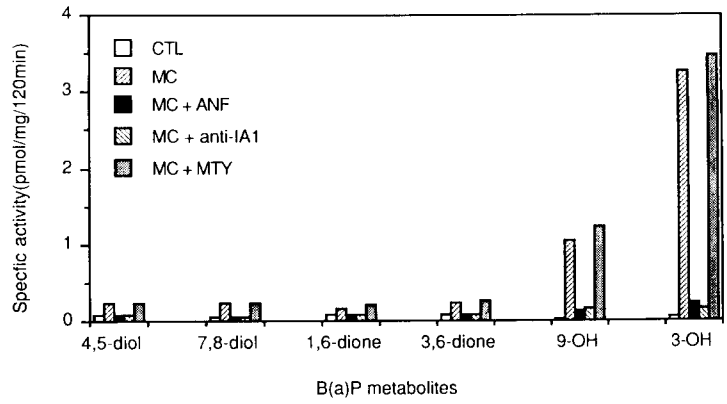


Fig. 5. Hydroxylation of benzo[*a*]pyrene in incubated preparations containing homogenates of conceptuses at day 11 of gestation. Reaction conditions are described in Materials and Methods.

Table 2. Effects of anti-P-450IA1 antibodies and chemical inhibitors on ethoxyphenoxazone deethylase activities catalyzed by embryo and yolk sac preparations from MC-pretreated rats at days 12 and 14 of gestation*

| | Activity (% of control) | | |
|---|---|---|---|
| Enzyme source | Anti-IA1 | α-Naphthoflavone | Metyrapone |
| Day 12 embryos | 54 | 54 | 99 |
| Day 14 embryos | 61 | 56 | 97 |
| Day 12 yolk sacs | 24 | 19 | 102 |
| Day 14 yolk sacs | 28 | 25 | 99 |
| Maternal liver (MC) | 2 | 5 | 98 |
| Maternal liver (control) | 98 | 71 | 97 |

* Anti-P-450IA1 IgG was added to the reaction cuvettes at a ratio of 0.01 to 0.5 mg IgG/ml of the reaction mixture. Data presented here were obtained using ratios that provide maximal inhibition. The reaction mixture was preincubated with IgG for 15 min at 25° before NADPH was added. Final concentrations of ANF and MTY in the reaction cuvettes were 1 μM and 1 mM respectively. For these inhibitors, the reaction mixture was preincubated for 5 min at 37° before NADPH was added to initiate the reaction. Values are percentages of activities observed under identical conditions, except in the presence of an equal volume of inhibitor vehicle (DMSO) or of an equal quantity of preimmune IgG. Effects of inhibitors on microsomal preparations from MC-induced and control maternal livers are presented for comparison. No significant inhibition by any of the three inhibitors was observed in preparations from untreated conceptuses. One hundred percent control activities (mean ± SD, $N > 4$) are as follows (pmol/mg protein/min): 1.597 ± 0.073 (MC day 12 embryos); 1.247 ± 0.08 (MC day 14 embryos); 7.39 ± 1.23 (MC day 12 yolk sacs); 6.92 ± 1.85 (MC day 14 yolk sacs); 2868 ± 196 (MC maternal liver); and 17.7 ± 2.2 (control maternal liver).

ANF effectively inhibited rates of each of the measurable oxidation reactions, whereas MTY did not inhibit at the concentrations employed (1.0 μM to 1.0 mM).

In further experiments with anti-P-450IA1 antibody, ANF, and MTY as diagnostic inhibitors (Table 2), it was found that the antibody and ANF effectively inhibited MC-induced deethylase activities but, at equivalent molar concentrations, did not detectably inhibit deethylase activity in experiments with uninduced conceptuses. With tissues from induced conceptuses, anti-P-450IA1 antibody and ANF reduced ethoxyphenoxazone deethylase activities to approximately the same levels as those measured in the corresponding tissues of uninduced conceptuses. At 0.1 mM concentrations, MTY failed to detectably inhibit any of the reactions investigated in these studies, even though a profound inhibition of pentoxyphenoxazone depentylase activity (>90%) in hepatic microsomes of phenobarbital-induced adult rats was observed in separate control experiments with 1.0 μM concentrations. The degree of inhibition observed with antibody or ANF (Table 2) was consistent with the idea that an MC-inducible, conceptal isozyme(s) could be totally inhibited by the investigated diagnostic inhibitors, but that a separate, constitutive conceptal deethylase(s) was unaffected by the same inhibitors.

The data from measurements of enzymic activities and immunoinhibition suggested that it might be feasible to immunodetect and possibly also immunoquantitate P-450IA1 in conceptal tissues. Immunoblotting experiments demonstrated that no signal was detectable in any of the investigated tissues of

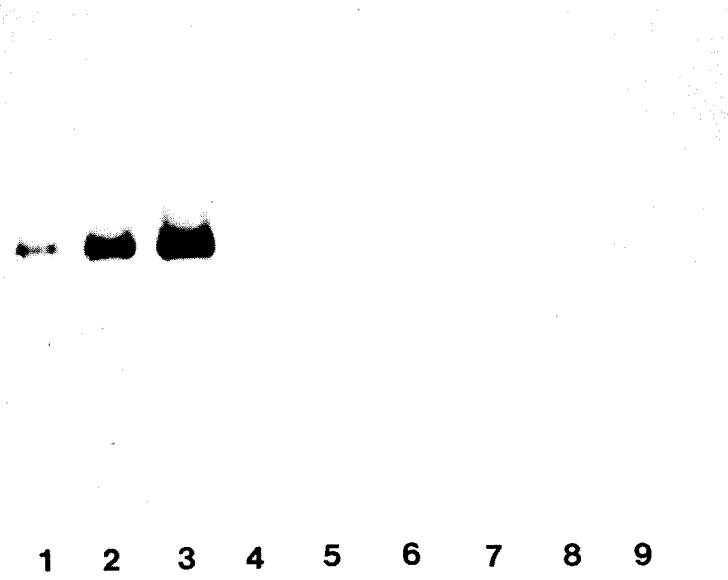


Fig. 6. Immunoblots of purified P-450IA1 and supernatant fractions (600 *g*, 5 min) of ectoplacental cones from conceptuses at days 11, 12 and 14 of gestation. Shown are 0.09, 0.18 and 0.36 pmol purified P-450IA1 in lanes 1, 2 and 3 respectively. Lanes 4 through 9 contained 400 μg of protein from 600 *g* supernatant fractions of ectoplacental cones as follows: day 11, untreated (lane 4); day 11, MC-induced (lane 5); day 12, untreated (lane 6); day 12, MC-induced (lane 7); day 14, untreated (lane 8); and day 14, MC-induced (lane 9). Specific content for the MC-induced chorioallantoic placenta at day 14 was 0.33 pmol P-450IA1/mg protein. For all other preparations, values were below the limits of detectability (0.05 pmol P-450IA1/mg protein).

uninduced conceptuses but that readily detectable signals were evident in the chorioallantoic placenta (Fig. 6) and yolk sacs (Fig. 7) of MC-induced conceptuses. In yolk sacs, immunodetection was demonstrated at all stages of gestation investigated. Immunodetection was shown for chorioallantoic placentas from MC-induced animals at day 14, although the signal was relatively low (Fig. 6) and could not be detected in the ectoplacental cone at earlier stages of gestation (days 11 and 12). A detectable signal could not be elicited for the embryo proper, even after MC induction and loading of extremely large quantities of protein (up to 1.6 mg) onto the gels at the latest stage of gestation studied (day 14). Gel scanning provided quantitative data for P-450IA1 present in the yolk sacs (Fig. 7) and chorioallantoic placentas (Fig. 6), and values are given in the respective figure legends. In general, quantities of P-450IA1 measured immunoquantitatively were well correlated with and predictable from analyses of anti-P-450IA1 antibody-inhibitable or ANF-inhibitable ethoxyphenoxazone deethylase activities in the respective tissues.

## DISCUSSION

The results of these experiments demonstrate that either P-450IA1 or a very closely related inducible isozyme can be detected as a functionally active protein in tissues of the rat conceptus during the critical stages of organogenesis. Although the isozyme was not detectable with immunoblotting procedures in the embryo proper, there seemed little doubt that it was also present in MC-induced embryonic tissues. Evidence is derived from studies with diagnostic substrates (phenoxazone ethers), selective inhibitors (anti-P-450IA1 antibodies and ANF) and a relatively specific inducer (MC). It was concluded that, in the embryo proper, the quantity of the isozyme was below the detection limits of the immunoblot assay, and the conclusion is supported by the herein reported experiments with diagnostic substrates and inhibitors. For example, detection with immunoblots was achieved only in those tissues exhibiting the highest MC-induced and anti-P-450IA1- and ANF-inhibitable activities. In the order of quantities assessed, these were the visceral yolk sac at days 12, 14 and 11 and the chorioallantoic placenta at day 14 respectively. Anti-P-450IA1- and ANF-inhibitable enzymic activities followed exactly the same order.

Although measurable amounts of the isozyme in these tissues were clearly very low (especially in the embryo proper), previous results have shown [8–10] that extremely low activities can generate sufficient reactive intermediates to elicit profound dysmorphogenic effects on the developing embryo. Ratios of activation/inactivation (rather than absolute rates) appear to play a very decisive role in these instances. Systematic investigations of the capacity of developing embryos to inactivate generated reactive embryotoxic intermediates have not been reported yet although some beginnings have been made [reviewed in Refs 1–4]. Results reported thus far would tend to suggest that embryonic tissues lack some of the crucial inactivating enzymes (e.g. glucuronyl transferases) and that activation/inactivation

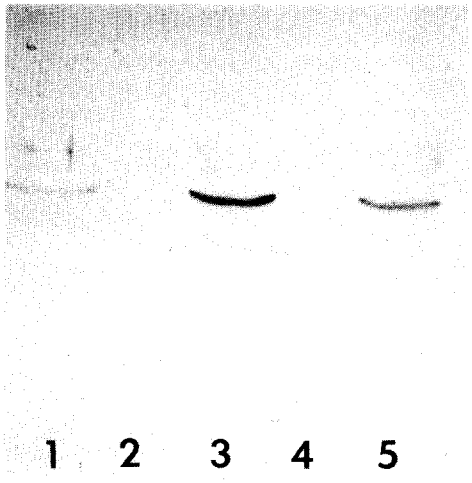


Fig. 7. Immunoblots of supernatant fractions (600 *g*, 5 min) of visceral yolk sacs from conceptuses at days 11, 12 and 14 of gestation. Each lane contained 400 μg of visceral yolk sac supernatant protein as follows: day 11, MC-induced (lane 1); day 12, untreated (lane 2); day 12, MC-induced (lane 3); day 14, untreated (lane 4); and day 14, MC-induced (lane 5). Immunoblots from day 11, untreated were consistently negative. Specific contents for the MC-induced visceral yolk sacs at days 11, 12 and 14 were 0.41, 1.21 and 0.96 pmol P-450IA1/mg protein respectively. For all other preparations, values were below the limits of detectability (0.05 pmol P-450IA1/mg protein).

ratios may be relatively high, particularly after exposure to certain inducers. These aspects, however, will require much more intensive future investigations.

As indicated above, the period of organogenesis for the rat conceptus extends roughly from day 9.5 to day 16 of gestation. The conceptuses investigated in this study ranged from day 10 to day 14 of gestation and thus did not cover the latest stages of organogenesis so defined. Recent data published by Shiverick *et al.* [32], however, strongly suggest that the trends observed in these studies would continue at days 15 and 16 of gestation. We therefore propose that, after exposure to MC or similar inducing agents (dioxins, planar polyhalogenated biphenyls, certain flavones), P-450IA1 or a very closely related isozyme(s) is both present and functionally active in rat conceptal tissues throughout the entire course of organogenesis. All of the data presented here and by Shiverick *et al.* [32] appear to support such a proposal. A caveat to this tentative conclusion, however, remains. Investigations reported by Giachelli and Omiecinski [33] have indicated that the mRNA for P-450IA1 could not be detected in MC-treated rats until several days subsequent to parturition. It may be possible that the hybridization analyses in those studies were not sufficiently sensitive to detect extremely small quantities of P-450IA1 mRNA, although this seems somewhat unlikely in view of the potential sensitivity of such assays. Conversely, the short hybridization probe utilized may have been sufficiently selective that it failed to hybridize with mRNA(s) coding for a very closely related embryonic P-450. Other possible explanations may also be tenable but, until a satisfactorily explanatory and definitive experimental delineation of those observations is provided, we are unwilling to rule out the possibility that the isozyme(s) tentatively identified as P-450IA1 in these studies may be a closely-related, immunologically cross-reacting isozyme(s) with very similar substrate/inhibitor specificity and inducing properties. That the isozyme is not P-450IA2 is very strongly indicated by a lack of capacity of conceptal tissues to catalyze O-demethylation of methoxyphenoxazone or N-hydroxylation of AAF, previously shown [22, 29] to be selectively catalyzed by the IA2 isozyme. The possibility should be entertained that certain isozymic P-450 forms may exist during embryonic development but do not exist postnatally. A similar scenario is known for hemoglobins, has been suggested with respect to fetal P-450 isozymes [34], and is consistent with our previously reported observations [22, 35].

In this study, increases in inducibility observed as a function of gestational age are consistent with observations of numerous previous investigators. Such studies have dealt primarily with fetal hepatic tissues but have also been demonstrated in rodent and human placental tissues [36]. Reasons for diminished inducibility at earlier stages of gestation remain speculative but may be a function of the state of cellular differentiation, i.e. inducibility of P-450IA1 and cellular differentiation sometimes appear to be positively correlated [36].

In a previous publication, we provided evidence for the presence of at least four separate P-450 isozymes (three constitutive and one inducible) in tissues of the rat conceptus during early gestation [22]. The results presented here are in harmony with that concept and may also suggest the possibility of an additional MC-inducible isozyme(s) capable of efficiently catalyzing the 7-, 5-, and 3-hydroxylation of AAF and the 3-hydroxylation of BaP. This idea is suggested by the quite remarkable increases in rates of these latter reactions as compared with relatively modestly increased rates of ethoxyphenoxazone deethylation after *in utero* induction with MC. Additional interest in, and importance of, the AAF-related aspect pertain to the finding that the 7-hydroxy metabolite of AAF appears to be a proximate dysmorphogen capable of eliciting abnormal neurulation in developing embryos [37, 38]. The findings reported here further indicate that pre-induction of embryos with MC-type inducing agents can enable embryos to generate the proximate 7-hydroxy-AAF metabolite with relatively good efficiency that also appears disproportionate to induction of P-450IA1.

Finally, the remarkably high enzymic activities and inducibility observed in the visceral yolk sac during the course of these studies suggest that this organ may play a critical role in determining or modulating the effects of foreign organic chemicals on the conceptus during organogenesis. It would seem that much greater attention should be accorded this organ in future toxicologic investigations.

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