Cytochrome P-450 metabolic activity in embryonic and extraembryonic tissue lineages of mouse embryos

(benzo[a]pyrene/mammalian development/sister chromatid exchange)

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ABSTRACT Mouse morulae, blastocysts, and embryonic and extraembryonic tissue layers were examined for benzo[a]pyrene metabolism by cytochrome P-450, using the sister chromatid exchange assay. Benzo[a]pyrene exposure in vitro increased sister chromatid exchanges in blastocysts of all genetically responsive mice examined (BALB/cDub, C3H/AnfCum, and outbred Dub:(ICR) strains) but not blastocysts of the nonresponsive AKR/J strain. Benzo[a]pyrene treatment of responsive 7½ - and 8½-day (postimplantation-stage) embryos, either intact or as separate tissue layers, increased sister chromatid exchanges in tissues of both embryonic and extraembryonic lineages—i.e., in the embryo proper, in isolated embryonic ectoderm, and in yolk sac, chorion, extraembryonic ectoderm, and extraembryonic endoderm layers. These results indicate that cytochrome P-450 is active in most or all tissues of the early mammalian embryo. It could metabolize xenobiotic molecules reaching the conceptus near the onset of morphogenesis and organogenesis, or it could have another as yet undefined role in normal development.

Benzo[a]pyrene and other polycyclic aromatic hydrocarbons are metabolized in cells by cytochrome P-450 monoxygenases whose synthesis they induce. The degree of inducibility in mice varies among strains: both cytochromes P4-450 and P4-450 are regulated by the Ah locus (1, 2), whose gene product is a receptor that binds to DNA after forming a complex with an inducer molecule (3, 4). In genetically responsive animals, exposure to benzo[a]pyrene leads to the expression of cytochrome P-450 structural genes coding for aryl hydrocarbon (benzo[a]pyrene) hydroxylase (RH), reduced flavoprotein:oxygen oxidoreductase (RH-hydroxylating), EC 1.14.14.1) and to the expression of other genes (5).

Recent evidence suggests that midgestation mouse embryos can metabolize certain xenobiotic molecules into mutagenic or teratogenic products (6, 7). Cultured mouse embryos of responsive strains can metabolize benzo[a]pyrene to products that cause sister chromatid exchanges (SCEs), indicating the expression of cytochrome P-450 activity at 7½ to 8½ days of gestation (8). Preimplantation embryos at the blastocyst stage can metabolize benzo[a]pyrene, as assessed by analysis of embryo culture medium using high-pressure liquid chromatography (9). Is benzo[a]pyrene metabolized by all tissue lineages that develop in the early postimplantation mouse embryo, including those that give rise to the fetus and placenta, or only by certain embryonic tissues, as suggested by Filler and Lew (9)?

Here we confirm that benzo[a]pyrene is metabolically activated in genetically responsive mouse blastocysts, and we characterize the distribution of cytochrome P-450 activity in the embryonic and extraembryonic tissue lineages of early postimplantation-stage embryos. The embryonic half of the 7½-day early postimplantation mouse egg cylinder already has several layers: primitive endoderm, mesoderm, and ectoderm. Embryonic ectoderm gives rise to the entire fetus (10). The extraembryonic half of the egg cylinder consists of visceral yolk sac, allantois, chorion, and ectoplacental cone. The latter three fuse to form the chorioallantoic placenta at about 9 days of gestation (see ref. 10 for review).

To determine cytochrome P-450 metabolic capacity, we used the SCE assay to detect reactive intermediates of benzo[a]pyrene. SCE is a sensitive, albeit indirect, indicator of DNA lesions that produce exchanges between sister chromatids during DNA synthesis (11, 12). These can be detected by incorporating the thymidine analogue 5-bromodeoxyuridine (BrdUrd) for two cycles of DNA synthesis, followed by fluorescence-plus-Giemsa staining of fixed chromosomes (13). The SCE assay is particularly suitable for early mammalian embryos, which contain limited numbers of cells (8).

MATERIALS AND METHODS

Materials. Benzo[a]pyrene was obtained from Aldrich; dimethyl sulfoxide and Colcemid from Sigma; organ culture plastic dishes and tubes from Falcon; microwell plates from Costar (Cambridge, MA); BrdUrd from Calbiochem; Hoechst 33258 stain from Hoechst Pharmaceutical (Somerville, NJ); Gurr Giemsa stain from Biomedical Specialties (Santa Monica, CA); pregnant mare's serum gonadotropin from Teikoku Zoki Pharmaceuticaels (Tokyo, Japan); human chorionic gonadotropin from Ayerst Laboratories (New York); fetal bovine serum from Sterile Systems (Logan, UT); purified crystalline trypsin from Worthington; and pancreatic from Difco.

Animals. AKR/J mice were obtained from The Jackson Laboratory. BALB/cDub and Dub:(ICR) (outbred ICR-devised Swiss-Webster mice) were obtained from Dominion Research Animals (Dublin, VA); C3H/AnfCum mice were from Cumberland Farms (Clinton, TN).

For preimplantation embryos, female mice (6–10 weeks old) were induced to ovulate by intraperitoneal injections of 5 international units [Dub:(ICR)] or 2.5 international units (other strains) of pregnant mare's serum gonadotropin followed in 45–48 hr by an equivalent dose of human chorionic gonadotropin. Each female was caged overnight with a male, and those that mated were killed at 3½ days of gestation to obtain blastocysts. (Mating was indicated by the presence of a copulatory plug the following morning; noon of this day was considered "gestational day 1/2.") In some cases females were killed at 1½ days of gestation to obtain embryos at the 2-cell-stage. For postimplantation embryos, two or three fe

Abbreviations: SCE, sister chromatid exchange; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin. "Cytochrome P-450" as used here designates all forms of inducible CO-binding hemeprotein associated with polycyclic aromatic-inducible aryl hydrocarbon hydroxylase activity (EC 1.14.14.1).

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males were caged overnight with a single male (spontaneous ovulation). All studies of postimplantation embryos were done in the C3H/AnfCum strain because of poor embryo yields in other inbred strains.

**Culture and Treatment of Embryos.** Preimplantation embryos were flushed from the reproductive tract either at the two-cell stage with modified Hanks' balanced salt solution [flushing medium I (14)] or at the blastocyst stage with flushing medium II (14). Two-cell embryos were cultured in standard culture medium (14) to the morula stage before treatment. For benzo[a]pyrene treatment beginning at 74–76 hr (morulae) or 98–100 hr (blastocysts) after injection of human chorionic gonadotropin, embryos were incubated at 37°C in the dark in modified Eagle's medium (14) containing 5% fetal bovine serum, 0.5 μM BrdUrd, and 0.2, 1.0, or 2.5 μM benzo[a]pyrene. Benzo[a]pyrene was dissolved in dimethyl sulfoxide at 400× the final concentration; an equivalent volume of dimethyl sulfoxide (2.5 μl/ml) was added to control cultures. Cultures were incubated for 33 hr with Colcemid (0.2 μM) present for the final 3.5 hr before fixation.

Postimplantation embryos were dissected from the uteri at gestational day 7½ as previously described (8), then incubated at 37°C in the dark in modified Eagle's medium containing 10% fetal bovine serum. Two to four intact, cleaned embryos were treated in 1 ml of medium in a 12 × 75 mm plastic tube, which was rotated at 0.2 rpm on a tissue drum (Lab-Line, Melrose Park, IL) in a humidified atmosphere of 5% CO2 in air. Pure tissue layers of 7½- or 8½- day embryos were obtained by dissection before culture by using the trypsin/pancreatin procedure (15). These tissue fractions were treated in modified Eagle's medium in organ culture dishes. In both approaches, BrdUrd was added to a final concentration of 0.15 mM. This higher concentration of BrdUrd was necessary to detect SCE in embryonic and extraembryonic ectoderm, as a result of the lower levels of BrdUrd incorporation in these tissues (unpublished observations). After 21.5-hr culture, Colcemid (0.2 μM) was added and incubation was continued for 2.5 hr before chromosome fixation.

**Chromosome Preparation and Staining.** Each group of preimplantation embryos was exposed for up to 30 min to 0.5% (hypotonic) sodium citrate, then transferred into fixative [methanol/glacial acetic acid, 1:1 (vol/vol)], and onto a slide as previously described (16). Chromosome preparations were stained by modification 2 of the fluorosence-plus-Giemsa technique (8), using long-wavelength ultraviolet (black) light.

Intact postimplantation embryos were exposed after benzo[a]pyrene treatment to freshly prepared 0.075 M hypotonic KCl for 5–10 min, then dissected into embryonic and extraembryonic regions with glass needles. Tissue fractions dissected before treatment were exposed to hypotonic KCl in the same way. Two to four whole embryos or their isolated tissues were pooled for each point, then fixed for 10 min in methanol/acetic acid (3:1, vol/vol). Chromosomes were spread (8) and stained as described for preimplantation embryos.

SCEs were counted in well-spread metaphases to determine the number of exchanges per chromosome and per cell (normalized to a diploid value of 40 chromosomes). The estimate of variance was based on an assumption of a Poisson distribution, in which the variance is equal to the mean. The standard error was then calculated as the [(no. of SCEs)²/ no. of chromosomes] × 40. Differences were evaluated on the basis of a Z statistic for differences between Poisson populations, using the standard normal distribution (17).

**RESULTS**

Our studies confirmed the conclusion of Filler and Lew (9) that the capacity to metabolize benzo[a]pyrene first appears at the blastocyst stage. Embryos from the responsive outbred strain Dub:(ICR) that were cultured from the morula stage with 0.4–2.5 μM benzo[a]pyrene showed no increase in SCE over control values (data not shown). When embryos from three responsive strains were cultured from the blastocyst stage with 0.4 or 1 μM benzo[a]pyrene, they showed statistically significant increases in SCE (Table 1); however, these increases in the BALB/cDub and C3H/AnfCum strains were much smaller than those observed in blastocysts of the Dub:(ICR) strain or in 7½-day embryos at comparable concentrations of benzo[a]pyrene (8). Blastocysts of the nonresponsive inbred strain AKR/J showed no increase in SCE when cultured with benzo[a]pyrene. There was considerable variation in SCE levels between strains in controls and at threshold benzo[a]pyrene concentrations.

**Benzo[a]pyrene Metabolism in 7½-day Tissues.** In our previous work (8), embryos of responsive strains [BALB/cDub, C3H/AnfCum, Dub:(ICR), and others] were shown to metabolize benzo[a]pyrene at 7½ and 8½ days of gestation. In the present study, we dissected the conceptus into embryonic and extraembryonic regions and into specific tissues (18). When whole embryos were exposed to 0.25–2.5 μM benzo[a]pyrene at gestational day 7½, benzo[a]pyrene induced SCE in both the extraembryonic region and the embryonic region of C3H/AnfCum embryos (Table 2). There was a 6- to 7-fold increase in the extraembryonic region, and a 3- to 4-fold increase in SCE in the embryonic region. When individual tissues were exposed, extraembryonic endoderm had more SCE than extraembryonic ectoderm, but the result was based on very few cells and the relative increase was similar in the two tissues (Table 2). Because pure embryonic endoderm was difficult to obtain, only embryonic ectoderm was evaluated. When exposed in isolation, this tissue also showed a 2-fold increase above control values, the smallest increase observed. Tissues were exposed to benzo[a]pyrene after dissection so that there would be little, if any, difference in their accessibility to benzo[a]pyrene in the medium.

**Table 1.** Benzo[a]pyrene-induced SCE in cultured mouse embryos at the blastocyst stage

<table>
<thead>
<tr>
<th>Benzo[a]pyrene, μM</th>
<th>Dub:(ICR)</th>
<th>BALB/cDub</th>
<th>C3H/AnfCum</th>
<th>AKR/J</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1409</td>
<td>15.5 ± 0.7</td>
<td>2935</td>
<td>8.9 ± 0.3</td>
</tr>
<tr>
<td>0.1</td>
<td>283</td>
<td>16.4 ± 1.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.25</td>
<td>584</td>
<td>13.2 ± 1.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.4</td>
<td>706</td>
<td>31.0 ± 1.3*</td>
<td>1997</td>
<td>11.2 ± 0.5*</td>
</tr>
<tr>
<td>1.0</td>
<td>1241</td>
<td>25.8 ± 0.9*</td>
<td>2705</td>
<td>15.6 ± 0.5*</td>
</tr>
<tr>
<td>2.5</td>
<td>192</td>
<td>24.8 ± 2.3*</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

n is the number of chromosomes scored; values were normalized to 40 chromosomes to obtain number of SCEs per cell. These values are expressed as mean ± SEM [SCEs/cell = SCEs/(chromosomes) × 40].

*Significantly greater than control (P < 0.001).
†Significantly greater than control (P < 0.01).
**Table 2.** Benzo[a]pyrene-induced SCE in cultured mouse embryos at 7½ days of gestation

<table>
<thead>
<tr>
<th>Benzo[a]pyrene, ( \mu M )</th>
<th>Extraembryonic region</th>
<th></th>
<th>Embryonic region</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Entire region</td>
<td>Dissected endoderm</td>
<td>Entire region</td>
<td>Dissected ectoderm</td>
</tr>
<tr>
<td></td>
<td>( n )</td>
<td>SCEs/cell</td>
<td>( n )</td>
<td>SCEs/cell</td>
</tr>
<tr>
<td>0</td>
<td>2486</td>
<td>6 ± 0.3</td>
<td>543</td>
<td>10.1 ± 0.9</td>
</tr>
<tr>
<td>0.25</td>
<td>1628</td>
<td>23 ± 0.8*</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>0.4</td>
<td>1702</td>
<td>31 ± 0.5*</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.0</td>
<td>3620</td>
<td>43 ± 0.7*</td>
<td>163</td>
<td>35.1 ± 2.9*</td>
</tr>
<tr>
<td>2.5</td>
<td>2576</td>
<td>36 ± 0.7*</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Embryos of C3H/AnfCum mice were treated with benzo[a]pyrene before dissection for analysis of entire regions and were treated after dissection for analysis of tissues. Each sample represents 3–12 experiments, each with tissues from 2–4 embryos. Values of SCEs/cell are expressed as mean ± SEM, as for Table 1; \( n \) = number of chromosomes scored.

*Significantly greater than control (\( P < 0.001 \)).

**DISCUSSION**

Consistent with the observations of Filler and Lew (9), our results show that blastocyst-stage mouse embryos convert benzo[a]pyrene into SCE-inducing metabolites. Studies of other cultured cells show that the most potent SCE-inducing metabolites are the mutagenic and carcinogenic metabolites of benzo[a]pyrene (11, 12). At the morula–blastocyst transition in mice, many enzyme activities increase—e.g., those involved in transport systems and macromolecular synthesis (reviewed in ref. 19). Thus, cytochrome P-450 activity appears during increased overall metabolic activity before implantation. After implantation, there is a relatively uniform rate of energy metabolism in mouse embryos (20).

Precisely when do mouse embryos acquire their ability to metabolize benzo[a]pyrene? The SCE assay detects DNA lesions incurred before or during the first and second S phases after addition of BrdUrd. Morulae would complete their second S phase 2–3 hr before the end of culture (i.e., the length of \( G_2 \) (21). By this time, our cultured morulae had attained 32 or more cells and had formed their blastocoel cavity, yet they did not show benzo[a]pyrene-induced increases in SCE. Therefore, embryos of responsive strains must have acquired their ability to metabolically activate benzo[a]pyrene sometime between the early blastocyst stage (74 hr after injection of human chorionic gonadotropin plus 30 hr culture) and the late blastocyst stage (98 hr after human chorionic gonadotropin plus 30 hr culture). Filler and Lew (9) state that benzo[a]pyrene metabolites are first found in embryos of C57BL/6 mice cultured to the “late blastocyst” stage. They also report a small but significant amount of benzo[a]pyrene metabolism in the nonresponsive DBA/2J strain. We previously found slightly increased SCE in embryos of the AKR/J and DBA/2J strains that were treated with benzo[a]pyrene at 7½ days of gestation (8), although in this study AKR/J embryos had no detectable increase in SCE at the blastocyst stage. Nonresponsive mice typically show some cytochrome P-450 inducibility in extrahepatic tissues, although their induced hepatic enzyme activities are less than 1/10th of those in responsive strains (22). The relatively low levels of benzo[a]pyrene-induced SCE at the blastocyst stage in embryos of the BALB/cDub and C3H/AnfCum strains [compared to Dub:(ICR)] may result from genetic variations in the rate of early development (23, 24).

Our major conclusion from analysis of postimplantation embryos is that, in genetically responsive mice, both the tissue lineages giving rise to the fetus and those giving rise to the placenta have acquired the ability to metabolize benzo[a]pyrene. The only embryonic tissue lineage that was not examined in isolation was mesoderm; because levels of SCE in intact tissues containing mesoderm equalled those in tissues dissected free of mesoderm, it is likely that mesoderm also has cytochrome P-450 metabolic activity. Thus, the cytochrome P-450 metabolic capacities of all fetal and placental tissues could be decisive factors in the developmental risk of exposure to polycyclic aromatic hydrocarbons during early gestation.

Because dissected endoderm had higher SCE values than dissected ectoderm, even when both tissues were exposed equally to benzo[a]pyrene, endoderm may have higher lev-

**Table 3.** Benzo[a]pyrene-induced SCE in cultured mouse embryos at 8½ days of gestation

<table>
<thead>
<tr>
<th>Benzo[a]-pyrene, ( \mu M )</th>
<th>Extraembryonic region</th>
<th>[embryonic region]</th>
<th>Dissected endoderm</th>
<th>Dissected ectoderm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Entire yolk sac</td>
<td>Entire chorio</td>
<td>Dissected endoderm</td>
<td>Dissected ectoderm</td>
</tr>
<tr>
<td></td>
<td>( n ) SCEs/cell</td>
<td>( n ) SCEs/cell</td>
<td>( n ) SCEs/cell</td>
<td>( n ) SCEs/cell</td>
</tr>
<tr>
<td>0</td>
<td>373</td>
<td>3.0 ± 0.6</td>
<td>369</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>0.4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1.0</td>
<td>303</td>
<td>45.3 ± 2.4*</td>
<td>530</td>
<td>19.0 ± 1.2*</td>
</tr>
<tr>
<td>2.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Embryos of C3H/AnfCum mice were treated with benzo[a]pyrene before dissection for analysis of entire yolk sac, choriion, or embryo and were treated after dissection for analysis of dissected tissues. Values of SCEs/cell are expressed as mean ± SEM, as for Table 1; \( n \) = number of chromosomes scored.

*Significantly greater than control (\( P < 0.001 \)).

†Significantly greater than control (\( P < 0.01 \)).
els of cytochrome P-450 activity. Furthermore, endoderm was probably responsible for the high SCE values observed in the intact 7½-day regions studied here and in the whole embryos studied previously (8). This is interesting in view of the similarity of yacht sac biochemical differentiation with the fetal liver (e.g., α-fetoprotein synthesis) (25). Mouse embryonal carcinoma cells, which are believed to be analogous to those of early postimplantation embryos (26), are also capable of metabolizing benzo[a]pyrene to mutagenic forms (27, 28).

The nature and disposition of benzo[a]pyrene metabolites within the conceptus might depend on the metabolic activity of the placental and fetal tissues. Placental detoxication (e.g., polyoxygenation, conjugation) alone could conceivably provide some protection from reactive products of benzo[a]pyrene metabolism, but it cannot confer protection from parent compound that reaches the embryo proper, since embryonic tissues are also metabolically competent. In the intact maternal–fetal–placental unit, other factors besides metabolic activation could also influence the embryotoxicity of benzo[a]pyrene. As demonstrated by Schreck and Latt (29), SCE frequency did not correlate with Ah genotype in adult tissues of mice treated with benzo[a]pyrene in vivo. They observed a similar disparity between activities of phenobarbital-induced drug-metabolizing enzymes (not associated with the Ah locus) and SCE frequencies in mice treated with cyclophosphamide (30). Thus, it appears that the induced SCE frequency in mice in vivo is influenced by genetic differences not only in drug activation but also in drug detoxication or absorption. For example, oral administration of benzo[a]pyrene to nonresponsive pregnant mice leads to greater embryotoxicity in nonresponsive embryos than in responsive embryos, which is the opposite of the results obtained after intraperitoneal administration (7, 31–33).

The capacity for DNA repair also varies among certain strains of mice, as indicated by the differences in the frequency of SCE induced by residual lesions, when eggs of these strains were fertilized by ultraviolet-irradiated sperm (34). However, consistently high levels of benzo[a]pyrene-induced SCE in embryos of strains high in repair capacity (C57BL/6) or low in repair capacity (BALB/c) (8) argue against DNA repair as a major factor determining the differences in benzo[a]pyrene-induced SCE between strains.

Our earlier work (8) also addresses the association between mouse embryonic metabolism of benzo[a]pyrene and the Ah locus. High frequencies of benzo[a]pyrene-induced SCE were seen in embryos from three responsive strains, with little or no SCE induction in two nonresponsive strains. In addition, a responsive recombinant inbred strain (B6NXAKN-12) showed high SCE levels, whereas a nonresponsive strain (B6NXAKN-3) showed no benzo[a]pyrene-induced SCE. The excellent correlation between Ah genotype and benzo[a]pyrene-induced SCE in the seven strains studied implied that the cytochrome P-450 form(s) responsible for embryonic metabolism of polycyclic aromatic hydrocarbons was regulated by the Ah locus. Analysis of benzo[a]pyrene metabolism in embryos of additional recombinant inbred strains and use of recombinant DNA probes (2) would provide a more definitive assessment of this correlation and would indicate the specific form(s) of cytochrome P-450 present at early embryonic stages.

Some investigators have suggested that the regulatory gene product of the Ah locus may have a role in normal development (35, 36). This concept is based primarily on the extreme toxicity and teratogenicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which binds specifically to the receptor product of the Ah locus (3, 37, 38) and leads to cleft palate and other defects in Ah-responsive mice (39, 40). Even though cytochrome P-450 induction is a major consequence of TCDD exposure in Ah-responsive mice, enzymes controlled by the Ah receptor are not necessarily responsible for the effects of TCDD; rather, persistent occupation of the Ah receptor itself may interfere with essential cellular regulatory events within the developing embryo or fetus, as postulated by Poland et al. (35). One implication of this idea is that there may be an endogenous ligand for the Ah receptor. The observation of benzo[a]pyrene metabolism in all tissue lineages examined at 7½ to 8½ days of gestation, and in blastocysts (refs. 10, 41, and this study), implies that the Ah locus is expressed throughout the earliest morphogenetic events in mammalian development.

If the sole function of the cytochrome P-450 expression in the early embryo were environmental surveillance, one might expect other drug-metabolizing systems to be similarly active. However, there is no detectable activity of the phenobarbital-induced cytochrome P-450 system (not associated with the Ah locus) in 9-day rat embryos (42) or in cultured rat yolk sac cells (43). Furthermore, predominance of quinone formation in mouse embryos treated with benzo[a]pyrene at 4 days of gestation (9) or at 10 days of gestation (33) suggests that detoxication enzymes are relatively inactive at early stages of development. Metabolism of benzo[a]pyrene by cytochrome P-450-mediated oxidation is thus the only system known to be active very early in gestation. Similar cytochrome P-450 metabolic activities are also detectable at very early stages (locus 3 of incubation) of chicken embryo development (44) and in fish embryos at later stages (45). This early expression may be evidence for a role of the Ah locus in normal mammalian development other than metabolism of xenobiotics. However, further work is needed to identify the cytochrome P-450 form(s) active in early vertebrate development and to characterize their normal function.

A preliminary report of this work was presented at the Fifth Symposium on Prenatal Development, May 7–10, 1981, in Berlin (41). We thank Dr. Paul Perry and Dr. Daniel Nebert for their contributions and encouragement during early phases of this work, Dr. Philip Iannaccone and Dr. Michael DiBartolomeis for comments on the manuscript, and Ms. Mary McKenney and Ms. Leslie Roberts for their editorial assistance. This work was supported by the U.S. Department of Energy, contract no. DE-AC03-76SF001012.