TOXICOLOGY AND APPLIED PHARMACOLOGY 80, 137–146 (1985)

Effects of 3-Methylcholanthrene and Phenobarbital on the Capacity of Embryos to Bioactivate Teratogens during Organogenesis[1]

MONT R. JUCHAU,[2,3] CECILIA M. GIACHELLI, ALAN G. FANTEL,[3] JEAN C. GREENAWAY,† THOMAS H. SHEPARD,[3] AND ELAINE M. FAUSTMAN-WATTS*

*Departments of Pharmacology and Pediatrics, School of Medicine; and Department of Environmental Health, *School of Public Health, University of Washington, Seattle, Washington 98195*

Received January 7, 1985; accepted April 15, 1985

Effects of 3-Methylcholanthrene and Phenobarbital on the Capacity of Embryos to Bioactivate Teratogens during Organogenesis. JUCHAU, M. R., GIACHELLI, C. M., FANTEL, A. G., GREENAWAY, J. C., SHEPARD, T. H., AND FAUSTMAN-WATTS, E. M. (1985). *Toxicol. Appl. Pharmacol.* **80**, 137–146. Pregnant Sprague–Dawley rats were divided into four groups and given ip injections of 3-methylcholanthrene (MC) in corn oil, corn oil only, phenobarbital (PB) in Hank's balanced salt solution (HBSS), or HBSS only. Maternal animals were killed on Day 10 of gestation, and embryos from each group were explanted in medium containing cyclophosphamide (CP), 2-acetylaminofluorene (AAF), or dimethylsulfoxide vehicle. After a 24-hr culture period, embryos from dams treated with HBSS, corn oil, or PB/HBSS exhibited no increase in abnormalities (as compared with controls) when either CP or AAF were added to the media. However, embryos transplacentally preexposed to MC and subsequently treated during culturing with AAF (but not CP) exhibited striking increases in malformation incidence. Commonly observed malformations included abnormally open neural tubes, abnormal flexure rotation, and prosencephalic defects. Homogenates of Day 10 embryos transplacentally preexposed to MC exhibited readily measurable oxidative biotransformation of AAF as assessed with HPLC. Biotransformation of AAF by embryos from the other three groups was virtually undetectable. Incorporation of exogenously supplemented bioactivating systems from livers of mature animals indicated that postmitochondrial supernatant fractions (S-9) from male, MC-pretreated rats effectively catalyzed the conversion of AAF (but not CP) to embryotoxic metabolites. Conversely, hepatic S-9 from adult, male, PB-pretreated rats was highly effective in converting CP (but not AAF) to embryotoxic metabolites. The results indicated the inducer-specific occurrence of embryonic bioconversion of AAF to embryotoxic metabolites via MC-inducible, *P*-450-dependent, embryonic enzyme systems.

In recent years, we and other investigators have studied the embryotoxic effects of several drugs and other foreign organic chemicals on cultured whole rat embryos and have demonstrated the importance of biotransformational factors as critical determinants of the causality of malformations for several of those chemicals. A review of these aspects of reproductive toxicology has recently appeared in the literature (Shepard *et al.*, 1983). Some of the chemicals studied have exhibited embryotoxicity only in the presence of an exogenously supplemented source of *P*-450-dependent monooxygenase activity. Cyclophosphamide (Fantel *et al.*, 1979; Mirkes *et*

[1] This investigation was supported in part by NIH Grants HD-04839, OH-01281, HD-12717, EH-07032, and HD-00836. Portions of this work were presented at the 1984 annual meeting of the Society of Toxicology in Atlanta, Georgia and have appeared in the *Toxicologist* **4**, 89 (1984).

[2] To whom all correspondence should be addressed: Department of Pharmacology, School of Medicine (SJ-30), University of Washington, Seattle, Wash. 98195.

[3] Affiliates of the Child Development and Mental Retardation Center, University of Washington.

† Deceased.

0041-008X/85 $3.00

al., 1981; Greenaway *et al.*, 1982) and 2-acetylaminofluorene (Faustman-Watts *et al.*, 1983, 1984; Giachelli *et al.*, 1984a); have been the most intensively examined (hereafter referred to as CP and AAF, respectively)[4] and were the substrates chosen for the present investigation. Both chemicals have been intensively studied in terms of the relationship of their biotransformation to certain toxic effects that they elicit (e.g., cytotoxicity, mutagenicity, carcinogenicity, covalent binding to biological macromolecules). It is reasonably well established that bioactivation of CP by *P*-450-dependent hepatic enzymes in adult test animals is markedly increased following induction by phenobarbital (PB) but not by 3-methylcholanthrene (MC) whereas the opposite appears to hold true for bioactivation of AAF (Hales and Jain, 1980; Thorgeirsson and Nebert, 1976). With exogenously added hepatic postmitochondrial supernatant (S-9) fractions from adult mice, we have found that induction with phenobarbital appeared to enhance the bioconversion of CP to embryotoxic metabolites in embryo culture systems (Greenaway *et al.*, 1982). The capacity of *P*-450-dependent, mammalian embryonic enzyme systems to catalyze the generation of sufficient reactive intermediates to produce observable malformations in the same embryos has not been demonstrated although studies from several laboratories have suggested that this might be possible (Shum *et al.*, 1979; Juchau *et al.*, 1980; Filler *et al.*, 1981; Hoshino *et al.*, 1981 Legraverend *et al.*, 1984). For this study, we have selected CP and AAF as model chemicals which depend on bioactivation to become embryotoxic and MC and PB as prototype inducing agents to examine the potential of embryonic enzymes to catalyze the conversion of chemicals to metabolites that can act to malform the same embryos. To avoid the complications of maternal biotransformational/dispositional factors, we selected the whole-embryo culture for the investigation. The advantages of the use of this system have been discussed (Shepard *et al.*, 1983). The results indicate that transplacental induction of *P*-450-dependent systems by MC will occur in Day 10 embryos and that the induction will enable embryos to catalyze the conversion of AAF (but not CP) to malforming metabolites. Administration of PB, however, failed to result in significant embryonic bioactivation of either CP or AAF.

[4] Abbreviations used: CP, cyclophosphamide; PB, phenobarbital; MC, 3-methylcholanthrene; AAF, 2-acetylaminofluorene; S-9, homogenate 9000*g* supernatant fraction; AF, 2-aminofluorene; NOF, 2-nitrosofluorene; HPLC, high-pressure liquid chromatography; G6P, glucose 6-phosphate; G6PDH, glucose 6-phosphate dehydrogenase; HBSS, Hank's balanced salt solution.

METHODS

Chemicals. 2-Aminofluorene (AF) and AF-9-one were synthesized by methods described earlier (Fletcher and Namkung, 1958; Pan and Fletcher, 1958). AAF and AAF-9-one were subsequently synthesized by acetylation of AF and AF-9-one with acetic anhydride as previously described (Fletcher and Namkung, 1958). 2-Nitrosofluorene (NOF) also was synthesized in our laboratory according to methods described by Pan and Fletcher (1958). Each of these chemicals was purified by recrystallization until a constant melting point was obtained. The final purity was >99% as assessed with HPLC. Hydroxylated AAF metabolite standards (Table 1) were acquired from the Chemical Repository of the National Cancer Institute. AAF and its chemically related derivatives are potentially hazardous substances and were handled in accordance with the guidelines set forth by OSHA. CP was obtained from Mead Johnson and Company (Evansville, Ind.); glucose 6-phosphate (G6P), G6P dehydrogenase (G6PDH), NADPH, and MC were purchased from Sigma Chemical Company (St. Louis, Mo.); 9-^{14}C-AAF (52 mCi/mmol, 98% purity) was purchased from New England Nuclear Corporation (Boston, Mass.) and was further purified by preparative HPLC with a Whatman Partisil ODS-2 Magnum (M9 10/25) column and eluting (Micromeritics HPLC, Model 7000) isocritically with methanol:water (80:20). Final purity was >99.5% as determined by analytical HPLC. PB was obtained as the pure powdered sodium salt from the University Hospital Pharmacy (Seattle, Wash.). All other chemicals utilized as reagents were of the highest purity commercially available.

Experimental animals. Virgin Sprague–Dawley (Wistar-derived) rats weighing 250 to 300 g were mated by and obtained from Tyler Laboratories, Bellevue, Washington. Mating was checked by the presence of the positive

TABLE 1

ELUTION OF AAF AND REFERENCE STANDARD AAF METABOLITES BY HPLC

Reference compound or metabolite	Retention time (min)	Fraction No.[a]
NOF	41.1	82
AF	37.6	75
Unknown A[b]	31.5	63
AAF	23.8	48
N-Hydroxy-AAF	20.6	41
1-Hydroxy-AAF	18.1	36
3-Hydroxy-AAF	16.0	32
AAF-9-one	12.7	25
5-Hydroxy-AAF	7.8	16
9-Hydroxy-AAF	6.7	13
7-Hydroxy-AAF	5.2	10
Unknown B[b]	3.5	7
Unknown C[b]	2.5	5
Solvent front	1.4	3

[a] Fraction in which the peak concentration appeared for each reference standard.

[b] These three metabolites appeared consistently in the metabolite profiles but did not exhibit retention times close to any of the reference standards.

sperm smears as well as by the presence of vaginal copulatory plugs on the morning after mating. The morning following overnight mating was designated as Day 0 of gestation. The pregnant animals were received on Day 3 of gestation and were housed 2 to 3 per cage in the animal facility of the Central Laboratory for Human Embryology. They were placed on crushed corncob bedding material (Sanicel), received Purina Rat Chow and water *ad libitum*, and were exposed to cycles of 14 hr light–10 hr dark each day. The pregnant rats were injected ip with MC, PB, or their respective vehicles as follows: MC was dissolved in corn oil and was injected as a single dose (40 mg/kg) on the morning of Day 8 of gestation. The same volume of corn oil was given to control animals at the same time. PB was dissolved in Hank's balanced salt solution (HBSS) and was injected (40 mg/kg) twice daily on Days 7, 8, and on the morning of Day 9 of gestation. The same volume of HBSS was given to control animals on the same treatment schedule. Induction of *P*-450 in the maternal liver by MC or PB was verified by monitoring absorbance changes in the CO-difference spectrum at 400 to 490 mm on a recording UV-VIS spectrophotometer (Beckman). PB pretreatment evoked the expected two- to threefold increases in total microsomal *P*-450 and MC-pretreatment resulted in the expected shift in absorption maximum from 450 to 448 nm. For experiments in which the hepatic postmitochondrial supernatant fractions (S-9) of adult, male Sprague–Dawley rats (Tyler Labs) were utilized as the enzyme source, the rats were treated with MC or PB with the same doses and treatment schedules as described above. Microsomal and S-9 fractions were prepared as previously described (Faustman-Watts *et al.*, 1983).

Explanation of embryos. At 0900 hr on the morning of Day 10 of gestation, maternal animals were killed by cervical dislocation and blood was collected from the abdominal aortas of animals not receiving MC or PB treatment. The pooled blood was centrifuged immediately and the red cells and fibrin clots were discarded. The serum was then heated at 56°C for 20 min. Uteri were removed to sterile petri dishes containing HBSS and the individual implanation sites were removed. Embryos were explanted by methods described by New (1978a,b) and Fantel *et al.* (1979) under a steroscopic dissecting microscope. Decidua, trophoblast remnants, parietal yolk sac, and Reichert's membrane were carefully removed, leaving the visceral yolk sac, ectoplacental cone region, and amnion intact. Embryos having 10 ± 2 somites were selected for culture. Other details of the culturing procedure were as described previously (Greenaway *et al.*, 1982). After 24 hr in culture, embryos were removed from the culture flasks and scored (without knowledge of treatment) for embryo viability, malformations, and measurements of embryonic length, somite numbers, and limb bud development. Embryos lacking yolk sac circulation and/or active heart beat were scored as nonviable. Protein (Bradford, 1976) and DNA (Labarca and Paigen, 1980) determinations also were performed on ultrasonically disrupted embryos. Representative embryos were photographed and fixed in glutaraldehyde (2.5% in 0.1 M sodium phosphate buffer) for histological examinations. They were then dehydrated, embedded in hydroxyethyl methacrylate, sectioned with glass knives at 2 to 3 μm with a microtome, and stained with toludine blue.

Analyses of embryonic AAF metabolism. The capacity of explanted embryos to catalyze the conversion of AAF to various metabolites was also assessed by homogenizing 50 to 60 Day 10 embryos (together with visceral yolk sac, ectoplacental cone region, and amnion) in 10 vol of potassium phosphate buffer (0.1 M, pH 7.4) and incubating the homogenate with AAF at 37°C for 2 hr in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2. Each incubation flask contained 1.9 mM NADPH, 1.01 μCi 9-^{14}C-AAF (42.4 μM, final concentration), 3.4 mM G6P, 2 units of G6PDH, approximately 2 mg protein from 15 embryos, and sufficient phosphate buffer to provide a total volume of 0.5 ml. Reactions were initiated by addition of labeled AAF and stopped by rapid addition of 1.5 ml of ice-cold, 1.0 M sodium acetate (pH 6.0). The mixture was then extracted four times with 10 vol of peroxide-free diethyl ether. Ring and *N*-hydroxylated AAF metabolites were extracted from the ether in 5 vol of 0.5 N NaOH, leaving all but traces of the substrate in the organic phase. An aliquot of the NaOH phase was

neutralized with HCl and subjected to scintillation counting. The remainder of the NaOH solution was then acidified and back-extracted four times with 10 vol of diethyl ether. The ether extracts were pooled and evaporated to dryness; the residue was redissolved in 20 μl of a 95% ethanol solution containing the metabolite standards (Table 1). Separation of the metabolites by HPLC was accomplished with modifications of the methods described by Smith and Thorgeirsson (1982) as follows: A 10-μl aliquot of the solution was injected onto a Zorbax C-8 column, utilizing a Beckman HPLC system. Initial composition of the mobile phase was 26:74 isopropyl alcohol:0.01 M acetic acid (v/v, pH 3.3). Each solvent contained 0.01% (w/v) deferoxamine mesylate (Desferal, Ciba). A constant flow rate of 1.2 ml/min was maintained throughout. The solvent ratio was changed linearly to 29:71 over the first 10-min period and then held at that composition for a period of 18 min. Over the next 10 min, the ratio was changed linearly to 54:46 and then held at that ratio for 7 min. Finally, a 100% concentration of isopropyl alcohol was achieved with a linear gradient over a 1-min period. Fractions were collected for 30 sec each and 90 fractions were obtained. Elution of standards (Table 1) was monitored by uv absorbance at 253 nm for each sample injected onto the HPLC column. Three milliliters of Aquasol (New England Nuclear) was added to each collected vial and the samples were counted in a Beckman, Model LS 8000 liquid scintillation counter for a time period sufficient to achieve less than a 2.5% error with 95% confidence intervals. Counting efficiency was 85%. Radioactivity remaining in the original aqueous incubation mixture (after the initial extractions with diethyl ether) was also quantified by liquid scintillation spectrometry.

Statistics. For parameters measurable on a continuous scale (protein and DNA concentrations, somite numbers, embryo length, and limb indices), Student's *t* test (Steele and Torrie, 1960) was used in ascertaining the statistical significance of differences between sample means. For quantal parameters (viability, malformations), ordered contingency tables were used to partition the overall χ^2 statistic to discern ordered trends and sources of variation (Everitt, 1977). The significance level chosen was $p < 0.05$. Numbers of embryos evaluated are given in tables.

RESULTS

Experiments designed to evaluate the capacity of cultured embryos to convert AAF to reactive metabolites that produce (in the same embryos) malformations detectable with the dissecting microscope yielded results that are presented in Table 2. Transplacental pretreatment of Day 8 embryos with MC resulted in a high percentage (60%) of malformed embryos following explanation on Day 10 of gestation and culturing for 24 hr in a medium containing AAF. Other measured parameters of growth and development also were affected significantly (Table 2). In contrast to results obtained with MC pretreatment, five injections of dams with phenobarbital on Days 7, 8, and 9 of gestation resulted in no statistically significant effects on explanted embryos cultured in the presence of added AAF. All attempts to evoke CP-induced malformations in cultured, CP-exposed embryos via transplacental pretreatment of embryos with either PB or MC (see Methods) were unsuccessful and the negative data are therefore not presented. A minimum of 14 embryos in each group was tested. All measured parameters of embryotoxicity were statistically indistinguishable from the corresponding controls.

The qualitative nature of the defects observed following the culturing of MC-preexposed embryos in AAF-containing media was indistinguishable from that observed in previous investigations in which an exogenous source of bioactivating enzymes from livers of MC-pretreated, adult male rats was added to the medium (Faustman-Watts *et al.*, 1983). Representative photographs of the malformed embryos are presented in Fig. 1. Approximately 90% of the malformed embryos exhibited abnormally open neural tubes. Those malformed embryos that did not display abnormally open neural tubes usually exhibited prosencephalic and/or mesencephalic hypoplasia. Approximately 29% of the malformed embryos also exhibited abnormal rotation of flexure. Histological examination of the exposed embryos also failed to distinguish the malformations observed in this investigation from those reported previously. All histological sections taken from malformed embryos displayed necrotic cells and debris in the neural tube region irrespective of the presence or absence of observable neural tube abnormalities. Necrotic cells were also present in the prosencephalic regions. Further

TABLE 2

EFFECTS OF TRANSPLACENTAL PREEXPOSURE OF EMBRYOS TO PHENOBARBITAL OR 3-METHYLCHOLANTHRENE ON SUBSEQUENT EMBRYOTOXIC EFFECTS OF 2-ACETYLAMINOFLUORENE IN AN EMBRYO CULTURE SYSTEM

Parameter	Transplacental pre-exposure[a]			
	Corn oil	3-Methylcholanthrene	Hank's solution	Phenobarbital
Embryos cultured	28	35	14	16
Viable embryos[b]	27	35	14	15
Malformed embryos[c]	2	21[d]	3	4
Mean somite number	20.0 ± 2.2	17.5 ± 2.4[e]	18.5 ± 1.9	18.1 ± 2.0
Mean protein/embryo (μg)	188 ± 51	128 ± 30[e]	176 ± 42	169 ± 47
Mean DNA/embryo (μg)	16.3 ± 2.1	13.6 ± 2.3[e]	15.4 ± 2.6	15.0 ± 3.1
Mean embryo length (mm)[f]	2.3 ± 0.3	2.1 ± 0.4	2.4 ± 0.2	2.3 ± 0.4
Mean limb index[g]	20.1 ± 1.8	19.5 ± 1.1	20.0 ± 1.6	19.3 ± 1.7

[a] Pregnant rats were treated with the indicated agents according to the dosage schedules described under Methods. Values in the table are $\bar{x} \pm$ SD.

[b] Presence of active yolk sac circulation and heartbeat were criteria for viability. Only viable embryos were further assessed.

[c] Approximately 90% of embryos malformed after preexposure to MC exhibited abnormally open neural tubes; 29% exhibited abnormalities of flexure rotation.

[d] Statistically different from vehicle control by the χ^2 criteria ($p < 0.05$).

[e] Statistically different from vehicle control by Student's t test ($p < 0.05$). Embryos from each treatment group were explanted into culture medium containing 282 μM AAF and 6.7 μl DMSO as vehicle. Embryos (13) exposed transplacentally to MC and cultured in the absence of AAF displayed no evidence of embryotoxicity.

[f] Measurements were made only on embryos not displaying flexure abnormalities. Maximal lengths were measured.

[g] Limb indices were measured as described by Hamburger and Hamilton (1951).

details, with photographs, were reported previously (Faustman-Watts *et al.*, 1983).

The relative capacities of various sources of exogenously added S-9 and cofactors to catalyze the conversion of CP or AAF to embryotoxic metabolites are presented in Table 3. The results showed that hepatic S-9 from adult male rats pretreated with MC were very effective in catalyzing the conversion of AAF (but not CP) to embryotoxic intermediates. By contrast, hepatic S-9 from adult male rats pretreated with PB were very effective in catalyzing the conversion of CP to embryotoxic metabolites, but were ineffective in catalyzing the conversion of AAF to malforming intermediates. Results obtained with S-9 fractions from vehicle-treated (corn oil or HBSS) adult male rats indicated that these preparations (at equivalent protein concentrations) were only minimally effective in converting either CP or AAF to embryotoxic metabolites in the embryo culture system utilized. The incidence of observable defects was not different statistically (χ^2 test) from that observed in control culture flasks.

Since the results presented in Table 2 suggested that the embryos themselves, and/or associated membrane structures, were bioactivating AAF in the embryo culture system, we performed investigations to determine whether embryonic biotransformation of AAF could be demonstrated more directly. Embryos, homogenized together with ectoplacental cones, visceral yolk sacs, and amnionic membranes, were incubated with AAF and cofactors for monooxygenation as described under Methods. The data (Table 4) show that cultured, Day 10 embryos whose mothers were treated with MC on Day 8 of gestation converted AAF to a variety of readily detectable, ether-extractable metabolites eluting on HPLC. Those embryos whose mothers were pre-exposed to PB, HBSS, or corn oil, however, converted only negligible

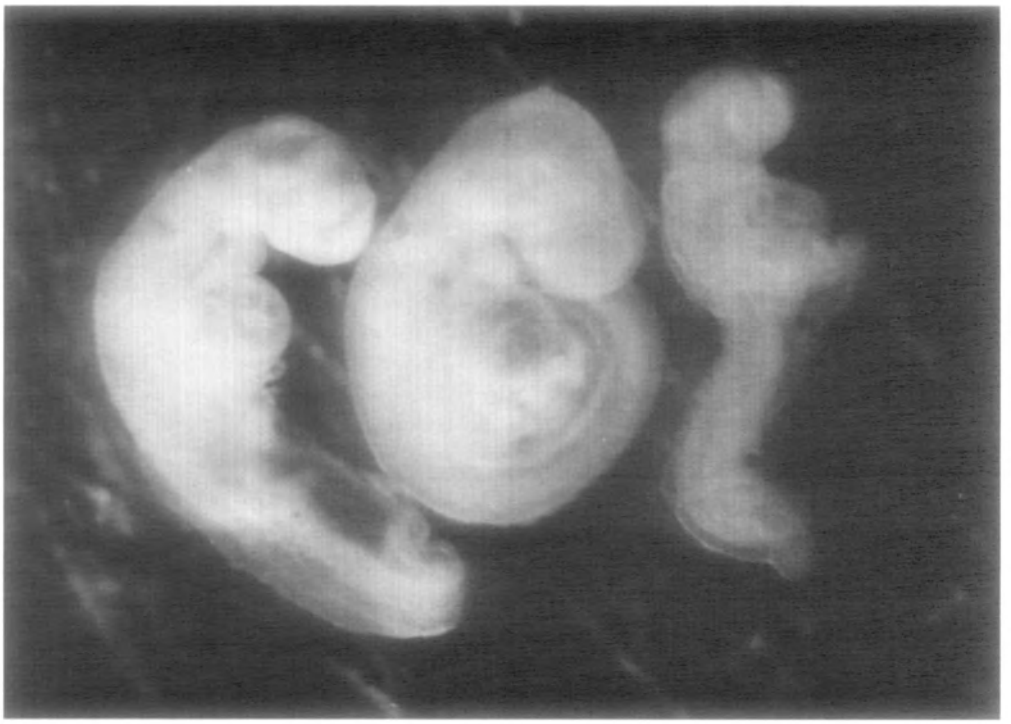

FIG. 1. Morphology of cultured rat embryos on Day 11. Day 10 embryos were cultured for 24 hr in medium containing AAF at an initial concentration of 282 μM. A control embryo which was preexposed to corn oil vehicle only (center) is contrasted with two embryos that were exposed transplacentally to a single dose of MC on Day 8 of gestation. Note abnormalities in the cephalic neural tube regions. Both MC-exposed embryos exhibited abnormally open neural tubes.

to barely detectable quantities of AAF to metabolites measurable by HPLC. A second set of experiments was run with very similar results obtained in each experiment.

DISCUSSION

In the field of xenobiotic biotransformation, a commonly held concept is that cytochrome *P*-450-dependent monooxygenation of foreign organic chemicals occurs at extremely low and often negligible rates during the early prenatal existence of common, small laboratory animals (e.g., rats, mice, rabbits, guinea pigs). Examples of reviews of developmental drug metabolism that foster this concept (Nau and Neubert, 1978; Leakey, 1983) have appeared recently. Accordingly, *P*-450-dependent bioactivation in rodents would be expected to occur minimally prenatally, particularly during the period of organogenesis during which the developing organism is most susceptible to the teratogenic effects of chemicals. Various studies (Filler and Lew, 1981; Legraverend *et al.*, 1984; Galloway *et al.*, 1980) have demonstrated that *P*-450-dependent biotransformation reactions can occur during early embryogenesis and prenatal life in rodents, but rates of such reactions appeared to be orders of magnitude less than those measured in hepatic preparations of mature animals. Such low rates lead to the expectation that the reactions would be of little if any biologic consequence. Nevertheless, studies in Nebert's laboratories (Shum *et al.*, 1979; Legraverend *et al.*, 1984) have demonstrated an apparent relationship between the capacity of mouse embryos to

TABLE 3

CAPACITY OF HEPATIC POSTMITOCHONDRIAL SUPERNATANT FRACTIONS FROM ADULT MALE SPRAGUE–DAWLEY RATS PRETREATED WITH PHENOBARBITAL OR 3-METHYLCHOLANTHRENE TO CATALYZE THE CONVERSION OF 2-ACETYLAMINOFLUORENE OR CYCLOPHOSPHAMIDE TO EMBRYOTOXIC METABOLITES DURING THE CULTURING OF WHOLE RAT EMBRYOS

	Percentage malformed[b]		
Treatment[a]	Cyclophosphamide[c]	2-Acetylaminofluorene[c]	No chemical
Corn oil	12 (17/18)	5 (19/20)	4 (23/25)
3-Methylcholanthrene	6 (17/18)	100[d] (17/20)	0 (18/20)
Hank's solution	6 (17/18)	7 (14/14)	7 (15/16)
Phenobarbital	100[d] (16/18)	21 (19/20)	11 (18/18)
No S-9	3 (37/39)	4 (53/56)	5 (73/76)

[a] Male Sprague–Dawley rats were given the indicated treatments according to the schedules described under methods.

[b] Malformations elicited by AAF included abnormally open neural tubes as the most common defect, abnormal flexure rotation, and prosencephalic defects. CP-elicited malformations were primarily hypoprosencephalia and hypomesencephalia. In all experiments, 40 μl of S-9 was added to the culture medium. Numbers in parentheses show the number of viable embryos/total embryos cultured.

[c] Final concentrations of CP and AAF were 12.5 and 63.0 μM, respectively.

[d] Statistically different from vehicle control by the χ^2 criterion ($p < 0.01$). S-9 from PB-pretreated animals elicited 100% malformations (CP-induced) when 40 μl was added to the medium. S-9 from MC-pretreated animals elicited 100% malformations (AAF-induced) when only 30 μl was added to the medium. CP-induced malformations have been described in detail by Greenaway *et al.* (1982). AAF-induced malformations have been described in detail by Faustman-Watts *et al.* (1983).

TABLE 4

QUANTITIES[a] OF AAF METABOLITES GENERATED IN INCUBATION FLASKS CONTAINING DAY 10 RAT EMBRYOS AS ENZYME SOURCE

Radioactivity eluting with	Treatment of pregnant rats[b]			
	Corn oil	3-Methylcholanthrene	Hank's solution	Phenobarbital
NOF	0.19	ND	0.23	0.15
AF	0.17	0.24	0.15	0.29
Unknown A	ND[c]	0.16	ND	0.21
N-Hydroxy-AAF	ND	0.34	ND	ND
1-Hydroxy-AAF	ND	0.84	ND	ND
3-Hydroxy-AAF	ND	14.91	ND	ND
AAF-9-one	ND	2.31	0.26	0.32
5-Hydroxy-AAF	ND	25.10	0.47	0.24
9-Hydroxy-AAF	0.42	2.11	0.33	0.29
7-Hydroxy-AAF	0.52	17.01	0.44	0.76
Unknown B	ND	0.83	ND	ND
Unknown C	ND	1.79	0.18	0.22

[a] Values in the table represent picomoles formed after a 2-hr incubation period, with a mean recovery of 75%. Protein concentrations in incubation vessels in these experiments were 1.8 to 2.2 mg/ml. A second set of experiments (with separate embryos) was performed in which the results were very similar.

[b] Pregnant rats were treated with the indicated agents according to the dosage schedule described under Methods.

[c] ND indicates that levels of radioactivity eluting with the respective standards could not be distinguished from radioactivity appearing when heat-inactivated (100°C, 10 min) tissue was utilized as enzyme source. Quantities less than 0.15 were regarded as nondetectable.

respond to inducers of *P*-450-dependent monooxygenases associated with the Ah locus and the incidence of malformations elicited by polynuclear aromatic hydrocarbons. This would suggest a role for the embryonic monooxygenation reaction(s) in the causality of the observed developmental defects. The results of the present investigation provide strong support for this concept. They suggest that for certain chemicals or classes of chemicals, e.g., AAF, the importance of the embryonic bioactivating reactions may far outweigh the importance of extraembryonic (e.g., maternal hepatic) bioactivation in the etiology of chemically induced malformations that occur *in utero*. By contrast, the results obtained with CP suggest that the importance of embryonic bioactivation is substrate-dependent and should not be generalized.

Although we cannot be totally positive that the transplacental effects of MC on AAF-elicited dysmorphogenesis were mediated via *P*-450-dependent bioactivation occurring in embryonic target tissues, the following observations would tend to support that viewpoint. (1) Previous studies (Faustman-Watts *et al.*, 1983) have shown that even very high concentrations of AAF elicited no embryonic defects in the absence of an active, *P*-450-dependent monooxygenase system. (2) In the presence of a bioactivating system added to the culture, AAF elicited morphologic abnormalities very similar to those observed during the presently reported studies when rates of endogenous, embryonic biotransformation of AAF were increased. Particularly striking was the relatively high incidence of abnormally open neural tubes observed in both cases. (3) Judged by the endpoint of dysmorphogenesis in cultured embryos, MC induced exogenous, adult hepatic as well as endogenous embryonic sources of AAF bioactivating enzymes. By contrast, PB enhanced only the exogenous adult hepatic enzyme source and affected primarily bioactivation of CP and not that of AAF. These data fit well with current knowledge concerning induced *P*-450-dependent monooxygenases and their corresponding substrate specificities.

Nevertheless it is likely that cytochrome *P*-450-independent reactions may participate in the bioactivation observed in these experiments. The importance of hydrolytic deacetylation in the conversion of AAF to mutagenic metabolites has been discussed frequently (Thorgeirsson *et al.*, 1982 and references therein). Because of embryotoxicity it was not possible to block the deacetylation reactions occurring in the embryo culture systems with sodium fluoride as it is commonly done in cell-free preparations. Thus, the role of deacetylation in elicitation of observed dysmorphogenesis in these experiments could not be evaluated.

It is also commonly believed that conjugation (particularly sulfation) of the N-hydroxylated (AAF) metabolite(s) plays a crucial role in the tumorigenicity of AAF (DeBaun *et al.*, 1970; King, 1974). Further, Boyd *et al.* (1983) have recently shown that arachidonate-dependent, cyclooxygenase-coupled, peroxidative reactions may be important in the bioactivation of deacetylated AAF. Whether some or all of these (or other) reactions are of importance in AAF-elicited dysmorphogenesis remains a question of interest.

The relative roles of target organ vs hepatic bioactivation are currently under intensive investigation in several laboratories. A recent study (Hix *et al.*, 1983) provides an example by showing evidence that the bladder urothelium might be more important than the liver in the bioconversion of various carcinogenic amines to reactive intermediates. In terms of the pathologic effects of bioactivable chemicals on extrahepatic tissues including embryos, the distinction between target cell vs hepatic bioactivation is important because of the differences among tissues in both complement and regulation of xenobiotic biotransforming enzymes. Also to be considered in connection with the results presented here are the enzymatic changes occurring during development and the regulation of such

changes during embryonic development. For example, recent preliminary studies (Giachelli *et al.*, 1984b) indicated that Day 10 rat embryos were very active in expressing mRNA sequences homologous to a cDNA probe for phenobarbital-inducible *P*-450. It may be possible that relatively large quantities of apocytochrome or otherwise inactive holocytochrome *P*-450 may be present in embryonic tissues and that they are regulated at the post-translational level. These aspects will require further investigation.

Previous studies also have demonstrated the capacity of human fetal and placental tissues to catalyze the bioactivation of various foreign components to mutagenic and covalently binding metabolites (Jones *et al.*, 1977; Juchau *et al.*, 1978). Activities were low, but, in view of the results presented here, it seems possible that even those low activities could be of considerable biologic/toxicologic consequence under certain conditions.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of Elizabeth Walker, Zamyat Kirby, Don Bark, and Hsueh-Ying Yang. Moses Namkung synthesized and purified 2-aminofluorene, 2-aminofluorene-9-one, 2-acetylaminofluorene, 2-acetylaminofluorene-9-one, and 2-nitrosofluorene and also performed the HPLC analyses. We also thank Cathy Manning for typing the manuscript.

REFERENCES

BOYD, J. A., HARVAN, D. J., AND ELING, T. E. (1983). The oxidation of 2-aminofluorene by prostaglandin endoperoxide synthetase: Comparison with other peroxidases. *J. Biol. Chem.* **258,** 8246–8254.

BRADFORD, M. M. (1976). A rapid, sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72,** 248–254.

DEBAUN, J. R., MILLER, E. C., AND MILLER, J. A. (1970). *N*-Hydroxy-2-acetylaminofluorene sulfotransferase: Its probable role in carcinogenesis and in protein-(methion-S-yl) binding in rat liver. *Cancer Res.* **30,** 577–583.

EVERITT, B. S. (1977). *The Analysis of Contingency Tables.* Chapman and Hall, London.

FANTEL, A. G., GREENAWAY, J. C., JUCHAU, M. R., AND SHEPARD, T. H. (1979). Teratogenic bioactivation of cyclophosphamide *in vitro*. *Life Sci.* **25,** 67–72.

FAUSTMAN-WATTS, E. M., GREENAWAY, J. C., NAMKUNG, M. J., FANTEL, A. G., AND JUCHAU, M. R. (1983). Teratogenicity *in vitro* of 2-acetylaminofluorene. *Teratology* **27,** 19–28.

FAUSTMAN-WATTS, E. M., YANG, H. L., NAMKUNG, M. J., GREENAWAY, J. C., FANTEL, A. G., AND JUCHAU, M. R. (1984). Mutagenic, cytotoxic and teratogenic effects of 2-acetylaminofluorene and reactive metabolites *in vitro*. *Teratogen. Carcinogen. Mutagen.* **4,** 273–283.

FILLER, R., AND LEW, K. J. (1981). Developmental onset of mixed-function oxidase activity in preimplantation mouse embryos. *Proc. Natl. Acad. Sci. USA* **78,** 6991–6995.

FLETCHER, T. L., AND NAMKUNG, M. J. (1958). Derivatives of fluorene IV. Raney nickel-hydrazine hydrate reduction of various mono- and dinitrofluorene derivatives; some new 9-substituted fluorenes. *J. Org. Chem.* **23,** 680–686.

GALLOWAY, S. M., PERRY, P. E., MENESES, J., NEBERT, D. W., AND PEDERSEN, R. A. (1980). Cultured mouse embryos metabolize benzo(α)pyrene during early gestation: Genetic differences detectable by sister chromatid exchange. *Proc. Natl. Acad. Sci. USA* **77,** 3524–3528.

GIACHELLI, C., FAUSTMAN-WATTS, E. M., AND JUCHAU, M. R. (1984a). An examination of 2-acetylaminofluorene (AAF)-induced dysmorphogenesis in rat embryos transplacentally exposed to 3-methylcholanthrene. *Toxicologist* **4,** 356.

GIACHELLI, C. M., JUCHAU, M. R., AND OMIECINSKI, C. J. (1984b). Expression of cytochrome *P*-450 and epoxide hydrolase messenger RNA in developing rat embryos. *Fed. Proc.* **43,** 342.

GREENAWAY, J. C., FANTEL, A. G., SHEPARD, T. H., AND JUCHAU, M. R. (1982). The *in vitro* teratogenicity of cyclophosphamide in rat embryos. *Teratology* **25,** 335–343.

HALES, B. F., AND JAIN, R. (1980). Effects of phenobarbital and β-naphthoflavone on the activation of cyclophosphamide to mutagenic metabolites *in vitro* by liver and kidney from male and female rats. *Biochem. Pharmacol.* **29,** 2031–2037.

HAMBURGER, V., AND HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88,** 49–92.

HIX, C., OGLESBY, L., MACNAIR, P., SIEG, M., AND LANGENBACH, R. (1983). Bovine bladder and liver cell and homogenate-mediated mutagenesis of *Salmonella typhimurium* with aromatic amines. *Carcinogenesis* **4,** 1401–1407.

HOSHINO, K., HAYASHI, Y., TAKEHIRA, Y., AND KAMEYANE, Y. (1981). Influences of genetic factors on the teratogenicity of environmental pollutants: Tera-

togenic susceptibility of benzo(α)pyrene and Ah locus in mice. *Congen. Anomalies* **21,** 97–103.

JONES, A. H., FANTEL, A. G., KOCAN, R. M., AND JUCHAU, M. R. (1977). Bioactivation of procarcinogens to mutagens in human fetal and placental tissues. *Life Sci.* **21,** 1831–1837.

JUCHAU, M. R., CHAO, S. T., AND OMIECINSKI, C. J. (1980). Drug metabolism by the human fetus. *Clin. Pharmacokinet.* **5,** 320–340.

JUCHAU, M. R., NAMKUNG, M. J., JONES, A. H., AND DIGIOVANNI, J. (1978). Biotransformation and bioactivation of 7,12-dimethylbenzo(α)anthracene in human fetal and placental tissues. *Drug Metab. Disp.* **6,** 273–281.

KING, C. M. (1974). Mechanism of reaction, tissue distribution and inhibition of arylhydroxamic acid acyltransferase. *Cancer Res.* **34,** 1503–1512.

LABARCA, C., AND PAIGEN, K. (1980). A simple, rapid and sensitive DNA assay procedure. *Analyt. Biochem.* **102,** 344–352.

LEAKEY, J. E. A. (1983). Ontogenesis. In *Biological Basis of Detoxication* (J. Caldwell, and W. B. Jacoby, eds.), Ch. 3, pp. 77–104. Academic Press, New York.

LEGRAVEREND, C., GUENTHER, T. M., AND NEBERT, D. W. (1984). Importance of the route of administration for genetic differences in benzo(α)pyrene-induced *in utero* toxicity and teratogenicity. *Teratology* **29,** 35–48.

MIRKES, P. E., FANTEL, A. G., GREENAWAY, J. C., AND SHEPARD, T. H. (1981). Teratogenicity of cyclophosphamide metabolites: Phosphoramide mustard, arcolein and 4-ketocyclophosphamide in rat embryos cultured *in vitro*. *Toxicol. Appl. Pharmacol.* **58,** 322–330.

NAU, H., AND NEUBERT, D. (1978). Development of drug-metabolizing monooxygenase systems in various mammalian species including man. In *Role of Pharmacokinetics in Prenatal and Perinatal Toxicology* (D. Neubert, H. J. Merker, H. Nau, and J. Langman, eds.), pp. 13–44, Georg Thieme, Stuttgart.

NEW, D. A. T. (1978a). Whole embryo culture and the study of mammalian embryos during organogenesis. *Biol. Rev.* **53,** 81–122.

NEW, D. A. T. (1978b). Whole embryo explants and transplants. in *Handbook of Teratology* (J. G. Wilson, and F. C. Fraser, eds.), Vol. 4, pp. 95–134. Plenum, New York.

PAN, H. L., AND FLETCHER, T. L. (1958). Derivatives of fluorene. V. 9-Hydroxyfluorenones; reduction of fluorenones in the presence of an aralkylidineamino group. *J. Org. Chem.* **23,** 799–807.

SHEPARD, T. H., FANTEL, A. G., MIRKES, P. E., GREENAWAY, J. C., FAUSTMAN-WATTS, F., CAMPBELL, M., AND JUCHAU, M. R. (1983). Teratology testing. I. Development and status of short-term prescreens. II. Biotransformation of teratogens as studied in whole embryc culture. In *Developmental Pharmacology* (S. M. MacLeod, A. B. Okey, and S. P. Spielberg, eds.), pp. 147–164, Raven Press, New York.

SHUM, S., JENSEN, N. M., AND NEBERT, D. W. (1979). The Ah locus: *In utero* toxicity and teratogenesis associated with genetic differences in benzo(α)pyrene metabolism. *Teratology* **20,** 365–376.

SMITH, C. L., AND THORGEIRSSON, S. S. (1982). An improved high-pressure liquid chromatographic assay for 2-acetylaminofluorene and eight of its metabolites. *Analyt. Biochem.* **113,** 62–66.

STEELE, R. G. D., AND TORRIE, J. H. (1960). Comparisons involving two sample means. In *Principles and Procedures of Statistics,* Ch. 5, pp. 67–87, McGraw–Hill, New York.

THORGEIRSSON, S. S., AND NEBERT, D. W. (1976). The Ah locus and the metabolism of chemical carcinogens and other foreign compounds. *Adv. Cancer Res.* **25,** 149–193.

THORGEIRSSON, S. S., WIRTH, P. J., STAINO, N., AND SMITH, C. L. (1982). Metabolic activation of 2-acetylaminofluorene. In *Biological Reactive Intermediates II-B* (R. Snyder, D. V. Parke, J. J. Kocsis, D. J. Jollow, C. G. Gibson, and C. M. Witmer, eds.), pp. 897–919. Plenum, New York.