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Generation of Reactive Dysmorphogenic Intermediates by Rat Embryos in Culture: Effects of Cytochrome *P*-450 Inducers[1]

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Generation of Reactive Dysmorphogenic Intermediates by Rat Embryos in Culture: Effects of Cytochrome *P*-450 Inducers. JUCHAU, M. R., BARK, D. H., SHEWEY, L. M., AND GREENAWAY, J. C. (1985). *Toxicol. Appl. Pharmacol.* **81**, 533–544. We have investigated the capacity of cultured whole rat embryos to convert 2-acetylaminofluorene (AAF) to reactive metabolites capable of eliciting dysmorphogenic effects in the same embryos. Cultured embryos (Sprague–Dawley) were exposed to AAF for periods of 2 or 24 hr, after which metabolites were isolated from the culture medium and identified with HPLC. Embryotoxic effects were evaluated in the same embryos. Day 10 embryos preexposed in utero to pregnenolone-16α-carbonitrile (PCN) exhibited marked increases in capacity to convert AAF to a variety of hydroxylated metabolites. 3-Methylcholanthrene (3MC) was also a very effective inducer in utero but Aroclor 1254 (PCB), and isosafrole (ISF) evoked only minimal induction while phenobarbital (PB) was not demonstrably effective. Exogenously added hepatic postmitochondrial supernatant (S9) fractions from adult male rats pretreated with PCB, 3MC, or ISF exhibited induced monooxygenase activities as well as increased capacity to convert AAF to dysmorphogenic intermediates in the culture system. PB and PCN displayed much lesser effects. PCN was a very effective inducer of hepatic monooxygenases of pregnant rats but, when this tissue was utilized as an enzyme source, no significant increase in malformations was observed. Embryos with relatively high monooxygenase activities also displayed a high incidence of embryonic abnormalities when cocultured with AAF. Malformation incidence was strongly correlated with hydroxy metabolite generation, suggesting that induction in utero of *P*-450-dependent, embryonic monooxygenases resulted in the production of embryotoxic metabolites by the embryos own enzymes. The data also indicated that endogenous bioactivation (within the conceptus) was considerably more effective than bioactivation effected by an exogenous (hepatic) enzyme source.

2-Acetylaminofluorene (AAF),[3] a highly potent model mutagenic/cytotoxic/carcinogenic aromatic amine, undergoes extensive cytochrome *P*-450-dependent biotransformation and bioactivation requisite to the elicitation of toxicologic sequelae. Previous reports from our laboratories have shown that AAF can also elicit significant dysmorphogenic effects in an embryo culture system but only when a *P*-450-dependent bioactivating system is cocultured with the explanted embryos (Faustman-Watts *et al.*, 1983). Subsequently, we have shown that various authentic metabolites of AAF produced malformed embryos in the absence of added enzymes but that the malformations produced differed qualitatively from those caused by AAF when S-9 fraction enzymes

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[3] Abbreviations used: AAF, 2-acetylaminofluorene; PCN, pregnenolone-16α-carbonitrile; 3MC, 3-methylcholanthrene; PCB, polychlorinated biphenyls; ISF, isosafrole; PB, phenobarbital; S9, hepatic 9000*g* supernatant fraction; HPLC, high-pressure liquid chromatography; G6P, glucose 6-phosphate.

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were added. Two deacetylated metabolites, *N*-hydroxy-aminofluorene and nitrosofluorene, produced abnormalities in flexure rotation as a principal defect (Faustman-Watts *et al.*, 1984a). *N*-Hydroxy-2-acetylaminofluorene and *N*-acetoxy-2-acetylaminofluorene elicited prosencephalic hypoplasia as the primary defect (Faustman-Watts *et al.*, 1984b). AAF, on the other hand, produced abnormally open neural tubes as the most common malformation. Recently, we showed that authentic 7-hydroxy-2-acetylaminofluorene produced abnormally open neural tubes when added to cultured whole embryos (Faustman-Watts *et al.*, 1985). No exogenous bioactivating system was required. On the basis of these results, we postulated that different sources of biotransforming enzymes could elicit qualitatively different abnormalities that might correspond with those produced by various tested AAF metabolites. As an approach to the investigation of the hypothesis, this study was designed to examine male and female rat hepatic enzyme sources after pretreatment of the animals with a number of prototypic inducing agents. In earlier experiments, we found (Giachelli *et al.*, 1984; Juchau *et al.*, 1985) that cultured embryos themselves appeared capable of converting AAF to reactive, dysmorphogenic intermediates. Therefore, an additional goal of this study was to examine further embryonic bioactivation and to compare tissues of the conceptus (endogenous) with bioactivation provided by hepatic S9 from adult rats (exogenous). A surprising and possibly very important finding reported here is that PCN was a highly effective inducer of AAF bioactivating enzymes in utero whereas, with the dosage regimens utilized, isosafrole (ISF) and PCB were not significantly effective in terms of endogenous embryotoxic bioactivation.

METHODS

Chemicals. NADPH, glucose 6-phosphate (G6P), and 3-methylcholanthrene (3MC) were purchased from Sigma Chemical Company (St. Louis, Mo.); isosafrole (ISF) was obtained from ICN Pharmaceuticals (Plainview, N.Y.); a mixture of polychlorinated biphenyls (Aroclor 1254, PCB) was obtained from Analabs Inc., (N. Haven, Conn.); phenobarbital was obtained as the pure powdered sodium salt from the University Hospital (Seattle, Wash.); [9-^{14}C]2-acetylaminofluorene (52 mCi/mmol, 98% purity) was purchased from New England Nuclear Corporation (Boston, Mass.) and was further purified by preparative HPLC using a Whatman Partisil ODS-2 Magnum (M9 10/25) column and eluting isocratically with methanol:water (80:20). Final purity was >99.5% as determined by analytical HPLC. 2-Aminofluorene and 2-aminofluorene-9-one were synthesized in our laboratory according to methods described by Fletcher and Namkung (1958) and by Pan and Fletcher (1958), respectively. AAF and AAF-9-one were subsequently synthesized by acetylation of 2-aminofluorene and 2-aminofluorene-9-one with acetic anhydride as earlier described (Fletcher and Namkung, 1958). These chemicals were purified by recrystallization until constant melting points were obtained. Final purification was by preparative HPLC on a Whatman Partisil 10, ODS-2 reverse phase column with a methanol:water linear gradient (80–100%, 30 min). Final purities were >99% as determined by analytical HPLC.

Experimental animals. Sprague–Dawley rats (Wistar-derived) were utilized in all experiments. Adult, male rats weighing 250 to 300 g were purchased from Tyler Laboratories (Bellevue, Wash.) and housed in the University Vivarium. Two to three animals were placed in single cages which contained hardwood shavings as bedding. The animals received Purina rat chow and water *ad libitum* and were exposed to cycles of 12-hr light–12-hr dark each day. They were injected ip with various inducers of *P*-450-dependent monooxygenases according to the following schedules: 3MC was dissolved in corn oil and administered as a single dose (40 mg/kg) 48 hr prior to termination; PB was dissolved in normal saline and administered once daily (80 mg/kg) for 4 days, the last dose 24 hr prior to termination; ISF was dissolved in corn oil and injected (150 mg/kg) once daily for 4 days, the last dose 24 hr prior to termination; PCN was dissolved in corn oil and administered (40 mg/kg) twice per day for 4 days and animals were killed 24 hr after the the last dose; PCB was dissolved in corn oil and given as a single injection (500 mg/kg) 5 days prior to termination; controls were given 0.2 ml of either corn oil twice daily or normal saline once daily for 4 days and killed 24 hr after the last injection. Animals were killed by cervical dislocation, and livers were surgically removed and homogenized in 2 vol of a 1.15% KCl solution in a glass homogenizer with a Teflon pestle (3 passes) at 4°C. The homogenate was centrifuged at 9000*g* for 20 min, and the supernatant (S9) fractions were utilized in various experiments as the exogenous enzyme source for catalysis of oxidative biotransformation of AAF in the embryo culture system. Pregnant rats mated by and obtained from the same vendor were housed in the animal facility of the Central Embryology Laboratory under identical conditions. Some pregnant rats were treated with inducing agents or vehicles on the same schedules as for males and were killed on Day 10 of gestation. S9 fractions were prepared as described for male rats and were likewise utilized for catalysis of AAF biotransformation in various experiments with the embryo culture system. Embryos taken from these and other (untreated) pregnant rats were cultured as described below.

Embryo culture system. The rat embryo explant culture system utilized in these investigations was developed by New (1973) and various modifications of the system as utilized in our laboratories have been described in detail (Fantel *et al.*, 1979, Greenaway *et al.*, 1982, Faustman-Watts *et al.*, 1983; Juchau *et al.*, 1985). The morning following mating of the rats was designated as Day 0 of gestation if copulation plugs were present. On Day 10 of gestation, uteri were removed under ether anesthesia at 0900 hr.

Prior to culturing, the medium was prewarmed and pregassed with 5% CO_2, 20% O_2, and 75% N_2 to maintain a pH of 7.3. After 20 hr of rotation at 37.5°C on a roller apparatus, the medium was regassed with 95% O_2 and 5% CO_2, and embryos were cultured for an additional 4 hr. At the end of the culture period, the embryos were removed from the culture flasks and examined under a dissecting microscope for abnormalities as well as for changes in indices of growth and development. Only those embryos with an active yolk sac circulation and heartbeat were defined as viable and examined further. Embryos were scored without knowledge of treatment for viability, malformations somite numbers, limb bud development, and embryonic length. Protein determinations were performed on ultrasonically disrupted embryos by the method of Bradford (1976). Other details of the embryo culture system and of methods of assessment of embryos have been published previously (Greenaway *et al.*, 1982; Faustman-Watts *et al.*, 1983).

HPLC Analyses of Metabolites. The generation of AAF metabolites within the embryo culture system was evaluated under several different conditions—the primary variable was the enzyme source. In some experiments, adult male or female rat hepatic S9 fractions were utilized as an exogenous enzyme source; 0.7 mg of S9 protein was added to the culture flasks. Additionally, 0.5 mM NADPH, 3.4 mM G6P, 11.4 μCi of [9-^{14}C]AAF, and a total concentration of 282 μM AAF were present as initial concentrations in a total of 15 ml of culture medium in those experiments. In other experiments, the cultured embryos served as the sole enzyme source. Eight embryos per bottle were explanted, no NADPH or G6P were added, concentrations of radioactive and nonradioactive AAF were the same as in the experiments in which S9 was added. For cases in which adult hepatic S9 served as exogenous enzyme source, reactions were initiated by addition of AAF and were allowed to continue for 2 or 24 hr at 37°C and pH 7.4 under an atmosphere of 75% N_2, 5% CO_2, and 20% O_2 (conditions for culturing of Day 10 embryos). Culture flasks were rotated constantly at 20 to 40 rpm. After 2 or 24 hr of incubation, embryos were removed, and the reactions were terminated by the addition of 1 vol of an ice-cold solution of sodium acetate (1 M) to the culture flasks and the mixtures were placed on ice. The pH was adjusted to 1.0 with 12 N HCl and metabolites were extracted from the culture medium 4 times with 25 vol each 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 parent AAF 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 each of diethyl ether. The ether extracts were pooled, evaporated to dryness, and 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 Åstrom *et al.* (1983) as follows: A 10-μl aliquot of the solution was injected onto a Nucleosil C-18 column (5 μm, 4.6 mm × 25 cm), utilizing a Beckman HPLC system. Initial composition of the mobile phase was 28:72 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 0.75 ml/min was maintained throughout. After 10 min, the solvent ratio was changed linearly to 38:62 over a 13 min time period and then held at that composition for a period of 9 min. Over the next 13 min, the ratio was changed linearly to 80:20 and then

TABLE 1

ELUTION OF 2-ACETYLAMINOFLUORENE AND REFERENCE STANDARD METABOLITES BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

Reference compound or metabolite	Retention time (min)	Fraction number[a]
Unk—3[b]	49.2	83
2-Acetylaminofluorene (AAF)	42.0	71
N-Hydroxy-AAF	38.1	64
1-Hydroxy-AAF	35.7	60
3-Hydroxy-AAF	32.8	56
9-Keto-AAF	28.4	48
Aminofluorene	25.9	44
5-Hydroxy-AAF	20.7	35
9-Hydroxy-AAF	17.3	30
7-Hydroxy-AAF	13.8	24
Unk—2[b]	9.5	17
Unk—1[b]	6.0	11
Solvent front	3.5	6

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

[b] These three metabolites appeared in many of the metabolic profiles but did not exhibit retention times close to those of any of the available reference standards.

held at that ratio for 10 min. Finally, a 100% concentration of isopropyl alcohol was achieved with a linear gradient over a 2-min period. Fractions were collected for 36 sec each and 100 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) were 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. Metabolite formation was calculated with the equation:

$$\mu\text{mol metabolite} = \frac{\text{total peak dpm} \times 4.23\ \mu\text{mol}}{2.5 \times 10^7\ \text{dpm} \times \text{fraction of dpm recovered}}.$$

Statistics. For parameters measurable on a continuous scale (protein concentrations, somite numbers, and embryo length), Student's *t* test (Steele and Torrie, 1960) was used to ascertain 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 $p < 0.05$. Correlation coefficients also were calculated according to procedures described by Steele and Torrie (1960).

RESULTS

Initial investigations were of the comparative capacities of hepatic S9 fractions from adult male rats pretreated with various inducing agents to catalyze the conversion of AAF to metabolic products when incubated within the embryo culture system. Quantitation of metabolites cochromatographing with authentic standards after incubation periods of 2 and 24 hr is given in Table 2. Three peaks of radioactivity did not cochromatograph with any of the eight reference standards and appeared on a regular basis in the metabolite profiles. These were designated as metabolites 1, 2, and 3 and are also presented in the table. Pretreatment of males with PCB resulted in S9 fractions with the highest monooxygenase activities and greatest quantities of generated hydroxylated metabolites. 3MC and ISF were also highly effective inducing agents, PB was somewhat effective, and PCN exhibited no significant inducing activity in male rats with AAF as substrate.

In general, S9 fractions from Day 10 pregnant rats treated with the same inducing agents displayed lower monooxygenase activities than those measured in their male counterparts (Table 3). By contrast, however, in the pregnant animals, PCN exhibited the greatest inducing activity of those inducers studied when assessed as a function of total hydroxylated metabolites generated within the first 2 hr of the culture period. Pretreatment with 3MC, PCB, and ISF each resulted in 9- to 11-fold increases in total hydroxylated products while increases produced by phenobarbital were less than 2-fold.

Evaluations of the embryotoxic effects caused by AAF and its metabolites in the same culture bottles in which metabolites were quantified (Tables 2 and 3) are given in Table 4. In general, S9 fractions generating the largest quantities of hydroxylated AAF metabolites were associated with culture flasks in which the greatest embryotoxicity also occurred. Only S9 fractions from ISF, MC, or PCB pretreated males elicited statistically significant increases in malformation incidence. In each case, the nature of the malformations elicited was very similar. Abnormally open neural tubes were commonly observed and occurred in 70 to 90% of all malformed embryos. Prosencephalic hypoplasia was also observed in 20 to 30% of the malformed embryos and abnormal flexure rotation in 15 to 25%. Detailed morphologic and histologic descriptions have been published previously (Faustman-Watts *et al.*, 1983, 1984a,b). Other measured parameters of embryotoxicity likewise appeared most severe in culture flasks in which the largest quantities of hydroxylated metabolites were generated. The incidence of embryolethality under the various culture conditions utilized could not be distinguished statistically. Hepatic S9 from 3MC- and PCN-pretreated pregnant rats caused AAF to elicit statistically significant reductions in embryonic length and

TABLE 2

GENERATION OF METABOLITES[a] OF 2-ACETYLAMINOFLUORENE IN EMBRYO CULTURES WITH ADDED HEPATIC ENZYMES FROM ADULT MALE RATS PRETREATED WITH VARIOUS INDUCING AGENTS

			Metabolite cochromatographing with										
Pretreatment[b]	N[c]	Incubation time (hr)	7-Hydroxy	9-Hydroxy	5-Hydroxy	9-Keto	3-Hydroxy	1-Hydroxy	N-Hydroxy	Aminofluorene	Unk-1[d]	Unk-2[d]	Unk-3[d]
Corn oil	5	2	7.9 ± 0.9	4.0 ± 0.9	0.5 ± 0.4	0.6 ± 0.3	0.7 ± 0.3	0.8 ± 0.4	0.2 ± 0.1	2.9 ± 1.4	0.5 ± 0.2	ND	ND
Corn oil	5	24	10 ± 1.7	4.2 ± 0.6	ND[e]	0.9 ± 0.2	1.4 ± 0.5	1.8 ± 0.3	0.4 ± 0.2	108 ± 42	ND	0.3 ± 0.1	ND
3MC	5	2	25 ± 5.3[f]	6.0 ± 0.9	16 ± 3.0[f]	0.8 ± 0.4	9.3 ± 1.7[f]	1.3 ± 0.6	1.9 ± 0.3[f]	0.8 ± 0.5[f]	2.1 ± 0.4[f]	1.1 ± 0.3[f]	0.1 ± 0.1
3MC	5	4	32 ± 4.5[f]	8.4 ± 0.7[f]	18 ± 1.3[f]	0.3 ± 0.2[f]	11 ± 1.2[f]	2.4 ± 0.7	2.1 ± 0.6[f]	66 ± 10	1.2 ± 0.9[f]	0.9 ± 0.6[f]	0.4 ± 0.3
PCB	5	2	64 ± 8.7[f]	18 ± 3.3[f]	47 ± 8.0[f]	0.7 ± 0.4	14 ± 2.4[f]	4.3 ± 0.5[f]	9.5 ± 2.2[f]	11 ± 1.8[f]	5.9 ± 1.6[f]	4.5 ± 1.1[f]	0.9 ± 0.7
PCB	5	24	75 ± 9.4[f]	16 ± 4.6[f]	49 ± 7.8[f]	0.2 ± 0.2[f]	10 ± 3.1[f]	3.9 ± 1.8[f]	8.2 ± 2.3[f]	76 ± 33	0.7 ± 0.6[f]	7.2 ± 2.2[f]	5.4 ± 4.1[f]
ISF	3	2	21 ± 4.3[f]	6.1 ± 0.8	11 ± 5.1[f]	0.5 ± 0.3	12 ± 3.3[f]	1.6 ± 0.4[f]	0.9 ± 0.3[f]	19 ± 9.2[f]	1.8 ± 0.7[f]	0.9 ± 0.5[f]	0.3 ± 0.2
ISF	3	24	26 ± 5.9[f]	9.2 ± 3.0[f]	15 ± 7.2[f]	0.3 ± 0.2[f]	12 ± 4.1[f]	1.9 ± 0.5	1.7 ± 0.7[f]	114 ± 29	3.3 ± 0.6[f]	1.3 ± 0.9[f]	0.8 ± 0.4
PCN	3	2	6.8 ± 1.7	2.9 ± 1.1	0.9 ± 0.5	0.7 ± 0.1	1.1 ± 0.5	0.6 ± 0.3	ND	7.0 ± 3.1[f]	ND	ND	0.3 ± 0.1
PCN	3	24	7.9 ± 3.4	5.1 ± 2.3	1.4 ± 0.5	0.8 ± 0.5	1.2 ± 0.4	0.9 ± 0.2[f]	0.4 ± 0.2	98 ± 19	0.3 ± 0.2	ND	0.5 ± 0.4
PB	4	2	19 ± 1.8[f]	9.1 ± 1.7[f]	0.7 ± 0.6	2.2 ± 0.6[f]	1.3 ± 0.3	1.4 ± 0.3	1.1 ± 0.1	1.8 ± 2.3	1.2 ± 0.5[f]	ND	ND
PB	4	24	19 ± 2.1[f]	10 ± 1.7[f]	10 ± 5.4[f]	3.7 ± 2.6[f]	1.7 ± 0.8	1.9 ± 0.6	0.4 ± 0.2	71 ± 27	ND	ND	ND

[a] Nanomoles generated (±SD) within the time period indicated. Culture vessels contained 2 mg of S9 protein, 1.9 mM NADPH, 3.4 mM G6P, and 11.4 μCi [9-^{14}C]AAF.

[b] Rats were pretreated according to schedules presented under Methods. Embryos were from untreated maternal rats.

[c] Number of experiments performed. Each experiment was performed wiith hepatic S-9 fractions pooled from 3 rats.

[d] Three peaks of radioactivity appeared on a relatively regular basis in metabolite profiles. These peaks were designated as Unks 1, 2, and 3. Retention times are in Table 1.

[e] ND indicates that amounts of radioactivity detected were not distinguishable from background amounts.

[f] Values differed statistically ($p < 0.05$) from those of the corresponding corn oil- or saline-treated controls.

TABLE 3

GENERATION OF METABOLITES[a] OF 2-ACETYLAMINOFLUORENE IN EMBRYO CULTURES WITH ADDED HEPATIC ENZYMES FROM DAY 10 PREGNANT RATS PRETREATED WITH VARIOUS INDUCING AGENTS

			Metabolite cochromatographing with										
Pretreatment[b]	N^c	Incubation time (hr)	7-Hydroxy	9-Hydroxy	5-Hydroxy	9-Keto	3-Hydroxy	1-Hydroxy	*N*-Hydroxy	Aminofluorene	Unk-1[d]	Unk-2[d]	Unk-3[d]
Corn oil	4	2	2.6 ± 1.0	0.5 ± 0.2	0.3 ± 0.1	ND[e]	ND	ND	ND	2.0 ± 1.1	ND	ND	ND
Corn oil	4	24	3.4 ± 1.1	0.9 ± 0.2	1.3 ± 0.6	ND	0.2 ± 0.1	ND	ND	46 ± 37	0.4 ± 0.2	ND	0.1 ± 0.1
3MC	3	2	17 ± 3.6[f]	2.9 ± 1.0[f]	5.0 ± 2.1[f]	0.2 ± 0.2	9.2 ± 3.1[f]	1.3 ± 0.7[f]	0.4 ± 0.2[f]	1.9 ± 1.1	0.2 ± 0.1	ND	0.1 ± 0.1
3MC	3	24	20 ± 4.3[f]	4.1 ± 1.3[f]	7.5 ± 3.4[f]	0.7 ± 0.5[f]	10.3 ± 3.9[f]	1.9 ± 0.9[f]	0.8 ± 0.4[f]	56 ± 20	0.6 ± 0.3	0.5 ± 0.2[f]	0.4 ± 0.2[f]
PCB	3	2	14 ± 3.6[f]	1.2 ± 0.4[f]	15 ± 4.8[f]	0.2 ± 0.1	1.2 ± 0.6[f]	0.3 ± 0.2	0.3 ± 0.1[f]	11 ± 5.6	0.4 ± 0.2[f]	0.2 ± 0.2	0.6 ± 0.2[f]
PCB	3	24	16 ± 5.8[f]	1.5 ± 0.3[f]	14 ± 4.7[f]	0.3 ± 0.2	1.0 ± 0.5[f]	0.3 ± 0.1[f]	0.4 ± 0.1[f]	23 ± 10	0.5 ± 0.2	0.6 ± 0.3[f]	0.5 ± 0.3[f]
ISF	3	2	13 ± 3.9[f]	0.9 ± 0.4	6.4 ± 2.1[f]	0.5 ± 0.3	8.9 ± 2.4[f]	1.0 ± 0.3[f]	1.2 ± 0.6[f]	14 ± 6.7	0.5 ± 0.3[f]	0.3 ± 0.2	0.3 ± 0.2
ISF	3	24	15 ± 4.8[f]	1.4 ± 0.7	6.0 ± 1.7[f]	0.8 ± 0.2[f]	6.4 ± 2.7[f]	1.6 ± 0.7	1.3 ± 0.5[f]	140 ± 51	0.6 ± 0.3[f]	0.6 ± 0.4[f]	0.5 ± 0.3[f]
PCN	3	2	24 ± 5.7[f]	2.1 ± 1.0[f]	13 ± 0.6[f]	0.3 ± 0.3	0.2 ± 0.0[f]	0.5 ± 0.2[f]	0.2 ± 0.2	13 ± 4.4	0.3 ± 0.2[f]	0.4 ± 0.3	0.4 ± 0.2[f]
PCN	3	24	21 ± 7.1[f]	2.4 ± 1.1[f]	17 ± 5.9[f]	0.5 ± 0.4	1.3 ± 0.8[f]	5.1 ± 1.9[f]	0.5 ± 0.3[f]	112 ± 39	0.5 ± 0.2[f]	0.6 ± 0.3[f]	0.5 ± 0.3[f]
PB	3	2	4.5 ± 2.0	0.4 ± 0.3	0.4 ± 0.2	ND	ND	0.4 ± 0.2	ND	7.1 ± 3.5	ND	ND	0.3 ± 0.2
PB	3	24	4.4 ± 1.3	0.7 ± 0.4	1.9 ± 0.3	ND	0.3 ± 0.2	0.6 ± 0.3	0.3 ± 0.2	145 ± 61	ND	0.3 ± 0.2	0.7 ± 0.4

[a] Nanomoles generated (±SD) within the time period indicated. Culture vessels contained 2 μg of S9 protein, 1.9 mM NADPH, 3.4 mM G6P, and 11.4 μCi [9-^{14}C]AAF.

[b] Rats were pretreated according to schedules presented under Methods section. Embryos were from untreated maternal rats.

[c] Number of experiments performed. Each experiment was performed with hepatic S-9 fractions pooled from 3 rats.

[d] Three peaks of radioactivity appeared on a relatively regular basis in metabolite profiles. These peaks were designated as Unks 1, 2, and 3. Retention times are in Table 1.

[e] ND indicates that amounts of radioactivity detected were not distinguishable from background amounts.

[f] Values differed statistically ($p < 0.05$) from those of the corresponding corn oil controls.

TABLE 4

EFFECTS OF VARIOUS P-450-DEPENDENT ENZYME SOURCES[a] ON THE CAPACITY OF 2-ACETYLAMINOFLUORENE TO ELICIT MALFORMATIONS IN CULTURED RAT EMBRYOS

Enzyme source	Pretreatment	N^b	Viability (%)	Malformations (%)	Protein content (μg/embryo)	Somite number	Length (mm)
Male liver	Corn oil	32	94	7.1	314 ± 47	22.6 ± 1.3	3.3 ± 0.3
Male liver	3MC	34	91	90[c]	231 ± 61[c]	19.7 ± 1.5[c]	2.9 ± 0.4[c]
Male liver	PCB	35	86	100[c]	217 ± 66[c]	18.4 ± 2.1[c]	2.8 ± 0.3[c]
Male liver	ISF	21	90	79[c]	258 ± 39[c]	20.2 ± 2.0[c]	3.0 ± 0.2[c]
Male liver	PCN	19	100	11	287 ± 44	21.9 ± 1.8	3.2 ± 0.3
Male liver	PB	20	95	21	294 ± 53	22.3 ± 1.6	3.3 ± 0.1
None	—	24	96	8.3	319 ± 52	22.5 ± 1.9	3.2 ± 0.2
Maternal liver	Corn oil	23	100	4.6	291 ± 67	23.0 ± 1.5	3.2 ± 0.3
Maternal liver	3MC	21	95	5.0	279 ± 52	22.1 ± 1.8	3.1 ± 0.2
Maternal liver	PCB	18	89	0	264 ± 71	21.3 ± 2.2	3.2 ± 0.3
Maternal liver	ISF	21	100	10	269 ± 43	21.9 ± 1.7	3.1 ± 0.2[c]
Maternal liver	PCN	20	95	16	255 ± 57[c]	21.2 ± 1.4[c]	2.9 ± 0.2[c]
Maternal liver	PB	23	100	8.7	283 ± 49	22.2 ± 1.6	3.2 ± 0.1

[a] Enzyme sources are the same as those utilized in Tables 2 and 3; embryos from the same culture flasks were evaluated. Culture flasks contained 2.0 mg of S-9 protein plus 1.9 mM NADPH and 3.4 mM G6P as initial concentrations.

[b] Total number of embryos explanted. Only embryos with active yolk sac circulation and heartbeat were scored as viable. Malformations and other parameters were assessed only in viable embryos.

[c] Statistically different ($p < 0.05$) from the corresponding control (corn oil) values. Viability and malformations were analyzed with the χ^2 test; proteins, somite numbers, and embryonic length with Student's t test.

the latter enzyme preparation was also associated with statistically significant decreases in embryonic protein content and somite numbers. The increased incidence of malformations, however, did not achieve statistical significance at the probability level chosen ($p < 0.05$).

The capacities of cultured embryos to generate measurable quantities of AAF metabolites are presented in Table 5. As indicated, quantities of metabolites generated by untreated or vehicle-pretreated embryos were virtually undetectable with the methods of analyses utilized. Pretreatment in utero with 3MC or PCN each resulted in marked increases in quantities of hydroxylated AAF metabolites present in culture medium after a 24-hr incubation period. (With the methods utilized, quantities could not be distinguished from background after a 2-hr period.) Pretreatment with PCB and ISF also resulted in increases in quantities of 7-hydroxy-AAF although the increases did not appear profound. Pretreatment with PB resulted in no apparent effect on embryonic capacity to biotransform AAF.

The embryotoxic effects produced by AAF in the same culture flasks in which the conceptuses served both as the targets for the embryotoxic effects of AAF as well as the enzyme source for AAF biotransformation (Table 5) are presented in Table 6. Again, the incidence of malformations and severity of other embryotoxic effects appeared to correlate with the quantities of hydroxylated metabolites detectable in the culture medium. In order to evaluate these relationships more carefully, a series of correlation coefficients was calculated and the data are presented in Table 7. As seen from the calculated coefficients, correlations between quantities of ring- or N-hydroxylated products and malformation incidence were highly significant statistically. The most striking correlations were between embryo-gener-

TABLE 5

GENERATION OF METABOLITES[a] OF 2-ACETYLAMINOFLUORENE BY CULTURED EMBRYOS PREEXPOSED[b] TO VARIOUS INDUCING AGENTS

Pretreatment[b]	N^c	Metabolite cochromatographing with										
		7-Hydroxy	9-Hydroxy	5-Hydroxy	9-Keto	3-Hydroxy	1-Hydroxy	N-Hydroxy	Amino-fluorene	Unk-1[d]	Unk-2[d]	Unk-3[d]
None	3	ND[e]	0.2 ± 0.1	ND	0.3 ± 0.2	ND	ND	ND	ND	ND	ND	ND
Corn oil	3	ND	0.1 ± 0.0	ND	0.2 ± 0.1	ND	ND	ND	0.3 ± 0.2	ND	ND	ND
Saline	1	ND	0.14	ND	0.17	ND	ND	ND	ND	ND	ND	ND
3MC	3	2.2 ± 1.1[f]	1.7 ± 0.5[f]	2.9 ± 0.9[f]	0.3 ± 0.1	0.5 ± 0.2[f]	0.2 ± 0.1[f]	0.4 ± 0.0[f]	0.2 ± 0.1	0.3 ± 0.2	0.2 ± 0.2	0.2 ± 0.1[f]
PCB	2	0.4 ± 0.1[f]	0.1 ± 0.1	ND	0.3 ± 0.2	0.1 ± 0.2	ND	0.1 ± 0.0[f]	ND	ND	ND	ND
ISF	2	0.4 ± 0.2[f]	0.1 ± 0.0[f]	ND	0.1 ± 0.1	ND	ND	ND	0.5 ± 0.2	ND	ND	ND
PCN	2	3.0 ± 0.5[f]	1.9 ± 0.4[f]	1.4 ± 0.7[f]	0.4 ± 0.2	0.6 ± 0.2[f]	0.2 ± 0.0[f]	0.3 ± 0.1[f]	0.7 ± 0.4	0.4 ± 0.1[f]	0.1 ± 0.0[f]	ND
PB	2	ND	0.1 ± 0.1	ND	0.2 ± 0.1	ND	ND	ND	ND	ND	ND	ND
No embryos	3	ND	ND	ND	0.3 ± 0.1	ND	ND	ND	ND	ND	ND	ND

[a] Nanomoles generated (±SD) during the 24-hr culture period. After 2 hr, amounts were indistinguishable from background. Culture flasks each contained 8 embryos from separate litters (randomized).

[b] Embryos were exposed to inducing agents prior to explanation by injecting pregnant rats according to schedules given under Methods.

[c] Number of experiments performed.

[d] Three peaks of radioactivity appeared on a relatively regular basis in metabolite profiles. These peaks were designated as Unks 1, 2, and 3. Retention times are in Table 1.

[e] ND indicates that amounts of radioactivity detected were not distinguishable from background amounts.

[f] Values differed statistically ($p < 0.05$) from those of the corresponding corn oil controls.

TABLE 6

EFFECTS OF 2-ACETYLAMINOFLUORENE ON CULTURED EMBRYOS[a] PREEXPOSED TO VARIOUS INDUCING AGENTS

Pretreatment	N[b]	Viability (%)	Malformations (%)	Protein content (μg/embryo)	Somite number	Length (mm)
None	24	96	8.7	306 ± 58	22.4 ± 1.7	3.2 ± 0.2
Corn oil	24	92	4.3	312 ± 63	22.9 ± 1.4	3.3 ± 0.3
Saline	8	100	13	304 ± 47	22.6 ± 1.7	3.4 ± 0.4
PCN	16	94	73[c]	231 ± 65[c]	20.4 ± 1.3[c]	3.0 ± 0.3
PCB	16	100	19	278 ± 74	21.1 ± 2.0	3.1 ± 0.4
3MC	24	87	68[c]	221 ± 81[c]	19.6 ± 2.3[c]	2.8 ± 0.3[c]
ISF	16	94	7.5	261 ± 49	20.9 ± 2.1	2.9 ± 0.3
PB	16	100	0	298 ± 32	21.7 ± 1.8	3.1 ± 0.1

[a] Embryos evaluated were those whose culture medium analysis is described in Table 5. NADPH and G6P were not added.

[b] Number of explanted embryos. Only viable embryos (footnote [b], Table 4) were further evaluated.

[c] See footnote *c*, Table 4.

ated hydroxylated metabolites and malformation incidence in the absence of an exogenous, hepatic biotransforming system. Similar correlations (coefficients not shown) were found for other parameters of embryotoxicity.

DISCUSSION

In the current work, we have established that induction in utero can stimulate the bioactivation of AAF to dysmorphogenic intermediary metabolites by enzymes present

TABLE 7

CORRELATIVE RELATIONSHIPS BETWEEN MALFORMATION INCIDENCES[a] AND APPEARANCE OF SPECIFIC METABOLITES OF 2-ACETYLAMINOFLUORENE[b] IN EMBRYO CULTURES[c]

	Enzyme source				
Metabolite	Hepatic S9 (males, $N = 6$)	Hepatic S9 (pregnant females, $N = 6$)	Embryos ($N = 8$)	Hepatic S9 (both sources, $N = 12$)	All sources ($N = 20$)
7-Hydroxy	0.77 (<0.1)	0.44 (>0.2)	0.92 (<0.01)	0.77 (<0.01)	0.63 (<0.01)
9-Hydroxy	0.60 (<0.2)	0.14 (>0.2)	0.95 (<0.001)	0.76 (<0.01)	0.69 (<0.001)
5-Hydroxy	0.81 (<0.05)	0.03 (>0.2)	0.95 (<0.001)	0.73 (<0.01)	0.66 (<0.01)
9-Keto	0.31 (>0.2)	0.37 (>0.2)	0.66 (<0.1)	0.23 (>0.2)	0.26 (>0.2)
3-Hydroxy	0.97 (<0.01)	−0.05 (>0.2)	0.95 (<0.001)	0.76 (<0.01)	0.65 (<0.01)
1-Hydroxy	0.70 (<0.1)	0.16 (>0.2)	0.96 (<0.001)	0.76 (<0.01)	0.65 (<0.01)
N-Hydroxy	0.69 (<0.2)	0.14 (>0.2)	0.99 (<0.001)	0.72 (<0.01)	0.66 (<0.01)
Aminofluorene	0.42 (>0.2)	0.37 (>0.2)	0.40 (>0.2)	0.26 (>0.2)	0.26 (>0.2)
Unk-1[d]	0.80 (<0.1)	0.08 (>0.2)	0.92 (<0.01)	0.85 (<0.001)	0.85 (<0.001)
Unk-2[d]	0.77 (<0.1)	0.57 (>0.2)	0.95 (<0.001)	0.79 (<0.01)	0.79 (<0.01)
Unk-3[d]	0.61 (<0.2)	−0.01 (>0.2)	0.75 (<0.1)	0.38 (>0.2)	0.38 (>0.2)

[a] Malformation incidences from Tables 4 and 6.

[b] Metabolite quantities from Tables 2, 3, and 5.

[c] Numbers in the table are calculated correlation coefficients. In parentheses are the statistical probabilities that regression slopes were equal to zero.

[d] See footnote [e], Table 2.

within the tissues of the conceptus itself and that these reactions occur to the extent that grossly manifested morphologic abnormalities can be elicited. The embryo culture system was selected for the investigations because it obviates the complications of maternal biotransformation and disposition. Other investigations, including some performed in our own laboratories, have provided evidence that prenatal tissues of commonly utilized experimental animals contained *P*-450-dependent biotransforming enzymes (Filler and Lew, 1981; Galloway *et al.,* 1980; Legraverend *et al.,* 1984; Juchau *et al.,* 1985). However, the extremely low activities observed raised doubts regarding their potential biologic significance. The investigations reported here, provide strong evidence that biotransformation of foreign organic chemicals in tissues of the conceptus can be of considerable biological/toxicological significance. They also show that several chemicals, including 3MC, PCB, ISF, and PCN, are effective inducers of the implicated embryonic bioactivating enzymes. Particularly interesting in this regard were the marked effects elicited by PCN, which, of the inducers examined, produced the largest increases in hydroxylated metabolites generated by cultured embryos as well as the highest incidence in AAF-elicited malformations in the same embryos following exposure in utero. Other research from this laboratory has shown (Namkung *et al.,* 1985) that PCN is a remarkably effective transplacental inducer of the fetal hepatic *P*-450-dependent monooxygenases that catalyze 2- and 4-hydroxylations of 17β-estradiol and 3-hydroxylation of benzo(α)pyrene. These observations suggest that PCN, and perhaps other steroids, may be highly effective for the induction of a broad spectrum of *P*-450-dependent monooxygenases peculiar to prenatal existence and point to the need for a detailed investigation of this possibility.

The initial hypothesis which served as the impetus for these studies was that enzymes added from different exogenous sources might generate different spectra of reactive AAF metabolites that would elicit qualitatively different malformations in the embryo culture system. This was not supported by our findings. The hypothesis was based on previously reported data (Faustman-Watts *et al.,* 1983, 1984a,b, 1985) which indicated that various directly added AAF metabolites could produce qualitatively different defects in the cultured rat embryos. Nitrosofluorene and 2-hydroxyaminofluorene each elicited abnormalities in axial rotation (flexure defects) as the predominant malformation, *N*-acetoxy-2-AAF and *N*-hydroxy-AAF were metabolites which produced prosencephalic hypoplasia as a characteristic abnormality, and 7-hydroxy-AAF produced a high incidence of abnormally open neural tube defects. The latter abnormality occurred frequently when AAF was incubated in the culture system with S9 fractions from 3MC-pretreated adult male rats (Faustman-Watts *et al.,* 1983). In the studies reported here, the qualitative nature of the malformations elicited by added AAF did not appear to depend on the enzyme source. Relative occurrence of abnormally open neural tubes, prosencephalic hypoplasia, flexure defects, or other less common abnormalities could not be distinguished among the various groups studied. Rather the source of enzyme appeared to affect the frequency of abnormalities in a quantitative manner; those enzyme sources most active in generating hydroxylated AAF metabolites were also responsible for the highest incidences of observed defects. A highly notable exception to this rule was observed when the embryos themselves served as the enzyme source for AAF biotransformation. Although quantities of metabolites generated by the embryos were very small, the malformation incidence was often considerable, particularly when embryonic enzymes were induced by PCN or 3MC. Surprisingly, PCB was a relatively ineffective inducer in utero although this may be explicable in terms of the schedule of administration. Embryos were exposed to PCB on Day 5 of gestation and it is not known whether embryonic enzymes respond well to *P*-450-dependent monooxygenase inducers at that stage of gestation. The studies of Filler and Lew (1981) would suggest that they do. Likewise, ISF and Pb did not display great effectiveness as inducers in utero but only one treatment schedule was employed. Whether effectiveness would increase with other injection schedules is unknown.

PCN is an effective inducer of aryl hydrocarbon hydroxylase in female (Wistar) rats but is only minimally effective in adult males (Gontovnick *et al.,* 1983). Thus, the results observed with PCN as an inducer in adult hepatic tissues were not unexpected. Although PCN was notably effective as an inducer of AAF biotransforming enzymes in the livers of 10-day pregnant females, use of S9 fractions from this source was not demonstrably effective in generating embryotoxic intermediates. The data did suggest marginal (but statistically insignificant) effects and it seems possible that greater added quantities could effect the appearance of significant malformations.

The importance of target tissue bioactivation of teratogenic chemicals is strongly evidenced in the data presented here. We cannot be absolutely certain that embryonic biotransformation of AAF was responsible for the observed defects. However, several lines of evidence support this supposition: (1) Several studies (Faustman-Watts *et al.,* 1983, 1984a,b, 1985) in our laboratory 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) When the bioactivating system was added, AAF caused morphologic abnormalities very similar in nature to those observed in the present studies when rates of embryonic AAF biotransformation were increased. It is particularly noteworthy that, in both instances a high frequency of abnormally open neural tubes was observed. (3) Very close correlations between quantities of hydroxylated metabolites generated by the embryos and the incidences of malformations in the same embryos were observed. The qualitative similarity of the malformations detected in PCN- and 3MC-preexposed embryos also is consistent with these ideas. These observations also suggest, but do not prove that the embryonic enzymes which catalyze the bioactivating reactions are cytochrome P-450-dependent. It is known that AAF undergoes a variety of P-450-independent bioactivation reactions and the possibility that various other of these could participate in embryonic bioactivation remains open for future studies.

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