Biochemical Pharmacology, Vol. 26, pp. 567–575. Pergamon Press, 1977. Printed in Great Britain.

INDUCTION OF HEPATIC MONO-OXYGENASE SYSTEMS IN FETAL AND NEONATAL RATS WITH PHENOBARBITAL, POLYCYCLIC HYDROCARBONS AND OTHER XENOBIOTICS*

THOMAS M. GUENTHNER† and GILBERT J. MANNERING
Department of Pharmacology, University of Minnesota Medical School, Minneapolis, MN 55455, U.S.A.

(*Received* 20 *May* 1976; *accepted* 27 *September* 1976)

Abstract—The induction of hepatic P-450 hemoprotein-dependent mono-oxygenase systems was studied in fetal and neonatal rats. The fetal liver was refractive to phenobarbital induction of aminopyrine and ethylmorphine *N*-demethylase, which are cytochrome P-450-dependent mono-oxygenases, but was not refractive to the 3-methylcholanthrene induction of benzo[a]pyrene hydroxylase, a cytochrome P_1-450-dependent mono-oxygenase. After parturition, all three enzyme activities were inducible. These and other observations suggest that a control mechanism operates in the fetal rat which selectively suppresses the induction of cytochrome P-450, but allows induction of cytochrome P_1-450. This selective suppression of phenobarbital induction in the fetus was reversed in part by the simultaneous administration of 3-methylcholanthrene. Several other inducing agents also partially reversed the suppression of phenobarbital induction in fetal livers: dibenz-[a,c]anthracene, 2-diethylaminoethyl-2,2-diphenylvalerate (SKF 525-A), and 3β-hydroxy-20-oxopregn-5-ene-16α-carbonitrile (PCN). Other inducing agents were inactive: α-naphthoflavone, β-naphthoflavone, 1,1-bis[*p*-chlorophenyl]2,2,2-trichloroethane (DDT), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), dieldrin, chlordane and chlorpromazine.

Hepatic microsomal mono-oxygenases, the group of enzyme systems primarily responsible for the biotransformation of most pharmacologic agents, as well as many endogenous substances, are deficient in the fetus of many mammalian species [1–9]. Immediately after parturition their levels increase rapidly and then gradually rise to adult levels within weeks or months [10–15]. The absence of activity in the fetus and the low levels of activity in the neonate have been attributed to various causes, including a lack of stimulation of the enzymes responsible for the synthesis of the systems [14] and inhibition by substances of fetal, maternal or placental origin [6, 16].

The absence of cytochrome P-450 mono-oxygenase systems in the fetal liver may be due either to the lack of endogenous inducing agents or to the inability of the mechanisms responsible for synthesis of the systems to respond to endogenous inducing agents. In the latter case, it might be expected that the liver would also fail to respond to exogenous inducing agents such as phenobarbital, which are known to induce cytochrome P-450-dependent mono-oxygenase systems in postpartum livers. In fact, various mono-oxygenase activities normally induced by phenobarbital and other xenobiotics in the livers of adult and neonate rats are not inducible in the fetus [2, 5]. Aryl hydrocarbon hydroxylase activity is inducible in fetal liver by polycyclic hydrocarbons [4, 8, 17–19], but not by phenobarbital [4]. Since phenobarbital induces cytochrome P-450‡ and polycyclic hydrocarbons induce cytochrome P_1-450 [20], these observations suggest that cytochrome P_1-450-dependent mono-oxygenase systems (e.g. benzo[a]pyrene hydroxylase) of the fetal liver are responsive to appropriate inducing agents, whereas cytochrome P-450-dependent mono-oxygenase systems (e.g. aminopyrine and ethylmorphine *N*-demethylases) are not.

The experiments to be reported are concerned with the development of fetal and neonatal hepatic mono-oxygenases and with their responses to phenobarbital, selected polycyclic hydrocarbons, and other inducing agents to the mother singly or in combination.

MATERIALS AND METHODS

Materials. Ethylmorphine HCl was purchased from Mallinckrodt Chemical Works. 3-Methylcholanthrene

* This research was supported by USPHS Grant GM 15477. Part of this material has appeared in abstract form [*Pharmacologist* **16**, 239 (1974)] and in a thesis submitted by T. M. G. in partial fulfillment of the requirements for the degree of Doctor of Philosophy to the Department of Pharmacology, University of Minnesota, 1975.

† U.S. Public Health Service Predoctoral Trainee (GM 01117). Present address: Section on Developmental Pharmacology, National Institute of Child Health and Human Development, NIH, Bethesda, MD 20014.

‡ Throughout this paper cytochrome P-450 is considered to be the P-450 hemoprotein found predominantly in livers of untreated or phenobarbital-treated rats; cytochrome P_1-450 (also known as cytochrome P-448) is considered to be the P-450 hemoprotein found predominantly in livers of rats treated with certain polycyclic hydrocarbons, including 3-methylcholanthrene. We recognize that cytochrome P-450 and cytochrome P_1-450 may describe classes of P-450 hemoproteins rather than specific cytochromes. P-450 hemoprotein designates any cytochrome which gives a spectrum with a maximum of about 450 nm when it is reduced and complexed with carbon monoxide; this includes both cytochrome P-450 and cytochrome P_1-450.

was obtained from Eastman Organic Chemicals. Benzo[a]pyrene, α-naphthoflavone, β-naphthoflavone, dibenz[a,c]anthracene and benz[a]anthracene were purchased from Aldrich Chemical Co. Aminopyrine came from K & K Labs., Inc. Sodium phenobarbital was obtained from Merck, Sharp & Dohme. γ-Chlordane was a gift from Velsicol Chemical Corp. 2,4,5-Trichlorophenoxyacetic acid (2,4,5-T) was a gift from Dow Chemical Co. Dieldrin was obtained from Shell Chemical Co. 3-β-Hydroxy-20-oxopregn-5-ene-16α-carbonitrile (PCN) was a gift from the Upjohn Co. SKF 525-A (2-diethylaminoethyl-2,2-diphenylvalerate HCl) and chlorpromazine HCl were supplied by Smith, Kline & French Laboratories. DDT (1,1-bis[*p*-chlorophenyl]-2,2,2-trichloroethane) was a gift from Geigy Chemical Co. Isocitric acid, isocitric dehydrogenase (type IV, partially purified), $NADP^+$, NADPH, NADH and cytochrome *c* (horse heart, type IV) were supplied by Sigma Chemical Co.

Animals. Pregnant Sprague–Dawley rats (200–300 g) were received from BioLab Co., St. Paul, MN, 1–4 days after being bred. Pregnancy was ascertained by vaginal inspection for sperm. Animals were bred overnight; the following day was considered day 1 of pregnancy. Animals were isolated 1 day prior to parturition; each was kept with its litter until the offspring were killed. Fetal livers were collected on days 13, 16 and 18–22 post conception. Neonatal livers were obtained on days 23–25. Control rats received 0.5 ml of 0.9% saline or 0.5 ml of corn oil intraperitoneally once daily for 4 days. Preliminary experiments showed equal amounts of monooxygenase activity in both control groups. The final injection was given approximately 15 hr before the animals were killed. Pregnant animals received intraperitoneal injections of 0.5 or 1 ml of corn oil or saline containing the appropriate drug once daily for 4 days. The following drugs were dissolved in saline and given in the indicated dosages (mg/kg): sodium phenobarbital, 40; SKF 525-A, 50; and chlorpromazine, 40. In one experiment, pregnant females received 80 mg/kg of sodium phenobarbital for 4 days in two daily doses of 40 mg/kg each. Other drugs were dissolved or suspended in corn oil and given in the following doses (mg/kg): 3-methylcholanthrene, 16; dieldrin, 25; chlordane, 25; DDT, 25; 2,4,5-T, 25; α- and β-naphthoflavone, 40; PCN, 40; benzo[a]pyrene, 20; and dibenz[a,c]anthracene, 20.

Liver preparation. Animals were stunned, decapitated and exsanguinated. Fetuses were removed and decapitated. The nonperfused fetal livers were removed, pooled by litter, and weighed. Livers from neonatal (1–3 days postpartum) animals were processed like fetal livers.

Whole fetal liver homogenates were prepared by suspending 80–120 mg of pooled fetal livers/ml of isotonic KCl–0.2 M phosphate buffer (pH 7.4), using a Potter–Elvehjem homogenizer fitted with a motor-driven Teflon pestle. P-450 hemoprotein levels, aminopyrine and ethylmorphine *N*-demethylase and benzo[a]pyrene hydroxylase activities, and phenobarbital tissue levels were determined using dilutions of these whole homogenates.

Microsomal preparations were used in determining NADPH cytochrome *c* reductase activities of fetal and neonatal livers. They were prepared as described previously for adult livers [21], except that two washes with 50 mM Tris buffer, pH 8.0 at 4°, were employed to remove excessive hemoglobin. All liver suspensions were prepared and used on the same day.

Determination of P-450 hemoproteins. The P-450 hemoprotein content of the whole liver homogenates from fetuses and neonates was determined using an Aminco DW2 spectrophotometer by the method of Schoene *et al.* [22], which eliminates spectral interference by contaminating hemoglobin. Accordingly, the previously described whole liver homogenates were diluted twenty times with 0.2 M PO_4 buffer, pH 7.4, saturated with CO, and placed in two cuvettes. After establishing a spectral baseline, the contents of the sample cuvette was reduced with dithionite and the characteristic cytochrome P-450 difference spectrum was recorded.

Enzyme assays. Benzo[a]pyrene hydroxylase activities of homogenates were determined by the method of Wattenberg and Leong [23].

Ethylmorphine and aminopyrine *N*-demethylase activities were measured by the method of Schoene *et al.* [22] with the following modifications: the incubation was conducted for 15 min in 0.5 ml of 0.1 M PO_4 buffer (pH 7.4) containing 12.5 mM semicarbazide. Amounts of other components were adjusted accordingly. Fetal or neonatal liver homogenate equivalent to 15–25 mg of liver served as the source of enzyme. Saturating concentrations of substrate were used. Enzyme activity was linear throughout the incubation period. Colorimetric determinations were made using microcuvettes in a Gilford spectrophotometer at 412 nm.

Cytochrome *c* reductase activity was measured by the method of Williams and Kamin [24]. Microsomal protein concentration in the cuvette was 0.1 mg/ml. The rate of reduction was calculated from the initial linear portion of the recorder tracing using an extinction coefficient of 2.11 $mM^{-1}\,cm^{-1}$ at 550 nm for reduced cytochrome *c*.

Assay of hepatic phenobarbital levels. Hepatic phenobarbital levels were measured by multiple ion mass fragmentography. Approximately 100 mg liver from fetuses whose mothers were given 40 or 80 mg sodium phenobarbital or 40 mg sodium phenobarbital plus 16 mg 3-methylcholanthrene/kg for 4 days was homogenized in 0.3 ml of 4.0 M NaH_2PO_4 buffer (pH 7.6). Hexobarbital was added at this time as an internal standard. The homogenate was extracted three times with 400 μl ethyl acetate. An aliquot of the combined extracts was injected along with an equal volume of commercial 0.2 M tetramethylanilinium hydroxide solution (Pierce Chemical Co.) onto a Finnegan model 6500 gas chromatograph mass spectrometer equipped with a programmable multiple ion monitor. A silonized OV-1 column was used at 190°. Injection temperature was 275°. Carrier gas flow was 6.5 cm^3/min. The detector was adjusted to monitor masses 232.3 (phenobarbital) and 235.3 (hexobarbital) simultaneously. Retention time for both peaks was approximately 7.5 min. Recovery of phenobarbital added to liver homogenates from non-pregnant female adults was 76 per cent.

Statistical analysis. Statistical analysis utilized one- and two-way analysis of variance programs written for the Control Data Cyber 70 model 72 computer.

Statistical differences ($P < 0.05$) between individual treatment groups were detected using Duncan's new multiple range test [25].

Protein determination. Protein contents of liver preparations were determined by the method of Lowry *et al.* [26] using bovine serum albumin as a standard.

RESULTS

Mono-oxygenase activity in fetal and neonatal livers. Homogenates from the livers of control fetuses did not metabolize aminopyrine (Fig. 1). *N*-demethylase activities appeared in 1-day-old neonates and increased rapidly during the next 2 days. Phenobarbital did not induce ethylmorphine or aminopyrine *N*-demethylase activities in prepartum livers.

3-Methylcholanthrene induced a small increase in aminopyrine metabolism in fetuses on the day of parturition, but did not elicit ethylmorphine demethylation (Fig. 2). Although phenobarbital and 3-methylcholanthrene showed no inductive activity in fetuses when given separately, in combination they produced an increase in aminopyrine metabolism as early as 2 days prepartum. Postpartum control livers showed increases in demethylase activities throughout the 3 postnatal days. Phenobarbital and the combination of phenobarbital with 3-methylcholanthrene-induced activities to levels above those obtained with controls, but 3-methylcholanthrene alone was without inductive effect.

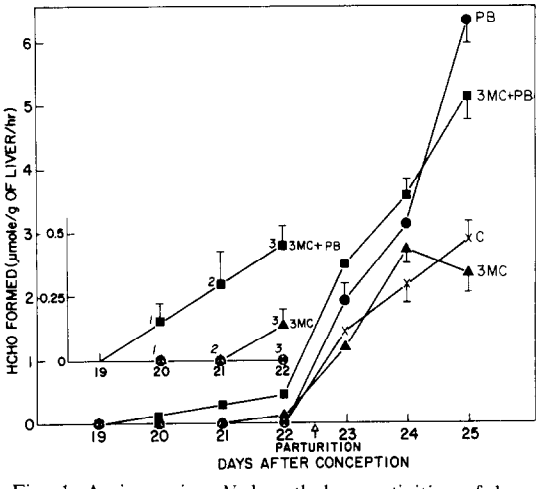

Fig. 1. Aminopyrine *N*-demethylase activities of homogenates of fetal and neonatal livers from pregnant rats given phenobarbital (PB), 3-methylcholanthrene (3MC) or both agents. Pregnant rats were injected daily for 4 days on days 9–21 after conception with NaPB (40 mg/kg), 3MC (16 mg/kg) or the same doses of NaPB + 3MC. Animals were killed 15 hr after the last injection, and fetal or neonatal homogenates of livers were assayed for aminopyrine *N*-demethylase activity on the days indicated. C = control (injected with saline only). Insert shows values obtained on days 18–22 on an expanded scale. Bars indicate S. E. Points labeled with the same numerals are significantly different at the $P < 0.05$ level. N for each point = 4. No *N*-demethylation of aminopyrine was observed with any of the liver homogenates obtained on days 13, 16, 18 and 19 after conception. Parturition occurred on day 22 after conception; all values shown for day 22 were obtained from fetal livers; all those for day 23 were obtained from neonatal livers.

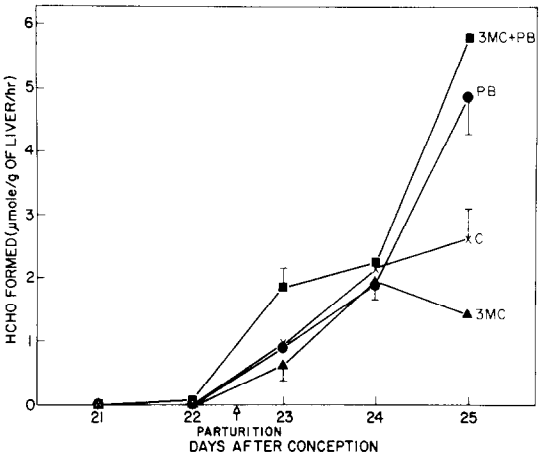

Fig. 2. Ethylmorphine *N*-demethylase activities of homogenates of fetal and neonatal livers from pregnant rats given phenobarbital (PB), 3-methylcholanthrene (3MC) or both agents. Pregnant rats were injected daily for 4 days on days 9–21 after conception with NaPB (40 mg/kg), 3MC (16 mg/kg) or the same doses of NaPB + 3MC. Animals were killed 15 hr after the last injection, and fetal or neonatal homogenates of livers were assayed for ethylmorphine *N*-demethylase activity on the days indicated. C = control (injected with saline only). Bars indicate S. E. Points labeled with the same numerals are significantly different at the $P < 0.05$ level. N for each point = 4. No *N*-demethylation of ethylmorphine was observed with any of the liver homogenates obtained on days 13, 16, 18 and 19 after conception. Parturition occurred on day 22 after conception; all values shown for day 22 were obtained from fetal livers; all those for day 23 were obtained from neonatal livers.

P-450 hemoprotein levels (Fig. 3) correlated well with aminopyrine *N*-demethylase activities in neonatal livers and in fetal livers that were 21 days old or younger, but there was no such correlation in 22-day-old fetal livers (compare Fig. 3 with Figs. 1 and 2). For example, the combination of phenobarbital and 3-methylcholanthrene treatments induced a level of P-450 hemoprotein at 22 days, which was almost half that observed at 25 days, but aminopyrine *N*-demethylase activity was only about one-tenth that observed at 25 days and no ethylmorphine *N*-demethylase activity was seen. The low levels of P-450 hemoprotein seen prepartum in control animals were not increased by treatment with phenobarbital. While still low, P-450 hemoprotein levels after 3-methylcholanthrene treatment were about double those seen in control livers. The simultaneous administration of phenobarbital and 3-methylcholanthrene produced levels of P-450 hemoprotein greater than the sum of the inductions produced by either agent alone. After birth, control livers exhibited a rapid increase in enzyme levels, and this was enhanced by treatment with either inducer. Thus, both metabolic activities and P-450 hemoprotein levels were induced by phenobarbital postnatally, but not prenatally.

The induction of mono-oxidases by polycyclic hydrocarbons results in a pattern of metabolic activity different from that produced by phenobarbital induction [20, 21]. Benzo[a]pyrene hydroxylase activity, a

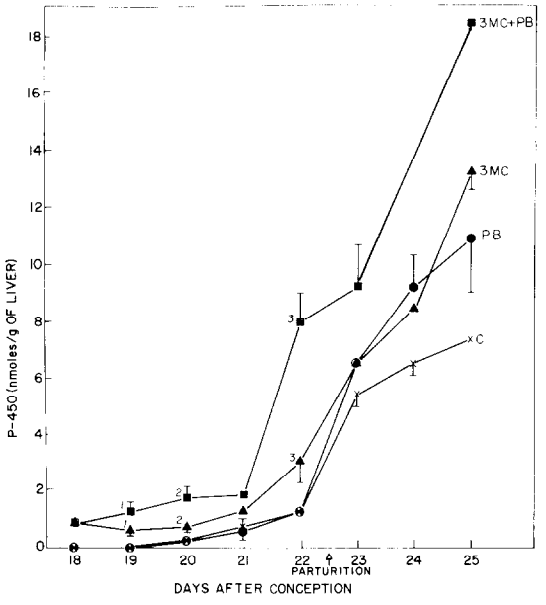

Fig. 3. P-450 hemoprotein contents of homogenates of fetal and neonatal livers from pregnant rats given phenobarbital (PB), 3-methylcholanthrene (3MC) or both agents. Pregnant rats were injected daily for 4 days on days 9–21 after conception with NaPB (40 mg/kg), 3MC (16 mg/kg) or the same doses of NaPB + 3MC. Animals were killed 15 hr after the last injection, and fetal or neonatal homogenates of livers were assayed for P-450 hemoprotein content on the days indicated. C = control (injected with saline only). Bars indicate S. E. Points labeled with the same numbers are significantly different at the $P < 0.05$ level. N for each point = 4. No P-450 hemoprotein was observed with any of the liver homogenates obtained on days 13–16 after conception. Parturition occurred on day 22 after conception; all values shown for day 22 were obtained from fetal livers; all those for day 23 were obtained from neonatal livers.

good indicator of induction by polycyclic hydrocarbons, was assayed to determine the response of fetal livers to 3-methylcholanthrene pretreatment (Fig. 4). Levels in prepartum control animals were very low. 3-Methylcholanthrene induced benzo[a]pyrene hydroxylase activity from day 19 until parturition, after which no further increase was seen. Phenobarbital was not effective as an inducer prepartum, but induced benzo[a]pyrene hydroxylase activity after parturition. The combination of inducers was also effective, but no more so than 3-methylcholanthrene alone. Activity of control livers rose rapidly during the first postnatal day, and then declined. This decline was largely prevented by phenobarbital administration. Effects of 3-methylcholanthrene plus phenobarbital in postpartum livers were about the same as those seen with 3-methylcholanthrene alone.

Activation of phenobarbital induction by other inducing agents. The data in Figs. 1 and 3 indicate that, when 3-methylcholanthrene is given to pregnant animals with phenobarbital, fetal livers respond to phenobarbital induction. This phenomenon cannot be explained as a simple additive effect of the second inducer, but seems to represent an alteration in the responsiveness of the fetal liver to induction by phenobarbital. Compounds known to induce adult liver mono-oxygenases were coadministered with phenobarbital to determine whether agents other than 3-methylcholanthrene might induce the inductive potency of phenobarbital in the fetal liver. None of the compounds listed in Table 1 promoted induction of aminopyrine demethylase activity by phenobarbital in the fetal liver. However, α- and β-naphthoflavone induced increases in P-450 hemoprotein levels. Since these two compounds induce benzo[a]pyrene hydroxylase activity in the adult [27], the hemoprotein induced here is probably cytochrome P_1-450, not cytochrome P-450, as would be expected if phenobar-

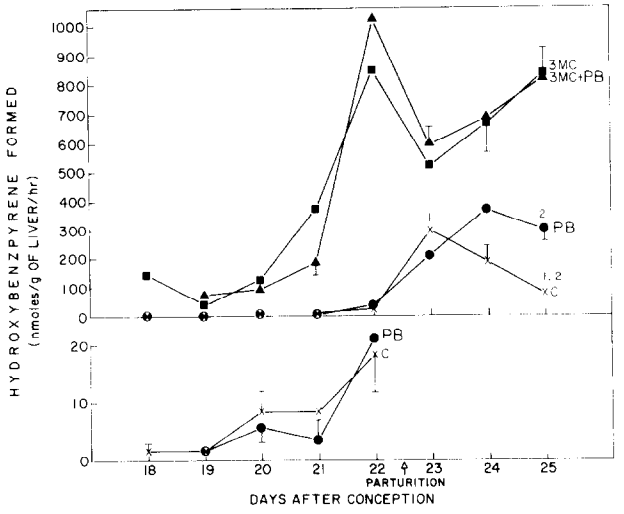

Fig. 4. Benzo[a]pyrene hydroxylase activities of homogenates of fetal and neonatal livers from pregnant rats given phenobarbital (PB), 3-methylcholanthrene (3MC) or both agents. Pregnant rats were injected daily for 4 days on days 13–21 after conception with NaPB (40 mg/kg), 3MC (16 mg/kg) or the same doses of NaPB and 3MC. Animals were killed 15 hr after the last injection, and fetal or neonatal homogenates of livers were assayed for benzo[a]pyrene hydroxylase activity on the days indicated. C = control (injected with saline only). The lower graph shows values from C and PB-treated animals on days 18–22 on an expanded scale. Bars indicate S. E. Points labeled with the same numerals are significantly different at the $P < 0.05$ level. N for each point = 4. Parturition occurred on day 22 after conception; all values shown for day 22 were obtained from fetal livers; all those for day 23 were obtained from neonatal livers.

Table 1. Effects of various agents on the response of fetal liver to induction by phenobarbital*

Treatment (mg/kg/day)	Aminopyrine *N*-demethylase activity (μmoles/g of liver/hr)	P-450 hemoprotein levels (nmoles/g liver)
Saline	<0.05†	0.94 ± 0.2
PB + corn oil	<0.05	0.98 ± 0.2
PB + dieldrin (25)	<0.05	0.95 ± 0.3
PB + chlordane (25)	<0.05	0.18 ± 0.4
PB + DDT (25)	<0.05	1.01 ± 0.3
PB + 2,4,5-T (25)	<0.05	1.08 ± 0.2
PB + chlorpromazine (40)	<0.05	1.11 ± 0.1
PB + α-naphthoflavone (40)	<0.05	1.25 ± 0.3
PB + β-naphthoflavone (40)	<0.05	2.54 ± 0.5
PB twice daily (total dose 80)	<0.05	1.09 ± 0.1

* NaPB (40 mg/kg) was given intraperitoneally to pregnant rats once daily for 4 days except in the one specified case where the same dose was given twice daily for 4 days. Numbers in parentheses are the dose of the agent given with phenobarbital (PB) in mg/kg/day (i.p.) for 4 days. Homogenates of fetal livers were assayed 15 hr after the last injection on day 21 of pregnancy.

† The lowest activity detectable was 0.05 μmole/g of liver/hr.

bital induction had been activated. No aminopyrine *N*-demethylase was induced after any of the treatments, although in adults, DDT, dieldrin, chlordane and chlorpromazine are known to induce mono-oxygenase activities similar to those induced by phenobarbital [28]. It is to be noted that an 80 mg/kg/day dose of phenobarbital did not induce demethylase activity, even though phenobarbital levels in the fetal livers are above those observed when the dose was 40 mg/kg/day (see Table 3).

Table 2 lists the compounds which, when given with phenobarbital, produced increases in cytochrome P-450 and aminopyrine *N*-demethylase activity above control levels. This group includes the steroid derivative, 3-β-hydroxy-20-oxopregn-5-ene-16α-carbonitrile (PCN), the alkylamine, SKF 525-A, and three polycyclic hydrocarbons. Benz[a]anthracene, a polycyclic hydrocarbon which is not an inducing agent [29], was included for comparison with the other polycyclic hydrocarbons, all of which are known to induce cytochrome P_1-450 [29, 30].

SKF 525-A and the three active polycyclic hydrocarbons caused increased cytochrome P-450 levels when given with phenobarbital. Concomitant increases in both P-450 hemoprotein levels and aminopyrine hydroxylase activity were interpreted to mean that phenobarbital-induced cytochrome P-450 was present. Aminopyrine demethylase activity was induced by PCN in the absense of phenobarbital.

Table 2. Effects of various agents on the response of fetal liver to induction by phenobarbital*

Treatment (mg/kg/day)	Aminopyrine *N*-demethylase activity (μmoles/g liver/hr)	P-450 hemoprotein levels (nmoles/g liver)
None	<0.05†	0.94 ± 0.2
PB	<0.05	0.98 ± 0.2
PB + SKF 525-A (50)	0.18 ± 0.1	1.80 ± 0.5
SKF 525-A (50)	<0.05	1.35 ± 0.4
PB + PCN (40)	0.83 ± 0.3	2.56 ± 0.6
PCN (40)	0.78 ± 0.3	1.93 ± 0.5
PB + benz[a]anthracene (20)	<0.05	2.50 ± 0.5
Benz[a]anthracene (20)	<0.05	1.99 ± 0.6
PB + benzo[a]pyrene (20)	0.12 ± 0.05	2.34 ± 0.7
Benzo[a]pyrene (20)	<0.05	1.77 ± 0.5
PB + 3-methylcholanthrene (16)	0.29 ± 0.1	2.40 ± 0.3
3-Methylcholanthrene (16)	<0.05	1.68 ± 0.3
PB + dibenz[a,c]anthracene (20)	0.35 ± 0.2	2.64 ± 0.5
Dibenz[a,c]anthracene (20)	<0.05	1.86 ± 0.6

* NaPB (40 mg/kg) was given intraperitoneally to pregnant rats once daily for 4 days. Numbers in parentheses are the dose of the agent given with phenobarbital (PB) in mg/kg/day (i.p.) for 4 days. Homogenates of fetal livers were assayed 15 hr after the last injection on day 21 of pregnancy.

† The lowest activity detectable was 0.05 μmole/g of liver/hr.

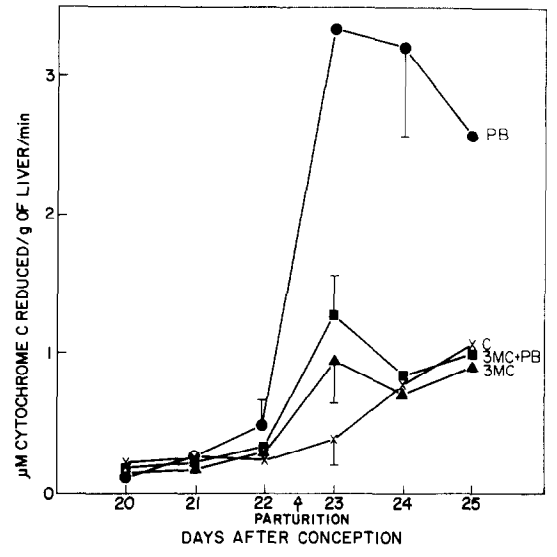

Fig. 5. NADPH-cytochrome *c* reductase activity of microsomes from fetal and neonatal livers from pregnant rats given phenobarbital (PB), 3-methyl-cholanthrene (3MC) or both agents. Pregnant rats were injected for 4 days on days 15–21 after conception with NaPB (40 mg/kg), 3MC (16 mg/kg) or the same doses of NaPB + 3MC. Animals were killed 15 hr after the last injection, and fetal or neonatal homogenates of livers were assayed for NADPH–cytochrome *c* reductase activity on the days indicated. C = control (injected with saline only). Bars indicate S. E. N for each point = 3. Parturition occurred on day 22 after conception; all values shown for day 22 were obtained from fetal livers; all those for day 23 were obtained from neonatal livers. Values are expressed in activity/g of liver equivalent to the mg of microsomal protein obtained from 1 g of fresh liver.

Phenobarbital induction was also activated by PCN, as was evidenced by the higher levels of P-450 hemoprotein and demethylase activity reached when PCN was given with phenobarbital.

NADPH-cytochrome c *reductase activities of fetal and neonatal livers.* Microsomal NADPH-cytochrome *c* reductase transfers electrons from NADPH to P-450 hemoprotein. The possibility was considered that the inability of fetal microsomes to metabolize drugs could be due to a deficiency of this reductase. NADPH-cytochrome *c* reductase was not induced in fetal livers by phenobarbital, 3-methylcholanthrene or their combination (Fig. 5). In accordance with expectations derived from studies with adult rats [31], phenobarbital induced activity in neonates whereas 3-methylcholanthrene produced no increase in activity above the moderate increase seen in controls after parturition. Unexpectedly, 3-methylcholanthrene suppressed phenobarbital induction of NADPH-cytochrome *c* reductase in neonates.

A comparison of Fig. 5 with Figs. 1 and 4 indicates that the comparatively low level of NADPH-cytochrome *c* reductase activity in fetal liver does not account for the deficiency of mono-oxygenase activity in this tissue. Reductase activities in 21-day fetuses are about equal in control and 3-methylcholanthrene and phenobarbital groups (Fig. 5), yet aminopyrine metabolism occurred in the latter group, but not in the former.

Levels of phenobarbital in fetal livers. The possibility was considered that the inability of phenobarbital to induce fetal livers might be attributable to failure of the fetal liver to accumulate phenobarbital in concentrations required for induction. Furthermore, the inductive potency of phenobarbital seen in the presence of methylcholanthrene could be due to a 3-methylcholanthrene-promoted increase in the uptake of phenobarbital by the fetal liver. To investigate these possibilities, 16-day pregnant rats were given one or two 40 mg/kg doses of sodium phenobarbital/day for 4 days. Fetal livers were removed on day 21, 15 hr after the last injection and analyzed for their phenobarbital content. Results summarized in Table 3 permit the following conclusions: (a) the absence of induction in fetal livers cannot be attributed to a failure of these livers to accumulate phenobarbital; phenobarbital levels in these livers were as high as those observed in adult livers where induction occurred[32]; (b) increasing the daily phenobarbital dose to 80 mg/kg raises phenobarbital levels in the fetal liver, but does not result in induction of cytochrome P-450; and (c) the administration of 3-methylcholanthrene does not increase the accumulation of phenobarbital in fetal livers.

DISCUSSION

Several studies of the development of the drug-metabolizing systems in fetuses and neonates have been published [2–6, 14]. The current study deals with the inductive effects of exogenous substances on the development of these systems in the livers of fetuses and neonates, particularly with reference to the role of P-450 hemoproteins. Our results indicate that the development of the mono-oxygenase systems is regulated by the development of the P-450 hemoprotein

Table 3. Correlation of hepatic phenobarbital level with aminopyrine *N*-demethylase activity and P-450 hemoprotein level in fetal liver*

Treatment (mg/kg/day)	Hepatic PB level (μg PB/g liver)	Aminopyrine *N*-demethylase activity (μmoles/g liver/hr)	P-450 hemoprotein level (nmoles/g liver)
PB (40)	85 ± 26	<0.05†	0.93 ± 0.19
PB (40)	149 ± 24	<0.05	1.09 ± 0.09
PB (40) + 3MC (16)	80 ± 13	0.29 ± 0.14	2.40 ± 0.3

* Sixteen-day pregnant rats were given phenobarbital (PB), 3-methylcholanthrene (3MC) or both agents daily for 4 days (the 80 mg dose of NaPB was divided into two 40 mg doses/day). Fetal livers were assayed on day 21 post conception. Values are ± S. E. of three experiments.

† The lowest activity detectable was 0.05 μmole/g of liver/hr.

components of those systems. The different responses of the fetal liver to the induction of cytochromes P-450 and P_1-450 reflects one aspect of this regulation.

Two mono-oxygenase systems may exist in the liver. One employs cytochrome P-450 as its terminal oxidase, the other, cytochrome P_1-450. Agents which induce hepatic mono-oxygenase systems can be grouped into two classes, those like phenobarbital, which induce the cytochrome P-450-dependent systems, and those like 3-methylcholanthrene, which induce the cytochrome P_1-450-dependent systems (reviewed by Mannering [33]). Zampaglione and Mannering [34] proposed that the P-450 systems, which are found primarily in the livers of untreated animals, are caused to appear as a result of endogenous factors, but may also be increased by exogenous factors such as phenobarbital, whereas the P_1-450 systems, which are found in portals of entry such as the lung and intestinal mucosa, as well as the liver, appear only when induced by a limited number of exogenous factors, including certain polycyclic hydrocarbons and some naturally occurring plant substances identified by Wattenberg *et al.* [32]. In accordance with this concept, the ability of fetal livers to respond to the inductive effects of 3-methylcholanthrene, but not to those of phenobarbital, suggests that the mechanism responsible for the synthesis of cytochrome P_1-450 in the fetal liver is intact, but that for the synthesis of cytochrome P-450 is not. Thus, cytochrome P-450 systems, as represented by aminopyrine and ethylmorphine *N*-demethylase activities, were not present in the fetal liver and administration of phenobarbital to the mother did not cause them to appear. This failure of the fetal liver to respond to the inductive effects of phenobarbital cannot be attributed to failure of phenobarbital to reach these livers; phenobarbital readily crosses the placenta [35], and in fact accumulates in the fetal liver to almost the same concentration observed in the adult liver (see Table 3 and Ref. 36).

Both mono-oxygenase systems are found after parturition and both respond to inducing agents. Thus the suppression of the fetal P-450 hemoprotein systems that occurred *in utero* is lost when the fetus is removed from the maternal environment. The gradual increase in the hepatic mono-oxygenase systems that occurs as neonates grow older might be viewed as a gradual removal of the suppression that occurred *in utero*. Alternatively, the gradual increase may reflect the response to increased amounts of either endogenous or exogenous inducing agents or both.

Suppression of the fetal mono-oxygenase systems appears to be directed at the processes which control the synthesis of cytochrome P-450. NADPH-cytochrome *c* reductase, the other major component of the mono-oxygenase systems, is present in sufficient quantity not to be rate limiting. This is evidenced by the observation that aminopyrine demethylase activity appears only after parturition, yet the reductase is present in about equal concentration before and after parturition. The advent of demethylase activity is concomitant with the appearance of cytochrome P-450. It is of some interest that δ-ALA synthetase is rate limiting in the synthesis of heme in the adult [37], but not in the fetus [38]. Suppression of cytochrome P-450 synthesis may conceivably be related to the rate-limiting step in heme synthesis, whatever that may be in the fetus. After parturition, a new rate limitation of heme synthesis may be established at the δ-ALA synthetase step, thereby relieving the suppression of P-450 hemoprotein synthesis.

The suppression of phenobarbital induction of cytochrome P-450 and aminopyrine *N*-demethylase activity was partially overcome by the coadministration of 3-methylcholanthrene or other agents listed in Table 2. This might be interpreted to mean that the potential for phenobarbital induction exists in the fetal liver, but that it cannot be expressed unless the mechanism of suppression is confounded by conditions created by the administration of 3-methylcholanthrene or these other inducing agents, which in themselves are not capable of inducing the cytochrome P-450-dependent systems in the fetus. It is to be emphasized that the coadministration of these agents with phenobarbital produces effects that are greater than the sum of the effects produced by either agent alone. The following polycyclic hydrocarbons are ranked in decreasing order of their ability to promote phenobarbital induction in fetal livers: dibenz[a,c]anthracene, 3-methylcholanthrene, benzo[a]pyrene and benz[a]anthracene. These compounds have been ranked in the same order for their ability to induce 3-methyl-4-dimethyl-aminoazobenzene *N*-demethylase, a cytochrome P_1-450-dependent system [29, 30].

Ethylmorphine *N*-demethylase is considered to be a cytochrome P-450-dependent, not a cytochrome P_1-450-dependent, mono-oxygenase system, yet it was not induced in fetal livers when a combination of 3-methylcholanthrene and phenobarbital was given. This would suggest that more than one cytochrome P-450 mono-oxygenase system may exist and that not all of these systems are responsive to the reversal of the suppression of phenobarbital induction by 3-methylcholanthrene.

The compounds which failed to show a 3-methylcholanthrene-like effect of the reversal of the suppression of phenobarbital induction in fetal livers (see Table 1) are mostly phenobarbital-like inducers in adult animals; as expected, they failed to induce mono-oxygenase activity in the fetus when given without 3-methylcholanthrene. α- and β-Naphthoflavone are cytochrome P_1-450 inducers in the adult [27], but they did not promote induction of aminopyrine *N*-demethylase activity in the fetus. PCN induced demethylase activity in the absense of phenobarbital, but did not activate phenobarbital induction of aminopyrine *N*-demethylase activity. SKF 525-A, which is both a potent inhibitor and an inducer of the hepatic mono-oxygenase system [39], was a weak activator of phenobarbital induction. Further evidence of phenobarbital activation by the compounds listed in Table 2 was offered by the observation that P-450 hemoprotein levels were always greater when phenobarbital was coadministered.

Some of the agents which activated phenobarbital in the fetal liver are structurally disparate. In an attempt to correlate certain properties of these agents with efficacy of activation of phenobarbital induction, the following observations are made.

First, all of the phenobarbital-activating agents are

known inducers of microsomal drug metabolism. This suggests that the site of activation is at or near the phenobarbital-sensitive site.

Second, the potency of the inducer in the adult does not necessarily reflect its potency as an activator of induction in the fetus. The polycyclic hydrocarbons employed in this study exhibited phenobarbital-activating potency commensurate with their effectiveness as inducers of cytochrome P_1-450-dependent metabolism in the adult, but α- and β-naphthoflavone exhibited no activation of phenobarbital induction although they are also potent inducers of cytochrome P_1-450-dependent metabolism in adults [27, 28].

Third, the lipophilicity of the compounds bears little relationship to efficacy. The list of active agents includes several polycyclic hydrocarbons with similar lipid solubilities, but widely different efficacies. PCN is quite active, yet more polar than several of the inactive compounds. SKF 525-A, an even more polar compound, was also active.

Fourth, the capacity of a compound to promote phenobarbital induction may be related to its capacity to bind to the endoplasmic reticulum. SKF 525-A and polycyclic hydrocarbons are known to bind avidly to the endoplasmic reticulum [20, 40]. It should be noted, however, that DDT and chlorpromazine, which also bind to the endoplasmic reticulum [20], had no effect on phenobarbital induction.

The possibility was considered that 3-methylcholanthrene might produce its observed effect on phenobarbital induction by increasing the ratio of parenchymal to hematopoietic cells in the fetal liver. This proved not to be the case; histological examination of 21-day fetal livers from mothers treated with 3-methylcholanthrene, phenobarbital or both agents by light and electron microscopy showed no difference in the cell population distinguishable from that observed with livers from untreated animals. Although unlikely, the possibility also existed that 3-methylcholanthrene might alter the accumulation of phenobarbital in the fetal liver. This proved not to be the case (see Table 3).

A mechanism which might explain both the resistance of the fetal liver to phenobarbital induction and the ability of other inducers to partially overcome this resistance evokes a potentially responsive cytochrome P-450-inducing system which is suppressed by an endogenous substance. In accordance with this hypothesis, phenobarbital-activating agents would interfere with the normal functioning of this substance. The presence of such a substance was suggested by Klinger *et al.* [41], who showed that extracts of fetal tissue inhibited induction of hepatic mono-oxygenase systems in weanling and adult female rats. If such a factor exists in the foeto-maternal environment, removal of the liver from this environment should permit the liver to respond to the inductive effects of phenobarbital. When fetal liver cells are cultured, they do in fact respond to phenobarbital with an increase in benzpyrene hydroxylase activity [20, 21, 42].

Regardless of speculation concerning its exact mechanism, a system controlling P-450 hemoprotein induction in the fetal liver must exist. This system suppresses cytochrome P-450 induction. Hepatic mono-oxygenase systems metabolize steroids. Suppression of steroid metabolism *in utero* may prevent a xenobiotically induced mono-oxidase system from inactivating vital endogenous substances. If this is the case, phenobarbital-activating agents might thereby exert secondary toxic effects by interfering with the normal protective function of this control mechanism.

Acknowledgements—We gratefully acknowledge the participation of Ms. Viola Abbott, who assisted with certain of the assays, and Mr. Adrian Swanson, who provided guidance for the mass spectrophotometric analysis of phenobarbital in the livers. We are indebted to Dr. J. G. White, Department of Pediatrics, University of Minnesota, for the histological studies.

REFERENCES

1. J. R. Fouts and R. H. Adamson, *Science, N.Y.* **129,** 897 (1959).
2. L. G. Hart, R. H. Adamson, R. L. Dixon and J. R. Fouts, *J. Pharmac. exp. Ther.* **137,** 103 (1962).
3. E. Bresnick and J. G. Stevenson, *Biochem. Pharmac.* **17,** 1815 (1968).
4. R. M. Welch, B. Gommi, A. P. Alvares and A. H. Conney, *Cancer Res.* **32,** 973 (1972).
5. R. L. Dixon and V. J. Willson, *Archs int. Pharmacodyn. Thér.* **172,** 453 (1968).
6. G. Feuer and A. Liscio, *Int. J. clin. Pharmac.* **3,** 30 (1970).
7. W. R. Jondorf, R. P. Maickel and B. B. Brodie, *Biochem. Pharmac.* **1,** 352 (1959).
8. E. Schlede and J. J. Merker, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **272,** 89 (1972).
9. A. Rane, M. Berggren, S. J. Yaffe and J. L. E. Eriesson, *Xenobiotica* **3,** 37 (1973).
10. T. K. Basu, J. W. T. Dickerson and D. V. Parke, *Biochem. J.* **119,** 54 (1970).
11. P. Th. Henderson, *Biochem. Pharmac.* **20,** 1225 (1971).
12. S. M. McLeod, K. W. Renton and N. R. Eade, *J. Pharmac. exp. Ther.* **183,** 489 (1972).
13. J. R. Fouts and T. R. Devereux, *J. Pharmac. exp. Ther.* **183,** 458 (1972).
14. J. R. Fouts and L. G. Hart, *Ann. N.Y. Acad. Sci.* **123,** 245 (1965).
15. S. El Defrawy El Masry, G. M. Cohen and G. J. Mannering, *Drug Metab. Dispos.* **2,** 267 (1974).
16. G. Feuer and A. Liscio, *Nature, Lond.* **223,** 68 (1971).
17. D. P. Bogdan and M. R. Juchau, *Eur. J. Pharmac.* **10,** 119 (1970).
18. M. Watanabe, V. R. Potter and H. P. Morris, *Cancer Res.* **30,** 262 (1970).
19. D. W. Nebert and H. V. Gelboin, *Archs Biochem. Biophys.* **134,** 76 (1969).
20. G. J. Mannering, in *Fundamentals of Drug Metabolism and Distribution* (Eds. B. N. LaDu, H. G. Mandel and E. L. Way), pp. 206–52. Williams & Wilkins, Baltimore Md. (1971).
21. N. E. Sladek and G. J. Mannering, *Molec. Pharmac.* **5,** 186 (1969).
22. B. Schoene, R. A. Fleischmann, H. Remmer and H. F. v. Oldershausen, *Eur. J. clin. Pharmac.* **4,** 65 (1972).
23. L. W. Wattenburg and J. L. Leong, *Cancer Res.* **25,** 365 (1962).
24. C. H. Williams and H. Kamin, *J. biol. Chem.* **237,** 587 (1962).
25. C. I. Bliss, in *Statistics in Biology*, pp. 253–57. McGraw-Hill, New York (1967).
26. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
27. L. W. Wattenberg, J. L. Leong and A. R. Galbraith, *Proc. Soc. exp. Biol. Med.* **127,** 467 (1968).

28. G. J. Mannering, in *Selected Pharmacological Testing Methods* (Ed. A. Burger), pp. 51–119. Marcel Dekker, New York (1968).
29. J. C. Arcos, A. H. Conney and N. P. Buu-Hoi, *J. biol. Chem.* **236,** 1291 (1961).
30. C. J. Parli and G. J. Mannering, *Molec. Pharmac.* **6,** 178 (1970).
31. J. R. Gillette and T. E. Gram, in *Microsomes and Drug Oxidations* (Eds. J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts and G. J. Mannering), pp. 133–48. Academic Press, New York (1969).
32. L. W. Wattenberg, W. D. Loub, L. K. Lam and J. L. Speier, *Fedn Proc.* **35,** 1327 (1976).
33. G. J. Mannering, *Metabolism* **20,** 228 (1971).
34. N. G. Zampaglione and G. J. Mannering, *J. Pharmac. exp. Ther.* **185,** 676 (1973).
35. W. J. Waddell and B. L. Mirkin, *Biochem. Pharmac.* **21,** 547 (1971).
36. T. M. Guenthner and G. J. Mannering, *Biochem. Pharmac.* **26,** 577 (1977).
37. S. Granick, *J. biol. Chem.* **241,** 1359 (1966).
38. A. B. Rifkind, S. Bennett, E. S. Forster and M. I. New, *Biochem. Pharmac.* **24,** 839 (1975).
39. M. W. Anders and G. J. Mannering, *Molec. Pharmac.* **2,** 341 (1966).
40. J. R. Gillette, *Proc. Second Int. Pharmac. Meeting* **4,** 9 (1963).
41. W. Klinger, G. Zwacka and H. Ankermann, *Acta biol. med. germ.* **20,** 137 (1968).
42. K. Bürki, A. G. Liebelt and E. Bresnick, *Archs Biochem. Biophys.* **158,** 641 (1973).