Biochemical Pharmacology, Vol. 34, No. 7, pp. 1007–1014, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

XENOBIOTIC IMPRINTING OF THE HEPATIC MONOOXYGENASE SYSTEM

EFFECTS OF NEONATAL PHENOBARBITAL ADMINISTRATION*

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(*Received* 31 *March* 1984; *accepted* 3 *July* 1984)

Abstract—The ability of phenobarbital (PB) to neonatally "imprint" or "program" the hepatic microsomal cytochrome P-450-dependent monooxygenase system (MOS) was investigated. Phenobarbital (30 mg/kg) was administered subcutaneously to neonatal rats of both sexes on days 1–5 postpartum. Various hepatic MOS activities were measured at 6, 22, 50 and 140 days of age. Six-day-old animals of both sexes displayed the increased hepatic microsomal protein levels and enzyme activities expected from the action of phenobarbital as a transitory MOS inducer. Most of these increases dissipated by 22 and 50 days of age. However, at 140 days of age rats of both sexes that had received neonatal phenobarbital showed increased levels of cytochrome P-450, as well as both P-450 and cytochrome *c* reductase, ethoxycoumarin *O*-deethylation, glucuronyl transferase activity, *in vitro* covalent binding of benzo[*a*]pyrene to DNA and *in vivo* covalent binding of aflatoxin B_1 to hepatic macromolecular fractions. Neonatal phenobarbital administration can alter the metabolic profile of rats in adulthood, apparently by a mechanism different from that responsible for either transitory PB induction or testosterone imprinting.

The presence of androgen during the neonatal period, particularly the first few days after birth, has been shown to influence masculine patterns of sexual behavior [1], patterns of hepatic steroid metabolism [2], androgen responsiveness of the liver [3], and sex differences in the activity of the P-450-dependent monooxygenase system (MOS) in adult rats [4]. Sex differences in MOS activity generally appear soon after weaning (22–24 days) and reach a maximum during the pubertal period (50–70 days) [5]. Castration of male rats immediately after birth results in either a diminution or abolition of the adult sex differences [3, 6, 7]. On the other hand, castration of adult male rats depresses MOS activity but does not decrease these activities to those measured in females. Administration of testosterone to castrated male rats during the neonatal period results in increased MOS activity at adulthood compared to non-testosterone-treated castrated controls [1, 3]. The effect of testosterone during the neonatal period, with the resultant changes observable in the adult life of the animal, has been termed "neonatal imprinting" [1].

Chung *et al.* [3], employing various MOS substrates, showed that neonatal androgen imprinting determines the developmental pattern of the hepatic MOS. Finnen and Hassall [8] have shown that the sex differences normally seen in the adult rat's metabolism of a dimethylated chlorocyclodiene epoxide could be abolished by castration of the males before 7 days of age, whereas castration after 14 days did not affect the normal metabolic profile. Levin *et al.* [7] found that the turnover of cytochrome P-450 was biphasic, consisting of a fast and a slow phase. Adult male rats had a lower ratio than female rats. However, males castrated at birth exhibited the female type ratio at adulthood. They also found testosterone hydroxylation rates at the 6β and 16α positions to be sex dependent, males having much higher activities than females, and that male castration at birth resulted in a feminine pattern of hydroxylation at adulthood.

The androgen imprintable MOS is also induced by various xenobiotics. Although enzyme induction is a transient phenomenon resulting in temporary increases in protein synthesis and enzyme activities, neonatal exposure to these inducers may result in permanent effects on hepatic metabolic activities, as does testosterone. In other words, the xenobiotics may also function as imprinting or programming agents.

Phenobarbital (PB), a classical barbiturate inducer of the MOS, has been used in various dosing protocols employing male rats and shown to imprint or program P-450 levels [9], arylhydrocarbon hydroxylase activity [9], the N-demethylation of ethylmorphine [10], the *in vitro* binding of benzo[*a*]pyrene to DNA [9], and the *in vivo* binding of aflatoxin B_1 to DNA [10].

This study was designed to gain a better understanding of the mechanism responsible for "xenobiotic imprinting" by addressing the following points. First, because the newborn animal appears most susceptible to androgen influence, PB was administered directly to neonatal rats during the first 5 days postpartum instead of to the lactating dam as in another study [10]. Second, PB was administered to

* Supported by Grant R01 ES03403 from the NIEHS.
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both sexes as neonates to allow a comparison of the "imprintability" of both the male and female hepatic MOS. This comparison is important if the mechanism of xenobiotic imprinting is associated with androgen imprinting, since androgen imprinting occurs only in males. Third, since "xenobiotic imprinting" requires exposure to the compound only during a short period of neonatal life, we examined the temporal response of certain enzyme activities from the neonatal period to adulthood.

METHODS AND MATERIALS

Male and female Sprague–Dawley rats, weighing approximately 350 g and 250 g, respectively, were purchased from Dominion Laboratories, Dublin, VA, for use as breeders. Males were housed in stainless-steel, wire-bottomed cages without bedding and females were housed in plastic cages with hardwood chip bedding. The animal room was maintained at 22–26° with a 12-h light–dark cycle. Animals were fed Purina Rodent Lab Chow (No. 3001) and tap water *ad lib.*

Individual females were mated with a randomly chosen male. The appearance of a vaginal plug was considered day 1 of pregnancy, at which time the female was returned to her home cage. Pups were born 21–23 days later. At birth the litters were culled to ten pups, keeping the ratio of males to females as close to one as possible. Litters were randomly assigned at birth to receive either 30 mg/kg PB (phenobarbital sodium from Baker) dissolved in 0.9% saline or 0.9% saline alone. The neonates were injected subcutaneously once each day on days 1 through 5 postpartum.

At 6 days after birth, assays were performed by utilizing livers pooled, by sex, from a single litter. For all other time points, one male and one female from each litter were used to avoid any intralitter effect.

Preparation of microsomes. Animals were killed by decapitation. Livers were removed and perfused with ice-cold 0.9% saline. All subsequent steps were performed at 0–5°. Microsomes from 6-day-old pups were isolated by the method of Cresteil *et al.* [11] which has been shown to result in higher microsomal activity in neonates. Microsomes from older rats were isolated as previously described [12].

Assays of MOS parameters. Microsomal protein was determined by the method of Lowry *et al.* [13] and adult cytochrome P-450 levels by the method of Omura and Sato [14]. Cytochrome P-450 reductase activity was measured as described by Peterson *et al.* [15] employing an Aminco DW-2 Spectrophotometer equipped with an Aminco–Morrow stopped-flow apparatus and a Bascon–Turner high speed data acquisition recorder. The final plateau levels of this reaction was used to calculate total NADPH-reducible cytochrome P-450 in 6-, 22- and 50-day-old rats. This same stopped-flow system was used to measure the rate of reduction of cytochrome *c* as follows: one syringe of the stopped-flow apparatus contained 250–500 μg of microsmal protein/ml in a solution of 0.1 mM cytochrome *c* in 0.1 M Tris, pH 7.6, at 25°. The second syringe contained a 0.2 mM solution of NADPH in 2 mM potassium cyanide. Absorbance was measured in the dual-wavelength mode at 550 nm vs 490 nm. Solutions were preincubated in the apparatus for 10 min at 37°. The rate of reaction was determined from the slope of the initial linear phase using an extinction coefficient of 18.5 mM^{-1}. The kinetics of the N-demethylation of ethylmorphine were determined as described by Mgbodile *et al.* [16]. Enzyme kinetic constants were determined by a computer program devised by Cleland [17]. Ethoxycoumarin-*O*-deethylase activity was measured by the method of Ullrich and Webber [18]. The rate of testosterone hydroxylation at the 6β, 7α, and 16α positions was determined by the method of Conney and Klutch [19]. Incubations were carried out at 37° in a 5 ml total volume with 0.05 M Tris–3.6 mM $MgCl_2$ buffer, NADPH-generating system, 2 mg/ml microsomal protein and 5 μl [^{14}C]testosterone (sp. act. = 1584 dpm/nmole). Background counts were obtained by counting a nonspotted section of the TLC sheet. Internal standards were obtained from Steraloids, Wilton, NH (6β + 7α) and Sigma (16α).

Glucuronyl transferase activity. Two substrates, 4-methylumbilliferone and testosterone, representing, respectively, the late fetal and early neonatal clusters as described by Wishart *et al.* [20], were used to measure glucuronyl transferase activity. 4-Methylumbilliferone glucuronidation was measured by the method of Aito [21]. Testosterone glucuronidation was measured by the method described by Lucier and McDaniel [22].

In vitro *covalent binding of benzo*[a]*pyrene to DNA.* [^{3}H]Benzo[*a*]pyrene, 0.1 mg in 10 μl (56.4 Ci/nmole), purchased from New England Nuclear, was incubated for 20 min with 3 mg calf thymus DNA (Sigma type IV), microsomes equivalent to 5 mg total protein and 0.5 ml of an NADPH-generating system containing 0.04 mmole glucose-6-phosphate, 2 μmoles NADP and 2.5 units of glucose-6-phosphate dehydrogenase in a total volume of 2.5 ml of 0.1 M PO_4 buffer, pH 7.4, at 37°. The reaction was stopped by the addition of 1 ml of saturated NaCl and frozen overnight. This reaction was found to be linear with time (to 30 min) and with protein concentration. Samples were thawed and centrifuged at 105,000 *g* for 60 min to remove the microsomes. The supernatant fraction was decanted into centrifuge tubes; 2 ml of saturated NaCl and excess acetone were added to precipitate the DNA. The precipitate was redissolved in water and reprecipitated with acetone and placed in a freezer overnight. This reprecipitation process was repeated until supernatant counts became negligible. The final precipitate was digested with 0.5 M perchloric acid at 70° for 20 min. Aliquots of the final solution were removed for quantitation of BP-DNA adducts by scintillation spectrometry. DNA was quantitated as described by Hubbard *et al.* [23].

In vivo *covalent binding of aflatoxin* B_1. [^{3}H]AFB_1 (sp. act. 8 Ci/mmole) was purchased from Moravek Biochemicals, Brea, CA. A dose of 1 mg/kg [^{3}H]-AFB_1 (sp. act. 0.11 mCi/mg AFB_1) was administered i.p. to 140-day-old male and female rats from both neonatally PB-treated and control litters. Animals were killed by ether 2 hr post-injection. Livers were removed and perfused with ice-cold saline to remove

Table 1. Effect of neonatal PB treatment on growth parameters and cytochrome P-450 levels in 6-day-old rats*

	Body wt (g)	Liver wt (g)	Liver wt/ 100 g body wt	Microsomal protein (mg/g liver)	Cytochrome P-450 (nmoles/mg protein)
Male					
Saline	14.0 ± 0.3	0.45 ± 0.01	3.20 ± 0.03	8.4 ± 1.4	0.327 ± 0.010
PB	13.8 ± 0.2	0.47 ± 0.02	3.33 ± 0.11	15.2 ± 0.8†	0.764 ± 0.055†
Female					
Saline	13.3 ± 0.3	0.43 ± 0.01	3.28 ± 0.07	8.8 ± 1.1	0.297 ± 0.026
PB	13.2 ± 0.2	0.46 ± 0.02	3.50 ± 0.10	15.2 ± 1.5†	0.798 ± 0.133†

* Livers from 6-day-old animals were pooled from each of four litters treated on days 1–5 after birth with 30 mg/kg PB and four litters treated on days 1–5 after birth with 0.9% saline. Each value represents the mean ± S.E.M.

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

the blood, and frozen. The method of Glazer and Weber [24] was used to isolate the macromolecular fractions, and the aflatoxin covalently bound to hepatic DNA, RNA, and protein was determined by scintillation spectrometry, as previously described [25]. DNA was quantitated by the diphenylamine method of Hubbard *et al.* [23], RNA by the method of Ceriotti [26] and protein by the method of Lowry *et al.* [13].

RESULTS

Effect of neonatal PB on 6-day-old rats. Animals were assayed at day 6 after birth to determine the early effects of neonatal PB administration. Table 1 indicates that PB treatment had no effect on either body or liver weight, indicating that PB treatment did not alter feeding time enough to result in altered growth. PB treatment increased hepatic microsomal protein by over 1.75-fold in both sexes and induced cytochrome P-450 levels by 2.34-fold in males and 2.69-fold in females.

Table 2 demonstrates the microsomal enzyme activities measured in 6-day-old pups. PB-treated animals of both sexes showed significant increases in ethylmorphine *N*-demethylase activity and significantly higher rates of testosterone hydroxylation at both the 7α and 16α positions. The rate of 16α hydroxylation showed the greatest response to PB induction with increases of 2.86-fold in males and 4.00-fold in females. Increased 16α hydroxylation is thought to represent induction of PB-type P-450s, where 7α hydroxylation represents constitutive P-450 activity [27]. The increase in 7α hydroxylation in response to PB cannot be accounted for at this time, but may represent either increases in constitutive P-450 activity or production of new forms of P-450. PB did not alter 6β hydroxylase activity. PB treatment induced cytochrome P-450 reductase activity by over 2.5-fold in both sexes.

Effect of neonatal PB on 22-day-old rats. Animals were weaned at 22 days after birth and one rat of each sex from each litter was removed for analysis. Hepatic microsomal protein contents are shown in Table 3. Protein levels were not significantly different between PB and control animals, whereas total P-450 hemoprotein levels were significantly higher in PB-treated animals (1.44-fold in males and 1.29-fold in females) although the degree of diffference dropped considerably from that observed at day 6.

Table 4 illustrates microsomal enzyme activities in 22-day-old rats. Ethylmorphine *N*-demethylase activity in the neonatally PB-treated animals had returned to control levels. There was no significant difference in P-450 reductase activity between the female treatment groups at 22 days of age. The P-

Table 2. Effect of neonatal PB treatment on microsomal enzyme activities in 6-day-old rats*

	Ethylmorphine *N*-demethylation (nmoles product/ mg protein/min)	Testosterone hydroxylation (nmoles product/mg protein/min)			P-450 reductase (nmoles P-450 red./mg protein/min)
		6β	7α	16α	
Male					
Saline	4.1 ± 0.9	727.5 ± 457.5	520.0 ± 182.5	87.5 ± 62.5	13.6 ± 1.3
PB	6.7 ± 0.7†	682.5 ± 102.5	780.0 ± 65.0†	250.0 ± 60.0†	32.7 ± 3.5†
Female					
Saline	3.9 ± 0.5	595.0 ± 260.0	365.0 ± 102.5	70.0 ± 35.0	11.8 ± 1.6
PB	8.0 ± 1.4†	665.0 ± 297.5	952.5 ± 225.0†	277.5 ± 82.5†	29.2 ± 3.9†

* Livers from 6-day-old animals were pooled from each of four litters treated on days 1–5 after birth with 30 mg/kg PB and four litters treated on days 1–5 after birth with 0.9% saline. Each value represents the mean ± S.E.M.

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

Table 3. Effect of neonatal PB treatment on microsomal protein and P-450 levels in 22-day-old rats*

	Microsomal protein (mg/g liver)	Cytochrome P-450 (nmoles/mg protein)
Male		
Saline	13.3 ± 0.7	0.220 ± 0.048
PB	13.5 ± 0.7	0.316 ± 0.025†
Female		
Saline	12.1 ± 0.6	0.258 ± 0.029
PB	13.0 ± 1.7	0.334 ± 0.012†

* Animals were injected subcutaneously with either 30 mg/kg PB or 0.9% saline on days 1–5 postpartum. At 22 days of age, one male and one female from each of four PB-treated and four saline-treated litters were removed for assay. Each value represents mean ± S.E.M.

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

450 reductase activity in PB males was higher than controls; however, the degree of difference was half that of day 6.

Effect of neonatal PB treatment on 50-day old rats. By 50 days of age, rats have reached puberty and sex differences become detectable [5]. Again, one rat of each sex from each of four neonatally PB-treated and four neonatally saline-treated litters was taken for analysis.

As illustrated in Table 5, at 50 days there were no statistically significant differences in growth parameters between the two treatment groups.

Table 6 illustrates the hepatic microsomal enzyme activities measured at 50 days. At this age, P-450 reductase activity showed no difference between PB-treated and control groups. Using cytochrome *c* as substrate for the flavoprotein, the females showed no treatment effect and there was a slight decrease (10%) in the activity in the neonatally PB-treated males compared to controls.

Testosterone hydroxylation showed no treatment-related alteration in the males. Females neonatally treated with PB did show higher hydroxylating activity at the 6β and 7α positions compared to controls, but not at the 16α position, which is thought to respond preferentially to PB treatment.

Ethylmorphine *N*-demethylation kinetics showed no treatment effects on the V_{max}, but there was a slight decrease in the K_m in the females.

Effect of neonatal PB administration on 140-day-old rats. At 140 days of age, one rat of each sex from each of four neonatally PB-treated and four neonatally saline-treated litters was employed. The results in Table 7 again emphasize that neonatal PB treatment did not produce permanent or latent effects on growth parameters. Although total hepatic microsomal protein was not affected, P-450 levels relative to total protein were increased 1.28-fold in both males and females treated neonatally with PB (Table 8). Table 8 also indicates that neonatal PB produced increased activity of the reductase flavoprotein. This is evident with both the endogenous substrate, cytochrome P-450, and with the exogenously added sub-

Table 4. Effect of neonatal PB treatment on microsomal enzyme activities in 22-day-old rats*

	Ethylmorphine *N*-demethylation (nmoles product/mg protein/min)	Testosterone hydroxylation (nmoles product/mg protein/min)			P-450 reductase (nmoles P-450 red./mg protein/min)
		6β	7α	16α	
Male					
Saline	3.8 ± 0.8	577.5 ± 100.0	657.5 ± 65.0	175.0 ± 37.5	5.0 ± 1.5
PB	4.1 ± 0.2	345.0 ± 82.5†	765.0 ± 115.0	67.5 ± 20.0†	8.8 ± 1.1†
Female					
Saline	3.7 ± 0.6	657.5 ± 115.0	600.0 ± 85.0	170.0 ± 25.0	5.4 ± 2.1
PB	3.6 ± 0.6	310.0 ± 40.0†	645.0 ± 52.5	72.5 ± 12.5†	8.0 ± 2.0

* Animals were injected subcutaneously with either 30 mg/kg PB or 0.9% saline on days 1–5 postpartum. At 22 days of age, one male and one female from each of four PB-treated and four saline-treated litters were removed for assay. Each value represents mean ± S.E.M.

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

Table 5. Effect of neonatal PB treatment on growth parameters and cytochrome P-450 in 50-day-old rats*

	Body wt (g)	Liver wt (g)	Liver wt/100 g body wt	Microsomal protein (mg/g liver)	Cytochrome P-450 (nmoles/mg protein)
Male					
Saline	244 ± 9	11.8 ± 0.7	4.8 ± 0.2	19.3 ± 2.1	0.297 ± 0.052
PB	246 ± 16	11.4 ± 0.8	4.6 ± 0.3	21.0 ± 1.0	0.258 ± 0.052
Female					
Saline	177 ± 11	7.5 ± 0.4	4.2 ± 0.1	17.3 ± 1.2	0.313 ± 0.045
PB	179 ± 6	8.1 ± 0.5	4.5 ± 0.2	20.8 ± 2.6	0.309 ± 0.058

* Animals were injected subcutaneously with either 30 mg/kg PB or 0.9% saline on days 1–5 postpartum. At 50 days of age one male and one female from each of four PB-treated and four saline-treated litters were removed for assaying. Each value represents mean ± S.E.M.

Table 6. Effect of neonatal PB treatment on microsomal enzyme activities in 50-day-old rats*

	P-450 reductase (nmoles P-450 red./mg protein/min)	Cytochrome *c* reductase (nmoles red./mg protein/min)	Testosterone hydroxylation (pmoles product/mg protein/min)			Ethylmorphine *N*-demethylation (nmoles product/mg protein/min)	
			6β	7α	16α	K_m (mM)	V_{max}
Male							
Saline	11.8 ± 1.8	265.5 ± 12.8	710.0 ± 110.0	470.0 ± 77.5	490.0 ± 92.5	0.129 ± 0.029	6.08 ± 1.83
PB	12.0 ± 1.9	239.0 ± 7.4†	987.5 ± 255.0	545.0 ± 95.0	520.0 ± 15.0	0.087 ± 0.008	5.95 ± 0.32
Female							
Saline	11.4 ± 2.4	209.6 ± 20.8	92.5 ± 2.5	145.0 ± 42.5	142.5 ± 90.0	0.131 ± 0.004	1.58 ± 0.36
PB	10.5 ± 1.4	216.7 ± 34.2	212.5 ± 45.0†	357.0 ± 50.0†	132.5 ± 32.5	0.115 ± 0.005†	2.15 ± 0.31

* Animals were injected subcutaneously with either 30 mg/kg PB or 0.9% saline on days 1–5 postpartum. At 50 days of age, one male and one female from each of four PB-treated and four saline-treated litters were removed for assay. Each value represents mean ± S.E.M.
† Significantly different from control ($P < 0.05$).

strate, cytochrome *c*. P-450 reduction was increased 1.37-fold in males and 1.97-fold in females. The reduction of cytochrome *c* was increased 1.27-fold in both sexes.

Table 9 demonstrates the effect of neonatal PB administration on MOS activity with specific substrates. Ethoxycoumarin O-deethylation was increased 1.30-fold in neonatally PB-treated males and 1.84-fold in similarly treated females. Testosterone hydroxylation showed no treatment effects at 140 days in any of the three positions of hydroxylation measured. The only statistically significant treatment effect on ethylmorphine *N*-demethylase kinetics was a 1.25-fold increase in the V_{max} of the PB-treated female rats.

Table 10 demonstrates the effect of neonatal PB treatment on glucuronyl transferase activity towards the substrates 4-methylumbilliferone and testosterone. These substrates were chosen because they represent the two classes or "clusters" of transferases based on the age at which maximal activity develops as described by Wishart *et al.* [20]. Maximal activity towards 4-methylumbilliferone occurs during the late fetal period, whereas maximal activity towards testosterone does not occur until the early neonatal period. Neonatal PB resulted in increased activity in both sexes towards both substrates. Glucuronidation of 4-methylumbilliferone was increased 1.61-fold in males and 1.51-fold in females. Testosterone glucuronidation was increased 1.25-fold in males and 1.43-fold in females.

Table 11 presents the data from studies of the effect of neonatal PB treatment on the covalent binding of carcinogens to macromolecules. Neonatal PB treatment increased the *in vitro* binding of benzo[*a*]pyrene to DNA by 2.00-fold in males and 2.93-fold in females.

In vivo covalent adduct formation between AFB_1 and hepatic DNA in males imprinted with PB was increased 2.32-fold and RNA adducts increased 1.68-fold. There were no statistically significant differences between the PB- and saline-treated females.

DISCUSSION

Our results demonstrate that neonatal PB administration directly to rat pups on days 1 through 5 postpartum imprinted, or programmed, not only constituents of the hepatic microsomal P-450-dependent monooxygenase system, but also the glucuronyl transferases in both male and female rats.

As is evident from this study of the time course of the effects of neonatal PB administration, the imprinting phenomenon was not an extension of the actions of PB as a transient inducer. Most of the activities that were increased at day 6 had returned to baseline by day 22, the rest by day 50, indicating that the transient induction had dissipated. The increases remaining at 22 days either may be due to slower absorption of PB when injected subcutaneously or it may be that PB triggers a precocious development of these systems in maturing animals. A second argument against prolonged transitory induction is that there was no observable treatment effect on total hepatic microsomal protein levels at day 140. This contrasts with classical PB

induction which results in increased hepatic microsomal protein levels [12]. A third argument against the imprinting mechanism being prolonged transitory induction comes from the *in vivo* AFB_1 binding data. PB induction in adult animals results in lower levels of covalent adducts than found in untreated controls [28]. However, both in our study and in the report of Faris and Campbell [10] it was found that PB-imprinted animals had higher levels of adducts than controls. A fourth consideration in eliminating classical PB induction as an imprinting mechanism is that the classical substrate metabolism patterns occurring after PB induction do not occur in PB-imprinted animals. For instance, ethylmorphine N-demethylation and 16α-testosterone hydroxylation activities are both sensitive indicators of PB induction, yet there was no effect on the hydroxylation of testosterone and only a slight effect on ethyl-

Table 7. Effect of neonatal PB treatment on growth parameters in 140-day-old rats*

	Body wt (g)	Liver wt (g)	Liver wt/ 100 g body wt	Microsomal protein (mg/g liver)
Male				
Saline	499.4 ± 6.0	14.9 ± 0.4	3.0 ± 0.1	23.2 ± 0.4
PB	478.2 ± 31.2	14.2 ± 1.0	3.0 ± 0.1	23.3 ± 2.4
Female				
Saline	304.5 ± 7.8	9.9 ± 0.9	3.2 ± 0.2	19.7 ± 0.6
PB	292.3 ± 14.1	8.8 ± 0.4	3.0 ± 0.1	19.4 ± 0.8

* Animals were injected subcutaneously with either 30 mg/kg PB or 0.9% saline on days 1–5 postpartum. At 140 days of age one male and one female from each of four PB-treated and four saline-treated litters were removed for assaying. Each value represents mean ± S.E.M.

Table 8. Effect of neonatal PB treatment on microsomal cytochrome P-450 and electron transport in 140-day-old rats*

	Cytochrome P-450 (nmoles/mg protein)	P-450 reductase (nmoles reduced/ mg protein/min)	Cytochrome *c* reductase (nmoles reduced/ mg protein/min)
Male			
Saline	0.450 ± 0.017	23.3 ± 2.4	172.3 ± 7.4
PB	0.579 ± 0.047†	31.7 ± 3.8†	218.0 ± 8.0†
Female			
Saline	0.398 ± 0.035	17.1 ± 1.9	133.2 ± 5.5
PB	0.507 ± 0.043†	33.6 ± 3.0†	169.4 ± 7.4†

* Animals were injected subcutaneously with either 30 mg/kg PB or 0.9% saline on days 1–5 postpartum. At 140 days of age, one male and one female from each of four PB-treated and four saline-treated litters were removed for assay. Each value represents mean ± S.E.M.

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

Table 9. Effect of neonatal PB treatment on P-450-dependent substrate metabolism activities in 140-day-old rats*

	Ethoxycoumarin O-deethylation (nmoles product/ mg protein/min)	Testosterone hydroxylation (pmoles product/mg protein/min)			Ethylmorphine N-demethylation (nmoles/mg protein/min)	
		6β	7α	16α	K_m (mM)	V_{max}
Male						
Saline	0.184 ± 0.009	312.0 ± 43.7	274.9 ± 48.0	342.4 ± 43.2	0.126 ± 0.037	5.3 ± 0.6
PB	0.238 ± 0.026†	306.7 ± 57.0	301.2 ± 38.9	441.6 ± 111.2	0.181 ± 0.029	6.0 ± 0.8
Female						
Saline	0.106 ± 0.012	64.5 ± 8.2	118.1 ± 13.9	161.0 ± 41.7	0.190 ± 0.027	1.3 ± 0.1
PB	0.195 ± 0.006†	65.8 ± 5.9	132.2 ± 24.5	139.4 ± 22.7	0.220 ± 0.045	1.6 ± 0.1†

* Animals were injected subcutaneously with either 30 mg/kg PB or 0.9% saline on days 1–5 postpartum. At 140 days of age one male and one female from each of four PB-treated and four saline-treated litters were removed for assaying. Each value represents the mean ± S.E.M.

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

Table 10. Effect of neonatal PB treatment on glucuronyl transferase activities in 140-day-old rats*

	4-Methylumbilliferone glucuronidation (nmoles/mg protein/min)	Testosterone glucuronidation (nmoles/mg protein/min)
Male		
Saline	2.52 ± 0.53	0.446 ± 0.034
PB	4.05 ± 0.60†	0.581 ± 0.059†
Female		
Saline	4.89 ± 1.09	0.386 ± 0.071
PB	7.37 ± 0.61†	0.551 ± 0.063†

* Animals were injected subcutaneously with either 30 mg/kg PB or 0.9% saline on days 1–5 postpartum. At 140 days of age one male and one female from each of four PB-treated and four saline-treated litters were removed for assaying. Each value represents the mean ± S.E.M.
† Significantly different from control ($P < 0.05$).

morphine kinetics in adult animals imprinted with PB as neonates. On the other hand, benzo[*a*]pyrene binding to DNA was greatly increased in the imprinted animals. The polycyclic hydrocarbon benzo[*a*]pyrene is preferentially metabolized by the polycyclic hydrocarbon-induced isozyme of P-450 (denoted P-448) [29, 30]. Simpson and Chung [9] also noted increased polycyclic hydrocarbon-induced type P-450 activities in their report of PB imprinting. However, it should be noted that 6β hydroxylation of testosterone was not elevated in PB-imprinted animals, yet hydroxylation at this position is reportedly responsive to P-448-type inducers [27].

It is also interesting to contrast the results of PB imprinting to those typically reported for androgen imprinting. For instance, androgen imprinting, which results in increased xenobiotic metabolism in male rats compared to females, manifests itself around 30 days of age [5]. On the other hand, PB imprinting does not manifest itself this early. Our results show that PB imprinting is not evident even at 50 days of age, but does become evident by 140 days. Faris and Campbell [10] observed this long latency effect when they examined the time course of AFB_1 binding in PB-imprinted rats. They did not observe imprinting until 118 days of age.

The metabolism of testosterone is also different between androgen- and PB-imprinted animals. Neonatal androgen results in increased 6β hydroxylation of testosterone [6, 7]. This activity was not altered in adult animals neonatally imprinted with PB.

Another difference between androgen and PB imprinting is seen when the responsiveness of the two sexes is compared. Testosterone is present postpartum in both male and female rats, albeit at lower levels in the female [31]. However, it appears that only males respond to the postpartum presence of androgens resulting in the sex differences measurable later in life [1, 2]. It has been suggested that males are sensitized to the presence of neonatal androgen by a sharp rise in testosterone levels in fetal males that does not occur in fetal females [31]. In contrast to testosterone, PB exposure during the neonatal period not only imprints the MOS of both sexes, but females seem to be more responsive than males. In addition, it has been shown that administration of testosterone to females during the neonatal period does not result in androgen imprinting or "mascu-

Table 11. Effect of neonatal PB treatment on levels of carcinogen binding to macromolecules in 140-day-old rats*

	In vitro binding of [^{3}H]benzo[*a*]pyrene to calf thymus DNA† (pmoles/mg DNA)	*In vitro* binding of aflatoxin B_1‡ (ng AFB/mg macromolecular fraction)		
		DNA	RNA	Protein
Male				
Saline	35.1 ± 11.4	281.4 ± 86.3	46.4 ± 10.7	5.4 ± 1.2
PB	70.3 ± 16.5§	653.1 ± 132.6§	78.1 ± 15.5§	7.5 ± 1.0
Female				
Saline	34.6 ± 10.2	180.7 ± 78.6	27.5 ± 1.4	3.8 ± 0.3
PB	101.4 ± 26.5§	271.5 ± 144.6	29.4 ± 3.9	3.3 ± 0.4

* Data represent mean ± S.E.M.
† *In vitro* binding assay was performed using microsomes from four animals of each sex treated neonatally with saline and four animals of each sex treated neonatally with PB.
‡ [^{3}H]AFB_1 (1 mg/kg) was injected i.p. into four 140-day-old animals of each sex and treatment (neonatal saline or PB). Animals were killed 2 hr after injection and livers were removed and frozen until time of extraction.
§ Significantly different from control ($P < 0.05$).

linization" of the MOS activity of the female. However, if these neonatally testosterone-treated females are ovarectomized at birth or 25 days of age, they respond with increased MOS activity as adults [32]. This contrasts with PB imprinting in females since neonatal PB evokes imprinting without ovarectomy.

The increase in MOS activity in imprinted females was often much larger than in imprinted males. In males, these MOS activities are also imprinted by neonatal androgen [3, 33]. PB administration to neonatal males has two interacting effects. Although neonatal PB may be additive to the androgen imprinting of these enzymes, at the same time it increases the metabolism and decreases the synthesis of testosterone [34]. This results in lower testosterone availability for androgen imprinting in the male PB-treated rats. Female rats have lower MOS activities due to the lack of androgen imprinting; therefore, PB imprinting is fully additive to control female levels of activity since PB is not decreasing any naturally occurring androgen imprinting. Imprinting appears to be a complex phenomenon and is not simply an interaction between PB and testosterone.

Chung *et al.* [35] have reported evidence for a novel type of P-450 which is present only in neonatally androgen-imprinted rats. It may be that either this P-450 or other P-450 isozymes different from the adult PB or hydrocarbon inducible P-450s are synthesized in response to neonatal PB and may be responsible for the increased activity found in imprinted rats. Further studies on the types of P-450 present in PB-imprinted animals are under way to ascertain the role of various P-450s in the mechanism of xenobiotic imprinting.

The phenomenon of xenobiotic imprinting may be important, especially when applied to the assessment of human susceptibility to xenobiotics. Many compounds require metabolic activation by the MOS to be converted to their toxic or carcinogenic form. Any process which increases the functioning of this activation system could increase the levels of the reactive toxic or carcinogenic species. The demonstration that PB-imprinted animals exhibit higher levels of covalent adducts to DNA serves as initial evidence for this proposition (see also Refs. 9 and 10).

In general, the realization that phenobarbital and perhaps other xenobiotics can act during the neonatal period to influence metabolic activation/detoxification enzymes later in life should be a concern in the treatment of pregnant mothers or newborn children with drugs which may have imprinting potential.

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