The work of Wong and Hseih [9] showed that $AFR_0$ is the major metabolite in rat plasma when $AFB_1$ is administered intravenously or orally, and that the concentration of $AFR_0$ in plasma is maximal at 5 min after dosing. Wong and coworkers postulated that the $AFR_0$ in rat plasma is produced by the action of liver cytoplasmic $AFB_1$-reductase. As a matter of fact, $AFB_1$-reductase activity in rat liver cytosol is relatively low compared with that of erythrocyte. Probably, the *in vivo* formation of $AFR_0$ in rat plasma is catalyzed by the action of erythrocyte $AFB_1$-reductase. Since $AFB_1$ is carried in the blood from the gastrointestinal mucosa to the target organ, the transformation of $AFB_1$ in hepatic tissues or in target organs may be modified by the action of enzyme(s) present in the erythrocytes. Recently, the $AFB_1$-reductase of chicken liver was partially purified by Chen *et al.* [10]. The molecular weight of the enzyme was estimated to be 46,500, and the activity of the enzyme was inhibited 50–70% by some steroid hormones. It seems that the $AFB_1$-reductase in the liver cytosol is attributable to a steroid-metabolizing enzyme. However, the intrinsic role of the $AFB_1$-reductase in erythrocytes *in vivo* warrants further investigation.

In summary, the rat erythrocyte was shown to be capable of catalyzing the interconversion of $AFB_1$ and $AFR_0$. The transformation of these toxins was modified by glucose, owing to the variation in the concentration of NADP and NADPH in the erythrocytes.

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# Xenobiotic imprinting of hepatic metabolic enzyme systems: effect of neonatal 3-methylcholanthrene administration*

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The cytochrome P-450-dependent polysubstrate monooxygenase system (PSMOS) is the primary hepatic enzyme system responsible for the activation and/or detoxication of both endogenous and exogenous substrates. In many cases, the detoxication is due to the fact that increased polarity of the product facilitates excretion. In other cases, the product may be highly electrophilic, resulting in covalent binding to nucleophilic sites on tissue macromolecules and thus in increased toxicity. The activity of this system can be increased or induced by many different classes of compounds. These inducers include the barbiturates, pesticides, steroids and polycyclic hydrocarbons, among others.

Certain endogenous steroids have major influences on this enzyme system. An often studied example of this is the role of testosterone in regulating PSMOS activity in the rat. An adult male rat has higher PSMOS activities than does the female. These sex differences usually appear after 30 days of age. Depriving male rats of testosterone by castration soon after birth prevents these sex differences [1, 2]. This property of testosterone, whereby its presence during the neonatal period results in alterations later in the life of the animal, is known as neonatal imprinting [3].

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This work with testosterone led to studies to determine if imprinting could be evoked by the administration of a nonsteroidal xenobiotic that is known to induce the PSMOS. A logical choice for such an agent was phenobarbital (PB). This barbiturate is one of the most extensively studied inducers of PSMOS. Using PB in various dosing protocols and determining PSMOS activity by different methods, it has been shown by us and others that PB administration during the neonatal period imprints or programs rat hepatic metabolic enzyme activities [4–6].

We previously reported that PB administration during the early neonatal period resulted in elevated enzyme activities in both male and female rats at 140 days of age [4]. These changes include higher P-450 levels, increased P-450 reductase activity, increased metabolism of ethoxycoumarin, higher rates of glucuronyltransferase activity, and increased binding of carcinogens to DNA both *in vitro* and *in vivo*.

Since evidence now exists that compounds from two different inducer classes, i.e. steroids and barbiturates, can imprint the PSMOS, the possibility exists that neonatal administration of a potent inducer from a different inducer class could program PSMOS activity in adulthood. We chose 3-methylcholanthrene (3-MC) as representative of the polycyclic hydrocarbons which induce forms of P-450 distinct from those induced by PB.

To compare this study with our PB study, 3-MC was administered during the same neonatal period followed by a determination of PSMOS variables at various ages.

*Materials and methods*

Male and female Sprague–Dawley rats weighing 350 and 250 g, respectively, were purchased from Dominion Laboratories, Dublin, VA, and used for breeding. Males were housed in wire-racked cages. Females were housed in plastic cages with hardwood chip bedding. The animal room was maintained at 21–24° with a 12 hr light/dark cycle. Purina lab chow No. 5001 and tap water were provided *ad lib*. Each female was mated with a randomly chosen male. The appearance of a vaginal plug was considered day 1 of pregnancy. Pups were born on day 22 or 23 of gestation. Litters were culled to ten pups with the ratio of males to females kept as close to one as possible. Litters were designated as either treatment or control and were administered either 20 mg/kg 3-MC (Sigma) in corn oil or an equivalent volume of corn oil. Administration was by subcutaneous injection once each day on days 1–5 postpartum.

Assays of growth variables and PSMOS activities were carried out at 6, 25, 50, 140 and 200 days of age. For day 6 assays, pups from each of five litters were pooled by sex due to the small liver size. At all other time points, one male and one female from each of four to five different litters were used to avoid any intralitter effects.

Animals were killed by decapitation. Livers were removed and perfused with ice-cold 0.9% saline and homogenized in 0.02 M Tris buffer–1.15% KCl, pH 7.4 at 4° using a glass homogenizing tube and Teflon pestle. Microsomes from 6-day-old pups were isolated according to the method of Cresteil *et al.* [7]. Microsomes from older animals were isolated by the method described by Hayes *et al.* [8]. All manipulations were carried out at 0–4°.

Microsomal protein was determined by the method of Lowry *et al.* [9]. Cytochrome P-450 content was quantified as described by Omura and Sato [10]. The activity of the P-450 reductase was measured using the stopped-flow procedure of Peterson *et al.* [11]. The N-demethylation of ethylmorphine (EM) was measured as described by Mgbodile *et al.* [12]. Ethoxycoumarin (EC) *O*-deethylase activity was measured by the procedure of Ullrich and Weber [13]. Benzo[*a*]pyrene (BP) hydroxylase activity was determined as described by Nebert and Gelboin [14].

*Results and discussion*

Table 1 shows the effects of 3-MC on male and female PSMOS variables at 6 days of age, the day following the last 3-MC administration. Although hepatic microsomal protein was not increased in the 3-MC animals, the amount of cytochrome P-450 relative to protein was nearly doubled. The lack of increase in protein along with the increase in P-450 levels was similar to that found in adult rats treated with 3-MC [15]. The O-deethylation of EC and the hydroxylation of BP were also induced by 3-MC in 6-day-old rat pups in a manner similar to adult rats. EC metabolism was increased 5-fold in the 3-MC animals and BP hydroxylation was elevated 4-fold. This inductive effect lasted until 24 days of age, probably due to the slow rate of absorption of the subcutaneously administered 3-MC (unpublished observations). However, at 50 days of age both the P-450 levels and metabolic activities in the 3-MC animals had returned to control levels, indicating that the inductive effect had dissipated (unpublished observations).

Table 2 shows the growth variables in 140-day-old rats, an age at which PB neonatal programming can be detected [4]. Neonatally the body and liver weights of 3-MC-treated females decreased relative to controls; however, the ratio of liver weight to body weight was not different from controls. The decreased body weight was probably due to solid encapsulated mammary tumors observed in 60% of the treated females. No control animals were observed to have tumors. There was no alteration in growth variables in the males; they also did not display tumors. In our report of PB imprinting, we observed no effects on growth variables at this age [4].

Neonatal 3-MC treatment had no effect on microsomal

Table 1. Effect of neonatal 3-MC treatment on various PSMOS variables in 6-day-old rat pups*

| Sex | Treatment | Microsomal protein (mg/g liver) | Cytochrome P-450 (nmoles/mg protein) | Ethoxycoumarin *O*-deethylation (nmoles/mg protein/min) | Benzo[*a*]pyrene hydroxylation (pmoles/mg protein/min) |
|---|---|---|---|---|---|
| M | 3-MC | 14.0 ± 0.4 | 0.595 ± 0.018† | 0.61 ± 0.03† | 964.5 ± 79.6† |
| | Corn oil | 12.8 ± 1.6 | 0.315 ± 0.042‡ | 0.14 ± 0.01‡ | 236.4 ± 48.3‡ |
| F | 3-MC | 13.4 ± 1.1 | 0.704 ± 0.103† | 0.53 ± 0.04† | 980.8 ± 130.5† |
| | Corn oil | 11.6 ± 1.4 | 0.330 ± 0.030‡ | 0.11 ± 0.03‡ | 222.0 ± 24.6‡ |

* Rats were injected s.c. with either 20 mg/kg 3-methylcholanthrene in corn oil or corn oil alone on days 1–5 postpartum.
†,‡ Different symbols indicate significantly different at $P \leq 0.05$, within each sex.

Table 2. Effects of neonatal 3-MC on growth variables in 140-day-old rats*

| Sex | Treatment | Body wt (g) | Liver wt (g) | Liver wt/100 g body wt. | Microsomal protein (mg/g liver) |
|---|---|---|---|---|---|
| M | 3-MC | 446 ± 32 | 15.9 ± 1.5 | 3.6 ± 0.3 | 18.1 ± 1.5 |
| | Corn oil | 482 ± 22 | 16.1 ± 1.0 | 3.3 ± 0.1 | 20.7 ± 1.4 |
| F | 3-MC | 259 ± 12† | 8.9 ± 0.5† | 3.4 ± 0.3 | 15.9 ± 2.2 |
| | Corn oil | 307 ± 21‡ | 10.4 ± 0.8‡ | 3.4 ± 0.1 | 15.8 ± 1.2 |

* Rats were injected s.c. with either 20 mg/kg 3-methylcholanthrene in corn oil or corn oil alone on days 1–5 postpartum.
†,‡ Different symbols indicate significantly different at $P \leq 0.05$, within each sex.

protein (Table 2), P-450 levels or on P-450 reductase activity (Table 3) in animals of either sex at 140 days of age. In contrast, our results with PB imprinting showed that, although microsomal protein was not increased, both P-450 levels and the activity of P-450 reductase were increased at 140 days.

The metabolism of various substrates in 140-day-old animals is shown in Table 3. The N-demethylation of EM was not altered by neonatal 3-MC. There was also no treatment-related effect on male EC O-deethylation; however, females that received neonatal 3-MC had significantly lower activity. This is in contrast to our finding with PB programming which increases EC metabolism in both sexes [4]. This decrease may be related to the high incidence of tumors in these females. BP hydroxylation, a reaction known to be induced by 3-MC, was also measured. The results in Table 3 show that neonatal 3-MC had no effect on this activity at 140 days of age. Although we did not measure this activity in our PB-imprinted animals, Simpson and Chung [6] showed that, under their protocol for PB programming, this enzyme activity is increased.

Because virtually no effect of neonatal 3-MC was seen at 140 days, another group of rats was assayed at 200 days of age. Three out of these five females and one out of the five males had mammary tumors at this time point.

Table 4 shows no treatment-related effect on growth variables in 200-day-old animals in spite of the tumor presence. It should be noted that, since the number of tumors at this age was approximately the same in the females as at 140 days, the 140-day-old females had much larger tumors. This may explain the discrepancy in weight alterations produced by neonatal 3-MC at these two ages.

Although total hepatic microsomal protein was not affected at this age (Table 4), there was a slight decrease in P-450 levels (Table 5) in these animals (although only statistically significant in the females). Table 5 also shows that, although P-450 reductase activity was not altered in the females, it was reduced by nearly 50% in the males which were treated neonatally with 3-MC.

Table 5 illustrates the metabolism of various substrates in 200-day-old rats. EM *N*-demethylase, BP hydroxylase and EC *O*-deethylase activities were decreased in both males and females treated neonatally with 3-MC.

By 200 days of age a total of seven out of ten females and one out of ten males had developed large, solid, encapsulated breast tumors, whereas control animals showed no evidence of tumor formation. It is possible that the tumor presence was responsible for the decreased PSMOS activity due to a compromised state of health in these animals. Our previous studies have shown that PB administered during the first 5 days of neonatal life results in elevated hepatic metabolic enzyme activities at 140 days of age. The current study indicates that neonatal administration of another potent PSMOS inducer, 3-MC did not increase cytochrome P-450 levels or metabolism at adulthood. Our data would therefore seem to argue against a direct relationship between induction and imprinting. On the other hand, neonatal 3-MC treatment may actually alter PSMOS activity in a negative direction, at least at the dose used in this study. We have seen (unpublished observations) and Faris and Campbell have reported [5] that, whereas lower doses of PB imprint the PSMOS resulting in higher adult activities, higher doses result in either no effect or in decreased activities. It is therefore possible that using lower 3-MC doses may result in a positive imprinting effect. Lower doses may also alleviate the adverse effects of the tumor burden in the animals, which could have masked a positive imprinting effect. However, at 140 days of age there were no tumors in neonatally 3-MC-treated males. At 200 days there was only one male with a tumor, yet neither age showed increased PSMOS activities in response to 3-MC. Therefore, these results support the conclusion that neonatal 3-MC probably does not imprint PSMOS

Table 3. Effects of neonatal 3-MC on PSMOS activities in 140-day-old rats*

| Sex | Treatment | Cytochrome P-450 (nmoles/mg protein) | P-450 reductase (nmoles/mg protein/min) | Ethylmorphine *N*-demethylase (nmoles/mg protein/min) | Ethoxycoumarin *O*-deethylase (nmoles/mg protein/min) | Benzo[*a*]pyrene hydroxylase (pmoles/mg protein/min) |
|---|---|---|---|---|---|---|
| M | 3-MC | 0.664 ± 0.062 | 25.3 ± 3.6 | 7.4 ± 1.1 | 0.16 ± 0.02 | 423.9 ± 98.8 |
| | Corn oil | 0.663 ± 0.044 | 19.4 ± 2.5 | 8.2 ± 1.0 | 0.15 ± 0.05 | 373.4 ± 91.0 |
| F | 3-MC | 0.432 ± 0.048 | 10.0 ± 1.2 | 1.3 ± 0.3 | 0.05 ± 0.01† | 254.7 ± 48.2 |
| | Corn oil | 0.482 ± 0.087 | 11.4 ± 1.2 | 1.9 ± 0.4 | 0.11 ± 0.02‡ | 257.9 ± 38.1 |

* Rats were injected s.c. with either 20 mg/kg 3-methylcholanthrene in corn oil or corn oil alone on days 1–5 postpartum.
†,‡ Different symbols indicate significantly different at $P \leq 0.05$, within each sex.

Table 4. Effects of neonatal 3-MC administration on growth variables in 200-day-old rats*

| Sex | Treatment | Body wt (g) | Liver wt (g) | Liver wt/100 g body wt | Microsomal protein (mg/g liver) |
|---|---|---|---|---|---|
| M | 3-MC | 481 ± 34 | 16.0 ± 1.6 | 3.4 ± 0.3 | 15.2 ± 1.9 |
| | Corn oil | 522 ± 15 | 16.3 ± 0.8 | 3.1 ± 0.1 | 13.2 ± 0.1 |
| F | 3-MC | 274 ± 15 | 9.0 ± 0.8 | 3.3 ± 0.2 | 14.8 ± 0.7† |
| | Corn oil | 300 ± 11 | 9.5 ± 0.7 | 3.1 ± 0.2 | 12.0 ± 0.9‡ |

* Rats were injected s.c. with either 20 mg/kg 3-methylcholanthrene in corn oil or corn oil alone on days 1–5 postpartum.
†,‡ Different symbols indicate significantly different at $P \leq 0.05$, within each sex.

Table 5. Effects of neonatal 3-MC administration on PSMOS activities in 200-day-old rats*

| Sex | Treatment | Cytochrome P-450 (nmoles/mg protein) | P-450 reductase (nmoles/mg protein/min) | Ethylmorphine *N*-demethylase (nmoles/mg protein/min) | Benzo[*a*]pyrene hydroxylase (pmoles/mg protein/min) | Ethoxycoumarin *O*-deethylase (nmoles/mg protein/min) |
|---|---|---|---|---|---|---|
| M | 3-MC | 0.728 ± 0.017 | 20.2 ± 1.5† | 5.1 ± 1.0† | 349.9 ± 51.1† | 0.15 ± 0.02† |
| | Corn oil | 0.810 ± 0.045 | 35.4 ± 3.0‡ | 7.9 ± 0.3‡ | 479.7 ± 29.3‡ | 0.20 ± 0.01‡ |
| F | 3-MC | 0.423 ± 0.016† | 19.4 ± 3.0 | 1.4 ± 0.2† | 253.5 ± 15.8† | 0.08 ± 0.01 |
| | Corn oil | 0.494 ± 0.020‡ | 18.8 ± 2.7 | 2.1 ± 0.1‡ | 314.4 ± 28.2‡ | 0.10 ± 0.02 |

* Rats were injected s.c. with either 20 mg/kg 3-methylcholanthrene in corn oil or corn oil alone on days 1–5 postpartum.
†,‡ Different symbols indicate significantly different at $P \leq 0.05$, within each sex.

activities under the conditions employed in this study. To further clarify this area, we are also currently examining the ability of a non-carcinogenic "P-448 type" inducer, β-naphthoflavone (BNF), to neonatally program the hepatic PSMOS. Preliminary findings indicate that neonatally administered BNF did not alter adult PSMOS activities.

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# Factors influencing drug sulfate and glucuronic acid conjugation rates in isolated rat hepatocytes: significance of preincubation time

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Certain phenolic drugs such as salicylamide and acetaminophen are metabolized by glucuronidation and sulfation *in vivo*. Both conjugations are bisubstrate reactions with UDP-glucuronic acid (UDPGA) in glucuronidation and with 3′-phosphoadenosine-5′-phosphosulfate (PAPS) in sulfation. To study these reactions *in vitro*, isolated hepatocytes apparently retaining the essential properties of intact liver for cosubstrate biosynthesis and enzyme activities are often used [1–9]. Under the best experimental conditions reflecting reactivity *in vivo*, it should be possible to determine the pharmacokinetics in drug conjugative metabolism from kinetic data in hepatocytes.

Hepatocytes, following preparation, are generally stored at 0–4° to maintain high viability [3, 5, 9, 10]. The cells are preliminarily incubated at 37° just before the reaction but the preincubation time may vary from 0 min until 20 min [1, 3, 4, 6, 7, 9]. Preincubation not only enhances the reaction temperature to 37° but activates various cells to function physiologically. In glucuronidation and sulfation, the extent of activation of the biosynthesis of UDPGA and PAPS in enzyme systems by preincubation should have important influence on reaction rates.

In this paper, the effects of preincubation time on $K_m$ and $V_{max}$ of the glucuronidation and sulfation rates of acetaminophen (APAP) in hepatocytes are studied and the best reaction conditions are proposed.

## *Materials and methods*

APAP was purchased from Wako Pure Industries Co. Ltd. (Osaka, Japan). APAP glucuronide (APAP-Glu) and APAP sulfate (APAP-Sul) were synthesized by the methods of Shibasaki *et al.* [11] and Burkhardt and Wood [12], respectively. All other chemicals and reagents were of analytical grade or better. Hepatocytes were isolated from male Wister rats weighing 230–285 g by a slightly modified method of Baur *et al.* [10] reported previously [9]. After isolation, the cells were suspended in a reaction medium containing 4% bovine serum albumin at $2 \times 10^6$ cells/ml and stored in ice-water. The reaction medium consisted of 137 mM NaCl, 5.2 mM KCl, 0.9 mM $MgSO_4 \cdot 7H_2O$, 0.12 mM $CaCl_2 \cdot 2H_2O$ and 5 mM glucose buffered with 3 mM $Na_2HPO_4$ and 15 mM HEPES (*N*-2-hydroxyethylpiperazone-*N*′-2-ethanesulfonic acid). The final pH was adjusted to 7.4 with NaOH. Trypan blue exclusion was more than 94% immediately after preparation and endogeneous cellular respiration was normal [9]. One and a half ml of the cell suspension stored in ice-water were first incubated in a 30 ml Erlenmeyer flask at 37° for 0, 5, 10, 20, or 30 min before starting the reaction. Following this, 1.5 ml of the APAP solution of the same reaction medium (0.04–10 mM) were added. The mixture was then incubated for 11 min at 37° since both conjugation reactions at all APAP concentrations are linear until 11 min. Incubation was performed in a metabolic shaker (90 oscillations/min) to ensure a complete mixing of the reaction medium. A supply of 95% $O_2$–5% $CO_2$ to the incubation mixture was unnecessary since this gas has no significant effect on the extent of conjugation [9]. The conjugation reaction was brought to a stop by adding 0.5 ml of the incubation mixture to 0.1 ml of 25% perchloric acid containing 1.8 mM 4-fluorophenol as the internal standard (IS). Then 0.2 ml of 0.8 M $BaCl_2$ solution was added to the mixture followed by centrifugation for 10 min at 3000 rpm. The supernatant was used for the reversed-phase HPLC assay. The HPLC apparatus and conditions were virtually the same as reported previously [13, 14]. The mobile phase was water:methanol:acetic acid (81:17.5:1.5 v/v/v) containing 100 mg/l $KNO_3$ and 26.4 mg/l tetrabutylam-