Biochemical Pharmacology, Vol. 25, pp. 2099–2101. Pergamon Press, 1976. Printed in Great Britain.

Hepatic steroid hydroxylase and aminopyrine *N*-demethylase activities in pregnant rats and rabbits, and the effect of phenobarbital

(*Received* 11 *August* 1975; *accepted* 13 *February* 1976)

The activities of certain hepatic mixed-function oxidases for drugs and steroids are altered by castration or administration of sex hormones [1–3]. Sex, age and species differences, and responses to various chemicals have been exploited to demonstrate that separate hemoproteins and groups of hepatic enzymes participate in different microsomal biotransformations [4–7]. Rates of the androgen-dependent microsomal 16α- and 7α-hydroxylases of dehydroepiandrosterone (DHA) and *N*-demethylase of aminopyrine in rat liver are increased by pretreatment with androgens and various drugs including phenobarbital (PB) [4, 8], which also increase levels of hepatic cytochrome P-450 [4, 9]. Different responses of DHA 16α- and 7α-hydroxylase and aminopyrine *N*-demethylase activities continue to accumulate [2, 3] with recent additional evidence for the greater sensitivity of the 16α-hydroxylase activity for androgen treatments [10]. The 16α-hydroxylation of testosterone and the *N*-demethylation of aminopyrine appear to be regulated by a common factor which differs from that for 7α-hydroxylation [11]. Since the endocrine state of the animals is an important variable in certain of these enzyme activities, we have studied *in vitro* the maternal hepatic oxidative metabolism of DHA and aminopyrine during normal pregnancies of rats and rabbits. The effects of phenobarbital treatments in these species during pregnancy were also investigated.

Non-pregnant (10–13 weeks of age) or pregnant Sprague–Dawley rats (A R Schmidt Co., Madison, Wis.) with dated gestations were maintained on Purina Laboratory pellets and water *ad lib*. The treated animals were alert, eating and drinking freely, although food consumption was not measured. Intraperitoneal (i.p.) treatments were for 4 consecutive days with sodium phenobarbital (Luminal, Winthrop Labs, New York, N.Y.; 40 mg/kg body wt on days 1 and 2, 80 mg/kg on day 3, and 120 mg/kg on day 4, a schedule devised experimentally because the usual 80 mg/kg dosage sedated the pregnant animals). Mature virgin or pregnant (3 and 8 days after mating) New Zealand rabbits were treated daily for 5 consecutive days with i.p. injections of PB (40 mg/kg body wt). Control animals received vehicle only. The day after the last treatment the animals were killed between 8:00 and 9:00 a.m. The livers were promptly removed, weighed and thoroughly perfused with isotonic saline, and microsomal fractions were prepared [12]. Final suspensions in Krebs–Ringer phosphate buffer, pH 7.4, contained the microsomes from 1 g liver/ml. Duplicate incubations were carried out in 25-ml incubation flasks containing 1.1 μmoles DHA as substrate in 0.1 ml propylene glycol, 12 μmoles of reduced NADP, 0.2 ml of microsomal protein (about 2 mg) in a final volume of 6 ml incubation medium [4]. The mixture was incubated in air at 37° for 30 min, and the reactions were stopped by placing the flasks in crushed ice. These conditions of excess substrate and NADPH produce linear increases of the transformation products with respect to tissue concentration or incubation time.

The incubation mixtures were extracted twice with 40 ml $CHCl_3$, and the combined extracts were washed with water, dried with anhydrous Na_2SO_4, filtered and evaporated *in vacuo*. The products of the reactions were isolated by thin-layer chromatography (ethylacetate–*n*-hexane–glacial acetic acid, 75:20:5). The plates were developed twice and the Silica gel in areas corresponding to the reference steroids was scraped off and eluted with 95% methanol. Recoveries of the metabolites ranged from 87 to 97 per cent. Duplicate portions were evaporated *in vacuo* for colorimetric analysis with the blue tetrazolium (BT) reaction for 3β,16α-dihydroxy-androst-5-en-17-one (16α-OH-DHA), and a modified Lifshutz reaction for the 7-hydroxylated isomers of DHA [4]. The *N*-demethylation of aminopyrine was quantitated by measurement of the complex formation of liberated formaldehyde with acetylacetone and NH_3 [13]. The microsomal content of cytochrome P-450 was measured as the carbon monoxide-induced difference in absorbance at 450 and 490 nm after dithionite reduction with Beckman DK-2A ratio-recording spectrophotometer using 91 $mM^{-1}\ cm^{-1}$ as the extinction coefficient [14]. The microsomal protein was measured with the biuret reaction [15], using crystalline bovine serum albumin as standard.

Body and liver weights of both species increased progressively during pregnancy. These data, the microsomal protein, and their responses to phenobarbital (PB) are summarized in Table 1. Liver weights of PB-treated rats were similar to control animals despite the heavier carcasses of the latter, but PB reduced the microsomal protein content at mid- and late-pregnancy. In rabbits, PB treatment increased the microsomal protein content at mid-pregnancy.

Hepatic cytochrome P-450 content and rates of hepatic oxidative metabolism of DHA and aminopyrine in pregnancy and after PB pretreatment are summarized in Table 2 (rats) and Table 3 (rabbits). In pregnant rats, the trend of hepatic microsomal cytochrome P-450 content (nmoles/mg of protein) was downward from the maximal value seen early in gestation (8 days), and its response to PB was blunted at term (22 days gestation). The rate of 7α-hydroxylation was maximal in early pregnancy (8 days), 0.95 ± 0.43 nmole/min/mg (mean ± S. D.), and decreased progressively to 0.26 ± 0.02, a rate significantly less than those of non-pregnant females. PB treatment stimulated the rates of 7α-hydroxylation in all groups. In contrast to the 7α-hydroxylation, the lower rates of 7β-hydroxylation did not change significantly with pregnancy, but this activity was also increased by PB. The rates of 16α-hydroxylation were initially suppressed by pregnancy, and increased to 0.21 ± 0.13 during mid-pregnancy (14–17 days), falling again near term (22 days) to 0.06, a mean level similar to those of non-pregnant females. The rates of 16α-hydroxylation responded to PB only at the end of pregnancy and in non-pregnant females. The *N*-demethylation of aminopyrine exhibited a similar trend to the 7α-hydroxylation, exceeding in early pregnancy the values of non-pregnant females, but decreasing thereafter toward term. PB treatment stimulated the *N*-demethylase activity at all gestational ages as well as in non-pregnant females, as was also observed for 7α-hydroxylation.

In rabbits, hepatic cytochrome P-450 content was decreased in early gestation, but PB increased the mean values of the hemoprotein in all groups. Similarly, early pregnancy decreased all of the enzyme activities which were subsequently restored. The rates of 7β-hydroxylation in this species were larger than those of 7α-hydroxylation, and the mean rates of the former transformation increased

Table 1. Effects of phenobarbital pretreatment on body and hepatic weights, and microsomal protein concentration of pregnant rats and rabbits

Day of gestation	Pretreatment	No. of animals	Body wt*	Liver wt (g)	Microsomal protein (mg g liver)
Rat					
0	None	8	262 ± 49	10.0 ± 0.5	5.9 ± 0.6†
	PB‡	6	238 ± 33	10.1 ± 0.9	6.8 ± 0.8
8	None	3	263 ± 15	10.6 ± 0.5	2.3 ± 0.3
	PB	2	239	12.0	3.9 ± 0.2
14–17	None	5	286 ± 29	12.4 ± 1.6	6.8 ± 2.8
	PB	5	264 ± 22	13.2 ± 1.6	3.8 ± 1.4
22	None	4	365 ± 29	13.2 ± 0.9	7.7 ± 1.0
	PB	2	276	13.0	5.8 ± 0.4
Rabbit					
0	None	3	3.7 ± 0.3	86 ± 12	6.3 ± 2.7
	PB	4	3.5 ± 0.2	117 ± 9	5.9 ± 0.3
8	None	6	3.8 ± 0.3	121 ± 11	7.7 ± 1.4
	PB	3	4.0 ± 0.2	107 ± 2	7.0 ± 1.5
14	None	2	3.9	125	3.5 ± 0.6
	PB	3	3.8 ± 0.3	120 ± 5	8.2 ± 1.6

* Body weight of the rats was measured in grams and that of rabbits in kilograms.
† Values are mean ± S. D. of duplicate data from each animal.
‡ PB = phenobarbital.

Table 2. Effects of phenobarbital pretreatment of pregnant rats on the hepatic cytochrome P-450 concentration and hydroxylase activities of DHA and *N*-demethylase activities of aminopyrine

Days of gestation	Pretreatment	Cytochrome P-450 (nmoles/mg)	DHA hydroxylation (nmoles/min/mg)			Aminopyrine *N*-demethylation (nmoles/min/mg)
			7α-	7β-	16α-	
0	None	0.38 ± 0.12	0.45 ± 0.20	0.05 ± 0.04	0.11 ± 0.10	2.98 ± 1.40
	PB*	0.70 ± 0.21†	1.25 ± 0.38†	0.38 ± 0.10†	0.26 ± 0.05†	6.79 ± 1.18†
	Δ*	+84	+177	+660	+136	+127
8	None	0.47 ± 0.06	0.95 ± 0.43‡	0.01 ± 0.01	0.02 ± 0.01‡	5.24 ± 1.54‡
	PB	NA*	2.21	NA	NA	9.10 ± 3.62
	Δ		+131.6			+73
14–17	None	0.39 ± 0.04‡	0.38 ± 0.23‡	0.02 ± 0.01	0.21 ± 0.13§	2.68 ± 1.14§
	PB	NA	1.60 ± 0.61†	0.29 ± 0.14†	<0.02	6.28 ± 2.47
	Δ		+321	+1350	<−90	+134
22	None	0.34 ± 0.06‡	0.26 ± 0.02‡,§	0.07 ± 0.05	0.06 ± 0.05	1.18 ± 0.60‡,§
	PB	0.48 ± 0.05†	2.04 ± 1.63†	0.25 ± 0.05†	0.57 ± 0.47†	2.86 ± 0.26†
	Δ	+41	+684	+177	+850	+142

* Abbreviations: PB = phenobarbital; Δ = per cent increase (+) or decrease (−) of mean values compared with comparable untreated controls; and NA = not available.
† Significantly different from the untreated controls of the same gestational age at $P < 0.05$ (Student's *t*-test).
‡ Significantly different from non-pregnant controls (0) at $P < 0.05$.
§ Significantly different from pregnant animals (8-day gestation) at $P < 0.05$.

Table 3. Effects of phenobarbital pretreatment of pregnant rabbits*

Day of gestation	Pretreatment	Cytochrome P-450 (nmoles/mg)	DHA hydroxylation (nmoles/min/mg)			Aminopyrine *N*-demethylation (nmoles/min/mg)
			7α-	7β-	16α-	
0	None	1.27 ± 0.23	0.12 ± 0.04	0.12 ± 0.04	1.31 ± 0.51	2.91 ± 0.93
	PB	1.84 ± 0.81	0.10 ± 0.09	0.15 ± 0.04	1.27 ± 0.37	3.47 ± 0.74
	Δ	+44	+11	+25	−3	+19
8	None	0.79 ± 0.28‡	0.03 ± 0.01†	0.13 ± 0.10	0.73 ± 0.32	2.06 ± 1.13
	PB	1.44 ± 0.23†	0.07 ± 0.06	0.36 ± 0.20†	1.44 ± 0.47†	5.78 ± 1.91†
	Δ	+82	+133	+176	+97	+180
14	None	1.34 ± 0.31§	0.09 ± 0.06§	0.52 ± 0.44§	1.33 ± 0.93	2.91 ± 0.94
	PB	2.22 ± 0.60	0.03 ± 0.01	0.42 ± 0.28	0.70 ± 0.38	6.29 ± 2.24
	Δ	+65	−66	19	−47	+116

* See the legend of Table 2. Abbreviations: PB = phenobarbital; and Δ = per cent increase (+) or decrease (−) of mean values compared with comparable untreated controls.
† Significantly different from the untreated controls of the same gestational age at $P < 0.05$. (Student's *t*-test).
‡ Significantly different from non-pregnant controls (0) at $P < 0.05$.
§ Significantly different from pregnant animals (8-day gestation) at $P < 0.05$.

4-fold by mid-pregnancy to 0.52. In rabbits, the rates of steroid transformation were stimulated 1 to 2-fold by PB only in early pregnancy, and in contrast to *N*-demethylation, they responded to this drug neither in non-pregnant animals nor at mid-pregnancy.

In the present study with rats, the hepatic microsomal content of cytochrome P-450 and the enzymic rates of *N*-demethylation of aminopyrine and 7α-hydroxylation of DHA were significantly increased early in pregnancy over non-pregnant levels, but by the end of pregnancy these values had decreased significantly below those of non-pregnant females. This correlation between drug- and steroid-metabolizing enzymes has previously been noted in non-pregnant rats [4, 10], and now appears in pregnancy, either with or without PB treatment. Previous workers have demonstrated that pregnancy in rats suppresses several drug-metabolizing enzyme activities, and that cytochrome P-450 content was slightly increased in comparison to non-pregnant animals [16, 17]. In rabbits, only the *N*-demethylase activity follows the changes in cytochrome P-450. All of the steroid hydroxylase activities are depressed by PB treatment of mid-pregnant rabbits. We find no published reports of steroid hydroxylase activities in the liver of rats or rabbits during pregnancy.

It is clear from these and other studies that pregnancy and species differences profoundly influence maternal hepatic steroid- and drug-metabolizing activities and their selective responses to PB in rats and rabbits. Nevertheless, gross inspection of the embryos and reproductive tracts of PB-treated animals revealed no abnormalities, and the mothers appeared healthy. An activating effect of antenatal PB therapy on bilirubin conjugation by human newborn has been observed repeatedly, although little attention has been given to its effects on maternal metabolism (see Ref. 18 for a review). Preliminary observations (unpublished) in human pregnancies complicated by erythroblastosis indicate that the major steroid biosynthetic pathway leading to the maternal excretion of estriol is not altered by antenatal PB therapy.

Acknowledgements—The valuable suggestions by Mont R. Juchau, Ph.D., Department of Pharmacology, during the preparation of this manuscript are appreciated. The technical assistance of Ms. Mary Waltman, Kimiko Fukushima and Robert Fallis, Ph.D., and the support of the Ford and The Lalor Foundations, and PHS Grant HD 03825 are gratefully acknowledged. The typing of the manuscript by Mrs. Ann Dow is also greatly appreciated.

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Biochemical Pharmacology, Vol. 25, pp. 2101–2102, Pergamon Press, 1976. Printed in Great Britain.

Effect of ingestion of *Lantana camara* L. on bile formation in sheep

(*Received* 17 *March* 1976; *accepted* 7 *April* 1976)

The triterpene acids lantadene A and icterogenin have been shown in rabbits to inhibit hepatic transport of porphyrins, bile pigments and bromsulphthalein [1], but little information is available on their effects on other transport processes which may be involved in bile formation. Some observations have been made on pathological changes in the sheep's liver and biliary tract after ingestion of the plant *Lantana camara*, an important source of lantadene A [2, 3, 4]. These pathological changes include jaundice, gall bladder distension, and proliferation of bile ductules [3]. In sheep, the major stimulants to bile secretion are the bile acid, taurocholic acid, and the hormone secretin [5, 6, 7]. These stimulants appear to act at different sites: the bile acid at the canaliculi, and secretin at the ductules. For this reason, they were used to analyse the effects of lantana on bile formation.

These studies were made on four crossbred sheep which had been operated on to remove the gall bladder, and to