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RATES OF PROGESTERONE OXIDATION BY RABBIT LIVER MICROSOMES BEFORE AND AFTER PHENOBARBITONE TREATMENT

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Summary—Liver sections, removed from outbred NZW rabbits under anesthesia, were used to determine the rates of progesterone oxidation prior to assessment of the effects of phenobarbitone(PB)-treatment. Progesterone 21-hydroxylase exhibited the same high affinity, low K_m kinetics before and after PB-treatment, whereas the V_{max} was significantly reduced. PB-treatment did not affect progesterone 21-hydroxylation by the adrenal microsomes of two groups of PB-treated and untreated rabbits. 21-Hydroxylase activity was detected for the first time with spleen microsomes but the majority of spleens examined lacked activity. 6β-Hydroxylation by control liver microsomes showed consistently low affinity, high K_m-values and was inhibited to a lesser, inconsistent degree than 21-hydroxylation by PB-treatment.

INTRODUCTION

The rabbit has been widely used to study the multiplicity of endogenous and induced cytochrome P-450 isozymes [1] and steroids have proved useful substrates for the evaluation of the regiospecificities of various oxidations [2–5]. Progesterone is oxidized at C6- [6–8], C16- [8–9] and C21- [7, 107] by rabbit liver microsomes. Considerable intraspecies variability in progesterone 21-hydroxylase activity has been documented with the outbred NZW rabbit [11, 12]. Selection of appropriate rabbits with high levels of hepatic 21-hydroxylase activity assisted substantially in the isolation of the associated cytochrome P-450 isozyme [13]. Variations in progesterone 6β-hydroxylase activity have also been observed, with one variant strain of inbred rabbit reported to be deficient [14]. Intraspecies variability in steroid oxidations presents problems for the precise assessment of the effects of potential inducers or inhibitors. The approach adopted in the present study assesses the *in vitro* oxidation of progesterone by individual rabbits by sampling liver slices from anesthetized animals in order that each animal may serve as its own control.

EXPERIMENTAL

Outbred NZW rabbits (3–4.5 kg) or both sexes were starved overnight and anesthetized with Innovar-Vet (fentanyl citrate; M.T.C. Pharmaceuticals, Canada) at a dose of 0.2–0.3 ml/kg body wt, given i.m. together with atropine sulphate (0.1 ml; Squibb, Canada Inc.) to maintain cardiac function. The operation was conducted under sterile conditions and one lobe of the liver (3–7 g wet wt) was removed through a midline incision. The incision was closed with surgical gut (plain 00; Ethicon) and the rabbits allowed a minimum of 2 weeks to recover.

Phenobarbitone sodium (0.1% w/v aqueous solution, pH 7.0; BDH Chemicals) was supplied to rabbits in drinking water for 5 consecutive days. On the second and fourth days phenobarbitone sodium (40 mg in 0.9% saline/kg body wt) was given i.p. Rabbits were allowed access to water for 24 h after 5 days and were starved overnight and then killed by cervical dislocation. Liver, adrenals and spleen were removed.

Preparation of microsomes

Liver microsomes were prepared by differential centrifugation. A Waring blender was used for the total liver (4 ml homogenization buffer/g wet wt) and a Potter–Elvjhem glass–Teflon homogenizer for the liver sections, adrenals and spleen. All other operations were scaled down from those previously reported [12] with the addition of a final wash of the 105,000 *g* sedimented microsomal pellets with sodium pyrophosphate to remove hemoglobin contamination [15]. Microsomes were suspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA and stored at −70°C until processed. Under these conditions steroid monooxygenase activity was preserved for 1–2 months. Microsomes were, however, routinely incubated within 1–2 days following preparation.

Kinetic studies

[4-^{14}C]Progesterone (0.1 μCi; 54.2 mCi/mmol; New England Nuclear Canada Ltd) was diluted with non-labeled progesterone to give 1–10 nmol progesterone

Abbreviations: PB, phenobarbitone sodium; DOC (deoxycorticosterone), 21-hydroxy-4-pregnen-3,20-dione; 6α/6β-OHP; 6α/6β-hydroxy-4-pregnen-3,20-dione; 6β-OHDOC, 6β,21-dihydroxy-4-pregnen-3,20-dione; 16α-OHP, 16α-hydroxy-4-pregnen-3,20-dione; NZW, New Zealand white.

per 20 μl aliquot of methanolic solution. Kinetic reactions were carried out for 5 min after 3 min preincubation at 37°C in potassium phosphate buffer (0.1 M; pH 7.4) in a shaking water bath. Sufficient microsomal protein was incubated to give 10–15% metabolism of the substrate and provide first-order reaction kinetics. Reaction was initiated by the addition of NADPH (500 μM) contained in 0.1 ml buffer to give a 1.0 ml total incubation volume. The reaction was terminated by extraction with ethyl acetate (2 × 5 ml) which removed an average of 95% of the radioactivity. The solvent was evaporated under a nitrogen stream and the residue spotted with u.v. absorbing reference steroids on Anasil-OF thin-layer plates (250 μm; 5 × 20 cm, Analabs, U.S.A.). The plates were developed 20 cm in chloroform–ethyl acetate (6:4, v/v) which effected separation of the following steroids, listed by R_f-value: progesterone (0.77); DOC (0.43); 6β-OHP (0.33); 6α-OHP (0.28); 6β-OHDOC (0.16); 16α-OHP (0.12). Radiometabolites corresponding to DOC and 6β-OHP standards were well-resolved and their identity confirmed as previously described [10], whereas radioactivity that corresponded to the 16α-OHP/6β-OHDOC standards (Zone 2) was less consistently well-resolved in this TLC system and was measured as a single peak. Radiometabolites were located with a radiochromatogram scanner (Model 7201) and the corresponding silica gel areas were scraped into glass vials containing a PCS–xylene scintillation mixture (10 ml; 1:1, v/v; Amersham, U.S.A.). The vials were vibromixed, allowed to stand in the dark until background scintillation decayed and were counted in a Beckman liquid scintillation counter (LS-3801).

RESULTS

Rates of progesterone oxidation by liver section microsomes

Microsomes prepared from liver sections removed from anesthetized rabbits metabolized progesterone at similar rates and gave similar metabolite spectra to the microsomes prepared from the total liver of the same rabbits following sacrifice. The rates of progesterone 21-hydroxylation by the liver section microsomes also showed wide intraspecies variability (0.09–7.2 nmol DOC formed·min^{-1}·mg protein^{-1}, $n = 22$) with no correlation discernible with sex, age or body weight. The rates of progesterone oxidation by liver section microsomes from four rabbits are given in Fig. 1. Although the V_{max} for 21-hydroxylation varied 5-fold in the rabbits shown, the microsomal enzymes all exhibited similar high affinity, low K_m kinetics. By contrast 6β-hydroxylation was consistently characterized by low affinity, high K_m kinetics. The latter was not accurately measurable and appeared to exceed the reported solubility of progesterone (60 μM) in aqueous buffer [8]. Hyperbolic plots of 6β-OHP formation

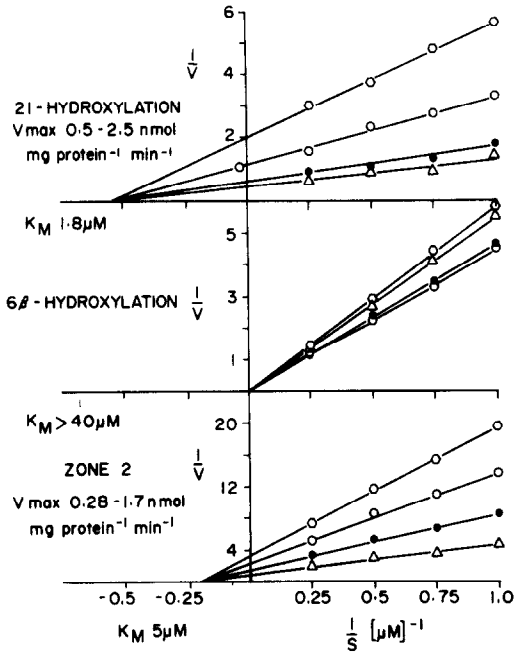

Fig. 1. Comparison of Lineweaver–Burk plots of progesterone hydroxylation by hepatic microsomes. Microsomes were prepared from liver sections removed from rabbits under anesthesia. [^{14}C]progesterone (1–4 nmol) was incubated with microsomes (1.0 mg protein) for 5 min and the radiometabolites quantitated after TLC. Zone 2 radiometabolites were not further resolved.

vs substrate concentration were not obtained and as shown in Table 1, 6β-hydroxylase activity, unlike 21-hydroxylase activity, was not saturated at a 20 nM substrate concentration. Figure 1 also gives the corresponding Lineweaver–Burk plots for the Zone 2 radioactive peak (R_f 0.15). Linear plots were obtained and a computed 5 μM K_m and a V_{max} range 0.28–1.7 nmol product·mg protein^{-1}·min^{-1} based on 16α-OHP.

Effect of PB-treatment on progesterone 21-hydroxylation

Treatment with PB induced a 2- to 2.5-fold increase in microsomal cytochrome P-450 content to levels of 2.3–2.9 nmol P-450·mg protein^{-1}. Figure 2 compares progesterone 21-hydroxylase activity in

Table 1. Rates of progesterone metabolism at different substrate levels

	nmol product·mg protein^{-1}·min^{-1} (mean ± SD)		
Product	1	10	20
6-OHP	0.197 ± 0.03	1.99 ± 0.41	3.64 ± 0.94
DOC	0.400 ± 0.22	1.07 ± 0.54	1.24 ± 0.63
Zone 2	0.116 ± 0.07	0.50 ± 0.15	0.88 ± 0.25

[^{14}C]progesterone, admixed with non-labeled progesterone to give the concentrations indicated, was incubated for 5 min and the radiometabolites separated by TLC. Zone 2 contained unresolved 16α-OHP/6β-OHDOC. Results are the mean of microsomes prepared from rabbits which exhibited significant 21-hydroxylase activity.

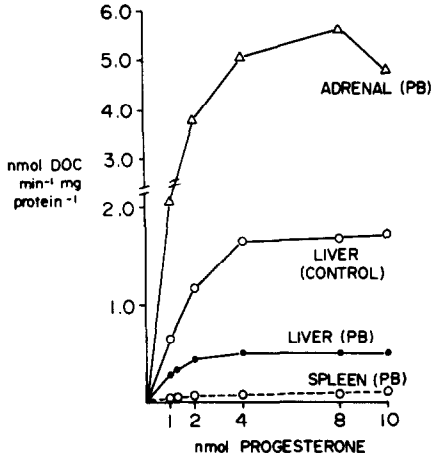

Fig. 2. Effect of substrate concentration on the rates of progesterone 21-hydroxylation. Adrenal (0.1 mg) liver (1.0 mg) and spleen (2.0 mg) microsomal protein was incubated with [^{14}C]progesterone (1–10 nmol) for 5 min and the radiometabolite peak corresponding to [^{14}C]DOC was separated by TLC and quantitated.

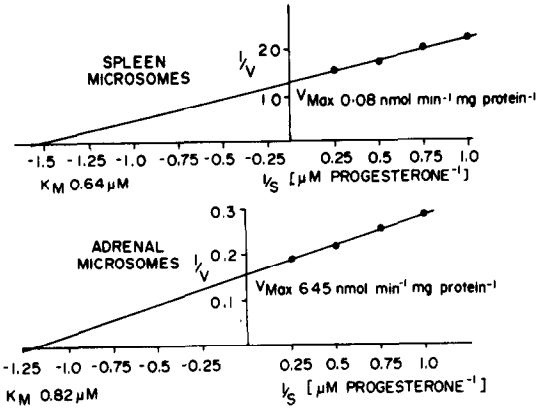

Fig. 4. Lineweaver–Burk analysis of DOC formation by adrenal and spleen microsomes. Adrenal (0.1 mg) and spleen (2.0 mg) microsomal protein, isolated from a PB-treated rabbit were incubated for 5 min with [^{14}C]progesterone (1–4 nmol) and the formation of [^{14}]DOC was quantitated after TLC.

liver microsomes before and after PB-treatment. Significant reduction occurred in all rabbits examined. Figure 3 gives Lineweaver–Burk plots of a typical preparation that shows a 70% decline in the V_{max} of DOC formation by PB-treatment whilst the K_m was unaltered. Several microsomal preparations with 21-hydroxylase activities ranging from 2.0–7.2 nmol DOC formed·mg protein^{-1}·min^{-1} were reduced by PB-treatment to V_{max}-values ranging from 0.70–0.75 nmol DOC formed·mg protein^{-1}·min^{-1}.

Figure 2 also compares the progesterone 21-hydroxylase activity of liver, adrenal and spleen microsomes from a PB-treated rabbit. The highest 21-hydroxylase activity was present in the adrenal microsomes. The effect of PB-treatment on adrenal microsomal 21-hydroxylase activity was considered by assaying activity with 3 rabbits which had been treated with PB and 3 untreated rabbits. No significant effect of PB-treatment was evident. V_{max}-values for DOC formation by adrenal microsomes ranged from 2.9–6.8 (5.1 ± 1.3 SD) and 4.54–7.14 (5.9 ± 2.0 SD) nmol·mg protein^{-1}·min^{-1} in the untreated and PB-treated rabbits, respectively.

Both adrenal and spleen microsomes exhibited high affinity, low K_m kinetics for progesterone 21-hydroxylation, though the V_{max} of the spleen was considerably lower (0.08 vs 6.45 nmol DOC formed·mg protein^{-1}·min^{-1} (Fig. 4). The detection of measurably progesterone 21-hydroxylase activity in the spleen microsomes of a PB-treated rabbit was also fortuitous since enzymatic activity was not de-

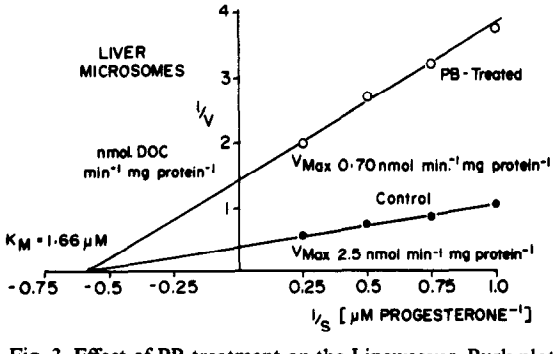

Fig. 3. Effect of PB-treatment on the Lineweaver–Burk plot of progesterone 21-hydroxylation. Microsomes (1.0 mg protein) prepared from rabbit livers before and after treatment with PB, were incubated with [^{14}C]progesterone (1–4 nmol) for 5 min and the formation of [^{14}C]DOC was quantitated after TLC.

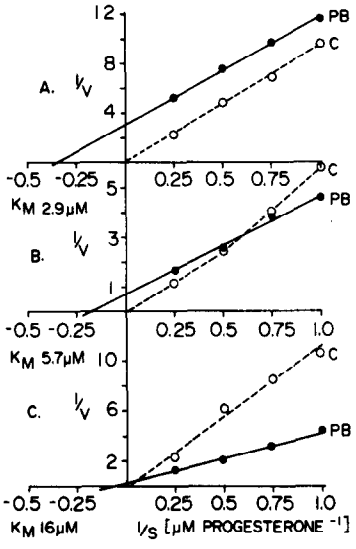

Fig. 5. Variable effects of PB-treatment on Lineweaver–Burk plots of progesterone 6β-hydroxylation. Microsomes (1.0 mg protein) from liver sections removed under anesthesia (control) are compared with microsomal preparations from the same rabbits after PB-treatment. A–C represent 3 different rabbits. Microsomes were incubated for 5 min and the radioactivity corresponding to 6β-OHP on the TLC was quantitated as described in the Experimental section.

Table 2. Effect of PB-treatment on progesterone oxidation by liver microsomes

	DOC			6β-OHP			Zone 2		
	C	PB	% Difference	C	PB	% Difference	C	PB	% Difference
	1.66	0.50	−70.0	0.90	0.64	−29	0.56	0.28	−50
	3.03	0.56	−81.5	0.42	0.91	+46	1.27	0.37	−71
	1.79	0.25	−86.0	0.46	0.19	−59	0.24	0.08	−67
Mean	2.16 ± 0.76	0.44 ± 0.16	−79.2 ± 8.3	0.59 ± 0.27	0.58 ± 0.36	—	0.69 ± 0.53	0.24 ± 0.15	62.7 ± 11.2

Microsomes (0.1 mg) from rabbits before and after PB-treatment were incubated for 5 min with 4 nmol progesterone.
Results are expressed as *nmol product·mg protein*$^{-1}$·min^{-1}, mean ± SD. Radioactivity corresponding to [^{14}C]DOC, [^{14}C]6β-OHP and Zone 2 was quantitated after TLC, as described in the Experimental section.

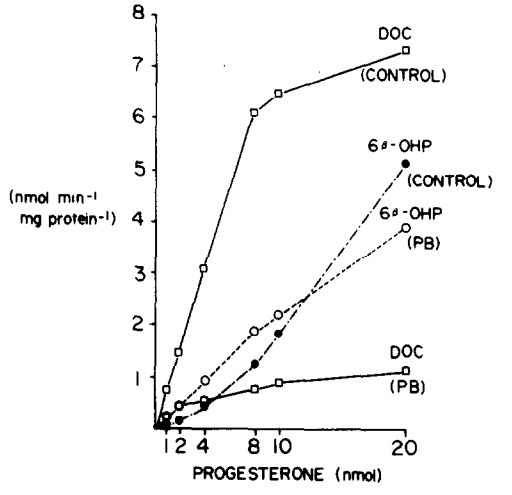

Fig. 6. Comparison of the effects of substrate concentration and PB-pretreatment on 21- and 6β-hydroxylation by rabbit liver microsomes. [^{14}C]progesterone plus non-labeled progesterone (1–20 nmol) was incubated with liver microsomes (1.0 mg protein) for 5 min and the formation of [^{14}C]6β-OHP and [^{14}C]DOC was quantitated after TLC.

tectably in the majority of spleens examined from both PB-treated and untreated rabbits.

Effect of PB-treatment on progesterone 21-hydroxylation

Figure 5 shows that PB-treatment had an erratic effect on 6β-hydroxylation, but tended to lower the K_m-value and reduce the reaction rate in 2 out of the 3 rabbits exemplified. The variable response to PB-treatment is summarized in Table 2. Inhibition of DOC and Zone 2 was consistent and comparable, whereas the 6β-hydroxylation response varied with the particular rabbit and the substrate concentration. Thus, 2 of the rabbits shown exhibited a decline in 6β-hydroxylase activity in the 1–4 nM substrate range, whereas the third rabbit did not. As shown in Fig. 6, the response of 6β-hydroxylation to PB-pretreatment in this rabbit was substrate dependent, and was inhibited by only 25% at substrate concentrations in excess of 10 nmol.

DISCUSSION

Wide intraspecies variability occurs in the level of hepatic microsomal progesterone 21-hydroxylase in outbred NZW rabbits [11, 12] that appears to parallel the specific content of cytochrome P-450 [11]. An accurate knowledge of the activity of this enzyme in a particular rabbit may be required for a meaningful interpretation of the actions of potentiators or inhibitors. Microsomes prepared from liver sections removed from rabbits under anesthesia provide a means of determining the monooxygenase activity so that individual animals may serve as their own controls, after an appropriate recovery period.

Kinetic analysis of the rates of progesterone hydroxylases in liver section microsomes showed that

for progesterone 21-hydroxylase, though the V_{max} of DOC formation may vary 5-fold, the apparent K_m remained the same with a value of 1.8 μM indicating a high affinity for progesterone. The apparent K_m-values for the 6β-hydroxylation of progesterone were too high to be accurately measured (>40 μM). In this respect these outbred NZW rabbits differed from those of another laboratory but resembled an incipient inbred strain (111VO/J) [8]. Unlike the latter, however, which exhibited a high K_m and low 6β-hydroxylase activity, the rabbit liver microsomes used in the present study catalyzed 6β-hydroxylation at a significant rate. The significance of the high K_m is uncertain since substrate saturation was not achieved at a substrate concentration of 20 nmol progesterone, a value that exceeds physiological concentrations. Nevertheless, 6β-hydroxylation plays a major role in progesterone metabolism as evidenced by the accumulation of 6β-OHP, and particularly 6β-OHDOC with liver microsomes having high 21-hydroxylase activity, when incubation times are extended beyond 5 min (details not shown). The level of 16α-hydroxylase activity was not precisely estimated in the present study since 16α-OHP and 6β-OHDOC were both present at 5 min incubation to variable extents and cochromatographed in Zone 2 in the TLC system used. Linear Lineweaver–Burk plots of Zone 2 radiometabolites were obtained with an apparent K_m of 5 μM for progesterone which is of a similar order reported for 16α-OHP formation [8].

Considerable species differences exist with respect to 6β- and 16α-hydroxylases. Orton and Philpot[16] compared testosterone oxidation by rat and rabbit liver microsomes and found apparent K_m-values for 6β-, 16α- and 2β-hydroxylations of 27, 29 and 19 μM, respectively, in the rat compared to values of 44 and 760 μM for 6β- and 16α-hydroxylation of testosterone by the rabbit. The response to PB-pretreatment was also species-dependent. It is well-established that pretreatment of experimental animals with PB results in an increase in cytochrome P-450 and early studies claimed increased formation of polar progesterone metabolites in the rabbit and guinea pig [17] and testosterone metabolites in the rat [18]. Recent studies indicate that PB-treatment may result in a redistribution of enzyme activities, with for example, an increased activity of 2β-, 6β-, 7α- and 16β-hydroxylation and a reduction in 16α- and 2α-hydroxylation in microsomes from PB-treated rats [19, 20]. Unlike the rat, rabbit liver microsomal monooxygenase activity has generally been inhibited by pretreatment. Thus, Orton and Philpot[16] reported reduced 2β-, 7α-, 6β- and 16α-hydroxylation (62.9–83.3% of control) for testosterone and Tabei and Heinrichs[21] a 19–66% reduction in the 7β-, 16α- and 7α-hydroxylation of dehydroepiandrosterone following PB-treatment of rabbits at 14 days of gestation. The present study shows a clear-cut inhibition by 72% in the V_{max} for DOC formation without a change in the apparent K_m. Whether this was the effect of reduced cytochrome P-450$_{21}$ Form 1 content in the microsomes has not been determined. Cytochrome P-450 content was increased 2- to 2.5-fold, whereas 21-hydroxylase activity was reduced 5-fold. In addition mere dilution of the cytochrome P-450 content or diminution of the hemoprotein pool for induction of the PB-specific cytochrome P-450$_{LM2}$ would not explain the failure to consistently reduce 6β-hydroxylase activity. The shift in apparent K_m-value may indicate the inhibition of the activities of other cytochrome P-450 isozymes which possess low 6β-hydroxylase activity and the partial expression of a higher affinity isozyme, which has been attributed to the P-450$_{LM3b}$ form [8, 14].

PB-pretreatment did not appear to have any effect on adrenal progesterone 21-hydroxylase activity which is in accord with a previous undocumented statement by Ichikawa *et al.*[22]. The non-identity of the bovine adrenocortical and hepatic microsomal cytochrome P-450 21-hydroxylases was also established immunologically by these workers. In particular, anticytochrome b_5 immunoglobulin was only effective against the hepatic 21-hydroxylase. We have previously found cytochrome b_5 to be stimulating with the reconstituted rabbit liver 21-hydroxylase system [23].

Extraadrenal progesterone 21-hydroxylase activity has been found in rabbit liver and kidney [12] and has now been extended to the spleen. The fact that it was found in sufficient quantities in the spleen of a PB-treated rabbit was fortuitous and allowed an apparent K_m-value of 0.64 μM for progesterone to be calculated. Winkel *et al.*[24] first reported progesterone 21-hydroxylase activity in guinea spleen microsomes with an appanent K_m of 0.405 μM and a V_{max} of 0.53 nmol·h^{-1}·mg protein^{-1}. The V_{max} of 0.08 nmol·min^{-1}·mg protein^{-1} obtained with rabbit spleen microsomes was considerably higher, but the majority of spleens examined failed to reveal any quantifiable 21-hydroxylase activity under the conditions employed.

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