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PRONOUNCED AND DIFFERENTIAL EFFECTS OF IONIC STRENGTH AND pH ON TESTOSTERONE OXIDATION BY MEMBRANE-BOUND AND PURIFIED FORMS OF RAT LIVER MICROSOMAL CYTOCHROME *P*-450

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Summary—The aim of this study was to determine the effects of ionic strength and pH on the different pathways of testosterone oxidation catalyzed by rat liver microsomes. The catalytic activity of cytochromes *P*-450a (IIA1), *P*-450b (IIB1), *P*-450h (IIC11) and *P*-450p (IIIA1) was measured in liver microsomes from mature male rats and phenobarbital-treated rats as testosterone 7α-, 16β-, 2α- and 6β-hydroxylase activity, respectively. An increase in the concentration of potassium phosphate (from 25 to 250 mM) caused a marked decrease in the catalytic activity of cytochromes *P*-450a (to 8%), *P*-450b (to 22%) and *P*-450h (to 23%), but caused a pronounced increase in the catalytic activity of cytochrome *P*-450p (up to 4.2-fold). These effects were attributed to changes in ionic strength, because similar but less pronounced effects were observed with Tris–HCl (which has $\sim\frac{1}{3}$ the ionic strength of phosphate buffer at pH 7.4). Testosterone oxidation by microsomal cytochromes *P*-450a, *P*-450b, *P*-450h and *P*-450p was also differentially affected by pH (over the range 6.8–8.0). The pH optima ranged from 7.1 (for *P*-450a and *P*-450h) to 8.0 (for *P*-450p), with an intermediate value of 7.4 for cytochrome *P*-450b. Increasing the pH from 6.8 to 8.0 unexpectedly altered the relative amounts of the 3 major metabolites produced by cytochrome *P*-450h. The decline in testosterone oxidation by cytochromes *P*-450a, *P*-450b and *P*-450h that accompanied an increase in ionic strength or pH could be duplicated in reconstitution systems containing purified *P*-450a, *P*-450b or *P*-450h, equimolar amounts of NADPH-cytochrome *P*-450 reductase and optimal amounts of dilauroylphosphatidylcholine. This result indicated that the decline in testosterone oxidation by cytochromes *P*-450a, *P*-450b and *P*-450h was a direct effect of ionic strength and pH on these enzymes, rather than a secondary effect related to the increase in testosterone oxidation by cytochrome *P*-450p. Similar studies with purified cytochrome *P*-450p were complicated by the atypical conditions needed to reconstitute this enzyme. However, studies on the conversion of digitoxin to digitoxigenin bisdigitoxoside by liver microsomes, which is catalyzed specifically by cytochrome *P*-450p, provided indirect evidence that the increase in catalytic activity of cytochrome *P*-450p was also a direct effect of ionic strength and pH on this enzyme. The mechanism by which an increase in ionic strength or pH caused a decrease in testosterone oxidation by cytochromes *P*-450a, *P*-450b and *P*-450h may involve an impairment of the interaction between these enzymes and NADPH-cytochrome *P*-450 reductase, because the effects of ionic strength and pH were diminished when purified *P*-450a, *P*-450b and *P*-450h were incubated with saturating amounts of NADPH-cytochrome *P*-450 reductase. The mechanism by which an increase in ionic strength or pH caused an increase in testosterone (and digitoxin) oxidation by cytochrome *P*-450p is unknown, but may involve a stimulation of the interaction between NADPH-cytochrome *P*-450 reductase and cytochrome b_5.

INTRODUCTION

Cytochrome *P*-450 is a family of membrane-bound hemoproteins that catalyze the biotransformation of innumerable xenobiotics (such as drugs, pesticides and chemical carcinogens) and certain endogenous substrates (such as steroid hormones and fatty acids) [1, 2]. Studies with purified proteins and inhibitory antibodies have established that some of the cytochrome *P*-450 isozymes in rat liver microsomes catalyze highly specific pathways of testosterone oxidation [3–10]. For example, cytochrome *P*-450a catalyzes the 7α-hydroxylation of testosterone and, to a lesser extent, the conversion of testosterone to 6α-

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Abbreviations—Androstenedione, 4-androstene-3,17-dione; 6-dehydrotestosterone, 4,6-androstadien-17β-ol-3-one, HPLC, high-performance liquid chromatography; testosterone, 4-androstene-17β-ol-3-one; Tris, tris(hydroxymethyl)aminoethane.

hydroxy- and 6-dehydrotestosterone.* Cytochrome *P*-450b converts testosterone to androstenedione, 16α-hydroxytestosterone and 16β-hydroxytestosterone. Only the latter metabolite is uniquely formed by cytochrome *P*-450b. The rate of testosterone 16β-hydroxylation and the levels of cytochrome *P*-450b are negligible in liver microsomes from untreated rats, but can be markedly induced (>30-fold) by treatment of rats with phenobarbital [13, 14]. Like cytochrome *P*-450b, cytochrome *P*-450h also converts testosterone to androstenedione and 16α-hydroxytestosterone. In addition, cytochrome *P*-450h catalyzes the 2α-hydroxylation of testosterone, whereas cytochrome *P*-450b catalyzes 16β-hydroxylation. The rate of testosterone 2α-hydroxylation and the levels of cytochrome *P*-450h increase markedly after puberty in male but not female rats [5]. Consequently, only liver microsomes from mature male rats contain high constitutive levels of cytochrome *P*-450h. Overall, these studies indicate that testosterone 7α-, 16β- and 2α-hydroxylation by rat liver microsomes are reliable markers for cytochromes *P*-450a, *P*-450b and *P*-450h, respectively.

The major pathway of testosterone oxidation catalyzed by rat liver microsomes is 6β-hydroxylation [4–7]. This reaction is catalyzed predominantly by cytochrome *P*-450p, which also catalyzes the 1β-, 2β-, 15β-, and 18-hydroxylation of testosterone, as well as the conversion of testosterone to 6-dehydrotestosterone [15]. Cytochrome *P*-450p is a steroid-inducible, developmentally regulated enzyme which, unlike cytochromes *P*-450a, *P*-450b and *P*-450h, is not an effective catalyst when purified and reconstituted with NADPH-cytochrome *P*-450 reductase and dilauroylphosphatidylcholine [16–18].

The purpose of this study was to determine the effects of ionic strength and pH on the various pathways of testosterone oxidation catalyzed by rat liver microsomes and by purified cytochromes *P*-450a, *P*-450b and *P*-450h. We initiated this study when we observed that incubating liver microsomes in Tris–HCl (50 mM, pH 7.4), instead of the potassium phosphate buffer typically used in our laboratory [7–9], significantly altered the profile of metabolites formed from testosterone. This study describes surprisingly large and differential effects of ionic strength and pH on the cytochrome *P*-450-dependent oxidation of testosterone.

EXPERIMENTAL

Chemicals

Mono- and dibasic potassium phosphate and Tris (Trizma base) were purchased from J. T. Baker Chemical Co. (Phillipsburg, N.J.) and Sigma Chemical Co. (St Louis, Mo.), respectively. Buffers were prepared at room temperature at twice the desired final concentration (each 1-ml incubation mixture contained 0.5 ml buffer). The pH of Tris–HCl at room temperature (22°C) was prepared 0.3 units above the desired pH at 37°C, to account for the effect of temperature on this buffer (−0.02 pH per °C). For example, Tris–HCl, pH 7.7 at room temperature was used to obtain a pH of 7.4 at 37°C. The sources of all other reagents are described elsewhere [7–9].

Animal treatment and preparation of liver microsomes

11-Week-old-male Sprague–Dawley rats (Sasco, Omaha, N.E.) were housed in polycarbonate cages with corncob bedding, in a temperature- and humidity-controlled room with a 12-h diurnal light cycle. The rats were allowed free access to water and Ralston Purina Rodent Chow 5001, and were treated i.p. with sodium phenobarbital (80 mg/kg) or isotonic saline (5 ml/kg) once daily for 4 consecutive days. Liver microsomes were prepared 24 h after the last injection as described by Lu and Levin[19], and were stored as a suspension in 0.25 M sucrose at −80°C.

Protein purification

Cytochromes *P*-450a, *P*-450b and *P*-450h, and NADPH-cytochrome *P*-450 reductase were purified to electrophoretic homogeneity from rat liver microsomes as previously described [3, 7–9, 20–22].

Testosterone oxidation

Testosterone oxidation was determined under conditions where product formation was proportional to incubation time and protein concentration [7]. Reactions were carried out at 37°C in 1-ml incubation mixtures containing potassium phosphate or Tris–HCl (at the final concentrations and pH indicated in Results), $MgCl_2$ (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 Unit/ml), testosterone (250 μM) and either liver microsomes (0.5 nmol cytochrome *P*-450/ml) or purified cytochrome *P*-450 (0.1 nmol/ml), at the final concentrations indicated. Unless otherwise indicated, each purified *P*-450 isozyme was reconstituted with an equimolar amount of NADPH-cytochrome *P*-450 reductase (0.1 nmol/ml) and optimal amounts of dilauroylphosphatidylcholine (15 μM). At each pH and buffer concentration, liver microsomes were incubated in quadruplicate, whereas the purified enzymes were incubated in triplicate. Samples were processed and metabolites analyzed by HPLC, as previously described [7, 8]. This HPLC method resolves 17 potential oxidation products of testosterone, namely 1β-, 2α-, 2β-, 6α-, 6β-, 7α-, 7β-, 11β-, 14α-, 15β-, 16α-, 16β-, 18- and 19-hydroxytestosterone, 16-ketotestosterone, androstenedione and 6-dehydrotestosterone [7, 8], but it does not resolve 6β- from

*The cytochrome *P*-450 nomenclature system of Ryan *et al.*[2, 3] is used throughout the manuscript. Cytochromes *P*-450a, *P*-450b, *P*-450h and *P*-450p are also known as cytochromes *P*-450 IIA1, IIB1, IIC11 and IIIA1, respectively [11, 12].

15α-hydroxytestosterone, nor 1α- from 1β-hydroxytestosterone. However, 15α-hydroxytestosterone represents only a minor pathway of testosterone oxidation by rat liver microsomes, whereas 6β-hydroxylation is the major pathway [4, 9]. Similarly, oxidation of testosterone at C_1 yields primarily 1β-hydroxytestosterone [4].

Digitoxin metabolism

The conversion of digitoxin to digitoxigenin bisdigitoxoside (dt_2) was determined by HPLC essentially as described by Plum and Daldrup[23], except estradiol 3-methyl ether (Sigma Chemical Co., St Louis, Mo.) was used as internal standard.

Other assays

Protein concentration was determined by the method of Lowry *et al.*[24], with bovine serum albumin as the standard. The concentration of cytochrome *P*-450 was determined by the method of Omura and Sato[25], from the carbon monoxide-difference spectrum of dithionite-reduced microsomes (or purified cytochrome *P*-450), based on an extinction coefficient of 91 $mM^{-1} cm^{-1}$. All spectra were recorded on a DW-2C spectrophotometer (SLM-Aminco, Urbana, Ill.).

RESULTS AND DISCUSSION

The liver microsomes used in this study were from mature male rats, which contain cytochromes *P*-450a, *P*-450h and *P*-450p, and from phenobarbital-treated rats, which also contain cytochrome *P*-450b. The rates of testosterone oxidation catalyzed by these liver microsomes under our standard assay conditions (50 mM potassium phosphate buffer, pH 7.4) are shown in Table 1. In this table (and in Figs 1–5), the metabolites are arranged to reflect the catalytic activity of cytochrome *P*-450a (6α- and 7α-hydroxylation), cytochromes *P*-450h and/or *P*-450b (2α-, 16α- and 16β-hydroxylation, and 17-oxidation to androstenedione), and cytochrome *P*-450p (1β-, 2β-, 6β-, 15β- and 18-hydroxylation and 6-dehydrotestosterone formation). It should be emphasized, however, that the only reliable indicators of the catalytic activity of microsomal cytochromes *P*-450a, *P*-450b and *P*-450h are testosterone 7α-, 16β- and 2α-hydroxylation, respectively [3–9]. For convenience the catalytic activity of cytochrome *P*-450p was measured as testosterone 6β-hydroxylase activity, although formation of certain minor testosterone metabolites (e.g. 2β- and 15β-hydroxytestosterone) is an equally reliable indicator of cytochrome *P*-450p levels [5, 7].

Tris–HCl versus potassium phosphate buffer

Table 1 shows the rate of testosterone oxidation catalyzed by liver microsomes from mature male rats incubated in either 50 mM Tris–HCl or potassium phosphate (both at pH 7.4 at 37°C). The change from

Table 1. Effects of potassium phosphate or Tris–HCl on testosterone oxidation by liver microsomes from control and phenobarbital-treated mature male rats

Microsomal sample[b]	Buffer (50 mM, pH 7.4)	Testosterone oxidation[a] (pmol metabolite formed/mg protein/min)										
		6α	7α	2α	16α	16β	A	1β	2β	6β	15β	18	6-DHT
Control	Tris–HCl	42 ± 1	314 ± 4	1570 ± 10	1580 ± 10	91 ± 1	1110 ± 10	61 ± 3 (NS)	127 ± 3	1670 ± 10	53 ± 1	41 ± 1	491 ± 7
Control	Potassium phosphate	38 ± 1	247 ± 2	1180 ± 20	1210 ± 10	52 ± 1	929 ± 16	54 ± 2	113 ± 3	1520 ± 20	58 ± 1	33 ± 1	424 ± 21
Phenobarbital	Potassium phosphate	76 ± 4	505 ± 4	573 ± 4	2990 ± 20	3370 ± 70	3550 ± 70	108 ± 2	495 ± 7	4090 ± 30	219 ± 2	188 ± 12	1160 ± 90

[a]Values represent the mean ± SE of 4 determinations. With the exception of A (androstenedione) and 6-DHT (6-dehydrotestosterone), the abbreviations denote the hydroxylated testosterone metabolite formed, e.g. 6α denotes 6α-hydroxytestosterone.

[b]Specific content of cytochrome *P*-450 was 1.05 nmol/mg protein for liver microsomes from control rats and 1.74 nmol/mg protein for those from phenobarbital-treated rats. Unless noted as not significant (NS), all comparisons with control values (potassium phosphate) are significantly different at $P < 0.05$ (Student's *t*-test).

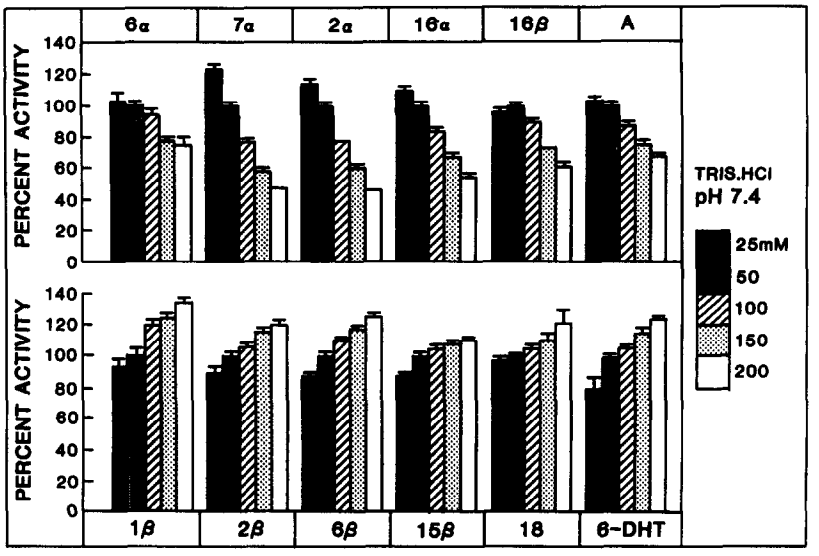

Fig. 1. Effects of Tris–HCl concentration (pH 7.4) on testosterone oxidation by liver microsomes from mature male rats. Each bar represents the mean ± SE of 4 determinations, expressed as a percentage of the rate of testosterone oxidation in 50 mM buffer, pH 7.4 (absolute values for which appear in Table 1).

phosphate to Tris buffer caused a significant increase in the catalytic activity of cytochrome *P*-450a, *P*-450h and, to a lesser extent, cytochrome *P*-450p. To determine whether these changes were due to differences in the ionic strength of Tris–HCl and potassium phosphate (which differ by a factor of about 3), we examined the effects of varying the concentration of Tris–HCl on testosterone oxidation. As shown in Fig. 1, the catalytic activity of cytochromes *P*-450a and *P*-450h both decreased by 60% with increasing buffer concentration (25–200 mM), whereas the catalytic activity of cytochrome *P*-450p increased 50%. The rates of testosterone oxidation at 150 mM Tris–HCl were similar to those at 50 mM potassium phosphate buffer. This result indicted that testosterone oxidation by rat liver microsomes is influenced by the ionic strength of the incubation buffer.

Effects of potassium phosphate concentration

The effects of potassium phosphate concentrations (25–200 mM, pH 7.4) on testosterone oxidation by

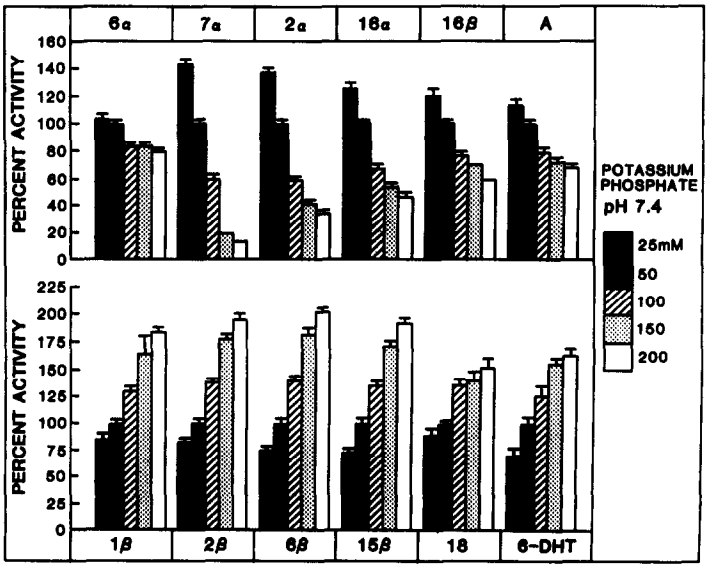

Fig. 2. Effects of potassium phosphate concentration (pH 7.4) on testosterone oxidation by liver microsomes from mature male rats. Each bar represents the mean ± SE of 4 determinations, expressed as a percentage of the rate of testosterone oxidation in 50 mM buffer, pH 7.4 (absolute values for which appear in Table 1).

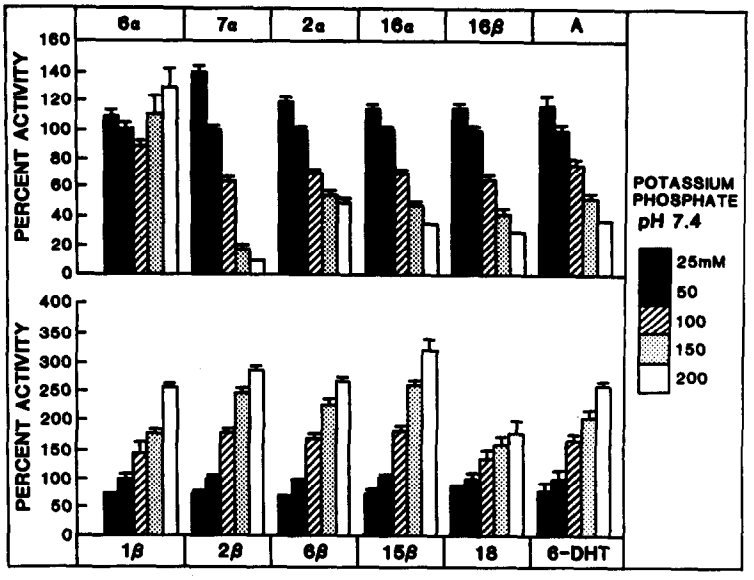

Fig. 3. Effects of potassium phosphate concentration (pH 7.4) on testosterone oxidation by liver microsomes from mature male rats treated with phenobarbital. Each bar represents the mean ± SE of 4 determinations, expressed as a percentage of the rate of testosterone oxidation in 50 mM buffer, pH 7.4 (absolute values for which appear in Table 1).

liver microsomes from mature male (control) rats are shown in Fig. 2. The results confirmed that the catalytic activity of cytochromes *P*-450a and *P*-450h declines with increasing ionic strength, whereas the catalytic activity of cytochrome *P*-450p increases. When the concentration of potassium phosphate was increased from 25 to 200 mM, the rate of testosterone oxidation by cytochrome *P*-450p increased 2.7-fold, whereas the rate catalyzed by cytochromes *P*-450a and *P*-450h decreased by 91 and 75%, respectively. Similar results were obtained with liver microsomes from phenobarbital-treated rats, as shown in Fig. 3. The results in Fig. 3 also established that the catalytic activity of cytochrome *P*-450b, like that of cytochromes *P*-450a and *P*-450h, decreased by 73% when the concentration of potassium phosphate was increased from 25 to 200 mM.

The results in Figs 2 and 3 suggest that testosterone oxidation by cytochrome *P*-450p would increase further if the potassium phosphate concentration were increased above 200 mM, whereas testosterone oxidation by cytochromes *P*-450a, *P*-450b and *P*-450h would increase further if the concentration of potassium phosphate were decreased below 25 mM. To test this possibility, testosterone oxidation by liver microsomes was examined at 0.2, 0.5, 1, 2, 5, 10, 250 and 300 mM potassium phosphate. The results indicated that the optimal potassium phosphate concentrations for testosterone oxidation by cytochromes *P*-450a, *P*-450b, *P*-450h and *P*-450p are 10, 25, 10 and 250 mM, respectively (Table 2).

Effects of pH

The effects of pH (6.8–8.0) on testosterone oxidation by liver microsomes from control and phenobarbital-treated rats are shown in Figs 4 and 5. The catalytic activity of cytochrome *P*-450a in liver microsomes from control rats was highest at pH 7.1, and decreased by 34% when the pH was increased to 8.0. In contrast, the catalytic activity of cytochrome

Table 2. Testosterone oxidation by rat liver microsomes incubated with potassium phosphate concentrations optimal for cytochromes *P*-450a (10 mM), *P*-450b (25 mM), *P*-450h (10 mM) and *P*-450p (250 mM)

Potassium phosphate mM (pH 7.4)	Testosterone oxidation[a] (pmol metabolite formed/mg protein/min) 7α (*P*-450a)	16β (*P*-450b)	2α (*P*-450h)	6β (*P*-450p)
Microsomes from mature male rats				
10 mM	474 ± 5	46 ± 2	1740 ± 40	651 ± 20
25 mM	354 ± 3	62 ± 2	1610 ± 20	1150 ± 50
250 mM	28 ± 3	33 ± 1	370 ± 5	3530 ± 90
Microsomes from phenobarbital-treated rats				
10 mM	854 ± 7	2900 ± 80	730 ± 7	1430 ± 30
25 mM	703 ± 9	3870 ± 60	685 ± 4	2890 ± 30
250 mM	47 ± 2	837 ± 20	267 ± 3	12,000 ± 110

Values are mean ± SE of 3 determinations. [a]The abbreviations denote the hydroxylated testosterone metabolite formed, e.g. 7α denotes, 7α-hydroxytestosterone.

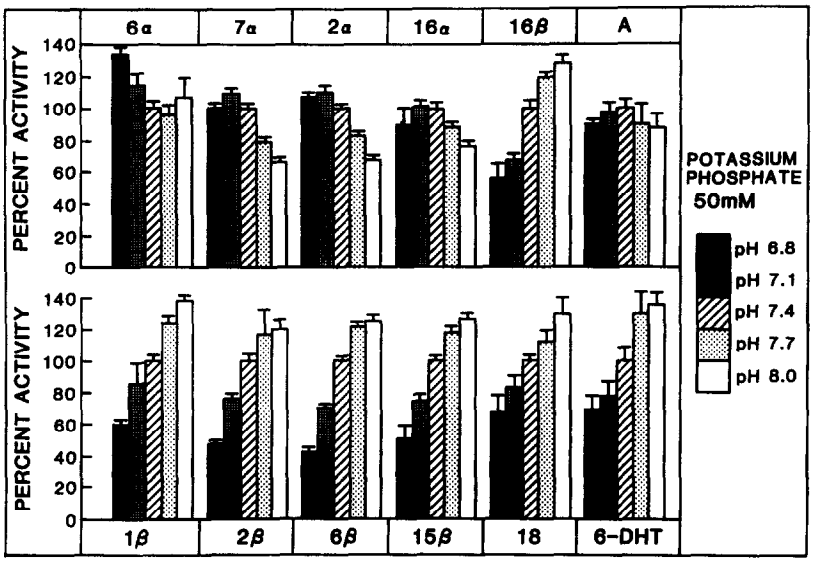

Fig. 4. Effects of pH of 50 mM potassium phosphate on testosterone oxidation by liver microsomes from mature male rats. Each bar represents the mean ± SE of 4 determinations, expressed as a percentage of the rate of testosterone oxidation in 50 mM buffer, pH 7.4 (absolute values for which appear in Table 1).

P-450p in liver microsomes from control rats increased 3-fold when the pH was raised from 6.8 to 8.0. Increasing the pH further (to 8.3, 8.6 and 8.9) caused a slight decrease in the rate of testosterone oxidation by cytochrome *P*-450p, hence, the pH optimum for this enzyme was ~8.0 (results not shown). Similar effects of pH on the catalytic activity of cytochromes *P*-450a and *P*-450p were observed with liver microsomes from phenobarbital-treated rats (Fig. 5). The pH optimum of testosterone oxidation by cytochrome *P*-450p was dependent on the concentration of potassium phosphate buffer. When the concentration of potassium phosphate was increased from 50 mM to 200 mM, the pH optimum shifted from 8.0 to ~7.25 (results not shown).

Based on the rate of testosterone 2α-hydroxylation, the catalytic activity of cytochrome *P*-450h in liver microsomes from control rats was highest at pH 7.1, and decreased by 38% when the pH was raised to 8.0 (Fig. 4). However, over this same pH range (7.1–8.0), the rate of formation of 16α-hydroxytestosterone and androstenedione declined

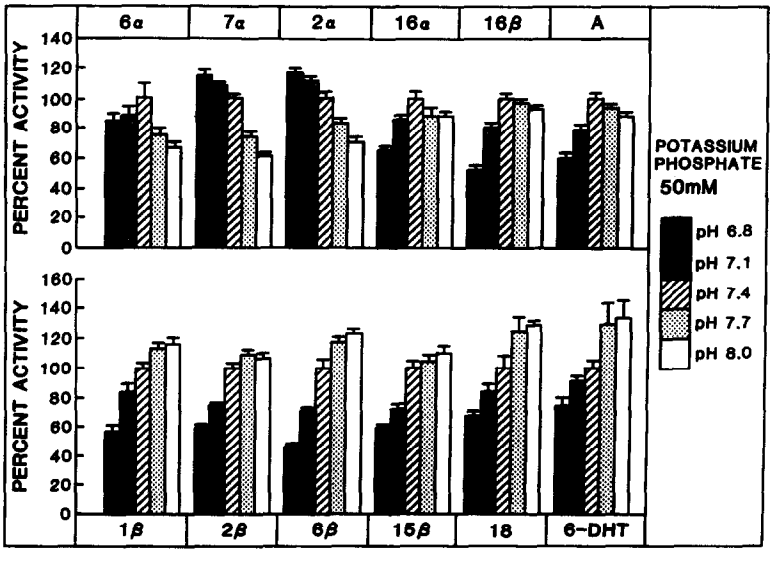

Fig. 5. Effects of pH of 50 mM potassium phosphate on testosterone oxidation by liver microsomes from mature male rats treated with phenobarbital. Each bar represents the mean ± SE of 4 determinations, expressed as a percentage of the rate of testosterone oxidation in 50 mM buffer, pH 7.4 (absolute values for which appear in Table 1).

only 24 and 8%, respectively. Although the interpretation of these results is complicated by the fact that enzymes other than cytochrome *P*-450h may contribute to the 16α- and 17-oxidation of testosterone by liver microsomes from mature male rats, the results raise the possibility that the regio-specificity of testosterone oxidation by cytochrome *P*-450h varies with changes in pH. This possibility was examined with purified cytochrome *P*-450h, and is discussed below.

The effects of pH on the catalytic activity of cytochrome *P*-450b, which was determined as testosterone 16β-hydroxylation by liver microsomes from phenobarbital-treated rats, indicated a pH optimum of ~7.4 (Fig. 5). Testosterone 16β-hydroxylation by liver microsomes from control rats did not peak at pH 7.4, but continued to increase up to pH 8.0 (as did those metabolites produced by cytochrome *P*-450p). These results suggest that the low rate of testosterone 16β-hydroxylation by liver microsomes from control rats is catalyzed, at least in part, by cytochrome *P*-450p, not cytochrome *P*-450b. We have recently established that 16β-hydroxylation is a minor pathway of testosterone oxidation catalyzed by cytochrome *P*-450p, and that antibody against cytochrome *P*-450p can inhibit 30–70% of this reaction catalyzed by liver microsomes from mature male rats, but not from phenobarbital-treated rats (unpublished results). It has also been shown that treatment of rats with inducers of cytochrome *P*-450p causes a small increase in testosterone 16β-hydroxylase activity without a detectable increase in cytochrome *P*-450b levels [7, 26].

Purified P-450 enzymes

Figures 6–8 show the effects of pH and potassium phosphate concentration on testosterone oxidation by purified cytochromes *P*-450a, *P*-450b and *P*-450h reconstituted with an equimolar amount of NADPH-cytochrome *P*-450 reductase and an optimal amount of lipid. The absolute rates of testosterone oxidation catalyzed by these purified *P*-450 enzymes under standard conditions (50 mM potassium phosphate, pH 7.4) are shown in Table 3. The catalytic activity of purified cytochrome *P*-450a decreased by 83% when the concentration of phosphate buffer was increased from 25 to 200 mM, and decreased by 51% as the pH was raised from 6.8 to 8.0 (Fig. 6). The effects of pH and ionic strength on testosterone oxidation by purified cytochrome *P*-450a closely resembled their effects on the microsomal enzyme (Figs 2–5). One notable difference is that changes in testosterone 6α-hydroxylation paralleled changes in 7α-hydroxylation by purified cytochrome *P*-450a (Fig. 6), but not by liver microsomes (Figs 2–5). We have reported previously that, whereas cytochrome *P*-450a is the principal catalyst of testosterone 7α-hydroxylation, enzymes other than cytochrome *P*-450a

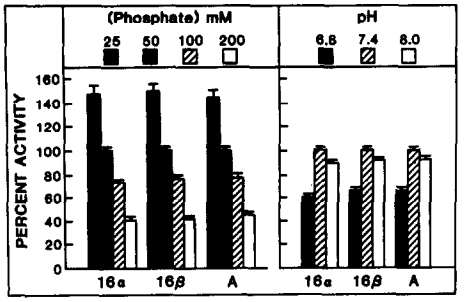

Fig. 7. Effects of pH and potassium phosphate concentration on testosterone oxidation by purified cytochrome *P*-450b. Purified cytochrome *P*-450b (0.1 nmol/ml) was reconstituted with an equimolar amount of NADPH-cytochrome *P*-450 reductase and 15 μM dilauroylphosphatidylcholine, as described in Experimental. Each bar represents the mean ± SE of 3 determinations, expressed as a percentage of the rate of testosterone oxidation in 50 mM buffer, pH 7.4 (absolute values for which appear in Table 3).

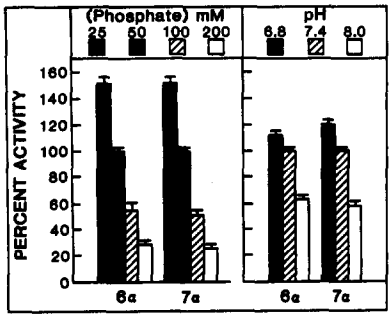

Fig. 6. Effects of pH and potassium phosphate concentration on testosterone oxidation by purified cytochrome *P*-450a. Purified cytochrome *P*-450a (0.1 nmol/ml) was reconstituted with an equimolar amount of NADPH-cytochrome *P*-450 reductase and 15 μM dilauroylphosphatidylcholine, as described in Experimental. Each bar represents the mean ± SE of 3 determinations, expressed as a percentage of the rate of testosterone oxidation in 50 mM buffer, pH 7.4 (absolute values for which appear in Table 3).

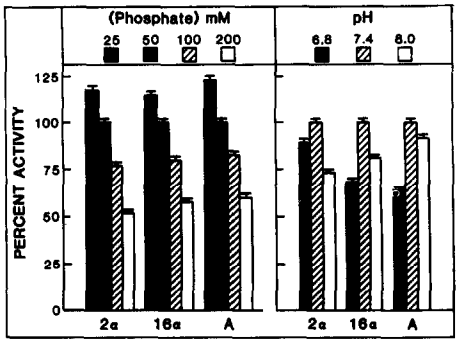

Fig. 8. Effects of pH and potassium phosphate concentration on testosterone oxidation by purified cytochrome *P*-450h. Purified cytochrome *P*-450h (0.1 nmol/ml) was reconstituted with an equimolar amount of NADPH-cytochrome *P*-450 reductase and 15 μM dilauroylphosphatidylcholine, as described in Experimental. Each bar represents the mean ± SE of 3 determinations, expressed as a percentage of the rate of testosterone oxidation in 50 mM buffer, pH 7.4 (absolute values for which appear in Table 3).

Table 3. Testosterone oxidation by purified rat cytochromes *P*-450a, *P*-450b and *P*-450h

Ratio of reductase to cytochrome *P*-450 (nmol:nmol)	Potassium phosphate mM (pH)	Testosterone oxidation[a] (nmol metabolite formed/nmol *P*-450/min)		
		6α	7α	
Cytochrome P-450a				
1:1[b]	50 mM (7.4)	0.28 ± 0.01	6.83 ± 0.14	
5:1[c]	10 mM (7.1)	1.25 ± 0.01	34.4 ± 0.2	
5:1	10 mM (7.4)	1.28 ± 0.01	34.6 ± 0.2	
5:1	50 mM (7.4)	1.24 ± 0.02	32.7 ± 0.6	
5:1	200 mM (7.4)	0.59 ± 0.01	15.6 ± 0.3	
		16α	16β	A
Cytochrome P-450b				
1:1[b]	50 mM (7.4)	2.11 ± 0.04	2.66 ± 0.04	1.73 ± 0.03
5:1	10 mM (7.4)	11.1 ± 0.1	13.7 ± 0.1	9.59 ± 0.04
5:1[c]	25 mM (7.4)	13.6 ± 0.1	16.1 ± 0.1	11.6 ± 0.1
5:1	50 mM (7.4)	13.9 ± 0.2	15.8 ± 0.3	12.4 ± 0.1
5:1	200 mM (7.4)	6.20 ± 0.02	7.32 ± 0.03	6.76 ± 0.02
		2α	16α	A
Cytochrome P-450h				
1:1[b]	50 mM (7.4)	1.75 ± 0.01	1.81 ± 0.01	1.03 ± 0.01
5:1[c]	10 mM (7.1)	4.01 ± 0.02	3.24 ± 0.02	2.55 ± 0.01
5:1	10 mM (7.4)	4.75 ± 0.05	4.33 ± 0.05	3.37 ± 0.04
5:1	50 mM (7.4)	5.55 ± 0.04	5.38 ± 0.04	4.15 ± 0.05
5:1	200 mM (7.4)	4.00 ± 0.08	4.29 ± 0.02	3.27 ± 0.05

Values are mean ± SE of 3 determinations. [a]The abbreviations denote the hydroxylated testosterone metabolite formed, e.g. 6α denotes 6α-hydroxytestosterone. [b]These values represent 100% in Figs 6–8. [c]This potassium phosphate concentration and pH supported the highest rate of testosterone oxidation by the corresponding enzyme in rat liver microsomes.

contribute significantly to the 6α-hydroxylation of testosterone catalyzed by liver microsomes from mature male rats [9].

The catalytic activity of purified cytochrome *P*-450b decreased by 72% when the concentration of phosphate buffer was increased from 25 to 200 mM, and was higher at pH 7.4 than either pH 6.8 or 8.0 (Fig. 7). As in the case of cytochrome *P*-450a, the effects of pH and ionic strength on testosterone oxidation by purified cytochrome *P*-450b closely resembled their effects on the microsomal enzyme (Figs 3 and 5). Changes in pH and potassium phosphate concentration had identical effects on the rate of formation of 16α-hydroxytestosterone, 16β-hydroxytestosterone and androstenedione by cytochrome *P*-450b, hence, the ratio of these 3 metabolites remained constant over the ranges examined.

The catalytic activity of purified cytochrome *P*-450h decreased by ~51% when the concentration of potassium phosphate was increased from 25 to 200 mM (Fig. 8), which closely resembled the effects of ionic strength on the catalytic activity of cytochrome *P*-450h in liver microsomes (Fig. 2). The magnitude of the effects of pH on the catalytic activity of purified cytochrome *P*-450h depended on which metabolite was considered. The rate of formation of 2α-hydroxytestosterone was comparable at pH 6.8 and 7.4, and declined when the pH was raised to 8.0. The rate of formation of 16α-hydroxytestosterone was greatest at pH 7.4, and declined when the pH was either lowered to 6.8 or raised to pH 8.0. The rate of formation of androstenedione was comparable at pH 7.4 and 8.0, and declined when the pH was lowered to pH 6.8. The results in Fig. 9 (which were derived from those in Fig. 8) show how the ratio of the 3 major testosterone metabolites produced by cytochrome *P*-450h varied with changes in pH. As the pH increased from 6.8 to 8.0, the relative amount of 2α-hydroxytestosterone decreased from 44 to 34%, the amount of 16α-hydroxytestosterone remained fairly constant (37 vs 39%), whereas the amount of androstenedione increased from 18 to 24%. These results, which complement those obtained with liver microsomes from mature male rats

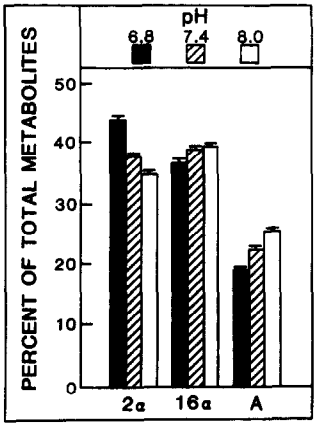

Fig. 9. Differential effects of pH on the regio-selective oxidation of testosterone by purified cytochrome *P*-450h. Each bar represents the mean ± SE of 3 determinations. The rate of formation of each metabolite is expressed as a percentage of the sum of the rate of formation of 2α-hydroxytestosterone, 16α-hydroxytestosterone and androstenedione. These data were derived from those in Fig. 8.

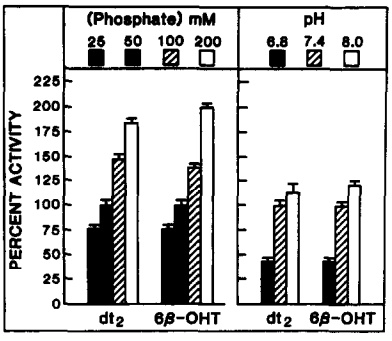

Fig. 10. Effects of pH and potassium phosphate concentration on digitoxin metabolism and testosterone 6β-hydroxylation by liver microsomes from mature male rats. Each bar represents the mean $\pm$ SE of 4 determinations, expressed as a percentage of the rate of product formation in 50 mM buffer, pH 7.4. Under these conditions, the rate of formation of digitoxigenin bisdigitoxoside (dt_2) was 113 ± 5 pmol/mg protein/min, and the rate of testosterone 6β-hydroxylation was 1520 ± 20 pmol/mg protein/min.

(Fig. 4), indicate that oxidation by cytochrome *P*-450h shifted from the A-ring to the D-ring of testosterone as the pH increased from 6.8 to 8.0. The reason for this change in regio-specificity is unknown.

Digitoxin metabolism

Experiments similar to those with purified cytochromes *P*-450a, *P*-450b and *P*-450h could not be carried out with purified cytochrome *P*-450p, because this enzyme is a poor catalyst of testosterone oxidation when purified and reconstituted with NADPH-cytochrome *P*-450 reductase and dilauroylphosphatidylcholine [7, 18]. Therefore, we were unable to address the question: Does the rate of testosterone oxidation by cytochrome *P*-450p increase with increasing pH and ionic strength due to a decrease in the catalytic activity of cytochromes *P*-450a, *P*-450b and *P*-450h? As an alternative approach to address this question, we examined the effects of pH and ionic strength on the oxidative metabolism of digitoxin by liver microsomes from mature male rats. Digitoxin was chosen because, unlike testosterone, it is converted primarily to a single product (namely digitoxigenin bisdigitoxoside), and this pathway is catalyzed by cytochrome *P*-450p [27, 28]. As shown in Fig. 10, the rate of formation of digitoxigenin bisdigitoxoside, like the rate of testosterone 6β-hydroxylation, increased 2.7-fold when the concentration of potassium phosphate was increased from 25 to 200 mM, and increased 2.5-fold when the pH was raised from 6.8 to 8.0. These results suggest, but do not prove, that the increase in the catalytic activity of microsomal cytochrome *P*-450p that accompanies an increase in ionic strength or pH is a primary effect, and does not result from a decrease in the catalytic activity of other *P*-450 enzymes.*

Mechanisms

The results obtained with purified cytochromes *P*-450a, *P*-450b and *P*-450h, which closely paralleled those obtained with liver microsomes, established that the effects of pH and ionic strength on the catalytic activity of these enzymes appears to be a primary effect. In other words, the results with the purified enzymes established that the catalytic activity of microsomal cytochromes *P*-450a, *P*-450b and *P*-450h probably did not decline with increasing pH or ionic strength simply due to an increase in testosterone oxidation by cytochrome *P*-450p. These results are consistent with those of our previous study, which argued against competing or inhibitory interactions between the *P*-450 isozymes involved in testosterone oxidation by rat liver microsomes [20]. Furthermore, we have recently shown that complete inhibition (>85%) of microsomal testosterone 2β-, 6β- and 15β-hydroxylation by a rabbit antibody against cytochrome *P*-450p does not stimulate testosterone oxidation by cytochrome *P*-450a, *P*-450b or *P*-450h [29, 43].

The mechanism by which an increase in ionic strength or pH caused a decrease in testosterone oxidation by cytochromes *P*-450a, *P*-450b and *P*-450h may involve an impairment of the interaction between these enzymes and NADPH-cytochrome *P*-450 reductase, because the effects of ionic strength and pH were diminished (but not abolished) when purified *P*-450a, *P*-450b and *P*-450h were incubated with a 5-fold molar excess (i.e. saturating amounts) of NADPH-cytochrome *P*-450 reductase (Table 3). The importance of the ratio of NADPH-cytochrome *P*-450 reductase to cytochrome *P*-450 on the magnitude of the effects of ionic strength and pH is interesting because rat liver microsomes contain less NADPH-cytochrome *P*-450 reductase than cytochrome *P*-450 [20]. This may explain why the effects of ionic strength on testosterone oxidation by cytochrome *P*-450a, *P*-450b and *P*-450h were slightly greater for the microsomal enzymes (Figs 2–5) than the purified enzymes, which were reconstituted with an equimolar amount of NADPH-cytochrome *P*-450 reductase (Figs 6–8).

The mechanism by which an increase in ionic strength or pH caused an increase in testosterone oxidation by cytochrome *P*-450p is unknown, but studies with digitoxin suggested that the catalytic activity of cytochrome *P*-450p did not increase simply because the catalytic activity of other *P*-450 enzymes decreased. However, the comparison between testosterone 6β-hydroxylation and the conversion of digitoxin to digitoxigenin bisdigitoxoside is potentially complicated by the existence of more than

*After this manuscript was submitted for publication, we established conditions to reconstitute testosterone oxidation by purified cytochrome *P*-450p. The effects of ionic strength and pH on testosterone oxidation by purified cytochrome *P*-450p mimicked the effects on microsomal cytochrome *P*-450p [43].

one form of cytochrome *P*-450p (30–32). Whether the same form(s) of cytochrome *P*-450p catalyze both reactions remains to be established.

It is possible that, in marked contrast to cytochromes *P*-450a, *P*-450b and *P*-450h, the interaction between cytochrome *P*-450p and NADPH-cytochrome *P*-450 reductase is facilitated by high ionic strength and pH, and this represents the mechanism by which high ionic strength and pH stimulate the catalytic activity of cytochrome *P*-450p. An alternative mechanism may involve a stimulation of the interaction between NADPH-cytochrome *P*-450 reductase and cytochrome b_5. We have recently determined that testosterone oxidation by purified, reconstituted cytochrome *P*-450p is highly dependent on the presence of cytochrome b_5, and similar results have been reported by Yamazoe *et al.*[33] and Imaoka *et al.*[34] for cytochrome *P*-450 PB-1 (which is a *P*-450p-related enzyme that also catalyzes the 6β-hydroxylation of testosterone). Furthermore, previous studies have shown that the reduction of cytochrome b_5 by NADPH-cytochrome *P*-450 reductase is stimulated by high concentrations of potassium phosphate or potassium chloride [35, 36]. Further studies will be necessary to delineate the extent to which cytochrome b_5 mediates the stimulatory effects of ionic strength and pH on testosterone oxidation by cytochrome *P*-450p.

Implications for measuring cytochrome P-450 induction

The results in Table 1 indicate that treatment of rats with phenobarbital caused a 65-fold increase in testosterone 16β-hydroxylation (due to the marked induction of cytochrome *P*-450b), a 2.0-fold increase in testosterone 7α-hydroxylation (due to the induction of cytochrome *P*-450a), and a 2.7-fold induction of testosterone 6β-hydroxylation (due to the induction of cytochrome *P*-450p). The rate of testosterone 2α-hydroxylation decreased after phenobarbital treatment (due to the suppression of cytochrome *P*-450h). These changes in testosterone oxidation are consistent with the results of immunochemical studies, which demonstrated that treatment of male rats with phenobarbital causes a marked induction of cytochrome *P*-450b (>30-fold), a modest induction of cytochromes *P*-450a and *P*-450p (2–5-fold), and a ~50% suppression of cytochrome *P*-450h [9, 13, 14, 37, 38]. The results in Table 4 show that the concentration of potassium phosphate in the incubation mixture can change the apparent degree of induction or suppression of cytochrome *P*-450 by treatment of rats with phenobarbital. This variation might be important when attempting to correlate changes in testosterone oxidation with changes in other enzyme activities or with changes in the absolute levels of liver microsomal cytochromes *P*-450a, *P*-450b, *P*-450h and *P*-450p.

Physiological implications

Studies with perfused livers from mature male rats have identified 2β-, 6β-, 7α- and 16α-hydroxylation as major pathways of testosterone oxidation [39, 40]. However, it is difficult to compare the results of our study with those obtained from liver perfusion studies, because the metabolism of testosterone in the latter system is a complex process involving oxidation by cytochrome *P*-450 and 17β-hydroxysteroid oxidoreductase, reduction by steroid 5α/5β-reductases and 3α/3β-hydroxysteroid oxidoreductases, and conjugation with sulfate, glucuronic acid or acetate [40, 41]. We have shown previously that some hydroxytestosterone isomers, such as 6β-hydroxytestosterone, are substrates for steroid 5α-reductase, whereas as others are not (e.g. 7α-hydroxytestosterone) [42]. Therefore, it is inappropriate to infer that the concentration of 7α-, 6β- and 16α-hydroxytestosterone in liver perfusate accurately reflects the catalytic activity of cytochromes *P*-450a, *P*-450p and *P*-450h, respectively, in an intact liver. In the absence of reliable information on the catalytic activity of cytochromes *P*-450a, *P*-450p and *P*-450h *in vivo*, we are unable to assess the possible physiological significance of the differential effects of pH and ionic strength on the cytochrome *P*-450-dependent oxidation of testosterone observed *in vitro*.

Despite marked changes in the rate of formation of individual metabolites, the overall rate of testosterone oxidation by rat liver microsomes varied less than 15% when the concentration of potassium phosphate buffer was varied from 25 to 200 mM, or when the pH was varied from 6.8 to 8.0 (results not shown). Therefore, the potential importance of the phenomena described in this paper is more likely to impact

Table 4. Effects of potassium phosphate concentration on the apparent inducibility of testosterone oxidation by treatment of male rats with phenobarbital

Potassium phosphate mM (pH 7.4)	Testosterone oxidation[a] 7α (*P*-450a)	16β (*P*-450b)	2α (*P*-450h)	6β (*P*-450p)
10 mM	2.0[b]	63	0.42	2.2
25 mM	2.0	62	0.43	2.5
50 mM	2.0	65	0.49	2.7
100 mM	2.5	55	0.58	3.2
150 mM	2.0	39	0.64	3.3
200 mM	1.8	33	0.71	3.5
250 mM	1.7	25	0.72	3.4

[a]The abbreviations denote the hydroxylated testosterone metabolite formed, e.g. 7α denotes 7α-hydroxytestosterone. [b]Ratios of rates of testosterone oxidation by liver microsomes from phenobarbital-treated and control rats at different potassium phosphate concentrations were compiled from Tables 1 and 2, and from Figs 2 and 3. Ratio (treated/control).

on the qualitative rather than the quantitative aspects of testosterone oxidation *in vivo*. The qualitative aspects of testosterone oxidation would be important if the hydroxytestosterone isomers formed *in vivo* have different biological properties. However, to the best of our knowledge, there has been no systematic investigation of the biologic effects hydroxytestosterone isomers.

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