Biochemical Pharmacology, Vol. 36, No. 22, pp. 3859–3866, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

STUDIES ON THE PREGNENOLONE-16α-CARBONITRILE-INDUCIBLE FORM OF RAT LIVER MICROSOMAL CYTOCHROME P-450 AND UDP-GLUCURONOSYLTRANSFERASE*

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(*Received* 26 *December* 1986; *accepted* 20 *April* 1987)

Abstract—Treatment of rats with pregnenolone-16α-carbonitrile (PCN) markedly induces rat liver microsomal cytochrome P-450p and UDP-GT-dt$_1$, a glucuronosyltransferase active towards the digitoxin metabolite, digitoxigenin monodigitoxoside. The present study characterizes the regulation of these two enzymes in rats treated with different xenobiotics. Like PCN, treatment of rats with dexamethasone, spironolactone, troleandomycin or erythromycin estolate markedly induced both UDP-GT-dt$_1$ and cytochrome P-450p (measured as erythromycin demethylase and testosterone 2β-, 6β-, 15β-, and 18-hydroxylase activities). However, compared to PCN and dexamethasone, both troleandomycin and erythromycin estolate preferentially induced cytochrome P-450p, whereas spironolactone preferentially induced UDP-GT-dt$_1$. Treatment of rats with the polychlorinated biphenyl mixture, Aroclor 1254, increased both cytochrome P-450p and UDP-GT-dt$_1$ activity to about 40% of that in liver microsomes from rats induced with PCN or dexamethasone. Treatment of rats with phenobarbital or chlordane caused a relatively small increase in cytochrome P-450p and UDP-GT-dt$_1$ activity. Neither enzyme was induced by treatment of rats with 3-methylcholanthrene, rifampin or digitoxin. The induction of cytochrome P-450p and UDP-GT-dt$_1$ by PCN followed similar dose–response curves. Although cytochrome P-450p and UDP-GT-dt$_1$ are differentially affected by the age and the sex of rats, the enzymes responded similarly, but not identically, to xenobiotic treatment. This suggests that cytochrome P-450p and UDP-GT-dt$_1$ are co-inducible but not coordinately regulated.

Pregnenolone-16α-carbonitrile (PCN)‡ has been shown to protect rats from the toxic effects of digitoxin and a wide variety of other structurally diverse xenobiotics, for which reason PCN is known as a catatoxic steroid [1]. The mechanism of protection by PCN and other catatoxic steroids, such as dexamethasone and spironolactone, is thought to involve the induction of liver microsomal drug-metabolizing enzymes [1–7]. Catatoxic steroids have been shown to induce a specific isozyme of rat liver microsomal cytochrome P-450, designated cytochrome P-450p [8–13], and a specific form of liver microsomal UDP-glucuronosyltransferase [14–18]. The latter enzyme can be distinguished from other forms of rat liver microsomal UDP-glucuronosyltransferase by its ability to catalyze the glucuronidation of the digitoxin metabolite, digitoxigenin monodigitoxoside (dt$_1$). This UDP-glucuronosyltransferase is designated UDP-GT-dt$_1$.

It has been shown by immunochemical techniques that cytochrome P-450p is responsible for >85% of the testosterone 6β-hydroxylase activity catalyzed by rat liver microsomes [12], and that the levels of cytochrome P-450p in liver microsomes correlate well with testosterone 6β-hydroxylase and erythromycin demethylase activities [12, 19]. We have shown recently that partially purified cytochrome P-450p catalyzes the 6β-hydroxylation of testosterone, as well as the 2β-, 15β-, and 18-hydroxylation [20].

The constitutive levels of cytochrome P-450p in liver microsomes are influenced markedly by the age and sex of rats [12, 20–23]. The constitutive levels of liver microsomal cytochrome P-450p decline with age in female but not in male rats. The age- and sex-dependent changes in cytochrome P-450p levels are paralleled by changes in testosterone 2β-, 6β-, 15β- and 18-hydroxylase activities and in the rate of digitoxin oxidation [12, 20, 21].

In contrast to cytochrome P-450p, liver microsomal UDP-GT-dt$_1$ is not sexually differentiated in

* This research was supported in part by Grants ES 03765 and ES 03192 from the National Institutes of Health. M. P. A. is supported by Training Grant ES 07079 from the National Institutes of Health. C. D. K. is a recipient of a Burroughs-Wellcome Scholar Award in Toxicology, A. P. is a recipient of a Research Career Development Award (ES 00166) from the National Institutes of Health.

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‡ Abbreviations and trivial names: PCN, pregnenolone-16α-carbonitrile; androstenedione, 4-androstene-3,17-dione; dt$_1$, digitoxigenin monodigitoxoside; CHAPS, 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; testosterone, 17β-hydroxy-4-androsten-3-one; and UDP-GT-dt$_1$, UDP-glucuronosyltransferase active towards digitoxigenin monodigitoxoside.

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either immature or mature rats. However, the activity of liver microsomal UDP-GT-dt_1 increases with age in both male and female rats (mature/immature ~ 10), which is in marked contrast to the age-dependent decline in cytochrome P-450p levels in female rats [18, 21]. The differential effects of age and sex on cytochrome P-450p and UDP-GT-dt_1 indicate that these two PCN-inducible liver microsomal enzymes have the potential to be regulated independently.

In addition to catatoxic steroids, various macrolide antibiotics, such as troleandomycin and erythromycin estolate, have been shown to induce rat liver microsomal cytochrome P-450p [19, 24]. These macrolide antibiotics not only induce cytochrome P-450p, but are converted to a metabolite that selectively binds to this hemoprotein, forming a stable complex that can survive the preparation of liver microsomes [19, 24]. Several nonsteroidal agents have been shown *in vitro* to induce UDP-GT-dt_1 in cultured hepatocytes [25]. The non-steroidal inducers include phenobarbital and certain organochlorine pesticides (e.g. chlordane) [25]. However, it is not known whether UDP-GT-dt_1 is inducible by macrolide antibiotics.

The aim of the present study was to investigate further the regulation of liver microsomal cytochrome P-450p and UDP-GT-dt_1 in rats treated with various steroids, macrolide antibiotics and other xenobiotics.

MATERIALS AND METHODS

Materials. Dexamethasone, erythromycin estolate, 3-methylcholanthrene, digitoxin, rifampin, UDP-glucuronic acid, D-saccharic acid-1,4-lactone, and glucose-6-phosphate dehydrogenase were purchased from the Sigma Chemical Co. (St. Louis, MO); corn oil from Matheson, Coleman & Bell (Gibbstown, NJ); Scinti Verse I liquid scintillation mixture from Fisher Scientific (Fair Lawn, NJ), and phenobarbital from the Department of Pharmacy, University of Kansas Medical Center (Kansas City, KS). The detergent, CHAPS, was purchased from Calbiochem (San Diego, CA). PCN was provided by the Upjohn Co. (Kalamazoo, MI) and Dr. P. S. Guzelian (Medical College of Virginia, Richmond, VA); troleandomycin by Pfizer, Inc. (Brooklyn, NY); and spironolactone by G. D. Searle & Co. (Skokie, IL). Tritium-labeled dt_1 was provided by Dr. M. C. Castle (Eastern Virginia Medical School, Norfolk, VA) and chlordane by the Velsicol Corp. (Chicago, IL).

Animals and treatment. Mature (56-day-old) female Long Evans rats (Blue Spruce Farms, Altamont, NY) were housed on corn-cob bedding, maintained on a 12-hr light–dark cycle, and allowed free access to Purina Rodent Chow 5001 and water. Rats were given a single intraperitoneal injection once daily for 4 consecutive days with digitoxin (1 mg/kg body weight), 3-methylcholanthrene (27 mg/kg), rifampin (50 mg/kg), chlordane (50 mg/kg), dexamethasone (50 mg/kg), PCN (1–50 mg/kg), phenobarbital (100 mg/kg), spironolactone (150 mg/kg), troleandomycin (500 mg/kg) or erythromycin estolate (500 mg/kg). Aroclor 1254 (500 mg/kg) was given as a single injection on day 1. Digitoxin was dissolved in ethanol (10 mg/ml), phenobarbital in phosphate-buffered saline, and troleandomycin and erythromycin estolate in dilute HCl (pH 4.0). All other compounds were administered in corn oil, which was also administered to control rats (5 ml/kg). Twenty-four hours after the last injection, livers were removed and microsomes were prepared as described by Lu and Levin [26], and stored as a suspension in 0.25 M sucrose at −80°. Microsomes were prepared from individual rats (three to seven per group) except for microsomes prepared from rats treated with Aroclor 1254 which were prepared from a pool of livers from twenty-five rats.

Enzyme assays. Testosterone metabolism was analyzed by an HPLC method capable of resolving sixteen potential oxidation products of testosterone, including fourteen isomeric hydroxytestosterones [20, 27]. Reactions were carried out at 37° in 1-ml incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 unit/ml), testosterone (250 μM), and liver microsomes (0.25 to 1.0 nmol cytochrome P-450/ml), at the final concentrations indicated. Reactions were started by the addition of an NADPH-generating system and stopped after 5 min by the addition of 6 ml dichloromethane. Metabolites were extracted and analyzed by HPLC as described [20, 27].

Erythromycin demethylase activity was determined at 37° in 1-ml incubation mixtures containing potassium phosphate buffer (100 mM, pH 7.4), $MgCl_2$ (3 mM), EDTA (1 mM), semicarbazide (5 mM), NADP (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 unit/ml), and liver microsomes (0.5 to 1.0 nmol cytochrome P-450/ml), at the final concentrations indicated. Unless otherwise indicated, a final concentration of 400 μM erythromycin was used. Reactions were started by the addition of the NADPH-generating system and stopped after 10 min by the addition of 0.4 ml perchloric acid (17%). Formaldehyde formation was determined spectrophotometrically at 412 nm by the method of Nash [28].

UDP-glucuronosyltransferase activity was assayed radiometrically with [^{3}H]dt_1 as substrate, essentially as described [17, 18, 21]. Microsomal protein (5–20 mg/ml) was solubilized for 15 min with an equal volume of 10% CHAPS in 400 mM Tris–HCl (pH 7.7 at room temperature) and 250 mM sucrose prior to its addition (in 50-μl aliquots) to incubation mixtures. The final incubation volume was 0.5 ml and contained Tris–HCl buffer (200 mM, pH 7.7), $MgCl_2$ (10 mM), EDTA (1 mM), D-saccharic acid-1,4-lactone (1.25 mM), UDP-glucuronic acid (4 mM), CHAPS (0.5%), [^{3}H]dt_1 (26 μM), and solubilized microsomal protein (0.25 to 1.0 mg/ml), at the final concentrations listed. Reactions were started with UDP-glucuronic acid and stopped after a 10-min incubation at 37° with 0.5 ml of ice-cold ethanol. Unreacted substrate was quantitatively (>99.9%) extracted with 5 ml chloroform. The amount of dt_1-glucuronide formed was determined from radio-

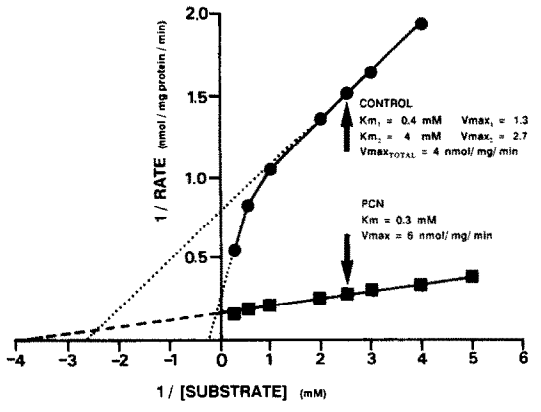

Fig. 1. Lineweaver–Burk plot of the rate of erythromycin demethylation as a function of substrate concentration by liver microsomes from control and PCN-treated rats. Liver microsomes from mature female rats treated with PCN or corn oil (control) were incubated with various concentrations of erythromycin (0.2 to 5.0 mM). Erythromycin demethylase activity was determined as the rate of formation of formaldehyde, as described in Materials and Methods. Arrows indicate the concentration of erythromycin (400 μM) used in subsequent studies.

activity in an aliquot (200 μl) of aqueous phase added to 5 ml of liquid scintillation fluid.

In all cases, preliminary studies were performed to establish conditions under which product formation was proportional to microsomal protein concentration and incubation time. Prior to each assay, liver microsomes from troleandomycin- or erythromycin estolate-treated rats were decomplexed by treatment with potassium ferricyanide, followed by dialysis to remove excess oxidant as previously described [20, 24].

Other assays. Protein concentrations was measured by the method of Lowry *et al.* [29], with bovine serum albumin as standard. The concentration of cytochrome P-450 was determined by the method of Omura and Sato [30], from the carbon monoxide-difference spectrum of dithionite-reduced microsomes based on an extinction coefficient of 91 mM^{-1} cm^{-1}. SDS-PAGE of liver microsomes was performed with a vertical slab gel apparatus from Hoefer Scientific Instruments (San Francisco, CA), according to the method of Laemmli [31]. The separating gel was 0.75 mm thick, 12 cm long and contained 7.5% acrylamide. Proteins were stained with Coomassie Blue R-250. Minor procedural modifications have been described elsewhere [32, 33].

RESULTS

Kinetics of erythromycin demethylation. The effect of substrate concentration on the rate of erythromycin demethylation catalyzed by liver microsomes from control and PCN-treated rats is shown in Fig. 1. The reaction catalyzed by liver microsomes from PCN-induced rats conformed to a linear Lineweaver–Burk plot, indicating that the reaction was dominated by a relatively high affinity enzyme or enzymes (K_m ~0.3 mM) with high catalytic turnover (V_{max} ~6 nmol/mg protein/min). In contrast, the reaction catalyzed by liver microsomes from control rats gave a non-linear Lineweaver–Burk plot, the shape of which suggested that at least two different enzymes (or groups of enzymes) catalyzed erythromycin demethylation. One was a high affinity (K_m ~0.4 mM) enzyme with low catalytic turnover (V_{max} ~1.3 nmol/mg protein/min). This enzyme was largely responsible for erythromycin demethylation catalyzed by control microsomes at low substrate concentrations (< 1 mM). The second was a low affinity enzyme (K_m ~4 mM) with comparatively high catalytic turnover (V_{max} ~2.7 nmol/mg protein/min). The second enzyme (or group of enzymes) contributed significantly to erythromycin demethylation catalyzed by control microsomes only at high substrate concentrations (> 1 mM).

The results in Fig. 1 indicate that the magnitude of the induction of erythromycin demethylase activity by PCN was dependent on the substrate concentration used. In subsequent experiments, a concentration of 400 μM erythromycin was used, as indicated by the arrow in Fig. 1. This corresponds to the concentration of erythromycin used by Wrighton *et al.* [24].

Effects of age and sex. Previous studies have shown that the age-dependent decline in cytochrome P-450p levels in female rats is paralleled by a decrease in testosterone 2β-, 6β-, 15β-, and 18-hydroxylase activities [20]. The effects of age and sex on liver microsomal erythromycin demethylase activity are shown in Table 1, together with testosterone 6β-hydroxylase activity for comparative purposes. Erythromycin demethylase activity did not vary more than 2-fold among liver microsomal preparations from immature or mature rats of either sex. In contrast, liver microsomes from mature female rats cata-

Table 1. Effects of age and sex of rats on liver microsomal erythromycin demethylase and testosterone 6β-hydroxylase activities*

Age and sex	Erythromycin demethylase (nmol HCHO/mg protein/min)	Testosterone 6β-hydroxylase (nmol/mg protein/min)
Immature female	0.90 ± 0.02	2.00 ± 0.10
Immature male	1.38 ± 0.11	2.10 ± 0.20
Mature female	0.68 ± 0.06	0.20 ± 0.02
Mature male	1.06 ± 0.06	2.30 ± 0.20

* Liver microsomes from immature (28-day-old) and mature (60-day-old) Long Evans rats of both sexes were assayed for erythromycin demethylase and testosterone 6β-hydroxylase activities as described in Materials and Methods. A final concentration of 400 μM erythromycin was used. Values are means ± SE of three to seven determinations.

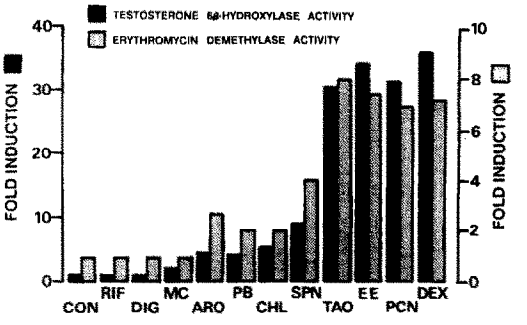

Fig. 2. Effects of various xenobiotic treatments on rat liver microsomal testosterone 6β-hydroxylase and erythromycin demethylase activities. Liver microsomes were prepared from mature female rats treated with corn oil (CON), rifampin (RIF), digitoxin (DIG), 3-methylcholanthrene (MC), Aroclor 1254 (ARO), phenobarbital (PB), chlordane (CHL), spironolactone (SPN), troleandomycin (TAO), erythromycin estolate (EE), pregnenolone-16α-carbonitrile (PCN) or dexamethasone (DEX). Testosterone 6β-hydroxylase and erythromycin demethylase activities were determined as described in Materials and Methods.

lyzed the 6β-hydroxylation of testosterone at one-tenth the rate catalyzed by the other microsomal preparations. Testosterone 2β-, 15β- and 18-hydroxylase activities also declined more than 90% with age in female, but not male rats (results not shown).

Induction of cytochrome P-450p. The effects of treating mature female rats with various xenobiotics on liver microsomal erythromycin demethylase and testosterone 6β-hydroxylase activities are shown in Fig. 2. Both activities were highly inducible by PCN, dexamethasone, troleandomycin and erythromycin estolate; moderately inducible by spironolactone and Aroclor 1254, and weakly but significantly ($P < 0.001$) inducible by phenobarbital and chlordane. In addition, treatment of rats with Aroclor 1254, phenobarbital or chlordane markedly induced (>30-fold) the 16α- and 16β-hydroxylation of testosterone and the 17-oxidation of testosterone to androstenedione, due to the induction of cytochrome P-450b [27, 32, 34]. At the doses tested, treatment of rats with rifampin, digitoxin or 3-methylcholanthrene had little or no effect on erythromycin demethylase or testosterone 6β-hydroxylase activity. Changes in testosterone 6β-hydroxylase activity were paralleled by changes in testosterone 2β-, 15β- and 18-hydroxylase activities (results not shown).

In Fig. 2, the catalytic activities shown for rats treated with troleandomycin or erythromycin estolate were determined after treatment of the microsomes with potassium ferricyanide followed by dialysis to remove excess oxidant. This treatment, which dissociates the cytochrome P-450-inducer complex [20, 24], caused a 3- to 4-fold stimulation of erythromycin demethylase and testosterone 2β-, 6β-, 15β- and 18-hydroxylase activities (results not shown).

Although changes in testosterone 6β-hydroxylase and erythromycin demethylase activities were qualitatively similar, the magnitude of the changes differed significantly (Fig. 2). For example, treatment of rats with PCN caused a 32-fold induction of testosterone 6β-hydroxylase activity, but only a 5-fold induction of erythromycin demethylase activity. The relatively low basal rate of testosterone 6β-hydroxylation catalyzed by liver microsomes from mature female rats (Table 1) accounted for the greater inducibility of this reaction. In male rats or immature female rats (which have a relatively high basal rate of testosterone 6β-hydroxylation), treatment with PCN caused a 4- to 6-fold induction of both testosterone 6β-hydroxylase and erythromycin demethylase activities (results not shown).

Induction of UDP-GT-dt$_1$. The effects of treating mature female rats with various xenobiotics on liver microsomal UDP-GT-dt$_1$ activity are shown in Fig. 3, alongside testosterone 6β-hydroxylase activity for comparative purposes. Those xenobiotics that had little or no effect on testosterone 6β-hydroxylase activity had little or no effect on UDP-GT-dt$_1$ activity. Treatment of rats with PCN or dexamethasone caused a 7- to 9-fold induction of UDP-GT-dt$_1$ activity and a 30- to 40-fold induction of testosterone 6β-hydroxylase activity. Troleandomycin and erythromycin estolate were equally as effective as PCN and dexamethasone at inducing cytochrome P-450p, but were less effective at inducing UDP-GT-dt$_1$. In contrast, spironolactone was equally as effective as PCN and dexamethasone at inducing UDP-GT-dt$_1$, but was less effective at inducing cytochrome P-450p.

The results from studies on mature female rats shown in Fig. 3 suggest that cytochrome P-450p is more inducible (30 to 40-fold) than UDP-GT-dt$_1$ (7 to 9-fold) by PCN and dexamethasone. However, the opposite result was obtained in immature rats, which have a low basal UDP-GT-dt$_1$ activity (which increased 24-fold in PCN-treated rats) and a relatively high basal testosterone 6β-hydroxylase activity (which increased 4.2-fold in PCN-treated rats), as shown in Table 2.

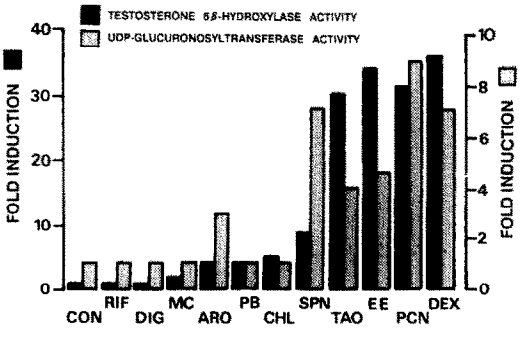

Fig. 3. Effects of various xenobiotic treatments on rat liver microsomal testosterone 6β-hydroxylase and UDP-glucuronosyltransferase-dt$_1$ activities. Liver microsomes were prepared from mature female rats treated with various xenobiotics, which are abbreviated as described in the legend to Fig. 2. The 6β-hydroxylation of testosterone and the glucuronidation of digitoxigenin monodigitoxoside were determined as described in Materials and Methods.

Table 2. Effects of xenobiotic treatment of immature male rats on testosterone 6β-hydroxylase and UDP-glucuronosyltransferase (dt_1) activities

Xenobiotic treatment	Testosterone 6β-hydroxylase (nmol/mg protein/min)	UDP-glucuronosyltransferase (nmol dt_1 glucuronidated/mg protein/min)
Control	2.1 ± 0.2	60 ± 30
Phenobarbital	3.9 ± 0.3* (1.9)	130 ± 60 (2.2)
Chlordane	4.6 ± 0.5* (2.2)	120 ± 32 (2.0)
3-Methylcholanthrene	1.2 ± 0.3 (0.6)	88 ± 30 (1.5)
Pregnenolone-16α-carbonitrile	8.9 ± 0.7* (4.2)	1450 ± 420* (24)

Liver microsomes from immature (28-day-old) male Long Evans rats were treated once daily for 4 consecutive days with various xenobiotics, and liver microsomes were prepared as described in Materials and Methods. Testosterone 6β-hydroxylase activity and UDP-glucuronosyltransferase activity towards dt_1 were assayed as described in Materials and Methods. Values are means ± SE of four to six determinations. Numbers in parentheses represent the fold increase over control.

* Statistically significant increase over control values at the 5% level of significance.

The catalytic activities of liver microsomes from troleandomycin- and erythromycin estolate-induced rats were determined before and after treatment with potassium ferricyanide, followed by dialysis. Unlike testosterone 6β-hydroxylase and erythromycin demethylase activity, which increased 2- to 5-fold following dissociation of the cytochrome P-450p–inducer complex, UDP-GT-dt_1 activity did not change following treatment of liver microsomes with potassium ferricyanide (results not shown).

Dose response. The dosage dependency of the induction of testosterone hydroxylase and UDP-GT-dt_1 activity by PCN is shown in Figs 4 and 5 respectively. Treatment of mature female rats with PCN at dosages from 1 to 50 mg/kg resulted in a progressive increase in testosterone 2β-, 6β-, 15β- and 18-hydroxylase activities. A dosage-dependent induction of 1-hydroxylase activity was also observed (Fig. 4), but the co-migration of 1α- and 1β-hydroxytestosterone in our HPLC system precluded an assignment of the stereochemistry of this reaction. In contrast, treatment of rats with PCN did not cause a dosage-dependent induction of pathways leading to the formation of 7α-hydroxytestosterone and androstenedione (results not shown). A dosage-dependent induction of UDP-GT-dt_1 activity by PCN, similar but not identical to that seen with testosterone hydroxylase activity, was also observed (Fig. 5).

Effects of co-administering PCN and troleandomycin. Administration of a single injection of troleandomycin (500 mg/kg) to rats pretreated for 4 consecutive days with PCN (50 mg/kg) caused a 40–50% increase in testosterone 2β-, 6β- and 15β-hydroxylase activities above that seen with PCN

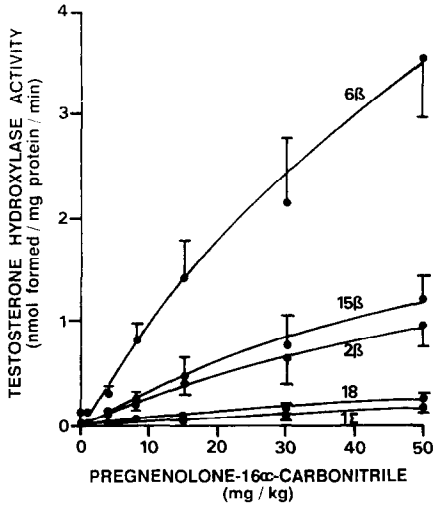

Fig. 4. Effects of various dosages of PCN on rat liver microsomal testosterone hydroxylase activity. Mature female rats were treated once daily for 4 consecutive days with PCN (1–50 mg/kg). Liver microsomes were prepared 24 hr after the last injection and incubated with testosterone as described in Materials and Methods. Testosterone metabolites were resolved and quantitated by HPLC [19, 27]. Abbreviations denote the hydroxylated metabolite formed, e.g. 6β denotes 6β-hydroxytestosterone. Values are mean ± SE of three determinations.

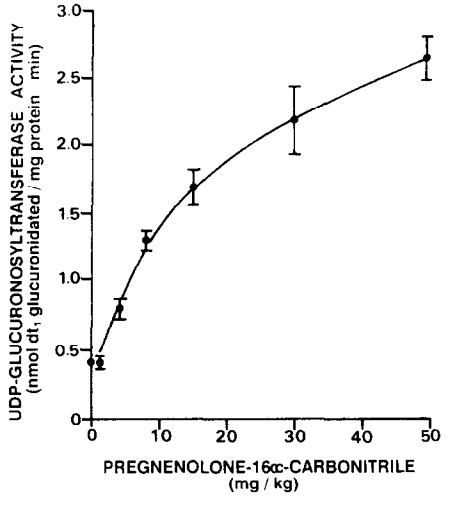

Fig. 5. Effects of various dosages of PCN on rat liver microsomal UDP-glucuronosyltransferase-dt_1 activity. Mature female rats were treated once daily for 4 consecutive days with PCN (1–50 mg/kg). Liver microsomes were prepared 24 hr after the last injection, and UDP-GT-dt_1 activity was determined as described in Materials and Methods. Values are mean ± SE of three determinations.

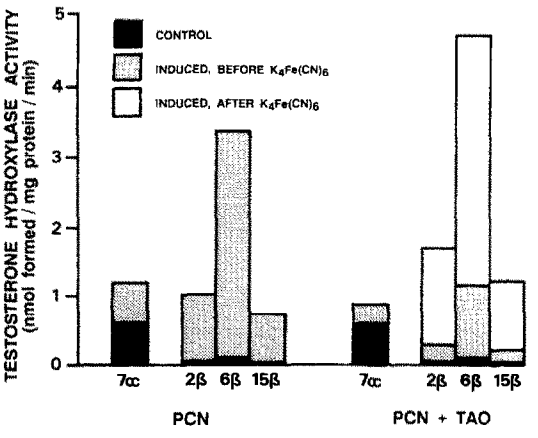

Fig. 6. Effects of co-administering troleandomycin (TAO) and PCN to rats on liver microsomal testosterone hydroxylase activity. Mature female rats were treated for 4 consecutive days with PCN (50 mg/kg) or corn oil (control). On day 4, half the PCN-treated rats (N = 5) received a single injection of troleandomycin (500 mg/kg), and all rats were killed 24 hr later. Liver microsomes were prepared and assayed for testosterone hydroxylase activity before and after treatment with potassium ferricyanide (followed by dialysis to remove excess oxidant), as described in Materials and Methods. Abbreviations on the abscissa denote the hydroxylated testosterone metabolite formed, e.g. 7α denotes 7α-hydroxytestosterone.

treatment alone (Fig. 6). Most of the cytochrome P-450p in the liver microsomes from rats treated with both PCN and troleandomycin was complexed with troleandomycin, as evidenced by the large activation in testosterone 2β-, 6β- and 15β-hydroxylase activities following treatment of the microsomes with potassium ferricyanide (Fig. 6). Dissociation of the cytochrome P-450p–inducer complex also increased the amount of cytochrome P-450 detectable by CO-difference spectroscopy [30], but had no effect on testosterone 7α-hydroxylase activity (Fig. 6). In contrast to its effect on cytochrome P-450p, administration of troleandomycin to PCN-pretreated rats resulted in no further increase in UDP-GT-dt_1 activity (results not shown).

SDS-PAGE. Liver microsomes from rats treated with the various xenobiotics shown in Figs. 2, 3 and 6 were subjected to SDS–PAGE, and the results are shown in Fig. 7. The ability of xenobiotics to induce cytochrome P-450p (i.e. testosterone 6β-hydroxylase and erythromycin demethylase activities) correspond with their ability to intensify a polypeptide of M_r 51,000. This polypeptide was electrophoretically distinct from cytochromes P-450a (M_r 47,000), P-450b (M_r 52,000) and P-450c (M_r 56,000) and epoxide hydrolase (M_r 49,000), and comigrated with partially purified cytochrome P-450p, isolated as described by Elshourbagy and Guzelian [9] or Shimada and Guengerich [35] (results not shown). Treatment of rats with phenobarbital or chlordane intensified a polypeptide that comigrated with cytochrome P-450b. Treatment of rats with 3-methylcholanthrene intensified a M_r 56,000 and M_r 52,000 polypeptide that comigrated with cytochromes P-450c and P-450d respectively. The pattern of microsomal polypeptides was apparently unaffected by treatment of rats with digitoxin and rifampin.

A distinct polypeptide that varied in intensity in accordance with changes in UDP-GT-dt_1 was not discernable by SDS–PAGE. It is possible that UDP-GT-dt_1 comigrates with cytochrome P-450p in the SDS–PAGE system, or is such a minor microsomal protein that changes in its concentration were undetectable by SDS–PAGE.

DISCUSSION

Previous studies have shown that cytochrome P-450p and UDP-GT-dt_1 are differentially affected by the age and the sex of rats [12, 18, 21], raising the possibility that these microsomal enzymes can be regulated (e.g. induced) independently. The results of the present study indicate a good correlation between the ability of xenobiotics to induce rat liver microsomal cytochrome P-450p (measured as erythromycin demethylase and testosterone 2β-, 6β-, 15β- and 18-hydroxylase activities) and their ability to induce UDP-GT-dt_1. Five xenobiotics, namely PCN, dexamethasone, spironolactone, troleandomycin and erythromycin estolate, were effective inducers of both enzymes, and their induction by PCN followed similar dose–response curves. Each of these five xenobiotics has been shown by immunochemical analysis to induce cytochrome P-450p [10, 24, 25]. Compared to PCN and dexamethasone, spironolactone preferentially induced UDP-GT-dt_1, whereas the macrolide antibiotics preferentially induced cytochrome P-450p. These results suggest that cytochrome P-450p and UDP-GT-dt_1 are co-inducible but are not coordinately regulated in rat liver microsomes.

The inducibility of testosterone 6β-hydroxylase activity in mature female rats closely resembled that of erythromycin demethylase activity (Fig. 2). However, the two activities were differentially affected by the age and sex of the rats. Only testosterone 6β-hydroxylase activity paralleled cytochrome P-450p levels, which have been shown by immunochemical techniques to decline markedly with age in female but not male rats [12]. Kinetic studies revealed that the demethylation of erythromycin catalyzed by liver microsomes from PCN-induced rats was dominated by a single enzyme (or group of enzymes). In contrast, liver microsomes from mature female rats contained at least two kinetically distinct enzymes (or group of enzymes) capable of demethylating erythromycin. Consequently, the inducibility of erythromycin demethylase activity by PCN was dependent on the substrate concentration used. These results suggest that forms of cytochrome P-450 other than cytochrome P-450p can contribute significantly to the demethylation of erythromycin catalyzed by rat liver microsomes.

We have shown recently that partially purified cytochrome P-450p catalyzes the 2β-, 6β-, 15β- and 18-hydroxylation of testosterone [20]. These four activities responded in unison to the effects of age, sex and treatment of rats with xenobiotics. Treatment of rats with PCN caused a dosage-dependent increase in all four activities, plus an increase in testosterone 1-hydroxylase activity (Fig. 4). Whether

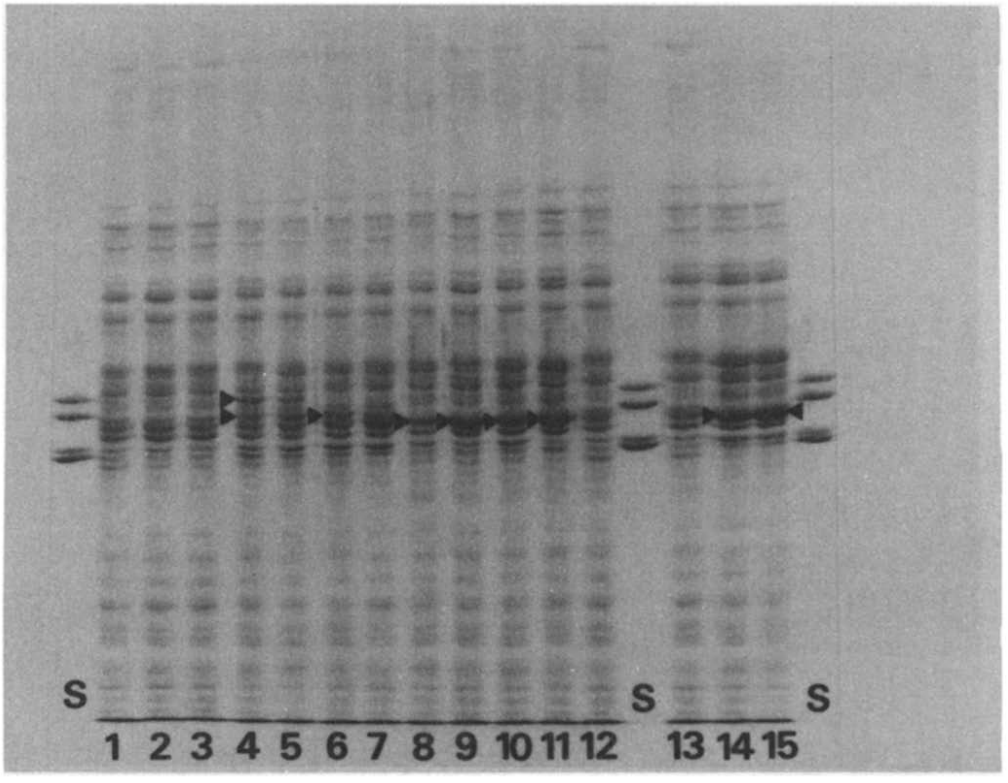

Fig. 7. Electrophoresis of liver microsomes from mature female rats treated with various xenobiotics. Liver microsomes (7 μg) from rats treated with corn oil (control) (lanes 1 and 12), rifampin (lane 2), digitoxin (lane 3), 3-methylcholanthrene (lane 4), Aroclor 1254 (lane 5), phenobarbital (lane 6), chlordane (lane 7), spironolactone (lane 8), troleandomycin (lane 9), PCN (lane 10) or dexamethasone (lane 11) were subjected to SDS–PAGE, as described in Materials and Methods. Lanes 13, 14 and 15 contained liver microsomes from rats treated with corn oil, PCN or a combination of PCN and TAO respectively. Lanes marked "S" contained a mixture (0.3 μg each) of cytochrome P-450a (M_r 47,000), epoxide hydrolase (M_r 49,000), cytochrome P-450b (M_r 52,000) and cytochrome P-450c (M_r 56,000), purified as described [32, 33]. Bands corresponding to cytochrome P-450c and cytochrome P-450d (lane 4), cytochrome P-450b (lane 6) and cytochrome P-450p (lane 8–11, 13 and 14) are indicated by a black arrow. Proteins were stained with Coomassie Blue R-250.

cytochrome P-450p catalyzes the 1-hydroxylation of testosterone remains to be determined. However, like 18-hydroxylation, 1-hydroxylation was a minor pathway of testosterone metabolism.

Although rifampin is the most effective inducer of rabbit liver microsomal cytochrome P-450 LM3c, the isozyme equivalent to rat liver microsomal cytochrome P-450p [24, 36, 37], rifampin is reportedly incapable of inducing cytochrome P-450p in rats [23]. The results of the present study indicate that, like cytochrome P-450p, liver microsomal UDP-GT-dt_1 is also refractory to treatment of rats with rifampin. Cytochrome P-450p and UDP-GT-dt_1 were also refractory to treatment of rats with digitoxin, despite the fact that digitoxin is a substrate for cytochrome P-450p, and that its metabolite, digitoxigenin monodigitoxoside, is a substrate for UDP-GT-dt_1 [14, 38–40].

Treatment of rats with phenobarbital has been shown previously to cause a 2-fold induction of rat liver microsomal UDP-GT-dt_1 activity [17, 25, 41]. The data in Fig. 3 indicate that treatment of mature female rats with phenobarbital did not induce UDP-GT-dt_1 activity. However, these data are expressed as per milligram of microsomal protein, whereas in previous reports these data were expressed as per gram liver. The data in Fig. 3 do not reflect a 1.6- to 1.8-fold increase in the amount of microsomal protein per g liver caused by treatment of rats with phenobarbital, which would account for the apparent discrepancy between this and previous reports [17, 25, 41]. In immature rats, mean UDP-GT-dt_1 specific activity did increase ~2-fold following phenobarbital or chlordane treatment, as shown in Table 2, although this was not statistically significant.

The ability of the macrolide antibiotics, troleandomycin and erythromycin estolate, to induce both liver microsomal cytochrome P-450p and UDP-GT-dt_1 is of particular interest. The mechanism by which these antibiotics induce cytochrome P-450p is thought to involve both an increase in rate of synthesis and a decrease in rate of degradation [19]; the latter being a consequence of the formation of a complex between cytochrome P-450p and a metabolite of the antibiotic [19]. The observation that troleandomycin and erythromycin estolate preferentially induced cytochrome P-450p over UDP-GT-dt_1 is consistent with this proposal, as is the

observation that administration of troleandomycin to PCN-induced rats caused a further increase in cytochrome P-450p activity, but had no effect on UDP-GT-dt_1 activity (Fig. 6).

The results of the present study provide further insight into the mechanism by which PCN, dexamethasone and spironolactone protect rats from digitoxin toxicity. Treatment of rats for 4 days with troleandomycin induced both cytochrome P-450p and UDP-GT-dt_1 activity. However, troleandomycin bound to and inhibited cytochrome P-450p, but did not form an inhibitory complex with UDP-GT-dt_1. We have shown previously that treatment of rats for 4 days with troleandomycin does not protect rats against the toxic effects of digitoxin [21]. These results indicate that induction of UDP-GT-dt_1 alone is insufficient to protect rats against the toxic effects of digitoxin. We have also shown that a single injection of troleandomycin administered to PCN-pretreated rats reverses the ability of PCN to protect rats against digitoxin toxicity [21]. This treatment also inhibited cytochrome P-450p but not UDP-GT-dt_1 activity (Fig. 6), which provides further evidence that induction of UDP-GT-dt_1 activity alone is insufficient to protect rats against digitoxin toxicity.

REFERENCES

1. H. Selye, *J. pharm. Sci.* **60**, 1 (1971).
2. B. Solymoss, S. Toth, S. Varga and H. Selye, *Toxic. appl. Pharmac,* **18**, 586 (1971).
3. S. H. Buck and G. L. Lage, *Archs. int. Pharmacody. Thér.* **189**, 192 (1971).
4. M. C. Castle and G. L. Lage, *Biochem. Pharmac.* **21**, 1449 (1972).
5. M. C. Castle and G. L. Lage, *Res. Commun. Chem. Path. Pharmac.* **5**, 99 (1973).
6. C. D. Klaassen, *J. Pharmac. exp. Ther.* **191**, 201 (1974).
7. W. C. Kershaw, P. Campbell and G. L. Lage, *Drug Metab. Dispos.* **13**, 635 (1985).
8. A. H. Lu, A. Somogyi, S. West, R. Kuntzman and A. H. Conney, *Archs. Biochem. Biophys.* **152**, 457 (1972).
9. N. A. Elshourbagy and P. S. Guzelian, *J. biol. Chem.* **255**, 1279 (1980).
10. D. M. Hueman, E. J. Gallager, J. L. Barwick, N. A. Elshourbagy and P. S. Guzelian, *Molec. Pharmac.* **21**, 753 (1982).
11. F. P. Guengerich, D. A. Dannan, S. T. Wright, M. V. Martin and L. S. Kaminsky, *Biochemistry* **21**, 6019 (1982).
12. D. J. Waxman, D. A. Dannan and F. P. Guengerich, *Biochemistry* **24**, 4409 (1985).
13. J. R. Gorski, M. P. Arlotto, C. D. Klaassen and A. Parkinson, *Carcinogenesis* **6**, 617 (1985).
14. L. G. Richards and G. L. Lage, *Toxic. appl. Pharmac.* **42**, 309 (1977).
15. M. C. Castle, *Biochem. Pharmac.* **29**, 1497 (1980).
16. A. Schmoldt and J. Promies, *Biochem. Pharmac.* **31**, 2285 (1982).
17. J. B. Watkins, Z. Gregus, T. N. Thompson and C. D. Klaassen, *Toxic. appl. Pharmac.* **64**, 439 (1982).
18. J. B. Watkins and C. D. Klaassen, *Drug Metab. Dispos.* **13**, 186 (1985).
19. P. B. Watkins, S. A. Wrighton, E. G. Schuetz, P. Maurel and P. S. Guzelian, *J. biol. Chem.* **261**, 6246 (1986).
20. A. J. Sonderfan, D. R. Dutton, M. P. Arlotto and A. Parkinson, *Archs. Biochem. Biophys.* **255**, 27 (1987).
21. M. P. Arlotto, A. J. Sonderfan, M. M. McKinney and A. Parkinson, *Archs. Biochem. Biophys.* **251**, 188 (1986).
22. D. Larrey, L. M. Distlerath, G. A. Dannan, G. R. Wilkinson and F. P. Guengerich, *Biochemistry* **23**, 2787 (1984).
23. S. A. Wrighton, E. G. Schultz, P. B. Watkins, P. Maurel, J. Bartwick, B. S. Bailey, H. T. Hartle, B. Young and P. S. Guzelian, *Molec. Pharmac.* **28**, 312 (1984).
24. S. A. Wrighton, P. Maurel, E. G. Schuetz, P. B. Watkins, B. Young and P. S. Guzelian, *Biochemistry* **24**, 2171 (1985).
25. E. G. Schuetz, G. A. Hazelton, J. Hall, P. B. Watkins, C. D. Klaassen and P. S. Guzelian, *J. biol. Chem.* **261**, 8270 (1986).
26. A. Y. H. Lu and W. Levin, *Biochem. biophys. Res. Commun.* **46**, 1334 (1972).
27. A. W. Wood, D. E. Ryan, P. E. Thomas and W. Levin, *J. biol. Chem.* **258**, 8839 (1983).
28. T. Nash, *Biochem J.* **55**, 416 (1953).
29. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
30. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2379 (1964).
31. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
32. D. E. Ryan, P. E. Thomas, D. Korzeniowski and W. Levin, *J. biol. Chem.* **254**, 1365 (1979).
33. D. E. Ryan, P. E. Thomas and W. Levin, *J. biol. Chem.* **255**, 7941 (1980).
34. P. E. Thomas, L. M. Reik, D. E. Ryan and W. Levin, *J. biol. Chem.* **256**, 1044 (1981).
35. T. Shimada and F. P. Guengerich, *Molec. Pharmac.* **28**, 215 (1985).
36. D. R. Koop, A. V. Persson and M. J. Coon, *J. biol. Chem.* **258**, 10704 (1981).
37. R. Lange, B. Balny and P. Maurel, *Biochem. Pharmac.* **33**, 2771 (1984).
38. M. C. Castle and G. L. Lage, *Toxic. appl. Pharmac.* **27**, 641 (1974).
39. A. Schmoldt, *Naunyn-Schmiedeberg's Archs. Pharmac.* **305**, 261 (1978).
40. L. von Meyerinck, B. L. Coffman, M. D. Green, R. B. Kirkpatrick, A. Schmoldt and T. R. Tephly, *Drug Metab. Dispos.* **13**, 700 (1985).
41. J. B. Watkins and C. D. Klaassen, *Drug Metab. Dispos.* **10**, 590 (1982).