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CHOLESTEROL 7α-HYDROXYLASE—EVIDENCE FOR A DISTINCT HEPATIC MICROSOMAL ENZYME SYSTEM*

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Abstract—The interrelationship of microsomal 7α-hydroxylation and drug oxidation reactions was studied in liver microsomes obtained from the male Wistar rat. When several compounds were administered to rats, a variety of changes ensued concerning the rate of cholesterol 7α-hydroxylation, ethylmorphine *N*-demethylase activity, and alterations in electron transport components. Cholestyramine and D-thyroxine administration resulted in significant increases in cholesterol 7α-hydroxylase activity. Both phenobarbital (PB) and spironolactone pretreatment did not produce an elevation of cholesterol 7α-hydroxylation, but significant increases in ethylmorphine *N*-demethylation over controls were realized. There was a lack of congruence with the rate of cholesterol 7α-hydroxylation and the content or activity of electron transport components whereas there was a demonstrable dependency of ethylmorphine *N*-demethylation on the monitored electron transport components for PB- and spironolactone-treated rats. Along with an elevation of cytochrome P448 content, 3-methylcholanthrene (3-MC) pretreatment reduced cholesterol 7α-hydroxylase activity and the rate of ethylmorphine *N*-demethylation. Concomitant treatment with 3-MC and cholestyramine caused no alteration of cholesterol 7α-hydroxylase activity. The inhibitory action of 3-MC on cholesterol 7α-hydroxylase activity was not due to competition for reducing equivalents in liver microsomes. The inhibitory phenomenon caused by NADH on microsomal cholesterol 7α-hydroxylase activity was reproducible and was dose-dependent at both subsaturating and saturating levels of NADPH. In conclusion, the results of this study indicate that the cholesterol 7α-hydroxylase enzyme system is distinctly different from that which catalyzes drug oxidations.

Several investigators have demonstrated that the 7α-hydroxylation of cholesterol is the initial and rate limiting step in the transformation of cholesterol to bile acids [1–3]. Cholesterol 7α-hydroxylase (EC 1.14) is located in the microsomal fraction of the rat liver homogenate and requires NADPH, molecular oxygen, NADPH-cytochrome *c* reductase and a form of cytochrome P450 [4–8]. These requirements are characteristic of the group of enzymes classified as mixed function oxidases (MFO) [9]. In general, the cytochrome P450 dependent MFO system of liver microsomes catalyzes the oxidation of a large number of xenobiotics and certain endogenous substrates such as steroids [10]. The MFO system is inducible as evidenced by the number of agents which when administered result in an enhanced ability to oxidize a wide variety of substrates [10, 11]. The 7α-hydroxylation of cholesterol appears to be likened to the MFO system described by Conney [10] and Gillette and associates [11] as evidenced by the fact that the K_m for oxygen for hepatic microsomal cholesterol 7α-hydroxylase has been determined to be 20 μM [7].

The participation of hepatic microsomal electron transport components in the 7α-hydroxylation of cholesterol in rat has been investigated by several laboratories [4–8]. However, the evidence for the involvement of cytochrome P450 in the 7α-hydroxylation of cholesterol still remains somewhat equivocal. In particular, there are contradictory views of the effect of phenobarbital (PB) on this hydroxylation process [12–17]. The unique properties of the cholesterol 7α-hydroxylase enzyme system, with respect to that catalyzing drug hydroxylation, become even more overt when animals are pretreated with several other compounds including: cholestyramine, D-thyroxine (D-T_4), pregnenolone 16α-carbonitrile (PCN) and 3-methylcholanthrene (3-MC) [5, 6, 18–20].

In this paper we have investigated the interrelationship of cholesterol 7α-hydroxylase and ethylmorphine *N*-demethylase employing several drug pretreatments to evaluate changes in these hydroxylations with alterations of hepatic microsomal electron transport components.

MATERIALS AND METHODS

Source of reagents. Chemicals were obtained as follows: cholesterol (standard for chromatography, >99%); cholesterol acetate (standard for chromatography, >99%); NADP-monosodium salt; NADPH-tetrasodium salt; NAD-monosodium salt;

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NADP-disodium salt; glucose-6-phosphate, disodium salt, glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (grade 2); pyruvic acid, sodium salt; cytochrome *c*, horse heart type 3; Coenzyme A; bovine albumin, fraction 5; EDTA, 3-methylcholanthrene; and spironolactone; hematoporphyrin, free base; from Sigma Chemical Co., St. Louis, MO. Tween 80, ICI–Atlas Chemical Co., Wilmington, DE. Aminopyrine; Merck and Co., Rahway, NJ; carbon monoxide, Matheson Gas Products, East Rutherford, NJ; ethylmorphine was a gift from Merck and Co., Rahway, NJ. Cholestyramine (Questran) was a gift from Mead Johnson, Evansville, IN; pregnenolone 16α-carbonitrile (PCN) was a gift from G. D. Searle, Chicago, IL; sodium dextrothyroxine was a gift from Flint Labs., Morton Grove, IL.

The following reference compounds were obtained from Steraloids, Hilton, NH: cholest-5-en-3β,7α-diol (7α-hydroxycholesterol), m.p. 183.5–184.5°, Rot.: −89°; cholest-5-en-3β,7β-diol (7β-hydroxycholesterol), m.p. 178–180°, Rot.: +7°; and cholest-5-en-3β-01-7-one (7-ketocholesterol), m.p. 174–177°, Rot.: −101.7°. Additionally, 7β-hydroxycholesterol was prepared by reduction of 7-ketocholesterol as described by Mosbach *et al.* [21]. Synthesis of 7α-hydroxy-cholesterol was accomplished via the photooxidation of cholesterol in the presence of hematoporphyrin described by Schenck *et al.* [22] and Lythgoe and Trippett [23]. Cholest-5-ene-3β,7α-diol diacetate was prepared by acetylation of 7α-hydroxycholesterol. A portion (50 mg) of 7α-hydroxycholesterol was dissolved in 2 ml of pyridine and allowed to react with 1 ml of acetic anhydride for 24 hr at 37°. Crystals of the diacetate were obtained after evaporation and extraction with methanol. 4-Dimethyl-aminopyridine (DMAP), Aldrich Chemical Co., Milwaukee, WI, was crystallized several times from hot benzene before use.

The following radiolabeled compounds were obtained from New England Nuclear Co., Boston, MA. [4-^{14}C]-cholesterol, specific activity 50–60 mCi/m-mole, was purified prior to usage by chromatography (t.l.c.) on silica gel plate (Analabs, New macia Fine Chemical, Piscataway, NJ) and eluted with dichloromethane, followed by thin layer chromatography (TLC) on silica gel plate (Analabs, New Haven, CN) developed in benzene–ethylacetate (7:13); [^{3}H]acetic anhydride, specific activity 100 mCi/m-mole, transferred and purified under vacuum as described by Henderson and co-workers [24], and [4-^{14}C]-cholest-5-en-3β,7α-diol, specific activity 0.41 mCi/m-mole was prepared and purified as previously described.

Animals. Male, albino Wistar rats (Harlan Laboratories, Cumberland, IN), weighing 150–170 g were fed Purina Chow and received water *ad lib.* All animals were maintained in a vivarium at 25–26° on an alternating 12-hr light and dark cycle. Before the initiation of any experimental procedures, the animals were allowed at least a period of 2–3 days to become acclimated to the animal facilities.

Drug preparation and pretreatment regimen. Cholestyramine was given by mixing it with the diet (5% w/w) unless otherwise stated. Rats were injected intraperitoneally (i.p.) with D-T_4 (0.5 mg/kg) dissolved in ethanol–distilled water once daily for 7 consecutive days. PB (80 mg/kg) was dissolved in 0.9% saline and administered i.p. once daily for 5 days in a volume of 0.2 ml/100 g body wt. Treatment with 3-MC (20 mg/kg) or an equal volume of corn oil was given i.p., once daily for 5 days. Injections were given in a volume of 0.2 ml/100 g body wt. Spironolactone and PCN were injected as microcrystalline suspensions stabilized with Tween 80 (2 drops/10 ml) i.p., twice daily for 5 consecutive days in a volume of 0.2 ml/100 g of body wt. Spironolactone was given in a dose of 120 mg/kg while PCN was administered in a dose of 35 mg/kg. All control animals received the appropriate vehicle in a volume coincidental with that of the drug-treated groups.

Preparation of hepatic microsomes. Animals were sacrificed between 8:00–9:00 a.m. on the day of the experiment. Their livers were excised immediately, rinsed in ice cold buffer, weighed, minced, and then homogenized in 0.1 M potassium phosphate buffer, pH 7.4, containing 1.15% (w/v) KCL and 1 mM EDTA, using a glass homogenizer equipped with a loosely fitting Teflon pestle. Unless otherwise stated, all steps were carried out at 0–4°.

The homogenates, 25% (w/v), were centrifuged at 9000 *g* for 10 min in a Sorvall RC2-B refrigerated centrifuge (Ivan Sorvall, IN., Norwalk, CN) (Sorvall type rotor, SS-34); the supernatant decanted off and recentrifuged at 9000 *g* for another 10 min period. The microsomal fraction was obtained by centrifuge (Ivan Sorvall, Inc., Norwalk, CN) (Sor- 1 hr in a Beckman Model L5-75 ultracentrifuge (Beckman Instruments; Inc., Palo Alto, CA) (Beckman-type 40 rotor). The 105,000 *g* supernatant was removed, and the microsomal pellet was washed and resuspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 5 mM $MgCl_2$. After resuspension, the microsomes were spun at 105,000 *g* for 30 min. The final pellet was resuspended in the appropriate buffer so that the final concentration of microsomal protein was 10 mg/ml.

N-Demethylase reactions. NADPH-mediated drug metabolism was determined in an incubation mixture consisting of 0.1 M potassium phosphate buffer, pH 7.4, with 5 mM $MgCl_2$, a NADPH generating system (0.65 mM NADP, 9.0 mM glucose-6-phosphate, two enzyme units glucose-6-phosphate dehydrogenase), 2.5–5.0 mg of microsomal protein and ethylmorphine, 3.0 mM, in a final volume of 2.0 ml. The incubation mixtures minus substrate were preincubated for 2.0 min at 37°. The reaction was initiated with addition of substrate and incubated in a Dubnoff metabolic shaker under air (unless otherwise stated) at 37° with a shaking speed of 100 oscillations per min (opm) for 12 or 15 min. The method of Nash [25] was used to estimate the amount of formaldehyde formed in the incubation mixtures.

Cholesterol 7α-hydroxylase assay. The assay for cholesterol 7α-hydroxylase activity is similar to that described by Mitropoulos and Balasubramaniam [26] and modified slightly for the isolation of the diacetylated metabolites by the method of Shefer *et al.* [14]. The standard incubation mixture contained, in a final volume of 2.0 ml, 0.1 M potassium phos-

phate buffer, pH 7.4, with 5.0 mM $MgCl_2$, an NADPH generating system (5.0 mM NADP, 25.0 mM glucose-6-phosphate, and four enzyme units of glucose-6-phosphate dehydrogenase), 2.5 mg of microsomal protein, and 6.5 μM [4-^{14}C]cholesterol. In every case the labeled cholesterol was added to the incubation mixture as a suspension in Tween 80 (0.5 mg of Tween 80/flask); the preparation of which has been described previously [27]. Incubation mixtures were allowed to preincubate for 2.0 min at 37° prior to the addition of [^{14}C]cholesterol. The incubations were carried out in air (unless otherwise specified) at 37° in a Dubnoff metabolic shaker with constant shaking (100 opm) for 20 or 30 min. It was determined that the formation of both the total mass of 7α-hydroxycholesterol and 7α[^{14}C]cholesterol were linear with respect to time and microsomal protein concentrations employed for this study.

The reaction was terminated and the incubation contents extracted several times with a total of 15 vol. of chloroform–methanol (2 : 1). Further analysis of the chloroform-methanol extracts and eventual quantitation of 7α-hydroxy[^{14}C]cholesterol was performed according to the procedures described previously [26]. Recoveries of [^{14}C]cholesterol and 7α-hydroxy[^{14}C]cholesterol were estimated from the microsomal incubations and found to be consistently > 98 and > 96 per cent, respectively.

For the acetylation of 7α-hydroxycholesterol, each tube contained 25 μl of 1% DMAP (resulted in a range of 91–93 per cent diacetylated product) in benzene (thiophene-free) and 25 μl of freshly distilled [^{3}H]acetic anhydride in benzene (16 μmoles of acetic anhydride/tube; specific activity of 1.5 μCi/μmole). The tubes were tightly stoppered with Teflon lined caps and the contents thoroughly mixed. After 36 hr at 40° the excess [^{3}H]acetic anhydride was removed by evaporation under a stream of N_2. The dried residue was redissolved several times with benzene–methanol (4 : 1) and finally evaporated to dryness. The acetylated sterols were dissolved in 25 μl of benzene and aliquoto spotted on aluminia t.l.c. plates, 250 μ thickness, 5 × 20 cm (Analabs, New Haven, CN.) which had been activated for 1 hr at 100°. The [^{3}H,^{14}C] cholest-5-en-3β,7α-diol diacetate formed was separated from the monoacetylated and non-acetylated sterols by allowing the t.l.c. plates to develop for a distance of 15 cm in an equilibrated chromatography chamber containing ethyl–ether-*n*-hexane (1 : 1) as the solvent. Reference standards were co-chromatographed with the radioactive sterols and detected as previously described [14].

Both the [^{14}C]sterols as well as the [^{3}H, ^{14}C]-diacetylated 7α-hydroxycholesterol were quantified by scraping the appropriate zones on the t.l.c. plates and determining the amount of radioactivity by liquid scintillation spectrometry using a xylene-based mixture (Kew Solve, OH). The silica gel or alumina scrapings were added directly to scintillation vials containing 10 ml of scintillation cocktail. The [^{3}H]–[^{14}C] ratio was determined by double isotope analysis and quenching was monitored by automatic external standardization. Counting efficiencies for [^{3}H] and [^{14}C] were 40 and 92–94 per cent; respectively.

Assays for microsomal electron transport components. The hemoproteins cytochrome P450 and cytochrome b_5 were estimated as described by Omura and Sato [28]. The amount of cytochrome P450 was determined from the absorbance difference (Δ 450–490) and the molar extinction coefficient of 91 mM^{-1} cm^{-1}. The amount of cytochrome b_5 was determined from the absorbance difference (Δ 409–424) and the molar extinction coefficient of 185 mM^{-1} cm^{-1}. NADPH-cytochrome *c* reductase activity was measured by the method of Phillips and Langdon [29] using a molar extinction coefficient of 19.1 mM^{-1} cm^{-1}. The procedure employed for determining NADPH-cytochrome P450 reductase was essentially that described by Gillette and associates [30–32]. The rate of cytochrome P450 reduction by NADPH was calculated from the initial rate of increase in absorbance to determine the effect of various drug pretreatments and the effect of addition *in vitro* of substrates on NADPH-cytochrome P450 reductase. Substrate addition *in vitro* and/or the diluting or suspending medium were added to the microsomal suspension during the initial gassing procedure. The rate of NADPH oxidation was determined by following the reduction of optical density at 340 nm of a solution containing 5.0 mg of microsomal protein, 1 μmole of NADPH, and enough 0.1 M potassium phosphate buffer pH 7.4 to make a final volume of 3.0 ml. All spectrophotometric measurements were made using a Gilford 240 spectrophotometer (Gilford Instrument Lab., OH) connected to a Honeywell electronik 15 recorder (Honeywell Incorporated, PA).

Protein determination. Microsomal protein was estimated by the method of Lowry *et al.* [33] with crystalline bovine serum albumin as a standard.

Statistical analysis. Where appropriate, statistical comparisons of independent sample means were made using the student '*t*'-test at the 95 per cent level of confidence.

RESULTS

A variety of changes ensued concerning the rate of cholesterol 7α-hydroxylation, ethylmorphine *N*-demethylase activity, and alterations in electron transport components after prior administration of several compounds. The results in Fig. 1 illustrate the magnitude of these changes when contrasted to their respective control groups. Cholestyramine fed to rats caused significant elevations over control animals in the NADPH-mediated reduction of cytochrome P450, 44 per cent and cytochrome *c*, 19 per cent, NADPH oxidation, 66 per cent and cholesterol 7α-hydroxylase activity, 131 per cent, in liver microsomes (Fig. 1a). D-T_4 (0.5 mg/kg) administration also resulted in a significant increase in cholesterol 7α-hydroxylase activity of 78 per cent over control animals (Fig. 1b). However, the enhancement of cholesterol 7α-hydroxylation *via* the treatments, cholestyramine, and D-T_4, was not consistent with changes observed in electron transport components or with variations associated with drug hydroxylation reactions (i.e. ethylmorphine *N*-demethylation). Whereas NADPH-cytochrome P450 reductase and NADPH-cytochrome *c* reductase activities were

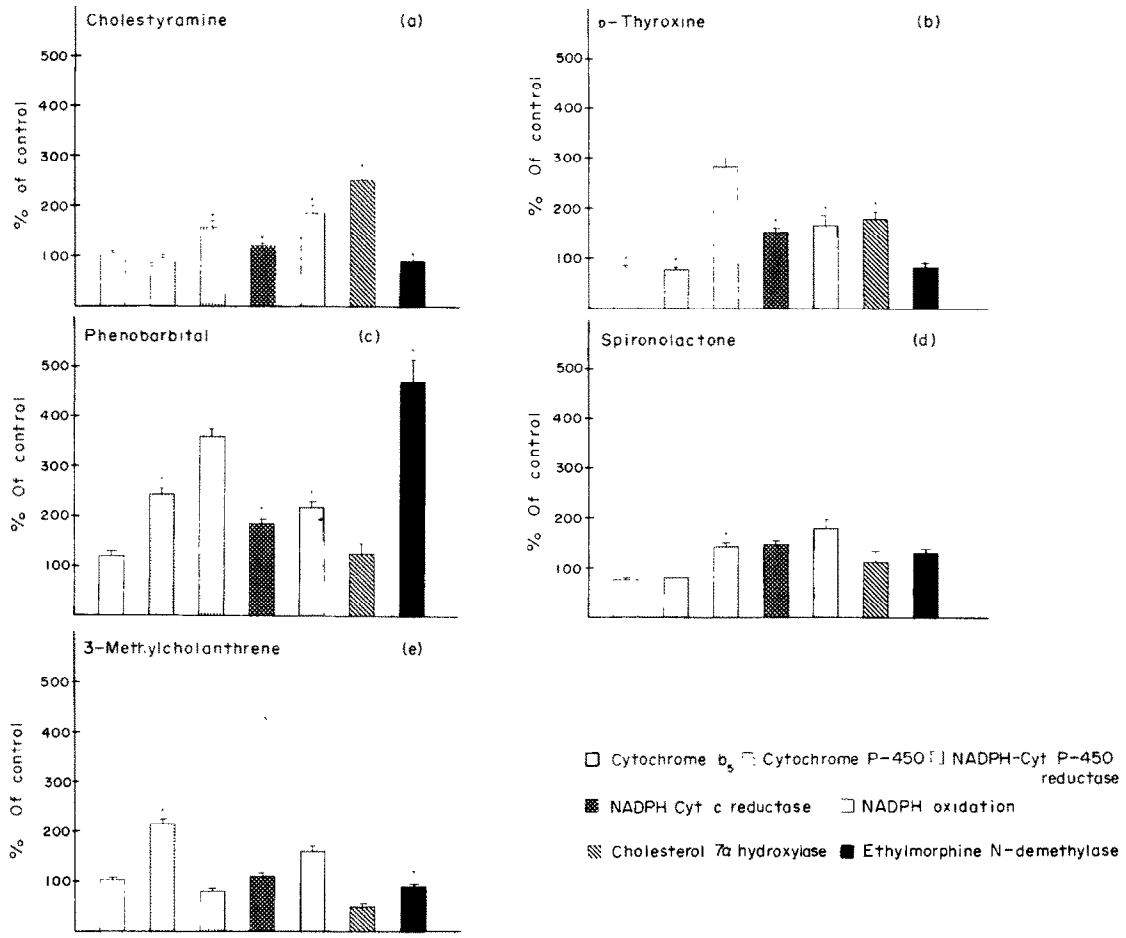

Fig. 1. The effect of several drug pretreatments on cholesterol 7α-hydroxylase activity, ethylmorphine *N*-demethylase activity, and on several microsomal electron transport components. Values represent the mean (expressed as per cent of control) ± S.E.M. of at least four animals. Cholesterol 7α-hydroxylase activity is measured from the total mass of 7α-hydroxycholesterol formed while ethylmorphine *N*-demethylase was estimated from the formaldehyde production as described in Methods. The mean with range of control values for each of the parameters presented is as follows: cytochrome b_5 content, 0.389 (0.377–0.405) nmoles/mg microsomal protein, cytochrome P450 content, 0.826 (0.781–0.863) nmoles/mg microsomal protein, NADPH-cytochrome P450 reductase activity, 3.13 (2.43–3.90) nmoles P450 reduced/mg protein/min, NADPH-cytochrome *c* reductase activity, 107.16 (97.37–112.90) nmoles cytochrome *c* reduced/mg protein/min, NADPH oxidase, 5.38 (4.30–6.24) nmoles NADPH reduced/mg protein/min, cholesterol 7α-hydroxylase activity, 16.79 (13.31–22.05) pmoles 7α-hydroxycholesterol formed/mg protein/min, and ethylmorphine *N*-demethylase activity, 2.78 (1.78–4.08) nmoles formaldehyde formed/mg protein/min. *Value obtained from drug pretreated animals is significantly different than the value obtained from control animals, $P < 0.05$.

increased via these pretreatments, cytochrome P450 levels were reduced by D-T_4, and unchanged with cholestyramine.

Upon the administration of the known hepatic enzyme inducers PB and spironolactone, ethylmorphine *N*-demethylase activity was greatly accelerated with PB (470 per cent of control) while spironolactone resulted in approximately a 40 per cent rise in activity compared to controls [10, 34, 35] (Figs 1c and 1d). On the contrary, neither agent stimulated cholesterol 7α-hydroxylase activity to any significant extent. A comparison of their ability to modify electron transport components reveals that while PB and spironolactone enhance NADPH-cytochrome P450 reductase and NADPH-cytochrome *c* reductase activities in conjunction with an overall stimulation of NADPH oxidation, their effect on heme content is dissimilar. PB treatment did not alter microsomal cytochrome b_5 levels but elevated cytochrome P450 content significantly (240 per cent of controls). On the other hand, spironolactone treatment reduced the heme content of both cytochrome b_5 and P450 in liver microsomes.

It is well recognized that the administration of 3-MC induces a different form of cytochrome P450 which has been designated as cytochrome P448 [36]. Administration of 3-MC results in yet another combination of alterations on drug and steroid hydroxylations as well as a profound modulation on microsomal electron transport components. Cholesterol 7α-hydroxylase activity is significantly decreased (49 per cent of controls) in addition to a lowering of the rate of ethylmorphine *N*-demethylation (91 per cent of control) (Fig. 1e).

Table 1. Effect of 3-MC (*in vivo* and *in vitro*) and cholestyramine pretreatment on microsomal cholesterol 7α-hydroxylase activity

Treatment	Cholesterol 7α-hydroxylation (pmoles/mg/min)
1. *In vivo* experiment	
Corn oil	15.06 ± 1.68
3-MC	7.65 ± 0.92*
Cholestyramine	41.05 ± 9.04*
3-MC + cholestyramine	11.48 ± 1.46
2. *In vitro* experiment	
Tween 80	29.10 ± 5.20
3-MC, 2.5 μM	29.02 ± 2.30
3-MC, 5.0 μM	31.15 ± 4.89
3-MC, 50 μM	24.24 ± 3.98

Cholestyramine (25 mg/day) was administered by oral intubation in a suspension containing 0.25% carboxymethyl cellulose for 5 days. Addition of 3-MC *in vitro* was accomplished by suspension in Tween 80 (0.8 mg/flask) as described by the method of Karaboyas and Koritz [27]. Incubations were carried out for a 30 min period. Each value represents the mean of three observations ± S.E.M.

* A significant difference from control rats, $P < 0.05$.

After 3-MC treatment, no significant alterations of cytochrome b_5 levels, NADPH-cytochrome P450 reductase, and NADPH-cytochrome *c* reductase occurred while elevations of cytochrome P448 and NADPH oxidation were observed.

An additional study was carried out to determine if prior administration of 3-MC could influence the effect of cholestyramine on the cholesterol 7α-hydroxylase enzyme system. A reduction in microsomal cholesterol 7α-hydroxylase activity (−45 per cent) was observed upon administration of 3-MC while a 2.7-fold increase resulted from cholestyramine treatment (Table 1). The combined treatment regimen of 3-MC and cholestyramine revealed enzyme activity that was not significantly different from the control group (Table 1). The observed reductions in cholesterol 7α-hydroxylase activity upon 3-MC pretreatment could be due to the altered heme state (i.e. P448 formation), or possibly to further changes in the electron transport system. Another mechanism by which 3-MC could possibly reduce the rate of cholesterol 7α-hydroxylation is by binding to the microsomal protein and acting as an alternative substrate for the cholesterol 7α-hydroxylase system. To this end, an experiment was conducted to resolve whether the addition *in vitro* of 3-MC would depress the rate of cholesterol 7α-hydroxylation. From the results in Table 1, it can be observed that 3-MC concentrations in the range of 2.5–50 μM cause no significant depression of cholesterol 7α-hydroxylase activity.

It was of interest to determine whether the stimulation of cholesterol 7α-hydroxylase activity was due to alterations in the microsomal fraction alone or whether the 105,000 *g* supernatant fraction was a necessary precursor for the inductive phenomenon. As can be seen in Fig. 2, microsomes derived from control rats did not differ in their cholesterol 7α-hydroxylating capacity irrespective of the source of the liver 105,000 *g* supernatant fraction. Furthermore, total augmentation of cholesterol 7α-hydroxylase activity occurs in the microsomal fraction when obtained from D-T_4-treated rats with no subsequent modification resulting from the source of the 105,000 *g* fraction.

The ability of cholesterol to interact with hepatic microsomal cytochrome P450 reductase activity is presented in Tables 2 and 3. As shown in Table 2, ethylmorphine (2.0 and 5.0 mM) stimulated the reduction of cytochrome P450 by NADPH to a value of 105 per cent above the rates observed in the buffer treated samples. Contrariwise, 0.125 mM or 0.250 mM cholesterol caused no significant change in the reduction rates of cytochrome P450 by NADPH (Table 2). Addition of either 0.125 mM or 0.250 mM cholesterol to microsomal suspensions containing

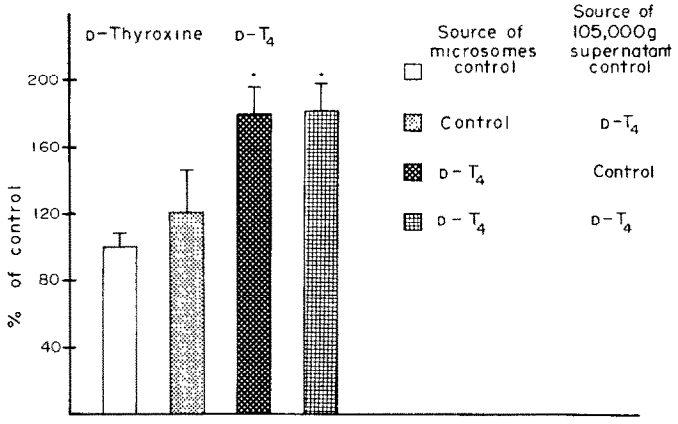

Fig. 2. The determination of 7α-hydroxy[^{14}C]cholesterol in microsomes plus the 105,000 *g* supernatant fraction from either control or D-T_4 (0.5 mg/kg) pretreated rats. The incubations contained 5.0 mg of microsomal protein (from control or D-T_4 treated rats), an NADPH-generating system described in Methods and 0.5 ml of the 105,000 *g* supernatant fraction derived from either control or D-T_4 treated rats. The incubations were carried out for a period of 30 min. Values represent the mean (expressed as per cent of control) ± S.E.M. for three animals. The mean value for control microsomes + control 105,000 *g* supernatant fraction for cholesterol 7α-hydroxylase activity was 1.24 pmoles/mg protein/min. The asterisk indicates that the level of 7α-hydroxylase activity is significantly different from incubations containing microsomes and the 105,000 *g* supernatant fraction from control animals, $P < 0.05$.

Table 2. Effect of ethylmorphine and cholesterol on NADPH-cytochrome P450 reductase activity in rat liver microsomes*

Compounds tested	Initial rate of cyt. P450 reduction by NADPH(nmoles reduced/mg protein/min)	% of Control
Buffer	2.27 ± 0.28	100
Tween 80-buffer	2.28 ± 0.18	100
Ethylmorphine, 2.0 mM	4.65 ± 0.08	205
Ethylmorphine, 5.0 mM	4.65 ± 0.38†	205
Cholesterol, 0.125 mM	2.86 ± 0.16	125
Ethylmorphine, 2.0 mM + Cholesterol, 0.125 mM	4.63 ± 0.44†	203
Ethylmorphine, 5.0 mM + Cholesterol, 0.125 mM	4.55 ± 0.36	200
Cholesterol, 0.250 mM	2.90 ± 0.20	127
Ethylmorphine, 2.0 mM + Cholesterol, 0.250 mM	4.45 ± 0.14†	195
Ethylmorphine, 5.0 mM + Cholesterol, 0.250	4.49 ± 0.44†	197

* The rates of cytochrome P450 reduction by NADPH in the presence or absence of the compounds were carried out as described in Methods. Ethylmorphine was dissolved in 0.1 M potassium phosphate buffer, pH 7.4, while cholesterol was suspended in buffer utilizing 2.0 mg of Tween 80. Results represent the mean of 3–4 determinations ± S.E.M.

† Values indicate a significant difference from control microsomes, $P < 0.05$.

2.0 or 5.0 mM ethylmorphine resulted in no alteration of the ethylmorphine-mediated stimulation of NADPH-cytochrome P450 reductase activity.

Preatment of rats with cholestyramine, PCN, and D-T_4 resulted in the stimulation of NADPH-cytochrome P450 reductase activity (Table 3). When ethylmorphine (5.0 mM) was added to control and drug pretreated microsomal suspensions, a stimulation of NADPH-cytochrome P450 reductase activity was observed in control, D-T_4, and PCN hepatic microsomes. Cholesterol concentrations of 0.250 mM failed to modulate cytochrome P450 reduction by NADPH in control or drug treated rats. Likewise, no alteration of ethylmorphine (5.0 mM)-mediated NADPH-cytochrome reductase activity was observed upon 0.25 mM cholesterol addition to either control or PCN treated preparations (Table 3).

Preliminary investigations revealed that 6.0 mM NADH caused a significant reduction of microsomal cholesterol 7α-hydroxylation whether in the presence of subsaturating concentrations of NADPH (1.5 mM) or in the presence of saturating conditions of NADPH (6.0 mM). Further investigation into the inhibitory effect of NADH on cholesterol 7α-hydroxylase activity revealed that the inhibition was dose-dependent in the presence of 1.0 and 6.0 mM NADPH (Table 4). To insure that NADH was stimulatory in drug hydroxylation reactions and that no nonspecific inhibition occurred as a result of high concentrations of this pyridine nucleotide (e.g. with nicotinamide), several concentrations of NADH were included in ethylmorphine *N*-demethylase assays. The results in Table 4 demonstrate that several concentrations of NADH (0.5–6.0 mM) increased the NADPH-mediated *N*-demethylation of ethylmorphine.

DISCUSSION

This investigation has been concerned with the interrelationship of the cholesterol 7α-hydroxylase enzyme system and the enzyme(s) responsible for drug oxidation found in the rat liver. It appears that cholesterol 7α-hydroxylase is highly regulated and plays an integral role in a metabolic sequence of physiological importance [1–3]. In this respect, cholesterol 7α-hydroxylation is different from most other known microsomal cytochrome P450-dependent reactions. As such, the factors controlling the activity of cholesterol 7α-hydroxylase might be expected to differ from those controlling the hydroxylations of nonphysiological compounds. For this reason, a series of experiments were imple-

Table 3. Effect of ethylmorphine and cholesterol on the reduction of cytochrome P450 by NADPH in control and drug pretreated rat liver microsomes*

Compound	Initial rate of cytochrome P450 reduction by NADPH (nmoles reduced/mg protein/min)			
	Control	D-Thyroxine	PCN	Cholestyramine
Tween 80-buffer	2.57 ± 0.11	3.45 ± 0.31‡	5.03 ± 0.23‡	4.03 ± 0.46
Ethylmorphine (5.0 mM)	5.14 ± 0.29†	4.84 ± 0.16†	15.16 ± 2.74†‡	5.00 ± 0.25
Cholesterol (0.25 mM)	2.90 ± 0.07	3.39 ± 0.08	5.26 ± 0.63	3.64 ± 0.20
Ethylmorphine (5.0 mM + Cholesterol (0.25 mM)	5.34 ± 0.30†	N.D.	15.27 ± 2.20†‡	N.D.

* The rates of cytochrome P450 reduction by NADPH in the presence or absence of the compounds were carried out as described in Methods. A total of 2–3 rats were pretreated with cholestyramine (5% in diet), PCN (35 mg/kg) or D-T_4 (0.5 mg/kg), and each group's livers pooled for the experimental procedure. Results represent the mean of 3–4 observations ± S.E.M.

† Significantly different from Tween 80-buffer values, $P < 0.05$.

‡ Significantly different from control values, $P < 0.05$.

Table 4. Effect of several concentrations of NADH on cholesterol 7α-hydroxylase and ethylmorphine *N*-demethylase activity at subsaturating and/or saturating NADPH levels in male Wistar rats*

NADH, mM	Cholesterol 7α-hydroxylation (pmoles/mg/min) NADPH 1.0 mM	NADPH 6.0 mM	Ethylmorphine N-demethylation (nmoles HCHO/mg/min) NADPH 1.5 mM
0	1.69 ± .05	2.63 ± .28	5.02 ± .06
0.5	1.63 ± .14	1.99 ± .04	6.64 ± .07§
1.5	1.43 ± .09	1.66 ± .18‡	7.08 ± .16§
3.0			6.94 ± .08§
6.0	0.63 ± .06†	0.80 ± .08‡	6.46 ± .03§

* Incubations were carried out for 30 min for cholesterol 7α-hydroxylation and 15 min for ethylmorphine *N*-demethylation. Each value represents the mean ± S.E.M. for 3–4 determinations.

† Value is significantly different from incubations containing NADPH, 1.0 mM, $P < 0.05$.

‡ Value is significantly different from incubations containing NADPH, 6.0 mM, $P < 0.05$.

§ Value is significantly different from incubations containing NADPH, 1.5 mM, $P < 0.05$.

mented to evaluate changes in the rate of cholesterol 7α-hydroxylation, ethylmorphine *N*-demethylation and several electron transport components.

Several studies in the past have indicated that there is no positive correlation with the fluctuations and/or modulations of cytochrome P450 content and cholesterol 7α-hydroxylase activity [5, 15, 17, 18, 37]. The data in the present study tends to corroborate these earlier findings (Figs 1a–e). In addition, there was a lack of congruence with the rate of cholesterol 7α-hydroxylation and the content or activity of electron transport components (i.e. cytochrome b_5, NADPH-cytochrome *c* reductase and NADPH-cytochrome P450 reductase activity) whereas there was a demonstrable dependency of ethylmorphine *N*-demethylation on the monitored electron transport components for PB, PCN, and spironolactone-treated rats. These results seem to indicate that a specific cytochrome P450 may be present (e.g. inducible upon cholestyramine or D-T_4 administration), however, in much less quantity than the total of the cytochrome P450 species in liver microsomes. Additionally, the terminal oxidase (cytochrome P450) may not be rate limiting for the cholesterol 7α-hydroxylase system.

While several laboratories have demonstrated cytochrome P450 involvement in the 7α-hydroxylation of cholesterol [5, 7, 12], the evidence for hemoprotein involvement still remains equivocal. In particular, the inconsistency of the effect of PB on cholesterol 7α-hydroxylase has been reported to be due to strain differences. Shefer *et al.* [38] have demonstrated that male rats of the Wistar strain are susceptible to PB stimulation of cholesterol 7α-hydroxylase whereas male rats that are Sprague–Dawley derived are not. In contrast to these findings, and in agreement with those of the present study, Boyd and associates [15, 17] have been unable to stimulate cholesterol 7α-hydroxylation with PB pretreatment utilizing male Wistar rats. These results lead to the conclusion that there may also be intrastrain differences of hepatic cholesterol 7α-hydroxylation.

The potential regulation of cholesterol 7α-hydroxylation via alterations of NADPH-cytochrome P450 reductase activity was examined by addition *in vitro* of cholesterol suspensions to both control and drug pretreated hepatic microsomes (Tables 2 and 3). The inability of cholesterol to alter the rate of cytochrome P450 reduction by NADPH in the absence and presence of ethylmorphine, might serve as an argument against the participation of cytochrome P450 reduction as being rate limiting for cholesterol 7α-hydroxylation. However, these concentrations may not have been sufficient to elicit observable changes in the rate of cytochrome P450 reduction by NADPH. Moreover, the cholesterol content in microsomal membrane fragments may be already saturated and, thus, additional exogenous cholesterol may not elicit an effect on NADPH cytochrome P450 reductase activity.

The findings that 3-MC administration causes a reduction in cholesterol 7α-hydroxylase activity (Fig. 1e) is in agreement with Wada *et al.* [5]. On the contrary, a study by Brown and Boyd [17] demonstrated no alteration in 7α-hydroxylase activity due to prior treatment with 3-MC. From the results given in Table 1, it is demonstrated that the inductive effect of cholestyramine on cholesterol 7α-hydroxylase activity is prevented by the coadministration of 3-MC. These results may be interpreted to mean that the newly formed cytochrome P448 cannot effectively act as the terminal oxidase for the cholesterol 7α-hydroxylase enzyme system. In addition, other electron transport components may have been altered in such a way as to negate any stimulatory effect by cholestyramine (e.g. neither NADPH-cytochrome P450 or cytochrome *c* reductase activities are affected by 3-MC), or that 3-MC may compete with cholesterol for reducing equivalents by binding to the microsomes. The latter argument can be eliminated as a possibility since addition *in vitro* of 3-MC did not alter cholesterol 7α-hydroxylase activity (Table 1). It remains to be seen whether the inhibitory activity of 3-MC is due to changes in terminal oxidase forms (i.e. P450 → P448) or in other components necessary to the electron transport system. Not until the purified components are isolated and purified will this knowledge be gained.

It was considered of interest to study whether NADH could substitute for NADPH as a pyridine nucleotide in the cholesterol 7α-hydroxylase enzyme system, and whether NADH addition would synergize the NADPH-mediated cholesterol 7α-hydroxylation reaction in liver microsomes from Wistar rats. The inhibitory phenomenon caused by NADH on cholesterol 7α-hydroxylase activity was dose-dependent at both saturating and subsaturating levels of NADPH (Table 4). This inhibitory effect of NADH is in direct contrast to those findings on the stimulatory activity of NADH on hepatic microsomal drug oxidations. In agreement with Correia and Mannering [39], NADH (0.5 mM – 6.0 mM) further stimulated ethylmorphine *N*-demethy-

lation (Table 4). Similarly, Björkhem and Danielsson [40], using Sprague–Dawley rats, demonstrated that NADH stimulated several steroid hydroxylations including the 7α-hydroxylation of cholesterol. These findings seem to indicate that strain selective differences exist for cholesterol 7α-hydroxylase activity. At least in Wistar rats, the cholesterol 7α-hydroxylase system is not capable of accepting reducing equivalents from NADH.

The metabolic reaction immediately following the 7α-hydroxylation of cholesterol in the bile acid biosynthetic sequence in rats is a NAD-dependent oxidation of 7α-hydroxycholesterol to 7α-hydroxy-4-cholesten-3-one [41]. The possibility that this reaction might be responsible for the apparent NADH inhibition has not been completely resolved. No positive correlation was found between NADH concentrations and the disappearance of 7α-hydroxycholesterol in liver microsomes in our laboratory (unpublished results). Thus, the mechanism of inhibition by NADH on the microsomal cholesterol 7α-hydroxylase system still remains to be elucidated. Further investigation into detection of subsequent metabolites of 7α-hydroxycholesterol stimulated by NAD or NADH may lend insight into the apparent lack of stimulation of NADPH-dependent cholesterol 7α-hydroxylation.

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REFERENCES

1. S. Shefer, S. Hauser and E. H. Mosbach, *J. Lipid Res.* **9**, 328 (1968).
2. H. Danielsson, K. Einarsson and G. Johansson, *Eur. J. Biochem.* **2**, 44 (1967).
3. K. Einarsson, *Eur. J. Biochem.* **6**, 299 (1968).
4. H. Danielsson and K. Einarsson, *Acta chem. scand.* **18**, 831 (1964).
5. F. Wada, K. Hirata, K. Nakao and Y. Sakamoto, *J. Biochem., Tokyo* **66**, 699 (1969).
6. J. R. Mitton, N. A. Scholan and G. S. Boyd, *Eur. J. Biochem.* **20**, 569 (1971).
7. G. S. Boyd, A. M. Grimwade and M. E. Lawson, *Eur. J. Biochem.* **37**, 334 (1973).
8. I. Björkhem, H. Danielsson and K. Wikvall, *Biochem. biophys. Res. Commun.* **61**, 934 (1974).
9. H. S. Mason, *Adv. Enzymol.* **19**, 79 (1957).
10. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).
11. J. R. Gillette, D. C. Davis and H. A. Sasame, in *Annual Reviews of Pharmacology* (Eds H. W. Elliott, R. Okun and R. George), p. 57. Annual Reviews, Inc., CA (1972).
12. F. Wada, K. Hirata, K. Nakao and Y. Sakamoto, *J. Biochem., Tokyo* **64**, 415 (1968).
13. E. H. Mosbach, in *Drugs Affecting Lipid Metabolism* (Eds W. L. Holmes, L. A. Carlson and R. Paoletti), p. 421. Plenum Press, NY (1969).
14. S. Shefer, G. Nicolau and E. H. Mosbach, *J. Lipid Res.* **16**, 92 (1975).
15. G. S. Boyd, N. A. Scholan and J. R. Mitton, in *Drugs Affecting Lipid Metabolism* (Eds W. L. Holmes, L. A. Carlson and R. Paoletti), p. 443. Plenum Press, NY (1969).
16. K. Einarsson and G. Johansson, *Eur. J. Biochem.* **6**, 293 (1968).
17. M. S. G. Brown and G. S. Boyd, *Eur. J. Biochem.* **44**, 37 (1974).
18. S. Balasubramaniam and K. A. Mitropoulos, *Biochem. Soc. Trans.* **3**, 964 (1975).
19. K. Einarsson and J. Gustafsson, *Eur. J. Biochem.* **32**, 197 (1973).
20. T. Honohan and T. M. Parkinson, *Biochem. Pharmac.* **24**, 899 (1975).
21. E. H. Mosbach, M. A. Rothschild, I. Berkersky, M Oratz and J. Mongelli, *J. clin. Invest.* **50**, 1720 (1971)
22. G. O. Schenck, K. Gollnick and O. A. Neumuller, *Annalen* **603**, 46 (1957).
23. B. Lythgoe and J. Trippett, *J. chem. Soc.* 471 (1959).
24. H. A. Henderson, F. Crowley and C. E. Faudette, in *Advances in Tracer Methodology* (Ed. S. Rothchild), Vol. 2, p. 83. Plenum Press, NY (1965).
25. T. Nash, *Biochem. J.* **55**, 415 (1953).
26. K. A. Mitropoulos and S. Balasubramaniam, *Biochem. J.* **128**, 1 (1972).
27. G. C. Karaboyas and S. B. Koritz, *Biochemistry* **4**, 462 (1965).
28. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
29. A. H. Phillips and R. G. Langdon, *J. biol. Chem.* **237**, 2652 (1962).
30. D. D. Davies, P. L. Gigon and J. R. Gillette, *Life Sci.* **8**, 85 (1969).
31. P. L. Gigon, T. E. Gram and J. R. Gillette, *Biochem. biophys. Res. Commun.* **31**, 558 (1968).
32. P. L. Gigon, T. E. Gram and J. R. Gillette, *Molec. Pharmac.* **5**, 109 (1969).
33. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
34. D. R. Feller and M. C. Gerald, *Biochem. Pharmac.* **20**, 1991 (1971).
35. B. Stripp, M. E. Hamrick, N. G. Zampaglione and J. R. Gillette, *J. Pharmac. exp. Ther.* **176**, 766 (1971).
36. G. J. Mannering, N. E. Sladek, C. J. Parli and D. W. Shoeman, in *Microsomes and Drug Oxidations* (Eds J. R. Gillette, A. H. Conney, G. J. Cosmides, R. W. Estabrook, J. R. Fouts and G. J. Mannering), p. 303. Academic Press, NY (1969).
37. S. D. Atkin, E. D. Palmer, P. D. English, B. Morgan, M. A. Cawthorne and J. Green, *Biochem. J.* **128**, 237 (1972).
38. S. Shefer, S. Hauser and E. H. Mosbach, *J. Lipid Res.* **13**, 69 (1972).
39. M. A. Correia and G. J. Mannering, *Molec. Pharmac.* **9**, 455 (1973).
40. I. Björkhem and H. Danielsson, *Biochem. biophys. Res. Commun.* **51**, 766 (1973).
41. I. Björkhem and H. Danielsson, *Molec. cell. Biochem.* **4**, 79 (1974).