The Selective Stimulation, Inhibition, and Physicochemical Alteration of the 7- and 16α-Hydroxylases of 3β-Hydroxyandrost-5-en-17-one and Drug-Metabolizing Enzymes in Hepatic Microsomal Fractions*

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ABSTRACT: The hepatic, microsomal hydroxylations of 3β-hydroxyandrost-5-en-17-one (DHA) at the 7α, 7β, and 16α positions have been compared with the activities of two drug-metabolizing enzymes, acetanilide *p*-hydroxylase and aminopyrine *N*-demethylase, and with the cytochrome P-450 content. Phenobarbital treatments to adult rats stimulated all the enzymatic activities in females but decreased the activity of the 16α-hydroxylase in males. Aminopyrine competitively inhibited only the 7α hydroxylation; SU-9055, a substituted pyridine, inhibited both the 16α hydroxylation and the N demethylation but increased the 7 hydroxylations. The amount of dithionite-reducible cytochrome P-450 in the microsomes was inversely related to the concentration of SU-9055, which also produced type II difference spectra (Schenkman, J. B., Remmer, H., and Estabrook, R. W. (1967), *Mol. Pharmacol. 3*, 90). Deoxycholate treatment of the microsomal fractions destroyed both the P-450 and the enzymatic activities but DHA, added prior to the treatment, partially protected the 16α-hydroxylase and increased the rate of *para* hydroxylation. These selective responses to phenobarbital stimulation, to inhibitors, and to physicochemical manipulation are further evidence of the dissimilarities in the enzymes that metabolize drugs and steroids at different positions.

Many hepatic steroid hydroxylases and drug-metabolizing enzymes are external mixed-function oxidases (Mason *et al.*, 1965a,b) that derive their reducing equivalents from the redox component, cytochrome P-450 (Estabrook *et al.*, 1966). Similarities among certain of these enzymes have prompted the generalization that steroid hormones are the physiological substrates for drug-metabolizing enzymes (Conney, 1967). However, several kinds of differences in the biological distributions of the enzymes (Conney, 1967; Heinrichs *et al.*, 1967) and some of their responses to "inducing agents" (Conney, 1967) have been difficult to reconcile with the concept of a single enzyme or mechanism for the reactions, which are often stereospecific. The data reported here indicate that individual enzymes or mechanisms are responsible for the 7α, 7β, and 16α hydroxylations of 3β-hydroxyandrost-5-en-17-one (DHA)[1] and for the *para* hydroxylation of acetanilide and the N demethylation of aminopyrine.

Experimental Procedure

Animals and Preparation of Tissue Fractions. Sprague–Dawley rats (10–13-weeks old) were maintained on Purina laboratory pellets taken *ad lib*, and were not fasted before being killing at approximately 8 AM on the days of the experiments. In some experiments, described in the text, intraperitoneal treatments with sodium phenobarbital (100 mg/kg; Winthrop Laboratories, New York, N. Y.) were given daily for 5 days. The treated animals were killed 24 hr after the last treatment. The preparation of microsomal fractions and subfractions was carried out with minimal modifications (Heinrichs *et al.*, 1967) of published methods (Mason *et al.*, 1965a,b; Fouts, 1961). Protein was measured with a biuret method (Gornall *et al.*, 1949). In experiments with sodium deoxycholate microsomal fractions were treated in the following manner.

1. DOC Only. Crystalline DOC (0.5 mg/mg of protein) was dissolved in the microsomal suspension by gentle shaking at room temperature. After 30 min, the

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: DHA, 3β-hydroxyandrost-5-en-17-one; androstenedione, androst-4-ene-3,17-dione; testosterone, 17β-hydroxyandrost-4-en-3-one; phenylbutazone, 4-butyl-1,2-diphenyl-3,5-pyrazolidinedione; phenobarbital, 5-ethyl-5-phenylbarbituric acid; acetanilide, acetylaminobenzene; aminopyrine, 4-dimethylamino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one; SU-9055, 3-(1,2,3,4-tetrahydro-1-oxo-2-naphthyl)pyridine; DOC, sodium deoxycholate.

clear solution was pipetted onto a Sephadex G-25 fine column (12.5 g, 2.2 × 15 cm; Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) and eluted with 0.1 M sodium phosphate buffer (pH 7.4). The midportion of the eluate (10–12 ml) was collected for the analyses. The other fractions were similarly prepared, except for preliminary treatments as follows.

2. N_2. A 15-ml centrifuge tube containing the microsomal solution was closed with a tight-fitting, multiple-dose, vial stopper penetrated by three stainless-steel needles. A constant flow of purified N_2, brisk enough to disturb the solution, was maintained. After 5 min the solution was drawn into a N_2-filled syringe containing the DOC crystals to dissolve them before the solution was returned to the centrifuge tube. The N_2 escaped through the third aperture until the needles were withdrawn. The DOC treatment consisted of gentle agitation for 30 min in the N_2 atmosphere. The clear solution was then chromatographed as described above.

3. Propylene glycol (0.25 ml), corresponding to the vehicle for the steroid substrates, was pipetted and stirred into appropriate microsomal fractions before the DOC treatment.

4. Steroid in Propylene Glycol. Either DHA or androstenedione (6.25 mg in 0.25 ml of propylene glycol), pipetted and stirred into the solutions, either was treated in air as in preparation 3 or was introduced into microsomes in a N_2 atmosphere. After the latter procedure, the solution was aspirated into a N_2-filled syringe containing the DOC, where it was mixed. For the 30-min reaction, the solution was reinjected into the centrifuge tube and again flushed with N_2 before the needles were removed. The chromatography and elution followed.

Enzymatic Methods

A. Steroid Hydroxylations. The experimental conditions for the incubation, extraction, thin-layer chromatography, and elution from the silica gel have been reported (Heinrichs *et al.*, 1966). The amounts of the products were measured in two aliquot parts of the methanolic extracts obtained from single incubation mixtures. Quantitation of 3β,16α-dihydroxyandrost-5-en-17-one was accomplished by oxidation with blue tetrazolium (Fotherby, 1958). The sensitivity and precision (Brown *et al.*, 1957) determined for the 2.3-ml reaction with reference steroids were 0.11 and 0.28 μg (1.9%) at the 15.2-μg level. The rates of steroid hydroxylation (millimicromoles per milligram per minute) were not corrected for losses. The standard deviations were calculated according to the formula $SD = \pm\Sigma X^2 - ((\Sigma X)^2/(N - 1))$ (Snedecor, 1952) and the significance of differences by the Student's *t* test.

Quantitation of 3β,7α-dihydroxyandrost-5-en-17-one and 3β,7β-dihydroxyandrost-5-en-17-one was also carried out spectrophotometrically by reading the absorbancies at 555, 580, and 605 mμ of a chromogen which developed at room temperature after adding 2 ml of the modified Pettenkofer reagent (Inagaki, 1960; Lifschutz, 1927; Usui and Yamasaki, 1960). After 10 min the chromogen was stable only for a 10-min period. The chromogenicity of both steroids obeyed Beer's law and the molar coefficients for the corrected absorbancies at 580 mμ were 17,580 and 19,800 cm^{-1} $mole^{-1}$ for the 7α and the 7β epimers, respectively. The sensitivity and precision of the method, calculated from uncorrected data collected from duplicate determinations of unpurified residues corresponding to the 7α epimer, were 0.13 and 0.20 μg (13.3%), respectively, at the level of 1–2 μg, and 0.25 and 0.49 μg (13.1%) at the level of 2–5 μg.

B. Drug Metabolism. The *para* hydroxylation of acetanilide (Krish and Staudinger, 1961) was measured by the Folin–Ciocalteu reagent in extracts from duplicate incubation mixtures prepared without nicotinamide. The maximum absorbance at 765 mμ of reference *p*-hydroxyacetanilide and the enzymatic product was the basis for the measurement. The sensitivity and precision were 0.35 and 0.45 μg (1.0%) at the 46.8-μg level.

The N demethylation of aminopyrine (Orrenius, 1965), also determined in duplicate incubation mixtures, was quantitated by the complex formation of the liberated formaldehyde with ethylacetone and NH_3 (Nash, 1953). The sensitivity and precision of the method were 0.11 and 0.13 μg (3.3%) at the 4.0-μg level, and 0.22 and 0.26 μg (3.3%) at the 8.0-μg level.

C. Cytochrome P-450. The microsomal content of cytochrome P-450 was determined with a Cary Model 14 recording spectrophotometer equipped with a scattered transmission attachment and a high-intensity quartz lamp operated at 70 V (Dallner, 1963; Omura and Sato, 1964). Solutions containing tartaric acid as a vehicle for the SU-9055 required 5 min after the bubbling of CO to obtain maximum absorbancy at 450 mμ.

Results

Stimulation of the Enzymatic Activities by Phenobarbital. The three products of the incubation of DHA with hepatic microsomes, previously identified as the 7α-, 7β-, and 16α-hydroxylated derivatives (Heinrichs *et al.*, 1967), were observed consistently after the animals had been treated with phenobarbital, and no new products were observed on the chromatograms after either the picric acid spray or ultraviolet examination.

The 7α and 7β hydroxylations by adult male rats increased whereas the 16α hydroxylation in the total microsomal fraction decreased (see Table I). This decrease appears to result from a reduction of 16α hydroxylation in the rough subfraction. The amount of protein was also reduced in the rough subfractions from treated male livers. In females, the increases in the 7-hydroxylation rates after phenobarbital treatment exceeded the response in the males and the rate of 16α hydroxylation increased. Nevertheless, all the rates in females were still lower than any of those in males. In microsomes from untreated adult males, the rates of DHA 7α and 16α hydroxylation and aminopyrine N demethylation were significantly higher ($p < 0.002$, 0.001, and 0.001, respectively) than in females. The

TABLE 1: Effect of Phenobarbital Treatment on the Activities of Steroid Hydroxylases and Drug-Metabolizing Enzymes and on the Cytochrome P-450 and Microsomal Protein Content in Total, Smooth, and Rough Microsomal Fractions from Adult Rats.[a]

Act.[b] or Content[c]	Total		Smooth		Rough	
	Control	Treated[d]	Control	Treated[d]	Control	Treated[d]
			Males			
7α-Hydroxylase	1.33 ± 0.82 (12)	0.96–3.11 (4)	n.a.[e]	n.a.	n.a.	n.a.
7β-Hydroxylase	0.09 ± 0.07 (12)	0.06–0.33 (4)	n.a.	n.a.	n.a.	n.a.
16α-Hydroxylase	1.41 ± 0.32 (12)	0.66–0.88 (4)	1.09 ± 0.44 (14)	0.79 –1.20 (4)	1.35 ± 0.33 (14)	0.31–0.63 (4)
p-Hydroxylase	0.28 ± 0.14 (12)	0.40–0.51 (4)	0.42 ± 0.19 (14)	0.27–0.91 (4)	0.33 ± 0.14 (14)	0.61–1.36 (4)
N-Demethylase	2.62 ± 0.25 (14)	6.68–9.18 (4)	2.18 ± 0.52 (14)	5.80–7.70 (4)	2.40 ± 0.54 (14)	6.40–6.90 (4)
Cytochrome P-450	0.48 ± 0.22 (12)	0.60–1.40 (4)	n.a.	n.a.	n.a.	n.a.
Protein	14.6 ± 3.2 (12)	19.1–24.8 (4)	1.9 ± 0.5 (14)	14.6–15.1 (4)	11.3 ± 1.5 (14)	4.4–10.2 (4)
			Females			
7α-Hydroxylase	0.41 ± 0.34 (8)	0.19–1.55 (3)	n.a.	n.a.	n.a.	n.a.
7β-Hydroxylase	0.08 ± 0.05 (8)	0.04–0.42 (3)	n.a.	n.a.	n.a.	n.a.
16α-Hydroxylase	0.10 ± 0.07 (8)	0.28–0.35 (3)	0.04 ± 0.02 (8)	0.16–0.27 (3)	<0.02 (4)	0.13–0.30 (3)
p-Hydroxylase	0.28 ± 0.14 (8)	0.79–0.86 (3)	0.40 ± 0.28 (8)	0.28–0.47 (3)	0.57 ± 0.05 (8)	0.36–0.41 (3)
N-Demethylase	1.70 ± 0.12 (8)	6.14–7.10 (3)	1.63 ± 0.25 (8)	4.82–5.80 (3)	1.84 ± 0.31 (8)	6.10–6.15 (3)
Cytochrome P-450	0.40 ± 0.21 (8)	0.43–0.92 (3)	n.a.	n.a.	n.a.	n.a.
Protein	11.3 ± 1.5 (8)	17.2–22.0 (3)	3.0 ± 1.8 (8)	6.05–10.80 (3)	5.9 ± 1.2 (8)	4.4–10.0 (3)

[a] The preparations of the total microsomal fractions and of the smooth and rough microsomal fractions are from separate groups of treated and control animals. The number of animals in each group is shown in parentheses. [b] Millimicromoles per milligram per minute plus and minus standard deviation (if given). [c] Millimicromoles per milligram of protein (cytochrome P-450) or milligram per gram of fresh tissue (protein). [d] Range. [e] n.a. = not available.

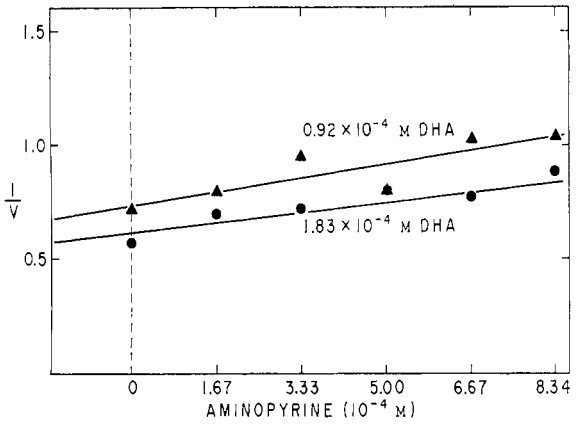

FIGURE 1: Inhibitory effect of aminopyrine on the 16α hydroxylation of 3β-hydroxyandrost-5-en-17-one (DHA) by hepatic microsomes from male rats.

corresponding male-female ratios of activities were also different for these enzymes: 3.2:1, 14:1, and 1.5:1 respectively. With the total fractions, we observed no sex differences in the protein or in the cytochrome P-450 content. However, the amount of microsomal protein in the rough subfractions from untreated males was noticeably greater ($p < 0.001$) than the amount recovered from the livers of females. The protein content of each cellular fraction, expressed as milligram per gram of fresh tissue, is of relative value only, since no effort was made to account for all of the protein. After treatments with 3-methylcholanthrene or with phenylbutazone, the enzymatic activities, the amount of cytochrome P-450, and the protein in the various fractions also increased as they had after the phenobarbital treatments, but the magnitude of the responses was less. Also, fasting the untreated animals for 16 hr did not significantly alter the activities of the 16α-hydroxylase or the drug-metabolizing enzymes (W. L. Heinrichs and A. Colás, unpublished data).

Inhibition of the Enzymatic Activities in Microsomes from Adult Male Rats. 1. INHIBITION OF N DEMETHYLATION BY DHA. The addition of different amounts of DHA (inhibitor) decreased the N demethylation in incubation mixtures incorporating aminopyrine (substrate) in two concentrations, 1.25 and 2.5 $\times$ 10^{-3} M (see Table II). The inhibition was about 60% complete with 1.38 $\times$ 10^{-4} M DHA, and it increased to about 80% for the highest concentrations of inhibitor.

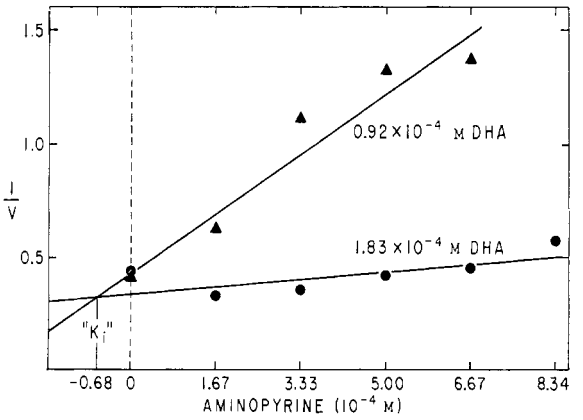

FIGURE 2: Inhibitory effect of aminopyrine on the 7α hydroxylation of 3β-hydroxyandrost-5-en-17-one (DHA) by hepatic microsomes from male rats.

TABLE II: Reciprocal Effects of DHA and Aminopyrine on Their Rates of Oxidation (millimicromoles per milligram per minute) by Hepatic Microsomes from Male Rats.

Inhibitor	Reaction: N Demethylation of Aminopyrine	
10^{-4} M DHA	1.25 $\times$ 10^{-3} M	2.5 $\times$ 10^{-3} M
0	3.42 (100)[a]	3.79 (100)
1.38	1.27 (37)	1.44 (38)
2.75	1.04 (33)	1.01 (27)
5.50	0.89 (26)	0.69 (18)
10.90	0.74 (22)	0.64 (17)
10^{-4} M Aminopyrine	16α Hydroxylation of DHA	
	0.92 $\times$ 10^{-4} M	1.83 $\times$ 10^{-4} M
0	1.40 (100)	1.77 (100)
1.67	1.27 (91)	1.44 (81)
3.33	1.08 (77)	1.41 (80)
5.00	1.27 (91)	1.26 (71)
6.67	0.97 (69)	1.30 (73)
8.34	0.97 (69)	1.25 (71)
	7α Hydroxylation of DHA	
0	2.43 (100)	2.36 (100)
1.67	1.63 (67)	3.08 (132)
3.33	0.98 (40)	2.88 (122)
5.00	0.77 (32)	2.54 (108)
6.67	0.74 (30)	2.41 (105)
8.34	2.11 (87)	1.75 (74)

[a] Per cent of control values in parentheses.

2. INHIBITION OF DHA 7α AND 16α HYDROXYLATIONS BY AMINOPYRINE. In flasks containing 0.92 or 1.83 $\times$ 10^{-4} M DHA (substrate), the addition of different amounts of aminopyrine inhibited the 16α hydroxylation, but the 7α-hydroxylation rates were actually increased in the set of flasks containing the greatest amount of substrate. These data show that DHA is a more effective inhibitor of the N demethylation of aminopyrine than the latter is of the 16α hydroxylation of DHA, particularly when the concentration ratios of inhibitor to substrate are considered. Plots of the reciprocals of the respective velocities *vs.* the inhibitor concentrations (Figures 1 and 2; Dixon and Webb, 1964) indicate that the inhibition of 16α hydroxylation was approximately linear at both concentrations of substrate, whereas aminopyrine competitively inhibited the 7α hydroxylation of DHA. The apparent K_i was 0.68 $\times$ 10^{-4} M.

TABLE III: Effect of SU-9055 on the 7- and 16α-Hydroxylases and the *N*-Demethylase Activities (A, B, and C, respectively) in Hepatic Microsomal Fractions from Male Rats.

	A		B		C	
M Su-9055	*a*	*b*	*a*	*b*	*a*	*b*
0	1.01	100	1.15	100	2.40	100
10^{-6}	0.96	96	0.93	81	1.88	78
2×10^{-6}	1.55	155	1.11	97	1.57	65
10^{-5}	1.29	129	0.91	79	Not available	
2×10^{-5}	1.10	110	0.83	72	1.45	60
10^{-4}	1.26	126	0.58	50	0.80	35
2×10^{-4}	0.65	58	0.56	49	0.52	21
10^{-3}	0	0	0.07	6	0.23	9

[a] Millimicromoles per milligram per minute. [b] Per cent of control value.

3. INHIBITION BY SU-9055. The rate of the 7 hydroxylation of DHA (the 7α and 7β epimers were measured together because of an inadequate chromatographic separation in this set of experiments) increased above the control rate when the inhibitor (SU-9055) concentration was less than that of DHA, 1.83×10^{-4} M (Table III). In contrast, when the SU-9055 concentration almost equalled that of the substrate, the 16α hydroxylation was inhibited about 50%. The inhibition of N demethylation, to an even greater extent than with the steroid hydroxylations, was 90% complete with the highest concentration of SU-9055.

4. INTERACTION OF SU-9055 AND CYTOCHROME P-450. The difference spectra of microsomal solutions after the addition of various amounts of SU-9055 to one of the samples were characterized by a trough at 383 mμ and a peak at 420 mμ (Figure 3), and the absorbancies, with one exception, were related to the concentration of SU-9055. Also, the amount of cytochrome P-450 that could be reduced by sodium dithionite, added after the SU-9055, was inversely related to the concentration of the inhibitor. Figure 4 is a plot demonstrating a decrement of the measurable cytochrome P-450 content (millimicromoles per milligram of protein) *vs.* the increasing concentrations of SU-9055. From the plot, we have calculated the equilibrium constant for the interaction of SU-9055 with cytochrome P-450 to be 1.7×10^{-6} M (Dixon, 1965).

Physicochemical Alteration of the Hepatic Microsomal Membranes from Adult Male Rats. The physicochemical alteration of the microsomal membranes was apparent after adding sodium deoxycholate because of an immediate change in the turbidity of the solutions and a greatly decreased residual cytochrome P-450 content (Table IV). The effect on the enzymatic activities was usually to reduce the 7α hydroxylation to the level of the sensitivity of the quantitation method (0.03 mμmole/mg per min). The 7β epimer was never recognizable on the chromatographic plates and was not quantitated. In comparison, the activity of the 16α-hydroxylase system was variable in different microsomal prepara-

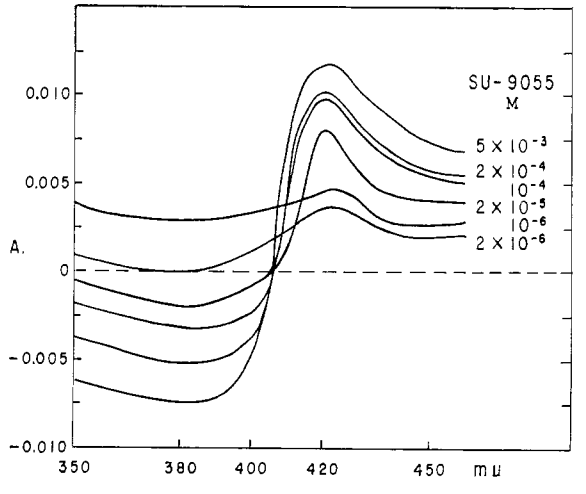

FIGURE 3: Difference spectra after addition of SU-9055 to hepatic microsomes from male rats.

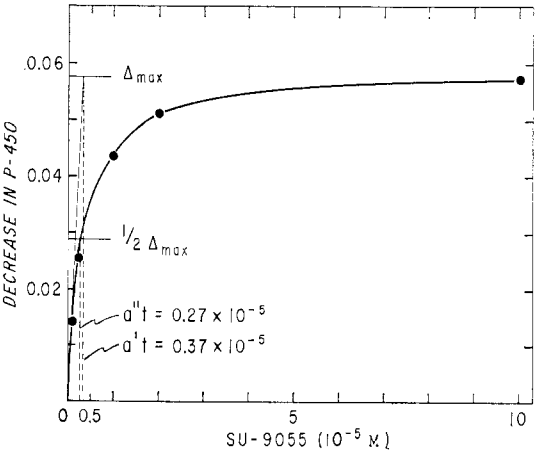

FIGURE 4: Graphic determination of the equilibrium constant, K_i, for the interaction of SU-9055 and P-450 in hepatic microsomes from male rats. The decrease in P-450 is given in millimicromoles per milligram of protein. Δ_{max} is the asymptotic decrement and a'_t and a''_t are the two parameters (Dixon, 1965) needed in the equation $K_i = 2a''_t - a'_t$.

TABLE IV: Effect of Deoxycholate Treatments of Microsomal Fractions on Cytochrome P-450 Content (A) (millimicromoles per milligram of protein), and on the 7α- and 16α-Hydroxylases, the *p*-Hydroxylase, and the *N*-Demethylase Activities (millimicromoles per milligram per minute) (B, C, D, and E, Respectively).[a]

Treatment	Expt	A	B	C	D	E
None	1	0.740 (100)	2.70 (100)	1.56 (100)	n.a.	n.a.
	2	0.562 (100)	1.79 (100)	1.00 (100)	0.15 (100)	2.70 (100)
	3	0.312 (100)	2.26 (100)	0.91 (100)	0.13 (100)	3.07 (100)
Deoxycholate (DOC)	1	0.047 (6)	0 (0)	0.05 (3)	n.a.	n.a.
	3	0.064 (21)	0.06 (3)	0.30 (33)	0 (0)	0.92 (30)
DOC + N_2	2	0.206 (37)	0.05 (3)	0 (0)	0.15 (100)	0.76 (28)
DOC + propylene glycol	3	0.392 (126)	0.06 (3)	0.14 (15)	0 (0)	0.67 (22)
DOC + propylene glycol + 3β-hydroxyandrost-5-en-17-one	2	0.186 (33)	0.04 (2)	0.42 (42)	0.49 (327)	0.74 (27)
	3	0.405 (130)	0.23 (10)	0.37 (41)	0.38 (292)	0.76 (25)
DOC + propylene glycol + androst-4-ene-3,17-dione	3	0.288 (92)	0.06 (3)	0.24 (26)	0.04 (31)	0.50 (16)
DOC + propylene glycol + 3β-hydroxyandrost-5-en-17-one + N_2	1	0.142 (19)	0 (0)	1.02 (65)	n.a.	n.a.
	2	0.374 (67)	0.12 (7)	0.44 (44)	0.37 (246)	0.14 (52)

[a] Values in parentheses are per cents of the control values for each experiment. n.a. = not available.

tions, but the prior additions of DHA partially protected (40% or more) the 16α-hydroxylase from the actions of deoxycholate. No such protection was conferred for the 7α-hydroxylase, and neither androstenedione nor the vehicle alone (propylene glycol) had the protective property. The prior addition of DHA actually stimulated the *para* hydroxylation of acetanilide after deoxycholate treatments of the membranes, but the decreased *N*-demethylase activity remained unaffected by DHA.

These data for the enzymatic activities after deoxycholate treatment were nearly duplicated in experiments in which untreated microsomes from female rats, having a low rate of 7α hydroxylation and even less 16α-hydroxylase activity, were added in the incubations of treated microsomes from male rats (W. L. Heinrichs and A. Colás, unpublished data). The purpose of the latter experiments was to see if cytochrome P-450 (or other essential components of the reactions) from female microsomes would replace the inactivated P-450 or other components in the male microsomes. We observed no increase in the 7α- or 16α-hydroxylation rates after those measured in the combined tissues were corrected for the native activity in the microsomes from females.

Discussion

The results presented here are further indirect evidence that individual enzymes hydroxylate steroids at specific sites of the molecules and oxidatively metabolize different drugs (Conney, 1967). In the absence of purified enzyme preparations to establish this hypothesis directly, the very selective biological distributions of a number of enzymatic activities according to species, sex, and age suggest a high substrate specificity of the enzymes. These biological distributions have been reviewed recently (Conney, 1967). To them can be added a sex difference for the 7α hydroxylation of DHA in rats.

Another type of biological difference, the selective localization of the enzymatic activities into the rough microsomal subfraction, appears to operate in the rat for steroid sulfatases (Burstein and Westort, 1967), for *N*-demethylases (Orrenius and Ernster, 1964; Dallner, 1963), now for DHA 16 α hydroxylation, and, possibly, for 7α hydroxylation. The 16α-hydroxylase system in rabbit liver resides in smooth microsomal subfractions prepared exactly like those of rat liver (W. L. Heinrichs and A. Colás, unpublished data). The answer to this species-localization discrepancy has been related to the method for the preparation of the fractions (Gram *et al.*, 1967a,b). We used the 8-hr centrifugation period in this study, but the tissue was never subjected to hypotonic solutions, a factor incriminated in the lability of enzymatic activities in rat microsomes prepared by certain methods.

The responses of hepatic steroid hydroxylases to phenobarbital treatments have been compared with the aminopyrine *N*-demethylase because the activity of the latter enzyme is increased greatly in rats by phenobarbital and with acetanilide *para* hydroxylation which is increased by chemical carcinogens (Conney, 1967; Krish and Staudinger, 1961). This experimental approach, and those of the drug-inhibition and physicochemical alteration of steroid hydroxylases, permitted

the demonstration of biochemical differences among the external mixed-function oxidases. Although the 7α- and 16α-hydroxylase systems responded similarly to treatments with immature rats (Kuntzman *et al.*, 1964; Conney *et al.*, 1966; Welch *et al.*, 1967), the use of adult rats enabled us to differentiate between the enzymes. The capacity of a wide variety of compounds, including phenobarbital and androgen hormones, to stimulate drug-metabolizing enzymes (Booth and Gillette, 1962; Gram and Fouts, 1966) decreases in mature rats (Chiesara *et al.*, 1967). The peak activity of the DHA 16α-hydroxylase in male rats (Heinrichs *et al.*, 1966) coincides with the peak secretion of testosterone (J. A. Resko, unpublished data), but the competitive effect of androgen hormones on the stimulation of hepatic enzymes by xenobiotic substances has not been studied.

The inhibition *in vitro* of oxidative drug metabolism by another steroidal hormone (DHA) is consistent with previous observations (Tephly and Mannering, 1964; Kuntzman *et al.*, 1965). However, the competitive inhibition of 7α hydroxylation by aminopyrine and a dissimilar response of the 16α-hydroxylase in the same flasks constitute additional evidence for the existence of separate enzymes. The completely different rates of inhibition of the latter enzymes produced by SU-9055 confirm the findings obtained with aminopyrine inhibition. SU-9055, a substituted pyridine compound, is known to inhibit the steroid 18-hydroxylase, 17-desmolase, 17α-hydroxylase, and 16α-hydroxylase activities in different tissues (Kahnt and Neher, 1962; Neher and Kahnt, 1965; Colla *et al.*, 1966) and to stimulate the adrenal 11β hydroxylation (Kahnt and Neher, 1962). Some basis of similarity between the group of enzymes that metabolize drugs and steroids is suggested by the greater inhibition of the *N*-demethylase compared to the steroid transformations. The observation that SU-9055 produces a type II spectrum (Imai and Sato, 1966a; Estabrook *et al.*, 1966; Schenkman *et al.*, 1967a) in the presence of microsomes, and decreases the reducible cytochrome P-450 as the concentration of SU-9055 increases, suggests that the hemoprotein is a common component for both groups of enzymatic activities. An excess of the SU-9055, however, reacted with a total of only 22.8% of the measurable cytochrome P-450. This finding might be interpreted as additional evidence for more than one species of P-450 (Imai and Sato, 1966b; Sladek and Mannering, 1966; Schenkman *et al.*, 1967b). Cytochrome P-450 has been ascertained by excellent, although indirect, methods, to be an active component in the hydroxylations of several steroids in different tissues (Estabrook *et al.*, 1966). The CO-inhibitory property of cytochrome P-450 has already been used to demonstrate that the testosterone 6β-, 7α-, and 16α-hydroxylases have selective rates of CO inhibition (Conney *et al.*, 1967). We have been able to confirm the different rates of 7α and 16α hydroxylation when DHA was the substrate and CO gas phases were employed for the incubations (W. L. Heinrichs and A. Colás, unpublished data). The serious limitation to valid conclusions from kinetic and inhibitory studies in crude preparations was partly circumvented in these experiments because the aminopyrine and SU-9055 inhibition studies (and the CO-inhibition studies, too) are relative, the different responses occurring in the same solutions. Deoxycholate or a variety of detergents or proteolytic enzymes convert cytochrome P-450 to P-420 (Mason *et al.*, 1965a,b) and simultaneously reduce the enzymatic activities (MacLennan *et al.*, 1967; Machinist *et al.*, 1966; Orrenius, 1965; Silverman and Talalay, 1967). Certain nonionic detergents or polyalcohols have prevented the alteration of P-450 or the enzymatic activities (Estabrook *et al.*, 1966; Narasimhulu *et al.*, 1965; Silverman and Talalay, 1967). In these experiments, the 7α hydroxylation was almost completely destroyed by deoxycholate, about 75% of the *N*-demethylase was destroyed, and less than 60% of the 16α-hydroxylase was destroyed (in the presence of DHA); *p*-hydroxylase was greatly stimulated and no proportionality with the cytochrome P-450 was apparent for any of the activities. Propylene glycol protected the cytochrome P-450 but did not protect the enzymatic activities. Androstenedione did not confer the protection. The decreasing enzymatic activities and hemoprotein content may reflect the progressively greater age of the rats used in successive experiments. These separate and apparently specific responses to drug treatment, inhibitors, and DOC treatments are evidence for a multiplicity of hepatic enzymes or enzymatic mechanisms which metabolize DHA at C-7 and C-16 or aminopyrine or acetanilide.

Acknowledgments

We are grateful to Dr. J. L. Gaylor for advice about the deoxycholate treatments and to Dr. H. S. Mason and Dr. R. B. Lyons for permission to use their equipment. SU-9055 was a gift from Dr. J. J. Chart, Ciba Pharmaceuticals, and phenylbutazone from Geigy Pharmaceuticals. The technical assistance of Mr. F. Herrera was invaluable. Dr. D. K. Fukushima and Professor R. M. Dodson kindly furnished the reference samples of 3β,7α- and 3β,7β-dihydroxyandrost-5-en-17-one.

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