Effect of Androgen and Estrogen on the Hydroxylation of Steroid Hormones by Rat Liver Microsomes

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Synopsis

Castration in male rats decreased the activities of testosterone and progesterone hydroxylations and reduced the magnitude of spectral change caused by testosterone and progesterone in liver microsomes, accompanying less marked decrease in microsomal P-450 content and NADPH-neotetrazolium reductase activities. The administration of testosterone or methyltestosterone to the castrated rats completely restored the hydroxylating activities and magnitude of the spectral change. The simultaneous injection of estradiol or diethylstilbestrol blocked the action of the androgens. These results suggest that androgen increases the binding capacity of P-450 with steroid hormones and makes an increase in the hydroxylating activities and that estrogen directly prevents the above action of androgen.

A number of foreign compounds of high lipid-solubility are oxidatively metabolized to more water-soluble compounds by the liver microsomal hydroxylase system, called drug-metabolizing enzymes, in the presence of NADPH and oxygen (Brodie *et al.*, 1958; Gillette, 1963). It has been well known that the activities of drug-metabolizing enzymes are higher in male rats than in female ones (Quinn *et al.*, 1958; Gillette, 1963; Kato and Gillette, 1965). These activities are stimulated by anabolic action of androgen and simultaneous administration of estrogen blocks the action of androgen (Kato *et al.*, 1962; Booth and Gillette, 1962; Kato and Onoda, 1969).

On the other hand, recent works of Conney and Klutch (1963), Kuntzman *et al.* (1964) and Conney *et al.* (1965) have shown that liver microsomal enzymes hydroxylate various steroid hormones in the presence of NADPH and oxygen and that there are many similarities between the natures of drug-metabolizing enzymes and those of steroid hydroxylating enzymes. The activities of both enzymes are

higher in microsomes from adult male rats than in those from adult female rats, and these enzyme systems have similar intracellular distribution, cofactor requirement and response to the administration of some drugs.

In the present communication, therefore, the effect of androgen and estrogen on the activities of testosterone and progesterone hydroxylations has been investigated. Since the involvement of cytochrome P-450 as the oxygen-activating enzyme and substrate-binding site in the hydroxylation of drugs and steroid hormones has been reported (Omura *et al.*, 1965; Kato, 1966; Imai and Sato, 1967; Schenkman *et al.*, 1967a), the effects of androgen and estrogen on the content of cytochrome P-450 and the spectral changes of P-450 caused by testosterone and progesterone have been investigated in relation to the examination of hydroxylating activity of testosterone and progesterone.

Materials and Methods

Animals

Male and female rats of the Wistar strain, weighing

about 160 and 180 g, respectively, were used.

Male rats were castrated 23 days before sacrifice and treated daily with testosterone propionate (5 mg/kg), methyltestosterone (5 mg/kg), estradiol benzoate (500 μ g/kg), diethylstilbestrol dibenzoate (500 μ g/kg) for 10 days before being killed. All the steroids were dissolved in olive oil and given subcutaneously with a volume of 1 ml per kg body weight. Control and castrated rats received only olive oil.

Chemicals

NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Böhringer and Soehne GmbH, Germany. Progesterone-4- 14 C (specific activity, 58.5 mc/mmole) and testosterone-4- 14 C (specific activity, 29.2 mc/mmole) were purchased from Daiichi Kagaku Co., Ltd., Japan. 16 α -Hydroxyprogesterone was kindly supplied by Prof. H. Solomon, 6 β -hydroxytestosterone, 7 α -hydroxytestosterone and 16 α -hydroxytestosterone were kindly supplied by Dr. A. H. Conney and Dr. A. Nehr. Other metabolites of progesterone and testosterone were purchased from Sigma, U.S.A. Other chemicals were of reagent grade and purchased from commercial sources.

Preparation of microsomal fraction

The rats were decapitated, and the liver was removed, chopped into small pieces, washed thoroughly and homogenized with 3 volumes of 1.15 % KCl in a Teflon-glass homogenizer. The homogenate was centrifuged at 10,000 Xg and the supernatant fluid was then centrifuged for 60 min at 105,000 Xg. The supernatant fluid and microsomal pellet were separated by decantation, and the microsomal pellet was rinsed once and suspended in 1.15 % KCl solution.

Assay of NADPH-neotetrazolium reductase

The activity of NADPH-neotetrazolium reductase was determined according to the method described in a previous paper (Kato and Takanaka, 1968).

Determination of microsomal protein and cytochrome P-450 contents

The protein content of microsomal fraction was measured according to the method of Lowry *et al.* (1952). The content of cytochrome P-450 was determined according to the method of Omura and Sato (1964) as described in the previous paper (Kato and Onoda, 1969). The content of cytochrome P-450 was expressed as μ mmole per mg microsomal protein according to Omura and Sato (1964).

Metabolism of testosterone and progesterone by liver microsomes

A typical incubation mixture consisted of microsomal suspension equivalent to 250 mg or 500 mg of

liver tissue, 20 μ moles of glucose 6-phosphate 1.0 μ mole of NADP, 1.5 international units of glucose 6-phosphate dehydrogenase, 50 μ moles of nicotinamide, 25 μ moles of $MgCl_2$, 1.4 ml of 0.2 M sodium phosphate buffer, pH 7.4, 1 μ mole (0.6 μ c) of testosterone or progesterone, 0.5 ml of 1.15 % KCl solution and water to a final volume of 5.0 ml. The mixture was incubated aerobically at 37°C for 10 min.

The steroids and their metabolites present in 5 ml portions of the incubation mixture were extracted with 30 ml of dichloromethane by shaking for 20 min. A 20-ml portion of the organic phase was evaporated to dryness by a rotary evaporator below 50°C and the residue was redissolved in 0.25 ml of ethanol. An exact portion (0.5 ml) of the ethanol solution was applied on Toyo No.50 filter paper for chromatography. Testosterone and its metabolites were chromatographed (descending) for 4 hr at 25°C with the upper phase of a toluene-methanol-water system (2:1:1 by vol.) as described by Conney and Klutch (1963). Progesterone and its metabolites were chromatographed (ascending) overnight at 25°C with the upper phase of a cyclohexan-dioxane-methanol-water system (100:25:100:10 by vol.) as described by Conney and Klutch (1963). The radioactivity on the chromatogram was measured by a radiochromatogram scanner (Packard 7201). Further separation and identification of the metabolites of testosterone and progesterone were carried out with thin-layer chromatography through the methods of acetylation by acetic anhydride and of oxidation by chromic acid as described in the previous papers (Kato *et al.*, 1970a; Kato *et al.*, 1970b) according to Shikita *et al.* (1964). In some experiments, the incubation under nitrogen atmosphere instead of air was carried out for the criteria of the hydroxylated metabolites.

Determination of substrate-induced difference spectra

The spectral changes caused by testosterone and progesterone were determined according to Schenckman *et al.* (1967b) as described in a previous paper (Kato and Onoda, 1969). The substrate-induced difference spectra were recorded at room temperature with a Hitachi EPS-3T recording spectrophotometer with an integral sphere attachment. The concentration of microsomal protein was about 1.2 mg per ml and the concentrations of testosterone and progesterone were 0.1 mM. The results were expressed by ΔOD per mg microsomal protein or μ mmole P-450 per ml.

Results

Effects of androgens and estrogens on

Table 1. Effects of androgens and estrogens on liver microsomal protein content, and the weight of seminal vesicle and musculus levator ani

Group	Microsomal protein (mg/g wet organ weight)	Seminal vesicle (mg/100g body weight)	Levator ani muscle (mg/100g body weight)
Male control	28.5 ± 0.4	238 ± 14	65 ± 4
Castrated	27.9 ± 0.4 -2%	23 ± 4 - 90%*	21 ± 3 -68%*
Castrated + TS	28.3 ± 0.3 -1%	598 ± 50 +151%*	98 ± 5 +51%*
Castrated + TS + ED	27.8 ± 0.5 -2% (-2%)	671 ± 61 +181%* (+12%)	102 ± 6 +57%* (+ 4%)
Castrated + TS + DES	28.4 ± 0.6 0% (0%)	650 ± 53 +173%* (+ 9%)	103 ± 8 +59%* (+ 5%)
Castrated + MT	28.8 ± 0.4 +1%	421 ± 43 + 77%*	85 ± 4 +31%*
Castrated + MT + ED	27.6 ± 0.3 -3% (-4%)	463 ± 45 + 95%* (+10%)	98 ± 6 +50%* (+15%)
Castrated + MT + DES	28.3 ± 0.5 -1% (-2%)	430 ± 36 + 81%* (+ 2%)	86 ± 5 +32%* (+ 1%)
Female control	28.0 ± 0.3 -2%	—	—

Male rats were castrated 23 days before the experiments, then treated for 10 days with testosterone (TS) (5 mg/kg, s.c.), methyltestosterone (MT) (5 mg/kg, s.c.), estradiol (ED) or diethylstilbestrol (DES) (500 µg/kg, s.c.) alone or combined as indicated in the table. The results are given as averages ± S.E. obtained from 6 determinations. The figures with + or - indicate the percentage difference from controls and the figures in the parentheses indicate the percentage difference from the castrated + TS treated rats or the castrated + MT treated rats. The asterisks in the table indicate the significant difference ($p < 0.05$).

liver microsomal protein, and weights of seminal vesicle and levator ani muscle

Castration in male rats and treatments with androgens and estrogens did not alter the ratio of liver weight to body weight and the content of microsomal protein (Table 1). Castration markedly decreased the weights of seminal vesicle and levator ani muscle, and the treatment with testosterone or methyltestosterone increased their weights over the control values (Table 1). The simultaneous administration of estradiol or diethylstilbestrol failed to block the action of the androgens. These results indicate that the amount of the estrogens used in the present experiments is not enough to prevent the action of the androgens on the target organs.

Effects of androgens and estrogens on

the content of cytochrome P-450 and activity of NADPH-neotetrazolium reductase

The content of cytochrome P-450 and the activity of NADPH-neotetrazolium reductase showed slight sex difference (Kato and Takahashi, 1968; Kato *et al.*, 1968), and the effects of androgens and estrogens upon the electron-transport system were investigated in the present study.

Castration in male rats decreased slightly the content of cytochrome P-450 and the activity of NADPH-neotetrazolium reductase (Table 2). The treatment with testosterone or methyltestosterone in the castrated rats restored the content of cytochrome P-450 and the activity of NADPH-neotetrazolium reductase to the control level. The administration of estradiol or diethylstilbestrol prevented the actions of

Table 2. Effects of androgens and estrogens on microsomal P-450 content and NADPH-neotetrazolium (NT) reductase activity

Group	P-450 content ($\mu\text{mole/mg protein}$)	NADPH-NT reductase ($\mu\text{mole/mg protein/min}$)
Male control	0.87 ± 0.03	108 ± 6
Castrated	0.65 ± 0.03 –25%*	73 ± 5 –32%*
Castrated + TS	0.89 ± 0.05 + 2%	107 ± 10 – 1%
Castrated + TS + ED	0.69 ± 0.04 –21%* (–22%)*	81 ± 4 –25%* (–24%)*
Castrated + TS + DES	0.73 ± 0.03 –16%* (–18%)*	86 ± 6 –20%* (–20%)*
Castrated + MT	0.85 ± 0.04 – 2%	101 ± 8 – 6%
Castrated + MT + ED	0.69 ± 0.03 –21%* (–18%)*	82 ± 5 –24%* (–19%)*
Castrated + MT + DES	0.72 ± 0.04 –17%* (–15%)*	85 ± 7 –21%* (–16%)*
Female control	0.60 ± 0.03 –30%*	66 ± 4 –39%*

See the legends for Table 1.

Table 3. Effects of androgens and estrogens on the metabolism of testosterone by liver microsomes

Group	Conversion of testosterone ($\mu\text{mole/mg protein/10 min}$)			
	Nonpolar peak	Polar peak T ₁	Polar peak T ₂	Polar peak T ₃
Male control	18.6 ± 1.3	12.5 ± 0.8	16.8 ± 0.5	33.1 ± 1.8
Castrated	47.7 ± 2.3 +156%*	5.4 ± 0.4 –57%*	5.9 ± 0.8 –65%*	13.7 ± 1.6 –59%*
Castrated + TS	19.4 ± 1.8 + 10%	11.2 ± 0.8 –10%	15.9 ± 1.1 – 5%	31.7 ± 1.3 – 4%
Castrated + TS + ED	43.2 ± 2.3 +132%*	6.5 ± 0.9 –48%*	7.5 ± 1.3 –55%*	16.4 ± 1.6 –50%*
Castrated + TS + DES	44.4 ± 2.7 +140%*	6.3 ± 0.9 –50%*	9.4 ± 1.3 –44%*	15.8 ± 1.5 –52%*
Castrated + MT	20.3 ± 3.1 + 9%	12.3 ± 1.3 – 2%	19.4 ± 1.0 +15%	29.3 ± 2.1 –11%
Castrated + MT + ED	45.2 ± 2.4 +143%*	7.1 ± 1.0 –43%*	7.9 ± 1.5 –53%*	16.2 ± 1.9 –51%*
Castrated + MT + DES	46.9 ± 2.5 +152%*	5.8 ± 0.7 –54%*	7.8 ± 1.3 –44%	14.8 ± 1.7 –55%*
Female control	50.1 ± 2.2 +169%*	5.0 ± 0.4 –60%*	5.1 ± 0.4 –70%*	13.5 ± 0.9 –59%*

See the legends for Table 1.

Fig. 1. Metabolism of testosterone by liver microsomes from male and female rats. After the incubation, testosterone and its metabolites were extracted with dichloromethane. The steroids were developed by descending paper chromatography with a toluene-methanol-water system (2:1:1 by vol.). The radioactivity was counted by a Packard radiochromatogram scanner. The mobility of authentic samples is indicated in the figure. (NT, nonpolar fraction; Test, testosterone fraction; T₁ to T₃, T₁ to T₃ fractions; Dione, 5 α -androstan-3,17-dione; Stanol, 5 α -androstan-17 β -ol-3-one; Test., testosterone; 6 β -ol, 7 α -ol and 16 α -ol, 6 β -, 7 α - and 16 α -hydroxytestosterone)

the androgens on the content of P-450 and the activity of NADPH-neotetrazolium reductase. These results indicate that the content of cytochrome P-450 may be a minor factor responsible for the sex difference in the hydroxylation of steroid hormones.

Effects of androgens and estrogens on the metabolism of testosterone

As shown in Figure 1 and Table 3, there is a clear difference between male and female rats concerning the metabolic pattern of testosterone by liver microsomes.

The polar peaks T₁, T₂ and T₃, which were not obtained in the incubation under nitrogen atmosphere, contain the hydroxylated metabolites of testosterone, such as 6 β -hydroxytestosterone, 7 α -hydroxytestosterone and 16 α -hydroxytestosterone, although T₁ was not identified and supposed to contain 2 α -hydroxytestosterone, and the radioactivities of

these polar peaks were higher in male rats than in female in agreement with the report of Kuntzman *et al.* (1964). The castration in male rats decreased the radioactivities of the polar peaks and the administration of androgens restored it to the level of intact male rats (Table 3). This action of the androgens was prevented by the simultaneous administration of estradiol or diethylstilbestrol.

As shown in Table 3, the activity of Δ^4 -reductase for testosterone was lower in microsomes from the males than in those from the females and the activity seemed to be regulated by androgen in agreement with the report of Hagen and Troop (1960).

Activity of the enzymes for hydroxylation of testosterone per P-450 was higher in microsomes from the males than in those from the females (Table 5). The castration in the males decreased the activity of total hydroxylation of testosterone per P-450 and the administration

Fig. 2. Metabolism of progesterone by liver microsomes from male and female rats. After the incubation, progesterone and its metabolites were extracted with dichloromethane. The steroids were developed by ascending paper chromatography with a cyclohexan-dioxane-methanol-water system (100:25:100:10 by vol.). The mobility of authentic samples is indicated in the figure. (NP, nonpolar fraction; Prog., progesterone fraction; P₁ to P₃, P₁ to P₃ fractions; Dione, 5 α -pregnan-3,20-dione; Prog., progesterone; 20 β -ol and 20 α -ol, 20 β - and 20 α -hydroxyprogesterone; 6 β -ol, 6 β -hydroxyprogesterone; 16 α -ol, 16 α -hydroxyprogesterone)

of androgens restored the hydroxylating activity to the level of the intact males. The action of the androgens was prevented to take place by the simultaneous administration of estrogens.

Since the combined administration of diethylstilbestrol or estradiol prevented the action of testosterone or methyltestosterone, the structure specificity for the actions of androgen and estrogen was assumed not to be highly estimated as already reported concerning the studies on the hydroxylation of drugs (Kato and Onoda, 1969). In addition, it is of interest to note that the action of androgens on the hydroxylating activity of liver microsomes is prevented by the low dose of estrogens which have failed to prevent the action of the androgens on the target organs.

Effects of androgens and estrogens on the metabolism of progesterone

As shown in Figure 2 and Table 4, there is

a clear difference between the males and females concerning the metabolic pattern of progesterone by liver microsomes.

The polar peaks P₂ and P₃, which were not obtained by the incubation under nitrogen atmosphere, contain the hydroxylated metabolites of progesterone, such as 6 β -hydroxyprogesterone and 16 α -hydroxyprogesterone, and the radioactivities of both polar peaks were higher in the males than in the females. The castration in the males decreased the radioactivities of both peaks and the administration of androgens restored the magnitude of the radioactivities to the level of the intact males (Table 4). The action of the androgens was prevented by the simultaneous administration of estrogens.

As shown in Table 4, the enzyme activity for 20-reduction of progesterone (polar peak P₁) was higher in microsomes from male rats than in those from the females; in contrast, the activity for Δ^4 -reduction of progesterone was

Table 4. Effects of androgens and estrogens on the metabolisms of progesterone by liver microsomes

Group	Metabolism of progesterone (nmole/mg protein/10 min)			
	Nonpolar peak	Polar peak P ₁	Polar peak P ₂	Polar peak P ₃
Male control	12.6 ± 0.8	6.7 ± 0.8	18.6 ± 1.2	15.6 ± 1.2
Castrated	20.6 ± 0.9	3.9 ± 0.5	5.8 ± 0.6	5.9 ± 0.9
	+63%*	-42%*	-69%*	-62%*
Castrated	11.7 ± 0.7	6.9 ± 0.9	21.2 ± 0.6	16.9 ± 0.5
+ TS	- 7%	+ 3%	+13%	+ 8%
Castrated	20.0 ± 1.0	4.0 ± 0.6	8.3 ± 0.8	5.3 ± 0.7
+ TS + ED	+59%*	-40%*	-55%*	-66%*
Castrated	16.8 ± 0.4	4.3 ± 0.8	9.6 ± 0.7	8.3 ± 0.5
+ TS + DES	+33%*	-36%*	-48%*	-47%*
Castrated	13.4 ± 0.9	6.5 ± 0.8	17.2 ± 0.9	14.5 ± 0.7
+ MT	+ 6%	- 3%	- 8%	- 7%
Castrated	19.4 ± 1.1	3.9 ± 0.7	8.0 ± 1.2	7.0 ± 1.1
+ MT + ED	+54%*	-42%*	-57%*	-55%*
Castrated	17.6 ± 1.3	4.2 ± 0.6	10.6 ± 1.3	8.8 ± 1.4
+ MT + DES	+40%*	-39%*	-43%*	-44%*
Female control	24.1 ± 0.8	2.8 ± 0.5	4.8 ± 0.5	5.4 ± 0.4
	+91%*	-58%*	-74%*	-65%*

See the legends for Table 1.

lower in the males than in the females. Both activities seemed to be regulated by androgen in agreement with the opinion of Hagen and Troop (1960).

The activity for total hydroxylation of progesterone per P-450 was higher in microsomes from the males than in those from the females (Table 5). The castration in the males decreased the activity for total hydroxylation of progesterone per P-450 and the administration of androgen restored the hydroxylating activity to the level of the intact males. The action of androgens was prevented by the simultaneous administration of estrogens. These results suggest that there may be other factor(s) responsible for the sex difference concerning the hydroxylation of steroid hormones, although the content of cytochrome P-450 is really involved in the sex difference.

Effects of androgens and estrogens on the magnitude of spectral changes caused by testosterone and progesterone

Recent studies have established that an unique hemoprotein called P-450 is involved as the oxygen-activating component in a number of NADPH-dependent monooxygenase reactions such as hydroxyations of drugs and steroid hormones. Imai and Sato (1967), and Schenkman *et al.* (1967a) have reported that a number of drugs and steroid hormones, i.e. substrates of hepatic microsomal monooxygenases, react with the microsomal cytochrome to give two characteristic types of spectral changes, which are indicative of substrate interaction with cytochrome P-450 presumably representing the primary binding of substrate for enzymic hydroxylation.

Moreover, Schenkman *et al.* (1967b) have observed that the magnitude of spectral change caused by hexobarbital is greater in microsomes from male rats than in those from female. In a previous paper (Kato and Onoda, 1969), we have described that the magnitude of spectral change caused by hexobarbital is increased by treatment with androgens and that estrogens

Table 5. Effects of androgens and estrogens on the activity of total hydroxylations of testosterone and progesterone

Group	Testosterone total hydroxylation**		Progesterone total hydroxylation***	
	per mic. prot.	per P-450	per mic. prot.	per P-450
Male control	62.4 ± 3.2	72.1 ± 3.6	34.2 ± 2.1	39.3 ± 2.5
Castrated	25.0 ± 2.4	38.1 ± 2.0	11.7 ± 1.3	18.0 ± 1.2
	–60%*	–43%*	–66%*	–54%*
Castrated	58.9 ± 2.3	66.1 ± 3.4	38.1 ± 1.0	42.8 ± 1.9
+ TS	– 5%	– 8%	+11%	+ 8%
Castrated	30.4 ± 2.7	44.1 ± 2.8	13.6 ± 1.5	19.7 ± 1.5
+ TS + ED	–51%*	–39%*	–60%*	–50%*
Castrated	31.5 ± 2.5	43.2 ± 2.3	17.9 ± 1.3	25.9 ± 1.9
+ TS + DES	–50%*	–41%*	–48%*	–34%*
Castrated	61.0 ± 4.8	71.7 ± 4.5	31.7 ± 1.5	37.2 ± 1.9
+ MT	– 2%	– 1%	– 7%	– 5%
Castrated	31.2 ± 3.4	45.2 ± 3.3	15.0 ± 1.6	21.7 ± 1.5
+ MT + ED	–50%*	–37%*	–56%*	–45%*
Castrated	28.4 ± 2.9	39.4 ± 3.2	19.4 ± 2.1	26.9 ± 2.5
+ MT + DES	–54%*	–45%*	–43%*	–32%*
Female control	23.6 ± 2.0	39.3 ± 2.5	10.2 ± 0.9	17.0 ± 1.4
	–62%*	–45%*	–70%*	–57%*

** Peak T₁ plus Peak T₂ and Peak T₃ in Table 3.*** Peak P₂ plus Peak P₃ in Table 4.The activity of total hydroxylation is indicated as μ mole hydroxylated metabolites formed per mg microsomal protein or per μ mole P-450 for 10 min.

See the legends for Table 1.

antagonize the effect of androgens.

In this connection, the effects of androgens and estrogens on the magnitude of spectral changes caused by testosterone and progesterone were investigated in the present study. As shown in Figure 3, a clear sex difference was observed in the spectral changes caused by testosterone and progesterone. The castration in the males decreased the magnitude of spectral change of cytochrome P-450 caused by testosterone (Table 6). The administration of androgens to the castrated rats restored the magnitude of the spectral change to the level of the intact males. The simultaneous administration of estrogens blocked the action of the androgens. Moreover, the magnitude of testosterone-induced spectral change per P-450 was also decreased by castration. The adminis-

tration of androgens to the castrated rats restored the magnitude of the spectral change and the simultaneous administration of estrogens blocked the action of the androgens. Furthermore, the similar results were obtained with progesterone-induced spectral change (Table 6). These results suggest that the capacity of P-450 to interact with steroid hormones is increased by androgens, and estrogens block the action of the androgens. The alterations in the magnitude of the spectral changes were similar to those in the hydroxylating activities, and this suggests that the sex difference in the binding capacity of P-450 with testosterone and progesterone is a major factor responsible for the sex difference in the hydroxylating activities of steroid hormones by rat liver microsomes.

Fig. 3. The difference spectra of cytochrome P-450 induced by progesterone and testosterone in rat liver microsomes. The difference spectra were recorded on addition of progesterone (0.1 mM) or testosterone into microsomal suspension (1.2 mg protein/ml) isolated from male and female rats.

Discussion

In a previous paper (Kato and Onoda, 1969), we have demonstrated that castration in male rats decreases markedly the activity of hexobarbital hydroxylation, and decreases slightly the activity of NADPH-neotetrazolium reductase and the content of P-450. The magnitude of spectral change caused by hexobarbital is also decreased by the castration. Moreover, the administration of testosterone or methyltestosterone restores the hydroxylating activity and the magnitude of the spectral change to the level of the intact males and the simultaneous administration of estradiol or diethylstilbestrol counteracts the action of the androgens. These results are identical with the present results obtained with testosterone and progesterone which were used as the substrate in place of hexobarbital. Thus, as shown in Table 6, the decrease in the binding capacity of P-450 with the steroid substrate seems to be a factor responsible for the decrease in the activities of testosterone and progesterone hydroxylations.

The administration of estradiol or diethyl-

stilbestrol to the castrated males or ovariectomized females did not result in any significant effect on the activities of testosterone and progesterone hydroxylations (Kato *et al.*, 1969). These results suggest that the estrogens prevent directly the action of the androgens in the males. It has been recently observed that the action of androgen to increase the binding capacity of P-450 with the steroid substrate and the activities of testosterone and progesterone hydroxylations have been able to be demonstrated only in rats, but not in mice and rabbits (Kato *et al.*, 1969). These results are consistent with our previous ones obtained as to the hydroxylation of hexobarbital (Kato *et al.*, 1968; Kato and Onoda, 1969), and suggest that there is an androgen-dependent regulating mechanism which is common for drug-metabolizing enzymes and steroid hormone hydroxylase(s). However, this mechanism seems to be unwarrantable genetically in other species of animals (Kato *et al.*, 1968).

At present, we have not known about the nature of the androgen-dependent regulating mechanism. According to the operon theory

Table 6. Effects of androgens and estrogens on the magnitude of spectral changes induced by testosterone and progesterone

Group	Testosterone-induced spectral change		Progesterone-induced spectral change	
	per mic. prot.	per P-450	per mic. prot.	per P-450
Male control	11.9 ± 0.7	13.6 ± 0.8	8.1 ± 0.6	9.3 ± 0.8
Castrated	5.7 ± 0.6	8.8 ± 0.7	3.6 ± 0.4	5.5 ± 0.6
	-52%*	-35%*	-56%*	-41%*
Castrated + TS	11.8 ± 0.9	13.3 ± 1.1	8.5 ± 0.8	9.5 ± 0.9
	- 1%	- 2%	+ 4%	+ 2%
Castrated + TS + ED	6.1 ± 0.6	8.8 ± 0.6	3.7 ± 0.3	5.4 ± 0.4
	-49%*	-35%*	-54%*	-42%*
Castrated + TS + DES	6.4 ± 0.7	8.8 ± 0.7	4.2 ± 0.4	5.7 ± 0.7
	-47%*	-35%*	-49%*	-39%*
Castrated + MT	11.8 ± 1.1	13.8 ± 1.0	7.8 ± 0.9	9.2 ± 0.9
	- 1%	+1%	- 4%	- 1%
Castrated + MT + ED	6.2 ± 0.7	9.0 ± 0.8	3.9 ± 0.5	5.7 ± 0.6
	-48%*	-34%*	-52%*	-39%*
Castrated + MT + DES	5.8 ± 0.6	8.0 ± 0.5	4.4 ± 0.4	6.1 ± 0.7
	-51%*	-41%*	-46%*	-34%*
Female control	5.4 ± 0.4	9.0 ± 0.4	3.3 ± 0.3	5.5 ± 0.5
	-55%*	-34%*	-59%*	-41%*

The change of absorbance between 420 and 500 μ m on the addition of testosterone (0.1 mM) was calculated from the testosterone-difference spectrum. The change of absorbance between 424 and 500 μ m on the addition of progesterone (0.1 mM) was calculated from the progesterone-difference spectrum. The magnitude of the spectral change is indicated as $\Delta OD \times 10^3$ per mg microsomal protein or per μ mole P-450 per ml.

See the legends for Table 1.

of Jacob and Monod (1961), it was assumed that androgen might act as an inactivator to bind with an endogenous repressor, which may repress the action of a regulator gene responsible for the binding capacity of P-450 with substrates of the microsomal hydroxylases. The additional administration of estradiol or diethylstilbestrol counteracts the stimulative action of testosterone or methyltestosterone on the binding capacity of P-450 with the substrates (Table 6). It is thus assumed that, if androgen plays a role as an inactivator of the repressor, estrogen may interfere the binding of androgen with the repressor. Since estradiol and diethylstilbestrol counteract, to the same extent, the stimulative action of testosterone and methyltestosterone, the structure specificity for the binding of androgen with the re-

pressor and interaction of estrogen with androgen are assumed not to be highly estimated.

Our further experiments have shown that the K_m (Michaelis constant) values for the hydroxylation of testosterone and progesterone and the K_s (spectral dissociation constant) values for the spectral changes caused by testosterone and progesterone were together lower in microsomes from the males than in those from the females (Kato *et al.*, 1969). The K_m and K_s values are, hence, supposed to be regulated by androgen (Kato and Onoda, 1969).

Our present results are in agreement with those observed as to hexobarbital hydroxylation and hexobarbital-induced spectral change (Kato and Onoda, 1969). We give, therefore, support to the view of Kuntzman *et al.* (1964) that the drugs and steroid hormones are hy-

droxylated by the similar enzymes under the same mechanism.

As in the present studies each hydroxylated metabolite of the steroid hormones were not separated quantitatively, the hydroxylating activity was indicated as total hydroxylation. Heinrichs *et al.* (1967) recently pointed out that there was no clear sex difference concerning the 7 α -hydroxylation of testosterone in rat liver microsomes. More recently, Jacobson and Kuntzman (1969) reported evidence of the different response upon 7 α -hydroxylation, 6 β -hydroxylation and 16 α -hydroxylation of testosterone in liver microsomes of rats with testosterone treatment. Their observations brought about the interesting relationships between the substrate binding with P-450 and hydroxylating activity for steroid hormones by rat liver microsomes in participation with the possible presence of cytochromes P-450 of more than two kinds and of terminal oxidases which determine the position of hydroxylation.

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