Testicular Sterols. IV. End-Product Steroid Inhibition of Lanosterol Demethylation*

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ABSTRACT: Homogenates of rat testicular tissue were incubated with biosynthetically labeled lanosterol. The rate of conversion of lanosterol (C_{30}) to C_{27}-sterols was measured by the rate of release of ^{14}C as CO_2 from the 4α-methyl group of lanosterol. Several C_{19} and C_{21} steroids added *in vitro* inhibited the demethylation; progesterone and testosterone were most active. Increasing concentrations of steroid resulted in more inhibition. Various mixtures of progesterone or testosterone produced similar degrees of inhibition. Reciprocal graphs of the effect of substrate concentration suggested noncompetitive inhibition. The K_i' was between 0.21 and 0.42 mM for progesterone and testosterone. Inhibition was reversed partially by removing the steroid from microsomes with Sephadex G-200. In addition, reversible inhibition was apparent because no effect on the rate of demethylation was observed during changes in the length of exposure of the enzyme to inhibitor and because the order of mixing enzyme, inhibitor, and substrate did not alter the kinetic values. The inhibition was associated more strongly with enzymes of the microsomes rather than with enzymes in the supernatant fraction. Apparently both the hydroxylation and dehydrogenation of demethylation were inhibited. Physiologically these results suggest that self-regulation of cholesterol formation in testicular tissue may be mediated by end-product inhibition of lanosterol demethylation.

Homogenates of testicular tissue catalyze the oxidative demethylation[1] of lanosterol (4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol) to cholesterol (cholest-5-en-3β-ol) and other C_{27}- sterols (Gaylor and Tsai, 1964). Demethylation is initiated by microsomal-catalyzed hydroxylation of the methyl groups (Olson *et al.*, 1957). Similar hydroxylations of steroidal 11-C, 21-C, and 20-C are inhibited by androstenedione (androst-4-ene-3,17-dione), testosterone (17β-hydroxyandrost-4-en-3-one), or pregnenolone (3β-hydroxypregn-5-en-20-one) (Sharma *et al.*, 1963; Sharma and Dorfman, 1964; Ichii *et al.*, 1963; Koritz and Hall, 1964). The demethylation of lanosterol proceeds by dehydrogenation of the hydroxymethyl group to the aldehyde, acid, and cleavage as CO_2 (Olson *et al.*, 1957). Testosterone and androstenedione inhibit dehydrogenation of Δ^5-3β-hydroxy steroids (Kowal *et al.*, 1964).

The effect of various steroids on the demethylation of lanosterol by testicular tissue was investigated. The present report describes the effect of steroids on the rate of demethylation of lanosterol, the nature of the inhibition, and the possible physiological significance of end-product inhibition of lanosterol demethylation in testicular tissue.

Experimental Procedures

Testicular tissue was dissected from adult rats (approx 250–400 g) (Holtzman Rat Co., Madison, Wis.), decapsulated, and ground in a glass homogenizer with 2 volumes of 0.1 M potassium phosphate buffer (*p*H 7.4) that contained 0.03 M nicotinamide. Homogenates were centrifuged for 20 minutes at 10,000 × *g* to remove mitochondria. Homogenate (4 ml; av, 72.6 mg protein) were incubated aerobically at 37° with 1.5 μmoles of DPN[2] and 8–200 mμmoles of labeled lanosterol (approx 200–350 dpm/mμmole). The final volume was 4.5 ml. Less than 0.1 ml of an alcohol solution of the steroid inhibitor was added before the substrate. Control samples contained equal amounts of alcohol. Labeled carbon dioxide was collected and radioactivity was determined as described previously (Gaylor and Tsai, 1964; Gaylor, 1964). From the specific activity of the labeled substrate, the amount of protein (Lowry *et al.*, 1951), and the length of incubation, the rate of demethylation was calculated (mμmoles/hour per 100 mg protein).

Labeled lanosterol was prepared from [2-^{14}C]-mevalonate by the method of Moller and Tchen (1961). The sterol was diluted with unlabeled lanosterol (mp 137–139°; $[\alpha]_D^{25}$ + 60.1° in $CHCl_3$ [*c*, 0.3]), and solutions

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[1] Demethylation is the oxidative loss of methyl groups 4α, 4β, and 14α of lanosterol (Olson *et al.*, 1957). Although other reactions accompany demethylation, only the rate of demethylation was measured.

[2] Abbreviations used in this work: DPN, diphosphopyridine nucleotide; HCG, human chorionic gonadotrophin; ACTH, adrenocorticotropic hormone.

TABLE I: Inhibition of Lanosterol Demethylation by Testosterone, Progesterone, and Steroid Metabolites.

Steroid Added	Demethylation[a] Normal (mμmoles/hour per 100 mg protein)	Demethylation[a] + HCG[b] (mμmoles/hour per 100 mg protein)
None[c]	1.28 ± 0.05 (7)[d]	4.05 ± 0.51 (7)
Testosterone[e]	0.22 ± 0.01 (3)	2.10 ± 0.51 (4)
Progesterone[e]	0.39 ± 0.04 (4)	2.11 ± 0.58 (4)
17α-Hydroxyprogesterone[f]	0.50 ± 0.10 (4)	2.89 ± 0.21 (4)
Androst-4-ene-3,17-dione[g]	0.56 ± 0.25 (4)	2.82 ± 0.46 (3)
Pregnenolone[h]	0.78 ± 0.24 (4)	3.36 ± 0.47 (4)
17α-Hydroxypregnenolone[i]	0.75 ± 0.10 (3)	3.01 ± 0.74 (4)

[a] Each flask contained homogenate (av 72.6 mg protein), DPN, lanosterol (approx 8.9×10^{-6} M), and steroid (approx 0.4 mM). [b] HCG (600 IU) was injected in three doses during the week before incubation. [c] 0.05 ml ethanol. [d] Standard error of the mean, and the number of samples. [e] Recrystallized steroids and chromatographically homogeneous progesterone (Calbiochem). [f] Chemed, Inc., Sigma (lot H49-17), Calbiochem (lot 501002). [g] Chemed, Inc., Sigma (lot A11B-54), chromatographically homogeneous (Calbiochem). [h] Sigma (lot P49-15), Calbiochem (lot 500475). [i] Chemed, Inc., Sigma (lot H80B-12).

of the substrate in buffer were prepared with Tween 80. The final concentration of Tween 80 in the incubation flasks was less than 1 mg/ml.

Testosterone (lot T11B-94, Sigma, and lot 43095, Calbiochem) was recrystallized from ethanol (mp 154–155°; $[\alpha]_D^{25}$ + 104.5° in ethanol [c, 0.1]; and λ_{max}^{EtOH} 241 mμ [ε 15,900 M^{-1} cm^{-1}]). Progesterone (lot 42445, Calbiochem) was recrystallized from ethanol (mp 129–130° [α form]; $[\alpha]_D^{25}$ + 180° in dioxane [c, 0.02]; and λ_{max}^{EtOH} 241 mμ [ε 16,600 M^{-1} cm^{-1}]). The chromatographic behavior of purified progesterone and testosterone on gas-liquid and thin-layer chromatography, and paper (Bush A) was compared with chromatographically homogeneous steroids from Calbiochem. Other steroids were used in one experiment without purification, and the commercial sources are indicated in Table I. Cofactors were purchased from P-L Biochemicals, Inc. (Milwaukee, Wis.). Labeled compounds were purchased from New England Nuclear Corp. (Boston, Mass.). Samples of crystalline human chorionic gonadotrophin (HCG) were generous gifts from E. R. Squibb and Sons (lot 90402) and Ayerst Laboratories, Inc. (lot K-28853).

Results

Effect of Various Steroids. Progesterone and testosterone added *in vitro* significantly inhibited the demethylation of lanosterol by homogenates of rat testicular tissue (Table I). Compounds with Δ^5-3β-hydroxy configuration (pregnenolone; 17α-hydroxypregnenolone) were less active inhibitors. Intraperitoneal injection of rats with HCG during the week before incubation stimulated the demethylation (Table I; Ying *et al.*, 1965). Addition of steroids *in vitro* decreased the rate of demethylation by homogenates of testicular tissue from HCG-injected rats. Testosterone and progesterone were the most active inhibitors of either preparation of tissue.

Increasing concentrations of progesterone or testosterone alone resulted in increasing inhibition (Table II, part A). Concentrations of steroid that produced 50% inhibition were between 0.24 and 0.34 mM for both compounds. The effect of concentration on the rate of demethylation was similar for each compound. In part B of Table II, the first sample of homogenate was incubated with 500 μg of progesterone per flask (0.354 mM). In the second sample, 100 μg of testosterone was substituted for 100 μg of progesterone, and increasing 100-μg amounts of testosterone were sub-

TABLE II: Effect of Concentration of Progesterone and Testosterone.

A[a]				B[a]		
Progesterone (mM)	(%)[b]	Testosterone (mM)	(%)[b]	Progesterone + Testosterone (mM)	(mM)	(%)[b]
0.065	17	0.065	17	0.354	0	57
0.130	43	0.130	35	0.283	0.078	55
0.320	67	0.320	65	0.212	0.155	60
0.650	81	0.650	80	0.141	0.232	62
				0.070	0.309	64
				0	0.386	62

[a] The concentration of substrate was 8.9×10^{-6} M. Each value is the average of four to eight samples. [b] % inhibition.

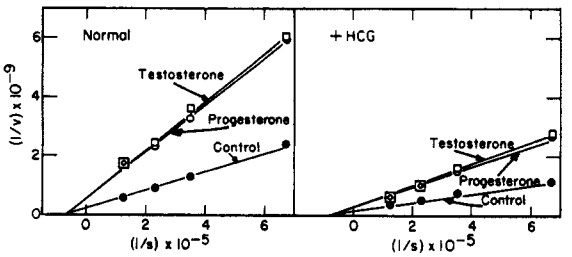

FIGURE 1: Noncompetitive inhibition by steroids. Normal homogenate of testicular tissue (4 ml) was incubated aerobically with 8–40 mμmoles of labeled lanosterol and DPN (1.5 μmoles) in a final volume of 4.5 ml. Testosterone or progesterone (0.38 mM) was added in ethanol solution (0.1 ml) to the experimental samples; 0.1 ml ethanol was added to the control samples. The figure indicates reciprocal rates plotted conventionally for extrapolation. Other groups of rats were injected three times during the week before incubation with a solution of HCG (200 IU/injection). Each point is the average of results from three separate experiments.

stituted similarly for equal weights of progesterone in the remaining samples. The extent of inhibition was essentially constant in all samples. The total concentration of inhibitor increased slightly because of the somewhat lower molecular weight of testosterone than of progesterone.

Type of Inhibition. Increasing concentrations of substrate from 8 to 40 mμmoles/flask were incubated with homogenate of normal rat testicular tissue. Similar concentrations of substrate were incubated with 0.38 mM progesterone or testosterone. Inhibition by the steroid was noncompetitive (Figure 1). The constants for normal and inhibited systems were: K_m, 1.4×10^{-5} M; K_i', 2.8×10^{-4} M; and V_{max}, 4×10^{-9} mole/hour per 100 mg protein.[3] Identical effects were observed with progesterone and testosterone. Rats were injected with HCG, and the testicular tissue was incubated with labeled lanosterol and progesterone or testosterone. Noncompetitive inhibition was observed: K_m, 1.4×10^{-5} M; K_i', 2.1×10^{-4} M; and V_{max}, 10×10^{-9} mole/hour per 100 mg protein.

Homogenate of rat testicular tissue (from 10,000 × *g*, 20 minutes) was centrifuged at 105,000 × *g* for 1 hour. The volume of microsomes was diluted to 25% of the volume of the original homogenate. Microsomes and soluble fraction were mixed with ethanol (control) or a solution of progesterone in ethanol (500 μg progesterone per ml microsomal suspension and 500 μg progesterone per 4 ml supernatant fraction). The microsomal fraction was centrifuged, suspended again and centrifuged, and diluted to 25% of the original volume. The supernatant fraction was dialyzed for 3 hours against 100 volumes of 0.1 M phosphate buffer.

[3] K_i' = apparent inhibitor constant.

Incubation of control supernatant fraction and control microsomal fraction with labeled lanosterol yielded maximal rates of demethylation (Table III). Treatment of either soluble enzymes or microsomes with progesterone resulted in inhibition when the sample was incubated with the other untreated fraction. No additional inhibition was observed with the mixture of

TABLE III: Effect of Treatment of Microsomes and Supernatant Fraction with Progesterone.

		Lanosterol Demethylated	
		Supernatant Fraction	
Expt.	Microsomes[a]	Control (%)	Progesterone (%)
1[b]	Control	100	80
	Progesterone	65	61
2[c]	Control	100	
	Sephadex	95	
	Progesterone	60	
	Progesterone + Sephadex	82	

[a] Microsomes were collected by centrifugation at 105,000 × *g* for 1 hour. [b] The microsomes and supernatant fraction were exposed to progesterone separately as described in the text. Samples of 1 ml microsomes and 3 ml supernatant fraction were incubated. The results are the average of three experiments. [c] Control supernatant fraction was incubated with microsomes from the various treatments described in the text. The results are the average of two experiments. The concentration of microsomes was adjusted to equal amounts by dilution.

both progesterone-treated enzyme fractions over that with progesterone treatment of only the microsomal fraction. When the enzyme fractions were mixed with similar concentrations of [4-^{14}C]progesterone, 50–65% and 60–85% of the labeled progesterone remained in the microsomes and supernatant fraction, respectively, after washing or dialysis.

Washed rat testicular microsomes (1.5 mg protein) were incubated with 250 μg of [4-^{14}C]progesterone for 15 minutes at 4°. The solution was transferred quantitatively to a column of Sephadex G-200 that was packed and equilibrated with water. Protein (microsomes) was separated from most of the labeled steroid (Figure 2).

Incubation of untreated microsomes with control supernatant fraction yielded maximal rates of demethylation (Table III, expt 2). Treatment of microsomes with ethanol and filtration through Sephadex G-200 decreased the activity to 95%. Treatment of the microsomes with progesterone depressed the rate of

demethylation. Filtration through Sephadex G-200 of the progesterone-treated microsomes (approx fractions 3–8, Figure 2) resulted in restoration of activity to within 13% of the normal Sephadex-treated microsomes.

The reversibility of inhibition was verified with other procedures. Mixtures of homogenate of guinea pig testicular tissue and ethanol (control) or progesterone (500 μg per each 4 ml of homogenate) in ethanol solution were maintained at 4°. After intervals of 0, 20, 40, 80, and 120 minutes, samples of homogenate (4 ml) from each treatment were incubated for 15 minutes with 40 mμmoles of labeled lanosterol. The ratio of demethylation activity in the progesterone-treated samples to that in similarly aged control samples remained constant regardless of the length of preliminary exposure of enzyme and progesterone. In previous studies homogenate and steroid inhibitor were mixed before the substrate was added. Other orders of mixing were investigated: enzyme, substrate, inhibitor; and inhibitor, substrate, enzyme. Identical kinetic values for noncompetitive inhibition were observed as reported in Figure 1.

Mixtures of microsomes that were washed three times with buffer and supernatant fraction that was precipitated between 30 and 60% saturated solution of ammonium sulfate and dialyzed against buffer were incubated without and with progesterone. The values for K_m and K_i' were identical with results of incubation of crude homogenates (10,000 × *g*) that were used in these studies. The partial purifications affected neither the activity of the demethylation nor the inhibition by steroid.

4,4-Dimethyl-5α-cholest-7-en-3β-ol was prepared from [^{14}C]methyl iodide by the method of Gautschi and Bloch (1958). The labeled sterol (800 mμmoles, 28,000 dpm) or labeled lanosterol was incubated aerobically with 16 ml of rat liver homogenate that contained 5 mM sodium arsenite (Gaylor, 1964). The homogenate was saponified, and the partially hydroxylated sterol was extracted. The solvent was evaporated, and the residue was dissolved in buffer with the aid of Tween 80. Samples were transferred to center-well flasks and incubated anaerobically for 15 minutes or 1 hour with 4 ml of fresh homogenate from guinea pig testicular tissue. Flasks contained alcohol (control) or 200 or 400 μg testosterone in alcohol solution. Less than 32 and 58% inhibition of the anaerobic release of labeled carbon dioxide was observed with the two concentrations of testosterone. Comparable concentrations of testosterone yielded approximately 40 and 65% inhibition of the overall aerobic demethylation (Table II).

Physiological Studies. The effect of various concentrations of progesterone on K_i' was studied. Homogenate of rat testicular tissue was incubated with 8–100 mμmoles of lanosterol and either 0.14 or 0.28 mM progesterone. The calculated K_i' was somewhat smaller for the higher concentration (Table IV). In addition, a further decrease in the value of K_i' was observed with 0.38 mM progesterone.

Progesterone or testosterone (1 mg) in ethanol solution was injected intraperitoneally into rats. Testicular tissue from the rats was removed after 2 hours, and the rate of demethylation was determined. Injection of progesterone did not result in a significantly slower rate of demethylation (Table V). However, injection of testosterone significantly inhibited the rate of demethylation *in vitro*.

Homogenates of rat liver were prepared as described previously (Gaylor, 1964). Three concentrations of progesterone, testosterone, and cortisone were added to the homogenate. The effect of progesterone and testosterone on testicular tissue is reported for comparison (Table VI). Concentrations of steroid that yielded 70–80% inhibition of lanosterol demethylation by homogenates of testicular tissue affected the rate of demethylation by liver homogenates only slightly. A 3-fold increase in concentration was required to yield

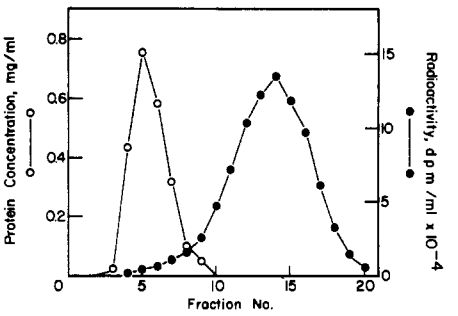

FIGURE 2: Protein content and radioactivity of washed microsomes. Washed microsomes from rat testicular tissue (1.5 mg protein) were incubated for 15 minutes at 4° with 250 μg of [4-^{14}C]progesterone (0.5 μc) in a final volume of 0.5 ml. The microsomes were collected by centrifugation and transferred (in 0.5 ml) to a column of Sephadex G-200 (200 mg) that was packed with water. The column was developed with water. The flow was 0.33 ml/min. Fractions of approximately 0.5 ml were collected. Protein was determined by the method of Lowry *et al.* (1951) and samples of the tube contents were assayed for radioactivity directly according to the procedure of Bray (1960).

TABLE IV: Effect of Progesterone Concentration on K_i'.

Concentration of Progesterone (mM)	K_i' (M × 10^4)	K_m (M × 10^5)
0.14	4.2	1.63
0.28	3.9	1.63
0.38[a]	2.8	1.4
0.38[b]	2.1	1.4

[a] From Figure 1, normal rats. [b] From Figure 1, HCG-injected rats.

TABLE V: Effect of Injected Testosterone and Progesterone on the Rate of Demethylation by Homogenates of Testicular Tissue.

	Lanosterol Demethylated[a] (mμmoles/hr per 100 mg protein)
Control	2.31 ± 0.11[b]
Progesterone	2.09 ± 0.13
Testosterone	1.61 ± 0.04

[a] Testosterone or progesterone (1 mg) in ethanol solution was injected into adult rats (approx 200 g) 2 hours before killing. Controls received the same volume of ethanol. Homogenates of testicular tissue from nine rats in each treatment were prepared separately and incubated with labeled lanosterol (40 mμmoles/flask). [b] Standard error of the mean.

TABLE VI: Effect of Steroids on the Demethylation of Lanosterol by Homogenates of Rat Liver.

Steroid Added	Concentration (mM)	Inhibition: Liver[a] (%)	Inhibition: Testicular Tissue[b] (%)
Progesterone	0.4	10	70
	0.8	55	
	1.2	84	
Testosterone	0.4	6	83
	0.8	51	
	1.2	80	
Cortisone	0.4	12	
	0.8	45	
	1.2	66	

[a] Homogenates of rat liver (4 ml) were incubated with DPN (1.5 μmoles) and 200 mμmoles of labeled lanosterol (63 dpm/mμmole). The results are reported as per cent inhibition from comparable controls (ethanol only). The results are the average of two values from separate experiments. [b] The approximate effect of steroids on the demethylation in testicular tissue is reported for comparison (see Table I). The concentration of protein was lower in homogenates of testicular tissue (60–85 mg/flask) than in liver (123–184 mg/flask).

similar extents of inhibition with homogenates of liver.

Discussion

The rate of demethylation of lanosterol by homogenates of rat testicular tissue is inhibited significantly by progesterone, testosterone, and related steroids. Δ^4-3-Ketosteroids appear to be more active, but extensive investigation will be required to establish structural requirements for maximal inhibitory activity.

Similar values for K_i' were observed by varying either the concentration of steroid (Table II) or the concentration of substrate (Figure 1; Table IV). Mean values of K_i' for noncompetitive inhibition were between 0.21 and 0.42 mM for both progesterone and testosterone by the two experimental procedures. Equal molar substitution of testosterone for progesterone did not alter the extent of inhibition. These observations suggest that the different steroids have similar effects on the enzymes.

The inhibition appeared to be both noncompetitive (Figure 1) and reversed by removal of the inhibitor (Table III). In addition, the reversibility was apparent in that extended exposure of the enzyme and inhibitor did not lead to additional inhibition. The order of mixing substrate, enzyme, and inhibitor affected neither the type of inhibition nor the kinetic values.

The effect of steroid inhibition apparently was associated more strongly with the microsomal fraction than with the supernatant fraction of testicular tissue homogenate. The similar effect of testosterone on the anaerobic release of $^{14}CO_2$ from the partially hydroxylated products from arsenite incubation and the effect on the overall process suggests that dehydrogenation may be inhibited more than hydroxylation. Others have demonstrated that end-product steroids may affect the rates of both hydroxylation (Sharma *et al.*, 1963; Sharma and Dorfman, 1964; Koritz and Hall, 1964) and dehydrogenation (Kowal *et al.*, 1964).

These results suggest a self-regulatory control of cholesterol synthesis. A strong self-regulation of cholesterol formation within testicular tissue may be particularly significant in the overall process of testicular steroid hormone biosynthesis. Morris and Chaikoff (1959) have demonstrated that most testicular cholesterol is formed *in situ*. Gerson *et al.* (1964) reported recently that injected β-sitosterol did not alter either the amount or the rate of biosynthesis of cholesterol in testicular tissue, whereas liver tissue was affected. This study lends further support to the evidence that testicular cholesterol is synthetized *in situ*. Accordingly, control of cholesterol formation *in situ* by feedback inhibition may have a direct effect upon the availability of this substrate for subsequent conversion into steroid hormones.

Gonadotrophin affects the kinetics of the demethylation reaction in a manner that suggests the removal of a noncompetitive inhibitor (Figure 1; Ying *et al.*, 1965), and end-product steroids produce noncompetitive inhibition. The decrease of K_i' (apparent dissociation constant of EI) with higher concentrations of inhibitor (Table IV) also may be ascribed to an endogenous steroid inhibitor that competes with the added in-

hibitor. In addition, the lowest value of K_i' was obtained from incubation of homogenate from HCG-injected rats (Table IV). Accordingly, the trophic hormone may act by increasing the rate of removal of the endogenous steroid from the enzymes. This mechanism was suggested recently by Koritz and Hall (1964) as a possible action of ACTH on enzymes that catalyze the conversion of cholesterol to pregnenolone in adrenal tissue. Direct spectrophotometric assay of the concentration of endogenous steroid has not been successful.

Inhibition does not indicate that the steroid is either a testicular metabolite or a normal precursor of testosterone. Indeed, feedback inhibition has been observed by end products of an alternate pathway (Sturani *et al.*, 1963).

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