Steroid 7α-Hydroxylase of Rat Testes*

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ABSTRACT: Previous identification of 7α-hydroxyandrostenedione as a testicular metabolite of androstenedione suggested the presence of 7α-hydroxylase activity in the testicular tissue of rat.

Reported in this communication are inter- and intracellular distribution of this enzyme, substrate specificity, pH and temperature dependence of the enzyme activity, and the effects of certain inhibitors on this enzyme. The specific activity per protein of the 7α-hydroxylase in the locally X-ray-irradiated testes of rat was found higher than in the intact testes, indicating the localization of the 7α-hydroxylase activity in the relatively radioresistant cells of the testicular tissue, or most likely the interstitial cells which contained the enzyme systems related to testosterone formation from pregnenolone. Intracellularly the enzyme activity was concentrated in the microsomal fraction (10,000–105,000*g* precipitate), and furthermore, was found to be relatively concentrated in the smooth-surfaced microsomal fraction. Among C-19 steroids such as androstenedione, testosterone, and dehydroepiandrosterone, the testicular 7α-hydroxylase exhibited a substrate preference for androstenedione. The optimal temperature and pH for the 7α-hydroxylase were 40° and pH 7.0, being different from the 17β-hydroxysteroid dehydrogenase which shared androstenedione as the common substrate. In the time-course study of the transformation of androstenedione with the testicular microsomal fraction, exponential consumption of the substrate was observed, and amount of 7α-hydroxyandrostenedione increased and reached a plateau 20 min after incubation, while testosterone was produced continuously during the elapsed time of incubation up to the 4th hr. As an atmospheric carbon monoxide was found inhibitory upon the 7α-hydroxylase activity, the cytochrome P-450 was demonstrated to be involved in the testicular 7α-hydroxylation system. The 7α-hydroxylase activity in the state of microsomal fraction was inhibited *in vitro* by addition of the following compounds to the incubation medium: sodium *p*-chloromercuribenzoate, disodium ethylenediaminetetraacetate, 3-(chloro-3-methyl-2-indenyl)pyridine, 3-(1,2,3,4-tetrahydro-4-oxo-7-chloro-2-naphthyl)pyridine, 2-methyl-1,2-dipyridyl-(3)-1-oxopropane, 3,3-bis(*p*-aminophenyl)-2-butane dihydrochloride, and 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride. The findings obtained in this study on testicular 7α-hydroxylase were compared to those on hepatic 7α-hydroxylase.

When androstenedione[1] was incubated in the presence of NADPH with the rat testicular microsomal fraction under an aerobic condition, two metabolites other than testosterone were isolated and identified, respectively, as 7α-hydroxyandrostenedione and 7α-hydroxytestosterone (Inano *et al.*, 1970b). The existence of 7α-hydroxylase activity in the rat testicular gland was thus demonstrated for the first time. Until the present time, a steroid-7α-hydroxylase system which converted dehydroepiandrosterone (Šulcová and Stárka, 1968) and testosterone (Conney *et al.*, 1969) was reported to be localized in the hepatic microsomal fraction.

In this paper, intercellular and intracellular distribution of the testicular 7α-hydroxylase activity was examined. Enzyme characteristics of the 7α-hydroxylase were studied with regard to the optimal pH and temperature, substrate specificity, and incubation time. Finally, the mode of inhibition with several compounds *in vitro* upon the 7α-hydroxylase activity was investigated.

Materials and Methods

Subcellular Fractionation. After male rats of the Wistar strain (3-months old) had been decapitated, the testicular glands were isolated and decapsulated. Pooled testes were weighed and homogenized with a loose-fitting glass-Teflon homogenizer in an ice-cold 0.25 M sucrose solution (pH 7.4). Subcellular fractions were obtained by sequential centrifugation of the homogenates, at 800*g* for 20 min (nuclei and cell debris fraction), at 10,000*g* for 20 min (mitochondrial fraction), and at 105,000*g* for 60 min (microsomal fraction). The mitochondrial and microsomal fractions were respectively resuspended in 0.25 M sucrose solution. A portion of 105,000*g* supernatant fluid was heated in a boiling-water bath for 10 min and then centrifuged at 10,000*g* for 20 min to remove the precipitate. This supernatant fluid was used as the heated supernatant fluid.

Following the method of subfractionating the hepatic microsomal fraction by Dallner *et al.* (1963), 4 ml of the testicular microsomal fraction containing 15 mM CsCl was placed in a layer on the top of 6.5 ml of 1.30 M sucrose solution containing 15 mM CsCl in a centrifuge tube. After centrifugation at 250,000*g* (Ultracentrifuge, Model B-60 with A-321 rotor, International Equipment Co., Needham Height, Mass.) for 60 min, a compact pellet was obtained at the bottom of the tube, and hereafter, will be designated as rough-

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[1] The following trivial names were used in the text: androstenedione, androst-4-ene-3,17-dione; 7α-hydroxyandrostenedione, 7α-hydroxyandrost-4-ene-3,17-dione; testosterone, 17β-hydroxyandrost-4-en-3-one; 7α-hydroxytestosterone, 7α,17β-dihydroxyandrost-4-en-3-one; dehydroepiandrosterone, 3β-hydroxyandrost-5-en-17-one; 7α-hydroxydehydroepiandrosterone, 3β,7α-dihydroxyandrost-5-en-17-one; cholesterol, cholest-5-en-3β-ol; 7α-hydroxycholesterol, cholest-5-ene-3β,7α-diol; pregnenolone, 3β-hydroxypregn-5-ene-3,20-dione; progesterone, pregn-4-ene-3,20-dione; 17α-hydroxyprogesterone, 17α-hydroxypregn-4-ene-3,20-dione; Metopyrone, 2-methyl-1,2-dipyridyl-(3)-1-oxopropane; SU-8000, 3-(chloro-3-methyl-2-indenyl)pyridine; SU-10603, 3-(1,2,3,4-tetrahydro-4-oxo-7-chloro-2-naphthyl)pyridine; Amphenone B, 3,3-bis(*p*-aminophenyl)-2-butane dihydrochloride; SKF-525A, 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride; EDTA, disodium ethylenediaminetetraacetate; and *p*-CMB, sodium *p*-chloromercuribenzoate.

surfaced microsomes. A fluffy layer was found near the position of the original boundary between the two sucrose solutions and will be designated as smooth-surfaced microsomes. Each subfraction was suspended in 0.25 M sucrose solution, and precipitated at 105,000*g* for 60 min. The two submicrosomal fractions together with the total microsomal fraction were subjected to the electron microscopic examination and as reported previously, it was revealed that smooth-surfaced microsomal fraction consists mostly agranular membrane structures without ribosomal particles on outer surfaces, and the rough-surfaced microsomal fraction consists granular membrane structure with ribosomes (Inano *et al.*, 1970a).

Steroids. 4-^{14}C-Labeled androstenedione (56.6 mCi/mmole) and testosterone (58.2 mCi/mmole) were purchased from the Radiochemical Centre, Amersham, England, and [4-^{14}C]-dehydroepiandrosterone (42.0 mCi/mmole) from New England Nuclear Corp. Boston, Mass. The purities of the radioactive steroids were confirmed by thin-layer chromatography immediately before use. These were diluted appropriately with the corresponding nonradioactive preparation before incubation, and then added to incubation system to insure saturation of the substrate to the enzyme system. Authentic 7α-hydroxyandrostenedione was kindly supplied by Dr. S. Kraychy, Searle Co., Chicago, Ill., and by Dr. D. H. Peterson and Dr. P. W. O'Connell, Upjohn Co., Kalamazoo, Mich.

Incubation. After the radioactive steroid solutions in volatile organic solvents as they were supplied were diluted with the same solvents, appropriate amount of the solution was transferred to the incubation flask. Two drops of propylene glycol per flask were added and then the solvent was removed under reduced pressure, shortly before the incubation. Incubation mixture generally consisted the substrate steroid (70–350 nmoles, 3.5–10 $\times$ 10^4 cpm), enzyme preparation, and NADPH (final concentration 240 μM, Boehringer und Soehne, Mannheim, Germany) in 0.25 M sucrose solution buffered as pH 7.4 with Tris (final concentration 10 mM) with HCl, and its final volume was adjusted as 5 ml. The mixture was incubated at 37° for 60 min in an atmosphere of a mixture of oxygen and carbon dioxide (95:5), unless otherwise mentioned. More detailed condition for incubation is stated later together with the corresponding results obtained.

Extraction and Isolation of the Metabolites. Immediately after incubation, enzyme reactions were arrested by addition of 15 ml of methylene dichloride and the mixture was vigorously shaken to extract the steroids. The methylene dichloride layer was collected by centrifugation of the mixture at 400*g* for 10 min. This extraction procedure was repeated two more times, extracts being pooled. The extracts were dried with anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure. At this stage, 90–98% of the radioactivity initially present was recovered into the extract. An aliquot of the radioactive steroid extract was chromatographed on a thin layer coated with a mixture of silica gel G and GF (4:1, wt/wt) (Merck A. G., Darmstadt, Germany), using a benzene–acetone (4:1, v/v) solvent system at 15–20°. After development of thin-layer plate, the spots of the carrier steroids on the chromatogram were detected by ultraviolet light (wavelength, 253 mμ). Radioactive spots were detected by an autoradiographic method by exposing X-ray film to the chromatogram for one week, and also by a windowless gas-flow counter with a scanning device (Vanguard Autoscanner 880 and 885, T. M. C., North Haven, Conn.). The detected spots of steroids were scraped off from the thin-layer plate, packed into a small glass column and steroids were eluted with a mixture of chloroform–ethyl alcohol (1:1, v/v) from the absorbent.

Measurement of Testicular 7α-Hydroxylase and 17β-Hydroxysteroid Dehydrogenase Activities. Activity of 7α-hydroxylase in the incubation mixture was defined as the amount of 7α-hydroxyandrostenedione obtained from the substrate for 60 min, unless otherwise stated. The 17β-hydroxysteroid dehydrogenase activity was expressed as the yield of the testosterone obtained for 60 min from the substrate androstenedione, and in a specific case, was expressed as the yield of androstenedione derived from the substrate testosterone for 60 min. All enzyme activities were expressed in terms of per mg of protein or per gland basis.

Quantitation of Radioactivities of the Products. Suitable aliquots of the extract were evaporated to dryness in 20-ml glass vials (Wheaton Glass Co. Millville, N. J.), and dissolved in 11 ml of a toluene solution containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene. The radioactivity was then measured with a liquid scintillation spectrometer (System 725, Nuclear-Chicago, Des Plaines, Ill.) for a sufficient time to reduce the counting error below 5%. Efficiency of ^{14}C counting was about 70%. The amounts of the products were estimated in nmoles, by dividing the recovered radioactivities by the specific activities of the substrate steroid.

Measurement of Protein. Protein concentrations in the enzyme preparations were determined by the copper–Folin method at 750 mμ, after diluting the samples to suitable concentration (Lowry *et al.*, 1951).

Results

Intercellular Distribution of Testicular 7α-Hydroxylase. Testes of adult 2-months old rats were irradiated locally with X-rays at total dose of 1000 rads by a X-ray generator (Shimadzu Co., 200 kVp, 20 mA filtered through 0.5-mm Cu and 0.5-mm Al; the dose rate, 95 rads/min). The following observations were made 1 month after the irradiation. Histological examination of a part of the testicular tissue revealed that, among the testicular cells, the cells related to spermatogenesis were severely damaged, while the interstitial cells and Sertoli cells remained relatively unaffected. Table I lists the 7α-hydroxylase and 17β-hydroxysteroid dehydrogenase activities as well as the weights of the testes and sexual accessory organs of normal and X-irradiated rats. When [4-^{14}C]androstenedione was incubated with the testicular microsomal fraction of normal and X-ray irradiated rats in the presence of NADPH, the specific activity per mg protein of 7α-hydroxylase in the X-irradiated testes was found higher than that of the intact testes. Similar tendency to the above was observed also of the 17β-hydroxysteroid dehydrogenase activity.

Intracellular Distribution of the 7α-Hydroxylase Activity in Rat Testes. [4-^{14}C]Androstenedione was incubated, respectively, with subcellular fraction of testicular tissue, equivalent to one animal. As shown in Table II, the 7α-hydroxylase activity in the testicular gland was concentrated in the microsomal fraction, when the enzyme activity was expressed on the basis of both organ and unit weight of protein. In the mitochondrial fraction, the specific activity of the 7α-hydroxylase was found to be one-half of the activity found in the microsomal fraction, while no significant activity of the enzyme was observed in the 105,000*g* supernatant fluid. The addition of the 105,000*g* supernatant fluid to the microsomal fraction

TABLE I: 7α-Hydroxylase and 17β-Hydroxysteroid Dehydrogenase Activities in Normal and X-Irradiated Rat Testes.[a]

Group	No. of Animals	Testes (g)	Seminal Vesicles (mg)	Ventral Prostates (mg)	7α-Hydroxylase: nmole of Product/mg of Protein	7α-Hydroxylase: nmoles of Product/Organ	17β-Hydroxysteroid Dehydrogenase: nmole of Product/mg of Protein	17β-Hydroxysteroid Dehydrogenase: nmoles of Product/Organ
Intact	15	2.67 ± 0.06	947 ± 52	378 ± 26	0.38	6.87	0.44	7.99
X-irradiated	15	1.09 ± 0.10	970 ± 48	353 ± 25	0.61	3.81	0.56	3.56
Difference by t test		$p < 0.01$	NS[b]	NS[b]				

[a] [4-^{14}C]Androstenedione (175 nmoles, 4.4×10^4 cpm) was incubated, respectively, with the microsomal fractions of the intact (36.5 mg of protein) and X-ray irradiated (12.7 mg of protein) testis for 60 min in the presence of NADPH (240 μM). [b] Not significant.

did not enhance the formation of 7α-hydroxylated androgens from androstenedione substantially.

Intramicrosomal Distribution of the 7α-Hydroxylase Activity. The testicular microsomal fraction was separated into two subfractions, or smooth-surfaced and rough-surfaced microsomes by the technique of sucrose density gradient centrifugation in the presence of CsCl. After incubation of [4-^{14}C]androstenedione with each submicrosomal fraction, the 7α-hydroxylated metabolites were determined quantitatively. As shown in Table III, the specific activity of the 7α-hydroxylase in the smooth-surfaced subfraction was 2.8 times that of the rough-surfaced subfraction. The sum of the enzyme activities measured separately for the two subfractions was about half of the activity of the total microsomal fraction prior to the submicrosomal separation. By adding the heated supernatant fluid which did not by itself retain the enzyme activity to the submicrosomal fractions, the apparent enzyme activities of the submicrosomal fraction were somewhat enhanced.

Influence of Atmospheric Carbon Monoxide upon the 7α-Hydroxylase Activity. When ^{14}C-labeled androstenedione was incubated with the testicular microsomal fraction in an atmosphere of a mixture of O_2–N_2 (20:80, v/v) or O_2–CO–N_2 (20:50:30, v/v) for 30 min, the production of the 7α-hydroxyandrostenedione in the CO-containing atmosphere was significantly smaller than in the atmosphere free from carbon

TABLE II: Intracellular Distribution of the 7α-Hydroxylase in Rat Testicular Tissue.[a]

Fraction	7α-Hydroxylase Activity: nmole of Product/mg of Protein	7α-Hydroxylase Activity: nmoles of Product/Organ
Mitochondrial fraction	0.39	0.53
Microsomal fraction	0.83	9.80
105,000*g* supernatant fluid	0.01	0.40
Mitochondrial fraction plus 105,000*g* supernatant fluid[c]	0.53[b]	0.66
Microsomal fraction plus 105,000*g* supernatant fluid[c]	0.96[b]	11.39

[a] [4-^{14}C]Androstenedione (70 nmoles, 8×10^4 cpm) was, respectively, incubated with the mitochondrial fraction (1.3 mg of protein), the microsomal fraction (12.0 mg of protein), and the 105,000*g* supernatant fluid (39.8 mg of protein) of rat testes in the presence of NADPH (240 μM) for 60 min. [b] These specific activities are expressed on the basis of milligram of protein of the mitochondrial or microsomal fraction alone, respectively. [c] Added amount of the 105,000*g* supernatant fluid was 79.6 mg of protein.

TABLE III: Intramicrosomal Distribution of 7α-Hydroxylase of Rat Testes.[a]

Fraction	7α-Hydroxylase Activity: nmoles of Product/mg of Protein	7α-Hydroxylase Activity: nmoles of Product/Organ
Total microsomal fraction	0.90	19.74
Smooth-surfaced microsomal fraction	2.65	7.42
Rough-surfaced microsomal fraction	0.96	0.44
Smooth-surfaced microsomal fraction plus heated 105,000*g* supernatant fluid	2.91[b]	8.15
Rough-surface microsomal fraction plus heated 105,000*g* supernatant fluid	1.91[b]	1.79

[a] [4-^{14}C]Androstenedione (70 nmoles, 5×10^4 cpm) was incubated, respectively, with total microsomal fraction (22 mg of protein), smooth-surfaced (2.8 mg of protein), and rough-surfaced microsomal fraction (1.5 mg of protein) in the presence of NADPH (240 μM) for 60 min. The protein content in the heated supernatant fluid was 12.3 mg/flask. [b] These activities are expressed on the basis of milligram of protein of smooth- or rough-surfaced microsomal fraction alone, respectively.

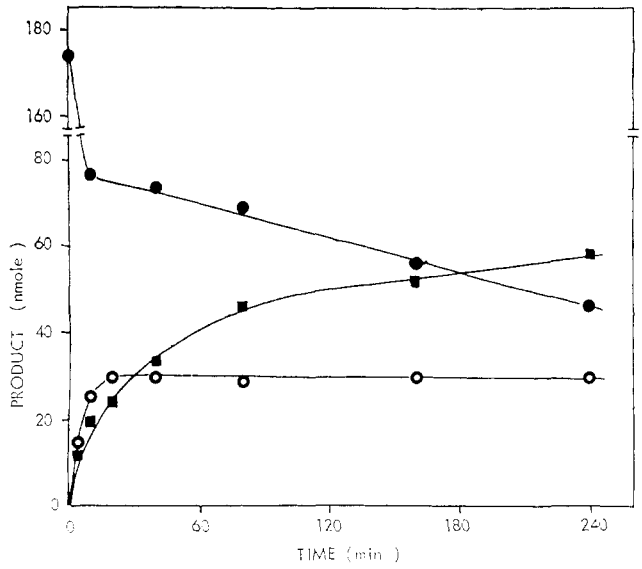

FIGURE 1: Time course of transformation of androstenedione with the testicular microsomal fraction. After [4-^{14}C]androstenedione (175 nmoles, 5.5 × 10^4 cpm) was incubated with the testicular microsomal fraction (32.5 mg of protein) in the presence of NADPH (240 μM), the amounts of androstenedione (●——●), 7α-hydroxyandrostenedione (○——○), and testosterone (■——■) isolated from the incubation flask are plotted against the time of incubation.

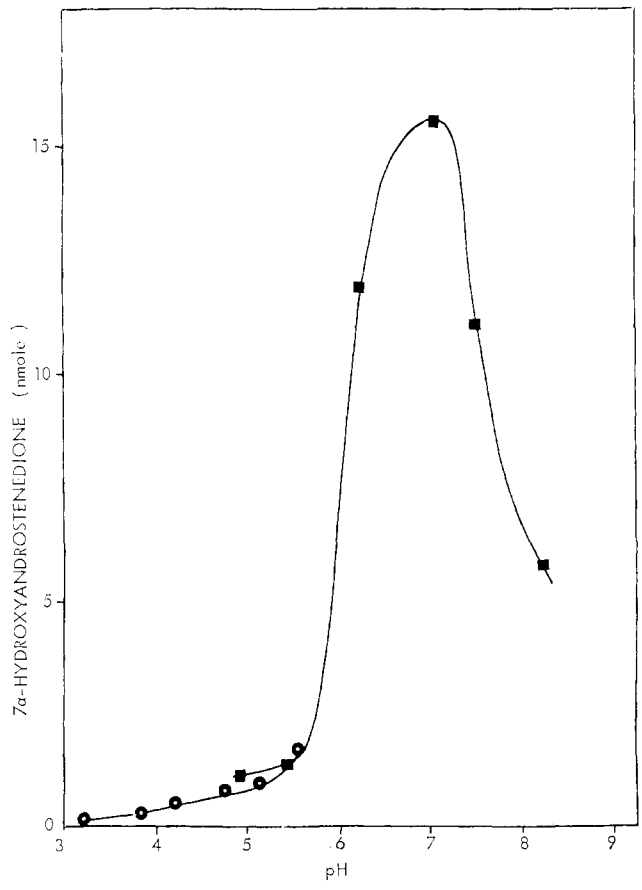

FIGURE 2: Influence of pH on the conversion of androstenedione to 7α-hydroxyandrostenedione. [4-^{14}C]Androstenedione (175 nmoles, 10 × 10^4 cpm) was incubated in the presence of NADPH (240 μM) with the testicular microsomal fraction (33.0 mg of protein) in the citric acid–Na_2HPO_4 buffer solution (○——○) and phosphate buffer solution (■——■) for 60 min at 37°.

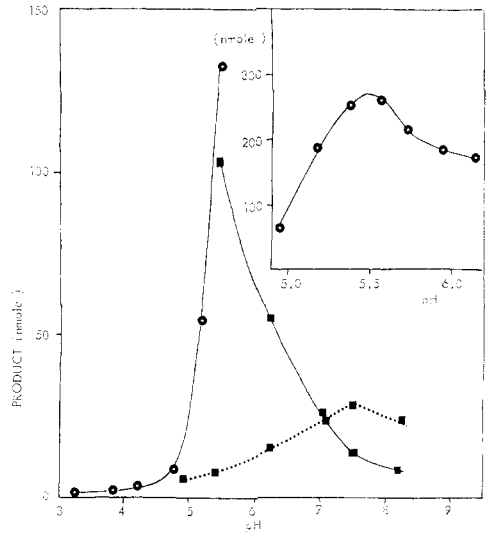

FIGURE 3: Influence of pH on oxidoreduction between androstenedione and testosterone. After [4-^{14}C]androstenedione (175 nmoles, 10 × 10^4 cpm) was incubated with the testicular microsomal fraction (33.0 mg of protein) in the following two buffer solutions for 60 min at 37°. The amounts of testosterone produced in citric acid–Na_2HPO_4 buffer (○——○) and phosphate buffer solution (0.067 M) (■——■) are shown. In the upper corner, amounts of the testosterone produced are indicated, when [4-^{14}C]androstenedione (350 nmoles, 10 × 10^4 cpm) was incubated with the enzyme preparation (18.6 mg of protein) in citric acid–Na_2HPO_4 buffer solution in air at 37° for 60 min. On the other hand, after [4-^{14}C]testosterone (174 nmoles, 5 × 10^4 cpm) was incubated with testicular microsomal fraction (33.0 mg of protein) in the phosphate buffer solutions (0.067 M) in the presence of NAD (272 μM) at 37° for 60 min, the amount of androstenedione (■·····■) obtained by oxidation of testosterone with 17β-hydroxysteroid dehydrogenase is plotted.

monoxide (Table IV). In contrast, the conversion of the substrate into testosterone was not influenced at all under this condition.

Substrate Specificity of the Testicular 7α-Hydroxylase. [4-^{14}C]Androstenedione, [4-^{14}C]testosterone, and [4-^{14}C]-dehydroepiandrosterone were incubated with testicular microsomal fraction. After incubation, 7α-hydroxylated metabolites were extracted from the incubation media, and acetylated with acetic anhydride and pyridine at room temperature overnight. 7α-Hydroxyandrostenedione 7α-monoacetate, 7α-hydroxytestosterone 7α,17β-diacetate, and 7α-hydroxydehydroepiandrosterone 3β,7α-diacetate were mutu-

TABLE IV: Influence of Atmospheric Carbon Monoxide upon the 7α-Hydroxylase.[a]

Gas Phase (%)			Metabolites (nmoles)	
O_2	CO	N_2	Testosterone	7α-Hydroxy-androstenedione
20	0	80	19.6	28.1
20	50	30	19.0	15.6

[a] [4-^{14}C]Androstenedione (105 nmoles, 3.5 × 10^4 cpm) was incubated with the testicular microsomal fraction (42.6 mg of protein) in the presence of NADPH (480 μM) for 30 min.

TABLE V: Apparent Substrate Specificity of Rat Testicular 7α-Hydroxylase.[a]

Metabolites (nmoles/Flask)	Substrate (nmoles/Flask) Androstenedione (175)	Testosterone (174)	Dehydroepiandrosterone (174)
Substrate recovered	115.2	140.0	9.4
Testosterone	23.6		18.6
Androstenedione		11.5	100.9
7α-Hydroxyandrostenedione	25.3[b]	2.5	12.8
7α-Hydroxytestosterone	0.7	12.8[b]	1.0
7α-Hydroxydehydroepiandrosterone			1.0[b]

[a] [4-^{14}C]Androstenedione (175 nmoles, 6.6 × 10^4 cpm), testosterone (174 nmoles, 9.9 × 10^4 cpm), and dehydropeiandrosterone (174 nmoles, 4.0 × 10^4 cpm) were incubated with the testicular microsomal fraction (30 mg of protein) in the presence of NADPH (240 μM) for 60 min. [b] Quantity of the direct product from each substrate by 7α-hydroxylase.

ally separated on the thin-layer chromatogram, using benzene–acetone (4:1, v/v) as the solvent system. As shown in Table V, androstenedione was converted into testosterone and 7α-hydroxyandrostenedione, while testosterone was transformed into androstenedione and 7α-hydroxytestosterone. With dehydroepiandrosterone, however, the substrate was mostly converted to androstenedione, and then to testosterone, but the production of 7α-hydroxydehydroepiandrosterone was very limited. So far as the amount of 7α-hydroxylated product directly derived from each substrate was concerned, 7α hydroxylation occurred with androstenedione to the greatest extent.

Time-Course Study on the Transformation of Androstenedione with the Testicular Microsomal Fraction. ^{14}C-Labeled androstenedione was incubated with testicular microsomal fraction in the presence of NADPH. During the incubation, the amounts of the substrate and the products were quantitatively analyzed at fixed time intervals. As shown in Figure 1, the substrate was consumed exponentially and the amount of testosterone produced still increased gradually even after 4 hr. On the other hand, formation of 7α-hydroxyandrostenedione reached a maximum 20 min after incubation, and then remained constant up to the end of experiment.

Influence of pH upon the Testicular Microsomal Conversion of Androgens. Androstenedione and testosterone were separately incubated with testicular microsomal fraction at various pH's at 37°, and the relationship of the amounts of the products to pH value was examined. As shown in Figure 2, the optimal pH for 7α hydroxylation of androstenedione at 37° was found to be approximately 7.0. The optimal pH for testosterone production from androstenedione was found to be 5.6, in contrast to the value (pH 7.5) for the reverse reaction from testosterone to androstenedione (Figure 3).

Influence of Temperature on the Androstenedione Transformation with Testicular Microsomal Fraction. As shown in Figure 4, 7α-hydroxyandrostenedione was produced in the highest yield at 40° (pH 7.4). On the other hand, the 17β-hydroxy-

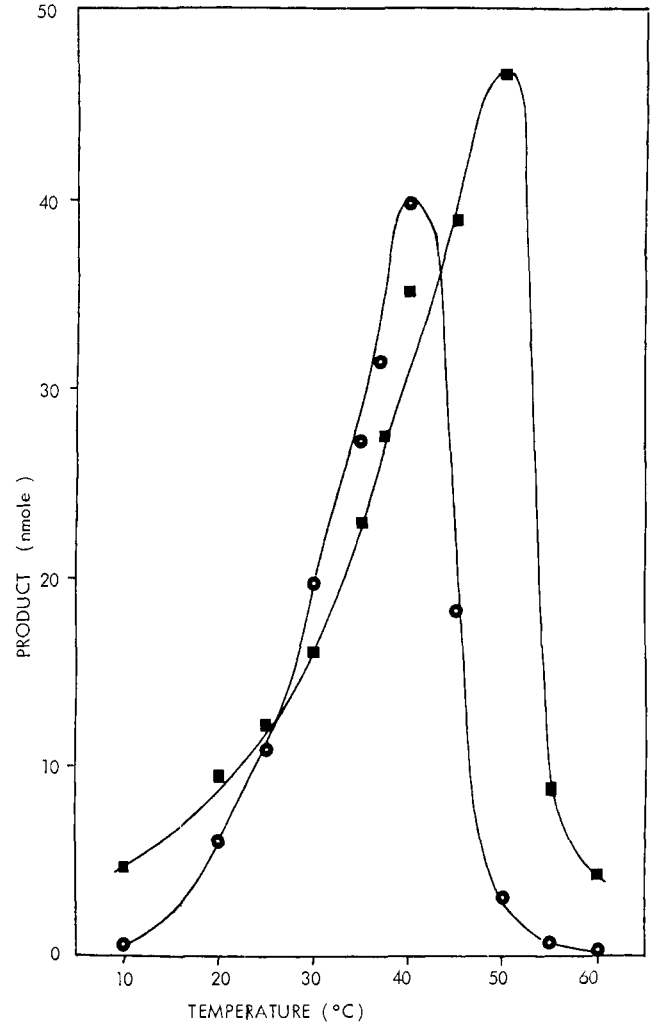

FIGURE 4: Influence of temperature on the testicular 7α-hydroxylase and 17β-hydroxysteroid dehydrogenase activities. The testicular microsomal fraction alone was warmed before the incubation to a fixed temperature for 2 min, and then [4-^{14}C]androstenedione (175 nmoles, 9.0 × 10^4 cpm) was incubated with the previously warmed testicular microsomal fraction (30.0 mg of protein) for 60 min. Testosterone (■——■) and 7α-hydroxyandrostenedione (○——○) obtained from androstenedione is shown at the various temperature.

steroid dehydrogenase which converts androstenedione to testosterone showed its optimal temperature at 50° (pH 7.4). At these temperature and pH, 7α hydroxylation of the substrate almost completely diminished.

Effects of Several Inhibitors upon the Testicular 7α-Hydroxylase Activity. As listed in Table VI, testicular 7α-hydroxylase was inihibited to various extents by chelating agents (α,α′-dipyridyl, EDTA, *o*-phenanthroline, and diethyldithiocarbamate), SH inhibitor (*p*-CMB), known steroid-hydroxylase inhibitors (Metopyrone, SU-8000, and SU-10603), and hydroxylase inhibitors (Amphenone B and SKF-525A).

Discussion

In the present experiment, the 7α-hydroxylase activity in rat testicular gland was demonstrated to be concentrated in the relatively radioresistant cells of the gland, or most likely in the interstitial cells which contained the enzyme systems involved in testosterone biosynthesis at least from preg-

TABLE VI: Effect of Inhibitors on the Testicular 7α-Hydroxylase Activity.[a]

Inhibitor	Final Concn (M)	Rate of Inhibn (%)
None		0.0
EDTA	1×10^{-3}	81.1
	1×10^{-4}	32.7
o-Phenanthroline	1×10^{-3}	15.9
	1×10^{-4}	26.9
α,α′-Dipyridyl	1×10^{-3}	20.2
	1×10^{-4}	9.0
Diethyldithiocarbamate	1×10^{-3}	9.3
	1×10^{-4}	10.5
p-CMB	1×10^{-3}	91.0
	1×10^{-4}	32.0
Metopyrone	1×10^{-3}	51.1
	1×10^{-4}	52.3
SU-8000	1×10^{-3}	82.9
	1×10^{-4}	37.2
SU-10603	1×10^{-3}	80.3
	1×10^{-4}	42.0
Amphenone B	5×10^{-3}	85.8
	5×10^{-4}	22.4
SKF-525A	5×10^{-3}	87.6
	5×10^{-4}	12.4

[a] [4-^{14}C]Androstenedione (175 nmoles, 6.6 × 10^4 cpm) was incubated with the rat testicular microsomal fraction (27.8 mg of protein) in the presence of NADPH (240 μM) and the inhibitors for 60 min.

nenolone (Inano and Tamaoki, 1968). And the testicular 7α-hydroxylase was localized in the microsomal fraction of the testicular tissue, being also similar to the intracellular distribution of the enzymes related to testosterone formation from pregnenolone in the testes of rat (Shikita and Tamaoki, 1965), guinea pig (Inano *et al.*, 1967), and patients with prostatic cancer (Murota *et al.*, 1966). By addition of the 105,000*g* supernatant fluid to the microsomal fraction, androgen formation from progesterone and 17α-hydroxyprogesterone was significantly enhanced (Shikita and Tamaoki, 1965), but the 7α-hydroxylase activity in the microsomal fraction was not increased significantly. The microsomal fraction of rat liver contained an enzyme system which hydroxylated dehydroepiandrosterone (Šulcová and Stárka, 1968) and pregnenolone (Stárka *et al.*, 1966) at the C-7 position. Distribution of the hepatic 7α-hydroxylase activities of rat between smooth- and rough-surfaced microsomal fractions was found about even (Heinrichs *et al.*, 1967). According to the present investigation, the microsomal 7α-hydroxylase activity was found to be enriched in the testicular smooth-surfaced fraction, in comparison with the activity in the rough-surfaced one. Other testicular enzyme activities related to testosterone production from pregnenolone and the cytochrome P-450 were reported to be concentrated also in the smooth-surfaced fraction (Inano *et al.*, 1970a). Concentration of the enzyme activities related to androgen production into the smooth-surfaced microsomes directly supports the cytological speculation by Christensen and Fawcett (1961) that, as agranular endoplasmic reticula were abundant in the testicular interstitial cells, testicular smooth-surfaced endoplasmic reticula would be the site of androgen biosynthesis. Under electron microscope, well-developed smooth-surfaced endoplasmic reticula had been observed in the other steroid-producing cells, such as adrenal cortex (Ross *et al.*, 1958) and corpus luteum (Enders and Lyons, 1964). In fact, the adrenal microsomal enzymes related to steroidogenesis were localized in the smooth-surfaced microsomal fraction (Inano *et al.*, 1969a,b).

The sum of the enzyme activities measured separately of each submicrosomal fraction per gland basis was lower than the activity of total microsomal fraction which was set aside before the separation procedure. A reason for such a significant difference may be that one or more components which were essential to display the enzyme activities were partially solubilized by treatment of the microsomal fraction with the hypertonic sucrose solution. As far as the 7α-hydroxylase was concerned, the solubilized and then lost principles were not complemented with the heated supernatant fluid at 105,000*g*. The activities of the 17α-hydroxylase, the C_{17}–C_{20}-lyase, and the 17β-hydroxysteroid dehydrogenase were, however, remarkably enhanced by addition of the heat-stable components in the soluble fraction (Inano *et al.*, 1970a).

The results of the study on the substrate specificity of the 7α-hydroxylase using the testicular microsomal fraction as the enzyme source (Table V) are complicated by the coexistence of the 17β-hydroxysteroid dehydrogenase, the Δ^5-3β-hydroxysteroid dehydrogenase and the Δ^5–Δ^4 isomerase in this subcellular fraction. The results given in Table V show the competitive consumption of the common substrate by two of the above enzymes. Despite of this complication, the 7α-hydroxylase appeared to have higher substrate specificity for androstenedione than for testosterone, since the consumption of these two substrates by the other enzymes was relatively small and did not lead to substantial decreases in the amounts of the substrates for the 7α-hydroxylase. The situation is different with dehydroepiandrosterone as the substrate. This steroid was mostly converted into androstenedione by the microsomal enzyme, whereas very limited amount of 7α-hydroxydehydroepiandrosterone was produced. Admitting that all the 7α-hydroxylated androstenedione and testosterone isolated would be derived from the 7α-hydroxydehydroepiandrosterone, the testicular 7α-hydroxylase was still concluded to prefer androstenedione to dehydroepiandrosterone.

It is possible to demonstrate the 17β-hydroxysteroid dehydrogenase activity alone as separated from the 7α-hydroxylase activity by setting the incubation condition at 50° and pH 7.4, where the latter enzyme activity contributes little to the consumption of substrate androstenedione (Figure 4). On the contrary, it has been impossible to demonstrate the 7α-hydroxylase activity without any influence by the other competitive enzymes present in the microsomal fraction. The activity of the known 7α-hydroxylase from hepatic microsomal fraction has not either been determined independently from testosterone 2β-, 6β-, and 16α-hydroxylases (Conney and Klutch, 1963; Lisboa *et al.*, 1968; Jacobson *et al.*, 1969).

The 7α-hydroxylase activity in the testicular microsomal fraction was inhibited by chelating agents, mostly EDTA. This result suggested that certain metal ions associated with the enzyme activity were removed from the enzyme by the chelating agent. The result obtained with diethyl dithiocarbamate which is a chelating agent for Ni and Cu ions requires particular attention. Šulcová and Stárka (1968) reported on the hepatic 7α-hydroxylase that a marked

increase in the production of 7α-hydroxydehydroepiandrosterone from dehydroepiandrosterone was caused by addition of 10^{-3} M sodium diethyldithiocarbamate. But in the present study, the activity of the testicular microsomal 7α-hydroxylase was not influenced by this chelating agent. Known inhibitors on steroid hydroxylases or SU compounds were also found to be inhibitory on the testicular 7α-hydroxylase. Amphenone B and SKF 525A which were competitively inhibitory upon rat testicular 17α-hydroxylase and C_{17}–C_{20}-lyase (Inano *et al.*, 1970a) was found to inhibit the 7α-hydroxylase activity. Hepatic 7α-hydroxylase activity in the homogenates was completely inhibited by Amphenone B (Stárka and Kůtová, 1962).

As both the cytochrome P-450 and the 7α-hydroxylase activity are distributed similarly within the cell structure and also within the microsomal fraction, the 7α-hydroxylase activity was suggested as involving the cytochrome in its enzyme system as the testicular 17α-hydroxylase did (Machino *et al.*, 1969; Inano *et al.*, 1970a). The inhibitory effect of carbon monoxide in the gas phase on the 7α-hydroxylase activity suggested that the reduced cytochrome P-450 reacted with CO to form an inactive complex of the pigment. The results of the previous experiment (Inano *et al.*, 1970b), in which 100% of gas phase for incubation was replaced with Ar or CO also support this conclusion; in the atmosphere of CO, the production of the 7α-hydroxylated steroids was reduced more severely than in the atmosphere of Ar, suggesting that 7α hydroxylation was reduced not only by the limited supply of molecular oxygen, but also by transformation of the cytochrome P-450 into the inactive CO complex. The metabolism of testosterone to 7α-hydroxytestosterone (Conney *et al.*, 1968) and of cholesterol to 7α-hydroxycholesterol (Wada *et al.*, 1969) by hepatic microsomal fraction were also inhibited by CO.

The role of the 7α-hydroxylase and its metabolite, 7α-hydroxyandrostenedione in connection to endocrinology are investigated, and will be reported elsewhere.

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