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Further Studies on 20α-Hydroxysteroid Dehydrogenase of Rat Testes*

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ABSTRACT: A 20α-hydroxysteroid dehydrogenase has been partially purified from rat testicular homogenates. This enzyme catalyzes the reduction of 17α-hydroxyprogesterone to 17α,20α-dihydroxypregn-4-en-3-one. The equilibrium of this reaction strongly favors the dihydroxy compound. Sulfhydryl compounds inhibit the enzyme while sodium ethylenediaminetetraacetate (EDTA-Na_2) stimulates it. The optimum temperature for the reduction reaction is between 30 and 42° with no sharp maximum, while the optimum pH for this reaction is about 7. The Michaelis constants were 6.3–6.7 $\times$ 10^{-5} M for 17α-hydroxyprogesterone and 2.0–2.5 $\times$ 10^{-5} M for reduced nicotinamide–adenine dinucleotide phosphate.

Soluble fractions of testicular tissue from several species of mammals contain a 20α-hydroxysteroid dehydrogenase which specifically catalyzes the reduction of the 20-carbonyl group of 17α-hydroxyprogesterone[1] (Shikita and Tamaoki, 1965). The substrate specificity of this testicular enzyme differs from that of the 20α-hydroxysteroid dehydrogenases present in hepatic (Recknagel, 1957), ovarian (Wiest, 1959), placental (Purdy *et al.*, 1964), and adrenal (Matthijssen *et al.*, 1964) tissues, and it is this unique specificity which prompted the present study.

Experimental Section

Tissue Preparation. Rats of the Wistar strain (age 2–3 months) were killed by a blow on the head. The testes were removed shortly thereafter, decapsulated, and homogenized in twice their weight of ice-cold 0.25 M sucrose. The homogenates were centrifuged by a conventional differential centrifugation method described elsewhere (Shikita and Tamaoki, 1965). The 105,000*g* supernatant solution was frozen and stored at −20° until sufficient material had been accumulated for the enzyme purification.

Assay of the Enzyme. 17α-Hydroxy[4−^{14}C]progesterone (10 μg, 30–50 $\times$ 10^3 cpm) in 1 ml of methylene dichloride was added to a 50-ml round-bottom centrifuge tube. Propylene glycol (2 drops) was added to this solution, and the methylene dichloride was removed first by a nitrogen jet at 40° and then under vacuum for 10 min at room temperature. Unless otherwise specified, 4 ml of 0.1 M Tris-Cl buffer (pH 7.4) containing 0.5 μmole of NADPH was added to each tube. Incubations were initiated by the addition of 1 ml of enzyme solution, and were preformed under aerobic conditions at 37° for 30 min with continuous shaking. The enzymatic reaction was stopped by the addition of 20 ml of methylene dichloride. After the organic phase was removed, the incubation mixture was extracted with two 20-ml portions of methylene dichloride. The 17α,20α-dihydroxy compound obtained by evaporating the combined extracts was purified by thin layer chromatography (silica gel G and GF) in a benzene–acetone (4:1, v/v) solvent system and detected by ultraviolet light. It was then extracted from silica gel with a mixture of ethyl alcohol and chloroform (1:1, v/v) and determined quantitatively by meas-

* From the National Institute of Radiological Sciences, Chibashi, Japan. *Received October 14, 1966.* This work was supported by Grant CA-07038 from the National Cancer Institute, U. S. Public Health Service, and presented in part at the 23rd International Congress of Physiological Sciences, Tokyo, Sept 1965.

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[1] The following are the systematic names for the steroids mentioned in the text: androstenedione, androst-4-ene-3,17-dione; 17α-hydroxyprogesterone, 17α-hydroxypregn-4-ene-3,20-dione; progesterone, pregn-4-ene-3,20-dione; $NADP^+$ and NADPH oxidized and reduced nicotinamide–adenine dinucleotide phosphates; NAD^+ and NADH, oxidized and reduced nicotinamide–adenine dinucleotides.

TABLE I: Reversibility of Reaction of Rat Testicular 20α-Hydroxysteroid Dehydrogenase.

Substrate	Cofactor	pH[a]	Steroid Recovered after Reaction (μg)	
			17α,20α-Diol	17α-Hydroxy-progesterone
17α,20α-Diol	$NADP^+$	7.4*	4.6	0.04
17α,20α-Diol	NAD^+	7.4	4.6	0.02
17α,20α-Diol	$NADP^+$	5.6**	4.5	0.02
17α,20α-Diol	$NADP^+$	6.5	4.5	0.05
17α,20α-Diol	$NADP^+$	7.4	4.4	0.14
17,α20α-Diol	$NADP^+$	8.5	4.3	0.22
17α,20α-Diol	NADPH	7.4*	4.4	0.02
17α,20α-Diol	NADH	7.4	4.4	0.02
17α-Hydroxyprogesterone	NADPH	7.4	1.9	2.6

[a] Substrate (5 μg) 17α-hydroxy [4-^{14}C] progesterone or 17α,20α-dihydroxy-[4-^{14}C]pregn-4-en-3-one (17α, 20α-diol) was incubated with 5 mg of enzyme for 30 min in 5 ml of 0.1 M Tris-Cl (*) or 0.1 M phosphate buffer (**) containing 0.5 μmole of the pyridine nucleotide cofactor as indicated in the table. Incubations with reduced form of the cofactors were carried out to show the recovery of the radioactive steroids by the method used in the present experiment.

uring its radioactivity with a liquid scintillation spectrometer (system 725, Nuclear-Chicago, Des Plaines, Ill.).

Protein Determination. Protein concentrations were determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin used as a standard without correction for moisture content.

Chemicals. 17α-Hydroxy[4-^{14}C]progesterone (43.8 μc/mg) was purchased from New England Nuclear Corp. (Boston, Mass.). 17α,20α-Dihydroxy[4-^{14}C]-pregn-4-en-3-one was prepared by incubating 17α-hydroxy[4-^{14}C]progesterone with the testicular 20α-hydroxysteroid dehydrogenase preparation. Identification and purification of this dihydroxy compound was described previously (Shikita and Tamaoki, 1965). Nonradioactive 17α,20α-dihydroxypregn-4-en-3-one was purchased from Zori (Tel Aviv, Israel) and then purified to remove the 20β epimer. $(NH_4)_2SO_4$ used for the enzyme purification was recrystallized shortly before use.

Results

Purification of the Enzyme. The 105,000*g* supernatant solution was acidified to pH 5.0 with 0.1 N HCl in an ice bath. The precipitate was removed by centrifugation at 10,000*g* at 3° for 20 min. Almost all of the initial enzyme activity was retained in the supernatant. After the solution was neutralized to pH 7.0 with 0.1 N NaOH, solid $(NH_4)_2SO_4$ was added to 30% saturation. The mixture was stirred mechanically for 30 min and then centrifuged for 20 min at 10,000*g*. The precipitate was discarded and the remaining supernatant solution was then brought to 50% saturation with $(NH_4)_2SO_4$. The resultant precipitate was collected by centrifugation as before and the supernatant solution was discarded. The precipitated protein, which virtually contained all of the enzyme activity of the 105,000*g* supernatant solution, was dissolved in 0.02 M Tris-Cl (pH 7.5). Subsequently, the enzyme solution was again fractionated by successive addition of solid $(NH_4)_2SO_4$. The precipitate obtained between 50 and 60% saturation of $(NH_4)_2SO_4$ contained approximately 60% of the initial enzyme activity. The precipitate was dissolved in a few milliliters of cold distilled water and dialyzed against distilled water for 5–6 hr at 5°. The dialyzed solution was lyophilized and the powder was stored in a desiccator over silica gel at −20°. The enzyme activity was stable for several months under these conditions. In a typical case, 23 mg of lyophilized powder possessing 40% of the initial enzyme activity was obtained from the 105,000*g* supernatant solution containing 460 mg of protein. Shortly before use, the lyophilized powder was dissolved in 0.1 M Tris-Cl (pH 7.5) or in 0.1 M phosphate buffer of various pH values. Some properties of the enzyme studied with this partially purified preparation will next be described.

Effect of pH. Aliquots (10 μg) of 17α-hydroxy[4-^{14}C]-progesterone were incubated with 5-mg portions of the enzyme preparation in 5 ml of 0.1 M phosphate buffer at pH 5.4, 6.3, 7.2, and 8.3 and yielded 3.0, 4.1, 4.3, and 2.9 μg of the 17α,20α-dihydroxy compound, respectively. Thus, the optimum pH for the reduction reaction is around pH 7.

Reversibility of Reaction. Incubation of 10 μg (0.03 μmole) of 17α-hydroxy[4-^{14}C]progesterone with 0.5 μmole of NADPH and 5 mg of the enzyme preparation in 0.1 M Tris-Cl (pH 7.4) for 540 min yielded 8.9 μg of radioactive 17α,20α-dihydroxy compound. The

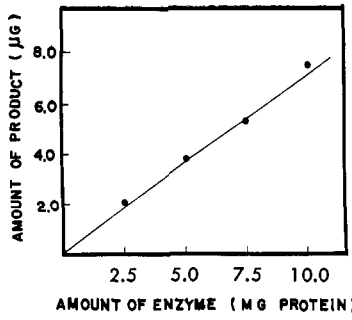

FIGURE 1: Reduction of the 20-ketone of 17α-hydroxy-[4-^{14}C]progesterone by the 20α-hydroxysteroid dehydrogenase of rat testes as a function of the amount of enzyme. Conditions of incubation are given in the text.

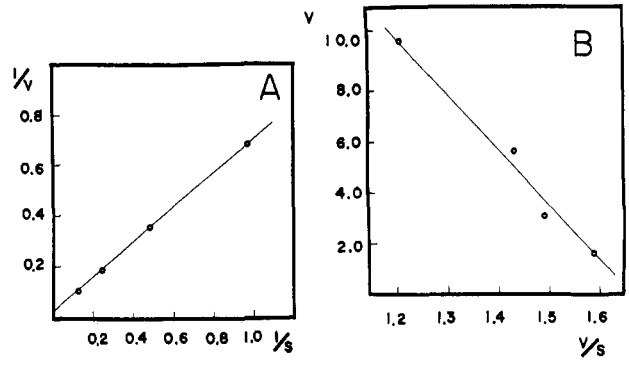

FIGURE 2: Determination of Michaelis constant for the substrate 17α-hydroxyprogesterone. Indicated amounts of substrate steroid were incubated with 3.0 mg of enzyme for 30 min. Other conditions of incubation are described in the text. The result obtained was plotted by two different graphical methods. (A) Reciprocal of substrate concentration (micrograms per milliliter) was plotted against reciprocal of the amount of the 17α,20α-dihydroxy compound produced (micrograms). Intersection with X axis ($1/V_{max}$) and slope (K_m/V_{max}) of the line were determined to be 0.03 μg^{-1} and 0.63 ml^{-1}, respectively. (B) The amount of the dihydroxy compound produced (micrograms) was divided by substrate concentration (micrograms per milliliter) and plotted against the ratio (milliliters) obtained. The slope of the line which represents $-K_m$ was found to be -22 μg/ml.

result suggests that the equilibrium is far in favor of the dihydroxy compound under these conditions. Thus, the reversibility of the enzyme reaction was studied precisely. It will be seen in Table I that approximately 90% of 17α,20α-dihydroxy[4-^{14}C]pregn-4-en-3-one was recovered unchanged after it was incubated with the enzyme in the presence of excess amount of either NAD^+ or $NADP^+$. It seems that virtually no oxidation of the dihydroxy compound occurred in these incubations. Incomplete recovery of the dihydroxy compound was due probably to loss of the steroid during the course of extraction and separation of the reaction products. Reversibility of the reaction was further examined in phosphate buffer of various pH values. In none of these incubations, oxidation reaction occurred in significant amounts, although there was a slight tendency for increased formation of 17α-hydroxyprogesterone with increase in pH as shown in Table I. It can be concluded that the reduction of 17α-hydroxyprogesterone by the testicular enzyme is practically irreversible in the pH range 5.6–8.5.

Effect of Temperature. 17α-Hydroxy[4-^{14}C]progesterone (10 μg) was incubated with 5 mg of the enzyme preparation and 0.5 μmole of NADPH for 30 min at various temperatures between 22 and 50°. Inactivation of the enzyme occurred rapidly at temperatures above 46°, while incubations at 42, 37, and 30° each gave approximately 5.2 μg of the dihydroxy compound. Thus, the enzyme has a broad temperature optimum in the region of 30–42°.

Kinetics of the Enzyme. The proportionality of the 20α-hydroxysteroid dehydrogenase activity with increasing enzyme concentration is shown in Figure 1. A linear increase in the formation of the dihydroxy compound was noted for the first 80 min of incubation with 1–5 mg of the enzyme preparation. Reduction of 17α-hydroxyprogesterone was also assayed at varying substrate and NADPH concentrations. Michaelis constants for the substrate and the cofactor were estimated by two graphical methods as shown in Figures 2 and 3 and summarized in Table II. The K_m for the pyridine nucleotide seems to be of the same order of magnitude as that for the steroid.

Inhibitors. The effect of several compounds on the enzyme activity is summarized in Table III. Sulfhydryl compounds, such as glutathione, *l*-cysteine, and β-mercaptoethanol, inhibited the enzymatic activity at concentrations of 10^{-3} M, and *p*-mercuribenzoate which reacts with the sulfhydryl groups of proteins was also inhibitory. Zinc and cobalt ions had either no effect or were somewhat stimulatory. EDTA-Na_2 increased the enzyme activity, while *o*-phenanthroline which is also a chelating agent was rather inhibitory.

TABLE II: The Michaelis Constant (K_m) of 20α-Hydroxysteroid Dehydrogenase of Rat Testes for Substrate 17α-Hydroxyprogesterone and for Cofactor NADPH.

	Method of Plotting[a]	
	$1/v$ *vs.* $1/s$	v *vs.* v/s
K_m for substrate (M)	6.3×10^{-5}	6.7×10^{-5}
K_m for cofactor	2.5×10^{-5}	2.0×10^{-5}

[a] Graphs are shown in Figures 2 and 3.

TABLE III: Influence of Various Substances on the Reduction of 17α-Hydroxyprogesterone by Rat Testicular 20α-Hydroxysteroid Dehydrogenase.[a]

Compounds Added	Final Concn of the Compd (M)	17α,20α-Diol Obtained (μg)
Glutathione	1×10^{-3}	0.19
l-Cysteine	1×10^{-3}	1.2
2-Mercaptoethanol	1×10^{-3}	1.5
p-Mercuribenzoate	1×10^{-4}	0.90
o-Phenanthroline	1×10^{-4}	1.4
Zinc chloride	1×10^{-4}	2.0
Zinc chloride	1×10^{-5}	1.9
Cobalt chloride	1×10^{-3}	1.9
Cobalt chloride	1×10^{-5}	1.8
EDTA-Na_2	1×10^{-2}	2.7
None	...	1.8

[a] Incubations were performed as described in the text with 5 mg of enzyme and 5 μg of substrate 17 α-hydroxy[4-^{14}C]progesterone.

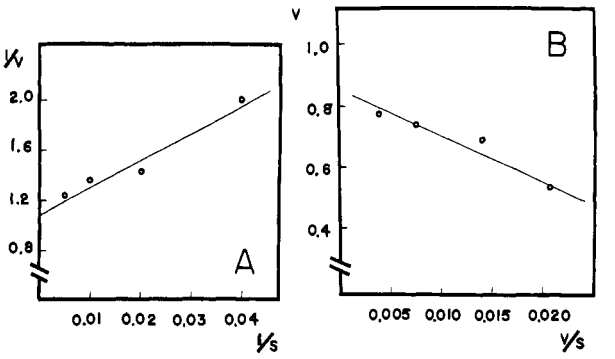

FIGURE 3: Determination of Michaelis constant for cofactor NADPH. 17α-Hydroxy[4-^{14}C]progesterone (5 μg) was incubated with 2 mg of enzyme with indicated amounts of NADPH (micrograms) for 30 min. (A) Reciprocal of NADPH concentration (micrograms per milliliter) was plotted against reciprocal of the amount of the dihydroxy compound produced (micrograms). The intersection and the slope were found to be 1.1 μg^{-1} and 20 ml^{-1}, respectively. (B) The same result is given in the product (micrograms) *vs.* product/NADPH concentration (milliliters) plot. The slope of the line was found to be −15 μg/ml.

Discussion

The result of the present experiment showed that the equilibrium of the testicular 20α-hydroxysteroid dehydrogenase reaction strongly favors the reduced form of the steroid even in the presence of excess amount of oxidized form of the pyridine nucleotide cofactors. This is consistent with a cruder observation of Matthijssen *et al.* (1964) on the adrenal 20α-hydroxysteroid dehydrogenase. Hydroxysteroid dehydrogenases usually catalyze reversible interconversions of hydroxyl and carbonyl groups of steroids, although optimum pH of oxidation reaction is sometimes largely different from that of reduction reaction (Langer and Engel, 1958; Wiest, 1959; Wilcox and Wiest, 1966). Difference in optimum pH between the two reactions is due, at least in part, to the fact that hydrogen ion accounts for one of the reductive hydrogens. The small but consistent increase in the oxidative reaction with increasing pH observed in the present experiment (Table I) also seems to be due partly to the direct participation of hydrogen ions in the reaction. On the other hand, the approximate irreversibility of the reaction may be due possibly to a partial positive charge at the C-20 carbonyl carbon of the substrate 17α-hydroxyprogesterone which may play a role in the interaction of the substrate and the enzyme. In the case of 17α-hydroxyprogesterone, a specific substrate of the enzyme, formation of a hydrogen bond between the C-20 carbonyl group and the adjacent hydroxyl group stabilizes the polarization of the carbonyl group, while the dihydroxy compound, the product of the reaction, lacks this type of strong polarization.

In the routine assay, the concentration of the pyridine nucleotide cofactor was sufficiently high to saturate the enzyme, but the concentration of the substrate steroid was less than saturating because of its low solubility in aqueous solution. Nonetheless, product formation was linear with respect to the amount of enzyme added.

The K_m value for 17α-hydroxyprogesterone estimated in the present experiment is not too different from that of rat ovarian 20α-hydroxysteroid dehydrogenase (4.4×10^{-5} M) (Wiest, 1959). The K_m for NADPH of the testicular 20α-hydroxysteroid dehydrogenase is of the same order of magnitude as those of hepatic 3α-hydroxysteroid dehydrogenase (1.5×10^{-5} M; Tomkins, 1956) and renal 16α-hydroxysteroid dehydrogenase (4.1×10^{-5} M; Meigs and Ryan, 1966). The K_m of placental 17β-hydroxysteroid dehydrogenase was evaluated at 3×10^{-5} M for NADH (Talalay *et al.*, 1958) and at 8×10^{-7} M for NADPH (O. Jarabak, unpublished data).

Stimulation of 17β-hydroxysteroid dehydrogenase activity by zinc ion was reported for the placental (Langer and Engel, 1958) and adrenal (Dahm and Breuner, 1964) enzymes. No significant stimulation was observed in the case of testicular 20α-hydroxysteroid dehydrogenase. EDTA in a concentration as high as 10^{-2} M did not inhibit testicular dehydrogenase activity but rather stimulated it. Langer and Engel (1958) also reported that EDTA in a concentration of 10^{-3} M had no effect on placental 17β-hydroxysteroid dehydrogenase activity. On the other hand, 16α-hydroxysteroid dehydrogenase activity is strongly inhibited by low concentrations of EDTA (Meigs and Ryan, 1966).

The physiological role of the testicular 20α-hydroxysteroid dehydrogenase is not clear at present time. Fevold and Eik-Nes (1962) have suggested that this enzyme is concerned with spermatogenic function of the testes of birds. The following observations on the 20α-hydroxysteroid dehydrogenase of rat testes suggest another interpretation. (1) The enzyme is present chiefly in the soluble fraction, while the majority of testicular enzymes related to steroid transformation are bound to mitochondria and microsomes (Shikita and Tamaoki, 1965). (2) The enzyme has a high substrate specificity for 17α-hydroxyprogesterone (Shikita and Tamaoki, 1965). (3) The equilibrium of the enzyme reaction strongly favors the formation of the 17α,20α-dihydroxy compound. (4) The product of the reaction, *i.e.*, the 17α,20α-dihydroxy compound, cannot be converted to androstenedione by the testicular microsomal enzyme system which cleaves the side chain of 17α-hydroxyprogesterone (Shikita and Tamaoki, 1965; Dominguez, 1966). (5) The dihydroxy compound competitively inhibits the side-chain-cleaving enzyme (Inano *et al.*, 1967). (6) No increase or, in fact, a small decrease was observed in 20α-hydroxysteroid dehydrogenase activity when the microsomal enzymes were stimulated by gonadotrophin injection (Schoen and Samuels, 1965; Shikita and Hall, 1967). (7) The specific activity of 20α-hydroxysteroid dehydrogenase is higher in testes damaged by X-ray irradiation than in intact testes, suggesting that the enzyme is related to androgen production in the testis rather than spermatogenesis (Schoen, 1964; Shikita and Tamaoki, 1965). These characteristics of the testicular 20α-hydroxysteroid dehydrogenase suggest that it acts to decrease formation of androgens in two ways: (1) by reducing the substrate for the side-chain-cleaving enzyme (*i.e.*, 17α-hydroxyprogesterone) and (2) by catalyzing the production of an inhibitor, the 17α,20α-dihydroxy compound, of the side-chain-cleaving enzyme. However, there is no direct evidence available for the physiological significance of this kind of regulation of androgen synthesis. In this connection it is interesting to note that there is increasing evidence to suggest that the possible function of 20α-hydroxysteroid dehydrogenase of ovarian tissue is to shunt the production of progesterone as discussed recently by Kidwell *et al.* (1966) and Pupkin *et al.* (1966).

Acknowledgment

The authors are grateful to Dr. Joseph Jarabak of the Johns Hopkins University for many helpful discussions.

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