20α-Hydroxysteroid Dehydrogenase of Testes*

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ABSTRACT: [4-^{14}C]Progesterone was incubated with teased issue of a rat testis. Among metabolites, 20α-hydroxy[^{14}C]pregn-4-en-3-one and 17α,20α-dihydroxy-[^{14}C]pregn-4-en-3-one were isolated and identified. The 20α-hydroxysteroid dehydrogenase activity was contained in the testicular 105,000 × *g* supernatant fluid but not in the microsomal fraction. After spermatogenic cells were completely destroyed by X-ray irradiation localized on the scrotum, the supernatant fluid of the damaged testes contained rather higher concentration of the dehydrogenase than that of the testes of intact animals. The enzyme activity was also found in the 105,000 × *g* supernatant of human, rabbit, and mouse testes. 17α-Hydroxyprogesterone was a much more reactive substrate for the dehydrogenase than progesterone. On the other hand, a hydroxy group at C-21 prevented the reduction of the 20-ketone. 17-Ketone of androst-4-ene-3,17-dione was not reduced by the enzyme. Reduced nicotinamide–adenine dinucleotide phosphate was required for the enzyme action, while reduced nicotinamide–adenine dinucleotide was much less effective.

The dehydrogenase activity of the supernatant fluid was stable at −20° for 2 months, but was destroyed at 50° within 15 minutes. 20-Keto-reduced derivatives of progesterone were not subsequently hydroxylated at C-17 by the testicular microsomal 17α-hydroxylase, nor cleaved to androst-4-ene-3,17-dione by the microsomal 17,20-desmolase. The 17,20-dihydroxy compounds were also much less susceptible to the enzymic cleavage of the side chain than 17α-hydroxyprogesterone.

The 20α-hydroxysteroid dehydrogenase seems to be present in various organs. Liver (Recknagel, 1957), uterine fibroblasts (Sweat *et al.*, 1958), ovary (Wiest, 1959; Sandor and Lanthier, 1960), placenta (Little *et al.*, 1959; Purdy *et al.*, 1964), adrenal (Matthijssen *et al.*, 1964), and skeletal muscle (Thomas *et al.*, 1960) are reported to contain the enzymic activity. As to testes, furthermore, some workers reported the reduction of 20-ketone of 17α-hydroxyprogesterone[1] *in vitro* (Lynn and Brown, 1958; Schoen, 1964). As 17α,-20α-dihydroxypregn-4-en-3-one and its 20β epimer were isolated from testes of young bulls (Neher and Wettstein, 1960), it was suggested that the 17,20-dihydroxy compound is the actual substrate of the 17,20-desmolase[2] which catalyzes the cleavage of the side chain of a C_{21} steroid (Wettstein, 1961). Sweat *et al.* (1960) and Axelrod and Goldzieher (1962) also described that both 17α-hydroxylation and 20-keto reduction are necessary for the cleavage of the side chain of progesterone in human ovarian tissue. In contrast, Lynn and Brown (1958), using guinea pig testicular microsomes, reported far less cleavage of the side chain of the 17,20-dihydroxy compounds than that of 17α-hydroxyprogesterone. This paper describes some properties o the 20α-hydroxysteroid dehydrogenase of the testicular tissue and the reactivity of the 20-keto-reduced derivatives of progesterone against the testicular 17α-hydroxylase and 17,20-desmolase.

Experimental

Tissue Preparation and Incubation. Male rats of the Wistar strain, about 3 months of age, were killed by exsanguination. Testes were removed immediately thereafter, weighed, chilled in twice their weight of ice-cold 0.25 M sucrose solution, decapsulated, and teased with forceps. In one experiment, this teased tissue was incubated with the substrate steroid. In other experiments, the teased tissue was homogenized with a loose-fitting glass-Teflon homogenizer and centrifuged at 10,000 × *g* for 10 minutes. The supernatant fluid was decanted and spun for 60 minutes at 105,000 × *g*. The 105,000 × *g* supernatant was again centrifuged at 105,000 × *g* for 60 minutes, and this supernatant was used as the soluble fraction.

The 105,000 × *g* precipitates were suspended in 0.25 M sucrose with a Teflon homogenizer driven by hand, and again sedimented by centrifugation. The precipitate was resuspended in 0.25 M sucrose. This suspension was centrifuged once more at 10,000 × *g* for 10 minutes and the supernatant was used as the microsomal frac-

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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; testosterone, 17β-hydroxyandrost-4-en-3-one; progesterone, pregn-4-ene-3,20-dione; 17α-hydroxyprogesterone, 17α-hydroxypregn-4-ene-3,20-dione.

[2] Abbreviations and trivial names used in this paper: NADPH, nicotinamide–adenine dinucleotide phosphate, reduced form; NADH, nicotinamide–adenine dinucleotide, reduced form; SU-10603, 3-(1,2,3,4-tetrahydro-4-oxo-7-chloro-2-naphthyl)pyridine; 17,20-desmolase, 17α-hydroxyprogesterone side-chain cleavage enzyme.

tion. Most of these operations were performed between 0° and 5°, and the whole procedure required about 5 hours. Electron microscopic examination showed that the microsomal fraction was not contaminated by mitochondria and other large particles.

The teased tissue or the subcellular preparations were mixed with the incubation medium and transferred to 100-ml Erlenmeyer flasks to which the radioactive steroid precursors had previously been added with 2 drops of propylene glycol. Unless otherwise stated, the incubation medium consisted of 0.25 M sucrose solution containing, besides substrates and the tissue preparation, 0.5 μmole of NADPH and 500 μmoles of Tris buffer, *p*H 7.4, in a total volume of 5 ml per flask. It was incubated at 37° in 95% O_2 and 5% CO_2 with continuous shaking for periods indicated in each case.

Extraction and Isolation of the Products. After the incubation, 80 ml of cold ethyl alcohol and about 100 μg each of progesterone, androstenedione, testosterone, 17α-hydroxyprogesterone, and 17α,20α-dihydroxypregn-4-en-3-one was added to each flask. The mixture was filtered through paper and the residue was washed exhaustively with ethyl alcohol. The filtrate and the washings were combined, concentrated at 40° under reduced pressure, and extracted with diethyl ether. The crude extracts were dried under nitrogen and chromatographed on paper in a *n*-heptane-benzene (2:1)/formamide system for 12 hours. The effluent was collected in a beaker. The effluent was chromatographed again in the same system for 2 hours. Carrier steroids on the paper chromatogram were located under an ultraviolet lamp as previously reported (Tamaoki and Shikita, 1963). The radioactive peaks were detected in a windowless gas-flow strip counter (Atomic Accessories, Bellerose, N.Y.). The areas of detectable radioactivity were eluted with ethyl alcohol–chloroform (1:1, v/v). The radioactive area corresponding to 17α-hydroxyprogesterone and testosterone and that corresponding to androstenedione were subjected to acetylation with acetic anhydride and pyridine (1:1, v/v) and rechromatographed in a cyclohexane-formamide system. By these procedures, progesterone, androstenedione, 20α-hydroxypregn-4-en-3-one (as its acetate), testosterone (as its acetate), 21-hydroxypregn-4-ene-3,20-dione (as its acetate), 17α-hydroxyprogesterone, 17α,20α-dihydroxypregn-4-en-3-one, 17α,20β-dihydroxypregn-4-en-3-one, and 17α,21-dihydroxypregn-4-ene-3,20-dione were separated. Of the radioactivity initially added, 80–90% was recovered from the regions of these steroids and other materials located between 17α,20α-dihydroxypregn-4-en-3-one and the starting line of the initial paper chromatogram.

The main radioactive products and the unchanged substrate were further identified by their behavior on thin-layer and paper chromatography, and by the result of repeated crystallizations after the addition of the corresponding authentic compounds. The criterion of the constancy of the specific activity of the crystals and the mother liquor was based on a coefficient of variation of less than 5% in three consecutive crystallizations. The details on the method of identification of [^{14}C]testosterone and [^{14}C]androstenedione were mentioned in a previous paper (Shikita and Tamaoki, 1964).

Radioactive Steroid Precursors. 17α-Hydroxy[4-^{14}C]-progesterone (43.8 μc/mg), [4-^{14}C]progesterone (83 μc/mg), 21-hydroxy[1,2-^{3}H]pregn-4-ene-3,20-dione (2.96 mc/mg), 17α-hydroxy[7α-^{3}H]progesterone (25 mc/mg), and 17α,21-dihydroxy[1,2-^{3}H]pregn-4-ene-3,20-dione (2.0 mc/mg) were purchased from New England Nuclear Corp. (Boston, Mass.). 17α,20α-Dihydroxy[^{14}C]pregn-4-en-3-one (43.8 μc/mg) was prepared by incubating 17α-hydroxy[4-^{14}C]progesterone with the testicular 105,000 × *g* supernatant fluid. 17α,20β-Dihydroxy[^{14}C]pregn-4-en-3-one (43.8 μc/mg) was prepared by chemical reduction of 17α-hydroxy-[4-^{14}C]progesterone with sodium borohydride. 20α-Hydroxy[^{14}C]pregn-4-en-3-one and its 20β epimer (83 μc/mg) were prepared by the sodium borohydride reduction of [4-^{14}C]progesterone.

Quantitation of Radioactive Compounds. Suitable aliquots of the radioactive compounds were transferred in 20-ml glass vials (Wheaton Glass Co., Millville, N.J.), evaporated to dryness, and dissolved in 10 ml toluene containing 4 g and 100 mg per liter of 2,5-diphenyloxazole (PPO) and 1,4-bis-2′(5′phenyloxazolyl)-benzene (POPOP). Radioactivity was measured with a liquid scintillation spectrometer (Tri-Carb, 314X, Packard Instrument Co., La Grange, Ill.) for a sufficient time to reduce the counting error to below 5%. Approximate efficiency was 70% for carbon-14 and 25% for tritium. In the double-tracer study, carbon-14 and tritium were measured as reported previously (Shikita and Tamaoki, 1964).

Quantitation of Protein. Protein concentrations in the testicular supernatant fluid were determined by the biuret method with a coefficient of 0.050 for 1 mg of protein in 5 ml total volume, 1 cm light path, at 530 mμ.

Results

Formation of 20α-Hydroxy[^{14}C]pregn-4-en-3-one and 17α,20α-Dihydroxy[^{14}C]pregn-4-en-3-one from [4-^{14}C]-Progesterone in Teased Tissue of Rat Testes. [4-^{14}C]-Progesterone (110 × 10^5 dpm, 60 μg) was incubated with the teased tissue (1.4 g) for 20 hours in 20 ml of Tris buffer (200 mM, *p*H 7.4) containing glucose-6-phosphate, nicotinamide, and $MgCl_2$ in final concentrations of 100 μM, respectively. In addition, penicillin G (5000 units) was added to the flask. More than 20 different radioactive substances were distinguished after the metabolites were separated by paper chromatography. Among them, a radioactive product coinciding with 17α,20α-dihydroxypregn-4-en-3-one was rechromatographed in the *n*-heptane-benzene (2:1)/formamide system for 48 hours. A total of 176 × 10^3 dpm of radioactivity (1.6% of the total radioactivity added) was recovered from the region of carrier 17α,-20α-dihydroxypregn-4-en-3-one.

Another radioactive area corresponding to 20α-acetoxypregn-4-en-3-one was eluted and hydrolyzed with 0.4% methanolic $KHCO_3$. After chromatography

on paper in the cyclohexane-formamide system for 5 hours, a total of 187 × 10^3 dpm (1.7% of the total radioactivity added) was recovered from the region of the 20α-hydroxypregn-4-en-3-one carrier and separated from the 20β epimer.

Further Identification of 17α,20α-Dihydroxy[^{14}C]-pregn-4-en-3-one. The radioactive substance was chromatographed on a thin layer of silica gel G (E. Merck AG., Darmstadt, Germany) in cyclohexane–ethyl acetate (4:6), chloroform–ethyl ether (5:1), and benzene–methyl alcohol (9:1) systems. In all cases, more than 95% of the radioactivity was recovered from the spot of the carrier 17α,20α-dihydroxy compound and separated from the 20β epimer. An aliquot of the radioactive substance was acetylated with acetic anhydride in pyridine at room temperature. The radioactive substance behaved identically with the carrier steroid in paper chromatography in the cyclohexane-formamide system as well as in thin-layer chromatography in a benzene-acetone (8:2) system. After hydrolysis with 0.4% methanolic $KHCO_3$, the dihydroxy compound was recovered with radioactivity. Another aliquot was treated with chromium trioxide–pyridine complex (Poos *et al.*, 1953) for 2 hours at room temperature. Radioactive products corresponding to 17α-hydroxyprogesterone and androstenedione were obtained in 24 and 32% yield, respectively. Similar oxidation for 1 hour produced 17α-hydroxy[^{14}C]progesterone in 60% yield. The products were pooled, mixed with respective standard steroid, crystallized from aqueous ethyl alcohol, and then crystallized twice from a mixture solution of methylene dichloride and *n*-heptane. Specific activities of the crystals and the mother liquor during the crystallizations were 904 ± 18 for 17α-hydroxy-[^{14}C]progesterone (Table I) and 774 ± 7 dpm/mg for [^{14}C]androstenedione. Oxidation of the substance with 1% periodic acid in aqueous dioxane also yielded [^{14}C]androstenedione.

Further Identification of 20α-Hydroxy[^{14}C]pregn-4-en-3-one Obtained. The radioactive substance with the carrier 20α-hydroxypregn-4-en-3-one was chromatographed on thin layer of silica gel G in benzene-acetone (8:2) and cyclohexane–ethyl acetate–*n*-heptane (4:6:1) systems. The substance was acetylated and chromatographed on silica gel G in a *n*-heptane–acetone–ethyl acetate (6:2:3) system. In each of these cases of thin-layer chromatography, more than 95% of the radioactivity was recovered from the region of the carrier steroid. Oxidation of an aliquot of the radioactive substance with 0.5% chromium trioxide in 90% aqueous acetic acid for 15 minutes yielded a radioactive product coinciding with progesterone in a yield of 56%. The oxidation product was chromatographed on the silica gel G layer in the benzene-acetone (8:2) system. Repeated crystallizations after the addition of carrier progesterone gave a constant specific activity of 203 ± 3 dpm/mg with regard to crystals and the mother liquor in four consecutive crystallizations from a mixture of ethyl acetate and *n*-heptane (Table I).

TABLE I: Crystallizations of the Radioactive Steroid Products.

	Specific Activity (dpm/mg)	
	17α,20α-Dihydroxy-[^{14}C]pregn-4-en-3-one	20α-Hydroxy-[^{14}C]pregn-4-en-3-one
Oxidized and Crystallized as:	17α-Hydroxy-progesterone	Progesterone
1st crystals	882	203
Mother liquor	991	201
2nd crystals	891	199
Mother liquor	910	197
3rd crystals	889	216
Mother liquor	859	201

Intracellular Distribution of the Testicular 20α-Hydroxysteroid Dehydrogenase. The rat testicular 105,000 × *g* supernatant fluid (2.5 ml, equivalent to approximately 1 g fresh tissue) was diluted with an equal amount of 0.25 M sucrose solution containing Tris (*p*H 7.4) and NADPH, and incubated for 2 hours with 17α-hydroxy-[4-^{14}C]progesterone (45.7 × 10^3 dpm, 5 μg). The 17α,20α-dihydroxy derivative was obtained in 58% yield and identified as mentioned earlier.

The rat testicular microsomal fraction contains a strong 17,20-desmolase activity (Shikita and Tamaoki, 1965). It is difficult, therefore, to demonstrate the presence of the dehydrogenase in this fraction, unless the desmolase activity is masked by a proper method. Accordingly, 17α-hydroxy[4-^{14}C]progesterone (66.6 × 10^3 dpm, 5 μg) was incubated for 1 hour with the microsomes (1 g wet weight tissue equivalent) in the presence of 1 μg/ml of SU-10603, which had been proved not to inhibit the dehydrogenase but strongly to inhibit the desmolase (Shikita *et al.*, 1965). No significant amount of the 17α,20α-dihydroxy compound was formed by this incubation, however, and most of the substrate 17α-hydroxyprogesterone was recovered unchanged. The result suggests that the microsomal fraction contained no 20α-hydroxysteroid dehydrogenase activity as contained in the supernatant.

Time Curve for Reduction of 20-Ketone of 17α-Hydroxy[4-^{14}C]progesterone. Similar incubations of the 105,000 × *g* supernatant with 17α-hydroxy[4-^{14}C]-progesterone (66.6 × 10^3 dpm, 5 μg) for periods of 20–150 minutes showed that the reaction proceeded linearly with time, and 80% or more of the substrate was reduced to the 17α,20α-dihydroxy compound within 3 hours (Figure 1).

Substrate Specificity of the 20α-Hydroxysteroid Dehydrogenase. As in the preceding experiment, the testicular 105,000 × *g* supernatant fluid was incubated for 90 minutes each with 17α-hydroxy[4-^{14}C]progesterone (66.6 × 10^3 dpm, 5 μg), [4-^{14}C]progesterone (60.5 × 10^3 dpm, 5 μg), and a mixture of 17α-hydroxy-[4-^{14}C]progesterone (46.7 × 10^3 dpm, 5 μg) and

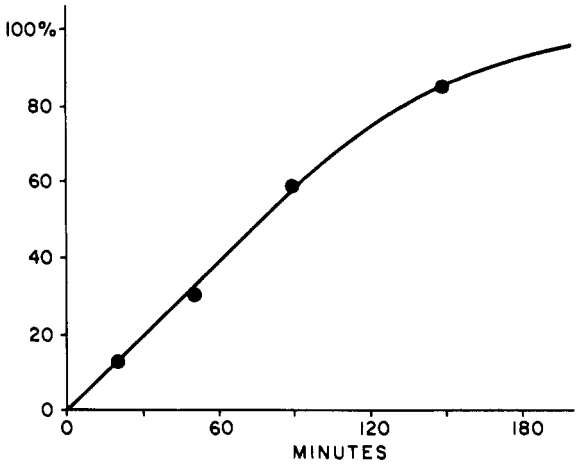

FIGURE 1: Time course of the reduction of 17α-hydroxy-[4-^{14}C]progesterone by the rat testicular supernatant enzyme. Percentage of the substrate 17α-hydroxy-[4-^{14}C]progesterone reduced to the 17α,20α-dihydroxy compound is plotted against time of the incubation.

TABLE II: 20-Ketone Reduction of 17α-Hydroxyprogesterone and Progesterone by the Rat Testicular 105,000 × *g* Supernatant.

Substrate	20-Ketone Reduced (%)	Substrate Unchanged (%)
[4-^{14}C]Progesterone	2.0[a]	82.0
17α-Hydroxy[4-^{14}C]-progesterone	59.5	15.3
[1,2-^{3}H]Progesterone[b]	3.0	72.0
17α-Hydroxy[4-^{14}C]-progesterone[b]	56.5	29.9

[a] Figures in the table represent the percentage of the radioactivity of the respective substrate initially added. [b] Incubated together in one flask.

[1,2-^{3}H]progesterone (75.3 × 10^{3} dpm, 5 μg). As shown in Table II, the carbonyl group of the side chain of 17α-hydroxyprogesterone was much more readily reduced than that of progesterone. The presence of progesterone did not inhibit the reduction of 17α-hydroxyprogesterone.

A hydroxy group at C-21 did not enhance the reduction of the carbonyl group at C-20, as did the hydroxy group at C-17, but rather inhibited the reduction. Most of the 21-hydroxy[1,2-^{3}H]pregn-4-ene-3,20-dione (356 × 10^{3} dpm, 5 μg) and of the 17α,21-dihydroxy-[1,2-^{3}H]pregn-4-ene-3,20-dione (325 × 10^{3} dpm, 5 μg) were recovered unchanged after the 50-minute incubation with the supernatant. Furthermore, [4-^{14}C]-androstenedione (339 × 10^{3} dpm, 5 μg) was also recovered unchanged in most part after the incubation, and only 2.5% was converted to [^{14}C]testosterone. These results suggest that the enzyme in the supernatant specifically reduces the carbonyl group of 17α-hydroxyprogesterone.

Cofactor Requirement of the 20α-Hydroxysteroid Dehydrogenase. Conversion of 17α-hydroxy[4-^{14}C]-progesterone to the dihydroxy compound by the testicular supernatant enzyme required the presence of NADPH. Without addition of the cofactor no significant amount of the steroid was reduced. Furthermore, NADH was far less effective than NADPH. In the presence of NADPH, 37.8% of 17α-hydroxy-[4-^{14}C]progesterone was reduced in 60 minutes, whereas only 6.0% was reduced in the case of NADH.

Stability of the 20α-Hydroxysteroid Dehydrogenase. The dehydrogenase activity of the rat testicular supernatant fluid showed no appreciable loss after having been stored for 2 months at −20° as a frozen solution in 0.25 M sucrose or as a lyophilized powder. It was, however, reduced by 10% or less after 12 hours at 5°, by 19% after 1 hour at 37°, and was completely inactivated at 50° within 15 minutes.

The 20α-Hydroxysteroid Dehydrogenase in Testes of Other Species. The 105,000 × *g* supernatant fluid of human (74 years old), rabbit (5 months old), and mouse (1 month old) testes also contained the dehydrogenase activity as observed in the case of rat testes. Relative activity is shown in Table III in μg of the dihydroxy compound produced from 17α-hydroxy[4-^{14}C]progesterone (55.7 × 10^{3} dpm, 10 μg) per mg of protein in 60-minute incubation.

TABLE III: 20α-Hydroxysteroid Dehydrogenase Activity in the Testicular 105,000 × *g* Supernatant.

Animal Species	Amount of Protein Incubated (mg)	Enzyme Activity[a] (μg/mg protein)
Rat	29	0.15
Rat (X-ray irradiated)[b]	13	0.25
Mouse	12	0.14
Rabbit	27	0.28
Man (a patient with prostate carcinoma)	25	0.05

[a] The enzyme activity is expressed in μg of the 17α,20α-dihydroxy compound produced from 17α-hydroxy[4-^{14}C]progesterone per mg of protein in 60-minute incubation. [b] Five weeks after irradiation (400 r) locally on scrotum.

The 20α-Hydroxysteroid Dehydrogenase in X-ray-

damaged Testes. Two-month-old rats received X-ray irradiation (400 r total) locally on the scrotum under Nembutal anesthesia. Five weeks thereafter, the weight of the testes had decreased by more than half, and histological examination showed that spermatogenic cells were completely destroyed. The 105,000 × *g* supernatant fluid obtained from these damaged testes, however, contained the dehydrogenase in higher concentration than the supernatant obtained from intact testes (Table III). This supports the result of Schoen (1964) and suggests that the dehydrogenase is localized to interstitial cells or other radioresistant cells rather than to the spermatogenic germ cells.

Action of the Testicular Microsomal Enzymes on the C-20-*Hydroxy Steroids.* The rat testicular microsomes (1 g wet weight tissue equivalent) were incubated for 25 minutes with 10 μg each of [4-^{14}C]progesterone (60.5 × 10^3 dpm), 17α-hydroxy[4-^{14}C]progesterone (66.6 × 10^3 dpm), 17α,20α-dihydroxy[4-^{14}C]pregn-4-en-3-one (62.5 × 10^3 dpm), 17α,20β-dihydroxy[4-^{14}C]-pregn-4-en-3-one-4 (62.9 × 10^3 dpm), 20α-hydroxy-[4-^{14}C]pregn-4-en-3-one (18.5 × 10^3 dpm), 20β-hydroxy[4-^{14}C]pregn-4-en-3-one (18.5 × 10^3 dpm), 21-hydroxy[1,2-^{3}H]pregn-4-ene-3,20-dione (356 × 10^3 dpm), and 17α,21-dihydroxy[1,2-^{3}H]pregn-4-ene-3,20-dione (325 × 10^3 dpm). The result shown in Table IV suggests that 17α-hydroxyprogesterone is the most favorable substrate for the side-chain cleavage by the microsomal desmolase. In the case of progesterone and 17α-hydroxyprogesterone, radioactive substances which were more polar than testosterone were obtained in 23.3 and 28.5% yield, respectively. Although the chemical structure of these substances was not elucidated, a large proportion of them are probably C_{19} steroids produced from testosterone by further metabolism. Therefore, the actual amount of progesterone and 17α-hydroxyprogesterone cleaved during the incubation was larger than the amount shown in the table.

TABLE IV: Cleavage of the Side Chain of Pregn-4-en-3-one Derivatives by Rat Testicular Microsomes.

Substrate[a]	Side Chain Cleaved[b] (%)	Substrate Unchanged (%)
Progesterone	44.3[c]	1.5[c]
17α-Hydroxyprogesterone	56.4	2.2
17α,20α-Dihydroxy-	8.9	70.0
17α,20β-Dihydroxy-	7.9	60.0
20α-Hydroxy-	2.0	75.8
20β-Hydroxy-	1.0	82.4
21-Hydroxy-20-keto-	1.5	63.0
17α,21-Dihydroxy-20-keto-	5.9	81.0

[a] Complete name and radioactivity of the substrate steroids are mentioned in the text. [b] Sum of the radioactivity recovered from the region of androstenedione and testosterone. [c] Percentages of the radioactivity initially added.

Double-tracer study using 17α-hydroxy[7α-^{3}H]progesterone (40.0 × 10^4 dpm, 10 μg) and 17α,20α-dihydroxy[4-^{14}C]pregn-4-en-3-one (25.7 × 10^3 dpm, 10 μg) again confirmed that 17α-hydroxyprogesterone is more rapidly converted to the C_{19} steroids than is the 17α,20α-dihydroxy compound (Table V). This result is consistent with that reported by Lynn and Brown (1958) and supports the view that the dihydroxy compound is not the intermediate substance in the course of the side-chain cleavage.

Discussion

In the present experiment, progesterone and 17α-hydroxyprogesterone were reduced to the corresponding 20α-hydroxy derivatives. The 20β epimers were formed only in a negligible amount. Predominance of the 20-ketone reduction to the α configuration has been reported with human ovarian slice (Lanthier and Sandor, 1962), homogenates of human testicular tumor (Dominguez, 1961), arrhenoblastoma tissue (Savard *et al.*, 1961), and human ovarian mince (Warren and Salhanick, 1961), although Lynn and Brown (1958) reported that 17α-hydroxyprogesterone was reduced mainly to the 17α,20β-dihydroxy derivative in guinea pig testes.

The results presented here showed that the 105,000 × *g* supernatant fluid of the testicular tissue retained the 20α-hydroxysteroid dehydrogenase activity. Similar intracellular distribution of the enzyme was observed with rat ovarian tissue (Wiest, 1959) as well as with human placenta (Little *et al.*, 1959). The adrenocortical steroid C-20-keto reductase in the rat liver, however, was reported to be firmly bound to the microsomes (Recknagel, 1957). The enzyme of the guinea pig testes was also found to be associated with the microsomes (Lynn and Brown, 1958). Physiological meaning of this difference in the intracellular distribution of the enzyme is not clear.

The 20-ketone of progesterone was reduced by the rat testicular enzyme to a much smaller extent than was that of 17α-hydroxyprogesterone. It seems that the rat testicular enzyme differs from the rat ovarian one, in which case 17α-hydroxyprogesterone was a less reactive substrate than progesterone (Wiest, 1959). Furthermore, it is different from the rat liver enzyme which acts characteristically on the 17α,21-dihydroxy-20-ketone side chain of adrenocortical steroids (Recknagel, 1957), and also from the placental enzyme which reduces 20-ketone as well as 17-ketone of C_{19} steroid (Purdy *et al.*, 1964). It is interesting that the 20α-hydroxysteroid dehydrogenases of various organs have different substrate specificity.

In the present experiment, incubation of [4-^{14}C]progesterone with the teased tissue of rat testes gave approximately equal amount of 20α-hydroxy and 17α,-

TABLE V: Double-Tracer Study on the Cleavage of the Side Chain of 17α-Hydroxy[7α-^{3}H]progesterone and 17α,20α-Dihydroxy[4-^{14}C]pregn-4-en-3-one by Rat Testicular Microsomes.[a]

Incubation (min)	Substrate Recovered				Products			
	17α,20α-Diol (%)		17α-OHP (%)		Δ^{4}-A-dione (%)		Testosterone (%)	
	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
5	90.6	1.1	nil	83.0	0.4	7.0	0.1	2.0
10	93.7	1.8	nil	70.2	0.2	11.8	0.1	3.0

[a] Figures in the table represent the percentage of the radioactivity of the respective precursor steroid initially added. Abbreviations in the table: 17α,20α-Diol, 17α,20α-dihydroxypregn-4-en-3-one; 17α-OHP, 17α-hydroxyprogesterone; Δ^{4}-A-dione, androstenedione.

20α-dihydroxy compounds. This seems to be contradictory to the observation on the substrate specificity of the 20α-hydroxysteroid dehydrogenase of the 105,000 × *g* supernatant. Rapid transformation of 17α-hydroxyprogesterone to androstenedione in the case of incubation with the teased tissue may explain the low yield of the dihydroxy compound as compared to that of the 20α-monohydroxy one.

Dominguez (1961) suggested that the reduction of 20-ketone precedes 17α-hydroxylation when progesterone is transformed to the 17α,20α-dihydroxy compound by the incubation with homogenates of testicular tumor. In the present experiment, however, the 17α-hydroxylase activity was separated from the 20α-hydroxysteroid dehydrogenase activity by differential centrifugation, and it was revealed that 17α-hydroxyprogesterone was more easily reduced than progesterone. Furthermore, it was shown that 20α- or 20β-hydroxypregn-4-en-3-one was not transformed to the 17α,20α- or 17α,20β-dihydroxy compound by the testicular microsomal 17α-hydroxylase. Therefore, it is suggested that the 17α-hydroxylation is followed by the reduction of 20-ketone in the course of formation of the 17α,20α-dihydroxy compound in the testicular tissue. This is consistent with the conclusion of Ball and Kadis (1964), who incubated 20α- and 20β-hydroxypregn-4-en-3-one with minced sow ovary.

Jones *et al.* (1952) presented evidence on the intramolecular hydrogen bond formation between the carbonyl oxygen at C-20 and the hydroxylic hydrogen at C-17 of 17α-hydroxyprogesterone. This hydroxy-carbonyl interaction seems to increase the fractional positive charge on the carbon atom at C-20. The polarization of the carbonyl group may be important for the side-chain cleavage by the desmolase, because the 17,20-dihydroxy compounds were not transformed to the C-19 steroid so readily as 17α-hydroxyprogesterone. According to Jones *et al.* (1952), the carbonyl oxygen at C-20 has no hydroxy-carbonyl interaction with the hydroxylic hydrogen at C-21. The present observation that the side chains of 21-hydroxypregn-4-ene-3,20-dione and 17α,21-dihydroxypregn-4-ene-3,20-dione were cleaved by the 17,20-desmolase to a much lesser extent than that of 17α-hydroxyprogesterone may be partly because the hydroxylic oxygen at C-21 attracted electrons and decreased the polarization of the C-20 carbonyl group. In any case, the report of Lynn and Brown (1958) that the 17,20-dihydroxy compounds were not subjected to the side-chain cleavage is supported by the results presented in this paper. Studies on purification and more detailed properties of the testicular 20α-hydroxysteroid dehydrogenase will be reported elesewhere in the near future.

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Reactivity and Function of Sulfhydryl Groups in Horse Liver Alcohol Dehydrogenase*

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ABSTRACT: Iodoacetate inhibits horse liver alcohol dehydrogenase (LADH) by selectively carboxymethylating two of the twenty-four SH groups of the molecule. DPNH protects the enzyme both against the inhibition [T.-K. Li and B. L. Vallee, 1961, *Biochem. Biophys. Res. Commun. 12*, 44] and the alkylation of these two cysteine residues. DPNH and ethanol still interact with the inactive carboxymethylated enzyme as shown by spectral and rotatory dispersion titration and by zinc exchange; hence these SH groups are not indispensible for coenzyme and substrate binding. However, both the dissociation constant of the carboxymethylated LADH·DPNH complex and the rate of zinc exchange are increased. This suggests that in the three-dimensional array of the enzyme the cysteinyl residues are situated in close proximity to the zinc and coenzyme binding sites.

Iodine similarly inhibits the enzyme when, on the average, six SH groups per mole of protein are oxidized, but DPNH and isobutyramide only *partially* protect the enzyme against inhibition. Optical rotatory dispersion and ultracentrifugation indicate that iodination denatures one fraction of the enzyme molecules; thus iodine reacts both with catalytically essential residues and with others involved in maintaining structural stability of the enzyme.

We have reported briefly (Li and Vallee, 1963) that preferential carboxymethylation of two out of twenty-four thiol groups[1] in liver alcohol dehydrogenase (LADH)[2] results in complete loss of catalytic activity. DPN^+ and DPNH protect the enzyme against inactivation and chemical modification indicating that these groups are situated at the active centers. Peptides containing these active center residues have been isolated and their sequences characterized (Li and Vallee, 1964a; Harris, 1964).

The present report describes the effects of this modification upon the binding of coenzymes and substrates to liver alcohol dehydrogenase as well as the enzymatic and physicochemical consequences of oxidation of the SH groups with iodine. Like iodoacetate, iodine preferentially reacts with the active center SH groups but, in addition, it also induces a pronounced change in the structure of the protein. A preliminary communication containing some of the data has been published (Li and Vallee, 1963).

Materials and Methods

Crystalline alcohol dehydrogenase of horse liver was

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[1] The sulfhydryl content of different preparations of the enzyme has varied from 20 to 28 groups per mole of protein. The sulfhydryl titer of the enzyme employed in this study was 24 moles SH per mole of protein.

[2] Abbreviations: LADH, liver alcohol dehydrogenase; CM-LADH, carboxymethylated liver alcohol dehydrogenase; [(LADH)$^{65}Zn_2$], liver alcohol dehydrogenase with the two functional zinc atoms replaced by ^{65}Zn; DPN^+ and DPNH, oxidized and reduced forms of diphosphopyridine nucleotide, respectively.