CONVERSION OF 4-ANDROSTENEDIOL AND 5-ANDROSTENEDIOL
TO TESTOSTERONE, AND CONVERSION OF DEHYDROEPIAN-
DROSTERONE TO 4-ANDROSTENEDIOL BY RAT TESTIS
IN VITRO*

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SYNOPSIS

The whole testis tissue preparation of rat or man was incubated with androst-4-ene-3 β ,17 β -diol (4ADL) (20 or 40 μ g in one flask containing 1 or 2 testes) for 2 hrs. in Krebs-Ringer bicarbonate buffer, pH 7.4, in 95% O₂-5% CO₂ gas phase. A major product was proved as testosterone by means of paper and gas chromatographic analyses and by the derivative formation. Human chorionic gonadotropin (50 or 100 U in 1 flask) was shown to have a minor influence on the conversion of 4ADL to testosterone by rat testis *in vitro*.

Androst-5-ene-3 β ,17 β -diol (5ADL) was also converted to testosterone in the same rate as observed for 4ADL. Conversion of these 2 androstenediols to androst-4-ene-3,17-dione (4ADN) was observed to very minor extent compared to the conversion of dehydroepiandrosterone (DHEA) to 4ADN.

4-C¹⁴-DHEA was incubated with rat testis tissue preparation for 2 hrs. and 8 radioactive compounds converted from DHEA were separated on paper by extensive chromatography. Among these compounds, testosterone and 4ADN were identified by recrystallization with carrier steroid to constant specific activity. A compound behaving very similarly to the reference 4ADL on papers was separated. Oxidation of this compound with chromium trioxide yielded 2 products which showed the same mobilities as 4ADN and testosterone. An aliquot of the compound was added with carrier 4ADL and recrystallization showed the nearly constant specific activity. Testosterone zone derived from the compound by oxidation with chromium trioxide was added with carrier testosterone for recrystallization and the constant specific activity was demonstrated.

Biosynthesis of testosterone from progesterone via 17 α -hydroxyprogesterone and 4-androstenedione *in vitro* was firstly demonstrated in 1956 (Slaunwhite and Samuels, 1956). Since dehydroepiandrosterone was isolated from hog testis (Neher and Wettstein, 1960), an alternative biosynthetic pathway to testosterone via dehydroepiandrosterone has been paid an attention by several workers (Kahnt *et*

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Trivial names and abbreviations used: 4-androstenediol: androst-4-ene-3 β , 17 β -diol, 5-androstenediol: androst-5-ene-3 β , 17 β -diol, 4-androstenedione: androst-4-ene-3, 17-dione, dehydroepiandrosterone: 3 β -hydroxyandrost-5-ene-17-one, 17 α -hydroxyprogesterone: 17 α -hydroxypregn-4-ene-3, 20-dione.

al., 1961; Eik-Nes and Hall, 1962; Eik-Nes and Kekre, 1963; Mahesh and Greenblatt, 1962).

Lately, conversion of 3-hydroxy-C₁₉-steroids to testosterone has been studied by several workers. Conversion of 4-androstenediol as well as pregnenolone to testosterone was demonstrated *in vitro* by minced tissue preparation of mouse testis (Grosso and Ungar, 1964) and by human adrenal tissue (Colla *et al.*, 1964). 5-Androstenediol was also reported to convert to testosterone in rabbit testis homogenate (Rosner *et al.*, 1964), in dog testis (Ibayashi, 1965) and in homogenates of human placenta and adrenal cortical tumor tissue (Baulieu *et al.*, 1963; Baulieu and Robel, 1963).

The present report demonstrates that 4-androstenediol and 5-androstenediol are converted efficiently to testosterone *in vitro* by the rat whole testis preparation and that 4-androstenediol might be an intermediate between dehydroepiandrosterone and testosterone. Human chorionic gonadotropin is also reported to have a minor effect on the conversion of 4-androstenediol to testosterone *in vitro*.

MATERIALS AND METHODS

4-Androstenediol and 5-androstenediol were supplied by the Takeda Pharmaceutical Co. (Osaka) and the purity was checked by paper and gas chromatography. 4-C¹⁴-Dehydroepiandrosterone, specific activity 0.01 mc/0.0709 mg, and 4-C¹⁴-progesterone, specific activity 0.067 mc/mg, were purchased from the New England Nuclear Corp. (Mass., U.S.A.) and purified by paper chromatography before use.

Testis for incubation was obtained from mature rat of the Donryu strain, (body weight 170~240 g). Following decapitation, testes were removed from the body, freed of surrounding tissue, and weighed. Immediately after weighing, the testis was decapsulated and thin thread of blood vessel was removed. Whole testis tissue was incubated in 2.0 ml of Krebs-Ringer bicarbonate solution containing 200 mg% glucose (pH 7.4) at 38°±1° in 95% O₂-5% CO₂ gas phase. After 1 hr., the medium was sucked out and the substrate in 0.05 ml of 95% ethanol and fresh bicarbonate solution (2.0 ml) were added into the incubation flask containing the tissue. In general, the testis of right side was incubated with a substrate and the testis of left side was incubated without a substrate as a control. After adding the substrate and fresh medium, additional incubation for 2 hrs. was carried out under the same condition as in the pre-incubation.

After incubation, the incubation mixture was homogenized in a glass homogenizer and centrifuged. Aqueous phase was decanted, saved and finally put together with the ethanol extract of the tissue residue. The tissue residue was extracted 4 times with 2 ml of 95% ethanol. Ethanol was evaporated from the combined aqueous ethanol extract under nitrogen stream (recovery at this stage was 92% averagely for testosterone) and partitioned between 70% methanol and light petroleum to remove lipid. Methanol was evaporated from 70% methanol fraction and extracted the aqueous phase with dichloromethane 4 times. The dichloromethane extract was dried over under nitrogen stream and put on the paper for chromatography.

Whatman No. 1 paper for chromatography was previously washed with benzene-methanol mixture in a Soxhlet apparatus for 4 days and used for the separation of steroids in dichloromethane extract. The ligroine-methanol-water (10:8:2 by volume) (Bush, 1954) was employed for the separation of testosterone. Ligroine-methanol-water, ligroine-benzene-methanol-water (33:17:40:10 by volume), and benzene-methanol-water (10:5:5 by volume) of Bush and ligroine-formamide system and cyclohexane-formamide system of Zaffaroni type (Zaffaroni *et al.*, 1950) were used for the separation of steroids converted from labelled progesterone and dehydroepiandrosterone.

Following chromatography ultraviolet absorbing spot corresponding to reference testosterone (R_f 0.26~0.3 in ligroine-methanol-water system) was located under an ultraviolet scanner and eluted. The eluate was then purified by microcolumn of alumina. The eluate was deposited on top of a column of 1 g of alumina (Merck, washed thoroughly with ethanol, dried and activated at 110°C for 3 hrs.) packed in Pasteur pipette with several portions of hexane containing a very small amount of dichloromethane. The column was first eluted with hexane (10 ml), then with 20% dichloromethane in hexane (10 ml), and finally with 25% methanol in dichloromethane (10 ml) which was saved for determination by ultraviolet absorption.

The amount of testosterone in the eluate purified through alumina was determined by ultraviolet absorption at 240 $m\mu$ with the Beckman type spectrophotometer. Methanol solution of the material (methanol was purified by refluxing on 2, 4-dinitrophenylhydrazine and redistilled) was placed in microcuvette and its absorptions between 220 and 260 $m\mu$ were read against a paper blank. The correction according to Allen was made using the absorptions at 220, 240 and 260 $m\mu$ (Allen, 1950) and the amount was determined against a known amount of standard testosterone.

Identification of the ultraviolet absorbing material as testosterone was made by paper chromatographic analysis in different systems for free, acetylated material (procedure of acetylation used in this report is consisted of adding several drops of acetic anhydride-pyridine mixture, 1:2 by volume, on the dried sample and standing overnight at room temperature), and oxidized material (oxidation was done with saturated chromium trioxide solution in glacial acetic acid for 5 mins.) and by gas chromatographic analysis for free and acetylated materials. Gas chromatography was performed on a 150 cm steel column using a hydrogen flame ionization detector (Shimazu, Japan).

In the experiments with radioactive substrates, radioactivity on paper strips was located with the aid of an automatic chromatogram scanner (Model JPC-101, Nihon Musen Co., Tokyo) and radioactivity in solutions was measured with a Tri-carb liquid scintillation spectrometer (Model 3224, Packard Instruments) using a standard toluene scintillation solution.

Table 1. Recrystallization of testosterone and 4-androstenedione converted from 4-C¹⁴-dehydroepiandrosterone by rat testis tissue

Sample	No. of recrystallization	mg	disintegrations/min.	disintegrations/min./mg
A (testosterone)	1	15.9	8315	523
	2	12.5	5887	471
	3	8.4	3856	459
	4	6.1	2751	451
B (4-androstenedione)	1	14.5	1218	84
	2	8.5	646	76
	3	6.6	475	72
	4	5.1	326	64

Sample A: 22 mg of testosterone added as carrier before 1st recrystallization to an aliquot of testosterone zone.

Sample B: 21.5 mg of 4-androstenedione added as carrier before 1st recrystallization to an aliquot of 4-androstenedione zone.

Solvents used for recrystallization: 1—hexane-dichloromethane, 2—methanol-water, 3—ethanol-water, 4—hexane.

Identification of radioactive steroids was based on the migration in several kinds of paper chromatography for free and acetylated steroid against reference steroid, and recrystallization with carrier steroid to constant specific activity within an error of 15% (Table 1).

RESULTS

In incubations of testis with 20 to 50 μ g of 4-androstenediol, 5-androstenediol, and dehydroepiandrosterone, a major spot absorbing ultraviolet ray on the paper corresponded in mobility to the reference testosterone run simultaneously. This ultraviolet absorbing material in testosterone zone was pooled and subjected to the identification work. R_f of the material was 0.3 in ligroine-methanol-water system which corresponded to 0.3 of the reference testosterone, and 0.64 in ligroine-benzene-methanol-water system, well agreeing the reference testosterone. Acetylation of the material changed its mobility to R_f 0.8 in ligroine-formamide system, which was the same as the reference testosterone acetylated. Oxidation product with chromium trioxide showed R_f 0.74 in ligroine-methanol-water system which coincided with 4-androstenedione and the reaction product of oxidized material with m-dinitrobenzene in alkaline medium showed the absorption maximum at 510 $m\mu$ indicating positive Zimmermann reaction. The ultraviolet absorption maximum of the material was at 240 $m\mu$ (Fig. 1) and the retention time of the

Fig. 1. Ultraviolet absorption spectra of testosterone converted from 4-androstenediol by the rat testis *in vitro*. Dotted line indicates the absorption spectrum of the reference testosterone.

Fig. 2. Tracings of gas chromatographic analyses of testosterone zone obtained from rat testis incubations with 4-androstenediol or 5-androstenediol. Gas chromatographic unit employed was Shimazu (Japan), Type HFD-1B with hydrogen flame ionization detector; steel column- 150 cm length by 4 mm I.D.; coating, 1.5% SE-30 on Chromosorb W, 80/100 mesh; gas- N_2 0.65 l/min., H_2 30 cc/min., air 50 cc/min.; temperatures: column $220^\circ C$, detector $250^\circ C$, vaporizer $300^\circ C$. Two microliters of sample injected. (1) Standard testosterone with cholestane. (2) Testosterone zone converted from 4- and 5-androstenediols. (3) Mixture of (1) and (2).

material in gas chromatography was the same as that of the reference testosterone (Fig. 2). From data indicated above, a major product from androstenediols and dehydroepiandrosterone by incubation with rat testis tissue was recognized as testosterone.

Table 2 illustrates the result of experiment in which 20 or 40 μg of 4-androstenediol was incubated with the whole testis tissue of a rat and man. The amounts of testosterone and 4-androstenedione produced were determined by ultraviolet absorption analysis. In the incubation of 40 μg of 4-androstenediol without tissue, there was no conversion to testosterone found and 26.2 μg of 4-androstenediol was recovered. 4-androstenediol gave yellowish product with ethanol-sulfuric acid (1:2 by volume) reagent, having absorption maximum at 410 $m\mu$ and this reaction was used for the determination of unchanged 4-androstenediol. In all incubations, the control testis (left side) incubated without substrate produced very small amount of testosterone during 2 hrs.' incubation (0.33~2.13 μg /testis). Testis of the other side incubated with 20 or 40 μg of 4-androstenediol, however, produced a quite detectable amount of testosterone (5.62~10.56 μg /testis). Testis from 75-year-old man with prostate hypertrophy was incubated with 40 μg of 4-androstenediol and produced 0.5 μg of testosterone. After treat-

Table 2. Conversion of 4-androstenediol (4 ADL) to testosterone *in vitro* by testis tissue of rat and man

Incubation No.	Testis from	Testis weight (g)	Substrate (μ g)	Steroid determined (μ g/testis)		
				Testosterone	4ADN*	4ADL**
1	Normal rat	0.94	No subst.	2.13	<0.5	
		0.94	4ADL 20	5.68	1.01	1.16
2	<i>ibid.</i>	1.01	No subst.	1.45	—	
		1.02	4ADL 40	5.62	0.71	13.21
3	<i>ibid.</i>	1.15	No subst.	1.60	—	
		1.15	4ADL 40	10.56	—	
4	<i>ibid.</i>	1.05	No subst.	0.33	—	
		1.05	4ADL 40	9.35	—	
5	<i>ibid.</i>	1.1	No subst.	0.79	—	
		1.1	4ADL 40	8.75	—	
6	<i>ibid.</i>	1.1	No subst.	0.91	—	
		1.15	4ADL 40	9.22	—	
7	Man, 75-year-old	3.2				
		1.0	No subst.	<0.5	—	
		1.0	4ADL 40	0.5	—	
		4.5***				
		1.0	No subst.	13.1	<0.5	
		1.0	4ADL 40	19.9	<0.5	
8	—	No tissue	4ADL 40	—	—	26.2
9	—	No tissue	Testosterone 40	27.2	—	—

* 4 ADN: 4-androstenedione.

** 4 ADL: 4-androstenediol unchanged.

*** Testis removed after treatment with 5,000 I.U. of human chorionic gonadotropin.

Table 3. Effect of human chorionic gonadotropin (HCG) on conversion of 4-androstenediol (4 ADL) to testosterone *in vitro* by testis tissue of hypophysectomized or normal rat

Incubation No.	Testis from	Testis weight (g)	Substrate (μ g)	HCG (U.)	Steroid determined (μ g/testis)		
					Testosterone	4ADN*	4ADL**
10	Normal rat	1.12	4ADL 50	No HCG	8.92	<0.5	
		1.11	4ADL 50	50	9.47	<0.5	
11	<i>ibid.</i>	0.99	4ADL 50	No HCG	9.31	1.12	11.75
		0.99	4ADL 50	50	11.41	1.56	8.09
12	Hypox rats	1.11+1.10	No subst.	No HCG	0.23	—	
		1.08+1.10	No subst.	100	1.76	—	
13	<i>ibid.</i>	0.98+1.0	4ADL 40	No HCG	10.3	<0.5	
		0.98+1.0	4ADL 40	100	13.1	<0.5	
14	Hypox rat	0.98	No subst.	No HCG	1.01	—	
		0.99	4ADL 40	50	6.44	0.76	10.81
15	<i>ibid.</i>	1.12	4ADL 20	No HCG	3.81	<0.5	
		1.12	4ADL 20	50	3.81	1.16	
16	—	No tissue	4ADL 40	No HCG	—	—	22.40

Hypophysectomized rat (Hypox rat) was decapitated 17–20 hrs. after operation.

* 4ADN: 4-androstenedione.

** 4ADL: 4-androstenediol unchanged.

ment with 5,000 units of human chorionic gonadotropin, testis of the other side was removed and incubated with and without substrate. Control incubation of 1.0 g testis tissue from 4.5 g of total mass without substrate produced 13.1 μ g of testosterone per testis and the amount increased to 19.9 μ g by adding 40 μ g of 4-androstenediol as substrate in the incubation flask.

Addition of 50 to 100 units of human chorionic gonadotropin (Schering) into the incubation medium seemed to give a minor influence to the conversion of 4-androstenediol to testosterone *in vitro*, as shown in Table 3.

Table 4 shows the conversion of 5-androstenediol and dehydroepiandrosterone

Table 4. Conversion of 5-androstenediol (5 ADL) and dehydroepiandrosterone (DHEA) to testosterone *in vitro* by rat testis tissue

Incubation No.	Testis from	Testis weight (g)	Substrate (μ g)	Steroid determined (μ g/testis)		
				Testosterone	4ADN*	5ADL**
17	Normal rat	1.10	No subst.	0.5	—	
		1.10	5 ADL 40	7.9	<0.5	
18	Hypox*** rat	1.21	4ADN 40	15.2	6.8	
		1.20	5ADL 40	12.6	0.5	
19	<i>ibid.</i>	1.20	No subst.	0.5	—	
		1.21	DHEA 40	12.4	6.3	
20	<i>ibid.</i>	1.16	4ADN 40	16.6	8.8	
		1.18	DHEA 40	10.1	6.8	
21	—	No tissue	5ADL 40	—	—	24.2
22	—	No tissue	4ADN 40	—	28.3	

* 4ADN: 4-androstenedione.

** 5ADL: 5-androstenediol unchanged.

*** Hypox rat: Hypophysectomized rat 17–20 hrs. after operation.

to testosterone by rat testis tissue *in vitro*, in which 7.9 and 12.6 μ g of testosterone were found in the incubates with 40 μ g of 5-androstenediol and these values were almost the same as 10.1 and 12.4 μ g found in the incubates with 40 μ g of dehydroepiandrosterone. These values, however, were less than 15.2 and 16.6 μ g of testosterone found in the incubates with 40 μ g of 4-androstenedione as substrate.

For the purpose of clarifying the position of 4- or 5-androstenediol in the biosynthetic sequence of testosterone in rat testis, 20.8×10^4 disintegrations/min. of 4-C¹⁴-dehydroepiandrosterone was incubated with 2 whole testis tissues for 2 hrs. By separation with several systems of paper chromatography, drawn schematically in Figure 3, 8 radioactive compounds were isolated from the incubation mixture (Table 5). Main product was testosterone, whose percentage conversion was 22.5% and which was finally identified by recrystallization to constant specific activity with carrier testosterone (Table 1).

A compound behaving like reference 4-androstenediol on different kinds of paper chromatography (Fraction 4) was isolated with 9.3% of conversion rate from dehydroepiandrosterone which was about twice more than 4.5% of 4-andros-

Fig. 3. Paper chromatographic separation of products converted from 4-C¹⁴-dehydroepiandrosterone by rat testis tissue.

(1) Cyclohexane-benzene (2:1)/formamide (2) Ligroine-methanol-water (10:8:2)
 (3) Ligroine-benzene-methanol-water(33:17:40:10) (4) Benzene-methanol-water
 (2:1:1) OR: origin SF: solvent front T: testosterone 4AND: 4-androstenedione
 4ADL: 4-androstenediol DEA: dehydroepiandrosterone 17P: 17 α -hydroxyprogesterone
 T.a.: testosterone acetate Ac.: acetylation.

tenedione. This compound was acetylated and showed the same mobility as the reference acetylated 4-androstenediol. The oxidized products of Fraction 4 with chromium trioxide moved with the reference testosterone and 4-androstenedione (Fig. 4).

An aliquot of Fraction 4 was added with cold 4-androstenediol (19.7 mg) and another aliquot was added with cold 5-androstenediol (19.7 mg), and recrystallizations were carried out using 3 different solvents. Testosterone zone derived from Fraction 4 by oxidation with chromium trioxide (Fig. 4) was added with carrier testosterone (15 mg) and recrystallization was performed using 4 different solvents. Table 7 shows the results of recrystallization. Specific activities in 4-androstenediol crystals were 244,189, and 194 disintegrations/min./mg and average was 209 disintegrations/min./mg. Although 244 was 16.7% higher than the aver-

Table 5. Steroids converted from 4-C¹⁴-dehydroepiandrosterone by rat testis tissue *in vitro*

Fraction No.	Radioactivity in final fraction (disintegrations per min.)	Rate of conversion (%)	Structure identified
1	9,570	7.63	Not identified
2	14,600	11.64	<i>ibid.</i>
3	15,620	12.42	<i>ibid.</i>
4	11,700	9.31	4-androstenediol
5	8,500	5.90	Not identified
6	32,210	22.49	Testosterone
7	6,710	5.49	Dehydroepiandrosterone unchanged
8	5,500	4.51	4-androstenedione

20.8 × 10⁴ disintegrations/min. of 4-C¹⁴-dehydroepiandrosterone was incubated with 2 testes of normal rat.

Fig. 4. Tracing of radioactivity on paper chromatogram of 4-androstenediol zone obtained from incubation of rat testis with 4-C¹⁴-dehydroepiandrosterone. Paper-chromatogram scanner employed was Nihon Musen Co. (Japan), Model JPC-101 with 4 π gas flow counters.

(A) Fourth chromatogram with ligroine-benzene-methanol-water (33:17:40:10) system, scale 1K, time constant 3, speed 12.5 mm/min.

(B) Fifth chromatogram after oxidation by chromium trioxide with ligroine-methanol-water (2:1:1) system, scale 0.3K, time constant 3, speed 12.5 mm/min.

OR: origin, SF: solvent front, 4ADL: 4-androstenediol.

Table 6. Paperchromatographic mobilities of Fraction 4 converted from 4-C¹⁴-dehydroepiandrosterone by rat testis tissue *in vitro*

System of Paperchromatography	Mobility (R _f)	
	Fraction 4	Reference 4-androstenediol
1. Ligroine-methanol-water (10:8:2)	on origin	on origin
2. Ligroine-benzene-methanol- water (33:17:40:10)	0.32	0.32
3. Benzene-methanol-water (2:1:1)	0.82	0.82
4. Ligroine-benzene-methanol- water (33:17:40:10) after acetylation	0.89	0.88

Table 7. Recrystallization of 4-androstenediol converted from 4-C¹⁴-dehydroepiandrosterone by rat testis tissue

Sample	No. of recrys- tallization	Solvent	Disinteg- rations/ min.	mg	disintegrations/ min./mg
A	1	Hexane	3708	15.2	244
	2	Methanol-water	2343	12.4	189
	3	Ethanol-water	1571	8.1	194
B	1	Hexane	1361	16.4	83
	2	Methanol-water	570	11.8	48
	3	Ethanol-water	197	9.4	21
C	1	Hexane-CH ₂ Cl ₂	2060	13.2	156
	2	Methanol-water	1733	11.4	152
	3	Ethanol-water	1098	8.2	134
	4	Hexane	950	6.5	146

Sample A : 19.7 mg of 4-androstenediol added as carrier before 1st recrystallization to an aliquot of 4-androstenediol zone.

Sample B : 19.7 mg of 5-androstenediol added as carrier before 1st recrystallization to an aliquot of 4-androstenediol zone.

Sample C : 15 mg of testosterone added as carrier before 1st recrystallization to an aliquot of testosterone derived from 4-androstenediol zone by chromium trioxide oxidation.

age, it might be considered that these values show fairly constant specific activities. Recrystallization of testosterone zone of Fraction 4 after oxidation with carrier testosterone revealed that this was testosterone. The other hand, constant specific activity was failed to show for 5-androstenediol.

In the other experiment, 20 μ g of cold 17 α -hydroxyprogesterone mixed with 10.4×10^4 disintegrations/min. of 4-C¹⁴-dehydroepiandrosterone was incubated with 2 whole testis tissues of rat for 2 hrs. Specific activities of 4-androstenedione and testosterone were determined after separation by paper chromatography, as shown in Figure 3. Specific activity found in testosterone was 3,369 disintegrations/min./

μg which was about 3 times higher than 1,074 disintegrations/min./ μg found in 4-androstenedione.

In the incubation of six whole testis tissues from 3 rats with 2 μC of 4- C^{14} -progesterone, neither 4- nor 5-androstenediol was isolated. As Δ^4 -3-hydroxysteroid, pregn-4-ene-3, 20-diol alone was nearly identified.

DISCUSSION

In our study, formation of testosterone from 4-androstenediol, 5-androstenediol, and dehydroepiandrosterone was readily demonstrated by incubation with whole testis tissue preparation from a rat and man. Formation of 4-androstenedione from these 2 androstenediols was observed to a minor extent compared to its formation from dehydroepiandrosterone. It was demonstrated by Grosso and Ungar that formation of testosterone occurred primarily and 4-androstenedione in minor amount from 4-androstenediol by incubation with mouse testis (Grosso and Ungar, 1964). Human adrenal tissue also had the capacity to convert 4-androstenediol to testosterone and 4-androstenedione (Colla *et al.*, 1964). An enzyme in supernatant fraction of rat liver homogenate could convert 4-androstene-3 α , 17 α -diol and 3 β , 17 β -diol to testosterone (Ungar *et al.*, 1957). Concerning the conversion of 5-androstenediol to testosterone, rat testis (Savard and Dorfman, 1954), human placenta and adrenocortical tumor tissues (Baulieu *et al.*, 1963; Baulieu and Robel, 1963), rabbit testis (Rosner *et al.*, 1964) and dog testis (Ibayashi, 1965) have been shown to have the capacity and 5-androstenediol has been postulated to be an intermediate in testosterone biosynthesis by these workers and others (Hagen and Eik-Nes, 1964; Ellis and Berliner, 1965). Conversion of dehydroepiandrosterone to testosterone became of interest since these steroids were isolated from hog testis (Neher and Wettstein, 1960). Biosynthetic sequence for the formation of testosterone from pregnenolone through 17 α -hydroxypregnenolone and dehydroepiandrosterone has been postulated (Eik-Nes, 1962; Mahesh and Greenblatt, 1962; and others).

As stated by Grosso and Ungar, the position of 4-androstenediol as well as 5-androstenediol in the metabolism of testosterone seems to be a quite problem. Previously, Δ^5 -3 β -hydroxysteroid dehydrogenase activity was demonstrated in rat testis (Samuels *et al.*, 1951). This enzyme can convert pregnenolone to progesterone, and dehydroepiandrosterone to 4-androstenedione. In rat testis, this enzyme would also convert 5-androstenediol to testosterone, and if specificity of the enzyme for substrate is not rigid, this might also be able to convert 4-androstenediol to testosterone. In rat testis, as in mouse testis (Grosso and Ungar, 1964), it seems that the oxidation of 17 β -hydroxy group to 17-ketone was not efficient or equilibrium of 17-ketone to 17 β -hydroxyl highly favored the reduced form. In our study, 4- and 5-androstenediol converted, to a very minor extent, to 4-androstenedione, and dehydroepiandrosterone converted, to greater extent than androstenediol, to 4-androstenedione by rat testis.

A question on 4-androstenedione as an obligatory intermediate in biosynthesis of testosterone has been raised recently by several workers. H^3/C^{14} was much higher in testosterone than in 4-androstenedione by incubation of rabbit testis homogenate (Rosner *et al.*, 1964), by infusion *in vivo* of dog testis (Hagen and

Eik-Nes, 1964) with a mixture of 7- H^3 -dehydroepiandrosterone and 4- C^{14} - 17α -hydroxyprogesterone. Biosynthesis of testosterone from 17α -hydroxypregnenolone not involving either dehydroepiandrosterone or 4-androstenedione in rat testis has been suggested by kinetic study (Slaunwhite and Burgett, 1965). In our study, specific activity in testosterone was always found to be much higher than that in 4-androstenedione in the incubations of whole testis tissue preparation of rat with a mixture of 17α -hydroxyprogesterone and 4- C^{14} -dehydroepiandrosterone. Moreover, 4- and 5-androstenediol were efficiently transformed, to great extent, to testosterone and a steroid very like 4-androstenediol was separated from incubation with 4- C^{14} -dehydroepiandrosterone. In the incubation with 4- C^{14} -testosterone, compound like 4-androstenediol was not separated. From these results we obtained, it might be possible that there exists a biosynthetic route to testosterone from dehydroepiandrosterone involving 5- and 4-androstenediol as intermediates, not involving 4-androstenedione in rat testis.

SUMMARY

Rat testis tissue was incubated with 4-androstenediol. Conversion to testosterone was proved by means of paper chromatographic and gas chromatographic analyses and derivatives formation. Conversion of 5-androstenediol to testosterone was also demonstrated in a rat testis. 4- and 5-androstenediol were converted to 4-androstenedione to very minor extent.

Minor stimulatory effect of human chorionic gonadotropin on the conversion of 4-androstenediol to testosterone was observed *in vitro*.

4- C^{14} -dehydroepiandrosterone was incubated with a rat testis tissue and a compound nearly identified as 4-androstenediol was demonstrated to be converted from dehydroepiandrosterone.

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