Norethisterone does not inhibit rat testis Δ^5- 3β-hydroxysteroid dehydrogenase

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1. INTRODUCTION

A key step in the biosynthesis of progesterone and of androgens is the production of Δ^4-3-ketosteroids from Δ^5-3β-hydroxysteroids [1]. This enzymatic conversion is a two step process in which dehydrogenation is followed by isomerization [2]. The first step is rate limiting, and the enzyme which mediates the dehydrogenation is Δ^5-3β-hydroxysteroid dehydrogenase (EC 1.1.1.145); it can be found in extracts of *Pseudomonas testosteroni* [3] and is localized in the microsomes and mitochondria of all steroid synthesizing organs such as adrenal, ovary, placenta and testis [4].

The enzymes from mammalian and bacterial sources were reported to exhibit similar properties [5] and were found to be inhibited by estrogens, progestins, androgens and steroidal analogues [5,16]. Genital defects such as hypospadias in male infants were reported to be associated with the administration of progestins and estrogens to pregnant women or female animals [9]. Inhibition by these steroids of Δ^5-3β-hydroxysteroid dehydrogenase has been assumed to cause hypospadias [9,10,17].

Here, we have studied the effects of some progestins and estrogens on rat testis Δ^5-3β-hydroxysteroid dehydrogenase activity using both dehydroepiandrosterone and pregnenolone as substrate.

2. MATERIALS AND METHODS

All steroids used in this study were obtained from Schering AG (Berlin) and were dissolved in ethanol and stored at – 20°C. The cyanoketone used was 2α-cyano- 4,4,17α-trimethylandrost-5-en-17β-ol-3-one. β-Nicotinamide adenine dinucleotide was purchased from Sigma Chem. Co. (St Louis MO). All other chemicals were of analytical grade and were obtained from Sigma, Boehringer or Merck AG (Darmstadt).

2.1. *Enzyme extraction from rat testes*

Testes of adult male rats of the Sprague-Dawley strain (Himberg; Oncins, specific-pathogen free, 200–250 g body wt were obtained from Forschungsinstitut für Versuchstierzucht, University of Vienna. The animals were used 2 weeks after shipment. The rats were housed in a temperature-controlled room lit for 12 h/day and unrestricted access was provided to food and water. The rats were killed by decapitation and the testes were dissected, decapsulated and rinsed with ice-cold buffer 1 (50 mM phosphate buffer, 0.25 M sucrose, 7 mM dithiothreitol, 0.5 mM EDTA, pH 7.4). All further procedures were done at 0–4°C. Tissue (1 g/ml buffer 1) was homogenized by 10 strokes at 1000 rev./min in a glass–Teflon Potter Elvehjem-type homogenizer. The homogenate was centrifuged at 800 × *g* for 10 min. The crude homogenate was centrifuged at 10 000 × *g* for 20 min and the supernatant was spun at 100 000 × *g* for 1 h to obtain the microsomal pellet. The microsomes were washed with buffer 1 and recentrifuged at 100 000 × *g* for 1 h. The pellet was resuspended by homogenizing it in 1 ml buffer 1/pair of testes to obtain 5–10 mg protein/ml. The suspension was stored at –20°C until used for enzyme assays.

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2.2. *Enzyme assay*

The enzyme preparation (300 μl) containing 1.5–4 mg protein was preincubated at 37°C with 750 μl buffer (0.2 M pyrophosphate, pH 8.9) 200 μl NAD solution (5×10^{-2} M, pH 7) and 1550 μl water. The reaction was started with dehydroepiandrosterone or pregnenolone as substrate dissolved in ethanol. The total volume of ethanol did not exceed 100 μl, and the blank contained all components except for the substrate. For the determinations of inhibition constants the reaction mixture was preincubated with the steroid to be tested prior to the addition of substrate. Absorbance was recorded at 340 nm by a Beckman spectrophotometer model DU-8 kinetic analysis system (Beckman Instr., Irvine CA) at 37°C. ΔA/min was registered 3 times through 3 min intervals and calculated as means of 3 such determinations.

2.3. *Other techniques*

Michaelis constants were determined by linear regression analysis of Lineweaver Burk [18] data and inhibition constants were calculated from Dixon-plot [19] data by linear regression on a Dec Data System 34/11 (Digital Equipment Corp., Maynard, MA). Protein measurements were done as in [20] using bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

Whether or not a single Δ^5-3β-hydroxysteroid dehydrogenase metabolizes various substrates such as dehydroepiandrosterone and pregnenolone has been studied in many laboratories. The present investigation was done to delineate possible differences between C-19 and C-21 testicular deydrogenases and to investigate whether Δ^5-3β-hydroxysteroid dehydrogenase is inhibited by estrogens and progestins. Data on the Michaelis constants (table 1) add further evidence to the suggestion [21–24] that Δ^5-3β-hydroxysteroid dehydrogenases mediating the dehydrogenation of C-19 and C-21 steroids in the testes are identical: equimolar mixtures of dehydroepiandrosterone and pregnenolone did not give additive rates, but maximal velocities intermediate between those of the separate substrates. However, multiple Δ^5-3β-hydroxysteroid dehydrogenases in bovine ovarian microsomes were proposed in [21]. Experiments on hydride-ion transfer from the 2 substrates to NAD^+ suggested the presence of 1 enzyme which metabolized only dehydroepiandrosterone and 1 enzyme which used both pregnenolone and dehydroepiandrosterone [21]. Evidence for multiple dehydrogenases may raise many im-

Table 1

Determination of Michaelis constants of Δ^5-3β-hydroxysteroid dehydrogenase from rat testes microsomes for various substrates

Steroid	K_m ($M \times 10^{-6}$)
Dehydroepiandrosterone	5.1
Pregnenolone	2.3
Dehydroepiandrosterone plus pregnenolone (equimolar)	3.4

Rat testes microsomal enzyme preparation (300 ml) containing 1.5–4 mg protein was incubated with various concentrations of different substrates in the presence of 5×10^{-3} M NAD. Data are given as means of 3 different experiments

Table 2

Determination of apparent inhibition constants (K_i) of Δ^5-3β-hydroxysteroid dehydrogenase for various steroids using dehydroepiandrosterone (5.78×10^{-6} M or 46.24×10^{-6} M) or pregnenolone (5.27×10^{-6} M or 42.12×10^{-6} M) as substrate

Steroid	K_i ($M \times 10^{-6}$)	
	Dehydroepiandrosterone	Pregnenolone
Norethisterone	n.i.	n.i.
Norethisterone acetate	150	175
Diethylstilbestrol	63	29[a]
Progesterone	n.i.	n.i.
Cyproterone acetate	47	n.i.
Cyanoketone	0.03	0.03[a]
Androstenedione	30	40
17β-Estradiol	n.i.	n.d.
17α-OH-Progesterone	n.d.	n.i.

[a] non-competitive

n.i., not inhibitory; n.d., not determined

Steroids were preincubated with 300 μl enzyme preparation containing 1.5–4 mg protein in the presence of 5×10^{-3} M NAD. Data are given as means of two different experiments

portant questions concerning the distribution of the enzymes in different cell types and the possibility of different effects of trophic hormones. These data suggest a single enzyme but examination of stereospecificity of hydride transfer may yield a more definitive answer to the question.

Of major interest is the observation that norethisterone does not inhibit testicular Δ^5-3β-hydroxysteroid dehydrogenase, when dehydroepiandrosterone or pregnenolone is used as substrate (table 2). Inhibition by progestins of this enzyme was proposed for the development of hypospadias in male infants born from females who were treated with norethisterone or other synthetic progestagens [17]. This assumption was made on grounds of results of experiments in which male fetuses of pregnant rats treated with these steroids were found to develop hypospadias. The effect of progestins on sexual organogenesis was thought to be analogous to that observed in infants born with a severe form of congenital adrenal hyperplasia due to genetic deficiency of Δ^5-3β-hydroxysteroid dehydrogenase.

In addition, injection of cyanoketone into pregnant rats was found to inhibit adrenal and testicular Δ^5-3β-hydroxysteroid dehydrogenase as noted by histochemical determinations and was registered to produce hypospadias [10]. But a recent review [25] concludes that adverse effects of synthetic progestins on the fetus had been shown in some, but not all, of the reports, and that any positive effects registered were small. Inhibition by norethisterone of testicular of Δ^5-3β-hydroxysteroid dehydrogenase activity could not be detected by the present assay system when dehydroepiandrosterone or pregnenolone were used as substrates (table 2). In addition, no great differences were noted between the

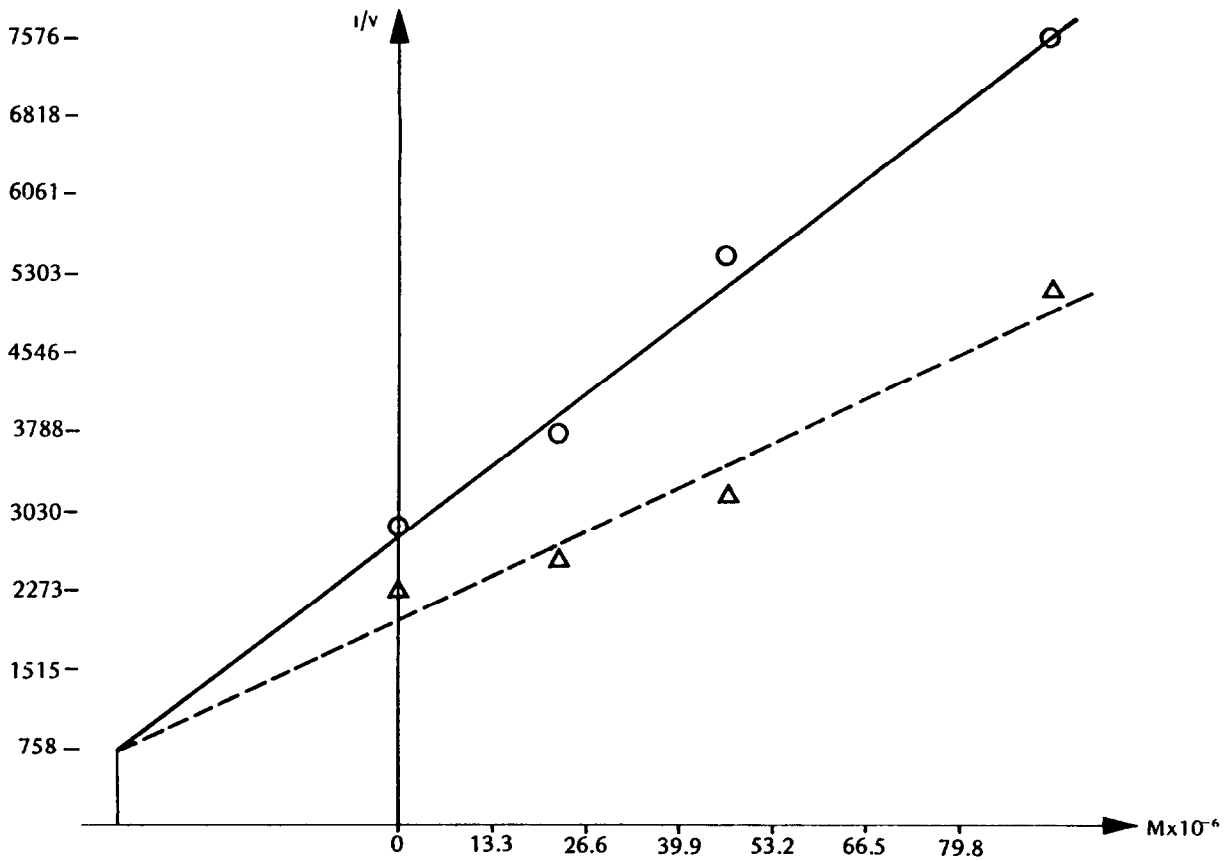

Fig.1. Dixon plot of inhibition by androstenedione of testicular Δ^5-3β-hydroxysteroid dehydrogenase: (○) 5.2×10^{-6} M pregnenolone; (△) 42.18×10^{-6} M pregnenolone: V = velocity in mU . min $^{-1}$. mg protein $^{-1}$.

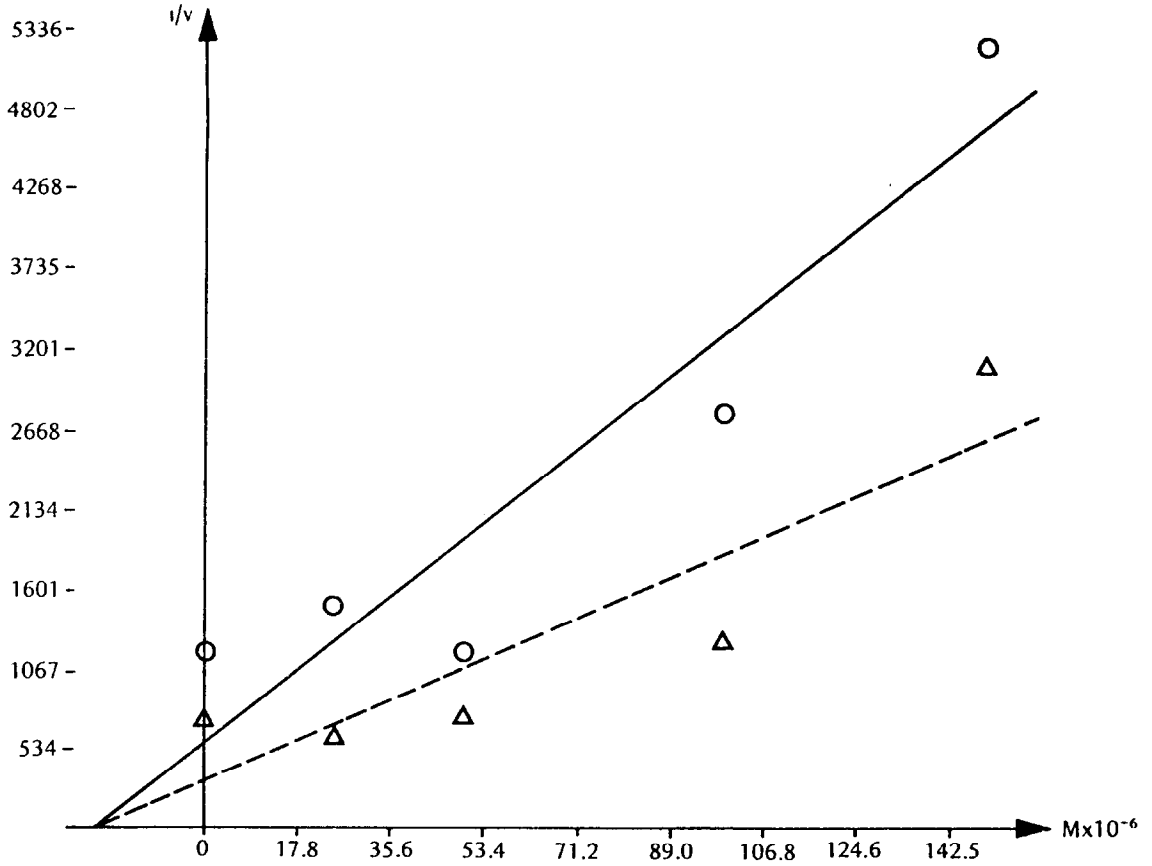

Fig.2. Dixon plot of inhibition by diethylstilbestrol of rat testis Δ^5-3β-hydroxysteroid dehydrogenase, pregnenolone: (○) = 5.2 × 10^{-6} M; (△) = 42.18 × 10^{-6} M; V = velocity in mU . min^{-1} . mg protein $^{-1}$.

apparent inhibition constants registered for the dehydroepiandrosterone and prenenolone system, respectively. The only apparent difference was found for cyproterone acetate which was slightly inhibitory when dehydroepiandrosterone was used as substrate, but no inhibition was observed for the pregnenolone system (table 2). A residual affinity of norethisterone acetate for Δ^5-3-β-hydroxysteroid dehydrogenase was observed. Since the affinity of norethisterone acetate is only 1% of the K_m and since the acetate is split very rapidly in vivo, no effect of the progestin on the enzyme system is expected to take place in vivo. In addition, androsteronedione was observed to competitively inhibit the enzyme system (fig.1). Accumulation of this intermediate could, therefore, act on both the dehydroepiandrosterone as well as pregnenolone system by feedback inhibition. Furthermore, a weak affinity of diethylstilbestrol for the enzyme was registered (fig.2) indicating that estrogenic substances may also inhibit testicular Δ^5-3β-hydroxysteroid dehydrogenase activity when pregnenolone is used as substrate. However, no inhibition was found for 17β-estradiol when dehydroepiandrosterone was used (table 2).

Both diethylstilbestrol (fig.2) and cyanoketone (fig.3) inhibited the enzyme in a non-competitive manner when the pregnenolone system was studied, whereas competitive inhibition was registered in the dehydroepiandrosterone assay system (table 2). These data and the differences found for cyproterone acetate may support the notion that more than one enzyme system is responsible for the conversion of C-19 and C-21 steroids. Non-competitive

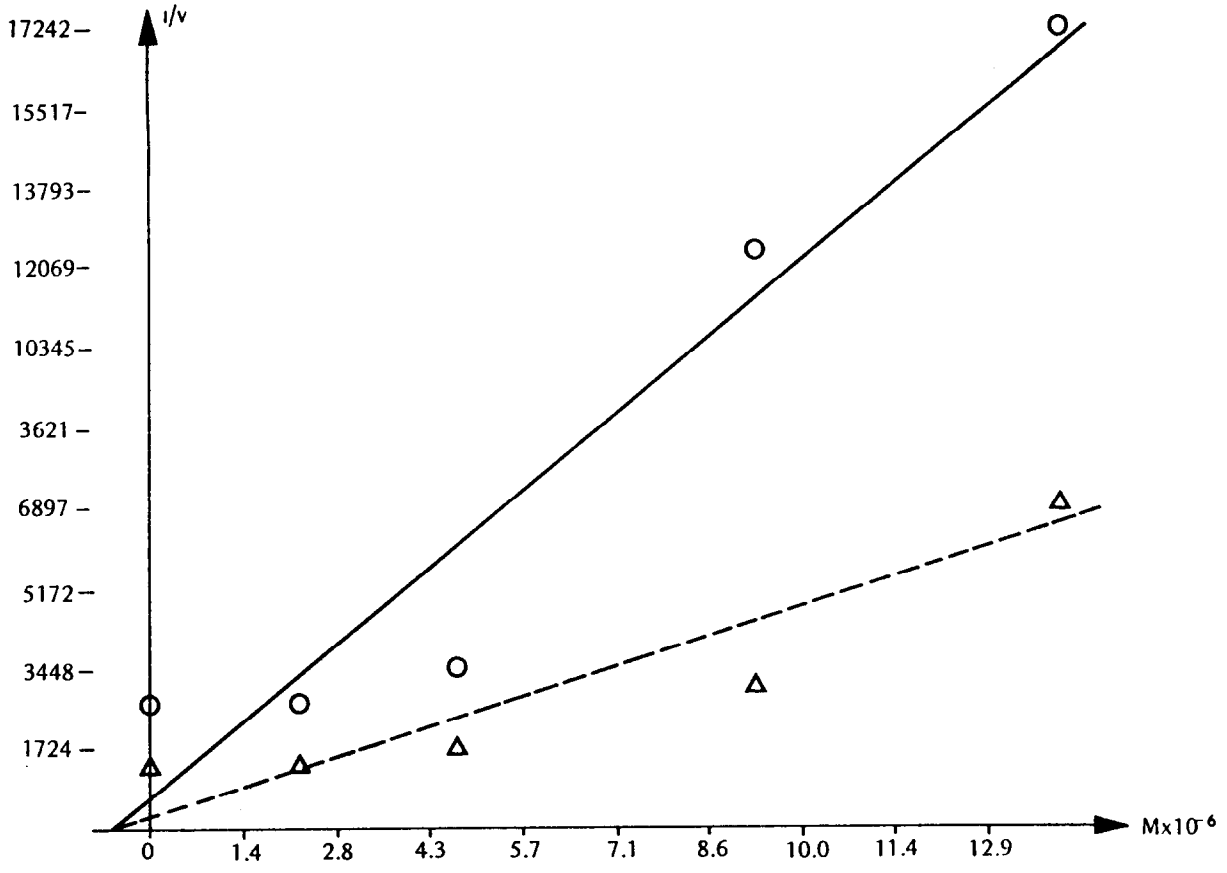

Fig.3. Analysis of inhibition by cyanoketone of testicular Δ^5-3β-hydroxysteroid dehydrogenase by Dixon plot. Pregnenolone: (○) 5.2 × 10^{-6} M; (△) 42.18 × 10^{-6} M; V = velocity in mU . min^{-1} . mg^{-1}.

inhibition by cyanoketone of *Pseudomonas testosteroni* Δ^5-3β-hydroxysteroid dehydrogenase was reported in [5].

These data combine to suggest that norethisterone does not inhibit testicular Δ^5-3β-hydroxysteroid dehydrogenase. Therefore, the notion that progestins are possible mediators of hypospadias by Δ^5-3β-hydroxysteroid dehydrogenase inhibition does no longer hold. In addition, whether there are >1 Δ^5-3β-hydroxysteroid dehydrogenases for the conversion of C-19 and C-21 steroids cannot be inferred from these data.

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