Stimulatory Effect of Estrogen Pretreatment on 3β-Hydroxysteroid Dehydrogenase Activity of Rat Testicular Tissue. (22574)

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Mammalian endocrine organs which produce steroidal hormones have been observed (1) to possess the enzyme 3-β-ol-dehydrogenase, which catalyzes the conversion of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids. This biosynthetic transformation is thought to be requisite for production of androgenic, gestogenic and adrenocortical hormones which possess in common the α, β-unsaturated ketone system in ring A of the steroid nucleus. Studies correlating the levels of 3β-ol-dehydrogenase activity of certain endocrine tissues with experimentally induced hormonal fluctuations would be expected to contribute information pertinent to a better understanding of biosynthetic mechanisms involving steroid hormone production.

The ability of testicular tissue from normal and estrogen pretreated rats to convert Δ^5-androstene-3β, 17β-diol to Δ^4-3-ketosteroid is here compared. Estrogen pretreatment was employed to effect what might be called a "chemical castration" induced via an inhibition of the pituitary output of gonadotrophins (2). The failure in testicular androgen production brought about by the estrogen pretreatment was evidenced by atrophy of the sex accessory organs. Consequently, a correlation may be made of the levels of 3β-ol-dehydrogenase activity of testicular tissue with the amount of androgenic hormone produced by that tissue.

Methods and materials. Estrogen Pretreatment. Male Sprague-Dawley rats ranging in weight from 160 to 195 g were divided into groups of 4 to 8 animals and fed commercial balanced diet and water *ad libitum*. All estrogenic substances were administered subcutaneously for 2 weeks. Diethylstilbestrol dipalmitate (DESDP) was given both as pellet implants and as a solution in cottonseed oil. The pellets were implanted under the skin in the lumbar region, one 5 mg pellet on each side of the animal. Estradiol-17β was injected as a solution in cottonseed oil. The dose of estrogen was administered in 0.2 ml of oil with injections being given daily except Sundays. On the 15th day of the experiment, the rats were sacrificed under chloroform. The testes, seminal vesicles and ventral prostates were excised, cleaned of adherent fat and weighed. The testes after weighing were immediately quick-frozen over dry ice and stored in a deep freeze chest until the 3β-ol-dehydrogenase activity could be determined. *Preparation of homogenates.* The connective tissue tunic of the testes was stripped off while the organ was in the frozen state. The testes from 2 or 3 rats were pooled, weighed and homogenized in the serum-buffer medium described below. After thorough homogenization, sufficient medium was added to adjust tissue concentration so that the desired amount of tissue to be incubated was contained in 1 or 2 ml of homogenate. *The 3β-ol-dehydrogenase activity* of testicular homogenates was ascertained on the basis of amount of α, β-unsaturated ketone formed during incubation with Δ^5-androstene-3β, 17β-diol as substrate. The α, β-unsaturated ketone was determined spectrophotometrically* by the intensity of absorption at 240 mμ. Testosterone was employed as a reference standard. One or 2 ml of the homogenate were added to flasks containing 25 ml of equal parts of bovine serum and phosphate buffer pH 7.4 to which was added 2 μM Δ^5-androstene-3β, 17β-diol, 6 μM DPN and 1 mM niacinamide. The flask contents were oxygenated for one minute, stoppered, and incubated in a water bath for 2 hours at 38°C on a revolving spoked drum. At the end of incubation period, the contents of the flask

* The assistance of Madelyn Becker in spectrophotometric determinations is gratefully acknowledged.

TABLE I. Effect of Estrogens on Reproductive Organs of the Male Rat.

Treatment	Dose, μg/kg/day	No. of animals	Body wt (g), mean and range Initial	Final	Organ wt (mean ± S.E.) Testes	Seminal vesicles	Ventral prostate
None		6	178 (160–195)	235 (218–266)	2660 ± 128 (1150 ± 84)†	191 ± 15 (82 ± 7)	158 ± 17 (68 ± 7)
DESDP	Pellet implant‡	7	177 (160–188)	181 (167–198)	1460 ± 322* (810 ± 176)	50 ± 8* (30 ± 4)*	35 ± 9* (19 ± 2)*
None		8	178 (154–190)	218 (191–235)	2770 ± 106 (1270 ± 68)	179 ± 8 (85 ± 6)	176 ± 12 (80 ± 5)
DESDP	114	5	176 (160–184)	203 (192–213)	2580 ± 92 (1280 ± 51)	78 ± 12* (39 ± 6)*	82 ± 19* (41 ± 9)*
None		6	170 (162–182)	223 (204–248)	2710 ± 95 (1230 ± 54)	240 ± 16 (113 ± 6)	217 ± 18 (90 ± 6)
Estradiol-17β	.5	5	176 (171–185)	215 (200–231)	2520 ± 101 (1190 ± 70)	212 ± 18 (99 ± 8)	173 ± 18 (82 ± 9)
	2	6	175 (170–180)	203 (198–208)	2690 ± 112 (1330 ± 53)	153 ± 13* (76 ± 5)*	129 ± 7* (64 ± 4)*
	8	6	174 (170–180)	202 (190–214)	2680 ± 133 (1330 ± 56)	124 ± 12* (61 ± 5)*	121 ± 8* (61 ± 4)*
	32	6	177 (170–188)	210 (200–222)	2470 ± 117 (1180 ± 52)	106 ± 22* (58 ± 6)*	107 ± 13* (51 ± 9)*

* Statistically significant at the 5% level or less from the respective control mean.
† mg/100 g body wt and S.E.
‡ Two, 5 mg pellets implanted subcut. in lumbar region.

were boiled to stop enzymatic action. Procedure for extraction and chromatographic separation of the α, β-unsaturated ketone was as described by Wiswell and Samuels(3) with modifications as suggested by Samuels (personal communication). After boiling, the flask contents were extracted 6 times with 15 ml portions of ether. The pooled ether extracts were evaporated to dryness under nitrogen and the dried extracts were taken up in 30 ml of hexane and chromatographed on aluminum oxide. The fractions were eluted according to the following scheme: Fraction I, 30 ml hexane; Fraction II, 10 ml 10% chloroform in hexane; Fraction III, 75 ml 25% chloroform in hexane; Fraction IV, 50 ml chloroform. The α, β-unsaturated ketone content of Fraction III after preliminary purification was determined spectrophotometrically in 95% ethanol.

Results. Effects of diethylstilbestrol dipalmitate. Table I gives data on the effects of treatment on growth of animals and their reproductive organs. Alterations in testicular 3β-ol-dehydrogenase levels for corresponding groups are given in Table II. The subcutaneous implantation of two 5 mg pellets of DESDP to young adult male rats brought about a marked suppression of growth of the animals. Whereas control rats gained an average of 57 g/rat during the test period, DESDP treated males gained only 4 g/rat. The suppression of growth was accompanied by atrophy of testes and sex accessory organs. It should be pointed out, however, that the degree of testicular regression was only slightly greater than concurrently retarded growth rate of the animals.

The 3β-ol-dehydrogenase activity was elevated in the testicular homogenates from the DESDP pellet treated animals. The increase/g of tissue was of the order of 1.7-fold. However, since testes from treated rats were considerably smaller than control gonads, the amount of ketone formed by an amount of homogenate equivalent to the total paired testicular weight was no greater than that calculated for the control. It seemed imperative to determine to what extent DESDP would affect the 3β-ol-dehydrogenase activity of testicular homogenates when dose levels of this estrogen were given which would not alter the gross morphology of the testes yet be sufficiently large to curtail androgen production

TABLE II. Effect of Estrogen Pretreatment on 3β-ol-Dehydrogenase Activity of Rat Testicular Homogenate.

Treatment	Dose, μg/kg/day	No. of determinations	α,β-unsaturated ketone formed (testosterone equivalents) μg/g tissue/2 hr	Ratio, control: test	μg/pair testes/2 hr	Ratio, control: test
None		2	459		1068	
DESDP*	Pellet†	2	776	1:1.7	1130	1:1.06
None		4	392		1087	
DESDP	114	4	662	1:1.7	1709	1:1.6
None		4	348		943	
Estradiol-17β	.5	2	363	1:1.04	915	1:0.97
	2.	2	754	1:2.2	2012	1:2.12
	8.	2	668	1:1.9	1790	1:1.9
	32.	2	1250	1:3.6	3090	1:3.3

* Diethylstilbestrol dipalmitate.
† Two, 5 mg pellets implanted subcut. in lumbar region.

as manifested by the degree of sex accessory organ atrophy. It had been previously observed(4) that a daily dose of DESDP equivalent to 114 μg kg (20 μg 175 g rat) brought about a statistically significant regression in seminal vesicle and ventral prostatic growth at the end of 2 weeks but did not measurably alter the mass of the testes. This dose was selected for the present study. The expected organ weight changes with the use of 114 μg/kg/day of DESDP were obtained (Table I). The degree of atrophy of the sex accessory organs in this case was not maximal as judged by a comparison with the organ weights in the pellet implanted series; nevertheless, it was statistically significant ($P < .05$). The conversion of Δ^5-androstene-3β, 17β-diol by the testicular homogenates was again demonstrated to be increased by the DESDP pretreatment. The extent of stimulation in terms of μg of α, β-unsaturated ketone formed per gram of tissue per 2 hours was, for all practical purposes, identical with that seen with the DESDP pellet implants. Since there was no change in total testicular weight due to the DESDP pretreatment, this change in 3β-ol-dehydrogenase activity is accepted as stimulation of the enzymatic mechanism.

Effect of estradiol-17β pretreatment. Experiments were performed in which natural estrogen, estradiol-17β, was administered subcutaneously in doses of 0.5, 2, 8 and 32 μg/kg/day, 6 days a week for 2 weeks. The growth of rats on these dose levels appeared to be slightly suppressed. No significant atrophy of testes was observed, but a regression in weight of sex accessory organs occurred which in degree was proportional to the dose. A remarkable stimulatory effect on the 3β-ol-dehydrogenase activity of testicular homogenates was effected by estradiol, particularly when given at 32 μg/kg. This amount was observed to bring about a 3- to 4-fold increase in the ability of testicular tissue to form α, β-unsaturated ketone when incubated with the substrate, Δ^5-androstene-3β, 17β-diol. At 0.5 μg/kg daily, estradiol-17β did not affect the enzymatic activity of the testes, nor was it capable of evoking a statistically significant curtailment in production of androgenic hormone by the testes. Estradiol-17β, at 2 and 8 μg/kg, exerted an intermediate effect which in terms of increased α, β-unsaturated ketone production was slightly greater than that found for DESDP pretreated rats.

Discussion. The enzyme 3β-ol-steroid-dehydrogenase, obtained from mammalian endocrine tissues, is assumed to participate in the biological synthesis of the steroid hormones which possess the α, β-unsaturated ketone structure in ring A of the steroid nucleus such as is typical of testosterone, progesterone and the adrenal cortical hormones. The finding that the capacity of testicular homogenates to convert Δ^5-androstene-3β, 17β-diol into α, β-unsaturated ketone was enhanced by estrogen pretreatment which concomitantly effected a

marked curtailment in testicular androgen production is pertinent to the general concept as stated above. The data presented indicate that the reaction catalyzed by 3β-ol-dehydrogenase is not the limiting step accounting for the marked interference in androgen production as the result of estrogen administration. From our studies the failure of hormonal synthesis would appear to occur at some step other than that which involves the formation of the α, β-unsaturated ketone precursors. A similar conclusion was reached by Huseby, Samuels and Helmreich(5) from studies correlating the 3β-ol-dehydrogenase activity and androgen producing capacity of estrogen-induced interstitial cell tumors.

The relative quantitative differences between DESDP and estradiol-17β in effectiveness in disrupting androgen production and on stimulating the 3β-ol-dehydrogenase activity of testicular homogenates should be pointed out. From this study it can be concluded that estradiol-17β is considerably more active than DESDP in accelerating the rate of α, β-unsaturated ketone formation by testicular homogenate. Whereas it would appear from the DESDP pellet implant experiments that a near 2-fold increase in 3β-ol-dehydrogenase activity is the maximal stimulating effect capable for this estrogen, it was observed that estradiol-17β evoked nearly a 4-fold increase in testicular enzyme activity at 32 μg/kg/day. Although the data do not permit an accurate estimate of relative potency, it was observed that estradiol-17β at 2 μg/kg exhibited a somewhat greater effect on the tissue enzyme content than did DESDP at 114 μg/kg. It would therefore appear that the natural estrogen is at least 50 times more active than the synthetic estrogen in respect to elevating tissue content of 3β-ol-dehydrogenase.

These data do not answer the question as to whether or not the estrogen effect on the enzyme is independent of the pituitary. The atrophy of the sex accessory organs of the estrogenized rats indicates a decreased output of pituitary gonadotrophin, which from the report of Samuels and Helmreich(6) would be expected to bring about a lowering of the testicular enzyme content. These authors observed that the 3β-ol-dehydrogenase activity of testicular tissue fell well below normal values within 3-5 days after hypophysectomy. The administration of 100 I.U. of chorionic gonadotrophin per day restored the enzyme levels to values more than double those of intact control rats. These findings provide some basis for eliminating the possibility that the estrogen-stimulating effect on the testicular 3β-ol-dehydrogenase activity is a result of the inhibitory influence of estrogen on pituitary gonadotrophic function.

The possibility exists that the estrogen-stimulating effect is directly on the 3β-ol-dehydrogenase system. Thus, Gordon and Villee(7) have shown that the rate of DPN reduction in an isocitric dehydrogenase system isolated from placental extracts is a function of the concentration of estradiol-17β. The reduced DPN was measured spectrophotometrically at 340 mμ. In their experiments estradiol-17β was approximately 1000 times more active than diethylstilbestrol. Estrone was as active as estradiol, but none of a number of other steroids tested possessed this activity. Talalay and Dobson(8) demonstrated that estradiol-17β, but not estradiol-17α, possessed a strong protective effect upon 3β-ol-dehydrogenase isolated and purified from cultures of certain species of *Pseudomonas*. Whether or not the stimulatory influence upon testicular 3β-ol-dehydrogenase activity brought about by estrogen pretreatment as reported here is a manifestation of these suggested mechanisms remains to be determined.

Summary. 1. Pretreatment doses of estrogens, which brought about marked curtailment in testicular androgen production as evidenced by sex accessory organ atrophy, increased the 3β-ol-dehydrogenase content of rat testicular homogenates. Estradiol-17β was approximately 50 times more active than diethylstilbestrol dipalmitate in this respect. 2. This evidence suggests that the reaction catalyzed by the 3β-ol-dehydrogenase system is not the limiting one with respect to disrupted androgen production induced by estrogen pretreatment.

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Toxicity of Hydroxamic Acid Analogues; Prophylactic and Therapeutic Efficacy Against Nerve Gas Poisoning in Mice.* (22575)

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Hydroxylamine HCl and a series of its analogues were shown to accelerate the hydrolysis of phosphoric acid esters by splitting off the phosphate groups(1). Some of the hydroxamic acid derivatives (HA) are capable also of reactivating cholinesterase that had been inhibited by reaction with phosphoric acid esters, such as nerve gases and certain of the insecticides. This is accomplished by releasing the phosphate bound in the reaction, thus freeing the enzyme(2-6).

These evidences of potential prophylactic and therapeutic use of HA compounds were tested in mice intoxicated with nerve gas or with phosphate insecticides(7). Only studies with the nerve gas, Sarin (isopropyl-methyl-phosphono-fluoride), are reported here.

Method. Hydroxylamine and analogues of hydroxamic acid (HA) were tested both for their individual toxicities and for their efficacies in reducing the toxicity of Sarin in albino mice. Each HA was made up as a solution in 0.85% NaCl, neutralized and adjusted so that 1 ml contained approximately one LD_{50} when administered intraperitoneally. For each analogue groups of 6 to 8 mice were injected with 4 to 6 different doses covering a range of approximately the interval LD_1-LD_{100}. The LD_{50} for each analogue was calculated by the method of Litchfield and Wilcoxon(8). These are listed in Table I. Mice generally died within one hour. A few succumbed during the second hour and those that survived for 2 hours seldom died during the subsequent 18 hours of observation. Only deaths within 2 hours were used in the calculation of the LD_{50}. Animals were observed

TABLE I. Approximate LD_{50}'s in Mice of An Analogous Series of Hydroxamates Injected Intraperitoneally.

Hydroxamate	Approximate LD_{50} (mg/g)
Hydroxylamine	.15
Salicyl-	.15
Nicotin- (benzobromide)	.16*
Polyacryl-	.16
Diglycolic di-	.17*
Picolin-	.3
Tartar-	.5
Sorb-	.7
Glycin-	.8
Acet-	1.3
Nicotin-	2.3
n-dimethyl glycin- (methochloride)	2.5 *
Glucuron-	3.0
Histidin-	3.7
Nicotin- (methiodide)	4.0
Serin-	6.5
Lact-	8.4
Mandel-	>5.0 †

* Tested against 2 × LD_{50} only (Table II).
† Limited by solubility.

* Synthesis of hydroxamates by Drs. T. Wagner-Jauregg, G. M. Steinberg and R. Plapinger, Medical Laboratories, Army Chemical Center, Md. Statistical analysis was done by Mr. I. A. DeArman, Medical Laboratories, Army Chemical Center, Md.