DANAZOL AS A STEROID ENZYME INHIBITOR IN RAT ADRENAL GLANDS

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ABSTRACT

DANAZOL AS A STEROID ENZYME INHIBITOR

IN RAT ADRENAL GLANDS

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The effect of Danazol, a synthetic steroid derivative, as an inhibitor of the enzymes of steroidogenesis, was studied in the adrenal glands of adult female rats in vivo. Analysis was made of adrenal weight, zona glomerulosa histology, and electrolyte concentration in the urine and blood plasma.

Changes in sodium, potassium, and chloride ion concentrations in the urine and plasma of Danazol injected rats with ovaries intact suggest the possible inhibition of the synthesis of aldosterone. However, castrated rats injected with Danazol exhibited no differences in the characteristics analyzed when compared to the castrate control group. This suggests that Danazol may affect the adrenal glands by way of its influence on estrogen release from the ovary. Another explanation may be that estrogens have a permissive action on the means by which Danazol affects the adrenals.

Accepted by: _____, Chairman

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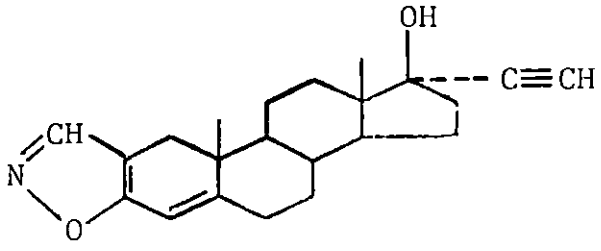
INTRODUCTION

Danazol, a 2,3-isoxazol derivative of ethinyl testosterone, was developed by Manson, et al. (1963) at Sterling-Winthrop Research Institute. The chemical name for Danazol (brand name Danocrine) is 17α-pregn-2,4-dien-20-yno-[2,3-d]-isoxazol-17-ol. It has the basic structure of testosterone along with an ethinyl group at C-17 and an isoxazol ring replacing the keto group at C-3 (Figure 1.).

According to Dmowski, et al. (1979) Danazol is currently approved by the Food and Drug Administration for use in the treatment of endometriosis. Clinically it has also been used for the treatment of chronic cystic mastitis, gynecomastia, precocious puberty, congenital angioneurotic edema, and as a male and female contraceptive. Danazol, if taken orally, is absorbed through the gastro-intestinal tract and metabolized quickly in the body, having a half-life of 4.5 hours in humans and reaching a peak in human female plasma two hours after administration. In monkeys and rats, however, plasma peak was reached four to eight hours after administration.



Chemical Structure of Danazol
Figure 1.

Danazol is metabolized in the liver by the cleavage of the isoxazol ring with over sixty of its metabolites having been isolated, the major one being 2-hydroxy-methylethisterone. According to Rosi, et al. (1977), Danazol, rather than one of its metabolites, appears to cause the biologic effects. None of the metabolites exhibited the antigonadotropic or androgenic activities of Danazol, and when Danazol was administered with its metabolic blockers, its biological activities greatly increased. In studies with radioactive Danazol, high concentrations of radioactivity were noted in the liver, kidneys, and adrenals when compared to the concentration in the blood plasma.

When Danazol was first marketed, advertising and pharmacological bulletins indicated that the primary

effect was suppression of the pituitary-ovarian axis by inhibiting the secretions of the gonadotropins FHS and LH from the pituitary. According to Dmowski, et al. (1977), Danazol appeared to have no progestational or estrogenic effect and only mildly androgenic side effects, which appeared in the form of acne, edema, mild hirsutism, and a decrease in breast size. Barbieri, et al. (1979) have stated that studies actually indicated that Danazol has at least four pharmacological properties: 1) direct inhibition of gonadotropin synthesis and/or release, 2) direct inhibition of the multiple enzymes of steroidogenesis, 3) interaction with androgen, glucocorticoid, and progesterone receptors in target tissues, and 4) alteration of endogenous steroid metabolism.

The antigonadotropic effect of Danazol was reported by Greenblatt and Dmowski (1971) because it had no effect when administered with exogenous gonadotropins. Greenblatt, et al. (1974) indicated the possible use of Danazol as a contraceptive agent because of the drop in FSH and LH levels at a dosage of 100 mg per day and, in particular, the prevention of the mid-cycle LH surge prior to ovulation. Van Dijk, et al. (1979) have used Danazol for treating unexplained infertility in women. Women with infertility diagnosed as unexplainable by all

known clinical tests were administered Danazol orally with a control group receiving placebos. Anovulation occurred, and upon cessation of treatment, a significant number of the treated group conceived within six months while none of the placebo group did. No explanation as to why conception occured was discernible. Franchimont and Cramilion (1977) reported that FSH and LH were the only hormones of the anterior pituitary inhibited, as no changes in the response of ACTH, TSH, or prolactin were noted.

Mild androgenic effects have been observed clinically, especially at higher doses, but more anabolic rather than androgenic effects seem to appear at these higher doses. Barbieri, et al. (1979) have done extensive work on the receptor binding ability of Danazol and have noted that Danazol displaces ^{3}H-dihydrotestosterone from the 8S androgen receptor of the prostate cytosol along with positive results of the androgen bioassay using ventral prostate and seminal vesicle weights. This receptor binding correlates with the mild androgenic side effects. Potts, et al. (1974) have referred to Danazol as an impeded androgen since a high dose of 672 mg/kg/day produced effects comparable to a low dosage of only 84 mg/kg/day.

Danazol exhibits no estrogenic effects. Barbieri, et al. (1979) reported slight gains in uterine weights in immature female rats, but when compared to the effect produced by the estrogen compound mestranol, Danazol produced less of a uterine weight gain at ten thousand times the concentration of mestranol. They also noted that no vaginal cornification occurred at a dosage of 40 mg/kg/day. In correlation Danazol bound only very weakly to the rat uterine cytosol in displacing ^{3}H-estradiol. Potts, et al. (1974) compared the slight uterine weight gain to that caused by androgens.

Whether Danazol is a progestational or anti-progestational agent still remains controversial. While Potts, et al. (1974) showed that Danazol has no progestational effect in the Clauberg assay, Dmowski, et al. (1971) and Wentz and Sapp (1978) have reported endometrial secretory changes with Danazol treatment, although the changes were atypical of those induced by progesterone. Creange, et al. (1974) developed a radioligand assay for Danazol using a protein which has a high specificity for progesterone. In determining the effect of Danazol as a luteolytic contraceptive, however, Wentz and Sapp (1978) reported a rise in progesterone levels following HCG injections after Danazol treatment, indicating that Danazol does not have the luteolytic effect of

progesterone. These facts plus the reported evidence by Barbieri, et al. (1979) that Danazol binds to the rat uterine cytosol receptor leave the progestational-antiprogestational controversy still be researched further.

Barbieri, et al. (1979) have demonstrated the glucocorticoid effect of Danazol by the liver glycogen assay and by the ability of Danazol to bind to the glucocorticoid receptor of the rat liver cytosol. Franchimont and Cramilion (1977) showed that serum cortisol levels were significantly lower in Danazol treated patients; however, Wentz, et al. (1975) have reported that Danazol has no apparent effect on the adrenal gland's ability to respond to ACTH. Their methods included the injection of the synthetic ACTH compound cosyntropin in Danazol treated patients as well as the injection of the 11β-hydroxylase inhibitor metyrapone. The cosyntropin challenge produced an increase in plasma cortisol levels, and the metyrapone challenge caused a rise in 17-hydroxysteroid levels.

Barbieri, et al. (1977) have reported the inhibition of steroidogenesis in hamster ovaries, rat testes, and rat adrenals in vitro. In rat adrenals the 3β-hydroxy-steroid dehydrogenase and 21-hydroxylase enzymes of the cytoplasm and the 11β-hydroxylase enzyme of the mito-

chondria are inhibited by Danazol. He mentioned that a number of previous reports of antigonadotropic effects by Dmowski, et al. (1971) and Potts, et al. (1974) in hemicastrates and castrates could actually have been caused by the inhibition of steroidogenesis. Sherins, et al. (1971) also noted changes in gonadal steroid levels in humans with no corresponding FSH or LH changes.

There are four main groups of adrenal cortical steroids (Greenberg, 1968): 1) the 11-oxygenated corticosteroids or glucocorticoids which affect carbohydrate metabolism and include primarily cortisol, corticosterone, 11-dehydrocorticosterone and cortisone, 2) the corticoids lacking oxygen at C-11 or mineralocorticoids which include 11-deoxycorticosterone (DOC), and 11-deoxycortisol, 3) aldosterone which is the most potent steroid involved in electrolyte metabolism and has an aldehyde group at C-18, and 4) the sex steroids.

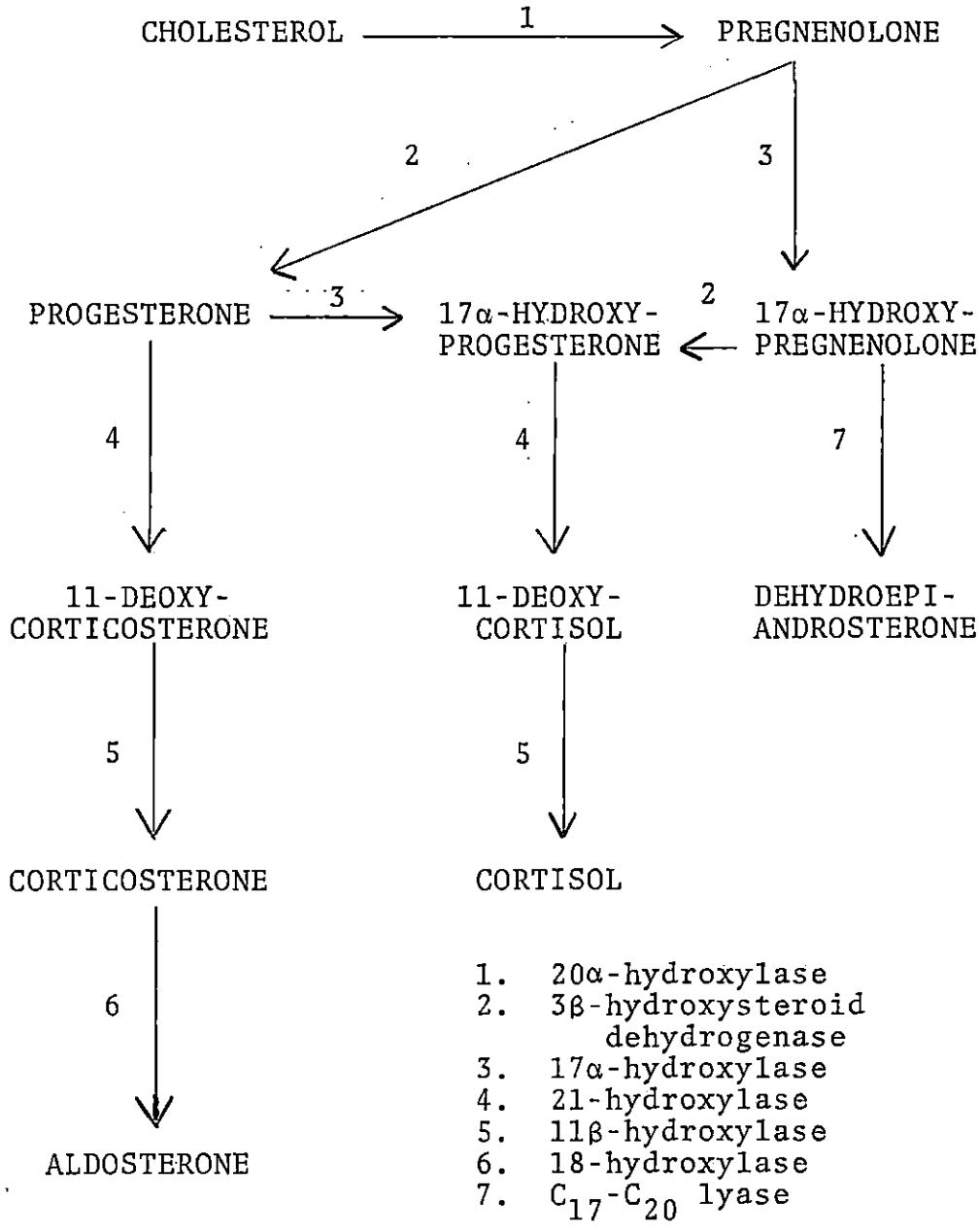
Since the adrenal cortex, testes, ovaries, and placenta are all centers of steroid synthesis, it has been shown that they all contain the same enzyme systems. The C-27 molecule cholesterol is the precursor of almost fifty different steroids produced by the adrenal cortex. The first step is the conversion of cholesterol to the C-21 molecule pregnenolone by a cytochrome P-450 enzyme of the mitochondria which requires NADPH and molecular oxygen

and cleaves the side chain from cholesterol. The side chain cleavage step appears to be the rate limiting step in steroid biosynthesis (Figure 2, Boyd, et al. (1978).

Pregnenolone can be converted to either progesterone or 17α-pregnenolone. The pathways from 17α-pregnenolone lead to synthesis of the androgens and estrogens, but only the pathways leading to the mineralocorticoids and the glucocorticoids will be discussed here.

Pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase requiring NAD^+ as a hydrogen acceptor and Δ^5-3-oxoisomerase which shifts the double bond from the numbers 5 and 6 carbons to the numbers 4 and 5 carbons. Progesterone can then be converted to 17α-hydroxyprogesterone by a 17α-hydroxylase associated with the cytoplasm. This pathway is prevalent in humans but does not exist in rats. A microsomal 21-hydroxylase then converts 17α-hydroxyprogesterone to 11-deoxycortisol, a mineralocorticoid which is then converted by the mitochondrial cytochrome P-450 11β-hydroxylase system to cortisol, the primary glucocorticoid in humans.

Another pathway from progesterone, the primary one in rats, has a 21-hydroxylase product, 11-deoxycorticosterone (DOC), a mineralocorticoid comparable to 11-deoxycortisol. DOC is then converted by the 11β-hydroxylase to



Some Major Enzymes in the
Biogenesis of Adrenal Cortical Hormones

Figure 2.

corticosterone, the primary glucocorticoid in rats. Corticosterone can then be converted to the very potent mineralocorticoid aldosterone by 18-hydroxylase and 18-hydroxy dehydrogenase found in the mitochondria. Once synthesized, aldosterone can be found either in the 18-aldehyde form or in the more common 11-18 hemiacetal form.

Sabatini and DeRoberts (1961) reported that, while 3β-hydroxysteroid dehydrogenase, 11β-hydroxylase, and 21-hydroxylase activity occur throughout the cortex, the 18-oxygenation to aldosterone is located only in the outer most zona glomerulosa, and the 17-hydroxylase activity is localized in the inner zona fasciculata or the zona reticularis. They originally reported the transformation of the zona glomerulosa into fasciculata cells upon chronic administration of ACTH, but more recently it has been shown that the zona glomerulosa and zona reticularis are independent of each other (Turner and Bagnara, 1976). Watanuki, et al. (1978) have provided evidence that the 11β-hydroxylase and 18-hydroxylase activities, which are part of the cytochrome P-450 complex, are actually catalyzed by only one enzyme.

It is the purpose of this research to further explore the effects of Danazol on the rat adrenal cortex. This will be done by studying the effects of Danazol

on adrenal weight, electrolyte concentrations in the urine and blood, and the histology of the zona glomerulosa. Electrolyte levels to be analyzed are sodium, potassium, and chloride concentrations. The first experiment will analyze the dosage level effect on these characteristics related to adrenal cortical steroidogenesis. From this an optimal dosage will be chosen to be used in a prolonged study of adrenal cortical response to Danazol.

MATERIALS AND METHODS

Animal Care

Two experiments were performed on adult female Sprague-Dawley rats. The rats were housed three per cage at 23° C. under a regimen of ten hours darkness and fourteen hours of artificial lighting. They were allowed access to water and Purina Rat Chow ad libitum.

Experiment I

Twelve rats were injected with Nembutol (4 mg/ 100 gm body weight) and ovariectomized. Four days following surgery, the rats were divided into four groups and injected in the hind leg with Danazol (Sterling-Winthrop Labs) dissolved in peanut oil with Groups 2,3, and 4 receiving 2,4, and 8 mg/kg body weight/day respectively and Group 1 receiving a corresponding injection of peanut oil as a control. Four groups of rats with ovaries intact were injected with doses corresponding to Groups 1,2,3, and 4. Injections of all eight groups were continued for ten days. On the tenth day urine samples were taken by applying pressure in the area of the kidneys. Urine samples were stored in disposable culture tubes and frozen. Twenty four hours following the final injection, the rats were removed from the rat room in groups of three, stunned and decapitated within two minutes of

removal from the quarters to prevent the influence of ACTH release into the bloodstream. Blood was drained from the necks into 400 ml beakers, transferred to 15 ml disposable centrifuge tubes, centrifuged for 15 minutes at 1550 rpm, and the plasma frozen. The right adrenal was removed and placed in fixative to prevent degradation of the zona glomerulosa. The left adrenal was weighed, as were the ovaries of the intact rats.

The right adrenal was treated for removal of mercury with a saturated iodine-alcohol solution, dehydrated in alcohol and toluene, embedded in paraffin, sectioned at ten microns, stained with Delafield's hematoxylin, and counterstained with eosin by the method of Humason (1972). The zona glomerulosa thickness was measured under 100X with an optical micrometer.

Ten lambda samples of urine and one hundred lambda samples of serum were measured for chloride ion concentration with a digital chloridometer (Buchler). Twenty lambda samples of urine and fifty lambda samples of serum were analyzed for sodium and potassium ion levels by flame photometry (Coleman Model 21).

Experiment II

Twelve adult female rats were ovariectomized as per Experiment I. Four days following surgery, six castrated

females were injected with peanut oil as a control, while six were injected with a dosage of 4 mg/kg/ body weight of Danazol dissolved in peanut oil. Two groups of six rats each with ovaries intact were given corresponding injections. Injections were continued for twenty days. Post treatment protocol was followed as in Experiment I.

Analysis of Data

All data was analyzed using the Student's t test.

RESULTS

Experiment I

Danazol caused no significant change in body weight in adult female rats, castrated or not, at the doses administered during the ten day experiment (Table 1).

A significant increase in adrenal weight was observed in the castrate control group compared to the intact controls (Table 1).

This experiment showed the most measurable effects of Danazol at a dosage of 4 mg/kg which corresponds to the reported dosage used by Dmowski, et al. (1971). At this dosage adrenal weight decreased compared to the control in castrates only, while intact rats showed a significant decrease in ovarian weight (Table 1). This dosage also caused an elevation of the serum Na^+ levels and a decrease in Na^+/K^+ ratios (Table 2), as well as an elevation of the urine Na^+ levels in intact rats compared to the intact controls (Table 2). Castrates at the 4 mg/kg dosage exhibited a lowering of the serum K^+ levels and an increase in the Na^+/K^+ ratios compared to the intact rats receiving this dosage (Table 2).

A dosage of 2 mg/kg produced an increase in the Na^+/K^+ ratios in castrates compared to intact rats receiving this injection (Table 2), while a dosage of

TABLE 1.
EXPERIMENT I

BODY WEIGHT CHANGE AND RELATIVE ADRENAL AND OVARIAN WEIGHTS

	Dosage (mg/kg body weight)	*Change in Body Weight (gm)	*Relative Adrenal Weight (mg/ 100 gm body weight)	*Relative Ovarian Weight (mg/ 100 gm body weight)
CASTRATE	0	+10 ± 19	10.7 ± 1.3[a]	
	2	+10 ± 21	8.0 ± 1.2	
	4	-7 ± 7	6.1 ± 1.4[b]	
	8	+3 ± 5	9.4 ± 2.3	
INTACT	0	-2 ± 4	6.9 ± 0.8	6.4 ± 0.9
	2	+3 ± 5	6.2 ± 1.0	4.5 ± 1.2
	4	-4 ± 4	7.8 ± 0.3	4.8 ± 0.5[c]
	8	+4 ± 7	6.2 ± 1.2	5.4 ± 1.1

* Mean ± Standard Deviation
([a] $P < .02$ compared to Intact Control)
([b] $P < .02$ compared to Castrate Control)
([c] $P < .05$ compared to Intact Control)

TABLE 2.
EXPERIMENT I

ION CONCENTRATIONS AND RATIOS IN THE BLOOD

	Dosage (mg/kg body weight)	Na^+ (meq/l) *	K^+ (meq/l) *	Cl^- (meq/l) *	Na^+/K^+ *
CASTRATE	0	109 ± 67	13.0 ± 4.6	81 ± 17	7.1 ± 3.4
	2	152 ± 33	12.0 ± 2.6	67 ± 16	12.7 ± 1.4
	4	150 ± 13	15.3 ± 2.0	80 ± 3	9.9 ± 1.5
	8	120 ± 26	19.7 ± 6.9	78 ± 8	6.4 ± 1.5
INTACT	0	118 ± 30	16.3 ± 2.3	81 ± 14	7.3 ± 0.8
	2	102 ± 28	16.7 ± 4.4	84 ± 15	6.4 ± 2.0[d]
	4	83 ± 8[a]	21.0 ± 0.0[b,c]	86 ± 8	4.0 ± 0.5[a,b]
	8	90 ± 10	21.0 ± 3.6	88 ± 14	4.4 ± 1.2

* Mean ± Standard Deviation
([a] $P < .01$ compared to dosage of 4 mg/kg in castrates)
([b] $P < .01$ compared to intact control)
([c] $P < .02$ compared to dosage of 4 mg/kg in castrates)
([d] $P < .02$ compared to dosage of 2 mg/kg in castrates)

TABLE 3.
EXPERIMENT I
ION CONCENTRATIONS AND RATIOS IN THE URINE

	Dosage (mg/kg body weight)	Na^+ (meq/l) *	K^+ (meq/l) *	Cl^- (meq/l) *	Na^+/K^+ *
CASTRATE	0	118 ± 71	74 ± 68	131 ± 127	1.9 ± 0.8
	2	179 ± 42	114 ± 46	186 ± 105	1.7 ± 0.4
	4	175 ± 13	175 ± 53	194 ± 45	1.0 ± 0.5
	8	156 ± 43	155 ± 22	227 ± 69	1.0 ± 0.5
INTACT	0	143 ± 19	174 ± 23	195 ± 18	0.8 ± 0.6
	2	171 ± 28	140 ± 40	196 ± 60	1.3 ± 0.4
	4	216 ± 27 [a]	184 ± 18	288 ± 70	1.2 ± 0.3
	8	191 ± 19 [a]	169 ± 31	263 ± 6 [b]	1.2 ± 0.1

* Mean ± Standard Deviation
([a] $P < .02$ compared to Intact Control)
([b] $P < .01$ compared to Intact Control)

eight mg/kg significantly elevated urine Na^+ and Cl^- levels in intact females only (Table 3).

Experiment II

Administration of Danazol over a twenty day period again produced no significant change in body weight at any of the doses administered (Table 4).

Injections of Danazol for twenty days caused a decrease in adrenal weights in both treated groups, but the castrate control group also exhibited a decrease in weight which was not significantly different from that of treated castrates (Table 4). Intact rats injected with Danazol exhibited a significantly lower ovarian weight compared to the intact control rats (Table 4).

Danazol also caused an elevation of serum K^+ levels and a decrease in the Na^+/K^+ ratios in the serum of intact rats (Table 5), but the effect of castration alone was to elevate the serum K^+ levels (Table 5) as well as the levels of urine chloride ions (Table 6).

Zona glomerulosa thickness decreased in both groups treated with Danazol compared to the intact control group, but castration alone also caused the glomerulosa layer to diminish in size (Table 7).

Finally it should be noted that in all the data obtained (adrenal weight, ion concentrations and ratios, and thickness of the zona glomerulosa) not one significant

difference was observed between Danazol injected castrates and control castrates.

TABLE 4.

EXPERIMENT II

BODY WEIGHT CHANGE AND RELATIVE ADRENAL AND OVARIAN WEIGHTS

Treatment	*Change in Body Weight (gm)	*Relative Adrenal Weight (mg/ 100 gm body weight)	*Relative Ovarian Weight (mg/ 100 gm body weight)
Intact Control	-1 ± 10	9.7 ± 0.2	11.4 ± 2.5
Intact Danazol	+8 ± 14	7.0 ± 1.3[a]	6.9 ± 1.6[b]
Castrate Control	-5 ± 15	7.1 ± 0.4[a]	
Castrate Danazol	-2 ± 10	7.4 ± 0.8[a]	

* Mean ± Standard Deviation
([a] $P < .001$ compared to Intact Control)
([b] $P < .01$ compared to Intact Control)

TABLE 5.

EXPERIMENT II

ION CONCENTRATIONS AND Na^+/K^+ RATIOS IN THE BLOOD

Treatment	Na^+ (meq/1) *	K^+ (meq/1) *	Cl^- (meq/1) *	Na^+/K^+ *
Intact Control	109.1 ± 9.2	13.5 ± 3.5	58 ± 8	8.2 ± 1.7
Intact Danazol	119.3 ± 7.6	21.3 ± 4.7 [a]	66 ± 6	5.7 ± 1.0 [a]
Castrate Control	117.0 ± 13.1	18.3 ± 3.2 [b]	65 ± 6	6.6 ± 0.7
Castrate Danazol	113.4 ± 9.5	17.3 ± 4.7	67 ± 9	6.9 ± 3.2

* Mean ± Standard Deviation
([a] $P < .02$ compared to Intact Control)
([b] $P < .05$ compared to Intact Control)

TABLE 6.

EXPERIMENT II

ION CONCENTRATIONS AND Na^+/K^+ RATIOS THE URINE

Treatment	Na^+ (meq/l) *	K^+ (meq/l) *	Cl^- (meq/l) *	Na^+/K^+ *
Intact Control	124 ± 49	174 ± 45	117 ± 48	.73 ± .21
Intact Danazol	129 ± 56	177 ± 18	134 ± 46	.71 ± .15
Castrate Control	181 ± 44	196 ± 31	211 ± 71	.93 ± .21
Castrate Danazol	195 ± 34	204 ± 8	288 ± 33[a]	.96 ± .19

* Mean ± Standard Deviation
([a] $P < .05$ compared to Intact Control)

TABLE 7.

EXPERIMENT II

ZONA GLOMERULOSA THICKNESS

Treatment	*Thickness (microns)
Intact Control	51.7 ± 5.1
Intact Danazol	40.5 ± 5.2 [a]
Castrate Control	33.0 ± 7.2 [b]
Castrate Danazol	37.3 ± 9.6 [a]

* Mean ± Standard Deviation

([a] $P < .01$ compared to Intact Control)

([b] $P < .001$ compared to Intact Control)

DISCUSSION

In neither experiment were significant changes in body weight observed, indicating no anabolic effect at the doses administered. It was mentioned previously that anabolic effects were usually more prevalent at higher doses according to Dmowski (1979).

Experiment I produced an unexpected increase in adrenal weight due to castration alone (Table 1). Ovariectomy has been shown by Kitay, et al. (1963) to have an atrophic effect on the adrenal glands due to the removal of the stimulatory effect of estrogens on the release of ACTH from the pituitary. Experiment II produced the expected decrease in adrenal weight. The unexpected result in Experiment I may be attributed to post operative stress and the subsequent release of ACTH which was still exhibiting a trophic effect on the day the animals were sacrificed. More polished surgical technique along with the longer post operative period before analysis accounts for the more expected observation in Experiment II.

Nonsignificant ovarian weight reduction in Experiment I at all doses and a significant reduction in Experiment II may be attributed to one of three possible

causes: 1) the antigonadotropic effect of Danazol by way of inhibition on the pituitary-ovarian axis as indicated by Dmowski (1979), 2) direct inhibition of steroidogenesis in the ovary as demonstrated by Barbieri, et al. (1977), or 3) a combination of the two.

The concentrations of sodium and potassium ions are essential elements in electrolyte metabolism. In the kidney nephron sodium tends to be retained in the body fluids while potassium tends to be eliminated into the urine. Chloride ion concentrations tend to correlate with sodium ion concentrations as sodium ions are usually retained in the blood or excreted in the urine as sodium chloride. Changes in the concentrations of these ions in the blood and urine may indicate an effect on aldosterone, either in terms of its synthesis, release, or mechanism of action.

In Experiment I the most measurable effects of Danazol were observed at a dosage of 4 mg/kg. Significant differences in ion concentrations and ratios were noted between Danazol injected intact rats and intact controls, while differences also occurred between castrate and intact rats receiving this same dosage (Table 2 and 3). The significant elevation of serum K^+ and urine Na^+ levels and a decrease in the Na^+/K^+ ratio in the blood along with a nonsignificant elevation in urine Cl^- levels indicates

that at a dosage of 4 mg/kg in intact rats, sodium chloride is being eliminated into the urine while K^+ is being retained in the blood to a greater extent than in control rats. These facts along with the significant reduction in ovarian weight observed at this dosage led to the selection of 4 mg/kg as the optimal dosage to be used in Experiment II.

In Experiment II changes in electrolyte metabolism in rat urine and serum (Tables 5 and 6) and the decrease in the thickness of the zona glomerulosa (Table 7) in intact Danazol treated rats suggests the inhibition of aldosterone synthesis. The electrolyte changes were indicated by a decrease in the Na^+/K^+ ratio in the blood as well as an elevation in blood K^+ levels (Table 5). If only electrolyte changes had been observed, influence on the renin-angiotensin system might have been suspected. The regression of the zona glomerulosa indicates that an inhibition of synthesis rather than an inhibition of the release of aldosterone is more probable.

Barbieri, et al. (1977) have shown that Danazol inhibits three of the enzymes of steroidogenesis leading from pregnenolone to the synthesis of corticosterone in rat adrenal cells in vitro. While data presented here suggests the inhibition of aldosterone in vivo, it does not indicate a mechanism of action. It is not evident

whether aldosterone synthesis is inhibited by Danazol's inhibition of its precursor production or whether Danazol directly inhibits the 18-hydroxylase activity. This could be explored *in vitro* by incubating rat adrenal cells with Danazol and radioactive corticosterone as Barbieri, *et al*. did in demonstrating the inhibition of the enzymes which produced the precursors of aldosterone. The data reveals a greater inhibition on the enzymes the closer you get to the synthesis of aldosterone, that is, 11β-hydroxylase is inhibited more than 21-hydroxylase, which is inhibited more than 3β-hydroxysteroid dehydrogenase (See Figure 2). Watanuki, *et al*. (1978) have indicated the very distinct possibility that the 11β-hydroxylase and 18-hydroxylase activities of the zona glomerulosa mitochondria are actually catalyzed by only one enzyme instead of two. If Danazol inhibits one of the enzyme's activities, there is a good possibility that it inhibits the other as well. To what extent this may occur is in need of further research.

According to Dmowski (1979) work with radioactive Danazol showing greater than plasma concentrations in the adrenal glands indicates possibly a direct influence of Danazol on the adrenal glands probably by means of binding to receptor sites. Rifka, *et al*. (1978) have reported the existence of an adrenal cytosol androgen receptor

which may mediate regression of the adrenals. Since Danazol is an androgen derivative, one possible explanation for adrenal weight loss upon treatment with Danazol is the binding of Danazol to this receptor. No significant differences were observed between the castrate controls, the castrated treated rats, or the intact treated rats. Therefore the effect observed between the intact treated and the intact controls may be related to the decrease in estrogens rather than a direct effect on the adrenal. Estrogens are currently believed to regulate the expression of certain genes and, as a result, subsequent protein formation (Turner and Bagnara, 1976). The lack of estrogens in castrated rats may cause a reduction of the receptors for Danazol, possibly the adrenal androgen receptor. Further research with radioactive Danazol's ability to bind to this androgen receptor and the determination of whether or not castration does, in effect, reduce the synthesis of these receptors would clarify this issue.

It should be noted that the length of time of administration affected the observed action of Danazol on electrolyte metabolism. In the ten day experiment at a dosage of 4 mg/kg, castrates exhibited significantly higher serum Na^+ and lower serum K^+ levels than intact rats receiving the same dosage (Table 2). Levels in

Experiment II, however, were not significantly different. The difference in Experiment I may be attributed to the post operative increase in aldosterone immediately following surgery due to fluid loss. The longer post operative period and reduction of post operative stress eliminated this effect in Experiment II as no differences in electrolyte concentrations or ratios were observed between intact or castrate treated rats (Tables 5 and 6).

Although research has shown Danazol's ability to inhibit steroidogenesis in vitro, this thesis presents data which suggests the inhibition of aldosterone synthesis by Danazol in vivo in rats. However, more research is needed on the in vivo inhibition of steroidogenesis by Danazol, particularly in humans. Wentz, et al. (1975) studied adrenal response in humans at a dosage of 800 mg/day which is the normal dosage recommended for treatment of endometriosis. They used a synthetic ACTH compound, cosyntropin, to test adrenal response as well as a metyrapone challenge which inhibits 11β-hydroxylation. These tests were run following chronic Danazol administration and showed normal cortisol elevation in the bloodstream compared to controls when injected with cosyntropin, and normal ACTH rise following the metyrapone challenge. At this dosage in humans, there appears to be no evidence of inhibition upon cortisol synthesis.

Because aldosterone is produced by a different pathway, evidence in this thesis indicates a need for research in the area of Danazol's effect on the human pathway leading to the synthesis of aldosterone. This research would be especially important in patients treated with Danazol who may engage in strenuous activities since they may have a tendency to lose large amounts of salt and water from their body during activity.

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