IN VIVO STUDY OF THE EFFECTS OF DANAZOL ON CYTOPLASMIC RECEPTORS IN THE FEMALE RAT

A Thesis

Presented to

the Faculty of the School of Sciences and Mathematics

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In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

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ABSTRACT

<u>IN VIVO</u> STUDY OF THE EFFECTS OF DANAZOL ON CYTOPLASMIC RECEPTORS IN THE FEMALE RAT

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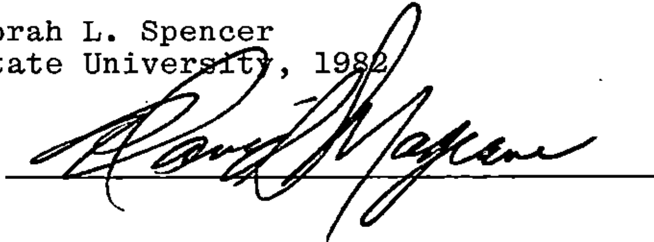
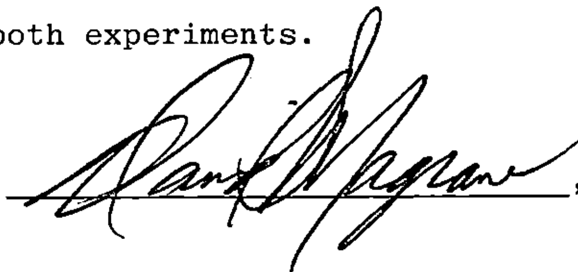


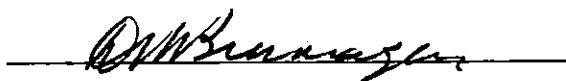
The <u>in vivo</u> effects of the synthetic antigonadotropin, danazol, on cytosol receptor binding in three groups of Sprague-Dawley rats were studied. Percent change in specific binding of estrogen, progesterone, dihydrotestosterone and corticosterone binding in uterine, mammarian, adrenal, hypothalamic and ovarian tissues was investigated. To evaluate specific binding, incubations of tissue cytosols with a 100-fold excess of 'cold' competitor was subtracted from the total binding of each incubate, and a hydroxylapatite micromethod assay was used to extract the steroid.

Long term, low dose (4 mg/kg/14 days) danazol therapy in adult female rats resulted in a non-significant decrease in all receptors compared to intact rats. Significant differences in estrogen receptor binding were noted (42% reduction). When ovariectomized rats received a continuation of danazol, a 45% reduction in estrogen binding occurred. Other steroid binding

concentrations were unaffected by ovariectomy. Mammary tissue was not significantly changed in its ability to bind steroid receptors.

A short term, higher dose (8 mg/kg/3 days) danazol therapy in pre-puberal female rats resulted in a non-significant decrease in binding of estrogen, progesterone and dihydrotestosterone in the uterus. In pooled adrenals and hypothalami, all but one receptor group decreased in specific receptor binding. The only increase was in estrogen binding in pooled adrenals. In addition, either antigonadotropic and/or androgenic effects of danazol were seen in relation to organ weights from both experiments.

Accepted by:  , Chairman



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INTRODUCTION

The binding of steroid hormones to the cytoplasmic protein receptor of target cells initiates a series of reactions leading to specific protein synthesis. Studies of the mechanism of action of steroid hormones is essentially the study of protein receptor molecules and the behavior of a steroid-receptor complex in activating enzyme synthesis (O'Malley and Means, 1974). Such receptor molecules have been found to be highly specific for a given hormone and are found in significant amounts only in target cells.

Early attempts to describe the molecular interactions of steroids with cell components were unsuccessful because physiological amounts of hormones could not be detected with the techniques then available. An important factor in establishing the current understanding of the mechanism of action of steroid hormones was the synthesis of isotopically labeled compounds. These provided the first molecular probes to discern the sequence of events that occur following the interaction of a steroid hormone with a target cell. In 1960, Jensen and Jacobson injected ^{3}H-estradiol of high radiospecific activity into rats and determined that the target organs, uterus and vagina, retained the label much longer than non-target tissues.

Since the non-target tissues incorporated the labelled steroid and then quickly lost it, this indicated there was no permeability barrier to the entry of estradiol into cells. Passage through the plasma membrane by the hydrophobic steroid was suggested to be by diffusion. The retention of estradiol in the uterus for at least six hours suggested that it must be bound by these target cells and detained from leaving the cell (Jensen and Jacobson, 1962).

Fractionation of uteri from rats previously injected with ^{3}H-estradiol showed that binding was stereospecific and that the label could be released after treatment with proteases. This suggested that the steroid was bound to a protein (Notebloom and Gorski, 1955). A clear demonstration that hormonally responsive tissues contained a particular macromolecule associating with the labeled ligand was reported by Toft and Gorksi (1966). These workers used sucrose gradient centrifugation to show that ^{3}H-estradiol associated with a cytoplasmic protein in the rat uterus having a sedimentation velocity of 8-9 Svedberg units (S). This peak was maximal within twenty minutes and could be blocked by adding a competing estrogenic molecule such as diethylstilbesterol. Presumably this inhibition was through competition of the same binding site with ^{3}H-

estradiol (Toft and Gorski, 1966).

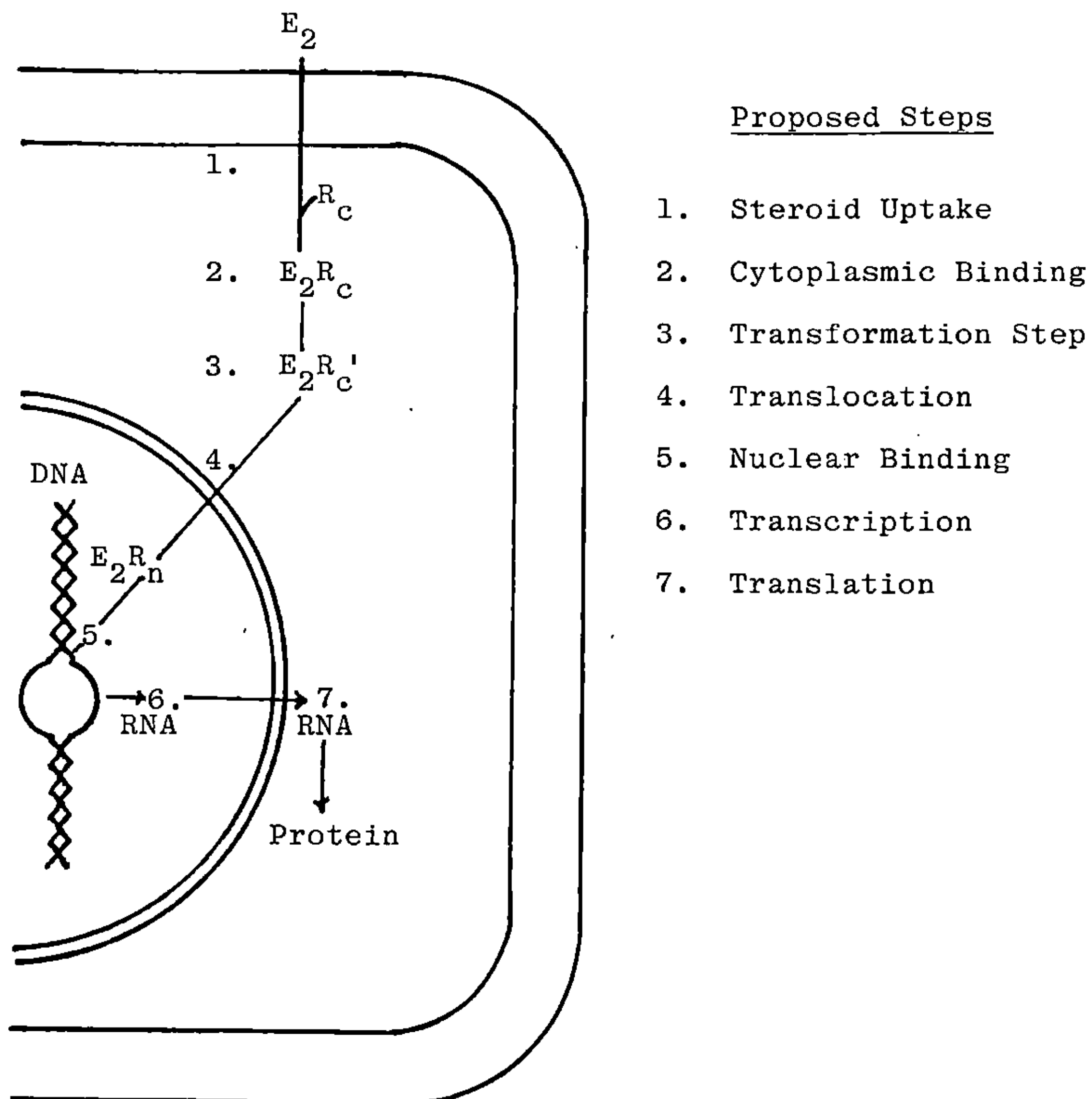
A smaller, 5S ^{3}H-estradiol-protein complex was extracted from uterine nuclei (Jensen, et al., 1967). This complex which showed maximum binding after one hour was recovered from isolated nuclei to which ^{3}H-estradiol was added in the presence of uterine cytoplasm (Jensen, et al., 1968). No nuclear uptake of ^{3}H-estradiol occurred in isolated nuclei in the absence of uterine cytosol. Furthermore, the amount of radioactivity in uterine cytosol decreased while the nuclear hormone concentration increased. This translocation of the steroid into the nucleus was shown to be temperature dependant (Jensen, et al., 1968).

The interaction of the steroid-nuclear receptor complex with chromatin was elucidated by O'Malley's lab in 1971 (Spelsberg, et al., 1971). Using the effect of progesterone on the chick oviduct, they demonstrated that the progesterone-receptor complex (PgR) bound to both non-histone acidic proteins and DNA. This high affinity binding by the PgR complex acts to relieve the constraints at specific regions on the DNA, leading to the transcription of specific messenger RNA and subsequent protein synthesis. Although an increase in the synthesis of a specific protein may be affected by changes in the cytoplasm, it is thought that an increase in the rate of

transcription of DNA is the major cause of the increase in the number of mRNA molecules, which is responsible for the increase in protein concentration (Roth and Grunfeld, 1981).

The experiments primarily from the labs of Jensen and Gorski, have led to a postulate of a two step model for the interaction of estradiol with its target cells in the rat uterus. The basic tenets of this model seem to hold true for all steroid hormone interactions. Figure 1 illustrates the model as developed by Jensen and DeSombre (1973) and updated by Wittliff (1974). Basically, estradiol enters the uterine cell by diffusion where it binds to an estrogen receptor protein forming the estradiol-receptor complex (E_2R_c). This complex undergoes a temperature dependant transformation to a new species called E_2R_c', which has the capacity to translocate into the nucleus and where it is now called E_2R_n. The E_2R_n binds to specific chromatin acceptor sites where activation of RNA polymerase occurs. New mRNA is transcribed with subsequent protein synthesis and eventual cellular growth and division.

Although the target cell contains the biologic capacity to associate with steroid hormone, this property does not obligate the cell to respond in an appropriate manner. This is evident in cancer cells. If cancerous



E_2 = Estradiol (Steroid Hormone)
R_c = Cytoplasmic Binding Protein (Receptor
E_2R_c = Estradiol-Receptor Complex (Cytoplasmic)
E_2R_c' = Estradiol-Receptor Complex (Nuclear)

Proposed Steroid Hormone - Target Cell Interaction (Jensen and DeSombre, 1973; Wittliff, 1974).

Figure 1

tissue is dependent on steroid hormone for growth and survival, steroid hormone receptors will be present in sufficient quantity to propagate the cancerous cells. If however, an interruption in the intracellular events can be accomplished, the cancerous cells will die. Therefore any method to inhibit estrogen binding in the cytoplasm of estrogen dependent tumor cells, should reduce the tumor. Huggins stated "the quality of hormone dependence resides in the tumor cells, whereas their growth is determined by the host's endocrine status. The cure of cancer after deprival of the dependent hormone results from the death of the cancer cell, whereas normal cells may shrivel but survive" (Huggins, 1979).

Erdos reported that uterine cytosol contained two types of estradiol binding sites (Erdos, et al., 1970). Binding to type A sites occurs at low estrogen concentration. The affinity is high, available binding sites are limited, and the complex formed is slowly reversible. Binding to type B sites occurs at higher estrogen concentrations where the affinity is low. In type B binding, the number of available binding sites is high and the complex formed is rapidly reversible (Erdos, et al., 1970). Hydroxylapatite, an inert and insoluble compound, has the ability to measure the concentration of estrogen-bound type A sites (Pavlik and Coulson, 1976). High affinity

binding, specific for cytoplasmic steroid receptors is reduced by non-steroidal competitors or 'cold', unlabeled hormone. In the presence of a 100-fold excess of unlabeled hormone, a percentage of binding is abolished indicating binding was saturable. This is a major criterion of receptor specificity. Thus specific binding of hormone receptor complexes can be determined by subtracting the non-specific binding from the total binding concentration.

In America and Europe, mammary cancer has the highest rate of incidence of any malignant tumor of either sex. Women are the most susceptible with greater than 99% of all mammary carcinomas. The incidence has remained stable even with women abandoning breast feeding and extensively using long-term steroids such as oral contraceptives (Huggins, 1979). Since normal breast tissue contains specific cytoplasmic binding sites for estrogens, progestogens, glucocorticoids and androgens, the usefulness of receptor studies for treatment of mammary cancer is being used as the basis for clinical trials with potential drug therapies.

Historically, Beatson made the first observation in 1896 that advanced mammary cancer could be induced to regress with surgical removal of both ovaries (Beatson, 1896). Subsequently, numerous ablative procedures were

performed including adrenalectomy and hypophysectomy. Urinary steroid levels were used at that time as an indicator of hormone levels and responsiveness to treatment. Radiation therapy was later used for endocrine ablation. Synthetic steroids were investigated as potential treatment since problems arose with the need for life-long hormone replacement in ablative therapy.

The use of aromatic carcinogens in albino rats has altered the course of research on breast cancer. The Sprague-Dawley rat which easily develops mammary acinar cancer with exposure to cancer-inducing drugs such as 7, 12-dimethylbenzanthrene (DMBA) has been used by researchers such as Huggins in breast cancer research. Many rat mammary cancers are completely hormone dependent and thus reverse with hormone therapy. In human breast cancer, the relationship between estrogen-binding capacity of the tumor and the patients' response to therapy is shown in Tables 1 and 2. Table 1 is a list of investigators compiled by Wittliff showing response to therapy (Wittliff, 1974). From the total number of patients, it can be stated that of those patients with tumors containing estrogen receptors, 78% exhibited objective remission following additive or ablative hormone therapy. Only six percent of patients with tumors lacking estrogen receptors showed any response with hormone therapy.

TABLE 1

RELATIONSHIP BETWEEN ESTROGEN-BINDING CAPACITY OF BREAST TUMORS AND PATIENTS' RESPONSE TO THERAPY

Investigators	Number of patients exhibiting objective remission following additive or ablative hormone therapy	
	Tumors containing estrogen receptors	Tumors lacking estrogen receptors
Jensen, *et al*.	10/16	1/29
Maass, *et al*.	6/7	0/14
Leung, *et al*.	12/12	2/10
Engelsman, *et al*.	14/17	2/20
Savlov, *et al*.	2/4	0/7
Totals	44/56	5/80

(Wittliff, 1974)

TABLE 2

RELATIONSHIP BETWEEN ESTROGEN-BINDING CAPACITY OF BREAST TUMORS AND PATIENTS' RESPONSE TO THERAPY

ER (fm/mg)	Response	% PgR+
3	4/47 = 8 %	10
3-10	11/24 = 45%	30
11-100	15/41 = 36%	50
100	22/36 = 61%	80

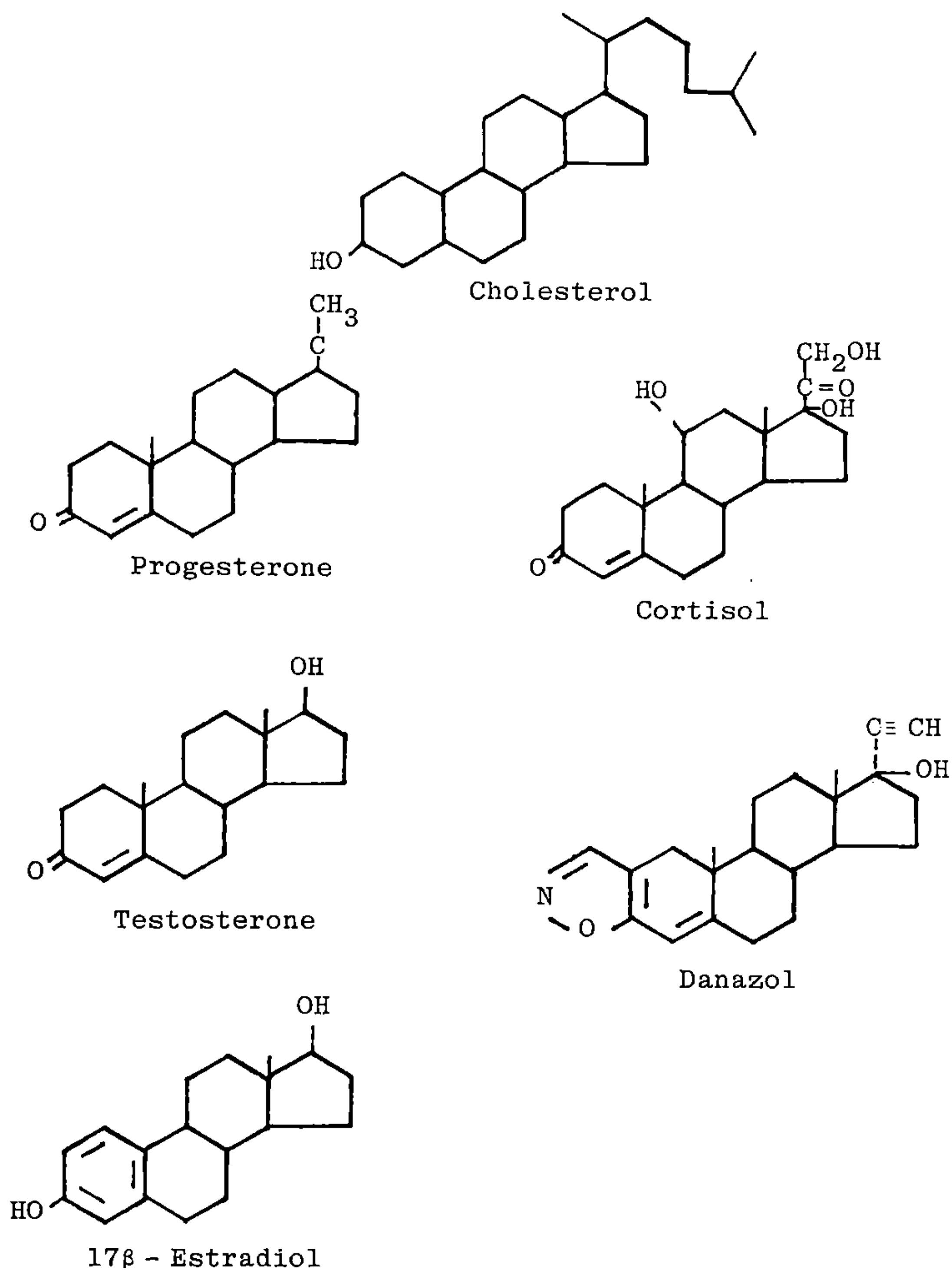
(McGuire, 1975)

ER = estrogen receptor
fm = femptomoles (10^{-15} moles) per mg protein
PgR+ = progesterone receptor complex, positive

Table 2 represents work by McGuire basing response rate on ER concentration (McGuire, 1975). To be considered ER positive, a tumor must have a concentration of equal to or greater than 7 femptomoles/milligram (fm/mg) cytosol protein. ER negative tumors contain less than 3 fm/mg cytosol protein. Table 2 shows that the overall response rate increases with the increasing ER concentration. Those tumors containing more ER complex also have a higher percent of PgR+ concentration. Perhaps the progesterone receptor which is thought to be inducible by the action of the ER complex on genomic sites would be a more appropriate indicator of response to endocrine manipulation (McGuire, 1975).

Synthetic steroids are being used as endocrine therapy for numerous carcinomas based on receptor concentrations. One synthetic steroid being considered for treatment of breast carcinoma is Danocrine, the brand of danazol released in 1976 by the Sterling-Winthrop Research Institute Laboratory. Figure 2 shows the chemical structure of danazol as well as other biologically important steroids for comparison of structural similarities and differences.

Danazol is a heterocyclic steroid related chemically to 17α-ethinyl testosterone. Dmowski reported danazol to be antigonadotropic, antiestrogenic, antiprogestational



Comparison of Danazol With
Some Biologically Important Steroids

Figure 2

and to have mild androgenic activity (Dmowski, 1979). The antigonadotropic properties of danazol were shown by the lowering of plasma follicle stimulating hormone (FSH) and luteotropic hormone (LH) levels through the inhibition of hypothalamic LH releasing hormone secretion (Dmowski, 1979). Dmowski further stated that danazol at low dosage (4 mg/kg body weight) produced antigonadotropic effects while at high dosage (12 mg/kg body weight) an androgenic effect was seen. The marked separation of pituitary gonadotropin inhibitory activity from overt hormone activity makes danazol unique and available for more clinical use.

In reviewing danazol's mode of action, Jenkin concluded that danazol may have antigonadotropic effects and steroid enzyme inhibitory effects but neither of these effects were sufficient to account for the complete mechanism of action (Jenkin, 1980). Danazol's effect on reported inhibition of cytoplasmic binding of hormone receptors warrants further consideration for its possible use in treatment of mammary carcinoma (Musich, et al., 1981) (Cook and Gibb, 1980).

Whereas anti-estrogens, usually non-steroids, block the uptake of estrogen in target tissue by binding to the estrogen receptor, danazol as a mild androgen was shown by some researchers to bind to the androgen,

glucocorticoid and progesterone receptors, but not to the estrogen receptor (Barbieri, *et al.*, 1979). Based on this evidence and the fact that danazol is derived from 17α-ethinyl testosterone, danazol should be considered non estrogenic and non anti-estrogenic. Cook and Gibb described danazol as a possible competitive inhibitor of estradiol binding by competing with ^{3}H-labeled estradiol for uterine receptors (Cook and Gibb, 1980). However patients were administered up to an 800 mg daily oral dosage of danazol in these studies.

In vivo receptor studies of danazol binding have been conducted by Barbieri, *et al.* (1981), Musich, *et al.* (1981), Chamness, *et al.* (1980), and Wood, *et al.* (1975). In studies by Musich, *et al.*, after short-term danazol administration a small but significant increase in uterine cytosol estradiol receptors was noted. Long-term danazol administration showed a marked decrease in uterine estradiol receptor binding. The uterotropism of danazol may have been due to actual receptor modification and nuclear receptor studies may elucidate this possibility (Musich, *et al.*, 1981).

Danazol has been approved by the Food and Drug Administration for treatment of endometriosis and fibrocystic breast disease. Clinical trials using danazol for treatment of breast carcinoma were based on

convincing studies by Peters, et al. (1979). In 59 DMBA-treated female rats, 75% developed mammary cancer. Of those that were then treated with danazol (100 or 400 mg/kg/day) 66% showed tumor regression and 36% showed complete regression. Of 50 rats injected with danazol (100 or 400 mg/kg/day) only 14% developed tumors after DMBA administration, while 84% of the control rats developed mammary cancer. The results of this study indicate that in animals with established tumors, danazol was effective in altering tumor growth and that the results were not dose dependent (Peters, et al., 1979).

Based on the review of literature and the need for more danazol-binding studies to be documented, two in vivo experiments were conducted studying the effects of two dosage levels of danazol on cytoplasmic receptors of estrogen, progesterone, dihydrotestosterone and glucocorticoids in uterine, mammarian, hypothalamic, and adrenal tissue. Although this research is done on non-cancerous tissue, research will be beneficial in future study of female rat and human mammary carcinoma.

MATERIALS AND METHODS

Animal Care

Two experiments were performed on female Sprague-Dawley (S/D) rats. The rats were housed in cool quarters under a regimen of ten hours darkness and fourteen hours of artificial lighting. They were allowed Purina Rat Chow and tap water *ad libitum*.

Experiment I

Twenty-four female S/D rats were divided into two groups. The first group was given daily intramuscular (IM) injections of danazol (4 mg/kg body weight) suspended in Planters Peanut Oil. This treatment continued for fourteen days. The control group received daily IM injections of peanut oil only for fourteen days. On treatment day nine, bilateral ovariectomy was performed on eight of the twelve experimental and control rats, leaving four intact in each group. Surgery was performed under anesthesia using Sodium Nembutal (Abbot Laboratories), (4 mg/100 gm body weight) injected intraperitoneally (IP). Sodium Penicillin G 10 mg was injected IM into each post-operative rat. The ovariectomized group received an IM injection of 100 micrograms of estradiol benzoate on treatment day twelve.

Preparation of Tissue

All animals were sacrificed on treatment day fourteen. Tissues for assay were immediately removed, trimmed of excess fat, weighed to the nearest milligram, dropped into an iced, acetone-CO_2 pellet bath, blotted, and kept frozen at -20°C until assays were performed.

Preparation of Cytosol

All reagents used for preparation of cytosol are listed in Table 3. Cytosol fraction was prepared by the method derived by Heidemann and Wittliff for the Beckman "Airfuge" (Heidemann and Wittliff, 1979). All tissues were prepared in the same manner. The frozen minced tissue was homogenized in cold phosphate buffer (2.0 ml/gm tissue) with a Ten Broeck glass-glass homogenizer. All preparations were kept on ice at all times. Using an Oxford adjustable sampler micropipetting system, 0.175 ml of cytosol was added to pre-cooled cellulose nitrate tubes (Beckman Instruments, Inc.) and placed in the pre-cooled (4°C) head of a fixed-angle rotor type A-100 Beckman Airfuge (Beckman Instruments, (Inc.) and centrifuged at 110,000 x g (24 psi) for 10 minutes. The protein concentration of the supernatant was confirmed later by a modified Lowry method (Lowry, *et al.*, 1951).

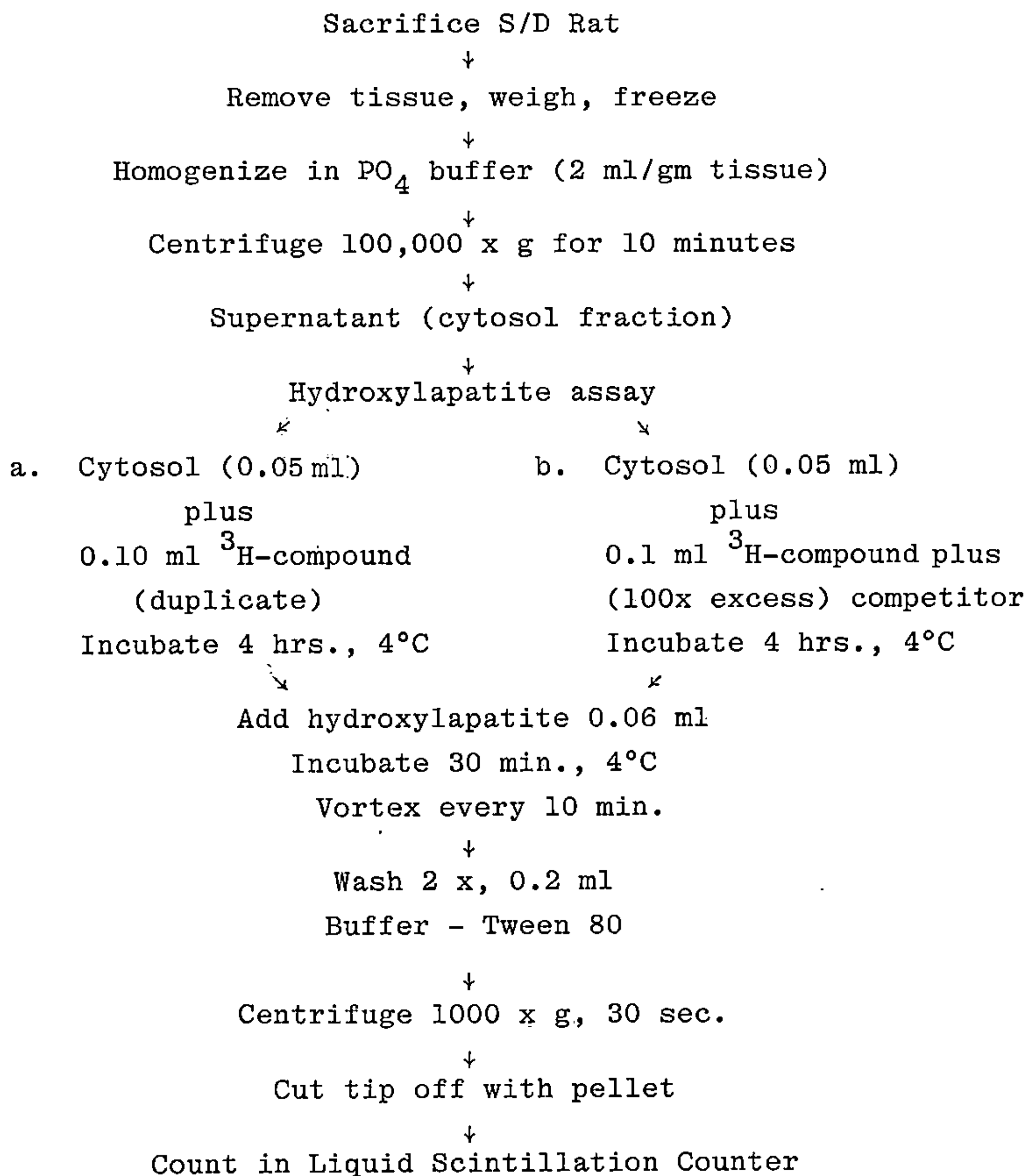
TABLE 3

REAGENTS

Danazol	Brandname Danocrine, gift of Sterling-Winthrop Research Institute.
Hormones	^{3}H-17β-estradiol, progesterone, dihydrotestosterone, corticosterone; dissolved initially in absolute alcohol then diluted with phosphate buffer (pH 7.4) to appropriate concentrations (New England Nuclear, Corp.).
	Diethylstibesterol, progesterone, dihydrotestosterone, corticosterone; dissolved initially in absolute alcohol then diluted to appropriate concentrations with phosphate buffer (pH 7.4) (Sigma Chemical, Co.).
Hydroxylapatite Suspension	100 ml TP buffer plus 2.5 gm DNA-grade Bio Gel HTP hydroxylapatite (Bio-Rad), pH adjusted to 7.2.
Phosphate Buffer	5 mM sodium phosphate, pH 7.4; 1 mM monothioglycerol; 10% glycerol.
Reagent A	2% Na_2CO_3 in 0.1 N NaOH.
Reagent B	0.5% $CaSO_4 \cdot 5\ H_2O$ in 1% Na or K tartrate.
Reagent C	25 ml reagent A plus 0.5 ml reagent B (mixed fresh daily).
Reagent D	1:1, Phenol Folin Reagent: distilled water (mixed fresh daily).
Scintiverse I ™	Universal LSC Cocktail (Fisher Scientific, Co.).
Tris Buffer	50 mM Tris, 10 mM KH_2PO_4, H 7.2 at 4°C.
Wash Buffer	Phosphate Buffer, pH 7.4; plus 1% (V/V) Tween 80.

Hydroxylapatite Micromethod

All reagents used for the hydroxylapatite micromethod are listed in Table 3. The Oxford adjustable micropipette system was used for all micropipetting. From pooled centrifuged supernatant (cytosol fraction), 0.05 ml aliquots were incubated in duplicate for four hours at 4°C in 0.45 ml polyethylene microtubes (Bio-Rad Laboratories) with 0.05 pmol (0.1 ml of 2×10^{-10}) ^{3}H-labeled hormone in 0.1 ml phosphate buffer. A parallel tube contained a 100-fold excess of competing unlabeled hormone. After incubation, 0.06 ml of hydroxylapatite (HAP) suspension was added to each microtube. After vortex, further incubation for 30 minutes was completed with gentle vortex every ten minutes. The microtubes were then centrifuged for 30 seconds in a pre-cooled Adams-Sero Fuge centrifuge (Clay-Adams, Inc.) at 1000 x g. The pellets were washed twice with 0.2 ml wash buffer, with centrifugation and gentle aspiration after each wash. After the final wash, centrifugation and aspiration, the resulting pellet in the end of the microtube was cut off and dropped into a scintillation vial. Scintiverse I (Fisher Scientific, Co.) or Insta Gel (Packard) scintillation Cocktail (10 ml) was added to each vial and all vials were counted for radioactivity in a Packard Tri-carb liquid scintillation counter (Model 300-C). This method is summarized in Figure 3.



Hydroxylapatite Micromethod

Figure 3.

Assay of Protein

All reagents used for the assay of protein are listed in Table 3. To 0.05 ml pooled supernatant cytosol placed in cellulose nitrate tubes was added 0.05 ml 10% trichloroacetic acid. After centrifugation in the Beckman Airfuge at 90,000 x g (20 psi) for five minutes, 0.10 ml 3N NaOH was mixed with the precipitated proteins and allowed to sit for 10 minutes at room temperature. The remainder of the determination for protein follows the standard Lowry procedure (Lowry, et al., 1951). Bovine serum albumin (1 mg/ml) was the standard. After the appropriate reagents were added, absorbance was read on the Spectronic-70 (Bausch and Lomb) at 750 nm. Protein concentration was established and expressed in mg/ml cytosol.

Experiment II

Ten female S/D rats were divided into two groups. The first group was given daily IM injections of danazol (8 mg/kg body weight) suspended in Planters Peanut Oil. This treatment continued for three days. The control group was given daily IM injections of peanut oil only, for three days.

Preparation of tissue, cytosol, and the hydroxylapatite microassay were identical to those of Experiment I.

Statistical Analysis

Analysis of data was by means of the Student's t test.

RESULTS

Experiment I

The effect of 4 mg/kg of danazol for 14 days can be seen in Table 4. Daily injections of danazol showed a highly significant reduction of uterine weight expressed either in absolute or relative weight terms in rats which had intact ovaries. Ovariectomized rats failed to show a further reduction in weight after danazol injections. The reduction in uterine weight after ovariectomy is also highly significant ($P < .01$).

Table 5 indicates that danazol has an effect on reducing pooled adrenal weight in intact rats but shows no additive reduction in adrenal weight after ovariectomy.

Rats receiving daily injection of danazol (4 mg/kg body weight) for nine days had mean ovarian weights of 130 ± 12 at the time of ovariectomy, compared to rats receiving only the vehicle (154 ± 20) as indicated in Table 6. This reduction in ovarian weight was highly significant ($P < .02$).

The effects of danazol injections on steroid receptors in the uterus is shown in Table 7. Danazol treated rats show a non-significant decrease in all receptors compared to intact rats with estrogen receptors showing a 28% reduction. However, significant differences

TABLE 4

EXPERIMENT I

EFFECT OF DANAZOL ON ABSOLUTE AND RELATIVE UTERINE WEIGHT

Treatment	Absolute Uterine Weight (mg)	Relative Uterine Weight (mg/100 gm body weight)
Intact Control (n = 4)	686 ± 30**	224 ± 14
Intact Danazol* (n = 4)	498 ± 50[a]	174 ± 16[a]
Ovariectomized Control (n = 8)	498 ± 85[b]	180 ± 28[a]
Ovariectomized Danazol* (n = 8)	489 ± 100[b]	176 ± 31[a]

*Danazol = 4 mg/kg/day for 14 days
**Mean ± Standard Deviation
[a] $P < .02$ compared to Intact Control
[b] $P < .01$ compared to Intact Control

TABLE 5

EXPERIMENT I

EFFECT OF DANAZOL ON ABSOLUTE AND RELATIVE POOLED ADRENAL WEIGHT

Treatment	Absolute Adrenal Weight (mg)	Relative Adrenal Weight (mg/100 gm body weight)
Intact Control	70.3 **	22.8
Intact Danazol*	64.8	23.5
Ovariectomized Control	57.3	22.6
Ovariectomized Danazol*	57.1	23.7

*Danazol = 4 kg/day for 14 days
**Mean adrenal weight calculated from pooled adrenals

TABLE 6

EXPERIMENT I

EFFECT OF DANAZOL ON ABSOLUTE OVARIAN WEIGHT

Treatment	Absolute Ovarian Weight (mg)
Control (n = 8)	154 ± 20**
Danazol*(n = 8)	130 ± 12[a]

*Danazol = 4 mg/kg/day for nine days
**Mean ± Standard Deviation
[a] $p < .02$ Compared to ovariectomized controls

TABLE 7

EXPERIMENT I

PERCENT CHANGE IN RECEPTOR BINDING OF ^{3}H-STEROIDS AFTER DANAZOL INJECTIONS AND OVARIECTOMY

Tissue	Treatment	E_2	DHT	PROG	CORT
Uterus	intact	100	100	100	100
	+danazol*	28 ↓ **	17 ↓	23 ↓	13 ↓
	ovarx	42 ↓ [a]	10 ↓	25 ↓	4 ↓
	+danazol	45 ↓ [a]	20 ↓	24 ↓	8 ↓
Mammary	intact	100	100	100	100
	+danazol	2 ↑	11 ↓	5 ↓	2 ↓
	ovarx	12 ↓	7 ↓	8 ↓	3 ↑
	+danazol	15 ↓	12 ↓	10 ↓	10 ↓

*Danazol injected at dose of 4 mg/kg/day for 14 days

**Arrows refer to increase ↑ or decrease ↓ in specific binding compared to intact control

[a] Significant from controls at $P < .05$

were seen for estrogen receptors when the rats were ovariectomized (42% reduction) and when these castrated rats received danazol (45% reduction). The ovariectomy failed to reduce significantly the concentration of the other steroid receptors. Mammary tissue was not significantly changed in its ability to bind steroid receptors.

Binding data for hypothalamic tissue and adrenal glands were incomplete since they were analyzed from pooled tissues. However, they demonstrated the general trend toward reduction of receptor binding.

Experiment II

Table 8 shows that there was a non-significant increase in uterine weights after injection of danazol (8 mg/kg/day for 3 days) in pre-puberal rats. Adrenal weights were not significantly changed from controls (Table 8).

Specific binding of estrogen was demonstrated by adding a 100-fold excess of DES which resulted in abolishing 78% of total estrogen binding. A 100-fold excess of unlabeled progesterone abolished total progesterone binding by 84%. Likewise, a 100-fold excess of unlabeled DHT abolished 84% of the radioactive counts of total DHT binding.

TABLE 8

EXPERIMENT II

EFFECT OF DANAZOL ON ABSOLUTE AND RELATIVE UTERINE AND ADRENAL WEIGHTS OF PRE-PUBERAL RATS

Tissue	Treatment	Absolute Weight (mg)	Relative Weight (mg/100 gm body weight)
Uterine	Control (n = 5)	220 ± 41 **	201 ± 35
	Danazol* (n = 5)	247 ± 52 (N.S.)	222 ± 41 (N.S.)
Adrenal	Control (n = 5)	21.4 ± 3.6	19.7 ± 3.3
	Danazol* (n = 5)	20.6 ± 2.4 (N.S.)	18.5 ± 1.4 (N.S.)

*Danazol = 8 mg/kg/day for 3 days
**Mean ± Standard Deviation
N.S. = Not significant from controls

In evaluation of the percent change of receptor binding, Table 9 shows that there is a non-significant reduction in the binding of estrogen, progesterone and dihydrotestosterone receptors in the uterus. Although danazol showed a 23% increase in estrogen receptor binding in pooled adrenals, DHT levels decreased 10% of controls. Estrogen and DHT receptor binding was decreased 8% and 22%, respectively, in pooled hypothalamic tissue.

TABLE 9

EXPERIMENT II

PERCENT CHANGE IN RECEPTOR BINDING OF ^{3}H-STEROIDS AFTER DANAZOL* INJECTIONS

Tissue	Estrogen	Dihydrotestosterone	Progesterone
Uterus (n = 5)	11 ↓** (N.S.)	2 ↓ (N.S.)	20 ↓ (N.S.)
Adrenals (pooled)	23 ↑	10 ↓	—
Hypothalamus (pooled)	8 ↓	22 ↓	—

*Danazol = 8 mg/kg/day for 3 days

**Arrows refer to increase ↑ or decrease ↓ in specific binding compared to controls as follows: $\frac{\text{Control} - \text{Danazol injected}}{\text{Control}} \times 100$

N.S. = Not significant from controls

DISCUSSION

Two *in vivo* experiments using female rats have been conducted. The possible effects of a dose-relationship of danazol and/or ovariectomy on four cytosol receptors in five tissues have been investigated in the rat utilizing the hydroxylapatite micromethod. Total binding was reduced by the non-specific binding using 100-fold excess of non-labeled hormone to obtain specific binding.

In Experiment I, specific binding in intact rats treated with danazol (4 mg/kg body weight) shows a non-significant reduction in all receptor binding in uterine cytosol (see Table 7). The estrogen binding in rats receiving danazol shows a 28% reduction from controls. Progesterone was reduced 23%, DHT reduced 17%, and corticosterone reduced 13% after danazol injections. These findings agree with similar studies done in the past several years. Barbieri found that danazol displaced DHT receptors in rat prostate, displaced progesterone in an estrogen-primed rat uterus, and that glucocorticoid receptors were displaced by danazol in rat liver cytosol (Barbieri and Ryan, 1981). In another study, Potts showed that intact rats, pre-treated with danazol, showed significant inhibition in the pituitary and hypothalamic uptake of ^{3}H-labeled estradiol. (Potts, 1977). Musich,

using a long term danazol at a high dosage, as opposed to low dosage as used in this study, showed a decrease in E-R binding which may have been due to the dose or the duration of treatment (Musich, et al., 1981). From a different viewpoint, Jenkin reported that danazol decreases the stimulatory effect on basal circulating levels of estrogen and progesterone to the uterus thereby also directly effecting estrogen receptors in the tissue (Jenkin, 1980). Jenkin also stated that danazol competed with the uterine cytosol receptor for the estradiol and progesterone receptors.

The effect of ovariectomy on receptor binding was only significant for estrogen receptors (see Table 7). The 42% reduction of estrogen binding in ovariectomized rats was reduced to 45% in rats that received danazol injections in addition to the ovariectomy. This agrees with studies by Bohnet, et al. (1981) who reported that danazol prevented a compensatory increase in LH and FSH after ovariectomy. Potts found that ovariectomized rats treated with danazol significantly inhibited pituitary and hypothalamic uptake and thus binding of ^{3}H-labeled estradiol (Potts, 1977). These findings disagree with findings by Wood who dimissed the local action by danazol on estrogen receptors when a 1000-fold excess of danazol did not effect the ^{3}H-estradiol binding to endometrial

cytosol (Wood, 1975). However, Wood's study was in vitro and many times in vivo and in vitro studies cannot be compared.

Other steroid receptor binding was not significantly reduced in Experiment I. The 25% reduction in progesterone binding after ovariectomy was not significant, a finding that differs from a study by Peters who reported that in ovarian hypofunction, a decrease in estrogen production would produce less progesterone receptors and thus reduced progesterone binding (Peters, et al., 1977). Danazol injections failed to influence a change in progesterone receptors after ovariectomy.

With respect to mammary tissue, these studies were investigated with the hope that some data reflecting the influence of danazol on mammary receptors would be enlightening. However, from the data presented in Table 7, mature female rats, either intact or ovariectomized, showed no significant change in the ability to bind steroid receptors.

The effect of danazol on the reduction of uterine and ovarian weights at low dose (4 mg/kg), long term (14 days), danazol treatment is consistant with that reported by other investigators (Jenkin, 1980) (Dmowski, 1971). This might be expected since the reported antigonadotropic effects of danazol (Dmowski, 1971) and

the direct effect of danazol on inhibiting steroidogenesis (Barbieri and Ryan, 1981) should lead to a reduction in uterine weight. Adrenal weight reduction is also consistent with Kitay's findings that estrogens have a stimulatory effect on the adrenal glands (Kitay, et al., 1963), and the more recent discovery that adrenals possess an androgen receptor which when bound by androgens, lowers adrenal weights (Rifka, 1978). Potts also reported a decrease in adrenal weights after danazol treatment (Potts, 1974).

After short term danazol administration at high dosage (8 mg/kg/3 days) as seen in Experiment II, it can be said that the weight data are fairly consistent with findings by Barbieri and Ryan (1981), Potts (1974) and Dmowski (1971). The increase in uterine weight of pre-puberal rats may be termed a uterotropic effect of the high dose of danazol. This uterotropic effect may be explained by a possible anti-estrogen mode of action of danazol at high dosage similar to that reported by Musich, et al. (1981), involving progesterone receptor studies of tamoxifen, an anti-estrogen. Effects here could be explained by the anabolic action of the androgens on the uterus which danazol could also produce at this dosage.

The results from Experiment II again show that in all cases but one, danazol produced a decrease in specific

binding of estrogen, progesterone and DHT in the uterus and hypothalamus (Table 9). Only in the adrenals of the pre-puberal rat did a 23% increase of estrogen binding take place, while at the same time, DHT binding decreased 10%.

The controversy over danazol's steroid binding to cytosol receptors remains but it has been seen that danazol has an effect on multiple classes of steroid receptors. Although discrepancies from established data have been noted, it should be remembered that in vivo receptor studies may reflect the indirect effects of danazol. Therefore it should not be surprising that inconsistant receptor data might be seen. Even with in vitro studies discrepancies have been reported (Chamness, et al., 1980). Another possibility for disagreement on receptor binding studies is that the metabolites of danazol are hormonally active and may play a part in receptor variability (Krey, 1981). These metabolites and their effect were not investigated.

Since steroid receptors are thermolabile, unstable proteins, possible variable results may be due to incorrect sample storage or assay conditions such as keeping the sample cold enough to enhance binding activity. Another consideration is that many receptor sites may be nuclear binding sites, so that cytoplasmic sites alone may

not be an accurate display of the influence of danazol and/or ovariectomy on receptor binding. In any event, receptor studies are a molecular index of endocrine function and dependency in a cell and improvements in quantitation of receptor complexes continue to be vital to hormone research and clinical therapy.

In summary, the results reported in this study have demonstrated the following with danazol injections of 4 mg/kg/body weight:

1). A highly significant reduction of uterine weight in ovariectomized and intact rats.

2). A highly significant reduction in uterine weight after ovariectomy.

3). A reduction in pooled adrenal weight (intact rats), with no additive weight reduction after ovariectomy.

4). A highly significant reduction in ovarian weights after nine days of danazol injections.

5). A non-significant decrease in all uterine receptors of danazol treated rats compared to intact rats.

6). A significant difference in ovariectomized rats for estrogen receptors.

7). Ovariectomy failed to reduce the concentration of other steroid receptors.

8). Mammary tissue was not significantly changed in its ability to bind steroid receptors.

9). A general trend toward reduction of receptor binding in hypothalamic and adrenal glands.

With danazol injections of 8 mg/kg body weight, the following results were demonstrated:

1). A non-significant increase in uterine weights but not adrenal weights from controls.

2). Specific binding of estrogen, progesterone and dihydrotestosterone were decreased by 'cold' steroids.

3). A non-significant reduction in the binding of estrogen, progesterone and dihydrotestosterone receptors in the uterus.

4). An increase in estrogen binding in pooled adrenals with a decrease in DHT levels.

5). A decrease in estrogen and DHT receptor binding in pooled hypothalamic tissue.

These results, though inconclusive, may contribute to a better understanding of the effects of danazol at the molecular level. The significance of this type of research might allow for a better utilization of clinically prescribed endocrine therapy.

LITERATURE CITED

Barbieri, R. L., H. Lee and K. Ryan. 1979. Danazol binding to rat androgen, glucocorticoid, progesterone, and estrogen receptors: correlation with biologic activity. Fertil. Steril. 31(2):182-185.

Barbieri, R. L. and K. J. Ryan. 1981. Danazol: endocrine pharmacology and therapeutic applications. Am. J. Obst. Gyn. 141(4):453-463.

Beatson, G. T. 1896. On the treatment of inoperable cases of carcinoma of the mamma: suggestions for a new method of treatment, with illustrative cases. Lancet 20:104-107.

Bohnet, H. G., J. Harker, K. Schweppe and H. Schneider. 1981. Changes of prolactin secretion following long term danazol application. Fertil. Steril. 36(6):725-728.

Chamness, G. C., R. H. Asch and C. J. Pauerstein. 1980. Danazol binding and translocation of steroid receptors. Am. J. Obst. Gyn. 136(4):426-429.

Cook, D. B. and I. Gibb. 1980. Competitive inhibition by danazol of oestradiol binding to rabbit and ovine uterine oestradiol receptors. J. Steroid Biochem. 13:1325-1329.

Dmowski, W. P., H. F. Scholer, V. B. Mahesh and R. B. Greenblatt. 1971. Danazol - a synthetic steroid derivative with interesting physiologic properties. Fertil. Steril. 22:9-18.

Dmowski, W. P. 1979. Endocrine properties and clinical application of danazol. Fertil. Steril. 31(3):237-251.

Engelsman, E., J. P. Persinjn, C.B. Korsten and F. J. Cleton. 1973. Oestrogen receptors in human breast cancer tissue and response to endocrine therapy. Br. Med. J. 2:750-752.

Erdos, T., M. Best-Belpomme and R. Bessada. 1979. A rapid assay for binding estradiol to uterine receptor. Ann. Biochem. 37:244-252.

Huggins, C. B. 1979. Experimental leukemia and mammary cancer - induction, prevention, cure. Univ. of Chicago Press, Chicago, p. 173.

Jenkin, G. 1980. The mechanism of action of danazol, a novel steroid derivative. Aust. N. Z. Obst. Gyn. 20:113-118.

Jensen, E. V. and H. I. Jacobson. 1960. In: Biological activities of steroids in relation to cancer. G. Pincus and E. P. Vollmer (eds.) Academic Press, N.Y.

Jensen, E. V. and H. I. Jacobson. 1962. Basic guides to the mechanism of estrogen action. Recent Progr. Hormone Res. 18:387-414.

Jensen, E. V., D. J. Hurst, E. R. DeSombre and P. W. Jungblut. 1967. Sulfhydryl groups and estradiol-receptor interaction. Science 158:385-389.

Jensen, E. V., T. Kawashima, W. E. Stumpf, P. W. Jungblut and E. R. DeSombre. 1968. A two-step mechanism for the interaction of estradiol with rat uterus. Proc. Nat. Acad. Sci. 59:632-639.

Jensen, E. V. and E. R. DeSombre. 1973. Estrogen-receptor interaction. Science 182:126-134.

Kitay, I. J. 1963. Pituitary-adrenal function in the rat after gonadectomy and gonadal hormone replacement. Endocrinology. 73:253-256.

Krey, L. C., R. Robbins and B. McEwen. 1981. Danazol suppression of lutenizing hormone secretion: the role for danazol-androgen interaction within the brain-pituitary complex. Fertil. Steril. 35(4):467-472.

Leung, B. S., W. S. Fletcher, T. D. Lindell, D. Wood and W. Krippaekne. 1973. Predictability of response to endocrine ablation in advanced breast carcinoma. Arch. Surg. 106:515-519.

Lowry, O. H., N. Rosebrough, A. Farr and R. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Maass, H., B. Engel, H. Hohmeister, G. Lehmann and G. Trams. 1972. Estrogen receptors in human breast tissue. Am. J. Obst. Gyn. 113:377-382.

McGuire, W. L. 1975. Current status of estrogen receptors in human breast cancer. Cancer 36:638-644.

Musich, J. R., S. J. Behrman and K. Menon. 1981. Estrogenic and anti-estrogenic effects of danazol administered in studies of estradiol receptor binding. Am. J. Obst. Gyn. 140(1):62-69.

Notebloom, W. D. and J. Gorski. 1965. Sterospecific binding of estrogens in the rat uterus. Arch. Biochem. Biophys. 111:559-568.

O'Malley, B. W. and A. R. Means. 1974. Female steroid hormones and target cell nuclei. Science 183:610-620.

Pavlik, E. J. and P. B. Coulson. 1976. Hydroxylapatite "batch" assay for estrogen receptors: increased sensitivity over present receptor assays. J. Steroid Biochem. 7:357-368.

Peters, T. G., J. D. Lewis, E. J. Wilkinson and T. M. Fuhrman. 1977. Danazol therapy in hormone-sensitive mammary carcinoma. Cancer 40:2797-2800.

Potts, G. O., A. Beyler and H. P Shane. 1974. Pituitary gonadotropin inhibitory activity of danazol. Fertil. Steril. 25(4):367-372.

Potts, G. O. 1977. Pharmacology of danazol. J. Int. Med. Res. (Suppl. 3) 5:1-5.

Rifka, S. M., G. B. Cutler, Jr., M. A. Sauer and D. L. Loriaux. 1978. Rat adrenal androgen receptor: a possible mediator of androgen-induced decrease in rat adrenal weight. Endocrinology 103(4):1103-1110.

Savlov, E. D., J. L. Wittliff, R. Hilf and T. Hall. 1974. Correlations between certain biochemical properties of breast cancer and response to therapy: a preliminary report. Cancer 33:303-309.

Spelsberg, T. C., A. W. Steggles and B. W. O'Malley. 1971. Progesterone-binding components of chick oviduct. J. Biol. Chem. 246:4188-4197.

Toft, D. and J. Gorski. 1966. A receptor molecule for estrogen: isolation from the rat uterus and preliminary characterization. Proc. Nat. Acad. Sci. 55:1574-1581.

Wittliff, J. L. 1974. Specific receptors of the steroid hormones in breast cancer. Sem. Oncol. 1(2): 109-118.

Wood, G. P., C. H. Wu, G. Flickinger and G. Mikhail. 1975. Hormonal changes associated with danazol therapy. Obst. Gyn. 45:302-304.