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CHARACTERIZATION OF DANAZOL BINDING TO SPECIFIC CYTOSOL RECEPTORS *IN VITRO*

A Thesis

Presented to

the Faculty of the School of Sciences and Mathematics

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In Partial Fulfillment

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Master of Science in Biology

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by

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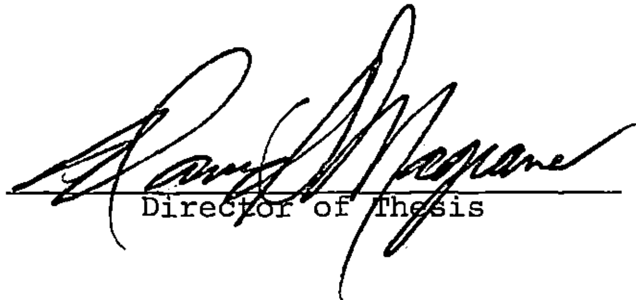

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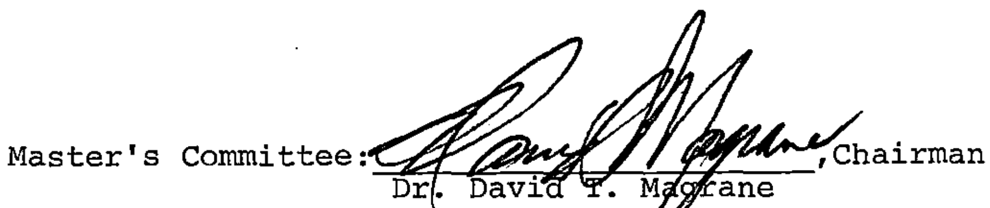

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ABSTRACT

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BINDING OF SPECIFIC
CYTOSOL RECEPTORS
IN VITRO

Gail Wright Russell, M. S.
Morehead State University, 1982

The binding of danazol to specific cytosol receptors *in vitro* was performed on uterine, mammary, adrenal, pituitary and hypothalamic tissue, using the hydroxylapatite assay. This study was designed to determine the specificity of danazol binding to cytosol receptors, evaluate danazol's dose responsiveness and determine the ability of danazol to translocate specific cytosol receptors into the nucleus. Results were obtained by using radioactively tagged estradiol, dihydrotestosterone, progesterone and corticosterone.

Danazol was shown to bind and translocate each steroid receptor, though not significantly, while showing the greatest success with the androgen receptor in all tissues. The dose responsiveness of danazol was effectively demonstrated by its increased competition at pharmacological concentrations. Furthermore, the results indicated that danazol was most effective in translocating the androgen receptor in mammary tissue.

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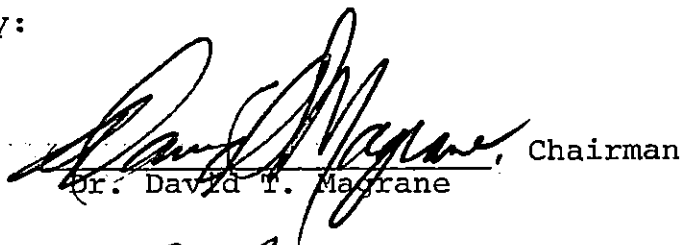

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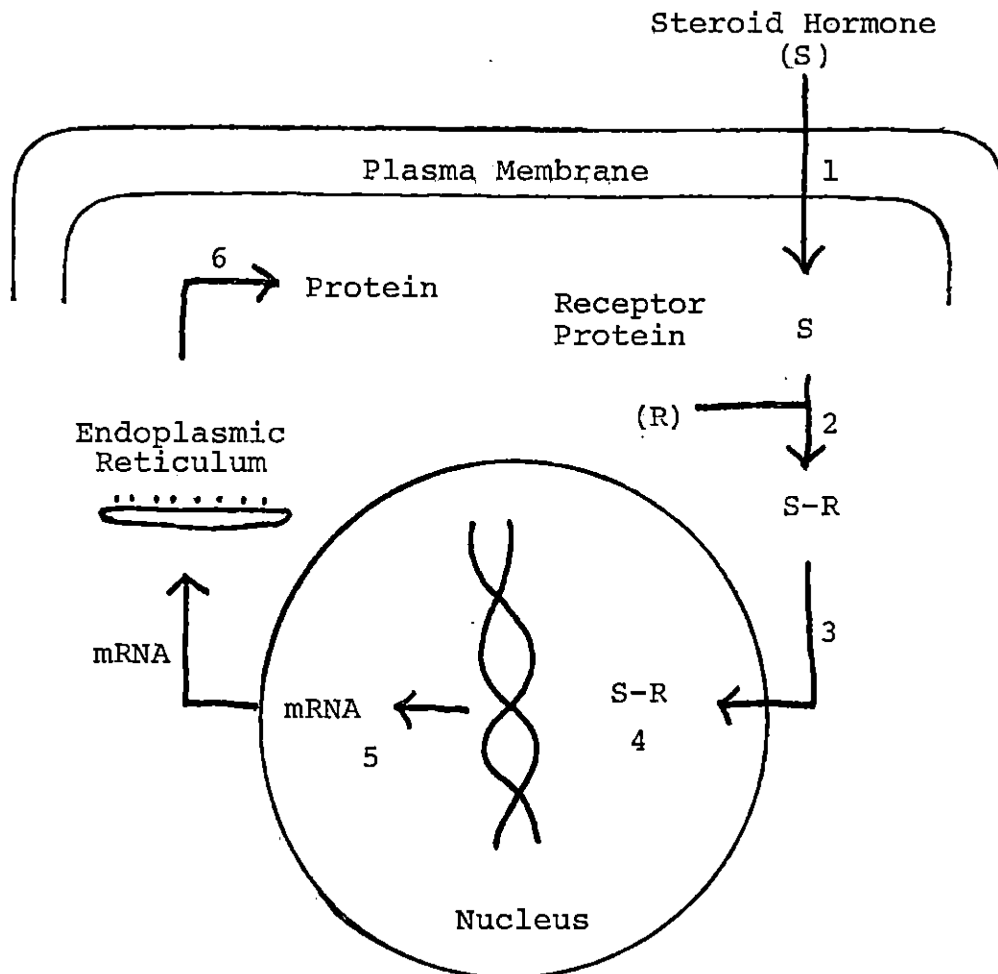
INTRODUCTION

The intracellular mechanisms underlying steroid regulation of target cells are not fully understood. However, important events in the regulation appear to be steroid entry into cells of the specific target tissue, interaction of the steroid with its specific cytosol receptor, and translocation of the steroid-receptor complex to the cell nucleus. Presumably this leads to initiate messenger RNA (ribonucleic acid) and protein synthesis (Figure 1).

An important factor in establishing the current understanding of the mechanism of steroid hormone action was the synthesis of isotopically labeled compounds, they provided the first molecular probes to discern the sequence of events that occur following the interaction of a steroid hormone with a target cell (Jensen and Jacobson,1962). The use of tritium (^3H) labeled compounds has also become very prominent in estrogen receptor studies involving breast cancer.

An increased incidence of new cases of breast cancer annually in the United States and numbers of deaths due to the disease has created a greater interest towards studies evaluating estrogen receptors and their role in treatment of malignant and benign tumors. Witliff (1977)



1. Steroid entry into cell
2. Receptor recognition
3. Transformation and translocation
4. S-R binding to chromatin
5. Transcription of specific mRNA
6. Protein synthesis

Figure 1. Mechanism of action of steroid hormones.

suggests that a tissue demonstrating estrogen receptors should be classified as an estrogen target and should require hormone for maintenance and growth. Removal of estrogen should result in regression or involution of the tissue. In contrast, tissue lacking estrogen receptors should not be effected by removal of endogenous hormone. The first evidence of a relationship between the association of ^{3}H hormones with a breast tumor and the clinical response of the patient to ablative hormone therapy was shown by Folca and associates in 1961 (Folca, Glascock, and Irvine, 1961). Other research also substantiates Witliff's proposal showing that 25-40% of patients with inoperable breast cancer have neoplasms that regress after either the administration or pharmacological concentrations of hormones, such as androgens and estrogens, or removal of endogenous hormones, such as by ovariectomy or adrenalectomy (Kennedy, 1974). These findings suggest that some malignant breast tumors may be hormone dependent.

The concept underlying endocrine therapy is that certain tumor cells have retained their ability to respond to the same hormonal stimuli as their normal progenitor cells (Witliff, 1977). It is therefore suggested that the presence of specific estrogen receptors in human breast carcinomas may be predictive of a patient's

response to endocrine therapy (Garola and McGuire, 1978, Witliff, 1977, and McGuire, 1975).

Other steroid receptors are also present in human breast cancer cells and may provide additional information. For example, Horwitz et al (1975) predict that the presence of progesterone receptors in a tissue should be an even more sensitive indicator of potential responsiveness to endocrine therapy than the estrogen receptor. The basis for such a prediction is the underlying concept that progesterone effects require estrogen priming, thereby inducing the synthesis of the progesterone receptor (Figure 2).

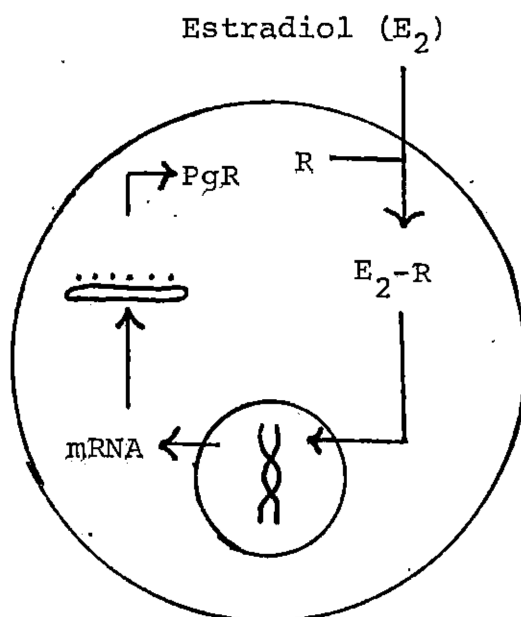


Figure 2. Induction of progesterone receptor by estradiol (McGuire et al, 1978).

Thus, the presence of progesterone receptors in a tumor would indicate that the entire sequence, involving estrogen binding to a cytoplasmic receptor, movement of the receptor complex into the nucleus, and stimulation of a specific end product, can be achieved in a tumor cell. This would rule out the existence of a defect beyond the binding step (McGuire *et al*, 1978). Therefore, the presence of progesterone receptors in cancerous tissue will show that the tumor remains under at least partial endocrine control and may be classified as endocrine responsive. In support of the prediction, research showed 56% of tumors with estrogen receptors also had progesterone receptors, and tumors without estrogen receptors also lacked progesterone receptors (Horwitz *et al*, 1975). Preliminary data show only those breast tumors with progesterone receptors regressed after endocrine therapy. Continuation of estrogen and progesterone receptor studies together with research directed toward the evaluation of other steroid receptors present in human breast carcinomas, such as androgens and glucocorticoids may give hope of treatment for those patients with estrogen-receptor positive but unresponsive tumors.

In research by McGuire (1977), it was shown in randomly tested human breast tumors that 75% were estrogen-receptor positive (ER^+), with 74% of the ER^+

tumors also having progesterone receptors (PgR) and only 9% of the ER^- tumors having PgR. Where primary versus metastatic tumors were examined, 77% of the primary tumors were ER^+ with 77% of them containing PgR and 66% of the metastatic tumors with ER^+ with only 59% of them having PgR (Figure 3).

Danazol is an isoxazole derivative of the synthetic steroid 17 α-ethinyltestosterone (Figure 4). It acts through the suppression of gonadal function (Chamness et al, 1980), and may possibly be used in future treatment of hormonally dependent cancers, particularly those of the breast and endometrium (Jenkin, 1980). Support for the use of danazol was strengthened by the reported increased incidence of breast cancer in patients with a history of benign breast diseases (Humphrey and Swerdlow, 1962, Davis et al, 1964, Veronesi and Pizzocaro, 1968, and Dmowski and Cohen, 1978), and the fact that danazol has been shown to cause relief of various disorders of the breast (Greenblatt et al, 1971, Lauersen and Wilson, 1976, Asch and Greenblatt, 1977, and Blackmore, 1977a). The attempts to show differences between estrogen receptors in normal human breast tissue and fibrocystic tissue have thus far been unsuccessful (Terenius et al, 1974).

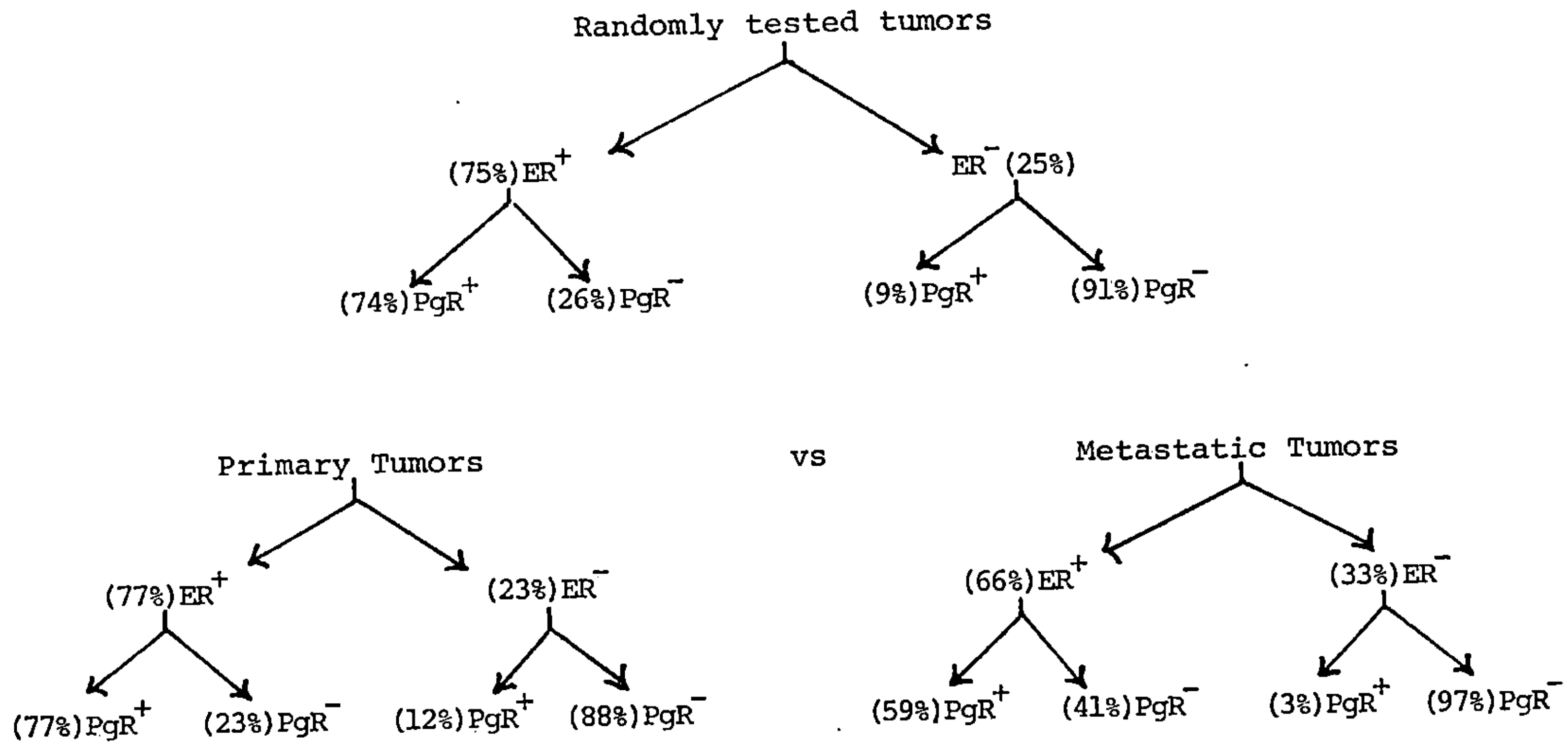
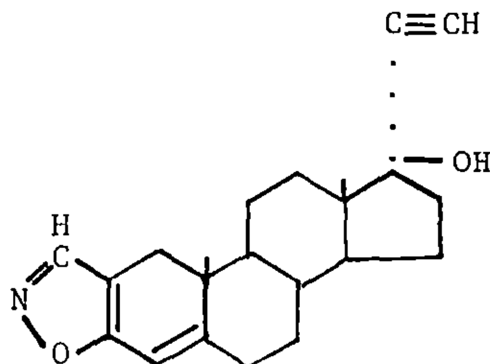


Figure 3. Summary of distribution of ER and PgR in human breast tumors (McGuire, 1977).



Danazol

Figure 4. Structure of Danazol.

However, Peters and Lewis (1976) showed that dimethyl-1,2-benzanthracene (DMBA) induced mammary cancer in rats was sensitive to danazol therapy. They found that danazol was effective in inhibiting carcinogenesis when given before DMBA and, furthermore, caused regression in established tumors. The authors concluded from their studies that danazol should be considered for clinical trials in the treatment of breast cancer.

Danazol was synthesized at the Sterling-Winthrop Research Institute by Manson and associates (1963), and marketed as Danocrine. In reference to danazol, Sterling-Winthrop Laboratories makes the following statement: "Danazol suppresses the pituitary-ovarian axis by inhibiting the output of gonadotropins from the pituitary gland. The only other demonstrable hormonal effect is weak androgenic activity." (Physicians Desk

Reference, 1982). Sterling-Winthrop suggests that, for as long as danazol is administered, it suppresses luteinizing hormone (LH) and follicle stimulating hormone (FSH) release. Without stimulation by these gonadotropins, ovulation and the full production of estrogen is prevented and thus, no corpus luteum or progesterone production occurs (Jenkin, 1980). Thus, danazol's therapeutic use would be advised for those patients in whom amenorrhea, suppression of gonadal steroids or inhibition of pituitary gonadotropin was expected to be beneficial. Accordingly, the widest clinical application for danazol has been in treatment of endometriosis (Dmowski, 1979, Dmowski and Cohen, 1978 and Greenblatt et al, 1971) where it alters normal and ectopic endometrial tissue, so it becomes inactive and atropic (Physicians Desk Reference, 1982). In benign breast disease, particularly fibrocystic disease, danazol usually produces partial to complete disappearance of nodularity. Due to danazol's suppression of the hypothalamic-pituitary axis, anovulation and thus ammenorrhea result. However, cyclic bleeding usually returns within 60-90 days (Physicians Desk Reference, 1982).

The androgenicity of the drug has been evident in the few side effects demonstrated. Weight gain was common, with relatively few patients showing mild hirsutism, acne or skin oiliness (Greenblatt et al, 1971).

In recent studies, danazol has been demonstrated to have four major pharmacologic effects: (1) direct inhibition of gonadotropin synthesis and/or release (Lauersen and Wilson, 1977, Wood et al, 1975, and Eldridge, Dmowski and Mahesh, 1974); (2) direct inhibition of multiple enzymes of steroidogenesis (Barbieri et al, 1977, and Barbieri, Camick and Ryan, 1977); (3) interaction with androgen, glucocorticoid and progesterone receptors in target tissues (Barbieri, Lee and Ryan, 1979, and Chamness, Asch and Pauerstein, 1980); and (4) alteration of endogenous steroid metabolism (Barbieri, Lee and Ryan, 1979, and Barbieri and Ryan, 1981). The majority of studies exclude binding to the estrogen receptor as a possible method of action (Krey, Robbins and McEwen, 1981, Chamness, Asch and Pauerstein, 1980, Barbieri et al, 1979, Creange, Potts and Schane, 1979, Woods et al, 1975, Dmowski et al, 1971, and Potts et al, 1974).

In further examination of danazol's antigonadotropic action, Chamness, Asch and Pauerstein (1980) studied its ability to bind and translocate androgen, estrogen and progesterone receptors both in vivo and in vitro in the rat. Their results showed danazol bound to the progesterone and androgen receptors, however, only the androgen receptor was translocated to the nucleus at effective antigonadotropic doses.

To further understand the mechanism of danazol's action in its target cells, the following study was performed to (1) determine the specificity of danazol binding to cytosol receptors by competitive binding experiments, (2) evaluate the dose response relationship of danazol; and (3) to determine the specificity for danazol to translocate receptors into the nucleus.

MATERIALS AND METHODS

Animal Care

Nineteen Sprague-Dawley female rats ranging 200-225 grams in weight, were obtained from Harlan Industries, Indianapolis, Indiana. Prior to being sacrificed, they were maintained for two weeks in animal quarters on Purina Lab Chow (Ralston-Purina Co.) and tap water *ad libitum*.

Tissue Preparation

The rats were stunned, decapitated, exsanguated and five tissues (uterus, mammary adrenals, hypothalamus and pituitary) were removed from each. Immediately upon excision, each tissue was placed on a chilled glass plate, trimmed of excess fat with a razor blade, weighed and quick frozen in dry ice and acetone. Each tissue was wrapped individually in foil, labeled and stored in a freezer for future competitive binding and dose-response experiments.

For *in vitro* nuclear translocation experiments, uteri from fourteen pre-puberal and thirteen post-puberal Sprague-Dawley rats were excised using the same method as mentioned above. After being trimmed of excess fat, the uteri were incubated in media consisting of 1 ml mimimal

essential media with Earle's salts and L-glutamine (Grand Island Biological Co.) and 1 ml of Kreb's Ringer Bicarbonate Glucose, pH 7.4 (Umbreit et al, 1964). Forty microliters of indicated steroids in 100% ethanol were added to give a final concentration of 2×10^{-8} M and the uteri were incubated for two hours at 37°C with gentle bubbling of 95% osygen: 5% carbon dioxide in a Dubnoff Metabolic Shaking Incubator (GCA Precision Scientific). Controls were incubated with 100% ethanol. After incubation, the tissue was blotted, weighed and quick frozen in dry ice and acetone for later assay.

Isolation of Cytosol and Nuclear Receptors

All procedures were done on ice and/or in a refrigeration unit at 4°C, unless specified otherwise. Centrifugations were carried out (depending on the procedure, sample size and rpm specification) in either a Beckman "Airfuge", International Refrigerated Centrifuge, Model B-20 (International Equipment Co.) or an Adams Sero-fuge. Where microliter volumes were required, automatic micro-pipets (Oxford laboratories) were used.

Isolated cytosol and nuclear receptors were prepared by a modification of the methods of Williams and Gorski (1974), Chamness et al (1979), and Pavlik and Coulson (1976). Frozen tissue was cut into small pieces and then homogenized in phosphate buffer [P-buffer (5mM sodium

phosphate, pH 7.4, 1mM thioglycerol, 10% glycerol)] in a seven milliliter Ten Broeck pyrex glass-glass tissue grinder (about 100-150 mg tissue/ml P-buffer). The homogenate was centrifuged (Adams Sero-fuge) eight minutes at 1000 x g; the supernatant was taken as the cytosol, while the pellet was resuspended in P-buffer and recentrifuged twice, then resuspended for one hour in buffer with 0.4 M potassium chloride to extract receptors from the nuclei. The pellet was then centrifuged for 10 minutes at 2000 x g (International Refrigerated Centrifuge) and the supernatant was assayed for nuclear receptors.

Receptor Incubations

Duplicate 200 µl aliquots of cytosol or nuclear extract were added to 50 µl of radioactive ligand, while another duplicate 200 µl aliquots were added to 50 µl of the same ligand with an excess of non-radioactive competitors for determination of non-specific binding. In addition, to measure total counts, duplicate 200 µl aliquots of P-buffer were added to 50 µl of radioactive ligand. Final concentrations of radioactive ligands (New England Nuclear) were 5×10^{-10} M [2,4,6,7,16,17-^{3}H(N)] estradiol (137.1 Ci/mmole), 5×10^{-10} M [1,2,4,5,6,7,16,17-^{3}H(N)] dihydrotestosterone (179.0 Ci/mmole), 5×10^{-10} M [1,2,6,7-^{3}H(N)] progesterone (101.0 Ci/mmole),

5×10^{-10} M [1,2,6,7-^{3}H(N)] corticosterone (105.0 Ci/mmole). Non-radioactive competitors were 1.67×10^{-7} M diethylstibesterol, 1.67×10^{-7} M dihydrotestosterone, 1.67×10^{-7} M progesterone, and 1.67×10^{-7} M corticosterone (all are final concentrations). Incubations for cytoplasmic receptors were all three hours at 0°C, while those for nuclear receptors were three hours at 30°C (estrogen) or 18 hours at 0°C (androgens, progestins and corticosteroids). Cytosol and nuclear extract were saved (50 µl samples) for protein determinations.

Binding Competition Experiments

Cytosol was prepared in P-buffer from uteri, mammary, adrenal, hypothalamic and pituitary tissue (assayed separately). Duplicate aliquots of 200 µl of cytosol were incubated with 50 µl of radioactive steroid in buffer plus 50 µl of varying concentrations of danazol. The danazol (Danocrine) was a gift from Sterling-Winthrop Research Institute. Binding was assayed by the hydroxylapatite method (HAP) and non-specific binding was subtracted.

Dose Response Experiments

Cytosol was prepared in P-buffer from uterine tissue. Triplicate aliquots of 200 µl of cytosol were

incubated with 50 µl of varying concentrations of radioactive ligand. Competitive binding was checked with 50 µl of non-radioactive ligand added to one aliquot. In addition, triplicate aliquots of 50 µl of radioactive ligand were incubated with varying microliter concentrations of cytosol, with 50 µl of non-radioactive competitor in one aliquot to check competitive binding. Assay for binding was by the HAP method and non-specific binding was subtracted.

Nuclear Translocation Experiments

Nuclear extract was prepared in P-buffer from uteri of pre- and post-puberal rats. In experiments with the pre-puberal uteri, triplicate 200 µl aliquots of the nuclear extract from control, danazol and dihydrotestosterone (DHT) incubated tissues were added to 50 µl aliquots of radioactive DHT with a triplicate also containing 50 µl of non-radioactive DHT.

Post-puberal uterine experiments were performed, using triplicate 200 µl aliquots of the nuclear extract from control, danazol, DHT, progesterone, corticosterone and estradiol incubated uteri, with a 50 µl aliquot of appropriate radioactive ligand. To check for competitive binding a triplicate aliquot of 200 µl nuclear extract from each incubation with 50 µl of radioactive steroid plus 50 µl of non-radioactive competitor was performed.

Hydroxylapatite Assay

One milliliter of hydroxylapatite suspension [2.5 grams of Bio-Rad HTP, DNA grade, per 100 ml of TP buffer (50 mM Tris and 10 mM KH_2PO_4)] was added to each tube at the end of incubation and kept suspended by occasional vortexing (Fisher Scientific Vortex-Genie) for 30 minutes. The HAP was then centrifuged 1.5 minutes at 1000 x g, resuspended in 1.5 ml of fresh P-buffer, and recentrifuged; this wash was completed twice more. One milliliter of 100% ethanol was then added to each pellet to extract the radioactive steroid, which was then counted in 5 ml of scintillation fluid (19 gm PPO and 1.9 gm POPOP/3.8 L toluene). All counts were made in a Hewlett-Packard Tri-Carb 300 scintillation counter at Maxey Flats Low-Level Nuclear Waste Disposal Site.

Protein Assay

Protein concentrations were determined by a modification of the Lowry Method (Lowry et al, 1951). To precipitate proteins, cold 10% trichloroacetic acid was added to 50 µl of cytosol or nuclear extract in a cellulose nitrate tube (Beckman, 175 µl capacity) and centrifuged in a Beckman Airfuge at 20 psi for 5 minutes. The supernatant was aspirated with a disposable transfer pipet. To the protein precipitate, 100 µl of 3N sodium hydroxide was added, stirred with a toothpick and set

aside for 10 minutes. Two concentrations (10 μl and 50 μl) of the protein mixture and of a bovine serum albumin standard (1 gm/ml) were added to separate test tubes and brought to 0.5 ml with distilled water. The remaining procedures were identical with those of Lowry *et al* (1951). Measurement of protein concentrations were made in a Bausch and Lomb Spectronic 70, and protein was expressed in mg/ml.

Statistical Analysis

Data were analyzed using the Student's t test.

RESULTS

To evaluate the validity of the HAP assay and to determine conditions to be used in later experiments, titration curves were run for both ^{3}H-estradiol and cytosol. Specific activity was measured in counts per minute/mg of protein. Figure five shows a dose-response relationship when the concentration of ^{3}H-estradiol was reduced in the incubate, in the presence of 200 µl of cytosol. Likewise, by the condition of increasing amounts of cytosol, the expected dose relationship was seen in specific activity. This indicates as the cytosol was increased, the amounts of cytosol receptors were increased, therefore the increase in specific binding. From these data, experimental protocol for all succeeding experiments called for the use of 10^{-9} M estradiol and 200 µl cytosol.

In the first series of experiments performed, the competition for steroid receptors with increasing doses of danazol were performed in various tissues. The results of these experiments are shown in figures six, seven, eight and table one, and are expressed as a percent of control binding. In all experiments, danazol exhibited a dose-response competition with steroid receptors. Figure six shows that in mammary tissue, competition with the androgen receptor exceeded all

other steroid receptors. Binding was 58% of the control at the physiological concentration of 10^{-9} M danazol compared to 88% of the control values of estradiol. Danazol was the most efficient competitor of the androgen receptor in uterine tissue, with a value of 80% of the control at 10^{-9} M (Figure 7). The competition for steroid receptors in pooled adrenal tissue is shown in figure eight. This figure shows poor competition with all steroid receptors at 10^{-9} M danazol and strong competition at the pharmacological dose of 10^{-5} M.

Due to the lack of cytosol for hypothalamic tissues, only two assays were performed. Using only the test dose of 10^{-9} M, results show in table one that danazol competed most effectively with the DHT receptor (73% of control) followed by progesterone (86%), corticosterone (90%) and estradiol (91%).

Table 1. Danazol competition for steroid receptors in hypothalamic tissue.

Incubation conditions	Estradiol	DHT	Progesterone	Corticosterone
Danazol (10^{-9} M)	91*	73	86	90

*Mean percent of cytosol alone which was taken as 100%.

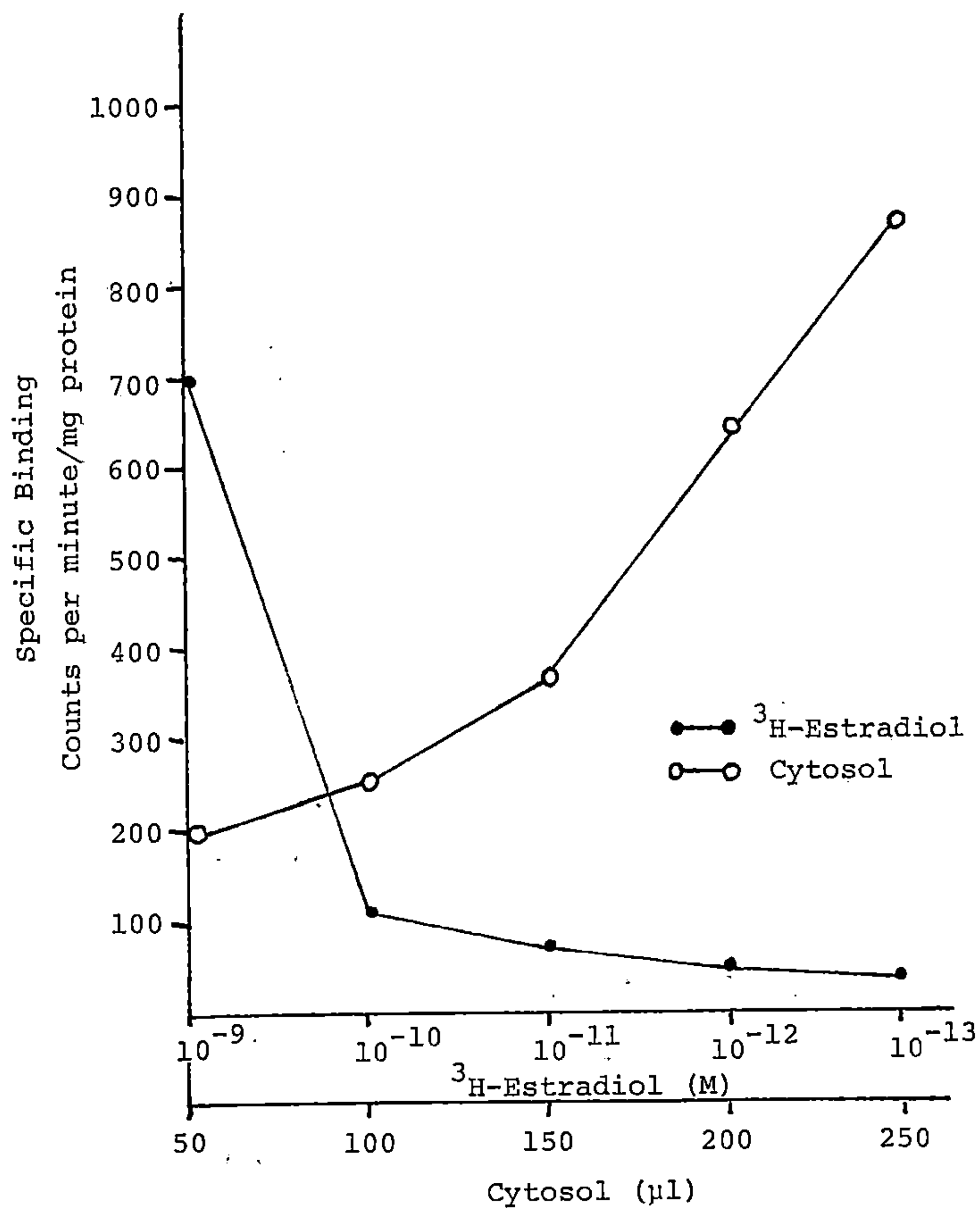


Figure 5. Titration curves of specific binding of ^{3}H-estradiol to cytosol receptors.

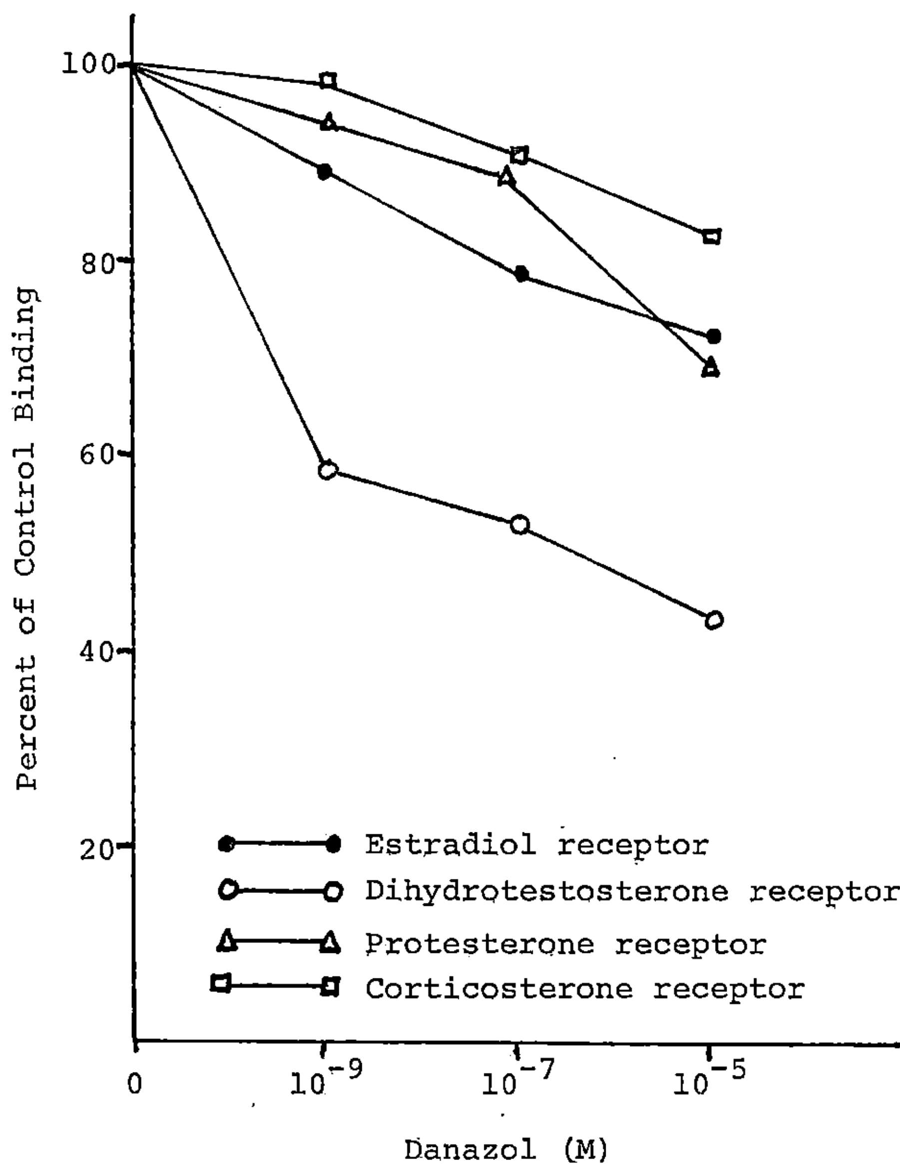


Figure 6. Danazol competition for steroid receptors in mammary tissue.

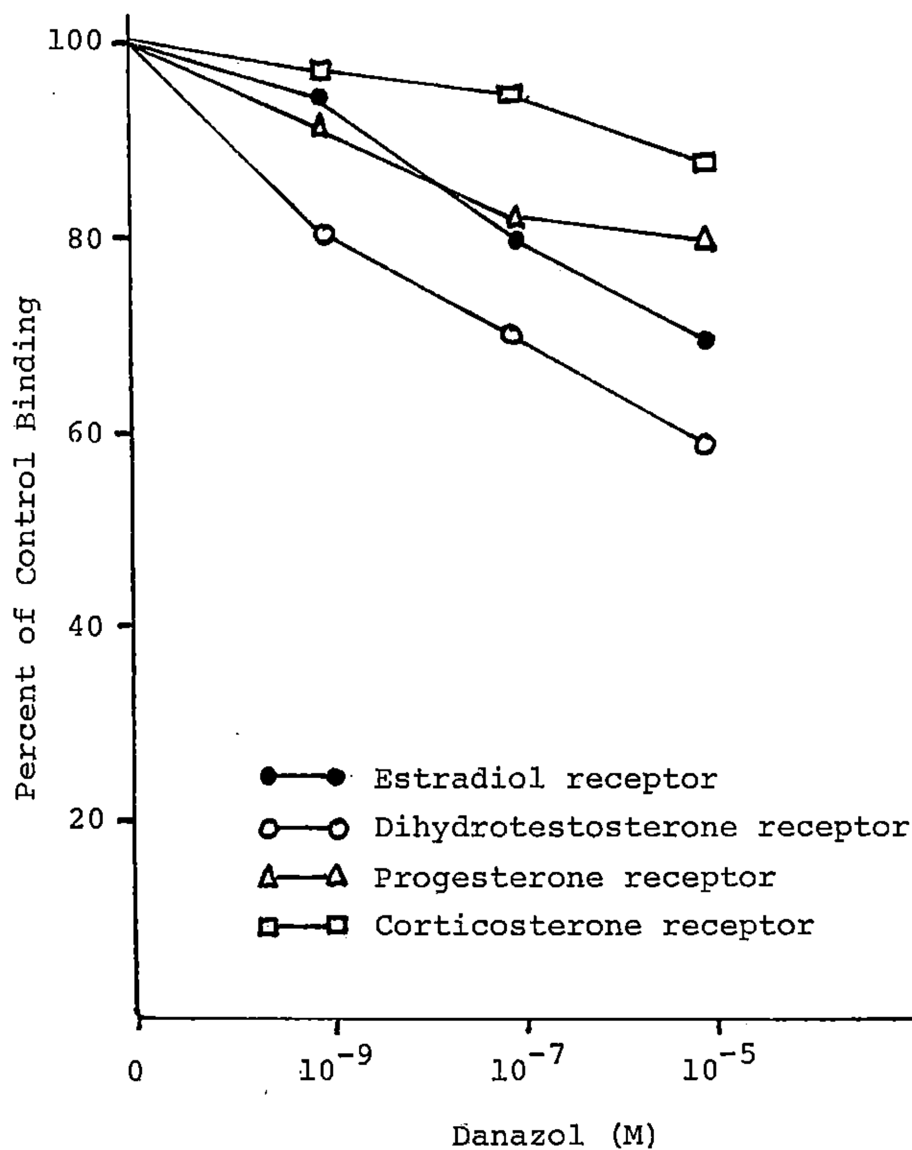


Figure 7. Danazol competition for steroid receptors in uterine tissue.

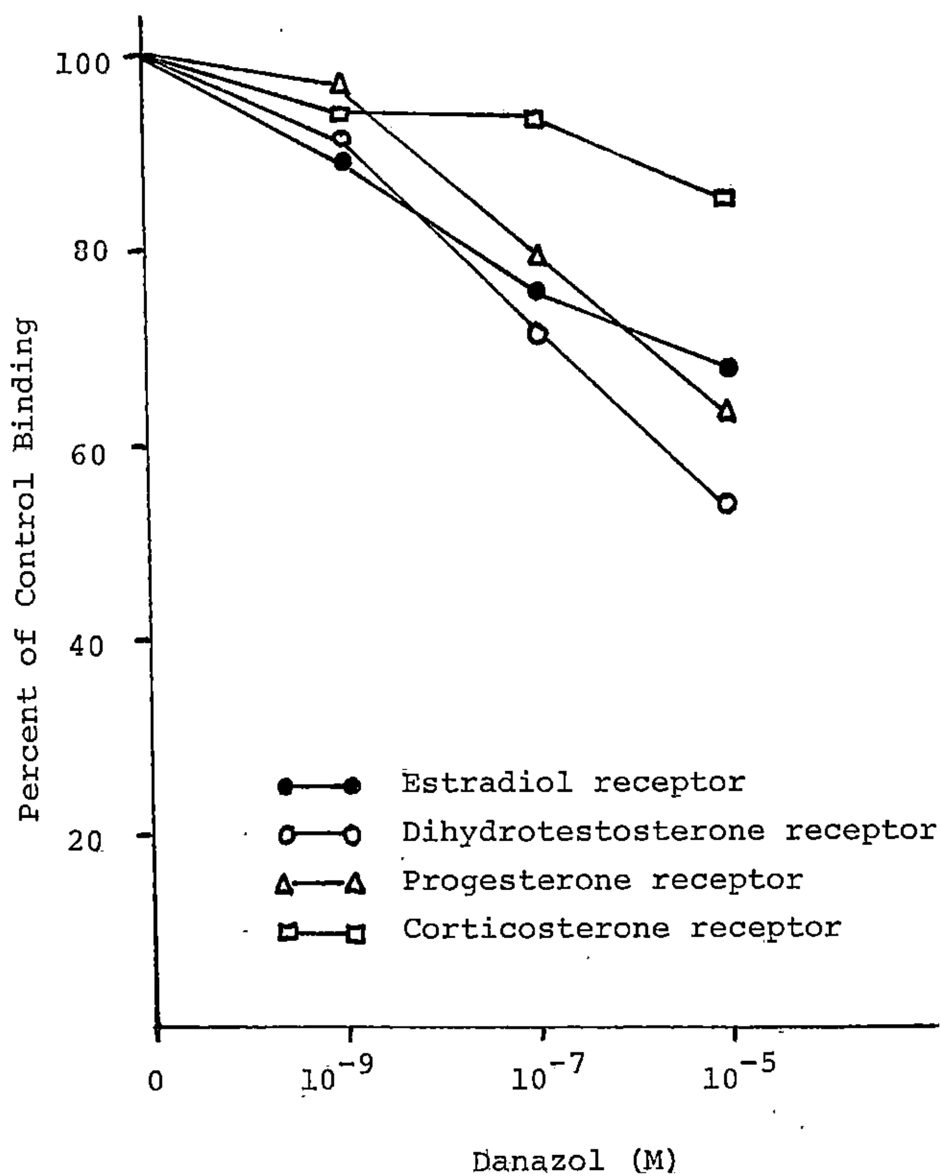


Figure 8. Danazol competition for steroid receptors in pooled adrenal tissue.

A single test was performed with pooled pituitary cytosol, showing that danazol at 10^{-9} M did compete with the DHT receptor (65% of control).

A comparison of steroid binding competition by danazol at 10^{-9} M is seen in table two. This table summerizes the data that danazol competes more favorably with the androgen receptor in all tissues with a mean of 76% of the control followed by estradiol (91%), progesterone (92%), and corticosterone (95%).

In vitro translocation experiments of steroid cytosol receptors were performed to better determine receptor specificity and biological function. The first experiments were performed on 25 day old, pre-puberal uterine tissue and the results are shown in table three. Compared to the control specific activity of 426 cpm/mg protein, incubation with 10^{-9} M DHT resulted in a 146% increase over the control with a mean specific activity of 1050 cpm/mg protein ($P<.1$). Danazol at 10^{-6} M translocated the androgen receptor 85% over the control.

In all experiments in which *in vitro* translocation was evaluated in adult uterine tissue, danazol did not signficantly elevate nuclear receptors in any of the steroids tested (Table 4). Danazol translocated the DHT receptor most successfully with a value of 68% increase over the control. Incubation with respective steroids

Table 2. Comparison of steroid binding competition by danazol at 10^{-9} M.

Competing Steroid Receptors	Uterus	Mammary	Adrenal	Hypothalamus	Mean
Estradiol	92*	87	92	91	91
DHT	80	58	93	73	76
Progesterone	89	95	98	86	92
Corticosterone	95	98	96	90	95

*Mean percent of control binding.

Table 3. *In vitro* translocation of uterine androgen receptor in pre-puberal rats.

Treatment N=6	Specific activity cpm/mg protein	Percent increase over control
Control	426±146*	
DHT (10^{-9} M)	1050±762[a]	146
Danazol (10^{-6} M)	790±483	85

*Mean ± standard deviation

a P<.1 from controls

Table 4. *In vitro* translocation of uterine steroid receptors in adult rats.

Treatment N=6	Specific activity cpm/mg protein	Percent increase over control
Control	873±200*	
Estradiol	1983±393[a]	127
Danazol	1041±491	19
Control	349±316	
DHT	971±354[b]	178
Danazol	585±692	68
Control	570±370	
Progesterone	1229±789	116
Danazol	694±528	22
Control	62±74	
Corticosterone	95±47	53
Danazol	68±64	10

* Mean ± standard deviation

a P<.001 from control

b P<.05 from control

demonstrated that the cytosol receptor was translocated, reflecting values that were significant for DHT ($P<.05$) and highly significant for estradiol ($P<.001$).

DISCUSSION

The mechanism of action of danazol at the subcellular level is not completely understood. However, according to current ideas of hormone action, danazol should bind to a specific cytosol receptor and translocate it to the nucleus of that particular target cell, where it would initiate protein synthesis. It has been well substantiated that danazol binds most efficiently to the androgen receptor in various target tissues (Chamness *et al*, 1980 and Barbieri *et al*, 1979). It is also suggested that danazol has a somewhat lower affinity for the progesterone (Chamness *et al*, 1980 and Barbieri *et al*, 1979) and glucocorticoid receptor (Barbieri *et al*, 1979), with little or no binding to the estrogen receptor (Krey *et al*, 1981, Chamness *et al*, 1980, Woods *et al*, 1975, Creange *et al*, 1979, Dmowski *et al*, 1971 and Potts *et al*, 1974). Results of this study are concurrent with these findings except for demonstrating a low affinity binding of danazol to the estrogen receptor. In support of these data, Creange *et al*, (1979) has shown danazol competition with estrogen receptors of the pituitary.

At physiological doses, danazol competed similarly with the estrogen, progesterone and glucocorticoid

receptor, showing a somewhat better competition with the androgen receptor (Table 2). In comparison, as concentrations of danazol were increased, there was an increased competition for all receptors (Figures 6, 7, and 8).

To check for receptor specificity and biological function, in vitro translocation experiments were performed on pre- and post-puberal rats. In vivo experiments by Chamness et al (1980) provides evidence that the androgen receptor is the only one effectively translocated to the nucleus by danazol. The findings of this research, in contrast, show a non-statistical tendency of danazol translocating the androgen receptor in pre-puberal and adult rats (Table 3 and 4) along with a somewhat lesser ability to translocate the estrogen, progesterone and glucocorticoid receptor (Table 4).

It is generally agreed that danazol has weak androgenic and no estrogenic or anti-estrogenic properties, while its glucocorticoid and progestational effects are still somewhat controversial (Dmowski, 1979, Barbieri, et al, 1979, Dmowski et al, 1971 and Potts et al, 1974). Therefore, it must be concluded from this study that the strong binding and translocation of the androgen receptor by danazol is indicative of its androgenic responses elicited in vivo. The lower binding affinity

for the estrogen, progesterone and glucocorticoid receptor and subsequent translocation of these receptors may suggest the action of danazol on these receptors is not sufficient to stimulate mRNA synthesis and thus biological activity.

Difficulty in obtaining good specific binding counts was a drawback of this procedure. It is suggested that higher counts may be obtained by a wash buffer of 1% Tween 80 in phosphate buffer, which has been shown to be more successful at diminishing the non-specific binding (Garola and McGuire, 1978) than phosphate buffer alone.

In summary, evidence presented in this research indicates that danazol binds to and translocates estrogen, progesterone, glucocorticoid and androgen receptors _in vitro_, though not significantly, while showing the greatest success with the androgen receptor in all tissues. The dose responsiveness of danazol was effectively demonstrated by its increased competition at pharmacological concentrations. Furthermore, the results indicated that danazol was most effective in translocating the androgen receptor in mammary tissue.

In light of this research, as well as evidence reported in the literature, the evaluation of receptor

binding and nuclear translocation data, will hopefully lead to a better understanding of normal steroid target cells, cancer modified cells and drug interaction with each cell type.

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