To the Graduate Council:

I am submitting herewith a dissertation written by Judy Ellen Stern entitled "Rabbit Endometrial and Myometrial Cytosolic Estrogen Receptors." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.

Gerald L. Vaughan, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

RABBIT ENDOMETRIAL AND MYOMETRIAL CYTOSOLIC ESTROGEN RECEPTORS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Judy Ellen Stern

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DEDICATION

This dissertation is dedicated to my parents, Sidney and Marilyn Stern. My father taught me to question and explore. My mother taught me to enjoy a challenge. Both parents have filled my life with love, honesty, and chicken soup, none of which could I have done without.

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I am grateful to the many students and faculty members of The University of Tennessee, Knoxville, who have enriched my life through their roles as friends and colleagues.

I must conclude by expressing my heartfelt appreciation to my husband, Joe, for putting up with me through the long hours and the tension involved with completing this degree. His patience, objectivity, and sense of humor have enabled me to complete my work while maintaining a semblance of sanity.

ABSTRACT

Numerous studies have been conducted into function, structure and mode of action of steroid receptors. Research has led to a partial understanding of interactions of receptors with hormones and the function of combined hormone-receptor complexes as inducers of transcriptional events. Despite investigation into those functions of receptors, little information is available on the significance of changes in receptor levels during different stages of the estrous cycle or pregnancy. Virtually none has been accumulated on the character of receptors found in isolated endometrial and myometrial uterine tissues. This study was initiated to test the hypothesis that endometrial and myometrial estrogen receptors of the rabbit respond differently to hormonal stimulation.

Changes in estrogen receptors were observed during the course of early pregnancy and under stimulation by steroid hormone. Estrogen receptor affinity (*Ka*) and titers were highest in the endometrium on day four of pregnancy. *Ka* reached 2.69 (± 0.29) x 10^{9} M^{-1} and titers reached 13.35 ± 0.45 fmol(ugP/ugDNA)$^{-1}$. Peak values for myometrial receptor affinity (2.43 ± 0.42 x $10^{9}M^{-1}$) and titers (5.34 ± 1.08 fmol(ugP/ugDNA)$^{-1}$) occurred on day one of pregnancy. Endometrial titers were significantly different from myometrial titers from day three of pregnancy onwards. Receptor titers in implantation-sites were reduced between day six (7.98 ± 1.66 fmol(ugP/ugDNA)$^{-1}$) and day nine (3.47 ± 0.59 fmol(ugP/ugDNA)$^{-1}$) of pregnancy.

Seasonal changes in estrogen receptor K_a but not in titer were observed. Lowest values occurred in early spring ($0.259 \pm 0.065 \times 10^{9}M^{-1}$) and highest values were observed in the fall ($2.21 \pm 0.21 \times 10^{9}M^{-1}$). Seasonality had not been anticipated in this species.

Estrogen treatment of ovariectomized animals resulted in reduction of K_a and titer over oil treated controls. Endometrial K_a was depressed by 48%, and endometrial titer by 86%. K_a of the myometrium decreased by 84%, and titer was reduced by 86%. Progesterone treatment decreased titers to a less appreciable extent (44% in endometrium and 75% in myometrium) and depressed affinity only in the myometrium (25%). Changes in affinity were probably caused by the presence of a competitive inhibitor which was present in the cytosol after steroid treatment.

This study served to demonstrate a difference in endometrial and myometrial estrogen receptor. In addition, an unexpected seasonal variation in receptor-hormone interaction was described. As a result of the data obtained, a model for control of receptor affinity was proposed. Modifications in titer and affinity might govern responsiveness of cytoplasmic estrogen receptor.

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I. INTRODUCTION

Pregnancy is characterized by changes in plasma levels of estrogen and progesterone which influence the uterus, effecting a wide variety of changes. In the rabbit, an induced ovulator, a fertile mating is followed seven days later by implantation of the blastocyst into a uterus which, through a precisely timed sequence of events, has been prepared for its arrival.

Changes in the uterus during pregnancy include: a change in vascularity (Hafez and Tsutsumi, 1966) and blood flow (Resnik et al., 1974), a change in cell junctions (Finn, 1977) or formation of a syncitium (Larsen, 1961), an accumulation of eosinophils (Tchernitchin et al., 1974), and histamine release (Szego, 1965). A uterine environment receptive to the blastocyst is developed. Blastokinin, found in rabbit uterine flushings (Daniel, 1976), blastocysts (Beier and Maurer, 1975), and in endometrial cells (Johnson, 1972; Kirchner, 1976) at the time of implantation, has been shown to enhance blastocyst development in vitro (Kirchner and Daniel, 1967; El-Banna and Daniel, 1972; Maurer and Beier, 1976).

Plasma levels of progesterone increase rapidly during the first half of a normal pregnancy and decline thereafter. In the rabbit these peak values occur on day twelve of a thirty-one day pregnancy (Hilliard et al., 1968; Challis et al., 1973). Estradiol and estrone levels remain fairly constant in this species (Hilliard and Eaton, 1971; Challis et al., 1973). In the rat, not an induced ovulator, estrogen

levels increase during early pregnancy (Heald, 1976). Progesterone is found in quantities from 200 to 400 times greater than those of estrogen.

Estrogen and progesterone synergise to produce much of the preparatory growth of uterine tissue. Normal decidual cell reaction (DCR) depends upon maternal tissue being properly prepared for implantation and the reaction will only occur within a limited time span (DeFeo, 1967; Finn, 1977). In the rat and mouse a precise sequence of steroid administration comprised of estradiol priming followed by treatment with estrogen and progesterone creates the most favorable environment for implantation (Pollard and Finn, 1972; Psychoyos, 1973; Finn and Martin, 1974). In studies on rabbits Kwun and Emmens (1974) and Denker (1972) both obtained implantation, in animals ovariectomized during pregnancy, by administration of progesterone alone; these experiments did not exclude the possibility that estradiol was needed for priming during the early days of pregnancy.

The blastocyst itself might provide hormonal stimulation to the uterus which could help prepare the uterine environment (Seamark and Lutwak-Mann, 1972; Bhatt and Bullock, 1974; Dickman et al., 1975; Dickman et al., 1977). Enzymes which participate in estrogen and progestin synthesis have been found in blastocysts (Dickman and Dey, 1975). Steroids other than estrogen and progestin have not been found (Borland et al., 1977).

Endometrium and myometrium respond to ovarian hormones with a sequence of developmental modifications which are different for the

two tissues. Unfortunately, few studies have been done which separate and compare the two tissue types.

Wet weight of the endometrium changes markedly under progestational stimulation. In the rabbit it increases from 10% of total uterine weight in atrophic uteri to 40% of total uterine weight under stimulation with progesterone (Telfer and Hisaw, 1957). Imbibition of water accounts for a large percentage of this increase. Water accumulates in uterine tissues four to six hours after doses of 0.035ug of estradiol without change in dry weight (Hisaw, 1959). Isolated endometrial cells in culture respond to estrogens with an accumulation of water and a change in the intracellular concentration of Na^+ after two hours (Pietras and Szego, 1975).

An increase in cell number also serves to increase wet weight. Endometrium is composed of a luminal epithelium, glandular epithelium and stroma. Finn and Martin (1974) have shown that in the mouse mitosis occurs in these regions at different times as pregnancy proceeds. About 30% of glandular epithelial cells are undergoing mitosis at day three as compared to less than 5% on day four. The stromal increase goes from about 5% of the total cells undergoing division to 40% over the same time period. Luminal cells are most prolific on or before day one (Finn and Porter, 1975). Stimulation by estrogens leads to proliferation of the stroma in preference to epithelial portions of the endometrium while progesterone stimulation leads to proliferation of the epithelium (Finn and Martin, 1974).

DNA of rat endometrium (as measured by ^{3}H-thymidine incorporation) increases during early pregnancy (Heald, 1976). Endometrial nucleic acid in the rabbit increases between days three and five (Psychoyos, 1973). Estrogen causes tritiated thymidine incorporation into DNA in all portions of the endometrium of rabbits, progesterone stimulates incorporation only into epithelium (Koseki and Fujimoto, 1974).

Myometrium undergoes changes during pregnancy, though these changes are less dramatic than those of endometrium. There is enlargement of cells, cell division and production of proteins involved in muscle contraction (Hamoir, 1977). Collagen is deposited between cells, cells enlarge, nuclei enlarge, the numbers of ribosomes and glycogen granules increase, and sarcoplasmic reticulum becomes more extensive (Shoenberg, 1977). Myometrial response to estrogen includes a less appreciable change in wet weight and dry weight than that of the endometrium (Telfer and Hisaw, 1957).

Electrical potential and contractility of the myometrium are altered with pregnancy. Comparison of contractile properties of late pregnant and postpartum rabbit myometrium reveal a lower membrance potential in late pregnancy muscle cells. This accompanies decreased contractility as seen in asynchronous irregular contractions of pregnancy as opposed to the regular spontaneous contractions of postpartum cells (Kuriyama and Csapo, 1961). These changes reflect modifications of intracellular and extracellular ion content (Casteels and Kuriyama, 1965) and can be measured as differences in resting potential and action potentials (rabbit—Goto and Csapo, 1959;

rats—Casteels and Kuriyama, 1965; mouse—Kuriyama, 1961). Ca^{++} accumulates in the myometrium and action potentials of the myometrial cells increase in frequency in response to estrogen (Kuriyama and Csapo, 1961).

The effects of steroid hormones are mediated through hormone interaction with cytoplasmic receptors. These intracellular protein molecules bind the hormone in a specific manner with high affinity ($K\alpha$) ranging from $1 \times 10^{8} M^{-1}$ to $1 \times 10^{11} M^{-1}$. Their properties and functions have been reviewed extensively (Jensen and DeSombre, 1972; O'Malley and Hardman, 1975; O'Malley and Birnbaumer, 1978). The cytoplasmic steroid receptor, first demonstrated by E. V. Jensen and H. I. Jacobson (1962), has been analysed by sucrose density gradient centrifugation (Toft and Gorski, 1966) and found to be a protein molecule of approximately 4.5 Svedberg units when isolated in high salt (400mM) and 8 Svedberg units when isolated in low salt (50mM). It is possible that more than a single class of receptor sites is present in some tissues (Clark et al., 1978a; Smith et al., 1979), however, many cytosolic receptors for estrogen are immunologically similar (Greene et al., 1977).

The receptor undergoes a transformation after its binding with the hormone from the 4S form with a molecular weight of 80,000 daltons to a 5S form of 130,000 daltons. The change in molecular weight may reflect complexing of the hormone receptor to yet another cytoplasmic factor (Notides and Nielsen, 1974; Notides et al., 1975).

As the hormone-receptor complex enters the nucleus, cytoplasmic content of receptor is depleted. The nuclear content of receptor is depleted after a short period of time except for a small number of receptors which remain bound in the nucleus (Clark et al., 1978b).

Nuclear binding by transformed cytosolic receptor may be a saturable (Buller et al., 1975; Kuhn et al., 1975) or nonsaturable and nonspecific phenomenon (Higgins et al., 1973; Yamamoto and Alberts, 1976). Gannon et al. (1976) suggested a "cytoplasmic exclusion hypothesis" by which the receptor moves to the nucleus in a nonspecific fashion dependent upon the concentrations of estradiol present.

The hormone-receptor complex appears to influence the uterus by controlling transcription. The complex can bind to chromatin (chick oviduct—Spelsberg et al., 1971; Schrader et al., 1972; calf uterus—Puca et al., 1974; rabbit uterus—Chatkoff and Julian, 1973; Chatkoff et al., 1974; Borthwick and Smellie, 1975), increase RNA polymerase activity (Jensen et al., 1974; Schwartz et al., 1975), and induce the production of specific mRNA products (Chan et al., 1973; Rhoads et al., 1973). "Induced protein" (I.P.), the first known translational product of estrogen stimulation in the rat uterus (Katzenellenbogen and Gorski, 1975), may be produced in direct proportion to the number of estrogen receptor sites stimulated (Bhakoo and Katzenellenbogen, 1977a).

Changes in the quantity of cytoplasmic and nuclear steroid receptors in the uterus result from changes in hormonal stimulation and may be an important factor controlling all other changes in the

uterus. Autoradiography reveals that luminal epithelial cells bind less estrogen when stimulated with progesterone (Sar and Stumpf, 1976; Tchernitchin, 1976). In endometrial cells of the rat there is a daily circadian rhythm in estrogen receptor number in the nucleus which is influenced by pregnancy (Martel and Psychoyos, 1976). Steroid receptor numbers from decidual tissue change during development of deciduomata (Armstrong et al., 1977, Talley et al., 1977; Ward et al., 1978). The levels of progesterone receptor change in the rabbit uterus during pregnancy (Davies et al., 1974; Muechler et al., 1976).

The rat has provided a source of material for many studies of estrogen receptors. On the basis of a number of studies estrogen has been termed an agonist, increasing the titer of estrogen receptors, and progesterone an antagonist, decreasing the titer of these receptors (Mester et al., 1974; Hsueh et al., 1976; Pavlik and Coulson, 1976b; Bhakoo and Katzenellenbogen, 1977b; Coulson and Pavlik, 1977; Saiduddin and Zassenhaus, 1978). Progesterone, however, may increase estrogen receptor titer in the endometrium (Martel and Psychoyos, 1978), or it may have a direct affect on estrogen binding ability of the estrogen receptor (DiCarlo et al., 1978).

Estrogen receptors in the rabbit uterus during pregnancy have been studied by Lee and Keyes (1971) and by Muechler et al., (1974). These authors have reported a drop in uterine estrogen receptor titer between estrus and day ten of pregnancy which Jacobsen et al. (1972) have attributed to the corpus luteum and progesterone involvement. These studies used preparations from different days of pregnancy composed of

different percentages of endometrium and myometrium. Thus, interpretation of the changes in receptor levels and analysis of the influence of receptor-hormone interactions on subsequent modification of the individual tissues is difficult.

This study was initiated to examine possible differences between estrogen receptors of rabbit endometrium and myometrium. Information was to be gathered on: (1) receptor levels in the two tissues, (2) physical properties of the receptor as demonstrated by the hormone binding affinity, (3) implantation site and nonimplantation site receptor titer and affinity and (4) the effect of estrogen and progesterone on receptor number and affinity in the two tissues.

II. MATERIALS AND METHODS

Reagent grade chemicals were supplied by Fisher Scientific Company. Radioactively labeled estradiol-17β was obtained from New England Nuclear Corporation (#317). Other steroids were obtained from Sigma Chemical Company. DNA grade hydroxylapatite (HAP) was from Bio-Rad Laboratories.

Animals

Mature, virgin, New Zealand white rabbits weighing between 6 and 9 lbs. and of at least 6 months of age, were obtained from Graves Rabbitry of Knoxville and housed in an authorized animal facility for from one to two weeks in a 12 hour/12 hour light dark cycle until use. The animals were bred to males of proven fertility and killed on the appropriate day of pregnancy via embolism (10cc air injected into the ear vein). The uterine tissue was removed, freed of connecting fat, and placed on ice. Ovaries were checked for the presence of corpora lutea. Uteri were flushed with cold TEM buffer (40mM Tris, 1.5mM EDTA, 15mM monthioglycerol, 50mM KCl and 10%(v/v) Glycerol) and embryos were counted to further confirm pregnancy.

Tissue Preparation

Endometrial tissue was separated from myometrial tissue by short rapid scraping stokes of a scalpel. Myometrium was further scraped to insure minimal contamination by endometrium. Tissues were placed in ice cold TEM buffer, washed with 3 changes of buffer to eliminate blood,

blotted on filter paper, and weighed. All subsequent steps were done at 0-4°C unless otherwise stated.

Tissues were homogenized in TEM buffer using a hand operated ground glass conical homogenizer (at least 150 circular strokes) for endometrium and a Laboratory Waring Blender with five ten second bursts at low speed and 15 second cooling periods for myometrium. Homogenates were centrifuged at 800xg x 10 minutes in an International PR-2 refrigerated centrifuge at 4°C. Nuclear pellets were saved for DNA assay. Supernatent fractions were further centrifuged in a Beckman Model L ultracentrifuge at 100,000xg x 60 minutes to remove particulate material. These cytosols were diluted to a concentration of 130mg wet weight per ml TEM. This gave protein concentrations of between 1.5 and 2.5mg per ml. Cytosols were used in receptor assays after 5 minute treatment with Dextran coated charcoal (0.2% (w/v) each) to remove unbound endogenous hormone. The charcoal suspension was removed with two 13,000g x 10 minutes centrifugations at 4°C.

Implantation Site and Nonimplantation Site Separation

For days six, seven and nine of pregnancy the implantation sites and nonimplantation sites were separated. The entire uterus was cut into longitudinal strips with the embryos still in place so that the implantation site regions could be readily identified. Embryos were then removed, the endometrium was scraped from the myometrium as before, and the two regions were treated separately. Care was taken to avoid breaking day six and day seven embryos. Day nine embryos were always

broken during removal. Nine day embryos are larger than six and seven day embryos and the trophoblast of the nine day embryos is thinner than at earlier stages. After removal of the embryos, uterine strips from implantation sites and nonimplantation sites were immediately washed in TEM.

Assay for Cytosolic Estrogen Receptor

Samples of cytosol (100ul) were added to 13 x 75mm RTU tubes containing various concentrations of ^{3}H-estradiol-17β to give concentrations of from 1 x 10^{-14} moles per 100ul cytosol to 1 x 10^{-12} moles per 100ul cytosol. Another set of tubes contained these concentrations of ^{3}H-estradiol plus a 100 fold excess of each concentration of diethylstilbesterol (D.E.S.). D.E.S. concentrations ranged from 1 x 10^{-12} moles per 100ul to 1 x 10^{-10} moles per 100ul cytosol. Seven different concentrations were used and duplicate tubes were prepared for each concentration of radioactive label alone and radioactive label plus competitor.

After incubation for 16 hours at 0-4°C the receptor-bound hormone was removed by addition of 0.5ml DNA grade hydroxylapitite (HAP) (Pavlik and Coulson, 1976a). The HAP was prepared in TEM buffer at a concentration of 10gm per 100ml and stirred overnight to reduce particle size. "Fines" were decanted and the HAP resuspended to the same concentration. The pH of the wash was periodically checked and found to be approximately 7.2. Removal of the receptor bound hormone was accomplished after incubation (5 minutes) with the HAP slurry.

Free hormone from the first 2 washes with TEM was added to scintillation vials for counting. The HAP pellet was washed twice more and then bound hormone was extracted twice with 2ml of 100% ethanol. Ethanol extracts were pooled and counted.

Scintillation cocktail contained 4gms PPO, 0.5gms POPOP and 23% Triton X 100 per liter of scintillation grade toluene. Vials were counted in a Beckman 230 Liquid Scintillation Counter with CsCl external standard quench correction. Quench correction curves were determined for the buffer (free) quench and the ethanol (bound) quench separately.

Scatchard analysis of the data involved determining specifically bound hormone (BND_{sp}) as BND_{sp} = Total Bound—Bound in the presence of D.E.S. and free hormone obtained directly as described above. Plots were made of BND_{sp}/Free vs BND_{sp} with the affinity constant (K_a) determined from the negative of the slope of the line and total sites per sample from the x intercept (Scatchard, 1949).

For some studies Scatchard plots were not necessary and single point assays were done. A series of tubes containing 100ul cytosol and equal amounts of ^{3}H-estradiol were incubated with differing amounts of competitor, differing temperature treatments, differing times of incubation or other treatments which were applied to replicate samples as described under Section III, Results. Bound hormone was measured and compared for each of the different treatment groups.

Lowry Protein Assay

Protein contained in cytosol samples was assayed by the method of Lowry et al. (1951). Monothioglycerol, contained in the assay buffer,

interferes with this assay, and thus, certain modifications of the basic procedure were included. Standard protein solutions were composed of 30ul of the assay buffer plus bovine serum albumin (BSA) (between 20 and 100ug protein) in 0.5ml distilled water. Thirty microliters of the unknown samples in assay buffer were diluted to 0.5ml with distilled water. Blank tubes without protein or assay buffer were used. Alkaline cuprate solution (2ml) was added to each tube for 15 minutes and 1N Folin reagent (0.2ml) was added for 30 minutes. The samples were read at 750nm. When performed in this manner the standard curve for the assay had a slightly different slope and y intercept than when standards were mixed without monothioglycerol. The combination of dilution effect and use of constant amounts of the interfering component resulted in a repeatable straight line between 20ug and 100ug protein. Thirty microliter samples of the cytosol preparation always yielded protein concentrations within this range.

Keck DNA Assay

DNA was assayed by the method of Keck (1956). Nuclear pellets were boiled for 20 minutes in 10ml of 5% tricloroacetic acid (TCA). Hydrochloric acid (2.5N) and Indole (0.06%) were added to aliquots of the hydrolyzed DNA solutions and boiled for 10 minutes. The reacted samples were extracted twice with an equal volume of isoamly-acetate. DNA gave a straight standard curve at concentrations of between 5 and 100ug per sample. RNA did not react.

Surgical Procedure for Ovariectomy

Animals were given the analgesic, Innovar-Vet (Pittman-Moore), 10 minutes prior to surgery, at a concentration of 0.3cc per kilogram body weight. They were further anesthetized with 0.2cc (10mg) sodium pentobarbital. A midventral incision was made through the skin and the underlying muscle was cut along the linea alba. The fat and mesentary beneath the ovary were clamped with a curved Kelley hemostat and a double thickness of 00 silk was pulled through the fatty tissue. The thread was tied on either side of the mesentary securely restricting the blood vessels to the ovary. The ovary was then excised. The muscle was sutured with a blanket stitch using size 0 silk and the skin was sutured with single stitches using 0 silk. The skin sutures were further secured with suture clips (9mm). Each animal was given 300 units of antibiotic containing Benzathine Penicillin G and Procane Penicillin G, and allowed to recover from anesthesia under a heat lamp. A cardboard neck collar was attached to each animal to prevent biting of the stiches and removal of the suture.

Treatment of Ovariectomized Animals

Injections were given to each animal according to the schedule in Table 1.

Estradiol-17β (100ug/ml) and progesterone (3mg/ml) were dissolved in unrefined sesame oil previously filtered through Fisher filter paper. Gentle heating and stirring were required to fully dissolve the steroid in the oil. Oil for control groups was treated the same as estradiol and progesterone. All preparations were stored in the

refrigerator (0-4°C) between usages and allowed to warm to room temperature before injection. The diluted steroid was kept no longer than three weeks. Subcutaneous injections were given in the loose skin behind the neck using a 20 gauge needle. Dose was calculated on the basis of body weight (1ml/Kg) giving a concentration of 100ug/Kg/day for estradiol and 3mg/Kg/day for progesterone. Half of this dose was given at 10 a.m. and half at 5 p.m. on each day of injection.

Table 1. Hormone Injection Schedule* for Ovariectomized Rabbits.

Treatment Group	Day after Ovariectomy 1	2	3	4	5	6
	Time of Injection					
Sesame Oil	-	10am 5pm	10am 5pm	10am 5pm	10am 5pm	killed 12 noon
Estradiol-17β	-	10am 5pm	10am 5pm	10am 5pm	10am 5pm	killed 12 noon
Progesterone	-	10am 5pm	10am 5pm	10am 5pm	10am 5pm	killed 12 noon

*Hormones in oil, or oil alone were injected subcutaneously twice daily. Treatment lasted for four consecutive days and animals were killed on the sixth day after ovariectomy as indicated. Concentrations of hormone were 100ug/ml for estradiol and 3mg/ml for progesterone. Doses were 1ml/Kg body weight with half the dose administered at the times specified.

Purification of ^{3}H-Estradiol

^{3}H-estradiol was periodically purified through an LH_{20} mini column. Sephadex LH_{20} (900mg) was allowed to swell for 3 hours in elution fluid containing 90% benzene and 10% methanol (v/v). The column, a 2.5ml

glass syringe with a metal 23 gauge needle, was washed with methanol and stopped with a fiber glass filter. The slurried LH_{20} was added to it. The column was washed for 20 minutes with the elution fluid. ^{3}H-estradiol to be purified was added to the column in 0.3ml elution fluid. The column was eluted with a total volume of 22ml of the benzene methanol mixture and fractions collected at 1 minute intervals. Total elution time was approximately 90 minutes. Samples of 10ul were taken from alternate fractions for counting and determination of the location of the estradiol peak. Peak fractions were pooled, dried, and rediluted in ethanol.

III. RESULTS

The goal of this study was to determine whether differences exist between amounts and properties of cytoplasmic estradiol receptors found in endometrium and myometrium obtained from the uteri of rabbits. The study entailed the use of an assay procedure sensitive to measurements of receptor titer and able to give reliable measurements of the affinity of the receptor for the hormone (K_a). The hydroxylapatite (HAP) separation procedure, first suggested by Williams and Gorski (1975), as developed by Pavlik and Coulson (1976a) was chosen. This assay permitted the measurement of both bound hormone and free hormone through binding of the hormone receptor complex to HAP. This has advantages over the widely used dextran coated charcoal method which allows for direct determination of bound hormone only.

The effectiveness of HAP removal of bound hormone from rabbit cytosol was established and is shown in Figure 1. The most effective binding of the hormone receptor complex took place during a 5 minute incubation with HAP. This time period was therefore chosen for all subsequent incubations.

Figure 2 gives a comparison of the values obtained when the dextran coated charcoal (DCC) separation method was compared to the HAP binding procedure. The HAP method gave higher specific binding than did the DCC method. A difference in nonspecific binding accounted for this. Unbound hormone could be washed off of the HAP pellet. Variability was reduced using the HAP assay.

Figure 1. Time Course of Binding to Hydroxylapatite.

The effect of incubation time with HAP upon the recovery of specifically-bound estradiol was measured. Specifically bound DPM is shown on the ordinate and incubation time is shown on the abcissa. Points represent the mean of four determinations and vertical bars are S.E.M.

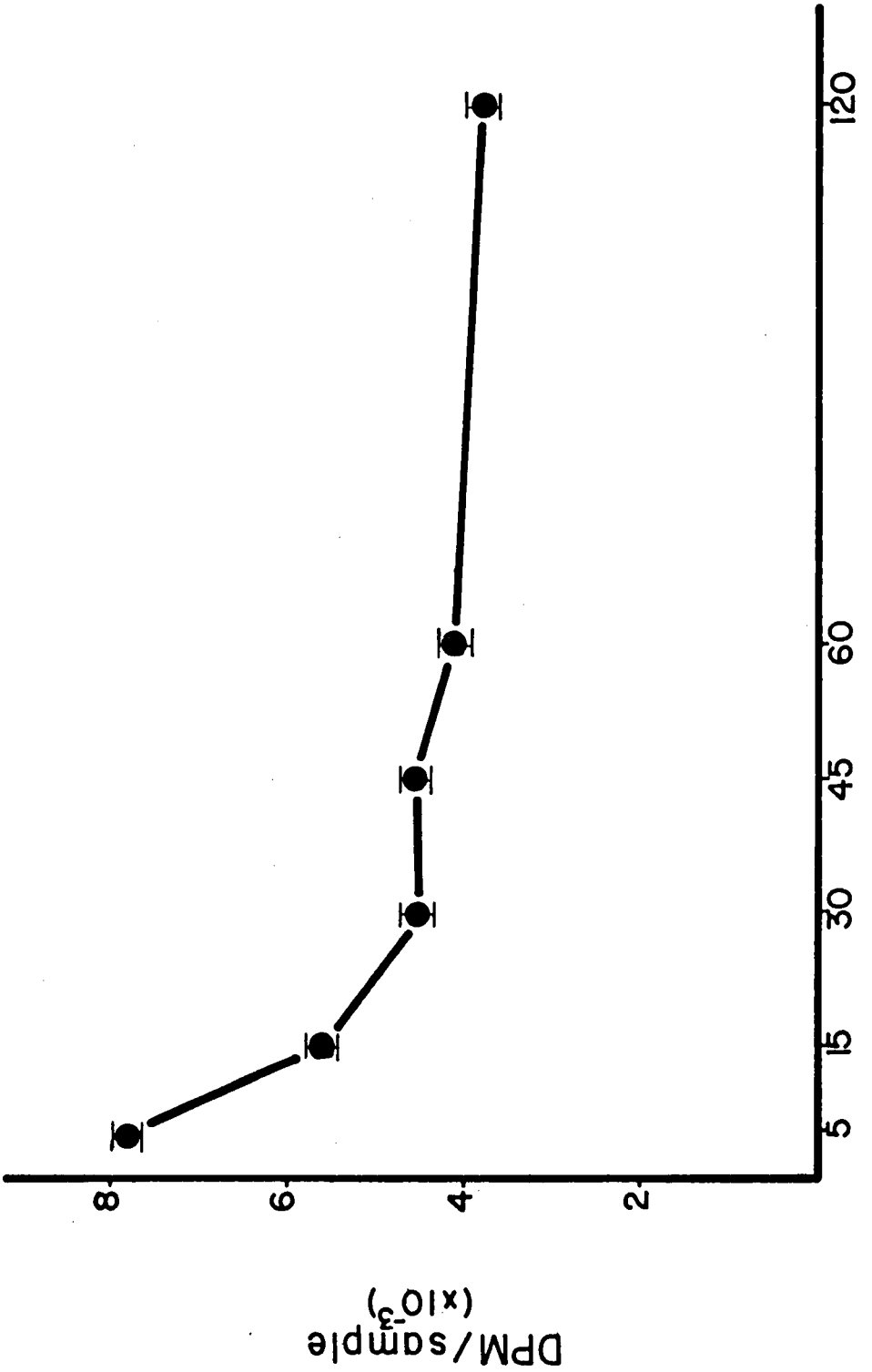
DPM/sample
(x10⁻³)
8
6
4
2
5
15
30
45
60
120
INCUBATION TIME
(minutes)

Figure 1

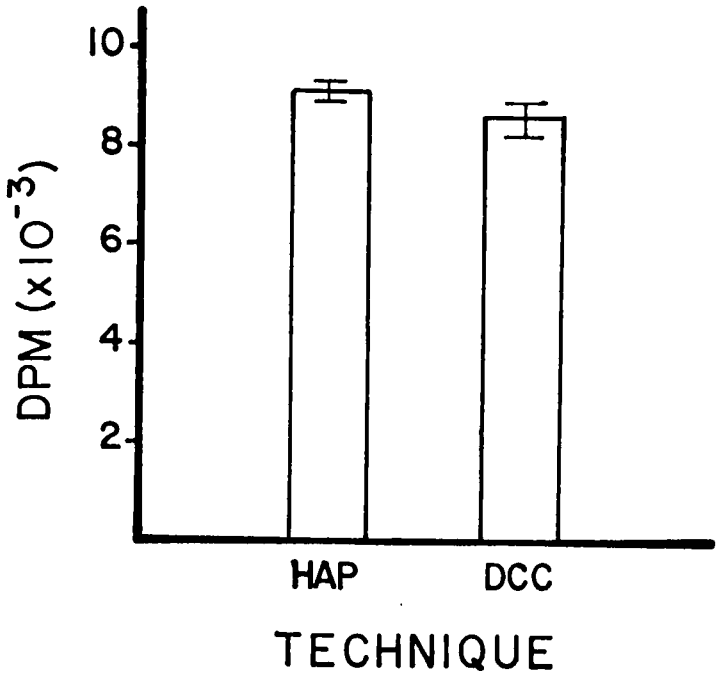

Figure 2. Comparison of Hydroxylapatite and Dextran Coated Charcoal Techniques for Measuring Specifically-Bound Hormone.

Two methods for determining specifically bound hormone were compared. Incubation with HAP was carried out as described in Section II, Materials and Methods. One ml of prewashed dextran coated charcoal suspension (0.025% dextran T-70 and 0.25% Norit A charcoal) was added to the cytosol, incubated (5 minutes), and removed by centrifugation (1,000gx 10 minutes). Four separate determinations make up the mean and S.E.M. in each case. DPM were corrected for nonspecific binding.

Incubation and Endogenous Hormone

A consideration not to be ignored when studying receptor concentrations in a tissue stimulated by endogenous hormone is the potential for this hormone to mask the binding sites present in the preparation. Estradiol binding sites previously bound by endogenous estrogens are not detected by many assay procedures. This problem has been treated by Anderson et al. (1972) who developed an exchange assay which makes use of temperature control to exchange estrogen already bound to receptor for radioactively-labeled steroid.

Figure 3 depicts the results obtained when endometrial and myometrial cytosols were incubated at 0°C for various periods of time up to 20 hours. A rapid increase in specifically bound hormone was seen during the first 2 hours of incubation. Maximal specific binding was obtained in both tissues after 8 hours and was constant through 20 hours of incubation.

After establishing equilibrium conditions at 0°C, an attempt was made to use an exchange assay procedure. Uterine cytosol composed of both endometrium and myometrium was incubated at 23°C, 30°C and 37°C and samples were taken from each at 5, 15, 30, 60 and 120 minutes. These were compared to values obtained from a 16 hour incubation at 0°C. Figure 4 shows the results. Although the binding obtained after 15 minutes at 23°C equalled that of a 16 hour incubation at 0°C, subsequent incubation of the preparation at this temperature did not significantly increase binding. Incubation at 30°C resulted in 96% of total binding occurring at 15 minutes, but binding decreased by

Figure 3. The Effect of Incubation Time on Specific Binding at 0°C.

Specific binding in endometrium and myometrium at 0°C was measured over a 20 hour period. Equilibrium was reached at 8 hours of incubation in both tissues. The concentration of ^{3}H-estradiol was 1×10^{-13} moles per 100ul. Vertical bars are S.E.M., means are for four samples.

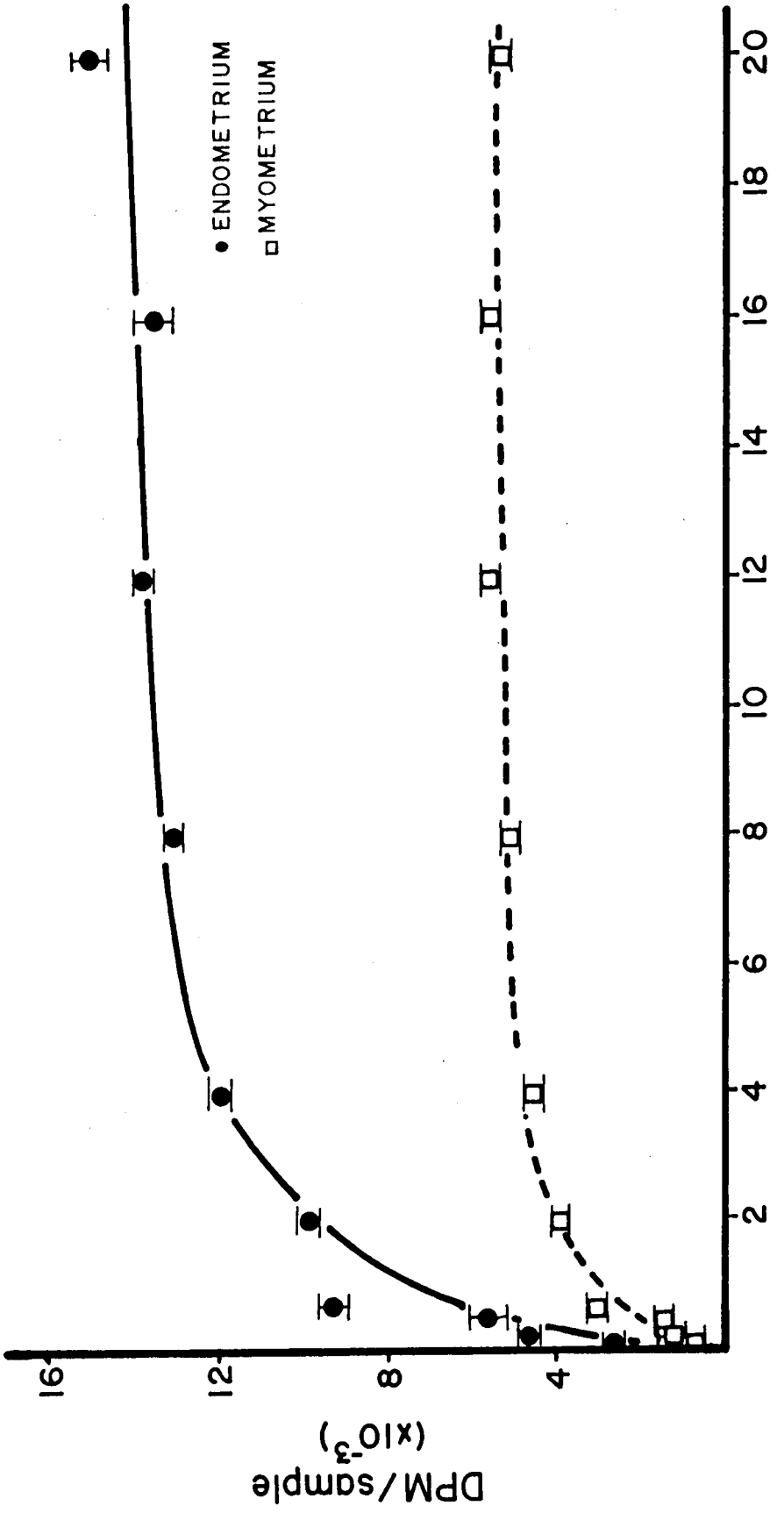
DPM/sample
(x10⁻³)
16
12
8
4
2
4
6
8
10
12
14
16
18
20
INCUBATION TIME
(hours)
• ENDOMETRIUM
□ MYOMETRIUM

Figure 3

Figure 4. The Effect of Temperature on Specific Binding.

Uterine cytosol was incubated with a constant concentration of ^{3}H-estradiol (see Figure 3) at various temperatures and specific binding was plotted as a percentage of the binding obtained in a 16 hour incubation at 0°C. Means and S.E.M. were calculated for four samples at each point.

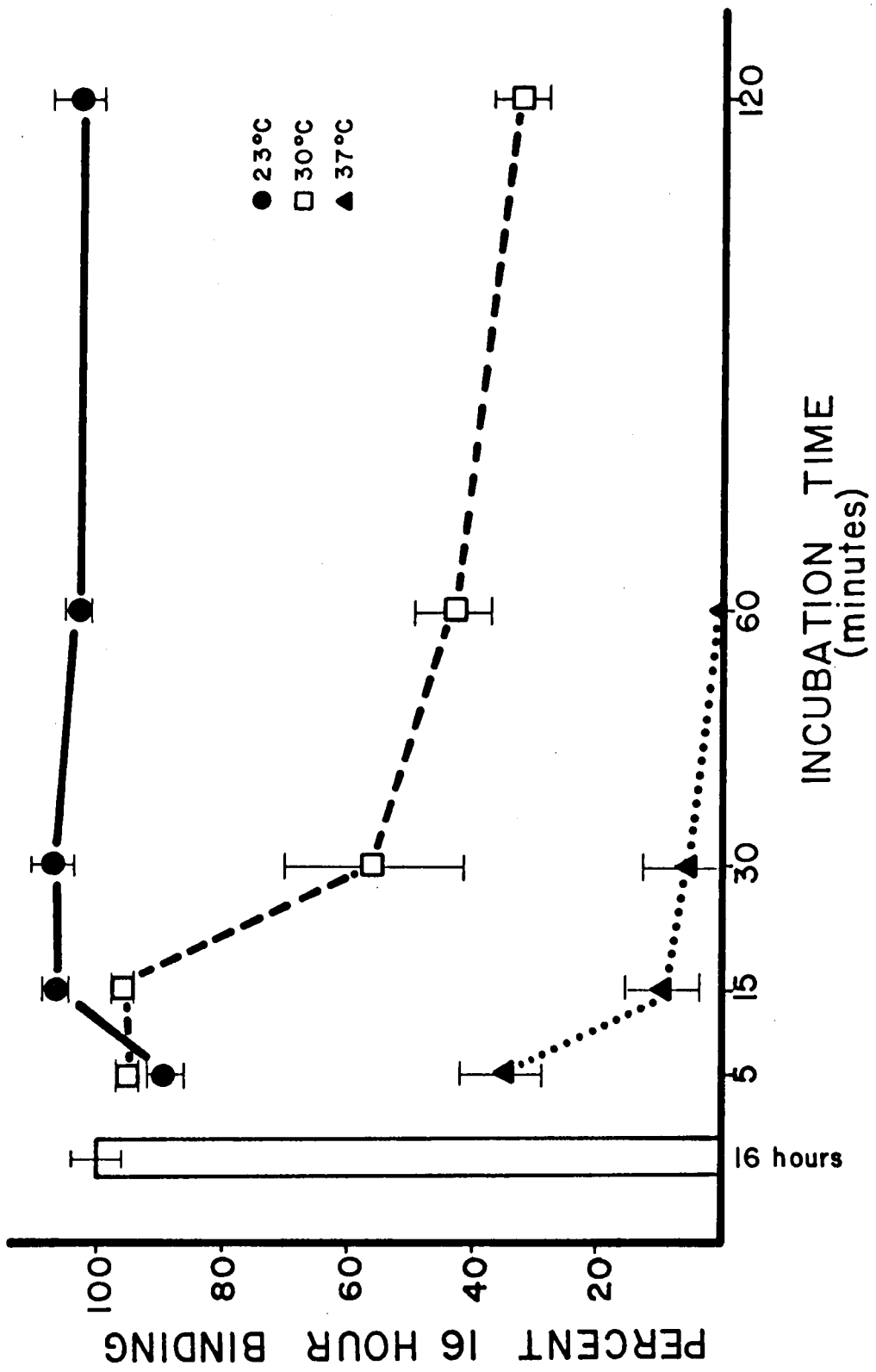
PERCENT 16 HOUR BINDING
100
80
60
40
20
16 hours
5
15
30
60
120
INCUBATION TIME (minutes)
23°C
30°C
37°C

Figure 4

30 minutes of incubation and had been reduced to 32% after 120 minutes. Specific binding was completely destroyed by a 60 minute incubation at 37°C. The decrease in specific binding at elevated temperatures might be the result of denaturation or degradation of the receptor. All subsequent studies were done using a 16 hour incubation at 0°C, the condition shown most likely to insure stability of the receptor.

The conditions chosen for the assay still did not correct for the problem of endogenous hormone in the cytosol. Dextran coated charcoal is known to remove unbound steroid without removing protein. This is the basis of the dextran coated charcoal steroid receptor assay. As indicated in Table 2 dextran coated charcoal (0.2% (w/v) each) removed approximately 99% of the unbound endogenous hormone from TEM buffer. Table 2 also presents values for the slight increase in binding seen when dextran coated charcoal was added to a cytosol preparation. The treated and untreated cytosols were assayed for receptor using a 16 hour incubation at 0°C. Although a 5 minute treatment with dextran coated charcoal did not remove hormone bound to the receptor sites, this treatment did insure that unbound endogenous hormone was removed. It was therefore routinely used in all subsequent assays.

Receptor Identity

Tissue specificity is an established criterion for receptor identity. High affinity, low capacity, and specificity for the hormone with which it interacts, are properties of receptors (Gorski et al., 1968). These parameters held for estradiol binding in rabbit uterine

Table 2. Pretreatment of Cytosol with Dextran Coated Charcoal.*

	DPM Added	% Removed by DCC
	3172.7	99.9%
	10374.1	99.2%
	63088.1	98.3%
	94320.1	99.2%
TOTAL BOUND NO DCC pretreatment Cytosol	100±	1.1%
TOTAL BOUND + DCC pretreatment	107.5±	2.9%

*Dextran coated charcoal (DCC) at a final concentration of 0.2% (w/v) Dextran T70 and 0.2% (w/v) activated charcoal, was incubated with cytosol or buffer + ^{3}H-estradiol. Incubation with DCC lasted for 5 minutes. Charcoal was removed by two 13,000gx 10 minute spins at 4°C. Values for specific binding ability of cytosol with and without DCC pretreatment are given plus or minus S.E.M.

endometrium and myometrium. Figure 5 shows the results of single point assays done on endometrium, myometrium and several control tissues. Bound radioactivity in disintegrations per minute (DPM) (the values shown on the ordinate) have been corrected for nonspecific binding and normalized to protein content. Both endometrium and myometrium bound greater amounts of estradiol than did liver, heart, lung or spleen. In the latter tissues competition was not greater than 50% of total binding, while in endometrium and myometrium D.E.S. competed 80% or more of the ^{3}H-estradiol added.

Where limited numbers of high affinity binding sites are present specific binding is a saturable process. Graded quantities of ^{3}H-estradiol were added to a constant quantity of cytosol obtained from endometrium or myometrium. The results were compared to the results obtained when ^{3}H-estradiol was incubated with bovine serum albumin, and with potential contaminants of the uterine preparation (plasma and uterine flushings). The data are shown in Figure 6. At equilibrium, specific binding in endometrium and myometrium increased with the concentration of estradiol until saturation was reached. In none of the controls was saturation observed. In control groups nonspecific binding was far greater than specific binding. These data are shown in Figure 7, plotted by the method of Scatchard (1949). Data were routinely plotted in this manner to determine total numbers of binding sites from the x intercept and affinity (Ka) from the slope of the line. In these examples the Ka for endometrium was $1.83 \times 10^{9} M^{-1}$, that for myometrium was $2.36 \times 10^{9} M^{-1}$. The correlation coefficients for the

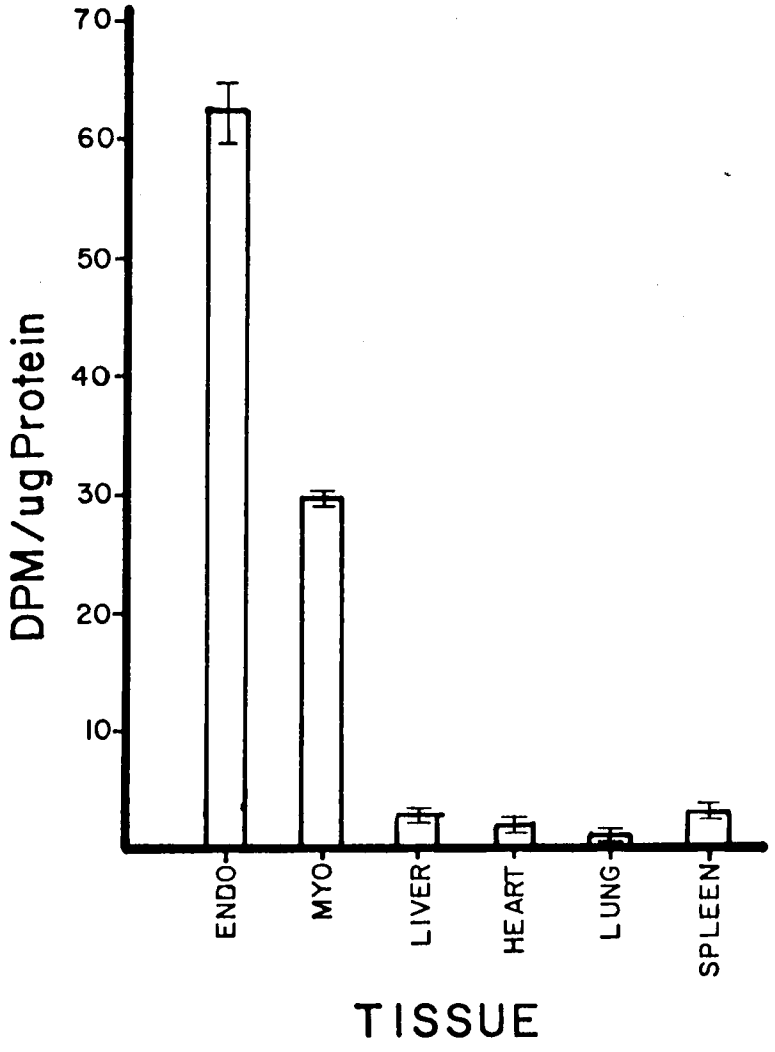

Figure 5. Specific Binding in Uterine and Control Tissues.

Cytosols from endometrium, myometrium and various control tissues were prepared as described in Section II, Materials and Methods. Radioactively labeled estradiol (1 x 10^{-13} moles/100ul cytosol) with and without D.E.S. as competitor was incubated with these cytosols at 0°C for 16 hours. DPM specifically-bound was normalized to protein content. Values represent the means and S.E.M. of four samples.

Figure 6. The Effect of Varying the Concentration of Estradiol on the Binding in Uterine Tissues and Controls.

Endometrial cytosol, myometrial cytosol, uterine flushings, bovine serum albumin and plasma were tested for their ability to bind estradiol over the range of concentrations used in Scatchard analysis. Two ml of plasma were diluted to 10 ml with TEM, and uterine flushings in 2 ml of this buffer were used. B.S.A. was assayed at a concentration of 140ug/ml TEM. Closed circles represent specific binding, open circles are total binding, and open squares are nonspecifically bound hormone. Each point represents duplicate determinations.

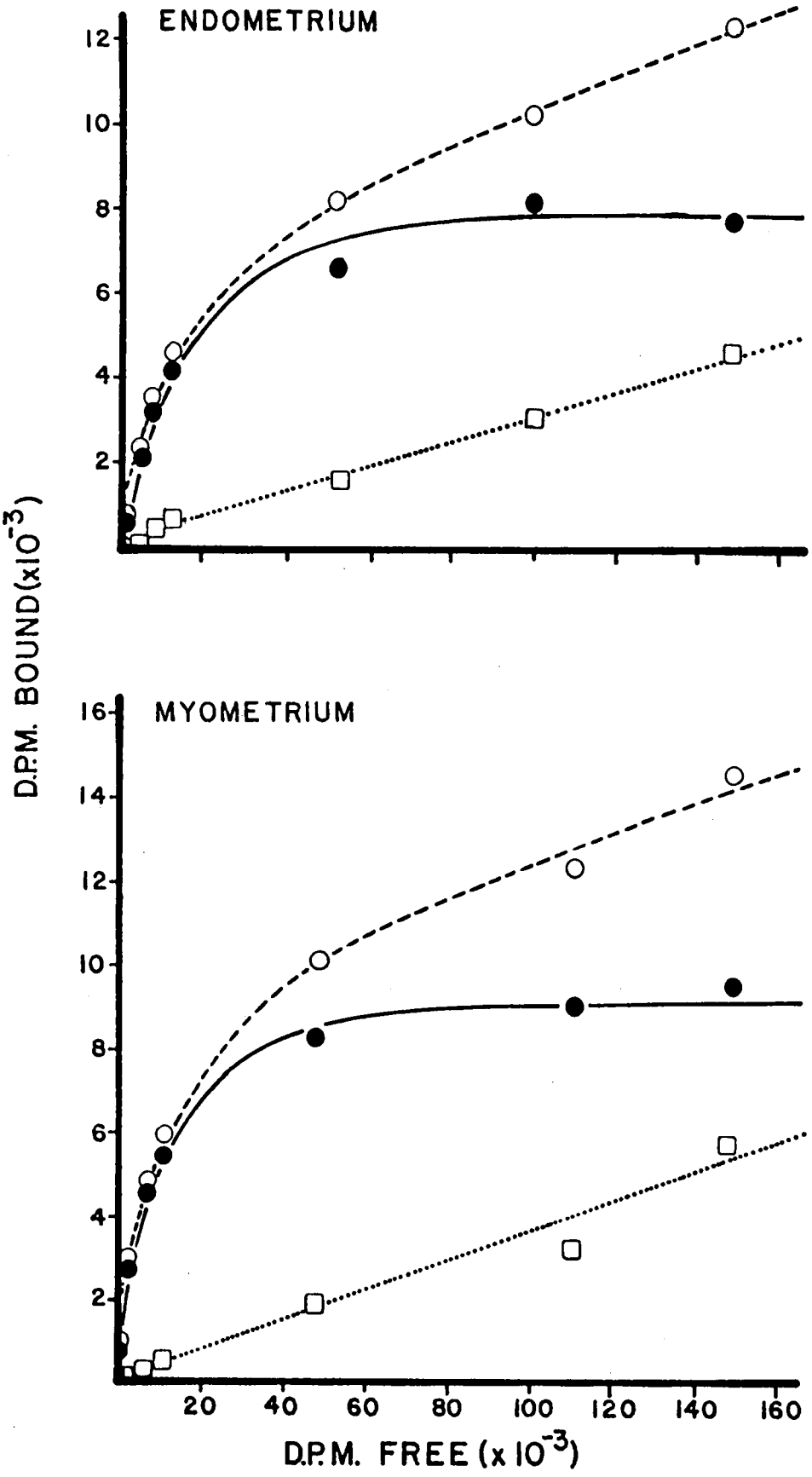
ENDOMETRIUM
MYOMETRIUM
D.P.M. BOUND(x10^{-3})
D.P.M. FREE (x 10^{-3})
2
4
6
8
10
12
14
16
20
40
60
80
100
120
140
160

Figure 6

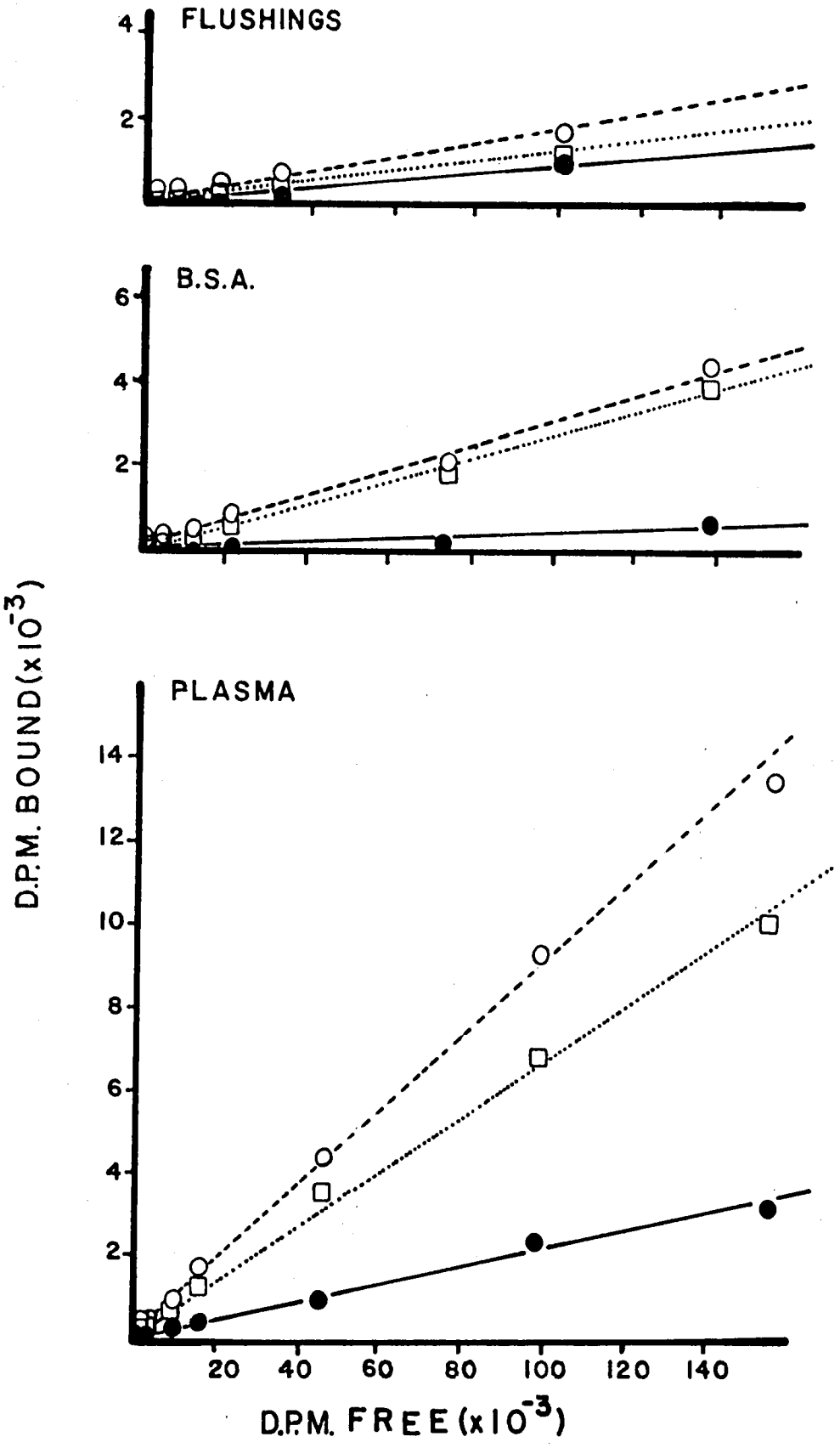

Figure 6 (continued)

Figure 7. Sample Scatchard Plots for Endometrium and Myometrium.

The data for endometrium and myometrium given in Figure 6 were analysed by Scatchard analysis and graphed. The ordinate is specifically-bound hormone over free hormone and the abcissa is specifically-bound hormone. Circles are endometrium and squares are myometrium. K_a was determined from the slope of the line and total sites were determined from the x intercept.

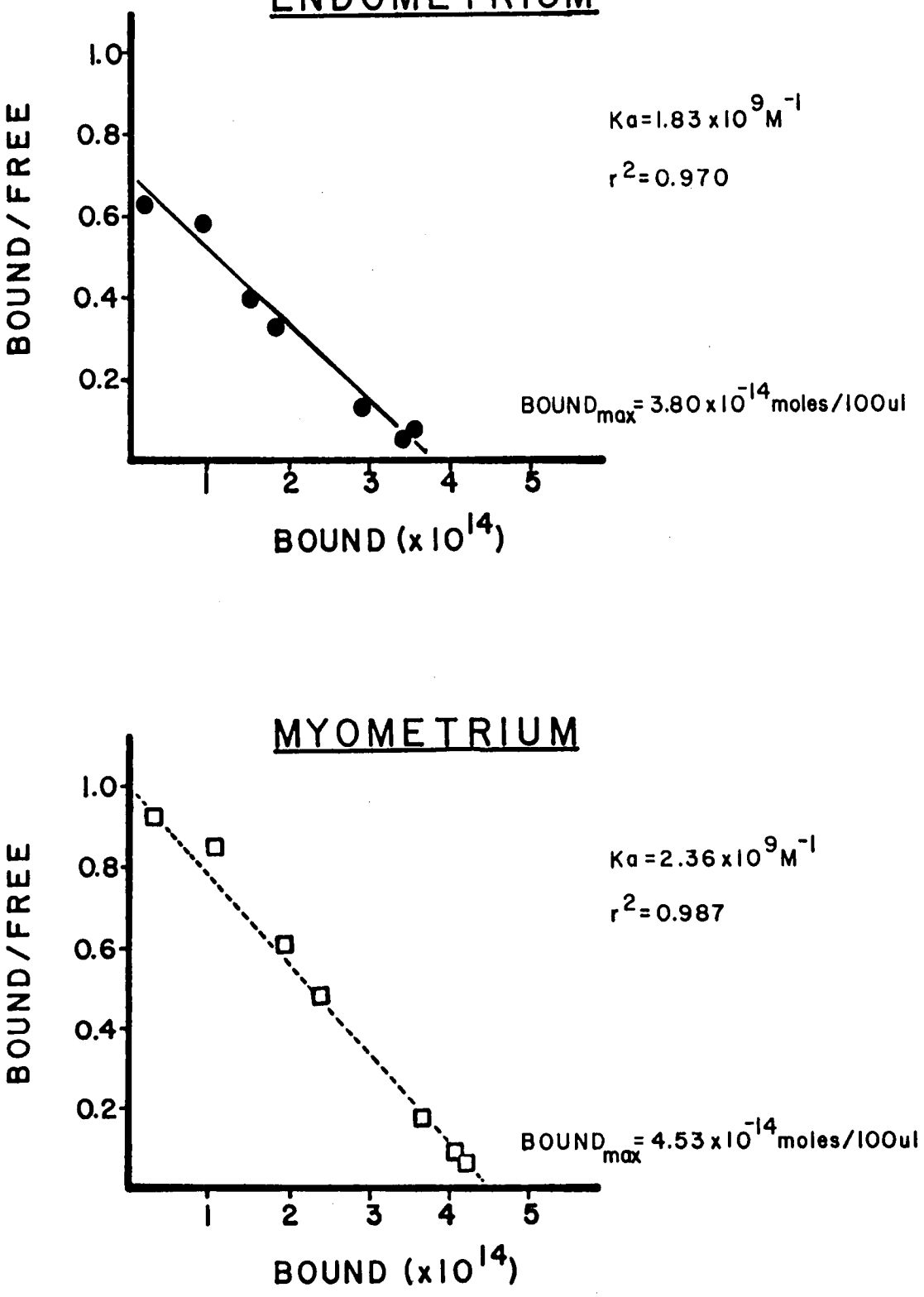
ENDOMETRIUM
BOUND/FREE
1.0
0.8
0.6
0.4
0.2
1
2
3
4
5
BOUND (x 10^14)
Ka=1.83 x 10^9 M^-1
r^2=0.970
BOUND_max= 3.80 x 10^-14 moles/100ul
MYOMETRIUM
BOUND/FREE
1.0
0.8
0.6
0.4
0.2
1
2
3
4
5
BOUND (x10^14)
Ka=2.36 x 10^9 M^-1
r^2=0.987
BOUND_max= 4.53 x 10^-14 moles/100ul

Figure 7

resultant straight lines were 0.970 and 0.987 for endometrium and myometrium respectively. Analyzing data in this manner will reveal heterogeneous binding sites and positive or negative cooperativity when present. These would be indicated by an abrupt change in the slope or curvature of the line. Neither of these characteristics were seen in the Scatchard plots constructed from this study. To further ascertain whether multiple sites were present the data were analyzed by Hill plots. With this method, the log [bound/bound total-bound] is graphed on the ordinate and the log [free] is graphed on the abcissa. The slope of the line (Hill coefficient) will be: (1) less than one in the case of negative coopertivity or heterogeneous binding sites, (2) one, when a single class of nondissociating binding sites exist, and (3) greater than one, when the binding sites display positive coopertivity (Boeynaems and Dumont, 1975). Hill coefficients were 0.964 and 1.02 for endometrial and myometrial samples shown in the above plots. Both tissues appeared to have a single class of high-affinity, low-capacity binding sites for estradiol.

The configuration of a receptor is such that only one group of similar compounds effectively binds with it (Gorski et al., 1968). Estradiol-17β and D.E.S., a synthetic estrogen, both bind to estrogen receptors while progesterone, testosterone, and dexamethasone do not. Figure 8 shows the results obtained when various competitors at 100 times the concentration of ^{3}H-estradiol-17β were added to cytosol in single point assays. Both endometrial and myometrial estrogen receptors were tested for specificity. Estradiol and D.E.S. removed

Figure 8. Receptor Specificity.

Cytosols from endometrium and myometrium were incubated with 1×10^{-13} moles ^{3}H-estradiol/100ul cytosol plus no competitor (none) or 100 fold excess of estradiol-17β (E_2), diethylstilbesterol (D.E.S.), progesterone (Prog.), testosterone (Test.) or dexamethasone (Dex.). Binding was recorded as a percentage of the binding obtained in the absence of competitor. Values represent four determinations each and vertical bars are S.E.M.

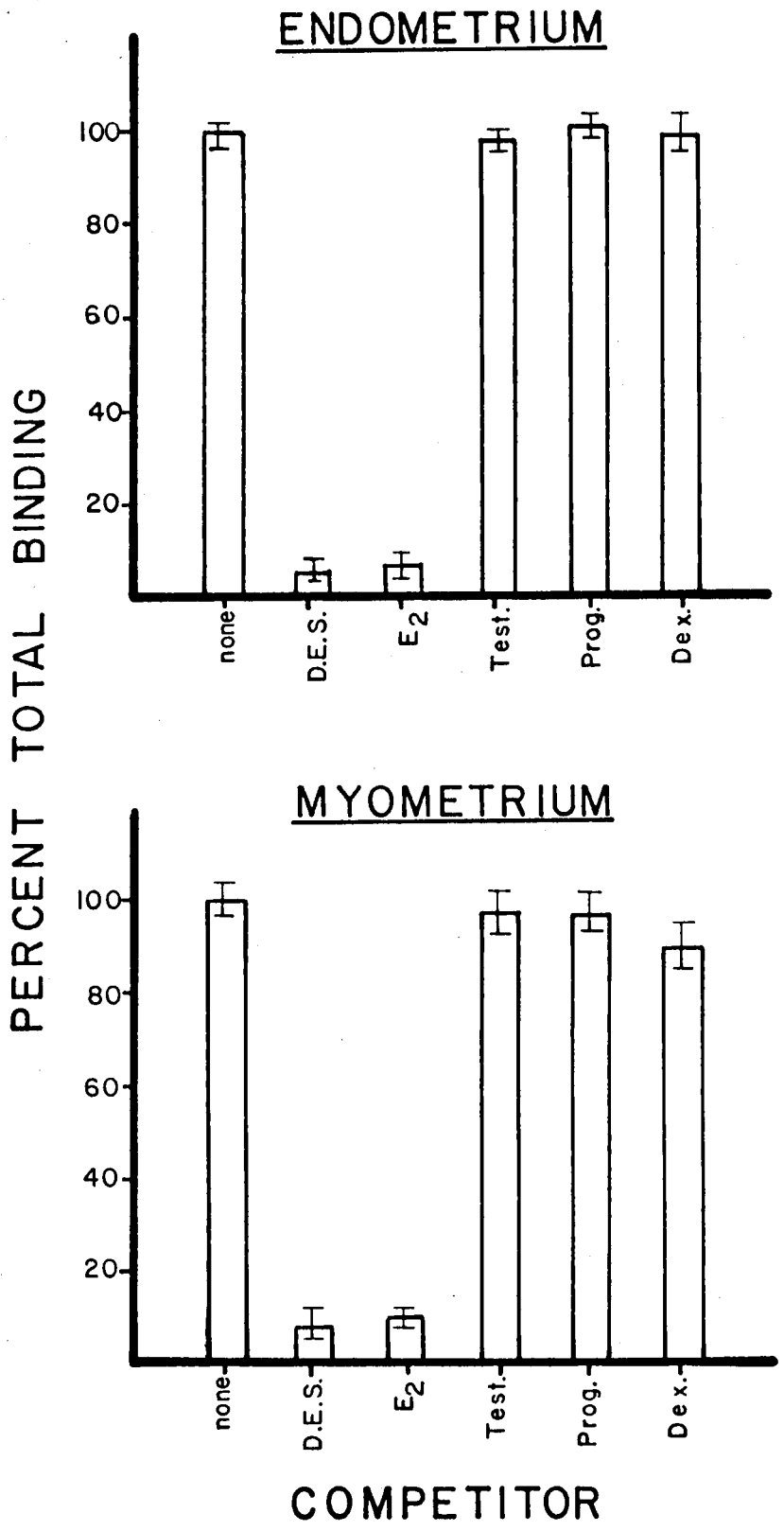
ENDOMETRIUM
MYOMETRIUM
PERCENT TOTAL BINDING
100
80
60
40
20
none
D.E.S.
E_2
Test.
Prog.
Dex.
COMPETITOR

Figure 8

over 90% of the bound labeled estradiol from both tissues. Progesterone, testosterone, and dexamethasone were not competitive for these binding sites.

Receptor Changes during Early Pregnancy

Cytosplasmic receptors bind steroid hormone after its entrance into the cell. The hormone may mediate the cellular response or its receptor may be converted into a responsive agent after interaction with the hormone. A responsive cell may be equipped with numbers of receptors or with receptors of appropriate properties which enable it to respond to the stimulus. Administration of hormone to different tissues and to tissues in different physiological states results in various responses. Modulation of cytoplasmic receptor is a potential mode of control by which a target cell can adjust its responsiveness to a hormone.

The properties of the cytoplasmic estrogen receptors of uterine endometrium and myometrium were studied during the period of early pregnancy when these tissues undergo marked changes. Endometrial receptor was compared to myometrial receptor as conditions changed.

Figure 9 depicts changes in estrogen receptor K_a during the first nine days of pregnancy in the rabbit. Endometrial receptor K_a remained high through the fourth day post coitum and then dropped through day nine ($P < 0.05$). Myometrial receptor K_a dropped between days three and seven of pregnancy ($P < 0.05$). There was no significant difference between endometrial and myometrial K_a on any day of pregnancy.

Figure 9. Estrogen Receptor Affinity (K_a) in Endometrium and Myometrium during Early Pregnancy.

The affinity of the estrogen receptor for estradiol was measured during early pregnancy. Cytosols from the endometrium and myometrium of pregnant rabbits were prepared and assayed as described in Section II, Materials and Methods. The data were analyzed by Scatchard analysis to obtain K_a. Values for from two to four animals were averaged for each day of pregnancy. Two values were used in one sample only (four day) and reflect the fact that the unexpected seasonal variation interfered with performance of the subsequent assays. Vertical bars are S.E.M. All determinations were made between August and December.

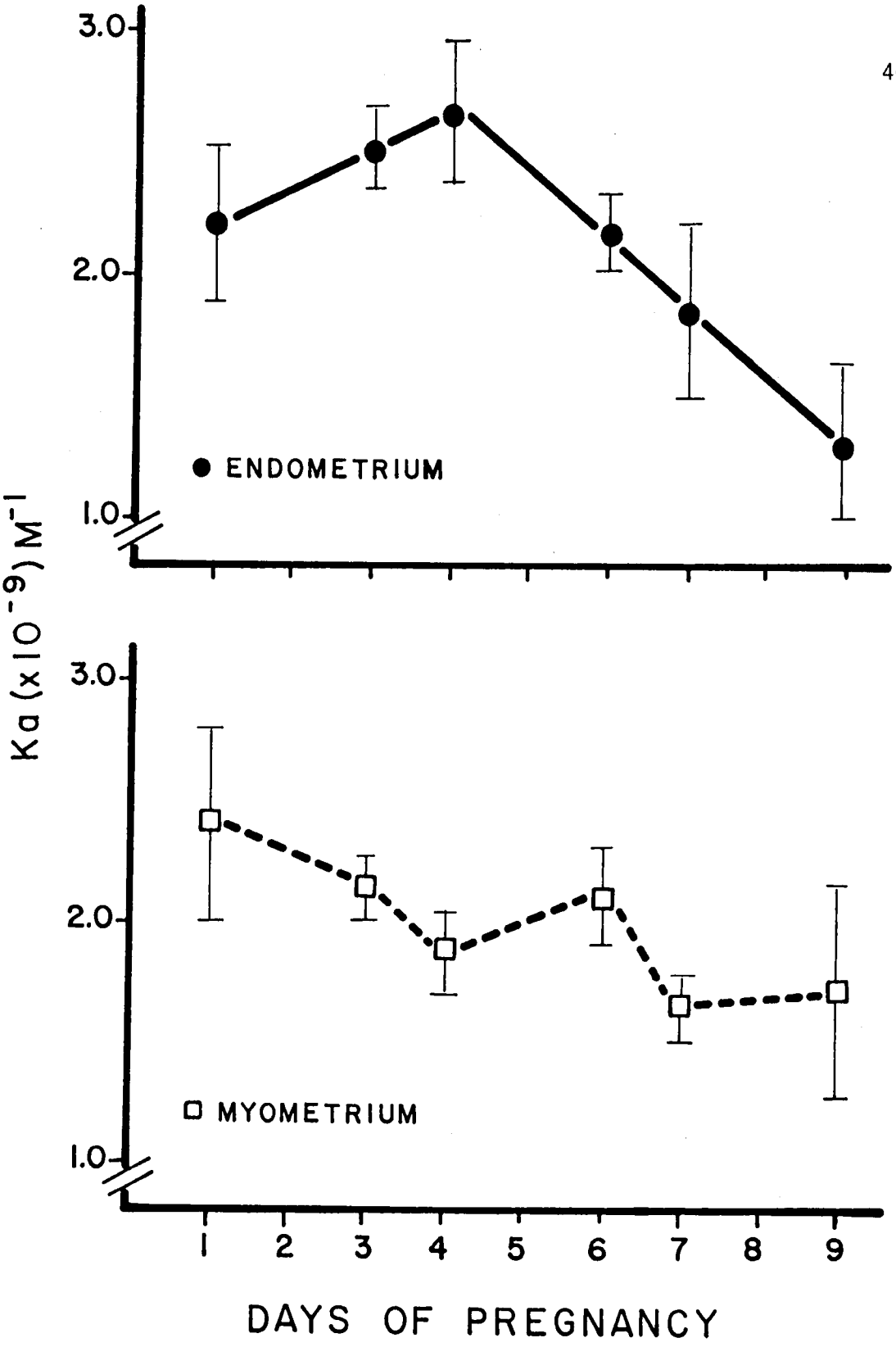
3.0
2.0
1.0
ENDOMETRIUM
3.0
2.0
1.0
MYOMETRIUM
Ka (x10⁻⁹) M⁻¹
1 2 3 4 5 6 7 8 9
DAYS OF PREGNANCY

Figure 9

Implantation occurs on, or around, day seven in this species. Localization of blastocysts over prospective implantation sites occurs on day 6. The drop in affinity of endometrial receptor occurred just prior to implantation and just after the entrance of the blastocysts into the uterus on day four.

Protein, DNA and wet weight of the uterine tissue increase markedly during the first nine days of pregnancy in the rabbit. To normalize receptor numbers to any one of these values could lead to conclusions based on changes in that value rather than on functional changes in receptor titer. A cellular unit, cytoplasmic-nuclear ratio, was chosen, therefore, as the basis for normalization. This is expressed as ug cytosolic protein per ug DNA and reflects cell size as well as cell number.

The data for receptor titer during the first nine days of pregnancy as a function of cytoplasmic-nuclear ratio are reported in Figure 10. Endometrial receptor number increased between estrus and the fourth day of pregnancy ($P < 0.005$) only to drop again by day seven ($P < 0.01$). In contrast, myometrial receptor declined steadily from day one onward. There was a significant difference between the levels of endometrial and myometrial receptor from day three through day nine.

It should be noted that the standard errors from endometrial samples were greater than those from myometrial samples. This may be a reflection of the greater variability inherent in endometrium. Endometrial protein, DNA and wet weight are changing much more rapidly than those in myometrium. Error would be potentiated by the fact that

Figure 10. Estrogen Receptor Titer in Endometrium and Myometrium during Early Pregnancy.

Estrogen receptor content was determined from Scatchard analysis (see Figure 9). Values were normalized per ug protein/ug DNA. There were from two to five rabbits in each group. Vertical bars are S.E.M.

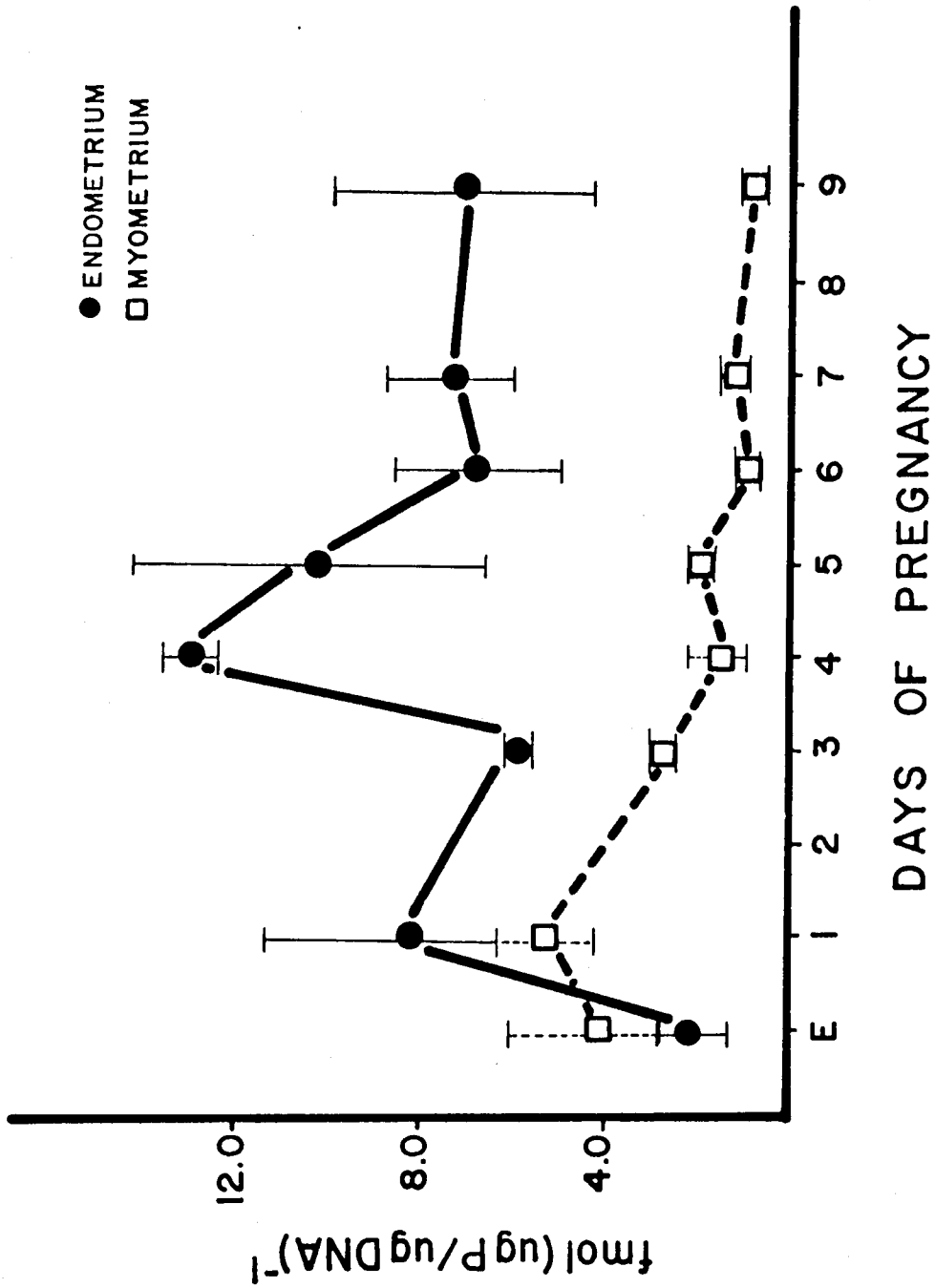
ENDOMETRIUM
MYOMETRIUM
12.0
8.0
4.0
fmol (ug P/ug DNA)-1
E
1
2
3
4
5
6
7
8
9
DAYS OF PREGNANCY

Figure 10

slight shifts in the time of fertilization (11-15hrs p.c.) (Kozma et al., 1974), the size and numbers of embryos, and the temporal sequence of other early reproductive events may have a more pronounced effect on the endometrium than on the underlying muscle.

Although functional receptor titer is best portrayed on the basis of cellular units, it is important to be able to compare the results obtained for these two tissues with data from uterine preparations where combined tissues were assayed. Table 3 shows values from several days of pregnancy normalized per ug DNA and compared to values from the literature. Receptor titer as a function of DNA did not change appreciably. Both Muechler et al. (1974) and Lee and Keyes (1971) reported a decline in receptor concentration through day six of pregnancy. Calculated values in Table 3 were determined on the basis of the change in the proportion of endometrium in the uterine preparations. When calculated on total tissue in this manner the receptor levels declined. This correlates with the previously reported values. A pitfall causing misinterpretation of data by using uterine cytosol rather than separate endometrium and myometrium for assay is clearly shown.

Table 4 gives the values for receptor number in terms of ug protein and total receptor. Endometrial protein content increases so rapidly during the early days of pregnancy that this method of normalization gives little useful information. Total receptor content of endometrium increased through day four as did functional receptor. Total myometrial receptor did not change.

Table 3. Estrogen Receptor Concentration Normalized per ug DNA; Comparison with Values in the Literature.*

Days of Pregnancy	n	fmol/ugDNA Endometrium	fmol/ugDNA Myometrium	Endometrial wt as % total	fmol/ugDNA Calculated	fmol/ugDNA Muechler et al., 1974	fmol/ugDNA Lee and Keyes 1971
Estrus	(5)	1.23 ± 0.39	5.09 ± 1.48	3%	5.31	5.59 ± 0.22	5.7 ± 3.0
1 day	(3)	1.27 ± 0.30	2.74 ± 0.67	13%	2.55		
3 day	(3)	1.55 ± 0.22	4.74 ± 1.75	28%	3.85	6.03 ± 0.68	
4 day	(2)	0.93 ± 0.18	4.12 ± 0.58	33%	3.07		3.9 ± 0.8
5 day	(5)	1.46 ± 0.52	3.44 ± 0.52	20%	3.04		
6 day	(4)	1.26 ± 0.24	3.21 ± 0.46	39%$^{\alpha}$	2.45	2.81 ± 0.18	
7 day	(4)	0.41 ± 0.05†	2.92 ± 0.28	40%$^{\alpha}$	1.92		
8 day							1.3 ± 0.2
9 day	(3)	0.38 ± 0.14	2.99 1.18	40%$^{\alpha}$	1.95		

*Estrogen receptor concentrations of endometrium and myometrium were normalized to DNA. Calculated concentrations were determined making corrections for the changing percentages of endometrium and myometrium in the preparations. Values from the literature are given which represent assays done on pooled endometrial and myometrial tissue from the appropriate days of pregnancy. Values in all cases are given plus or minus S.E.M. Endometrial and myometrial receptor titer were different.

$^{\alpha}$Approximate values.

†Significant from estrus values $P < 0.05$.

Table 4. Estrogen Receptor Concentration Normalized to Protein, and Total Estrogen Receptor in Endometrium and Myometrium.*

Days of Pregnancy	n	fmol Receptor/ugP		pmol/Total tissue	
		Endometrium	Myometrium	Endometrium	Myometrium
Estrus	(5)	0.131 ± 0.030	0.240 ± 0.075	0.41 ± 0.16	17.08 ± 3.64
1 day	(3)	0.362 ± 0.030††	0.265 ± 0.044	1.91 ± 0.64†	16.37 ± 5.59
3 day	(3)	0.239 ± 0.030†	0.227 ± 0.033	4.01 ± 1.11†	15.23 ± 3.69
4 day	(2)	0.235 ± 0.012†	0.152 ± 0.040	5.97 ± 3.26	11.05 ± 3.25
5 day	(5)	0.319 ± 0.093	0.158 ± 0.024	3.77 ± 0.92†	15.32 ± 2.65
6 day	(4)	0.210 ± 0.012†	0.119 ± 0.015	5.04 ± 1.71†	-
7 day	(4)	0.152 ± 0.013	0.138 ± 0.011	3.03 ± 0.72†	-
9 day	(3)	0.187 ± 0.020	0.145 ± 0.036	2.94 ± 0.26††	-

*Estrogen receptor normalized per ug Protein is given plus or minus S.E.M. for each day of pregnancy. Total receptor concentration from the two tissues is also shown for each day of pregnancy. Endometrial total estrogen receptor concentrations include receptor from both implantation site and nonimplantation site regions. Myometrial receptor from day six through day nine was not calculated since not all of the tissue from these days was analyzed. Total tissue values are approximate since some tissue loss occurred during the scraping procedure which was used to separate endometrium and myometrium.

†Significant from estrus values $P < 0.05$.

††$P < 0.01$.

The implantation site is marked by the presence of the blastocyst from the sixth day post coitum onwards in the rabbit. Tissue underlying and surrounding the blastocyst was used as implantation site. Nonimplantation site areas were designated as those found between the blastocysts. The implantation site is characterized by selective proliferation and changes in cell shape which may be caused by physical presence of the blastocyst, blastocyst enzymes such as acid phosphatase and alkaline phosphatase (Hafez and White, 1967; Hafez, 1972), or blastocyst estrogens (Seamark and Lutwak-Mann, 1972; Dickman et al., 1977).

The estrogen receptors in endometrium of implantation site and nonimplantation site regions were measured independently. The implantation sites of day nine rabbits contained less receptor than did the implantation site regions on day six (Figure 11). This may be a result of movement of more of these receptors to the nucleus or of masking of estrogen receptor in this particular region. Either explanation would be indicative of the presence of blastocyst estrogen. No difference in $K\alpha$ was seen between these regions, as is illustrated in Figure 12.

The corpus luteum is the major source of progesterone in the pregnant rabbit. In rabbits, from 5 to 15 ova mature at once producing an equal number of active corpora lutea. Significantly greater numbers of corpora lutea might produce larger total quantities of steroids than those found in animals with fewer corpora lutea. Greater numbers of steroid producing blastocysts found in these individuals might also

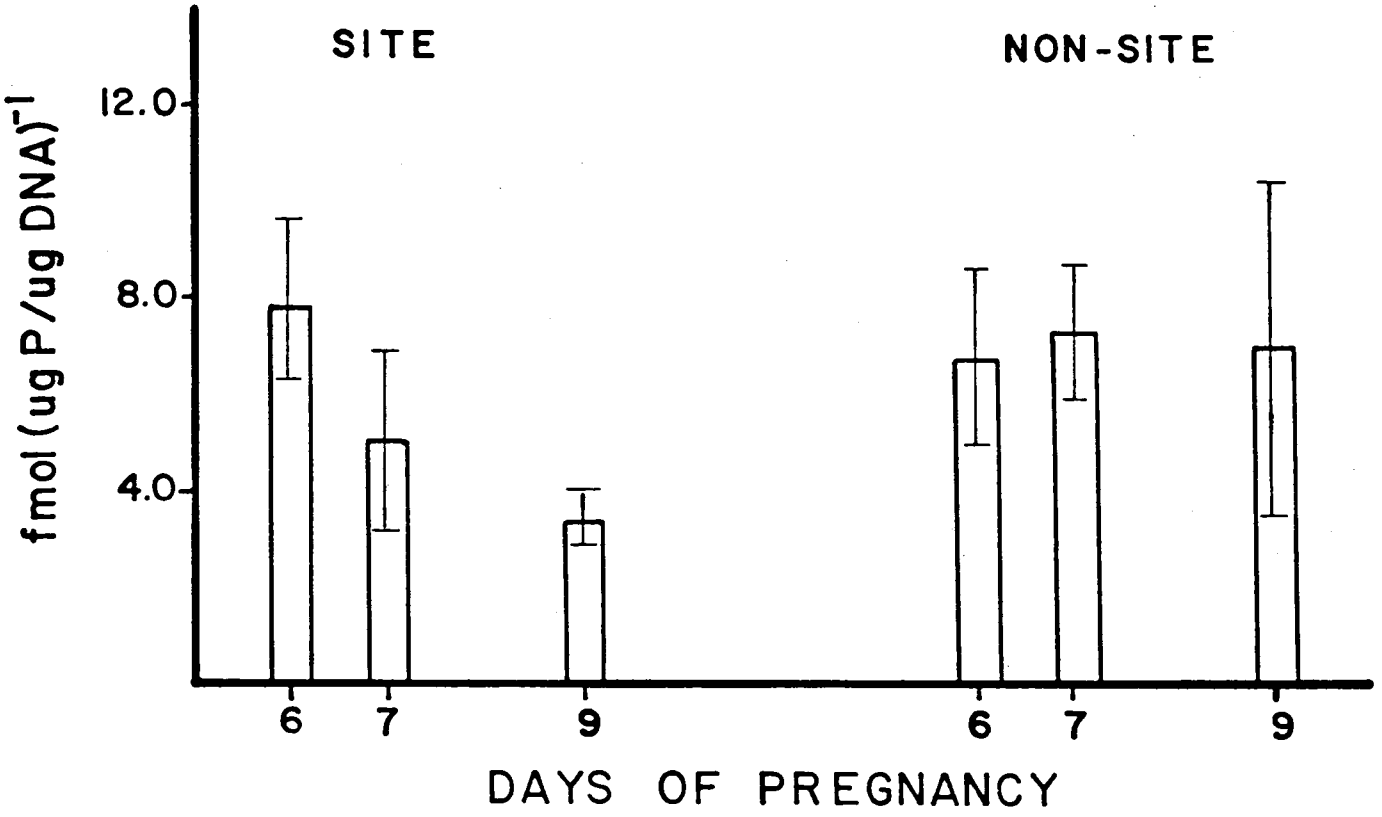

Figure 11. Comparison of Estrogen Receptor Titer of Implantation Site and Nonimplantation Site Regions of the Endometrium.

Implantation site (site) and nonimplantation site (non-site) endometrium were assayed separately for estrogen receptors. Receptor titer normalized to cytoplasmic nuclear ratio (ug P/ug DNA) is given. Values represent for from three to four separate determinations and S.E.M. is represented by the vertical bars.

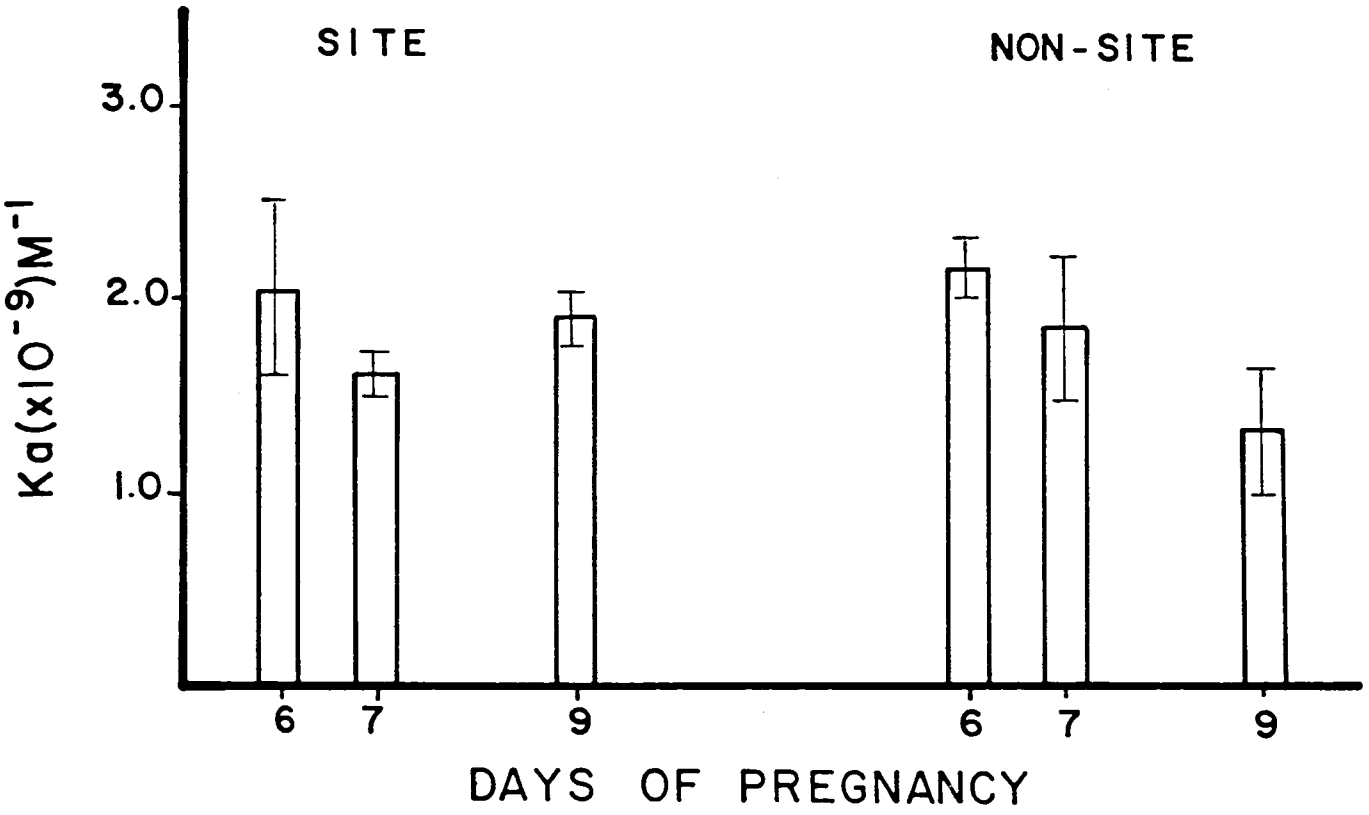

Figure 12. Comparison of Estrogen Receptor Affinity (Ka) of Implantation Site and Nonimplantation Site Regions of the Endometrium.

Estrogen receptor affinity was determined in two regions (see Figure 11). No differences between these regions were seen. Values represent for from three to four separate determinations and S.E.M. is represented by the vertical bars.

have an influence on receptor titer. No consistent correlation was found between the numbers of corpora lutea present and estrogen receptor affinity or titer.

Seasonal Changes

In addition to the changes already described, a phenomenon was noted which was not expected in an animal which can breed and produce viable offspring throughout the year. This was a seasonal change in receptor K_a (Figure 13). Receptor K_a was lowest in February, increased through early summer and then remained at constant high levels through late summer and fall. The values in Figure 13 represent data for from 2 to 15 determinations each. Both endometrial and myometrial values were included. Data were collected over a period of 16 months. Breeding is most successful in these animals between February and June (Reed, 1979).

Table 5 compares the affinities and titers of estrogen receptors of one group of fall and one group of spring animals. There were three animals per group. Although the K_a of the receptor was depressed during the spring in both tissues, the titers of receptors were not significantly different from winter values. Annual changes in affinity were not accompanied by changes in receptor titer.

Hormonal Modification of Receptor Properties

Having established that differences existed in receptor properties as tissues are influenced by pregnancy and season, factors influential in producing these modifications were sought. Estrogen increases

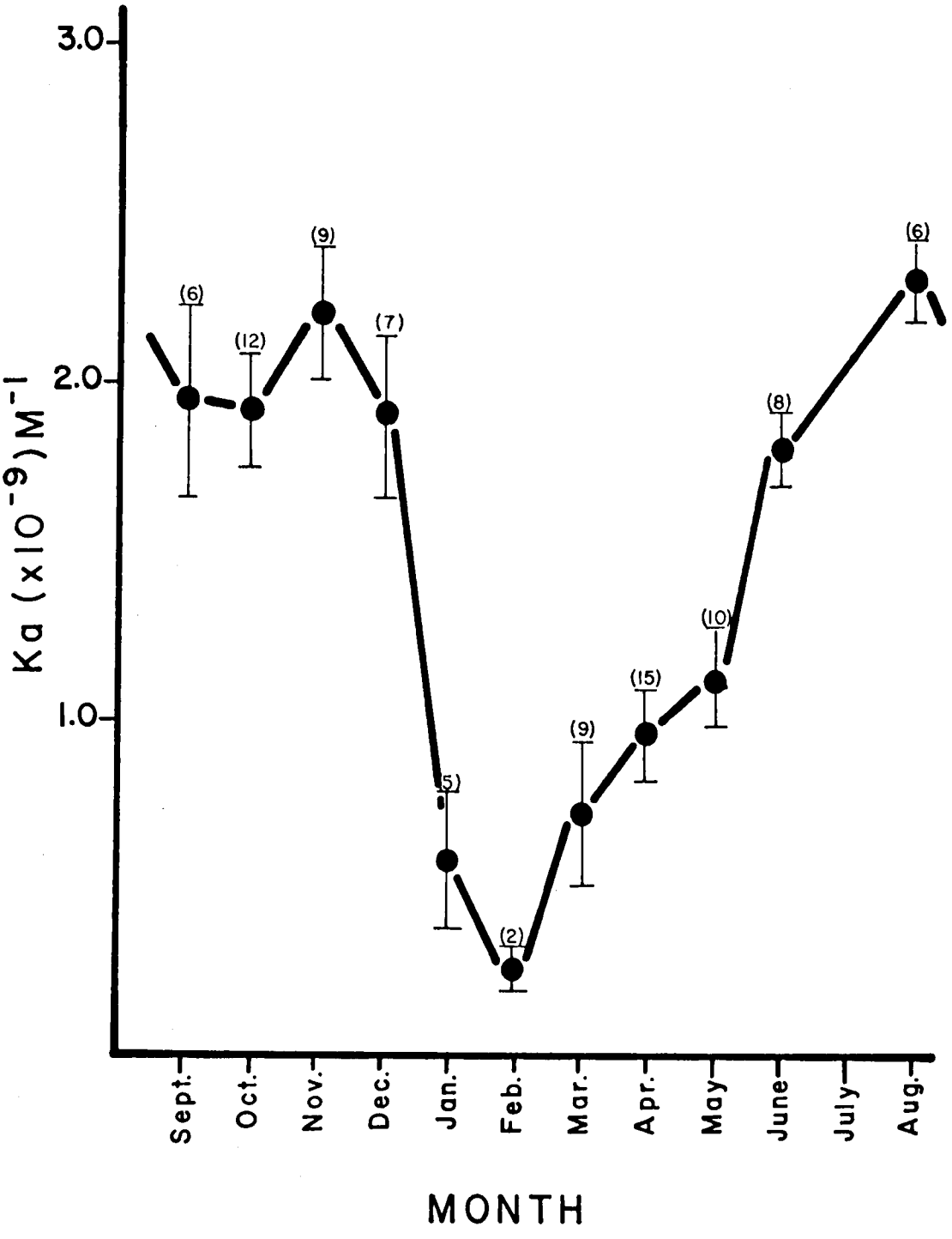

Figure 13. Seasonal Changes in Estrogen Receptor Affinity (Kα).

Receptor affinity from all samples assayed throughout a 16 month period were graphed by month. Endometrial and myometrial samples were included. Values represent for from two to fifteen individual assays. Numbers of assays are in parentheses above each point. Vertical bars are S.E.M.

Table 5. Comparison of Spring and Fall Titer and $K\alpha$ from One Day Pregnant Rabbits.*

Tissue	Season	$K\alpha(x10^9)$	P	fmol(ugP/ugDNA)$^{-1}$	P
Endometrium	Spring	2.23 ± 0.33	<0.05	8.27 ± 3.20	N.S.
	Fall	0.77 ± 0.51		10.90 ± 1.40	
Myometrium	Spring	2.43 ± 0.42	<0.010	5.34 ± 1.08	N.S.
	Fall	0.40 ± 0.17		5.67 ± 1.28	

*The titer and $K\alpha$ from one day pregnant rabbits killed in November (Fall) and in March and April (Spring) were compared. The probability of the two populations being the same, as calculated by Student's t test are given (P). $K\alpha$ differed with season in the two tissues and titer did not. Values are for three determinations each ± S.E.M.

estrogen receptor titer in rats and progesterone decreases estrogen receptor titer (Hsueh et al., 1976). Further these hormones have been shown to influence progesterone receptors in the rabbit (Rao et al., 1973; Rao and Katz, 1977). These steroids show the most marked changes of any hormones during pregnancy and they may influence seasonal behavior in those species with marked seasonal patterns. They were thus a logical choice to test in the rabbit when seeking an agent that influences the estrogen receptor.

Animals were ovariectomized on day zero and the hormone was injected subcutaneously according to the schedule shown in Table 1, page 15. Long term castrates were not used for the following reasons: (1) hormone levels drop rapidly after ovariectomy and thus low levels, though not baseline levels of the hormone could be obtained; (2) long term castration results in a continued degradation of endometrium until virtually one is left after ten to twelve weeks; (3) separation of the two tissue types becomes extremely difficult after 6 days of castration because the endometrium has deteriorated by this time (Telfer and Hisaw, 1957); and (4) long term castration in some animals results in the uterus becoming refractory to progesterone (Lee and Dukelow, 1972) and in order to restore progesterone responsiveness the uterus must be primed with estradiol. Long term castration would thus require estradiol priming in both control and treated groups, making it expedient to use short term castration. The two day interval after ovariectomy was included to insure recovery of the animals from anesthesia and the effects of surgery, and to permit the plasma levels of ovarian steroid to drop.

The effect of steroid treatment on wet weight of the uterus and the percentage of total weight which was endometrium and myometrium are shown in Table 6. Estrus values and 5 day values are included for comparison. The wet weight of the uterus six days after ovariectomy was reduced relative to estrus values ($P < 0.010$). The percentage of total weight which was endometrium was not reduced. Progesterone had a pronounced influence on the percentage of endometrium increasing it 16 times over ovariectomized values.

Table 6. Changes in Uterine Wet Weight and Percent Endometrium after In Vivo Hormone Treatment.*

Treatment or Condition	Uterine Weight (gms)	Percent Endometrium	Percent Myometrium
Oil	2.72 ± 0.27	2.46 ± 0.66%	97.54 ± 0.66%
Estradiol	6.56 ± 0.90†	13.80 ± 6.05%	86.20 ± 6.05%
Progesterone	10.46 ± 1.09†	40.43 ± 2.97%†	59.57 ± 2.97%†
Estrus	4.03 ± 0.32†	2.63 ± 0.48%	97.37 ± 0.48%
5Day	6.10 ± 0.61†	19.78 ± 3.99%†	80.22 ± 3.99%†

*Hormone treatment was administered as described in Section II, Materials and Methods. Uterine horns were removed free of fat, weighed, and used to calculate total uterine weight. Endometrium and myometrium were weighed after separation of the two tissues and percentage weight was calculated on the basis of tissue recovered. Estrus and five day pregnant values are shown for comparison. Significant relative to oil treated animals, $P < 0.05$.

†Significant relative to oil treated animals, $P < 0.05$.

Figure 14 depicts the influence of estrogen and progesterone treatment on estrogen receptor Ka for estradiol. Endometrial Ka was reduced by 48% ($P < 0.05$) in the estrogen treated group as compared

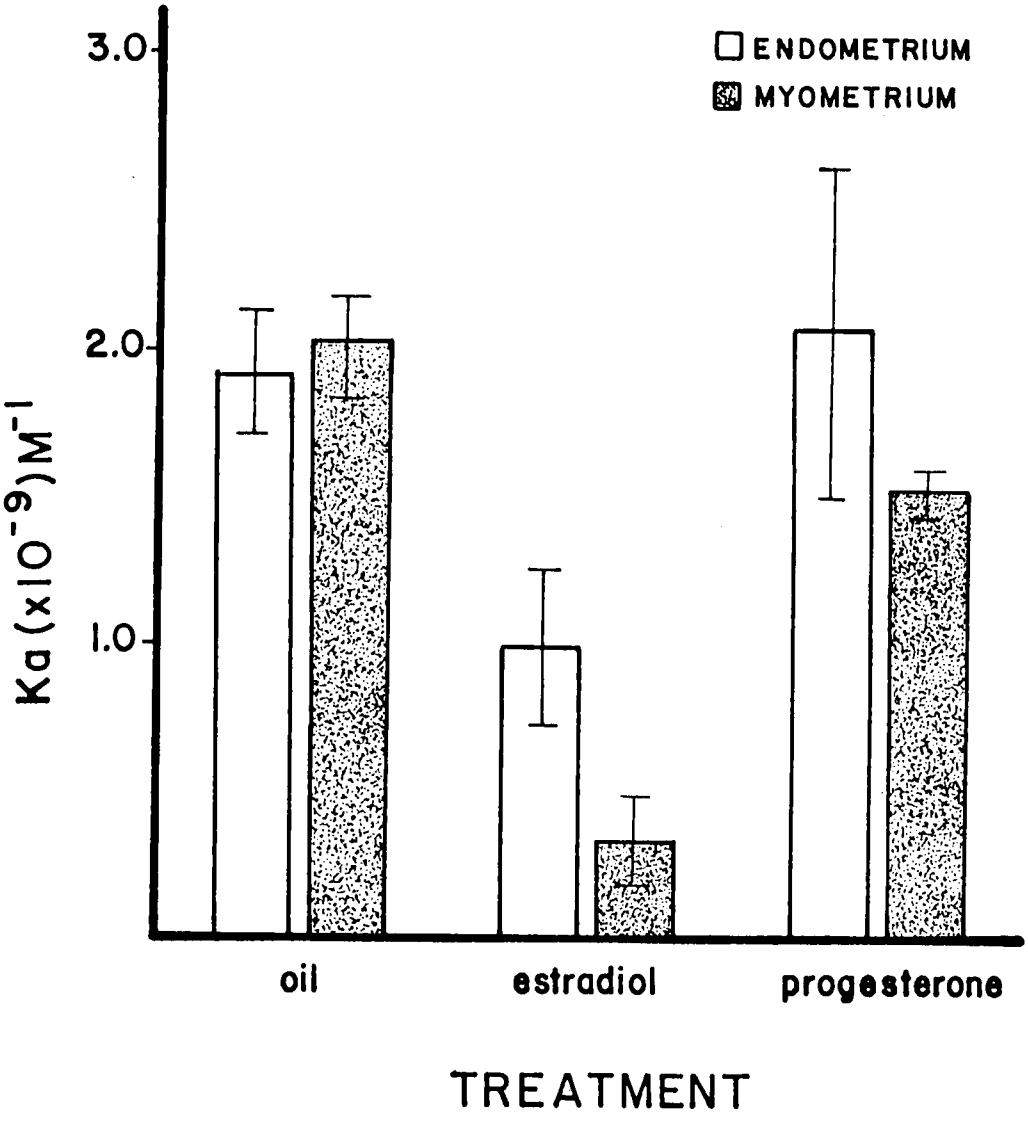

Figure 14. Influence of Estrogen and Progesterone In Vivo on Receptor Affinity (Kα).

Estrogen and progesterone were administered to ovariectomized animals at concentrations of 100ug/Kg/day and 3mg/Kg/day respectively. Treatment lasted for four consecutive days as described in Section II, Materials and Methods (see Table 1, page 15). Endometrium (open bars) and myometrium (shaded bars) were assayed separately. There were three samples per treatment group. Estrogen significantly depressed both endometrial and myometrial receptor affinity for estradiol.

to oil-injected controls, but did not change significantly under progesterone stimulation. There was a decrease of 84% in myometrial estrogen receptor K_a after estradiol treatment ($P < 0.005$). Progesterone stimulated a 25% reduction in K_a for estradiol in myometrium ($P < 0.025$). Under estrogen stimulation endometrial and myometrial K_a also differed ($P < 0.05$). After estrogen treatment K_a for estradiol was equal to or lower than K_a of spring rabbits. K_a in ovariectomized animals and the endometrium of progesterone treated animals reached or exceeded fall values. These data were collected during the last week in May and early in June.

The titers of estrogen receptors normalized to the cytoplasmic nuclear ratio are given in Figure 15. The highest values were obtained for the ovariectomized animals and these values were greater than the highest values found during pregnancy, being 143% greater than values from four days of pregnancy in endometrium ($P < 0.005$) and 78% greater than values for one day of pregnancy in myometrium ($P < 0.05$). Both steroids depressed the titer of receptors though estrogen was the more effective of the two. Myometrial receptor titer was less than endometrial receptor titer under all conditions.

Table 7 gives total uterine receptors and titers of receptors normalized to DNA or to protein. Relative to protein, these values showed a trend comparable to that seen using cytoplasmic-nuclear ratio. When normalized to DNA no differences were observed. Total receptor increased substantially in the endometrium under both treatments.

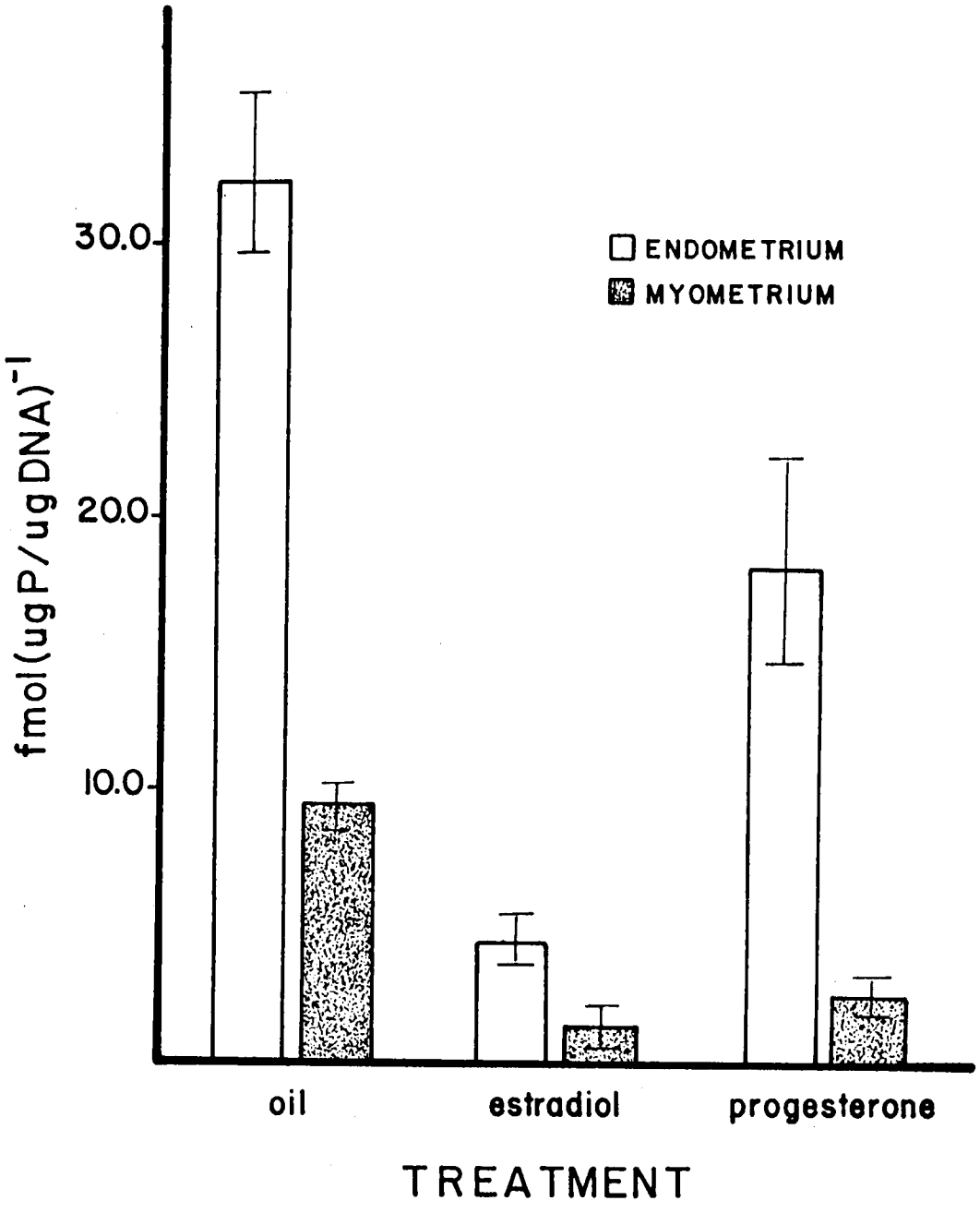

Figure 15. Influence of Estrogen and Progesterone In Vivo on Receptor Titer.

Hormones were administered as described in Figure 14. There were three samples per treatment group. Both hormones effectively depressed receptor titer.

Table 7. Estrogen Receptor Concentration after *In Vivo* Hormone Treatment; Total Receptor and Receptor Normalized to Protein and DNA.*

Treatment	fmol/ugP		fmol/ugDNA		pmol Total Receptor	
	Endometrium	Myometrium	Endometrium	Myometrium	Endometrium	Myometrium
Oil	0.624 ± 0.091	0.390 ± 0.027	1.21 ± 0.53	2.53 ± 0.13	2.4 ± 0.8	25.8 ± 2.1
Estradiol	0.125 ± 0.035	0.130 ± 0.084†	0.77 ± 0.34	2.63 ± 1.73	6.2 ± 0.2††	37.7 ± 5.4
Progesterone	0.450 ± 0.095	0.222 ± 0.056†	2.78 ± 0.74	5.31 ± 1.64	34.8 ± 10.6†	25.8 ± 8.1

*Estrogen receptor concentration from oil treated, estrogen treated and progesterone treated ovariectomized animals (Figure 15) were normalized per ug Protein and per ug DNA. Approximate values for total endometrial and myometrial receptor were calculated. Values are for three determinations plus or minus S.E.M.

†Significant from oil treated animals, $P < 0.05$.

††$P < 0.01$.

Having established an influence of estrogen and progesterone on receptor K_a, an attempt was made to elaborate upon the mechanism by which this influence could be exerted. Changes in K_a might be controlled by one of the following methods:

1. There could be a direct effect by these steroids on the receptor (estradiol could change receptor K_a as it binds to the high affinity binding sites and/or progesterone could change the K_a by binding to additional inhibitory sites on the receptor);

2. There might be synthesis of a different receptor molecule under the influence of these hormones, one property of which could be reduced affinity for estradiol; and

3. These hormones may induce the synthesis of some cytoplasmic factor which combines with or competes for the receptor to depress its K_a.

The first of these possibilities is overruled for estradiol by the following argument. If estradiol depressed the receptor K_a directly, then a decrease in K_a would have been reflected in curved Scatchard plots and in Hill coefficients of less than one. Scatchard plots were always straight lines for the estradiol treated animals and Hill coefficients were approximately one in all cases.

To determine whether progesterone had a direct effect, this hormone was added in vitro to a cytosol obtained from one day pregnant animals. Testosterone was added as an additional control. Figure 16 shows that these steroids did not depress receptor affinity. Thus the first hypothesis, direct effect by either hormone, seemed unlikely.

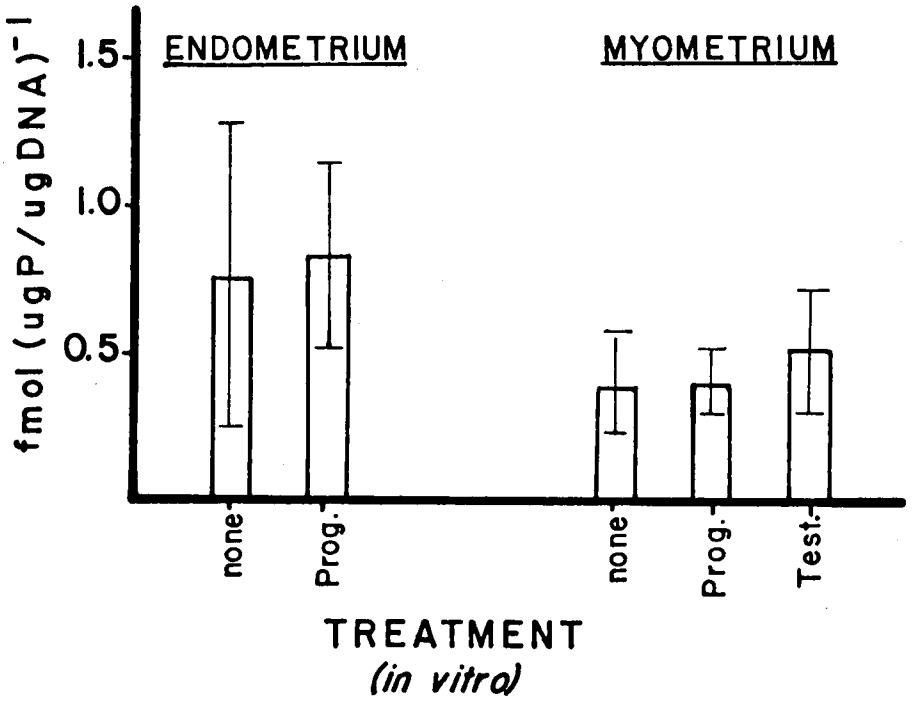

Figure 16. Lack of Effect of Progesterone and Testosterone In Vitro on Receptor Affinity (K_a).

Progesterone and testosterone (5×10^{-12} moles/100ul cytosol) were added to endometrial and myometrial cytosol and these when then assayed for receptor affinity. Values represent data for three separate determinations of affinity in each group. Vertical bars are S.E.M. No influence by these hormones was observed.

The second possibility, that of synthesis of a different receptor, would lead us to assume that the ovariectomized animals, the estrogen treated animals and the progesterone treated animals all had different receptors. If this was true, then pooling the cytosol from any one of these animals with any of the others would result in heterogeneous binding sites. Myometrial cytosol from ovariectomized oil treated, estrogen treated, and progesterone treated animals was pooled and

used for assay. Two ml of cytosol from an oil treated animal were combined with two ml of cytosol from an estrogen treated animal. In a similar manner cytosol from a progesterone treated animal was combined with each of the other two types. A sample Scatchard plot with its corresponding Hill plot obtained in this manner are shown in Figure 17. No break was obvious in the Scatchard plot and the Hill coefficient was 1.02. Table 8 shows the data for all of the combinations. Values are for two samples each and calculated affinities are the means of the original samples (oil, estrogen, and progesterone treated groups). For any of the samples, as for the original samples, Hill coefficients were approximately 1.00. Only one class of receptors appears to exist in each group.

Data from Figure 17 and Table 8 suggest that, not only was the hypothesis relying on synthesis of new receptor not possible, but that the third hypothesis (modification of receptor affinity by a cytoplasmic factor) was possible. Calculated affinity in each of the samples was approximately equal to that of the combinations. This would occur only if an inhibitor was present in one of the cytosols which could be diluted when that cytosol was mixed with one containing no inhibitor. The inhibitor would have to bind reversibly with the receptor.

Figure 17. Sample Scatchard and Hill Plots for Myometrial Cytosol Combined from Different *In Vivo* Treatment Groups.

Two ml of cytosol from an ovariectomized rabbit were combined with two ml of cytosol from an estrogen treated rabbit and an assay for receptor was performed. Data were analyzed by (a) Scatchard, or (b) Hill analysis. No break or dissociation was seen in the Scatchard plot. The Hill coefficient was 1.02. Each point represents two determinations.

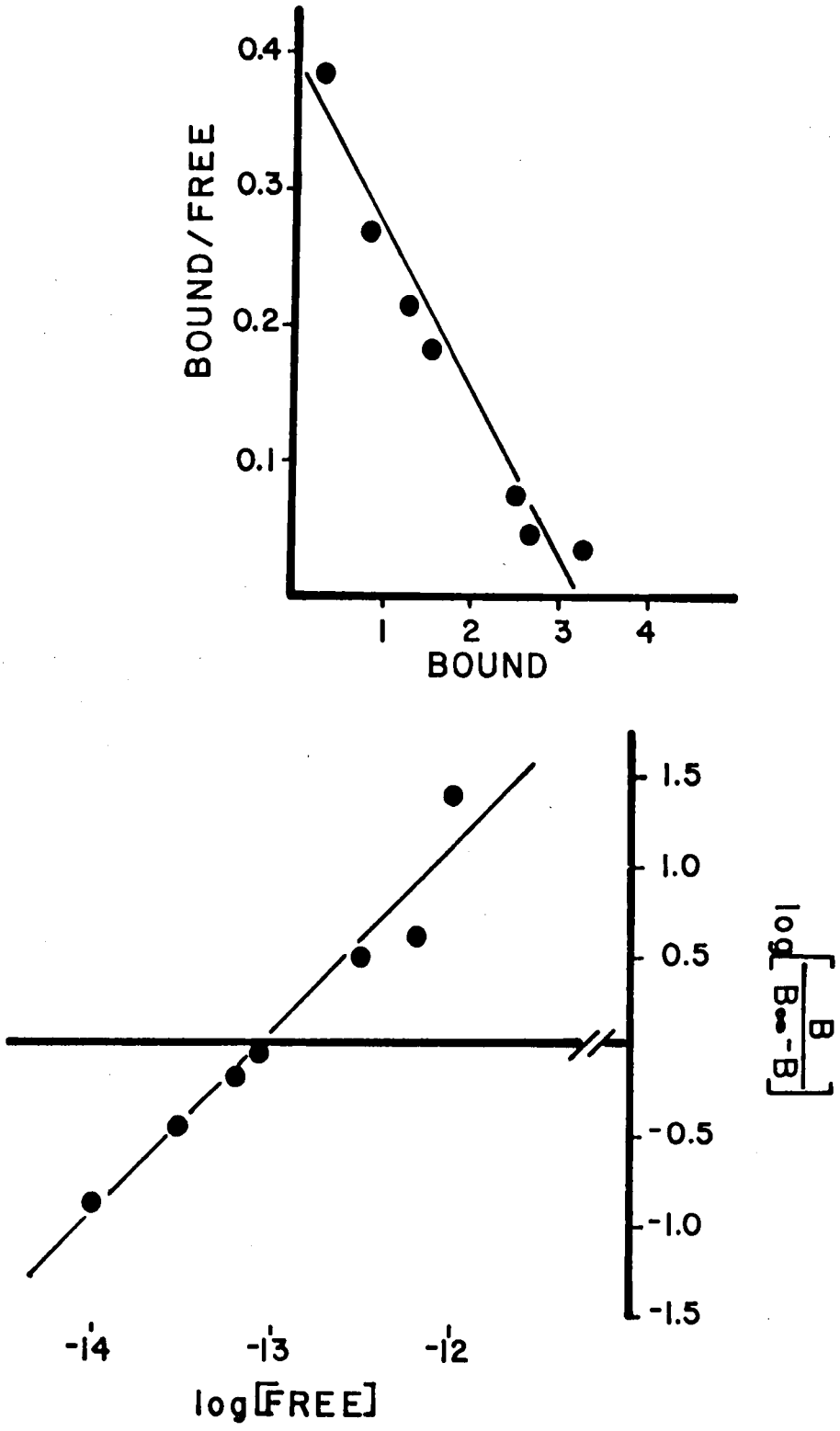
0.4
0.3
0.2
0.1
BOUND/FREE
1
2
3
4
BOUND
1.5
1.0
0.5
-0.5
-1.0
-1.5
log [B∞-B / B]
-1/4
-1/3
-1/2
log[FREE]

Figure 17

Table 8. Estrogen Receptor Affinity ($K\alpha$) and Hill Coefficients of Combined Myometrial Cytosols from In Vivo Treatment Groups.*

Treatment Combinations	$K\alpha(x10^9)M^{-1}$	Calculated $K\alpha(x10^9)M^{-1}$	Hill Coefficient
Oil + estrogen	1.20 ± 0.02	1.10 ± 0.05	1.03 ± 0.01
Oil + progesterone	1.58 ± 0.17	1.77 ± 0.10	1.10 ± 0.05
Estrogen + progesterone	1.08 ± 0.21	0.88 ± 0.12	0.87 ± 0.01

*Two milliliters each of myometrial cytosol from different in vivo treatment groups were combined as shown. Scatchard plots and Hill plots were constructed from assays run using these combined cytosols. $K\alpha$ was approximately equal to the mean of the original values (Calculated $K\alpha$) and Hill Coefficients were approximately one. Values are from two determinations each.

IV. DISCUSSION

The hypothesis upon which this study was based was confirmed; estrogen receptors from endometrium and myometrium, of the rabbit, responded differently to the hormones of early pregnancy. Titer of estrogen receptors and the estrogen binding affinity (K_a) of the receptor were altered differently in the two tissues.

The binding of estrogen to a tissue does not, of itself, signify the presence of estrogen receptor. Several criteria must be met for estrogen receptor identity to be established:

1. Binding must be specific, that is, competition with estradiol or a hormone of equal biological potency must remove this hormone from the binding sites;

2. Specific binding must take place only in target tissues;

3. Binding must be saturable so that when all of the receptor sites are filled no further specific binding takes place;

4. Binding should be of high affinity in the range previously reported for estrogen receptors; and

5. Steroids which are not biologically potent estrogens should not replace estradiol on the binding sites (Gorski et al., 1968; Katzenellenbogen and Gorski, 1975).

Each of these criteria were met for estradiol binding in both endometrium and myometrium of the rabbit. Figure 5, page 29, shows that specific binding, one of a competitive nature with D.E.S., was present in endometrium and myometrium. Liver, heart, lung and spleen did

not have the capacity to bind estradiol in this manner. The specific binding component of endometrium and myometrium was saturable. Uterine flushings, bovine serum albumin and plasma had no such binding abilities, and, in those substances, nonspecific binding accounted for most of the estradiol bound (Figure 6, pages 31-32). Binding took place with high affinity, well in the range of that expected for estrogen receptors (Figure 7, page 34). Progesterone, testosterone and dexamethosone did not compete for binding sites (Figure 8, page 37).

Substances other than receptor bind steroids. Immature rat uteri contain a plasma binding substance called alpha-fetoprotein which binds estradiol with a $K\alpha$ of $4.0 \times 10^{7}M^{-1}$ (Raynaud et al., 1971; Radanyi et al., 1977). Plasma of many animals contains estrogen-testosterone binding globulin (Rosner and Smith, 1975). In the rabbit, blastokinin found in uterine flushings (Daniel, 1976), and endometrium (Johnson, 1972, Kirchner, 1976) binds steroid (Rahman et al., 1975). Blastokinin, however, appears to bind progesterone specifically (Beato, 1976) and the affinity of binding ($K\alpha = 3 \times 10^{6}M^{-1}$) is much lower than that for receptor (Beato, 1974). The possibility that two binding components or receptors were being measured was eliminated by demonstration, through the use of Hill plots, that only a single class of binding sites were present. It was thus concluded that a specific cytoplasmic estradiol receptor was being measured.

Pregnancy in the rabbit is characterized by a precisely timed sequence of events. Ovulation occurs 10-12 hours after mating and

fertilization takes place 1-5 hours later. During the first several days postcoitum, the embryos remain in the oviduct undergoing cleavage. They enter the uterus between days three and four. On day six the blastocysts localize over presumptive implanation sites, and, on about the seventh day postcoitums implantation takes place. By day nine the embryos have enlarged considerably, and maternal tissue begins to grow around them.

Within the first several days of pregnancy a variety of hormonal events have taken place. Progesterone and estradiol levels drop significantly during the first 24 hours of pregnancy after an initial preovulatory peak (Hilliard and Eaton, 1971). Estrogen levels remain constant throughout most of pregnancy while progesterone levels increase rapidly until midpregnancy (Hilliard et al., 1968; Challis et al., 1973).

Within the changing milieu of early pregnancy the endometrium undergoes increases in wet weight, in synthesis (of DNA, RNA, and protein), and in cell number which are far more extensive than changes in the myometrium. The present study demonstrated that endometrial estrogen receptors also undergo more pronounced changes than myometrial estrogen receptors.

Endometrial estrogen receptor K_a remained constant between days one and four of pregnancy, then decreased. Myometrial affinity showed a slight reduction during this time (Figure 9, page 40). Muechler et al. (1974) observed no change in rabbit uterine estrogen receptor K_a during pregnancy. Myometrium comprises a large percentage of uterine

weight and it is likely that pooling of the two uterine tissues in that study served to mask the change in K_a of endometrial receptor. Progesterone receptor in the rabbit has previously been reported to be capable of undergoing changes in affinity under hormonal stimulation (Rao et al., 1973; Davies et al., 1974), thus modification of steroid receptor K_a may be an important and hitherto unexplored means of control of receptor function in this species.

Table 3, page 45, clearly demonstrates the difficulty in using total uterine tissue rather than endometrium and myometrium when studying receptor titer. The changing proportion of endometrium in uterine preparations has not been considered when analyzing the results obtained in previous studies. Total endometrial, but not myometrial, content of estrogen receptors increased through the fourth day of pregnancy (Table 4, page 46). The increase in total endometrial estrogen receptor, accompanied by a greater receptor titer per cellular unit (Figure 10, page 43), may represent an increase in responsiveness of the endometrium at this time. Myometrial responsiveness, as reflected in titer per cellular unit, decreased from day one.

Endometrial estrogen receptor K_a and functional concentration were greater during the preparatory phases of uterine activity, than during implantation itself. During these early days of pregnancy estrogen aids progesterone to increase mitotic index of the endometrium (Lee and Dukelow, 1972), to change the enzyme profile of the endometrium (Murdoch and Whte, 1969), to increase DNA synthesis in the endometrium (Psychoyos, 1973; Heald, 1976) and to change endometrial wet weight

(Telfer and Hisaw, 1957). The increased availability of receptor throughout this period could facilitate these actions of estrogen.

Dickman et al. (1975 and 1977), and Dickman and Dey (1975) suggested that rat blastocysts produce endogenous estrogen which in turn aids in implantation. Bhatt and Bullock (1974) demonstrated estrogen binding ability in blastocysts of the rabbit. That the implantation sites of nine day pregnant rabbits contained less estrogen receptor than those of six day pregnant rabbits (Figure 11, page 48) may have resulted from the presence of blastocyst estrogen influencing these regions or from the fact that proliferation of the cells of the implantation site occurred more rapidly than receptor synthesis. The presence of estrogen could have influenced the receptors in the implantation site, moving them to the nucleus or masking them from assay.

Domestic rabbits of the species Orytolagus cuniculus are commonly assumed to display no seasonal breeding behavior. Seasonal changes in brood size, percent pregnancies, and properties of the reproductive tract have, however, been reported in wild rabbits. In a study on the North American cottontail, Sylvilagus floridanus, ovarian weight and pituitary weight were greatest between the months of March and August, suggesting heightened reproductive activity during those months (Elder and Finerty, 1943). Marsden and Conaway in 1963 reported the onset of reproductive behavior in mid-February in cottontails from Missouri. Reproductive activity continued through August. Environmental conditions such as availability of food and changes in temperature appear to be the major factors influencing reproductive

success. Severe weather conditions served to reduce reproductive activity even after the onset of breeding behavior (Marsden and Conaway, 1963). Conaway et al. (1974) reported differences in the start of the breeding season in cottontails from different parts of the country. In 1965 in Louisiana the first litter was conceived on January 20. In Tennessee, the date on which the first litter was conceived was February 26, and in South Dakota, April 1. The dates of first conception varied somewhat with year. Litter size was also influenced by season.

Orytolagus cuniculus displays seasonal breeding patterns in the wild. In both New Zealand and British populations of rabbits, a far greater percentage of pregnancies occurred in one study between January and August than between September and December (Sadleir, 1969). Caged rabbits from southern California showed a higher percentage of conceptions when temperature was between 60 and 70°C than when temperature was between 70 and 90°C. There was also a larger litter size and percentage of live births during this time (Sadleir, 1969). The poorest period for breeding in these animals has been reported to be July to September when temperature is high (Asdell, 1964).

An article from the March/April 1979 issue of "Domestic Rabbits" (Reed, 1979) states:

> "Breeding Problems." This is a very common complaint in the late fall and early winter months. Rabbit raisers must remember that basically this is the sexually inactive time of the year for their rabbits. . . . Breeding problems can basically be characterized into two broad categories: (1) Those where the feamle animal is nonreceptive and copulation never takes place and therefore no offspring

result; or (2) Those where a female is receptive, copulation takes place, but no offspring result due to either failure for the sperm and egg to unite or early embryonic death.

Receptor affinity, in this study, was lowest in February, increased through June and remained at high levels throughout the late summer and fall. Tennessee has four distinct seasons with the summer months of July and August being particularly hot (high 80's and 90's), the winter mild, and spring beginning between late February and mid-March. The period of low receptor affinity in the animals used in this study correlates with the productive spring season.

Although breeding of animals during winter months may have been more difficult than in spring months, it must be remembered that all of the rabbits used in this study were reproductively active since all of the uteri were gravid at the time of assay. Differences in estrogen receptors might prove to be even more pronounced were random populations of nonpregnant rabbits to be sampled during the two seasons. A random sampling would include rabbits which refused copulation as well as those which were receptive to the male.

Photoperiod influences attainment of puberty, length of estrous cycles, duration of pregnancy, and onset of pregnancy in many mammalian species. These functions reflect hormone patterns and involve the function of the pineal gland (Hoffmann, 1973). In seasonal breeders, LH and FSH titers increase during the reproductively active months (Davis and Meyer, 1973; Freedman et al., 1979). In the present study, estrogen and progesterone decreased receptor Ka and titer. Seasonal changes occurred in Ka alone. One may speculate, therefore, that the

change in K_a caused by season was the result of changes in pituitary output, rather than ovarian output. It is possible that plasma titers of LH and FSH are higher in the spring than in the fall in domestic rabbits from Tennessee, and that these pituitary hormones influence receptor K_a in the uterus.

In the rat uterus estradiol increases the total numbers of estrogen receptors (Hsueh et al., 1976; Pavlik and Coulson, 1976b; Coulson and Pavlik, 1977; Martel and Psychoyos, 1978). The increase probably results from synthesis of new receptor molecules after estrogen stimulation. Despite estrogen induced movement of receptor to the nucleus, the cytosolic content of estrogen receptor increases under estrogen stimulation. In the rabbit, as shown by this study, estrogen treatment of ovariectomized animals increased the total cytosolic content of estrogen receptor in endometrium but not in myometrium (Table 7, page 58).

Despite an increase in total endometrial estrogen receptor and no change in that of myometrium under estrogen stimulation, the functional level of receptor dropped in both tissues after estrogen treatment (Figure 15, page 57). The uterus increased in weight after estrogen treatment (Table 6, page 54) and synthesis of estrogen receptor did not keep pace with synthesis of protein and DNA in the two tissues (Figure 15, page 57). Estrogen treatment decreased the available receptor within each cellular unit.

Estrogen receptors of the endometrium and myometrium responded differently to progesterone treatment. There was a substantial increase

in the amount of endometrium present after stimulation with this hormone (Table 6, page 54). There was a fifteen fold increase over oil treated controls in the total estrogen receptor in endometrium. Nevertheless, the receptor titer per cellular unit was reduced (Figure 15, page 57). Total myometrial receptor did not change although the functional levels of receptor (receptor per cellular unit) were decreased.

From the information available it is possible to speculate as to the mechanisms by which estrogen and progesterone alter receptor titer. The mechanisms may be different for endometrium and myometrium. Comparison of endometrial receptor titer of estrous animals and ovariectomized oil treated animals reveals receptor titer of oil treated animals to be 26 times the titer at estrus. Wet weight decreased only slightly and percent endometrium was the same. Restoring estrogen to ovariectomized animals reduced titer significantly (Figure 15, page 57). In progesterone treated animals, as in oil treated controls, titer was significantly greater than at any time during pregnancy. Estrogen is present in plasma during pregnancy and at estrus (Hilliard and Eaton, 1971). Thus, in the endometrium, denial of estrogen may stimulate an increase in receptors per cell. The reduction in titer seen in progesterone treated animals over oil treated animals may be an indirect effect of the large increases in protein, DNA, and wet weight in progesterone stimulated endometrium. The myometrium responded quite differently. There was no significant difference in total myometrial receptor during pregnancy nor under any

of the treatments (Table 4, page 46, and Table 7, page 58). Receptor titer of estrous animals and receptor titer of ovariectomized oil treated animals, as analyzed per cellular unit, were not significantly different. The reduction in functional receptor during pregnancy and under estrogen and progesterone treatment may be indirect; an apparent change as the result of changes in cell number or cell size in the myometrium.

Estrogen and progesterone, administered in vivo, elicited changes in estrogen receptor K_a (Figure 14, page 55). Synthesis of a unique receptor did not appear to be responsible for the change; rather, affinity may have been modified by a component present in the cytoplasm.

The affinity of the specific-binding site on the receptor for its hormone is a property of its shape as controlled by the tertiary configuration of the receptor molecule. From knowledge of enzymes there are two possible methods for modification of affinity by a component in the cytoplasm. One is a rarely observed mechanism caused by the presence of an uncompetitive inhibitor which alters the affinity and the number of total binding sites observed. Inhibitors of this type are often seen in multienzyme systems and are applicable to this system only in that the receptor itself is transformed after hormone binding. This type of inhibition is not common in single substrate reactions. Though this type of inhibition cannot be ruled out as acting in this receptor system, it is not probable since only a single substrate is present.

The second and more likely mechanism by which receptor affinity could be modified is through the presence of a competitive inhibitor. Through the experiments described in the previous section, estrogen itself and progesterone were eliminated as candidates for inhibitors producing this change. Thus, substances with the potential to modify receptor affinity through competitive inhibition are probable and should be sought.

Enzyme kinetics and receptor kinetics are similar. Competitive inhibition increases the apparent Km of an emzyme and thus would decrease the K_a (= $1/K_D$ or $1/Km$) of the enzyme (or receptor in the present discussion). The lower the apparent K_a, the more inhibitor would be present. Several characteristics can be assigned to a potential inhibitor from the studies already performed:

1. The inhibitor would have to compete for the estradiol binding site on the receptor. A steroid or other lipid is suggested;

2. Synthesis of the inhibitor would have to be induced by estradiol because estradiol treatment lowered the K_a of the receptor;

3. Under estrogen treatment the inhibitor would be expected to accumulate in myometrium in greater quantities than in endometrium because myometrial affinity was lower than endometrial affinity after estrogen treatment; and

4. Progesterone should stimulate the sequestration of the inhibitor in the myometrium, but not in the endometrium.

Modification of receptor affinity as a means for receptor control has not been studied. Possible roles of such a phenomenon can only be

speculated upon. Until recently, it was assumed that the steroid hormone itself was responsible for manifestations of control. The establishment of the presence of A and B subunits of cytoplasmic progesterone receptor (Schrader and O'Malley, 1972), establishment of receptor binding to nuclear acceptor sites (Schrader et al., 1972; Puca et al., 1974) and growing evidence for the obligatory role of receptor in steroid action increase the likelihood of an important role for this protein molecule. The hormone apparently acts as a trigger to subsequent events. A change in the shape of the receptor or its ability to bind hormone could have widespread effects on events following hormone binding. The interaction during transformation, which may involve binding of an additional cytoplasmic component (Notides et al., 1975; Notides, 1978), could be modified. Kinetics of movement to the nucleus could be changed since this may involve ATP-dependent enzymatic steps (Lohmar and Toft, 1975). Interactions with acceptor sites could be modified as the result of modification of receptor affinity and this could result in distinct patterns of transcription when modified cytoplasmic receptor was present. Changes in affinity of cytosolic estrogen receptor for its hormone, shown in this study, and changes in affinity of cytosolic estrogen receptor for its acceptor site, demonstrated previously in the rabbit (Chatkoff and Julian, 1973), might be an important means for control of steroid response in this species.

Endometrium and myometrium responded differently to the levels of estrogen and progesterone present during pregnancy and after <u>in vivo</u>

treatment with these hormones. Receptor titer and Ka were modified in both tissues, though more pronounced changes were observed in the endometrium. The changes in titer and Ka of the estrogen receptor may help control the response of these two tissues to estrogen, and reflect the fact that the response of the endometrium to estrogen is more extensive than the response of the myometrium to estrogen.

Seasonal changes in the Ka but not the titer of estrogen receptors were observed. These correlate with the period of greatest reproductive success in this species. Seasonal changes in receptor may result from seasonal changes in the levels of reproductive hormone.

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VITA

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