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I am submitting herewith a dissertation written by Edward John Pavlik entitled "Modulation of Estrogen 'Receptors' in the Female Sprague Dawley Rat." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Zoology.

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2

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MODULATION OF ESTROGEN "RECEPTORS" IN THE FEMALE SPRAGUE DAWLEY RAT

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee

Edward John Pavlik

December 1975

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ACKNOWLEDGMENT

This dissertation is joyfully dedicated to my mother and father, Mary and John, whose support, encouragement, patience and understanding have made its completion possible, and who have raised me with love and sacrifice to stand erect in a sometimes silly and insane world against things which "go bump in the nite."

ABSTRACT

A "batch" procedure which adsorbs the estrogen receptor to hydroxylapatite (HTP) is described and characterized with respect to washing efficiency, binding specificity, competition, adsorption time, sensitivity and stability against KCl ionic strength. This technique is extremely sensitive (capable of detecting estrogen receptor in cytosols containing as little protein as 20 μg/ml) and is run entirely at 0-2 C under conditions of buffer and pH which confer maximum stability to the binding protein. This technique was employed in a series of experiments designed to measure the effect of estrogen and progesterone on the estrogen receptor concentration in Sprague Dawley rats.

An approach which utilized the advantages of intact tissue conditions ("Estrogen Organ-Binding Assay") demonstrated that estrogen pretreatment for 48 hours increased estrogen binding per uterus. Specific assay of the estrogen receptor on HTP showed that low physiological doses of estrogen increased uterine and vaginal estrogen receptor concentration significantly within 48 hours. The administration of progesterone was able to completely block any increase in estrogen receptor concentration. Similar assays performed on pituitary and hypothalamic cytosols did not demonstrate any modulation of estrogen receptor concentration by estrogen or progesterone. Scatchard and Hill plot data indicated that progesterone neither competes for the estrogen binding site nor acts as a negative heterotropic effector at some allosteric regulatory site. The low steroid doses employed gave a biological response which was demonstrable by vaginal lavage cytology.

Thus, estrogen and progesterone exert different effects on receptor concentration in different tissue.

Examination of uterine modulation in time revealed three components: (1) an early return to baseline following initial depletion of receptor, (2) an increase above baseline estrogen receptor concentration after 20 hours, (3) a rapid decrease in estrogen receptor concentration mediated by progesterone. Experiments designed to examine receptor turnover established that neither receptor activation nor increased receptor degradation could be demonstrated as a result of estrogen or progesterone treatment.

Pulse-labeling studies performed with [^{35}S]-methionine revealed that both estrogen and progesterone stimulated a maximal utilization of uterine ribosomes. Furthermore, both estrogen and progesterone stimulated the amount of incorporation of [^{35}S]-methionine into cytosol and nuclear proteins and hence mediated increases in the rates of uterine protein synthesis. Thus, while both steroids increased the rates of protein synthesis, progesterone selectively decreased estrogen receptor concentration. As a consequence, estrogen and progesterone appear to exert differential effects on transcriptional and translational control. It is suggested that modulation of estrogen receptors is in part responsible for this differential. The hypothesis is presented that when estrogen increases estrogen receptor concentration, it may amplify molecular signals which contribute to the cascading growth observed in the uterus; conversely, progesterone may selectively limit the concentration of estrogen receptors and may thereby provide a specific molecular "brake" to estrogen responsiveness in target tissues.

PREFACE

THE CALF PATH

One day through the primeval wood
A calf walked home as good calves should;
But made a trail all bent askew,
A crooked trail as calves all do.
Since then three hundred years have fled
And I infer the calf is dead.
But still he left behind his trail,
And thereby hangs my moral tale.
The trail was taken up next day
By a lone dog that passed that way;
And then a wise bellwether sheep
Pursued the trail o'er vale and steep
And drew the flock behind him, too;
As good bellwethers always do.
And from that day, o'er hill and glade
Through those old woods a path was made.
And many a man wound in and out,
And dodged and turned and bent about,
And uttered words of righteous wrath
Because 'twas such a crooked path;
But still they follow--do not laugh--
The first migrations of that calf.
This forest lane became a road,
There many a horse with his load
Toiled on beneath the burning sun
And travelled some three miles in one.
And thus a century and a half
They trod the footsteps of that calf.
The years passed on in swiftness fleet;
The road became a village street;
And this, before men were aware,
A city's crowded thoroughfare,
And men two centuries and a half
Trod in the footsteps of that calf.
A hundred thousand men were led
By one calf near three centuries dead.
For men are prone to go it blind
Along the calf paths of the mind
And work away from sun to sun
To do what other men have done.
They follow in the beaten track
And out and in, and forth and back
And still their devious course pursue,
To keep the path a sacred groove
Along which all their lives they move,
But how the wise old wood gods laugh
Who saw that first primeval calf.

"The Calf Path"; Author Unknown

I have been profoundly influenced by the mountains of Colorado and Tennessee, and "mountains" have generally come to reflect much of life to me. This dissertation is an excursion up steep previously untreaded biochemical hillsides and along the rocky ridges of molecular biology. When in the mountains, it is necessary to discriminate between "hiking" or "hill walking," and "climbing." To pass for a mountain climber, one must load himself down with 200 feet of climbing rope, together with enough slings and avalanche cord, slung slantwise from shoulder to waist, to look authentically respectable. It is necessary to decorate the rest of your costume with sufficient assorted pitons, carabiners, bongs, hexentrics, hammers, picks and crampons to appear thoroughly magnetic. Attired thusly, you are entitled to a "subtly" condescending attitude toward any hikers you might encounter even on the levelest trail or in mountain valleys. "Hikers" on the other hand remain "hikers" even when their "walk" changes into a crawl up a 70° slope which is attached to the mountain by loose gravel and rocks interspersed with lonesome wisps of wild oats and orchard grass and the occasional shrub oak or rhododendron.

Biochemically this is a hiker's dissertation because I have taken fundamental approaches which have not required sophisticated apparati (those which operate dependably only when buffers are accurate to 0.00001 pH units and yield data only through digital computer interfaces). When confronted by a rock face without prominent foot or handholds, I have not assembled an assault team to lay seige to the mountain with drills, slings and rope ladders, but have instead backed off and approached the summit by a more accessible, albeit less

glamorous route. Hence, I have wandered in the lower elevations, sometimes lost in a fog of inapproachability. Nevertheless, I have become somewhat of a backwoods expert on false trails and biochemical pitfalls. With the work for this dissertation completed I am able to look back on the practical knowledge I have gained on how to attempt a safe biochemical ascent of the more accessible physiological hills and perhaps every now and then generate a glimpse of what a mountain must truly be.

This dissertation is itself not free of error since no one has ever written a treatise without mistakes. It is my hope that the mistakes are not numerous nor the writing obscure, and that the faults which are evident be more of omission than commission.

The purpose of the preface is to allow the author to thank those who have been helpful and instrumental in the completion of the finished work. My heaviest debts are owed to Professor Ronald Reed Cowden who taught me to discern between good science and good men, who set examples for me (not all of which have I faithfully followed), who ultimately provided the encouragement and opportunities to "get it back together again," and to Patricia Coulson, who after taking me in, worked hard to provide me with financial support, encouragement, warmth, an intellectual atmosphere and of course the commodities of equipment and supplies without which nothing can be accomplished. She shall ever remain "Pat," loved by all of us who were privileged to work with her. I thank Joseph C. Daniel, Jr., for support both financial and structural during these studies, and for his attempts to catalyze somewhat discrepant academic components into a genuine intellectual chemistry. I am

indebted to Kwang Jeon and Finnie Murray for providing me with examples of integrity and perseverance and for orienting my direction at the University of Tennessee. Both have been instrumental in providing me with various insights into both men and molecules. I thank Ronald Fraser for somehow always finding room in the budget for the seemingly endless lists of supplies that I required and for the informal chats we often had, not all of which centered on the grim status of the annual budget. I thank Arthur Jungreis for many informal talks, for his criticism both solicited and unsolicited, for his almost superhuman tolerance of the mispronunciation of his name, and for providing a personality almost hasidic in honest outlook which seemed to challenge the ferment in the department. I thank the remaining members of my graduate committee, Mary Ann Handel and Robert Murphree, for their effort and participation, both formal and informal in bringing these graduate studies to completion. I am grateful to Francis T. Kenney for helpful discussions and for performing the role of ex-officio adviser. Throughout these studies, I am thankful for my association with Jim Ireland, Tony Calandra and Seth Schor and sincerely appreciate their friendship. Lastly, I would be remiss if I did not acknowledge the contributions of my companion and friend, Bardwell.

Edward J. Pavlik
Knoxville, Tennessee
July 1975

and in chasin' what I thought were moonbeams
I have run into a couple of walls . . .
But in looking back at the faces I've been
I would sure be the first one to say
When I look at myself today
Couldn'ta done it any other way.
["The Hard Way Every Time"; Jim Croce, 1973]

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GENERAL INTRODUCTION

The ideas for this dissertation conceptually arose from the speculation that any component which reacted early with a chemical stimulus was important in the relaying of chemical messages. Proteins which were able to bind estradiol were not only one of the first cellular components with which the sex steroid reacted, but were also translocated as a hormone:binding protein complex to the nucleus. Reviews by Jensen and DeSombre as well as by O'Malley and Means present in greater detail current concepts involving these binding proteins in the mechanism of steroid hormone action and are referenced elsewhere in this dissertation. Because of their eventual nuclear destination, it is easy to speculate that estrogen-binding proteins may have an allosteric role reminiscent of repressor proteins in procaryotic systems. While it may be premature to attempt to construct experiments which directly test the link between estrogen-binding proteins and transcriptional controls involving derepression or gene activation, it is appropriate to attempt to correlate changes in estrogen-binding protein concentration with changes in hormonally triggered growth. In particular, does the concentration of estrogen-binding protein increase as estrogen-mediated growth rapidly progresses? Secondly, what is the status of estrogen-binding protein concentration upon terminating estrogenic development and initiating progestational development? If estrogen-binding proteins increase in response to estrogen administration, a convenient explanation for accelerating uterine growth could be constructed in terms of molecular amplification. Conversely, if progestational steroids terminate

estrogenic events by decreasing estrogen-binding protein concentration, control could be expanded to include a biochemical "brake" which operates through progesterone to limit estrogen receptor concentration. Hence, the events associated with estrogen could be potentiated through increased estrogen-binding protein concentrations, while these same events could be antagonized by progesterone through restriction of estrogen-binding protein concentration.

This dissertation describes a series of experimental tests designed to determine if estrogen-binding proteins can be modulated to positive increases in concentration or activity by estrogen, and if they can be modulated in a negative sense by progesterone.

At the time the work comprising this dissertation was begun (1971-1972) there were only a few techniques available for assaying estrogen receptors. Parameters which described how estrogen receptors could be maintained in reasonably stable condition were then at best scant.

It quickly became apparent that in order to perform the measurements which we wanted to obtain, it would be necessary to make substantial improvements in the assay of estrogen-binding proteins. The desired improvements needed to involve increased sensitivity, enhanced reproductibility, improved adaptability to concentration and volume conditions of high yield homogenization and decreased time of performance. Designing such an assay system became the first requirement for this work. The description of this assay which depends upon adsorption of the binding protein to hydroxylapatite comprises Part 1 of this dissertation. Subsequent to the design of this assay, the concurrent efforts of other

laboratories, which had also recognized the need for an improved assay, began to appear in the literature. Currently there are several alternative methods for assaying estrogen-binding proteins; however, after having tried all the techniques presently described with the exception of protamine sulfate precipitation, it is this experimenter's (somewhat biased?) opinion that the technique described in this dissertation demonstrates superior sensitivity, reproducibility and ease of performance.

Regrettably, as new assay techniques began to appear, the values obtained by such varied approaches began to reflect empirical "drift." Since the estrogen-binding proteins are not indifferent to the effects of temperature, pH, ionic strength, time, charge, absorptive surface, etc., it should be expected that assay conditions, physical and chemical, have a pronounced influence both quantitatively and qualitatively upon the estrogen-binding protein. Unfortunately, a clear and complete understanding of the contributions made by various assays to the measurements so obtained has not been undertaken for any assay. In addition, the chemical and physical interactions which mediate the separation of estrogen-binding proteins remain ambiguous in many assay systems. It has been tacitly assumed that the separation of bound from free steroid was equivalent by any system, and that any differences were *probably* of minor consequence. Results collected from the literature and presented in Part 1 of this dissertation emphasize that such an assumption is both unwarranted and untrue. While the nature of the physicochemical interaction between hydroxylapatite and estrogen-binding proteins remains obscure, this assay is performed under conditions of temperature, pH,

ionic strength, handling time and charge which attempt to provide optimum stability to the estrogen receptor.

Having obtained an assay system which would adequately measure the estrogen-binding protein, experimental manipulations and determinations were begun to examine changes in binding-protein activity. It was first necessary to establish if changes in estrogen-binding protein activity occurred in response to steroid administration. Evidence in favor of modulation of estrogen receptors by estrogen and progesterone is presented in Part 2 of this dissertation.

Effort was combined with caution in order to avoid the pitfalls of premature or erroneous interpretation. A spectrum of phenomena might account for increases or decreases in estrogen receptor activity. Possible contributions by competitive inhibition, allosterism, receptor activation and receptor degradation were each individually tested. Alternative contributions by competitive inhibition and allosterism are considered in Part 1 of this dissertation, while aspects of receptor turnover are considered in Part 3.

After verifying that modulation of estrogen receptor concentration did occur, effort was directed at close observation of the time course of these events. Additional experiments were performed to determine if increases in estrogen-binding protein concentration mediated by estradiol were a part of a general hypertrophic response or were themselves a distinct mechanism responsible in part for hypertrophy. The temporal sequence of events involving changes in receptor concentration as well as changes in rates of protein synthesis is presented in Part 3 of this dissertation.

The terms "estrogen-binding protein" and "estrogen receptor" have been used interchangeably to this point. Purists may once again be chagrined at this exploitation of the term "receptor," but its use arises from simple historical perspective. The molecule in question satisfies all the classical sedimentation requirements of the so-named "estrogen receptor." Despite Jensen's recent attempts to rectify the nomenclature with the introduction of an additional proper name "estrophilin" (*Symposia for the Society of Developmental Biologists*, June 1975, Orono, Maine), the posture of this dissertation is to recognize Shakespeare's influence on Bertrand Russell: what is in a name as long as it is clear of what is being spoken? Hence, the terms "estrogen-binding protein" and "estrogen receptor" are used interchangeably throughout this dissertation.

PART 1: HYDROXYLAPATITE "BATCH" ASSAY FOR ESTROGEN RECEPTORS: INCREASED SENSITIVITY OVER PRESENT ASSAYS

I. INTRODUCTION

Steroid hormone receptors are soluble proteins which very likely play key roles in the mechanism of action of steroid hormones (for review see Jensen and DeSombre [1]. Measurement of the steroid-receptor complex requires the separation of free steroid from steroid bound to the macromolecular receptor. A wide variety of techniques have been employed to this end including: density gradient centrifugation, gel chromatography, charcoal adsorption of free steroid, dialysis, adsorption to glass and silica mesh, protamine sulfate precipitation, binding to DEAE filters, *in vitro* concentration by free cells and adsorption to hydroxylapatite [1-23, and p. 25]. Disadvantages exist in any procedure and improvement usually implies a gain in sensitivity, in performance time, in reproducibility or in ease of application.

A "batch" adsorption assay for the estradiol-17β-receptor (E_2R_c) complex using hydroxylapatite (HTP) which is a modification of the technique of Erdos *et al.* [17] is described and characterized in this section. "Batch" adsorption should provide an improved method of obtaining large numbers of statistically significant points for quantitative analysis. In addition, this approach is easy to apply and has an operational range capable of detecting specific estrogen binding in uterine cytosol samples containing *as little as* 5-500 μg protein.

In short this technique involves: (a) adsorption of receptor bound [^{3}H]-estradiol complex to hydroxylapatite at an operational temperature of 0-2 C and (b) removal of unbound steroid by sequential washes with buffer.

II. EXPERIMENTAL

Animals

Immature female Sprague Dawley rats were obtained from Sprague Dawley, Madison, Wisconsin. Upon reaching maturity, rats were bilaterally ovariectomized. These animals are referred to as "long-term castrate" females. Experimental animals were injected no sooner than 4 weeks postoperatively, and sacrificed by cervical dislocation under light ether anesthesia.

Cytosol Preparation

Uterine horns were immediately removed, dissected free of fat and connective tissue and placed in Hank's balanced salt solution (HBSS) on ice. All procedures were performed as outlined in the literature [4, 6, 10] at 0-2 C unless otherwise specified. Homogenization in glass homogenizers was performed in buffer containing TRIS (40 mM, pH 7.2), EDTA (1.5 mM), mercaptoethanol (14 mM) and KCl (50 mM). This buffer is referred to as $TEMK_{50}$ where KCl is the only variable (Fig. 1). The homogenate was centrifuged for 15 minutes at 800 g to pellet the nuclear-myofibrillar components. This low speed supernatant was then centrifuged for 90 minutes at 106,000 g in a titanium (50 Ti, Beckman Industries) fixed-angle rotor yielding the high speed supernate or receptor-containing cytosol fraction. A homogenization ratio of 4 uterine horns/ml (2 rat uteri/ml) was routinely used unless otherwise specified. The receptor-containing "cytosol fraction" was diluted as described in individual experiments. Protein determinations

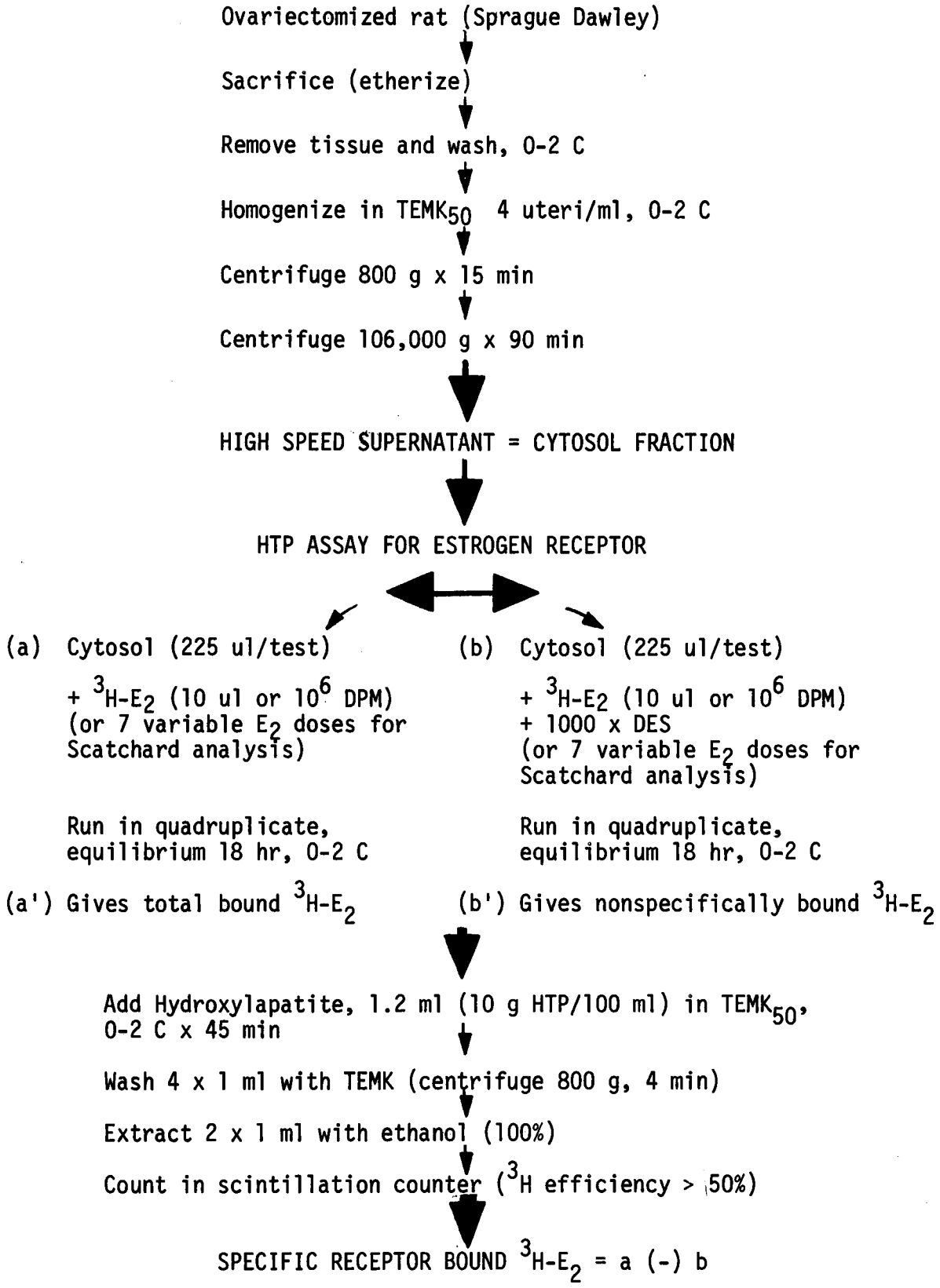

Figure 1. Hydroxylapatite assay of estradiol 17β-receptors.

were performed on the cytosol fraction according to the procedure of Lowry *et al.* [24].

Hydroxylapatite Preparation

Analytical grade hydroxylapatite (HTP or DNA grade HTP, Biorad Inc.) was mixed in $TEMK_{50}$ buffer for 48 hours (10 g/100 ml). The "fines" were routinely decanted, although comparison with undecanted HTP showed no detectable difference. The HTP solution was then stored at 0-2 C and used within one week after preparation. Prior to use the HTP mixture was resuspended by stirring for 30-60 min at 0-2 C and divided into 1.2 ml aliquots. Procedures for adsorption and washing HTP-E_2R_C complex free of unbound ligand are detailed in the Results and Discussion section. Bound [^{3}H]-estradiol ([^{3}H]-E_2) was removed from the HTP with two ethanol extractions with an extraction efficiency of 95% or greater. Liquid scintillation counting was performed with a PPO-POPOP fluor (2,5 diphenyloxazole and 1,4 Bis[2-(5-phenyloxazolyl)] benzene) in a Beckman LS-230 liquid scintillation counter. Counting efficiency for [^{3}H] was 30-40%, and external standard quench correction was obtained for each sample.

Cytosol Incubation Conditions

For all studies 225 µl of cytosol was incubated with 20 µl ethanol containing either [^{3}H]-estradiol-17β (80-100 Ci/mM; Amersham Searle or New England Nuclear) or [^{3}H]-estradiol-17β ([^{3}H]-E_2) and diethylstilbesterol (DES, from Sigma Chemical Corporation). For equilibrium studies, incubation times of 18 hours at 0-2 C were employed. The purity of the [^{3}H]-E_2 was checked by thin layer chromatography, and did

conform to the manufacturer's specification. Incubation was terminated with the introduction of 1.2 ml of HTP solution (0-2 C) followed by vortex mixing for 5 seconds. The E_2R_c complex adsorbed to HTP was then washed with $TEMK_{50}$ buffer by sequential resuspension and centrifugation.

III. RESULTS

A fundamental requirement for any system used to adsorb the E_2R_c complex is that it can be washed clean of free steroid. The degree to which unbound steroid can be washed free from pure HTP is demonstrated in Fig. 2. There is no difference in "washout" between HTP and finely powdered DNA grade HTP. DNA grade HTP was routinely selected for further use because its fine particle size allowed tighter packing by centrifugation. Four washes were sufficient to reduce supernatant wash activity below 1% of the input activity, and to reduce the activity remaining in the HTP (i.e., ethanol extracted activity) to below 1% of the input activity.

Another obligatory requirement placed on a system used to separate the E_2R_c complex is the demonstration of high affinity or specificity for $[^3H]$-E_2 binding as distinct from low affinity or non-specific steroid binding. High affinity binding specific for the E_2R_c is reduced (Fig. 3) by the known non-steroidal estrogenic competitor (DES) as well as by nonradioactive estradiol-17β. Increasing concentrations of DES rapidly reduced the total amount of bound activity. Hence, as demonstrated by Williams and Gorski [6] and as shown here, non-specific binding appears to be an unsaturable process. In addition diethylstilbesterol and estradiol at 100- or 1000-fold excess appear as indistinguishable competitors. Specific binding to the E_2R_c can be determined as originally suggested by Williams and Gorski [6] by the difference between the total activity bound in the absence of any competitor and the unsaturable amount of non-specific $[^3H]$-E_2 binding which occurs in

Figure 2. Retention of free [^{3}H]-estradiol by hydroxylapatite.

$TEMK_{50}$ buffer (225 µl) containing a known concentration of [^{3}H]-estradiol-17β was incubated for 45 minutes in the presence of HTP. Adsorption was terminated by centrifugation for 5 minutes at 800 g. The supernatant containing unbound steroid was collected and counted. The pellet was resuspended in 1 ml of $TEMK_{50}$, and successive 1 ml washes with $TEMK_{50}$ were also collected and counted. The activity which had not been removed by washing with $TEMK_{50}$ was determined by extracting the HTP two times with ethanol and counting. Retention of [^{3}H]-E_2 by HTP and finely powdered DNA grade HTP were compared. Results were expressed as CPM in the supernatant wash as well as a percentage of the input activity recovered from the extracted HTP pellet after the fifth wash.

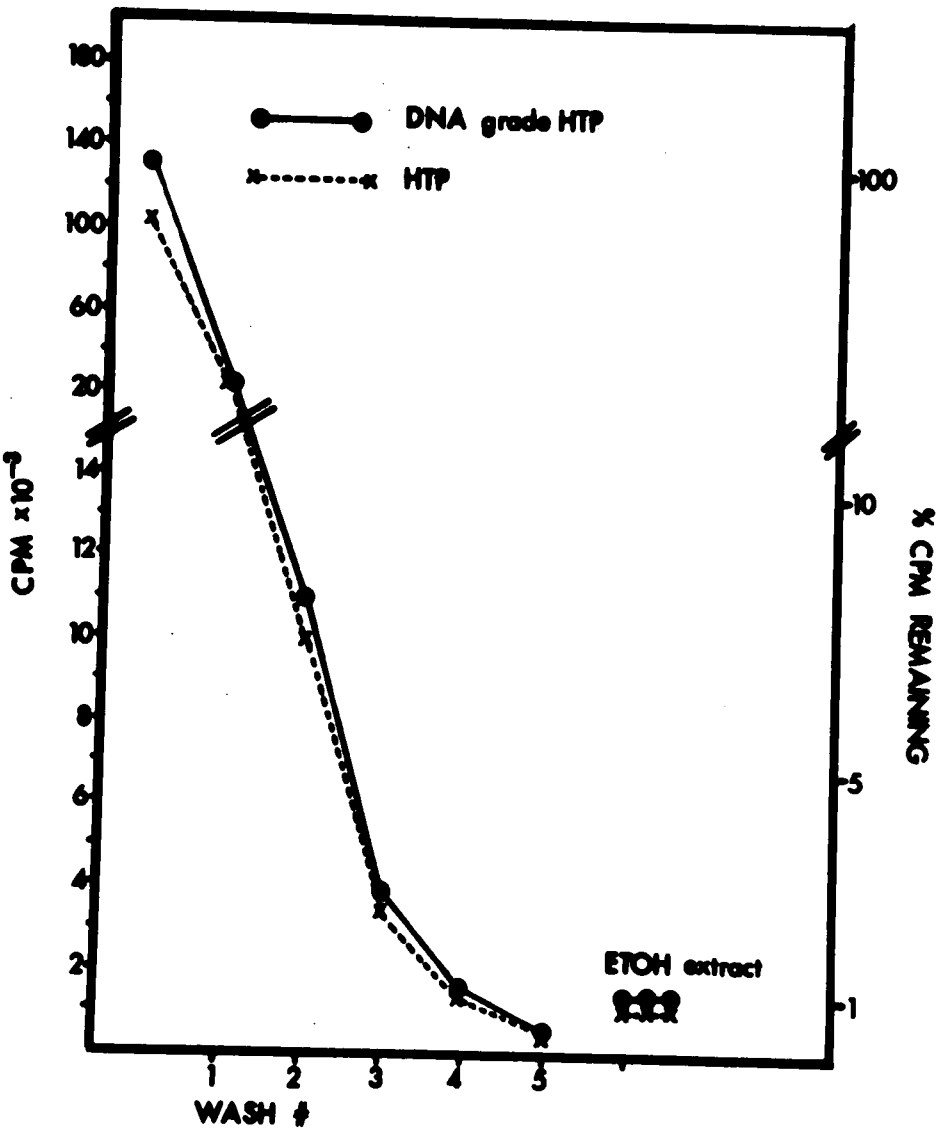
DNA grade HTP
HTP
ETOH extract
CPM x10⁻³
% CPM REMAINING
WASH #
180
140
100
60
20
14
12
10
8
6
4
2
100
10
5
1
1
2
3
4
5

Figure 2

Figure 3. Adsorption of the E_2R_c to HTP and competition by diethylstilbesterol.

The receptor containing cytosol was incubated for 5 hours at 0-2 C with a saturating amount of [^{3}H]-E_2 (=450 x 10^3 CPM/225 µl cytosol). The tissue to buffer ratio consisted of 14 uterine horns homogenized in 12 ml of $TEMK_{50}$ buffer. All determinations were run in replicates of four and mean values are expressed ± the standard error of the mean. Competition was performed so that there were no effects due to dilution or volume change. The receptor was adsorbed to HTP by incubating for 45 minutes at 0-2 C. Adsorption was followed by four 1 ml washes with $TEMK_{50}$ and double extraction of the HTP pellet with ethanol. Competition by nonradioactive E_2 in 100- and 1000-fold excess yielded: 43,193.8 ± 2284.7 and 42,690 ± 778.1 DPM bound, respectively. This level of competition was indistinguishable from that achieved with 100- and 1000-fold excess diethylstilbesterol which yielded: 43,834.5 ± 1772.4 and 42,437.8 ± 751.8 DPM bound, respectively.

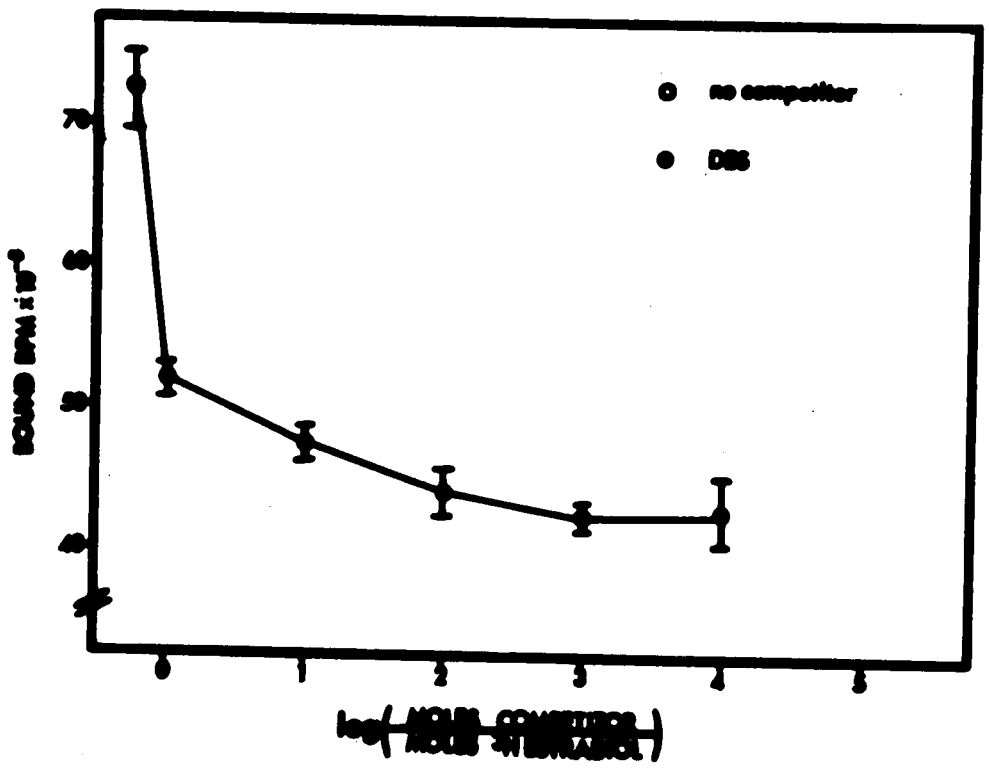

Figure 3

the presence of excess competitor (Fig. 1, p. 9), or: specific [^{3}H]-E_2R_c equals [^{3}H]-E_2 bound without nonradioactive competitor minus non-specific [^{3}H]-E_2 bound in the presence of 1000-fold excess nonradioactive DES.

Therefore, in all determinations of specific [^{3}H]-E_2 receptor binding, additional aliquots were run simultaneously containing a 1000-fold excess of nonradioactive competitor in order to accurately estimate non-specific binding.

Optimal and efficient adsorption are also important considerations. The time necessary for maximum adsorption was determined (Fig. 4) and was found to plateau within approximately 30 minutes. While relatively the same mean amount of specific adsorption occurred after shorter adsorption times, the variability, as judged by reduced standard error, was seen to decrease after 30 minutes. Thus, all subsequent determinations were routinely adsorbed for 45 minutes.

The assay sensitivity is described in Fig. 5. Aliquots containing various dilutions of cytosol were assayed. The highest concentration of receptor was 1 uterine horn 225/µl and was tested over a dilution range of 1:10,000. The dilution sensitivity is easily demonstrable over a 50-100 dilution range as shown in Fig. 5. Thus, the HTP method is well suited for detecting estrogen receptor in target tissue homogenates which contain 5-500 µg protein.

Other assay techniques are dependent upon low ionic strength conditions for adsorbing the receptor [4, 5, 8]. HTP adsorption is examined as a function of ionic strength in Fig. 6. Buffer concentrations were designed so that only the concentration of KCl varied. All aliquots

Figure 4. Adsorption time for binding E_2R_c complex to HTP.

Conditions were as described in Fig. 3, p. 15. The tissue-buffer ratio consisted of 16 uterine horns homogenized in 12 ml of $TEMK_{50}$. Replicates of four were run, and results expressed as mean ± standard error of the mean. The receptor was adsorbed to HTP for varying periods of time. Adsorption was followed by four 1 ml washes with $TEMK_{50}$ and double extraction with ethanol. Specific binding was determined by subtracting the DPM bound in the presence of a 1000-fold excess of DES from the total DPM bound.

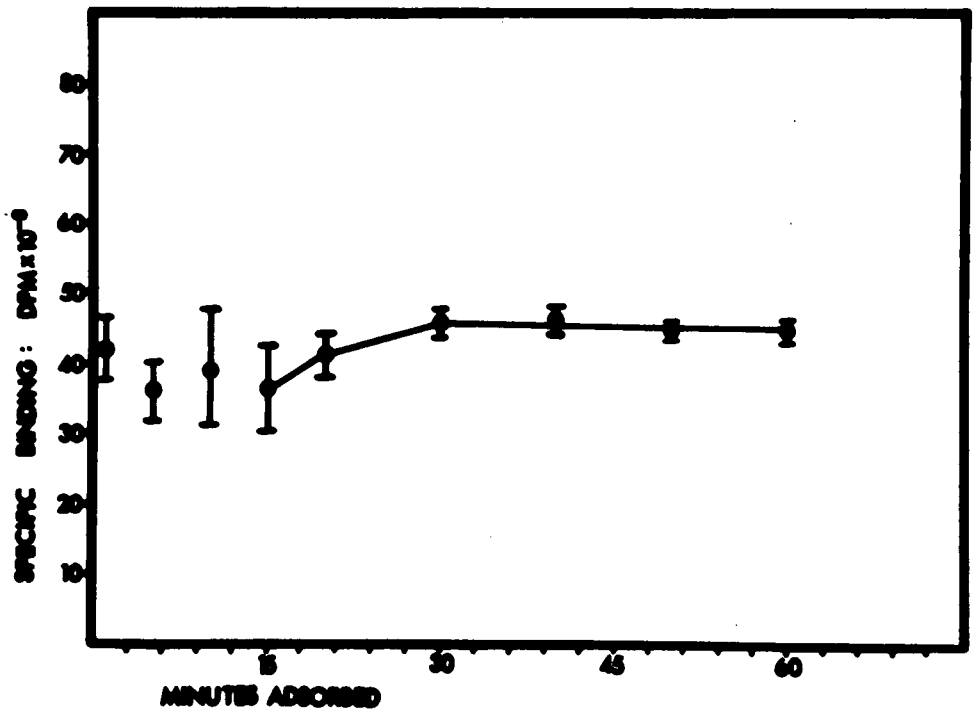
SPECIFIC BINDING: DPM x 10⁻³
80
70
60
50
40
30
20
10
15
30
45
60
MINUTES ADSORBED

Figure 4

Figure 5. Adsorption sensitivity of HTP for E_2R_c binding.

Conditions were as described in Fig. 3, p. 15. The tissue-buffer ratio consisted of 22 uterine horns homogenized in 5 ml of $TEMK_{50}$. Adsorption to HTP was performed for 45 minutes at 0-2 C. Replicates of four were used and results were expressed as mean ± standard error of the mean. Correction for non-specific binding was made by subtracting $[^3H]$-E_2 bound in the presence of a 1000-fold excess of DES from the total DPM bound in the absence of competitor. The soluble protein content in this experiment averaged 525.4 ± 31.3 µg protein per uterine horn. Specific binding is clearly shown over a 50-fold dilution range. Specific binding could be detected down to 1:100 dilution, but disappeared at 1:10,000 dilution.

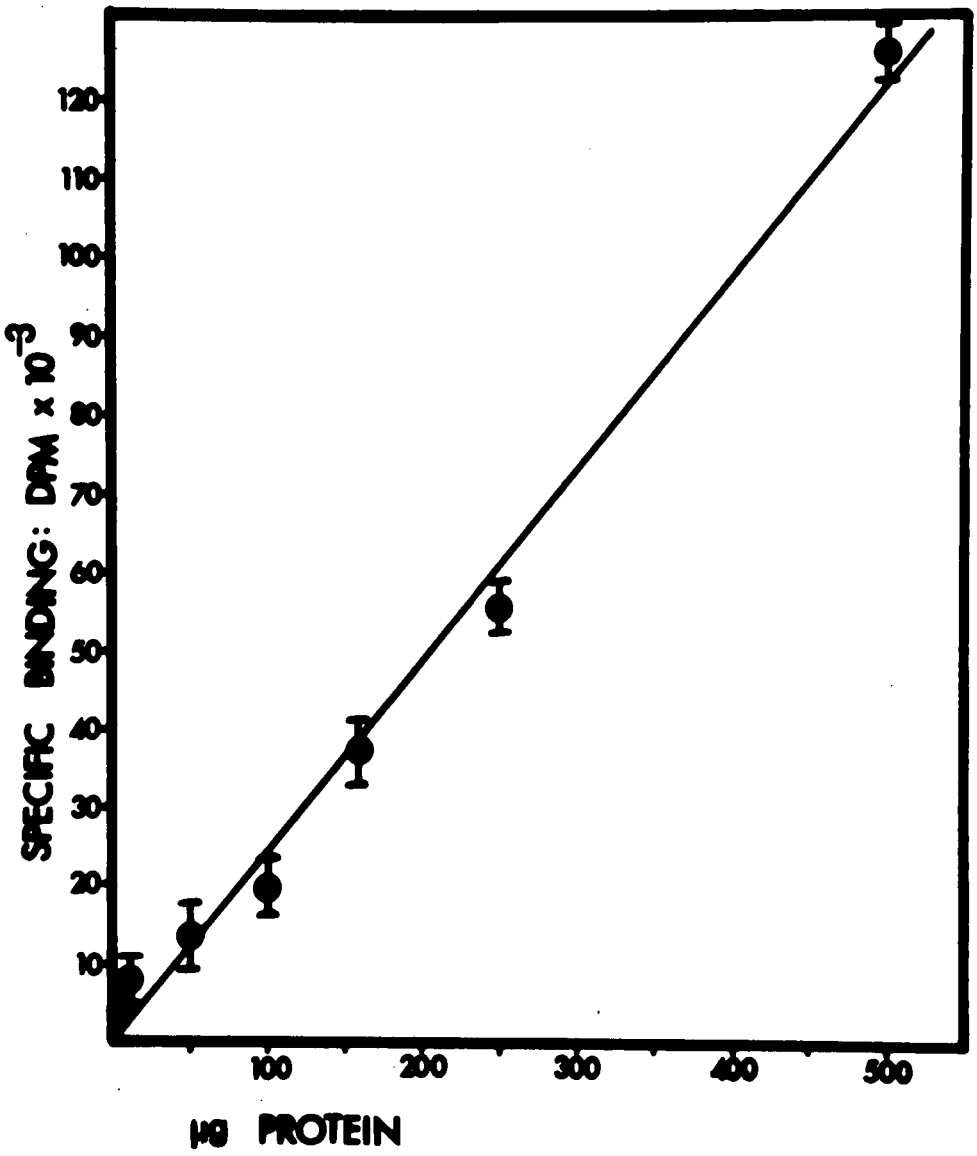
SPECIFIC BINDING: DPM x 10^{-3}
120
110
100
90
80
70
60
50
40
30
20
10
100
200
300
400
500
µg PROTEIN

Figure 5

Figure 6. Adsorption of E_2R_c to HTP: KCl ionic strength insensitivity.

Conditions were as described in Fig. 3, p. 15. Replicates of four were run and results were expressed as mean ± standard error of the mean. Dark bar: Receptor was adsorbed to HTP for 45 minutes at 0-2 C. Adsorption was followed by five 1 ml washes with $TEMK_{50}$ and a double ethanol extraction. Open bar: Following the fifth wash with $TEMK_{50}$, the receptor-containing HTP was washed sequentially with buffer of increasing KCl concentration ($TEMK_{111}$ to $TEMK_{2500}$) and then double extracted with ethanol. Supernatants were collected after each wash for monitoring [^{3}H]-E_2 washed free due to increasing KCl ionic strength. Recovery was never less than 95%.

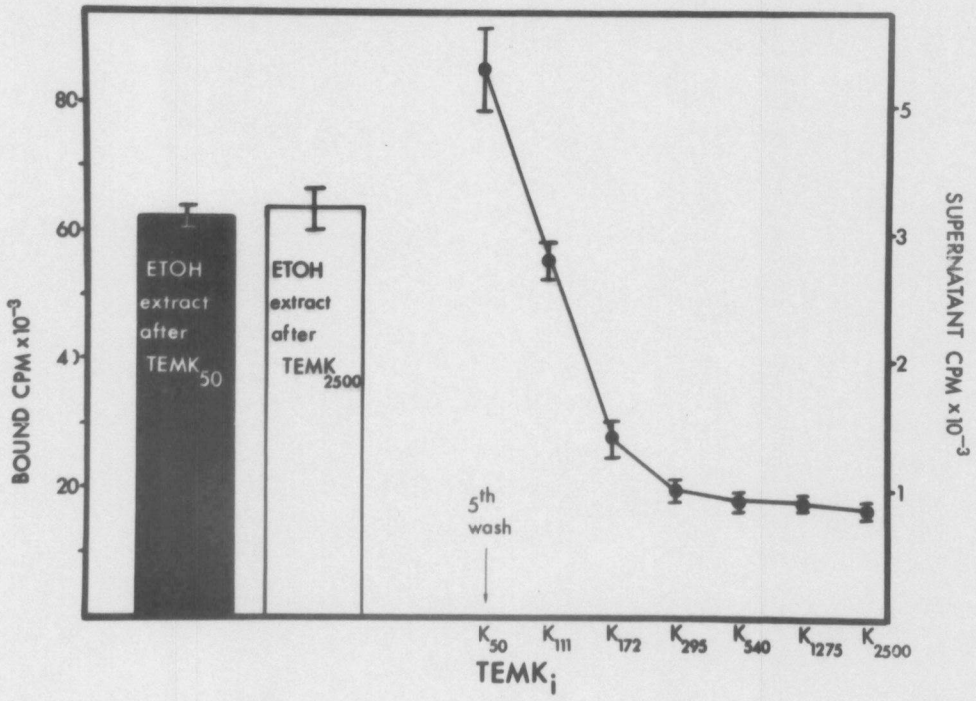
BOUND CPM x10⁻³
80
60
40
20
ETOH extract after TEMK50
ETOH extract after TEMK2500
5th wash
SUPERNATANT CPM x10⁻³
5
3
2
1
K50 K111 K172 K295 K540 K1275 K2500
TEMKi

Figure 6

were washed five times with $TEMK_{50}$. The first group was double ethanol extracted while the second group was washed with buffers of increasing KCl concentration. After washing the samples with $TEMK_{2500}$ (the final wash of 2500 mM KCl ionic concentration), a double ethanol extraction was performed. Wash supernatants were collected and counted. No differences in counts retained by HTP or counts recovered in the supernatant could be detected between aliquots subjected to washes of increasing ionic strength versus those washed in $TEMK_{50}$. These data indicate that the $[^3H]$-E_2R_c complex adsorbed to HTP is unaffected by increasing KCl ionic strength. In addition, since the nuclear estrogen receptor is generally defined as extractable in buffers containing 400-600 mM KCl [21-23], this adsorption technique should prove particularly valuable in quantitating the nuclear estrogen receptor in the presence of high KCl concentration.

Recent studies by Bresciani *et al.* [25] confirm the ability of HTP to bind the E_2R_c complex while not retaining the nuclear E_2-acceptor which is apparently a more basic protein. DNA binding to HTP has been well characterized, but the DNA contamination in these cytosol preparations was very low. Any E_2R_c bound to a DNA-HTP complex would have been eluted off at concentration of KCl above 300 mM [26] and therefore does not appear to be a contributing factor in this E_2R_c HTP assay (Fig. 6).

In an effort to compare this assay with E_2R_c assays currently in use, a survey of the literature was made (Table 1) to document values for association constants and receptor concentration in the rat uterus. It is clear that a wide range of K_a values have been reported. This wide range may reflect not only the technique and the temperature used, but also the method of correction for non-specific binding.

Table 1. Comparison of Published Values for Association Constant (K_a) of Estrogen Receptors

Association Constant $K_a \times 10^{10} M^{-1}$	Receptor Conc. (Moles/unit)	Protein Conc. of Cytosol	Uterus/ Tissue	Technique	Correction for Non-Specific Binding	Sources
2.40	$1\text{-}8 \times 10^{-15}$ m/mg tissue	--	rat	dextran-coated charcoal	Analytical a,b temp. dissoc.	Mester *et al.* 1970 (21)
0.14	0.89×10^{-12} m/uterus	--	immature rat	*in vitro* nuclear binding	temp. dissoc.[b]	Shymala & Gorski 1969 (2)
3.6	5.8×10^{-10} M/L	5.2 mg/ml	adult rat	dialysis, partial purification	analytical[a]	Ellis & Ringold 1971 (3)
4.5	4.0×10^{-10} M/L	15 mg/mg	adult rat	dialysis, partial purification	analytical[a]	Ellis & Ringold 1971 (3)
0.8-19.0	--	--	--	--	--	Ellis & Ringold 1971 (3)
0.03	0.9×10^{-12} M/uterus	--	immature rat	glass bead binding	temp. dissoc.[b,e]	Clark & Gorski 1969 (4)
0.06	$0.56/10^{-12}$ M/mg protein	--	adult rat	Silica mesh	temp. dissoc.[b,e]	Notides 1970 (8)
0.22	--	--	immature rat, free cells	glass beads, Sephadex G-25	Competitive saturation, (100xexcess estradiol-17β)[c]	Williams & Gorski 1973 (6)

Table 1 (continued)

Association Constant $K_a 10^{10} M^{-1}$	Receptor Conc. (Moles/unit)	Protein Conc. of Cytosol	Uterus/ Tissue	Technique	Correction for Non-Specific Binding	Sources
0.04	--	--	rat	DEAE filters	Competitive saturation (100xexcess estradiol-17β)[c]	Santi *et al.* 1973 (7)
4-6.7	$1.3\text{-}2.2 \times 10^{-14}$	--	hypothalamus adult rat	Protamine precipitation	Competitive saturation (100xexcess E_2)[c]	Korach & Muldoon 1974 (9)
1.0	--	--	--	dextran-coated charcoal	differential dissociation	Alberga *et al.* 1970 (31)
0.12	$3.2\text{-}3.7 \times 10^{-10}$ m/mg protein	700 µg/ml	long-term castrate rat	HTP	Competitive saturation (100xexcess E_2)[c]	Pavlik & Coulson 1975
0.14	1×10^{-12} M/mg protein	8.6 mg/ml	immature rat	Sucrose gradient	N.C.[d]	Toft *et al.* 1967 (10)
0.77	--	--	rabbit	dextran-coated charcoal	N.C.[d]	Korenman & Rao 1968 (11)
70.0	--	-	rat endo-metrium	*in vitro* incubation	N.C.[d]	Alberga & Baulieu 1968 (12)
0.13-0.35	$3.2\text{-}6.5 \times 10^{-13}$ M/mg protein	--	calf (partial purification)	Sephadex G-25	N.C.[d]	Puca & Bresciani 1969 (13, 28)

Table 1 (continued)

Association Constant $K_a 10^{10} M^{-1}$	Receptor Conc. (Moles/unit)	Protein Conc. of Cytosol	Uterus/ Tissue	Technique	Correction for Non-Specific Binding	Sources
0.025-0.5	--	5.4 mg/ml	immature rat	Sephadex G-25	N.C.[d]	Giannopoulos & Gorki 1970 (14)
0.3	3.0/kg tissue	--	immature & adult rat	*in vivo* infusion	N.C.[d]	DeHertogh *et al.* 1971 (15, 16)
0.88-1.10	$0.39\text{-}1.54 \times 10^{-9}$ M/g prot./L	--	calf	HTP	N.C.[d]	Erdos *et al.* 1970 (17)
0.102	45×10^{-15} M/mg protein	--	immature	Sephadex G-25 & G-100	N.C.[d]	Godefroi & Brooks 1973 (18)
0.45	--	--	mature rat	Biogel P-10	N.C.[d]	Eisenfeld 1974 (19)
0.77	0.3×10^{-12} M/mg protein	--	rat (partial purification)	Protamine precip.	N.C.[d]	Steggles & King 1970 (32)
1.5-1.7	$0.2\text{-}2 \times 10^{-8}$ M/L	--	calf	HTP	N.C.[d]	Best-Belpomme *et al.* 1970 (29)

[a]Analytical correction obtained by least squares solution of two linear equations, one representing high affinity binding, the other low affinity binding.

[b]Elevated temperature if believed to cause more rapid dissociation of ligands at low affinity sites than at high affinity sites. Incubation was at 30 C for 30 min.

[c]Excess nonradioactive ligand cannot saturate low affinity sites. Thus, total activity - activity in the presence of excess unlabeled ligand = high affinity binding.

[d]N.C. = no correction.

[e]Assay conditions include warming to 25 C, 30 min.

The HTP adsorption approach has been employed in an equilibrium saturation experiment which is evaluated by Scatchard plots [27]. These results, obtained by linear regression analysis, are presented in Table 2 for the long-term castrate rat uterus and are well within the range of values present in the literature (Table 1).

Table 2. Hydroxylapatite Adsorption of Estrogen Receptor (E_2R_c) from Long-Term Castrate Rat Uterus

Association Constant	E_2R_c Concentration	E_2R_c Specific Activity
$K_a \times 10^{10} M^{-1}$	$E_2R_c \times 10^{-12}$ moles/uterus	$E_2R_c \times 10^{-12}$ moles/mg protein
0.12±0.01	1.53±0.32	3.72±0.27

Note: Correction was made for non-specific binding by subtracting counts from similar aliquots equilibrated in the presence of 1000-fold excess DES. Equilibrium time for binding was 18 hours at 0-2 C. Results were analyzed according to Scatchard (27) and subjected to simple linear regression analysis.

IV. DISCUSSION

The application of hydroxylapatite "batch" adsorption for quantitative studies of the uterine E_2R_c has been described. There are several advantages in the "batch" method which are improvements over other established techniques in the literature:

1. Hydroxylapatite [$3Ca_3(PO_4)_2 \cdot Ca(OH)_2$] is chemically homogenous and obtainable in highly purified, analytical grade quantities of uniform particle size.
2. Adsorption of estrogen receptor to HTP can be performed at 0-2 C in contrast to techniques which require warming [4, 5, 8] with concomitant increases in dissociation of the steroid receptor complex.
3. HTP has little affinity for free estradiol, so that free [^{3}H]-E_2 can be quickly washed away.
4. DNA grade HTP (Biorad) can be tightly packed by centrifugation for 2 minutes at 800 g and is easily resuspended by vortexing.
5. Due to low affinity between free estradiol and HTP, estimation of free steroid in the presence of receptor bound activity can be made by directly sampling the supernatant.
6. The sensitivity of detection was found to exist over a 50-100 fold range and is capable of detecting specific binding in aliquots of rat uterus cytosol containing as little as 5 µg protein.
7. HTP adsorption appears insensitive to changes in KCl ionic strength. Such fortuitous insensitivity should enable the

quantitation of nuclear estrogen receptor after high salt extraction from the nuclear fraction.

8. The modification described here does not require HTP to be packed in columns. Hence, more determinations with greater statistical significance can be made.

The procedure and advantages of a modification of Erdo's original hydroxylapatite technique [17] for extracting E_2R_c complexes has been outlined. "Batch" processing provides large numbers of points which lend statistical significance to quantitative determinations. In addition, the large range of K_a values reported in the literature suggests that the choice of technique, method of correction for non-specific binding, temperature at which the assay binding is performed, and the receptor yield may all influence these values.

Factors influencing the determination of equilibrium K_a are very complex. For example, ionic strength influences the sedimentation values of the E_2R_c [11, 13,]2], but does not appear to affect the association constant [14]. Hence, the buffer contribution to the value of the association constant may be minimal. Changes in K_a due to short warming temperatures, however, may be quite important. For prolonged warming periods there is no change in K_a measured under equilibrium conditions; however, with increasing temperature dissociation is kinetically more rapid than association [24, 28]. The paradoxical result is that association constants calculated by association-dissociation kinetics must decrease with increasing temperature. Hence, provided equilibrium is reached, there may be no measurable effect on K_a values, but increased temperature for short times without

complete "re-equilibrium" might cause association constants to behave in a fashion similar to those which are kinetically derived. The role of temperature effects should not be ignored in these determinations and may help explain the variability seen in Table 1, p. 25.

Even more striking is the range of values found for receptor site concentration in the literature [Table 1 and 29, 30]. Jensen [21] has postulated a variable receptor yield after processing as a possible explanation, but procedural variability may also be responsible. Therefore as a simple precautionary tactic, it seems wise to avoid temperature changes during quantitative determination and to include in the assay design an empirical correction for non-specific binding either by excess competitor or by heat inactivation of the receptor [20].

In summary an in-depth validation is reported for a new assay for uterine E_2R_c which will yield multiple determinations, an increase in sensitivity over current assay methods, and a low background. This assay is easy to perform, sensitive to changes in KCl ionic strength, and reproducible. It should be especially useful in the future quantitation of either cytosol or nuclear receptors.

Addendum. A similar HTP batch procedure has recently been reported by Williams D. and Gorski, J., *Methods in Enzymology* (1975), edited by B. W. O'Malley and J. G. Hardman, Academic Press, N.Y., pp. 275-283. The characterization of the HTP assay described here gives a more complete analysis and is deliberately designed with increased sensitivity and avoidance of warming temperatures as foremost considerations.

V. SUMMARY

A "batch" procedure for the adsorption of the uterine estradiol-17β receptor complex is described. Characterization with respect to washing efficiency, binding specificity, competition, adsorption time, sensitivity and stability against increasing KCl ionic strength were included. Equilibrium parameters obtained by Scatchard analysis were compared to the range of values found in the literature. The K_a and receptor site concentration per uterus obtained by this "batch" technique were found to be well within these reported values.

This technique is particularly advantageous due to its wide range of operational sensitivity (capable of detecting specific estradiol-17β binding to a cytosol fraction containing 5 μg protein per 225 μl). The assay is run entirely at low temperature (0-2 C). In addition, this technique depends on a homogeneous insoluble chemical, hydroxyl-apatite (which can be obtained in analytical grade quantities of uniform particle size), shows little affinity for free steroid, can be readily packed and resuspended and appears independent of changes in concentration of KCl up to 2500 mM.

The implications of temperature increases during assay are discussed as well as the importance of empirical correction for non-specific binding.

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PART 2: MODULATION OF ESTROGEN RECEPTORS IN FOUR DIFFERENT TARGET TISSUES: DIFFERENTIAL EFFECTS OF ESTROGEN AND PROGESTERONE

I. INTRODUCTION

Estrogens are concentrated by specific target tissues (uterus, vagina, pituitary, hypothalamus) which contain a macromolecular binding protein called the estrogen receptor [1-5]. Estrogen treated uteri respond by undergoing hypertrophy and mitosis. However, the progestational uterus, under the influence of both estrogen and progesterone, becomes distinct from the estrogen dominated uterus showing a decreased epithelial cell division and an increased or "differentiated" secretory function [1, 6, 7]. Since the steroid-receptor interaction is one of the very early events which must occur in each target tissue in order for the tissue to respond, it is possible that the response is limited or potentiated by the availability of the cytosolic estrogen receptor.

The term "replenishment" refers to restoration of estrogen receptor concentration in the first 0-16 hours after estrogen treatment [2, 10] while the term "modulation" will be used to describe the changes in estradiol-17β receptor (E_2R_c) concentration determined via Scatchard analysis. This part of the dissertation considers the changes in receptor concentration which occur after replenishment of E_2R_c to pretreatment levels. Factors involving synthesis, degradation, or sequestrational changes in E_2R_c cannot be separately distinguished by current methodology.

To date little or no information is available to correlate the known effects of progesterone on the rat estrous cycle with the potential effect of progesterone on E_2R_c concentrations in the four primary target tissues. Here the "modulation" of estrogen receptor concentration

is examined in uterine, vaginal, pituitary, and hypothalamic tissue following stimulation by estradiol-17β (E_2) and progesterone (P_4) for 24-48 hours. The results are consistent with a differential effect of the sex steroids on estrogen receptor concentration.

II. EXPERIMENTAL

Animals

Female Sprague Dawley rats (190-250 gm) were bilaterally ovariectomized and used four weeks post-operatively. The atrophied uterus of the four week castrate is referred to as the "long term castrate" uterus. Animals were sacrificed under light etherization with cervical dislocation. The four target tissues (uterus, vagina, pituitary and hypothalamus) were removed and placed in Hank's balanced salt solution at 0-2 C. The "hypothalamus" was defined to extend posterior from the mammillary bodies to the preoptic region, approximately 3 mm anterior of the optic chiasma, bounded laterally by its obvious morphological termination. This tissue was excised to a depth of approximately 2 mm. The pituitary was teased from the sella turcica with its intermediate lobe left intact.

Cytosol Preparation

Tissue homogenates were prepared as previously described [8-12, 15]. Tissues were debrided of connective tissue and fat. Small pieces of minced tissue were homogenized in $TEMK_{50}$ buffer (0.04 M Tris, pH 7.2, 0.0015 M EDTA; 0.014 M mercaptoethanol; 0.05 M KCl) with a tissue to buffer ratio of (N=4)/ml for uterine and (N=number of animals used per determination) vaginal tissue, and (N=8)/ml for pituitary and hypothalamic tissue. Procedures were performed at 0-2 C except where indicated. The homogenate was centifuged for 15 minutes at 800 g. The pellet was saved for DNA determinations performed according to Burton [13]. The low speed supernatant was then centrifuged for 90

minutes at 106,000 g in a 50 Ti fixed angle rotor (Beckman Industries) yielding the high speed supernatant or "cytosol" fraction. Protein determinations were performed according to Lowry *et al.* [14] on this cytosol.

Sucrose gradient analysis was performed on cytosols which were similarly prepared except for the buffer. A Tris buffer (0.01 M Tris; 0.001 M EDTA; 0.4 M KCl) was used for homogenization and made to 0.004 M CaCl for the buffered 5-20% sucrose gradients in order to stabilize the 4S binding subunit [12]. Gradients were centrifuged for 24 hrs at 38,000 rpm at 0-2 C in a SW-39 rotor (Beckman Industries).

HTP Assay

Cytosols were assayed for E_2R_c via the "hydroxylapatite E_2R_c assay" [15] as described in Part I of this dissertation, employing modifications of the techniques of Erdos *et al.* [16] and Clark and Gorski [17]. Cytosolic aliquots (225 µl) were incubated with various concentrations of [^{3}H] estradiol-17β ([^{3}H]-E_2), specific activity = 80-100 Ci/mM (Amersham-Searle) for 18 hrs at 0-2 C. Hydroxylapatite (DNA grade Biogel HTP, BIORAD Inc.) was prepared in $TEMK_{50}$ buffer (10 gm/100 ml) and mixed overnight. Cytosolic receptors were then adsorbed to the hydroxylapatite (HTP) by adding 1.2 ml of HTP to each aliquot of cytosol and shaking at 0-2 C for 30 min. The HTP was then centrifuged (800 g, 4 min) and washed six times with $TEMK_{50}$ buffer at 0-2 C. Bound estradiol was removed from the HTP with two 4 ml ethanol extractions. The ethanol extract was air dried in scintillation vials and liquid scintillation counting was performed in a Beckman LS-230 liquid

scintillation counter utilizing a PPO-POPOP fluor (2,5 diphenyl-oxazole and 1,4 Bix [2-(5-phenylosxazolyl) benzene]. Quench correction was determined with an external standard. Multiple samples (4x) were run for each steroid concentration with seven concentrations being generally employed. Correction was made for non-specific binding using excess nonradioactive competitor. Saturation was achieved for at least two of the concentrations employed.

In Vitro E_2 Organ-Binding Assay

In vitro manipulations were performed in Hank's buffered MEM (GIBCO), with 2% Penn-Strep (GIBCO) at 37 C under an atmosphere of 95% O_2 and 5% CO_2. Tissues were assayed for ability to retain $[^3H]$-E_2 by incubating tissue minces with varying concentrations of $[^3H]$-E_2 for 2 hours in the presence and absence of 100-fold excess nonradioactive estradiol-17β. Tissue was washed in three changes of MEM at 0-2 C and carefully homogenized. After homogenization steroidal uptake was determined by double extraction with equal volumes of ethyl acetate. Extraction efficiency was found to be at least 95%. Correction was made for non-specific binding components by the use of excess competitor.

Biological Response

Vaginal lavage cytology was determined according to the following classification: large numbers of cornified epithelial cells = estrus; cornified epithelial cells + leucocytes = diestrus 1; large numbers of leucocytes = diestrus 2; large numbers of round epithelial cells = proestrus. Whenever cornified cells were observed their presence

was recorded irrespective of the final classification of the lavage type.

In vivo experiments on long term castrate rats consisted of three groups: a saline injected control group, a second group receiving 25 µg or 0.1 µg E_2 (I.P.) in 0.5 ml saline per rat, and a third group receiving E_2 (I.P.) at 0 hrs plus 1 ml progesterone (S.C.) in 0.5 ml propylene glycol at 24 hours. All rats were sacrificed 48 hrs after treatment.

III. RESULTS

In Vitro E_2 Organ-Binding Assay

Long term castrate female rats were injected with estradiol-17β (25 μg E_2, I.P.) in order to test the "priming" effect of estradiol on receptor concentration (Table 1). After 48 hours, the animals were sacrificed, and the tissues subjected to an "E_2 Organ-Binding Assay" *in vitro*. This approach being performed on intact tissue, at 37 C, with the receptor in a physiological microenvironment. In this assay the [^{3}H]-E_2 tracer ± nonradioactive competitor E_2 (a) was bound to the receptor, (b) was allowed to reach equilibrium and (c) was washed free of unbound E_2 prior to the homogenization step. This sequence obviates any concern for receptor loss during homogenization of processing. This is especially important since the E_2R_c is more labile in the absence of steroid [3]. The results were plotted according to Scatchard [18] and subjected to linear regression analysis on an IBM 360-91 computer.

Under the conditions of the E_2 Organ-Binding Assay (Table 1), it is clear that estrogen binding per uterus is increased from 1.86 to 4.53 picomoles by 48 hours of prior exposure to nonradioactive estradiol *in vivo*. Only a slight decrease in association constant was seen perhaps due to competition from nonradioactive estradiol. However, the presence of nonradioactive competitive hormone (E_2) as a consequence of the E_2 pretreatment does not affect the numbers of binding sites when determined by Scatchard analysis [18]. It should be noted that the events described in Table 1 are tissue phenomena and may (or may not) reflect specific molecular or synthetic events. These tissue phenomena or binding

Table 1. *In Vitro* E_2-Organ Assay for Estradiol Tissue Binding in Uterus[a]

Tissue	*In vivo* Treatment	$K_a x10^{10} M^{-1}$	Binding $x10^{-12}$ moles	R
Uterus	0.5 ml saline (I.P.)	0.50±.0112	1.86±0.32	-0.937
Uterus	+25 µg estradiol-17β in 0.5 ml saline (I.P.)	0.20±.0050	4.53±0.84	-0.927

[a]Minced uterine tissue was incubated in Hank's MEM for 2 hrs at 37 C with varying concentrations of [^{3}H]-estradiol-17β. K_a: tissue association constant. Binding: concentration of tissue binding sites. R: correlation coefficient. Values are expressed ± the standard error of the mean.

events [19] could include changes in cytoplasmic receptor concentration, in nuclear acceptor site concentration, in receptor affinity and cooperativity, or in some set of events which can alter the manner in which estradiol is partitioned within the uterus [20].

Biological Response In Vivo to a Lowered E_2 Dose

Since the "priming" doses of nonradioactive estrogen used in the previous experiment appeared to influence the K_a values, it was desirable to decrease the pretreatment steroid dosage. E_2 dosages of 0.1 µg/50 gm rat body weight have been shown to influence E_2R_c in the uterus [3, 22]; however, it was of special importance to be able to demonstrate a measurable biological response to these lower levels of steroid in order to justify further receptor "modulation" studies.

Vaginal lavage was monitored in two groups of long-term castrate animals under low E_2 stimulation. The first group received estradiol (0.1 µg E_2/0.5 ml saline, I.P.) at t=0. The effect of E_2 treatment

alone on vaginal cell types is shown in Fig. 1A. Round epithelial cells characteristic of a proestrous lavage cell type increased maximally by 50 hours after E_2 treatment with a concomitant decrease in the leucocytic (diestrus 2) lavage cell type. By 120 hours after injection all lavages were again leucocytic. The second group received 0.1 μg estradiol-17β in 0.5 ml saline (I.P.) at t=0 and an injection of 1000 μg progesterone 0.5 ml propylene glycol (S.C.) at t=24 hours (Fig. 1B).

Contrasting the estrogen treatment alone, cornified epithelial cells characteristic of the normal estrous lavage cell type, appeared in the vaginal lavage after treatment with estrogen and progesterone. While one animal showed an "estrous lavage" after E_2 treatment alone, more (n=9) displayed this type of lavage only after estrogen plus progesterone treatment. The presence of cornified cells leading to a lavage classification of "estrus" and "diestrus 1" became apparent only after progesterone exposure. While not mimicking exactly the temporal sequence of vaginal cell types, this treatment did succeed in inducing the morphological cell types associated with estrogenic and progestational events in the rat.

Assay for Cytosolic Receptors

Cytosolic receptors for long term castrate uteri can be demonstrated (Fig. 2A) by the conventional sucrose gradient technique *in vitro* [8]. However, sucrose gradients cannot provide the large number of data points necessary for the Scatchard analysis (Fig. 2B) or the statistical significance necessary to evaluate association constants (K_a), numbers of estradiol binding sites (EBS), etc. Therefore the newer cytosolic receptor assay using hydroxylapatite (HTP)

Figure 1. Biological response to steroid treatment.

(A) Thirty long-term castrate animals received a 0.1 μg intraperitoneal injection of estradiol-17β in saline at t=0. (B) Thirty long-term castrate animals received a 0.1 μg (I.P.) injection of estradiol-17β in saline at t=0 and a subcutaneous injection of 1000 μg progesterone in propylene glycol at t=24 hours. Animals were smeared at the times indicated and lavage types classified with respect to types seen in the cycling animal. Only one animal displayed a cornified cell lavage after estrogen treatment alone. Following progesterone treatment an increase in cornified cell types occurred in all lavage types.

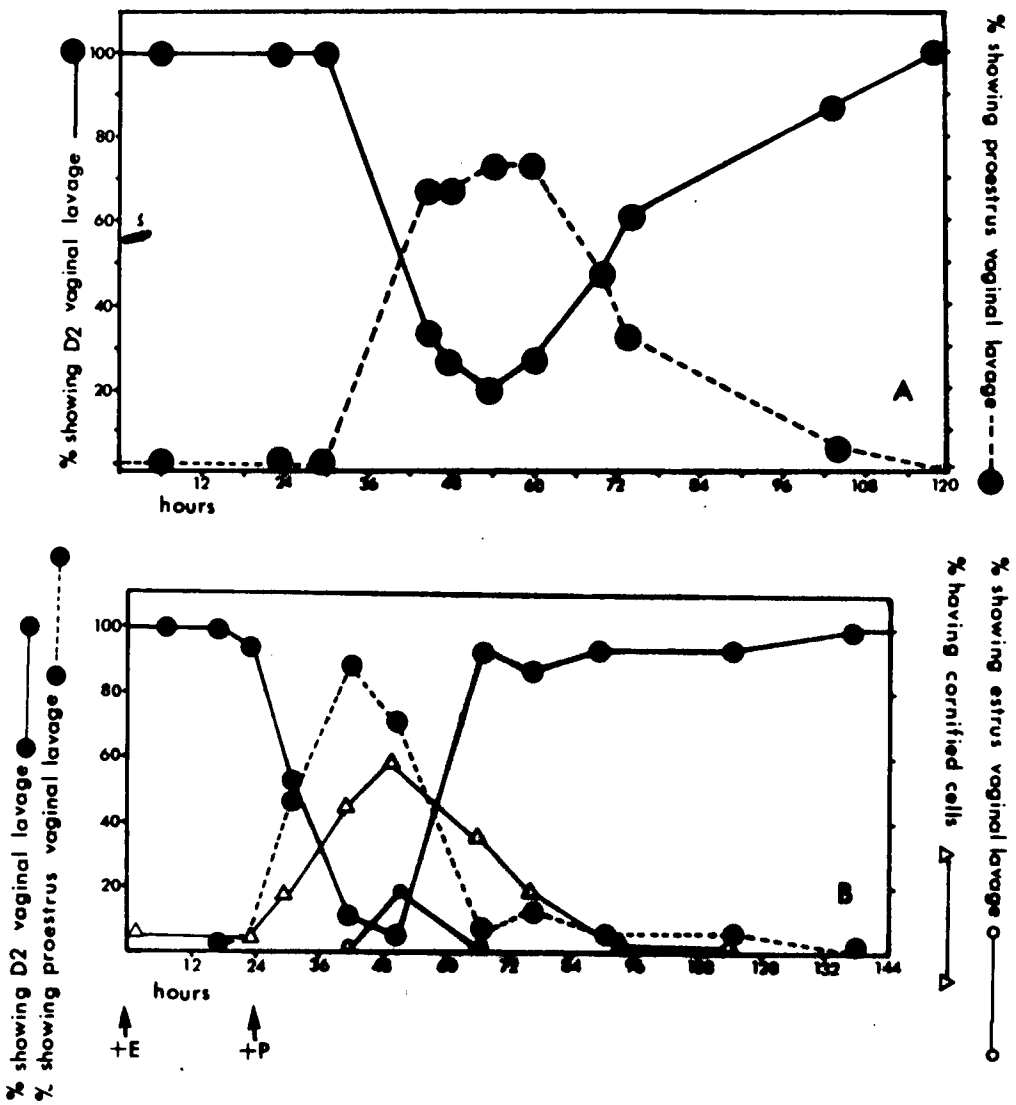
% showing D2 vaginal lavage
% showing proestrus vaginal lavage
hours
A
B
+E
+P
% having cornified cells
% showing estrus vaginal lavage

Figure 1

Figure 2. Sucrose density gradient pattern and Scatchard plot for the uterine cytosolic receptor.

A. Sucrose density gradient pattern. Five uteri were pooled per group. The long-term castrate uteri were incubated *in vitro* for 1 minute at 37 C with [^{3}H]-estradiol-17β (1 μCi/ml). Uteri were homogenized in $TEMK_{50}$ containing 0.4 M KCl and the high speed cytosol was centrifuged in a Beckman Model L ultracentrifuge using a SW-39 rotor for 24 hours at 0-4 C at 38,000 rpm. *In vivo* and cell free *in vitro* preparations yielded similar profiles on these 5-20% sucrose gradients. Fraction 1 is at the bottom of the tube. The arrow identifies the bovine serum albumin peak. The 4S peak at fraction 29 can be eliminated with excess nonradioactive E_2.

B. Scatchard plot. The receptor was separated by adsorption to hydroxylapatite. Each point represents a mean of 4 data points. The line was fitted by linear regression so that the arrow marks an extrapolated value for the receptor of 3.74 picomoles per 225 μl aliquot of cytosol with a correlation coefficient of -0.993. Fifteen animals were used for each determination. Similar plots were obtained for vaginal, pituitary and hypothalamic tissue. The K_a for this plot was calculated to be $0.12 \times 10^{10} M^{-1}$.

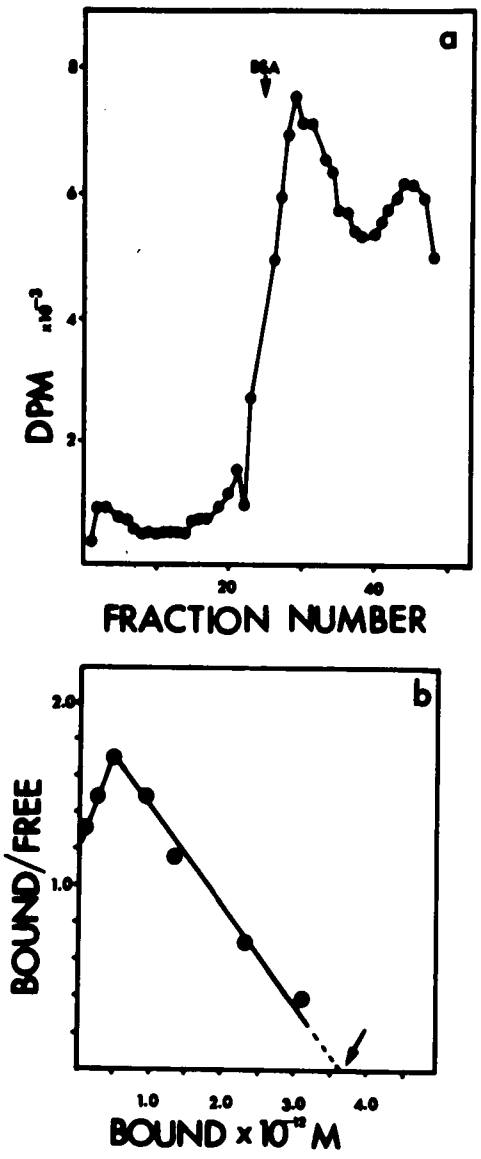

Figure 2

adsorption as described in Part I was employed [15]. The uterine cytosolic E_2R_c in untreated long-term castrate rats is described in Fig. 2B and is representative of all other Scatchard plots.

In these experiments the dose of estradiol-17β was reduced from 25 μg/animal (Table 1) to 0.1 μg/animal in order to approximate a "physiological" dose. Progesterone (1000 μg/animal) was included in the treatment schedule in order to correlate the biological or vaginal lavage changes with the effect of this steroid on cytosolic E_2R_c concentrations. Uterine cytosolic receptor concentrations in saline-injected long-term castrate controls, determined either by "HTP Assay" or by the *in vitro* "E_2 Organ-Binding Assay," were not significantly different. The long-term castrate HTP receptor concentration [15] was $1.53 \pm 0.32 \times 10^{-12}$ moles/uterus versus *in vitro* E_2 Organ-Binding Assay for E_2R_c which was $1.86 \pm 0.32 \times 10^{-12}$ moles/uterus.

Statistical analysis of the mean values in Table 2 and 3 was simplified by a least squares regression analysis performed on Scatchard plots. The mean Scatchard determination, degrees of freedom and student's "t" are indicated for each of the four experimental groups. Tables 2 and 3 summarize the Scatchard analysis results for E_2R_c. Due to the surgery performed four weeks earlier and to the variable amounts of internal scar tissue formed around the oviducts, uterine horns tended to be of different lengths. Thus, normalization was based on protein and DNA content rather than on number of uteri.

When uterine cytosolic receptor content (Table 2, Section A) was expressed per mg protein there was no difference between treatments. However, when normalization was based on DNA content, the estradiol

Table 2. Uterine and Vaginal Cytosol Estradiol Receptor HTP Assay[b]

	Treatment I		Treatment II		Treatment III	
	Saline	"t" Test (I vs II)	0.1µg E_2, 48hrs	"t" Test (II vs III)	0.1µg E_2,48hrs 1.0µg P_4,24-28hrs	"t" Test (I vs III)
A. Uterus: n=35/group						
$K_a x 10^{10} M^{-1}$	0.12±0.01[a]	(0.000)	0.12±0.02	(0.800)	0.10±0.02	(1.208)
EBS$x10^{-12}$moles/mg protein	3.72±0.27	(0.248)	3.81±0.24	(1.345)	3.27±0.32	(1.080)
EBS$x10^{-12}$moles/mg DNA	47.92±3.33	(8.297)*	115.17±7.39	(6.967)*	52.57±5.11	(0.762)
Protein/DNA	12.88±1.88	(9.172)*	30.24±1.58	(8.042)*	16.01±0.97	(2.049)
Correlation coefficient=R	-0.993		-0.948		=0.953	
B. Vagina: n=21/group						
$K_a x 10^{10} M^{-1}$	0.07±0.01	(1.333)	0.05±0.01	(3.098)	0.27±0.07	(2.834)
EBS$x10^{-12}$moles/mg protein	4.94±0.39	(0.138)	4.83±0.70	(1.897)	3.46±0.19	(3.394)
EBS$x10^{-12}$moles/mg protein	119.12±9.43	(0.138)	660.40±95.23	(6.453)*	45.65±2.54	(7.523)*
Protein/DNA	24.12±9.43	(6.691)*	160.89±20.44	(7.211)*	13.20±1.28	(8.439)*
Correlation coefficient=R	-0.968		-0.877		-0.905	

[a]Values are expressed ± standard error of the mean.

[b]Long-term castrate Sprague Dawley rats received treatments via injection *in vivo*. Hydroxylapatite Assay was used to retain hormone receptor complexes. Data were analyzed by linear regression analysis on Scatchard Plots. EBS: estrogen binding sites.

n: Number of determinations used in Scatchard analysis.

*Student's "t" test significantly different at F<0.9995, p>0.0005; degrees of freedom = m+n-2: A. uterus DF = 18; B. vagina DF = 22.

Table 3. Hypothalamic and Pituitary Cytosol Estradiol Receptor HTP Assay

	Treatment I		Treatment II		Treatment III	
	Saline	"t" Test (I vs II)	0.1μg E_2, 48hrs	"t" Test (II vs III)	0.1μg E_2,48hrs+ 1.0μg P_4,24-48hrs	"t" Test (I vs III)
A. Hypothalamus: n=21/group						
$K_a 10^{10}{}_M{}^{-1}$	0.04±0.03[a]	(1.288)	0.15±0.08	(0.300)	0.12±0.06	(1.193)
EBSx10^{-12}moles/mg protein	0.35±0.17	(0.838)	0.52±0.12	(0.536)	0.62±0.15	(1.214)
EBSx10^{-12}moles/mg DNA	20.36±9.64	(0.818)	26.92±6.61	(0.971)	41.37±9.77	(1.531)
Protein/DNA	57.53±3.61	(0.032)	57.57±3.53	(2.996)	70.03±2.20	(2.910)
Correlation coefficient=R	-6.48		-0.744		-0.732	
B. Pituitary: n=21/group						
$K_a 10^{10}{}_M{}^{-1}$	0.11± 0.02	(0.282)	0.12±0.03	(1.342)	0.21±0.06	(0.063)
EBSx10^{-12}moles/mg protein	1.67±0.31	(1.871)	1.14±0.33	(0.055)	1.16±0.15	(1.470)
EBSx10^{-12}moles/mg DNA	113.00±22.40	(2.010)	57.12±16.46	(1.742)	93.01±12.39	(0.781)
Protein/DNA	67.63±2.22	(2.498)	50.12±6.15	(2.012)	80.05±13.31	(0.924)
Correlation coefficient=R	-0.961		-0.893		-0.859	

[a]Values are expressed ± standard error of the mean.

n=Number of determinations used in Scatchard analysis. Conditions are as described in Table 2. Degrees of freedom = m+n-2: A. Hypothamalus DF = 14; B. Pituitary DF = 14.

receptor concentration increased 2-fold in response to a 48 hour pre-treatment with estradiol. This suggests that the increase in estradiol receptor is not due to a simple increase in cell number but parallels an increase in cell size or protein content. When progesterone is present for the last 24 hours of steroid pretreatment, the receptor concentration remained at the original untreated level (Table 2, Section A). Similar findings have been reported for the estrogen stimulated increase in progesterone receptors in the chick oviduct [17].

Even greater changes (5-6 fold) can be seen in rat cytosolic E_2R_c concentration from vaginal tissue (Table 2, Section B). The ratio of protein/DNA in uterine and vaginal tissues parallels the cytosolic estrogen receptor response. This agrees with earlier work on protein/DNA changes at 24-48 hours after estrogen treatment reported by Hamilton *et al.* [21]. These data suggest that the vaginal tissue has a greater hypertropic response to estradiol than uterine tissue or that the uterine tissue has a greater mitotic response than vaginal tissue. It is also possible that a greater percentage of the total vaginal cell population responded to the estrogen treatment. Good correlation coefficients were obtained for these data.

Similar assays were performed on hypothalamic and pituitary cytosols. Table 3 shows that the hypothalamic tissue contained 1/5 to 1/10 as many EBS/mg DNA compared to the pituitary tissue. This correlates well with recent reports in the literature [19, 24]. There were no significant differences in the brain tissue K_a or in the absolute binding site concentration of E_2R_c after 24-48 hours of steroid treatment normalized to either protein or to DNA (Table 3). The protein/DNA ratio remained statistically unchanged.

This evidence suggests that receptors in the hypothalamus and pituitary respond in a manner distinct from the system operating in the uterus or the vagina. Another explanation may be that the techniques used in this, and other studies, were still not sensitive enough to demonstrate the changes which may occur. The correlation coefficients for the hypothalamic determinations were lower than those observed for uterine and vaginal tissue and may reflect the extremely small amounts of material which were being assayed or the possible interfering effect of high levels of lipid typically found in nervous tissue.

Recent reports by several groups have shown no effect on pituitary E_2R_c following ovariectomy even though the fall in uterine E_2R_c was quite significant [23]. Other studies suggest that gonadectomy and/or sex differences do not affect the K_a, the EBS concentration, or the molecular characteristics of the pituitary or hypothalamic E_2R_c concentration [24]. This adds support to these results suggesting that the K_a for all four E_2R_c containing tissues are almost identical.

Many factors may be responsible for the decrease in apparent estrogen receptor concentration when progesterone is present. One plausible explanation is that progesterone interacts with the estrogen receptor and thus changes the estrogen binding site on the receptor. Although progesterone does not compete directly for the estrogen binding site, it could be acting as a negative heterotropic effector and may demonstrate negative cooperativity. Since cooperativity has been reported for estrogen receptors [16, 20, 26], and deviations from linearity (reported to reflect cooperativity) can be observed in these Scatchard plots (Fig. 1B, left hand side), the data have been reevaluated using

the "Hill Plot" equation in order to obtain the Hill coefficient of cooperativity" [25, 27] seen in Table 4. Statistically there are no significant differences in cooperativity for the estradiol cytosol receptor under estradiol pretreatment compared to estrogen plus progesterone. Thus, progesterone is not reducing the affinity of the estrogen receptor for estradiol by some type of heterotropic or allosteric interaction. Correlation coefficients for this data are very good ($R > 0.97$).

Table 4. Hill Coefficients for Uterine and Vaginal Tissue[a]

Treatment	Hill Coefficient	S.E.	R
A. Uterus			
(1) 0.5 ml saline (I.P.)	1.069	0.028	0.998
(2) +0.1 μg E_2-17β 48 hrs	1.142	0.084	0.987
(3) +0.1 μg E_2-17β 48 hrs; +1.0 mg progesterone hrs 24-48	1.070	0.068	0.990
B. Vagina			
(1) 0.5 ml saline (I.P.)	1.023	0.044	0.996
(2) +0.1 μg E_2-17β 48 hrs	1.079	0.101	0.979
(3) 0.1 μg E_2-17β 48 hrs; +1.0 mg progesterone hrs 24-48	1.069	0.061	0.992

[a]Conditions are as described in Table 2, p. 51.

R = Correlation Coefficient.

S.E. = Standard Error of the Mean.

Hill coefficients for uterine and vaginal E_2R_c measured by the HTP assay (range = 1.023 to 1.142) at 0-2 C demonstrate little positive cooperative effect. These results are in contrast to the Hill coefficients reported for uterine cytosolic receptors measured by equilibrium dialysis (range = 1.40 to 1.620) at 0-2 C [26]. Hill coefficients for pituitary and hypothalamic estrogen receptors are of comparable magnitude. None of the tissues show negative cooperativity in the presence of progesterone.

IV. DISCUSSION

Progesterone-mediated decreases in E_2R_c concentration first seemed possible when uterine cytosols from estrogen-progesterone treated long-term castrate rats demonstrated decreased binding on sucrose gradients as compared to cytosols from both untreated controls and estrogen treated long-term castrates. Since 10,000 times more progesterone than estradiol, an amount 10^6 times in excess of a 3 picomole uterine receptor capacity, was being injected it was possible that the vast excess of progesterone competed successfully against [^{3}H]-estradiol for binding to the receptor.

When Scatchard analysis was employed to determine competition as well as concentration of E_2R_c, the competitive contributions by progesterone were found to be negligible. Two separate techniques, each subject to Scatchard analysis, were employed. Both the *in vitro* "E_2 Organ-Binding Assay" and the "Hydroxylapatite E_2R_c Assay" yielded similar values for the receptor concentration per uterus in the saline-injected controls. However, K_a values obtained *in vitro* assay at 37 C were about 4.0 times greater than those determined on hydroxylapatite at 0-2 C. Contributions to the affinity constant by low affinity binding proteins and partition effects would be expected to depress the affinity constant rather than increase it. In fact, an increase in K_a after correction for "non-specific" binding is common to almost all assay techniques, notably charcoal adsorption [15].

The difference in K_a values between these two techniques is interpreted as a consequence of "physiological" conditions. In the

in vitro E_2 Organ-Binding Assay the binding conditions occur at physiological temperature, cellular concentration, and are not terminated by tissue disruption. It does not seem unduly optimistic that binding affinity is tighter under intact conditions.

While nuclear estrogen receptor concentration has not yet been determined, it is rationalized that nuclear translocation has easily occurred after 48 hrs [10, 28]. Thus, effects due to nuclear vs cytoplasmic compartmentalization will not be sufficient to explain these phenomena. Two recent reports support these observations with regard to rat uterine cytosol receptors. These findings under different dose schedules and using different assay techniques, extend progesterone-mediated increases in estrogen receptor concentration to the immature rat uterus [30] and to the monkey oviduct [31].

These data on binding cooperativity remove the possibility of progesterone acting as a negative heterotrophic effector. It supports the emerging concept of independence between binding sites for estrogen in the uterus and vagina and extends this independence to pituitary and hypothalamic estrogen receptors. The clear deviations from linearity in the Scatchard plots (Fig. 2B, left side, p. 47) could be explained in several ways: (a) interference from other cytosolic proteins is maximized at low ligand concentrations, (b) equilibrium times may be greater at low ligand concentrations, especially in heterogeneous cytosolic preparations, (c) the receptor, "protected" by bound steroid, is actually labilized at low "unprotected" ligand concentrations, thus altering the equilibrium parameters of the assay. The deviations from linearity in Scatchard plots do not appear to be indicators of

positive cooperativity when the data are reevaluated as the Hill coefficient (Table 4, p. 54).

Milgrom *et al.* [29] have detailed control mechanisms mediated by estrogen and progesterone for the cytosolic progesterone receptor. They report that progesterone limits the half life of its own progesterone receptor; thus, progesterone may program uterine cell populations in some terminal fashion.

Cidlowski and Muldoon [19] recently reported replenishment phenomenon for pituitary and hypothalamus. It is emphasized that their results cannot be directly compared to these experiments. Their report deals with early events (1-14 hrs) after steroid treatment with estradiol only and their "replenishment" never exceeds control levels. Also their experimental design using a charcoal adsorption assay without Scatchard analysis for E_2R_c measured "relative" rather than "absolute" E_2R_c binding site concentration [19]. Studies are currently underway to apply the HTP E_2R_c assay and Scatchard plotting to the entire time period covering 0-72 hrs to get a better comparison of the sequence of events.

Recent data by Clark *et al.* [32] suggest that other known estrogen antagonists (nafoxidine, CI-628, and clomiphene) mimic the estrogen stimulated increases in polymerase I and II, as well as the increases in RNA, protein, and DNA synthesis. Also, they block or fail to stimulate the "replenishment" of the cytosolic estrogen receptors. Work by several groups [10, 19] using cycloheximide demonstrated an inhibition of receptor replenishment in the absence of protein synthesis. Thus, while it is tempting to speculate about receptor synthesis,

transcriptional control mechanisms, and other processes such as receptor activation or decreased degradation, none can be eliminated at present. Further work is necessary to demonstrate that protein synthesis is actually involved in the replenishment process.

The variability in the comparisons of various data on E_2R_c currently in print [15] serve to re-emphasize the danger in trying to compare numerical values between different animals, target tissues, experimental designs, and assay systems. It is particularly significant, however, that when all four target tissues for estrogen are compared under the same experimental conditions they do not respond to sex steroid feedback or modulation in the same fashion. Indeed, growth and division should not be expected to occur as a response in neural tissue. The principal response in the pituitary or hypothalamus for instance is probably low level synthesis and secretion, hypertrophy or even depolarization, but not mitosis. Thus, it would seem logical that estrogen receptor content in the hypothalamus and pituitary remains unchanged with respect to either protein or DNA after steroid treatment.

Vaginal tissue responds in the same relative fashion as uterine tissue but perhaps with greater hypertrophy and less cell division as shown in Table 2, p. 51. These data indicate an estrogen stimulated increase of estrogen receptor sites from 47.92 to 115.17 x 10^{-12} moles/mg DNA in the uterus; concomitantly, receptors increased from 119.12 to 660.40 x 10^{-12} moles/mg DNA in the vagina.

These experiments performed on mature, long-term castrate rats illustrate that the capacity of reproductive tissues to respond to low levels of sex steroids (estradiol-17β and progesterone) either by

in vivo biological or *in vitro* E_2R_c criteria has been retained even in highly atrophied uterine and vaginal tissue. While this response is characterized by estrogen-mediated increases in cytosolic E_2R_c concentration in uterine and vaginal tissues, it has no effect on long-term pituitary or hypothalamic E_2R_c concentration. Progesterone opposes the E_2-induced increases in E_2R_c. Values for K_a in all four target tissue studies were very similar and were not affected by the steroid treatment.

V. SUMMARY

The effect of pretreatment with estradiol-17β with or without progesterone on cytosolic estrogen receptors was investigated in four different target tissues from long-term castrate Sprague Dawley rats. Receptor assay was performed by hydroxylapatite adsorption and Scatchard analysis. Treatment with estradiol (0.1 μg/rat) for 48 hours *in vivo* increased absolute cytosolic receptor concentrations per mg DNA 3-5 fold for uterine and vaginal tissue, but no increase was observed in pituitary or hypothalamic tissue. These increases in estradiol receptor concentration after 48 hours of estrogen exposure should be distinguished from "replenishment" phenomenon after shorter time periods (15-20 hours) which demonstrate recovery to baseline of "available" estrogen receptors.

The increase in cytosolic estrogen receptors in uterine and vaginal tissue in response to estrogen is opposed by *in vivo* treatment with estrogen plus progesterone. This opposition cannot be explained by competition from progesterone or negative cooperative interaction with progesterone when analyzed by Scatchard plots and Hill coefficients. Deviations from linearity in Scatchard plots are not explainable in terms of positive or negative cooperativity between estrogen and progesterone. These results support the concept of a single, non-interacting binding site for estrogen on the estrogen receptor. Similar K_a values were obtained for receptors in all four target tissue studied.

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PART 3: EFFECTS OF ESTROGEN AND PROGESTERONE ON ESTROGEN CYTOSOLIC RECEPTOR AND RATES OF PROTEIN SYNTHESIS IN RAT UTERUS

I. INTRODUCTION

Uterine tissue contains a high affinity estrogen-binding protein which is reported to be involved in the mechanism of steroid hormone action (for reviews see Jensen and DeSombre [1] and O'Malley and Means [2]). When cells are exposed to estradiol-17β, the E_2 passes across the cell membrane, binds to a specific uterine cytoplasmic protein (E_2R_c) and eventually moves as a "steroid-receptor" complex into the nucleus [1, 2]. As a result, the concentration of available E_2R_c declines and is gradually restored to the initial level of receptor concentration after 20 hours of treatment. Such short-term restoration is termed "replenishment" [3-6, 24]. Continuation of replenishment results in E_2R_c concentrations clearly elevated above basal levels after 24-72 hours. These long-term E_2R_c changes are described in this part of the dissertation. "Modulation" of E_2R_c concentration involves short-term "replenishment" and long term increases above control levels, as well as ddcreases in E_2R_c concentration mediated by progesterone [7, 8]. Progesterone (P_4) has been shown to oppose E_2 mediated increases in E_2R_c concentration by mechanisms other than competitive inhibition or negative cooperative effects on steroid binding [7, 8]. In this investigation steroid mediated modulation of E_2R_c concentration over a 48-hour period is described and the involvement of both E_2R_c activation and degradation during modulation was examined. Experiments were designed to compare modulation of E_2R_c concentration with the steroid mediated changes in rates of protein synthesis measured via [^{35}S]-methionine pulse-labeling in order to establish if estrogen or

progesterone mediated increases in E_2R_c concentration occur as part of a spectrum of hypertropic events or are each specific phenomenon which are selectively distinct from hypertrophy.

II. METHODS AND MATERIALS

Sprague Dawley rats (150-180 gm) were bilaterally ovariectomized and used 4 weeks post-operatively. These animals, referred to in the text as "long-term castrate" females, received either an injection of estradiol-17β (Sigma, 0.1 μg/0.5 ml saline) intraperitoneally or an injection of progesterone (Sigma, 1 μg/0.5 ml propylene glycol) subcutaneously at t=24 hours in addition to the original E_2 injection. Animals were killed by cervical dislocation while under light ether anesthesia. Uterine horns were dissected free of oviduct, vagina, fat or connective tissue and placed in Hank's calcium-free media (CMF) at 0-2 C. Uteri were sliced into 1-2 mm fragments and washed twice in CMF.

Pulse Labeling

Uterine fragments were incubated in Hank's minimal essential media (MEM, GIBCO) containing [^{35}S]-methionine (10 μCi/ml, 381.2 Ci/mM, New England Nuclear Corp.) for 30 minutes at 37 C under an atmosphere of 95% O_2 and 5% CO_2. A flow diagram of this procedure is shown in Fig. 1. Incubation was terminated by chilling (0-2 C). The tissue was immediately washed (4X) with Hank's CMF (4 ml) at 0-2 C in TEMK buffer containing TRIS (pH 7.4, 40 mM), EDTA (1.5 mM), mercaptoethanol (14 mL) and KCl (50 mM). A tissue/buffer ratio of 4 uterine horns/ml was maintained. The homogenate was centrifuged for 15 minutes at 800 g (0-2 C) and the nuclear-myofibrillar pellet assayed for DNA [9].

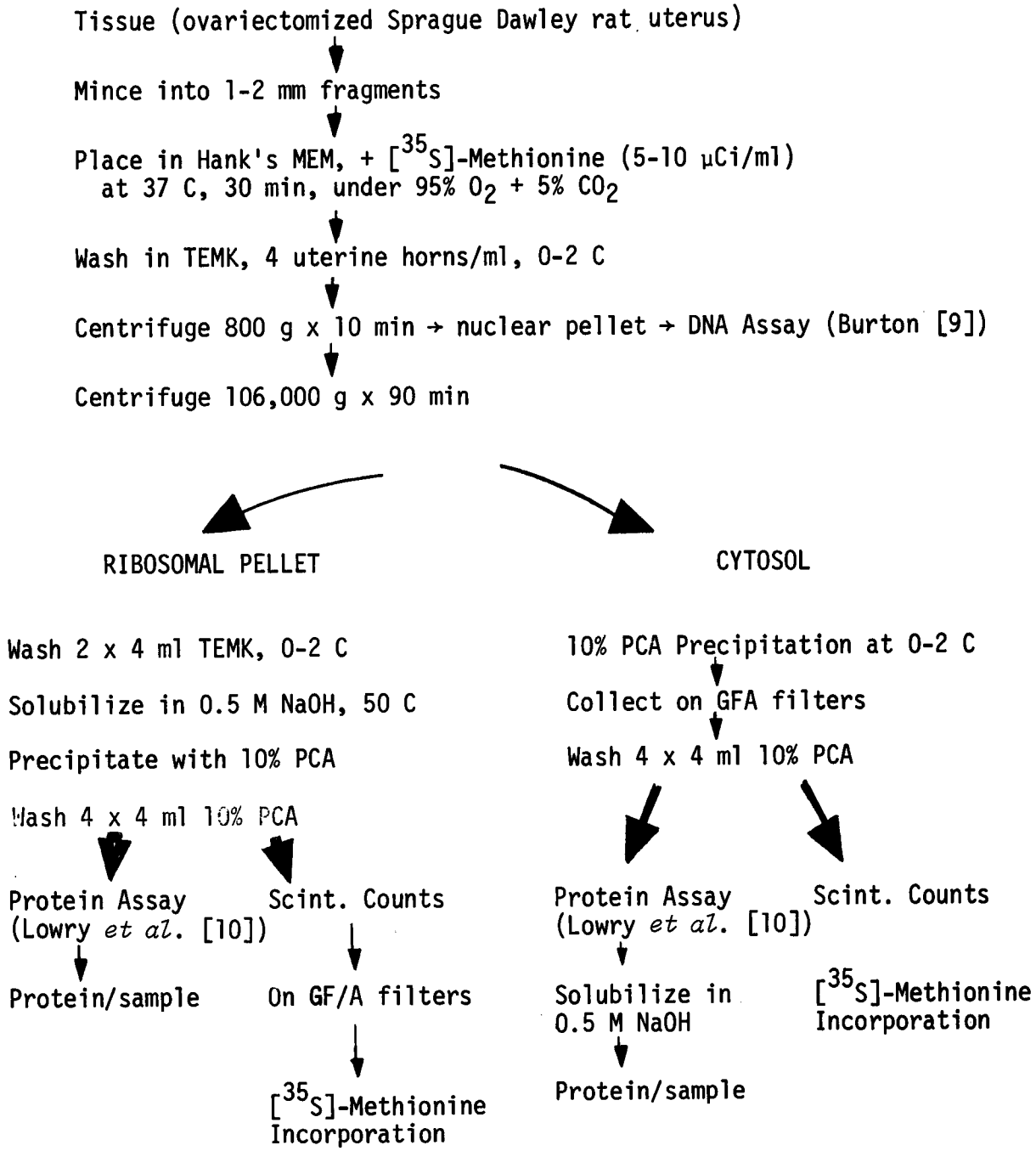


Figure 1. Pulse labeling with [^{35}S]-methionine.

The low speed supernatant was recentrifuged at 106,000 g for 90 minutes in a Beckman fixed-angle rotor yielding, a ribosomal-microsomal pellet, and a high-speed supernatant or "cytosol" fraction. The ribosomal pellet, containing some contaminating soluble protein, free [^{35}S]-methionine, lysosomes and other low density organelles, was washed two times in hypotonic TEMK buffer. The hypotonic treatments were sufficient to release both lysosomal and mitochondrial soluble protein, and to substantially reduce counts due to free [^{35}S]-methionine, [^{35}S]-methionine-tRNA, and aminoacyl transferase charged with [^{35}S]-methionine. The washed pellet was solubilized in 0.5 M NaOH at 50 C. Following a 1:1 dilution with TEMK buffer, the ribosomal protein was precipitated with 10% perchloric acid (PCA) onto Whatman GF/A glass filters and washed with 4 x 4 ml of 10% PCA. The filters were then transferred into liquid scintillation vials containing 22% Triton X100 (Rohm-Haas) and PPO-POPOP-Toluene fluor (2,5 diphenyloxazole [4 gm], and 1,4 bis [2-(5 phenyloxzoyl)] benzene [0.5 gm] in 1 liter toluene).

Preparation of Acid Insoluble Protein

The soluble proteins in the cytosol fraction were precipitated with an equal volume of 20% (PCA) at 0-2 C. The precipitates were collected on GF/A glass fiber filters (Whatman Industries), washed with 4 x 4 ml 10% PCA and counted directly in liquid scintillation vials. Protein determinations were made on samples redissolved in 0.5 M NaOH [10]. Duplicate experiments, in which soluble protein was precipitated with 20% trichloroacetic acid (TCA), washed twice with 20% TCA and three times with ether, gave isotope incorporation data which were

indistinguishable from the PCA precipitation. [^{35}S]-methionine could easily be distinguished during scintillation counting from [^{3}H]-estradiol because of its higher energy emission.

Preparation of the "Nuclear" Fraction

The nuclear-myofibrillar pellet, obtained by low speed centrifugation at 800 g, was resuspended in 0.5 M PCA and precipitated by centrifugation at 0-2 C. The pellet was then washed 1 x 4 ml with ethanol (95%), 1 x 4 ml ethanol:ether (3:1), and 2 x 4 ml 10% PCA. DNA was hydrolyzed by heating for 45 minutes at 80 C and assayed according to Burton [9]. The acid insoluble material was resuspended in 0.25 M NaOH and sampled for [^{35}S]-methionine incorporation into protein.

Assay of Estrogen Cytosolic Receptors

High speed cytosol fractions were assayed for E_2R_c concentrations using the "batch" hydroxylapatite (HTP) method described elsewhere [11]. Aliquots of cytosol (225 µl) were incubated with saturating amounts of [^{3}H]-estradiol-17β (80-100 Ci/mM, New England Nuclear Corp., 4 x 10^{-12} M per 225 µl cytosol). Incubation (0-2 C) was terminated after 10 hours by the addition of 1.2 ml of hydroxylapatite suspension [10 gm DNA grade hydroxylapatite (Biorad Industries) per 100 ml TEMK buffer)]. Adsorption of E_2R_c to HTP was allowed to proceed for 45 minutes at 0-2 C. HTP with E_2R_c adsorbed was then centrifuged, washed 5 x 1 ml with TEMK, and [^{3}H]-E_2 was extracted 2 x 1 ml with 100% ethanol.

Liquid scintillation counting was performed on the tritium-containing ethanol extract using the Triton X100 fluor described above and gave a counting efficiency of 30-40%. Individual quench correction

was performed on each sample with an external standard. Correction for non-specific binding was routinely made by subtracting [^{3}H]-E_2 bound in the presence of a 1000 fold excess of diethylstilbesterol (DES). All points were run in replicates of four [8].

Rates of Decay of Estrogen Receptors

Three experimental treatment groups containing 18 rats each (page 74) were designed. Group A (control) was injected with 0.5 ml saline (I.P.) at t=0; Group B received 0.1 µg E_2 in 0.5 ml saline (I.P.) at t=0; and Group C received 0.1 µg E_2 at t=0 plus 1000 µg P_4 in 0.5 ml propylene glycol (S.C.) at t=24 hours. All animals were killed after 36 hours. Mixed groups designated AB, BC and AC were formed by combining equal portions (1:1, v:v) of cytosol fractions from A, B, or C respectively. Homogenization was performed in 10 ml of TEMK buffer at 0-2 C followed by centrifugations at 800 g, and at 106,000 g as described (Fig. 1, p. 68). The high speed cytosol fraction was diluted to 37 ml with TEMK buffer and delivered into individual assay tubes. Incubation with saturating amounts of [^{3}H]-estradiol-17β (2×10^{-12} M/225 µl cytosol) was performed for 12 hours at 0-2 C. The six experimental cytosols were then tested for potential activation or degradation of E_2R_c at 0 C and at 22 C. Control cytosols were always included in the experimental design for all temperatures and times in order to compensate for the expected rate of E_2R_c decay ($t_{1/2}$ = 5-6 days [3]) during the course of the experiment and assay. All points were run as replicates of four and completed within ± 10 seconds per experimental time category. A post-experimental period (0-2 C for 12 hours) during which equilibrium

between free steroid and receptor could be re-established was included in order that receptor-bound activity would not reflect any accelerated dissociation which would be caused by elevated temperatures. Lowry assay for protein was performed [10].

III. RESULTS

E_2R_c Modulation by E_2 versus Time and Its Opposition by P_4

Comparisons between the time course of E_2R_c modulation and P_4-mediated opposition to these changes in receptor concentration are presented in Fig. 2A and 2B. Because protein and DNA concentrations are changing differentially in the uterus following initial E_2 stimulation, results were individually normalized for both parameters.

Specific activity or E_2R_c concentration per mg protein (Fig. 2A) remained below control levels for 24 hours in animals treated with estrogen alone and became maximal 36-40 hours after E_2 treatment. About 42 hours after E_2 treatment, specific activity of E_2R_c began to decline due to increases in uterine soluble protein. Increased E_2R_c/mg protein failed to occur in animals "primed" with E_2 and then treated with P_4 at t=24 hours.

E_2R_c concentration in rats injected with E_2 alone rose above control levels per mg DNA between 12-24 hours (Fig. 2B). After P_4 treatment (at t=24 hours) E_2R_c/mg DNA declined to below control levels within 4 hours. This rapid opposition to increased E_2R_c concentration may result from decreased E_2R_c synthesis, decreased E_2R_c replenishment, decreased activation of a "precursor" molecule, or increased E_2R_c degradation.

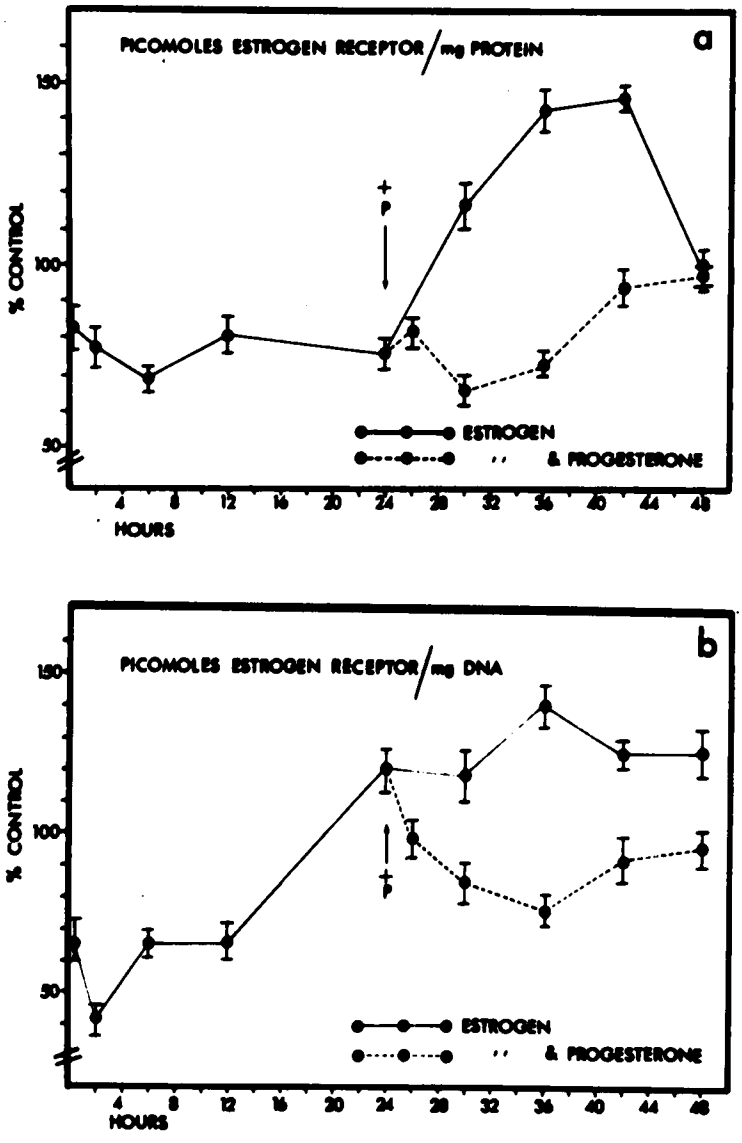
Estrogen Receptor Activation or Degradation

Receptor activation may explain long-term E_2R_c increases and E_2R_c degradation may explain progesterone mediated decreases in E_2R_c

Figure 2. Changes in estrogen receptor concentration following treatment with E_2 or E_2 and P_4.

At t=0 long-term castrate rats received 0.1 μg estradiol-17β in saline (solid line). At t=24 hours a second group of long-term castrate rats received 1000 μg progesterone (S.C.) in propylene glycol (dashed line) marked by the arrow (+P). Replicates of four were performed for each determination and results were expressed as mean ± standard error of the mean with the saline-injected control group representing 100%.

A. Results are expressed per mg soluble protein in the cytosol fraction.
B. Results are expressed per mg DNA.


a
PICOMOLES ESTROGEN RECEPTOR/mg PROTEIN
% CONTROL
150
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4 8 12 16 20 24 28 32 36 40 44 48
HOURS
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PICOMOLES ESTROGEN RECEPTOR/mg DNA
% CONTROL
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ESTROGEN
" & PROGESTERONE
4 8 12 16 20 24 28 32 36 40 44 48
HOURS

Figure 2

concentration. Both activation and degradation were therefore explored as possible processes responsible for modulation.

Uterine cytosol preparations were homogenized in a hypotonic buffer in order to swell and rupture most lysosomal particles and release their contents into the soluble fraction. The soluble fraction contained, therefore, both lysosomal and cytosolic factors.

If inactive E_2R_c precursors exist, cytosols from estrogen treated animals should be capable of activating these receptor "precursors" in control cytosols. Conversely, cytosols from P_4 treated animals might demonstrate increased degradation of E_2R_c when mixed with cytosols from control or estrogen treated animals.

Based on the information in Fig. 2, an experiment was designed to terminate after 36 hours of steroid treatment when differences in E_2R_c/mg DNA or E_2R_c/mg protein were at their greatest.

In this experiment both E_2 mediated increases as well as P_4 mediated decreases in E_2R_c (DPM/225 µl or DPM/mg protein) were apparent (Table 1). Thus, "modulation" was occurring in the system examined at 36 hours. Cytosol fractions labeled A, B, and C were examined individually as well as when combined in 1:1 proportions. All fractions were examined for changes in the rate of E_2R_c decay. Cytosols were either held for varying periods of time at 22 C, rapidly cooled and equilibrium re-established at 0-2 C for 12 hours, or cytosols were kept at 0-2 C and assayed for E_2R_c after varying intervals of time. The results of these experiments (Fig. 3) were subjected to linear regression analysis and are expressed as the linear equation:

$Y = bx + a$, where b = rate of decay and a = initial activity.

Table 1. Baseline Parameters for Rat Uterine Cytosol Fractions Used in Receptor Decay Determination

In vivo Treatment	I Cytosol Fraction µg protein/ml ± S.E.M.	II E_2R_c DPM*/225 µl ± S.E.M.	III Specific Activity E_2R_c DPM*/mg protein ± S.E.M.
A. Control	496.4 ± 15.9	49,664.3 ± 1,065.9	419,752.0 ± 9,012.5
B. Estrogen	884.7 ± 9.4	102,346.6 ± 1,616.9	538,383.0 ± 8,505.5
C. Estrogen and Progesterone	1024.0 ± 52.9	70,076.3 ± 414.5	303,886.8 ± 1,797.5

A. Saline Control, 0.5 ml/rat (I.P.)

B. Animals were injected with 0.1 µg estradiol-17β at t=0 and sacrificed after 36 hours.

C. Animals were injected with 0.1 µg estradiol-17β at t=0, received 1000 µg progesterone at 24 hours and were sacrificed at 36 hours.

*DPM refers to specifically bound [^{3}H]-estradiol (E_2R_c) after correction for non-specific binding using 1000X excess DES (± S.E.M. = standard error of the mean).

Figure 3. Receptor Decay Determination.

Decay rates for E_2R_C following 36 hours *in vivo* steroid treatment. Group A received saline only, Group B received 0.1 µg E_2 (I.P.) in saline, and Group C received 0.1 µg E_2 and 1000 µg P_4. Treatment was as described in Fig. 2, p. 74. Groups designated AB (Open circles = control + E_2), AC (crosses = control + P_4), BC (solid circles = E_2 + P_4) are 1:1 combinations of Groups A, B, or C. Decay was examined at 0 C as well as 22 C. Equilibrium was re-established following exposure at 22 C for periods of time shown on the X axis. Linear regression analysis was performed on all data points and is fitted to the equation shown. Compensation for the normally expected decay of E_2R_C over the course of the experiment was made by including parallel reference cytosols which were not subject to temperature manipulations. Replicates of four were made for all determinations.

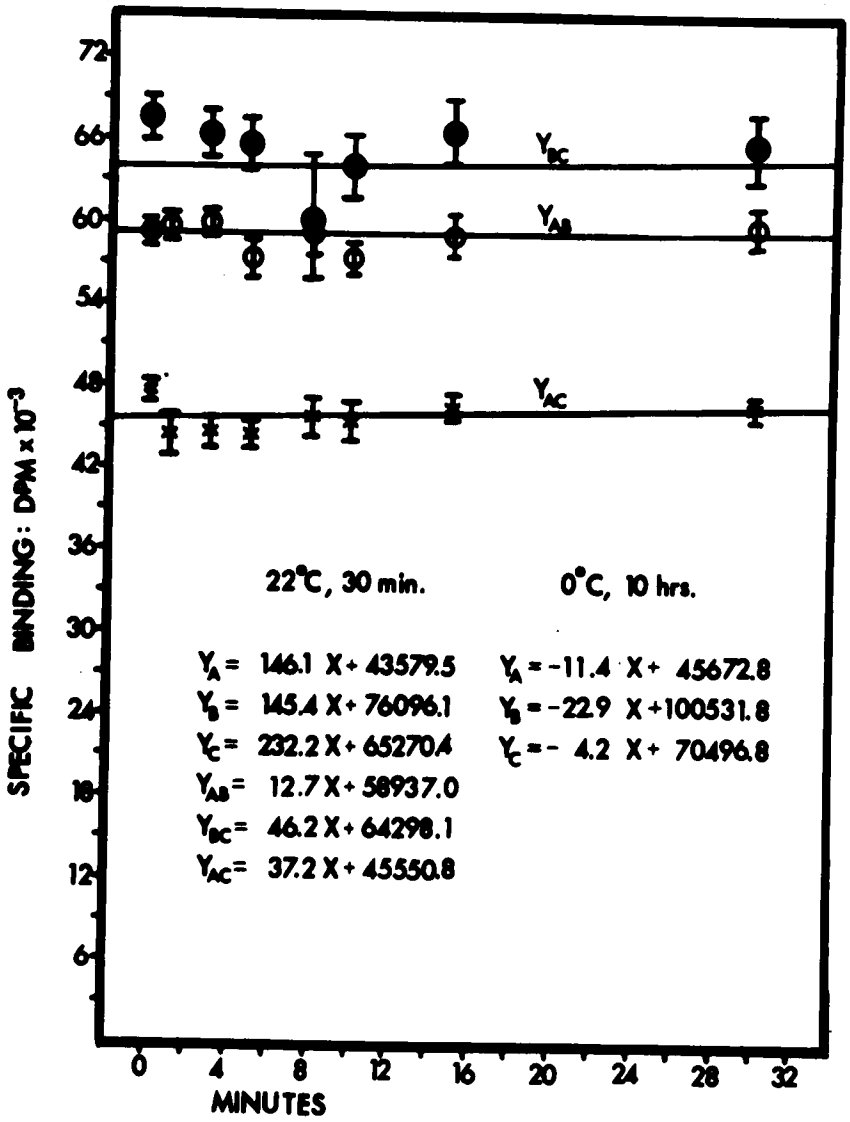
SPECIFIC BINDING: DPM x 10⁻³
72
66
60
54
48
42
36
30
24
18
12
6
0
4
8
12
16
20
24
28
32
MINUTES
Y_BC
Y_AB
Y_AC
22°C, 30 min.
Y_A = 146.1 X + 43579.5
Y_B = 145.4 X + 76096.1
Y_C = 232.2 X + 65270.4
Y_AB = 12.7 X + 58937.0
Y_BC = 46.2 X + 64298.1
Y_AC = 37.2 X + 45550.8
0°C, 10 hrs.
Y_A = -11.4 X + 45672.8
Y_B = -22.9 X + 100531.8
Y_C = - 4.2 X + 70496.8

Figure 3

Long term decay rates (0 C, 10 hours) for E_2R_c did not differ significantly between cytosols from any of the treatment groups (Y_A, Y_B, or Y_C) as judged by least squares regression analysis of the reaction slopes (Table 2, Section A). In fact, the P_4 treated group showed the slowest rate of E_2R_c decay (Y_C = -4.2 CPM/min).

Short-term decay rates (22 C, 30 minutes) of E_2R_c showed no detectable differences in decay betwwen cytosols from any treatment group. In fact, the least squares slope is slightly positive for all groups at 22 C and reflects the fact that no decay was detectable (Table 2).

In the combinatorial experiments performed at 22 C, no increase in E_2R_c decay could be detected (Fig. 3). A numerical reduction in least squares slope occurred for all groups at 22 C; however, this value changed the least for "P_4 + E_2" (Y_{BC}) and for "control + P_4" (Y_{AC}) combinatorials. None of the combinatorials yielded significantly different decay rates (Table 2, Section C, mixtures).

The results in Figure 3 indicate that soluble factors mediating activation or degradation were not functionally detectable. Values for combinatorials Y_{AB}, Y_{AC}, and Y_{BC} can be predicted by averaging intercepts from Y_A, Y_B, or Y_C at 22 C. A close correspondence was observed between predicted and empirical ("actual") intercept values. This correspondence is further supported by internal controls which clearly demonstrated that any decreased concentration in the raw data for E_2R_c over time could be explained by slow, expected rates of receptor decay equivalent to the reported $t_{1/2}$ for E_2R_c of 5-6 days [3].

Table 2. Statistical Comparison of Receptor Decay Rates

In vivo Treatment	Rate of Decay (DPM/min ± S.E.M.) Incubated at 0-2 C (df=4)	Incubated at 22 C (df=14)	Mixtures Incubated at 22 C (df=14)
A. Saline Control	-11.44 ± 5.39	146.12 ± 93.63	12.694 ± 46.70
"t" test	A vs B = 0.719	A vs B = 0.001	AB vs BC = 0.286
B. Estrogen	-22.88 ± 14.97	145.41 ± 531.54	41.16 ± 107.24
"t" test	B vs C = 1.205	B vs C = 0.155	BC vs AC = 0.077
C. Estrogen and Progesterone	-4.20 ± 4.05	232.15 ± 174.73	37.15 ± 47.21
"t" test	A vs C = 1.074	A vs C = 0.434	AB vs AC = 0.368

Treatment groups were the same as in Table 1, p. 78. Rates of decay were obtained from linear regression analysis of mean DPM specifically bound to receptor at different periods of time. Combinational cytosols are designated AB, BC, AC. Student's "t" comparisons were made as indicated, and no comparison was found to be significantly different at $F > 0.90$, $P < 0.1$ (df = degrees of freedom).

Therefore, soluble degradative factors were not responsible for the rapid decrease in E_2R_c concentration caused by progesterone treatment.

Effect of Estrogen and Progesterone Treatment on Translation

Ribosomal incorporation. Pulse labeling with [^{35}S]-methionine into total ribosomal protein began increasing 2-4 hours after E_2 treatment, continued to a maximum of 24 hours and then declined to control rates by 30 hours (Fig. 4A). Progesterone treatment prolonged the elevated level of [^{35}S]-methionine incorporation into the ribosomal fraction. The E_2 plus P_4-treated group showed an extended high level

Figure 4. Rate of incorporation of [^{35}S]-methionine into uterine ribosomal protein.

The *in vivo* animal treatment was described in Fig. 2, p. 74.

A. Total incorporation of [^{35}S]-methionine into ribosomal protein. Values expressed are CPM ± S.E.M. (N=4) in equivalent experimental aliquots and should be multiplied x 10 for values occurring in the uterus.

B. Specific incorporation [^{35}S]-methionine into the ribosomal protein. Values expressed are CPM/mg protein ± S.E.M. (N=4) contained in ribosomal protein after solubilization in 0.5 N NaOH at 50 C.

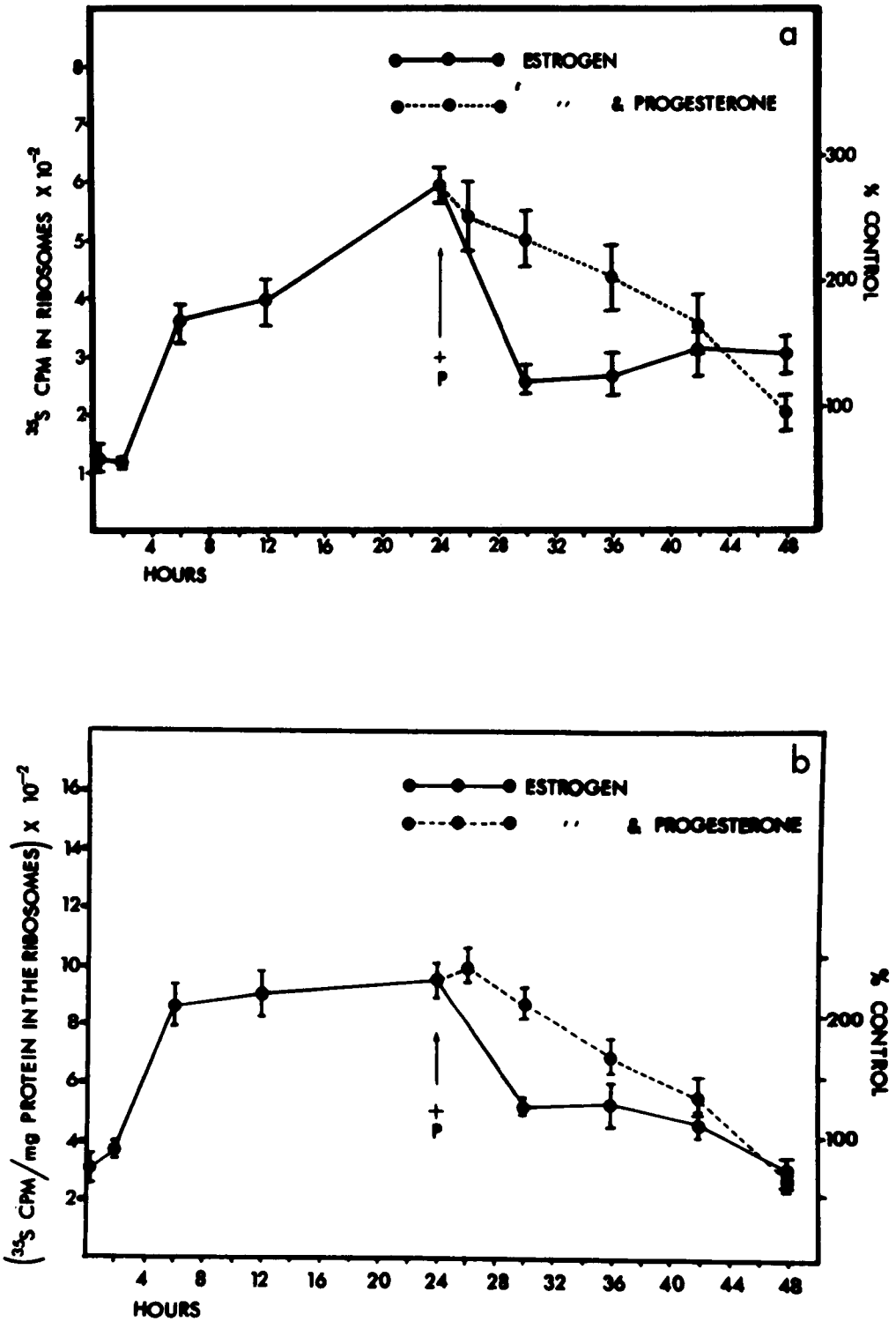
a
ESTROGEN
" & PROGESTERONE
^{35}S CPM IN RIBOSOMES X 10^{-2}
% CONTROL
HOURS
+ P
b
ESTROGEN
" & PROGESTERONE
(^{35}S CPM/mg PROTEIN IN THE RIBOSOMES) X 10^{-2}
% CONTROL
HOURS
+ P

Figure 4

of incorporation and returned to control rates only after 42 hours. Changes in synthetic activity very likely reflect changes in numbers of ribosomes, rates of polysome formation, or changes in functional capacity mediated through increased mRNA availability as has already been reported after E_2 treatment [12-15].

When the specific rate of [^{35}S]-methionine incorporation into the ribosomal fraction was examined (Fig. 4B), the incorporation of [^{35}S]/mg ribosomal protein rose within 6 hours to a constant maximum which was maintained for 24 hours after E_2 treatment. A decline in specific incorporation to baseline occurred by 48 hours. Treatment with P_4 at 24 hours prolonged the E_2-stimulated increase in [^{35}S]-methionine incorporated into the ribosomal fraction. These increases reflect increased synthetic parameters which utilize ribosomes in a constant near maximal manner. Hence, P_4 treatment at 24 hours prolonged a maximal utilization of the available ribosomal translational components. Since an early lag did occur in response to E_2 treatment it is likely that all translational components were not initially available for maximal ribosomal utilization.

Incorporation into Soluble Protein. Examination of the rate of incorporation of [^{35}S]-methionine into uterine, acid precipitable cytosolic protein showed an increase by 8 hours after E_2 treatment when normalized per mg protein (Fig. 5A) and also when normalized per mg DNA (Fig. 5B). In addition, for the first four hours following estradiol treatment, rates of protein synthesis were below saline control levels (=100%) as has already been reported [16, 17]. This correlates well with an initial fall following E_2 stimulation in the relative

Figure 5. Rate of incorporation of [^{35}S]-methionine into acid precipitable uterine cytosol proteins.

The *in vivo* animal treatment was described in Fig. 2, p. 54.

A. Results were expressed as CPM/mg protein ± S.E.M. (N=4).

B. Results were expressed as CPM/mg DNA ± S.E.M. (N=4).

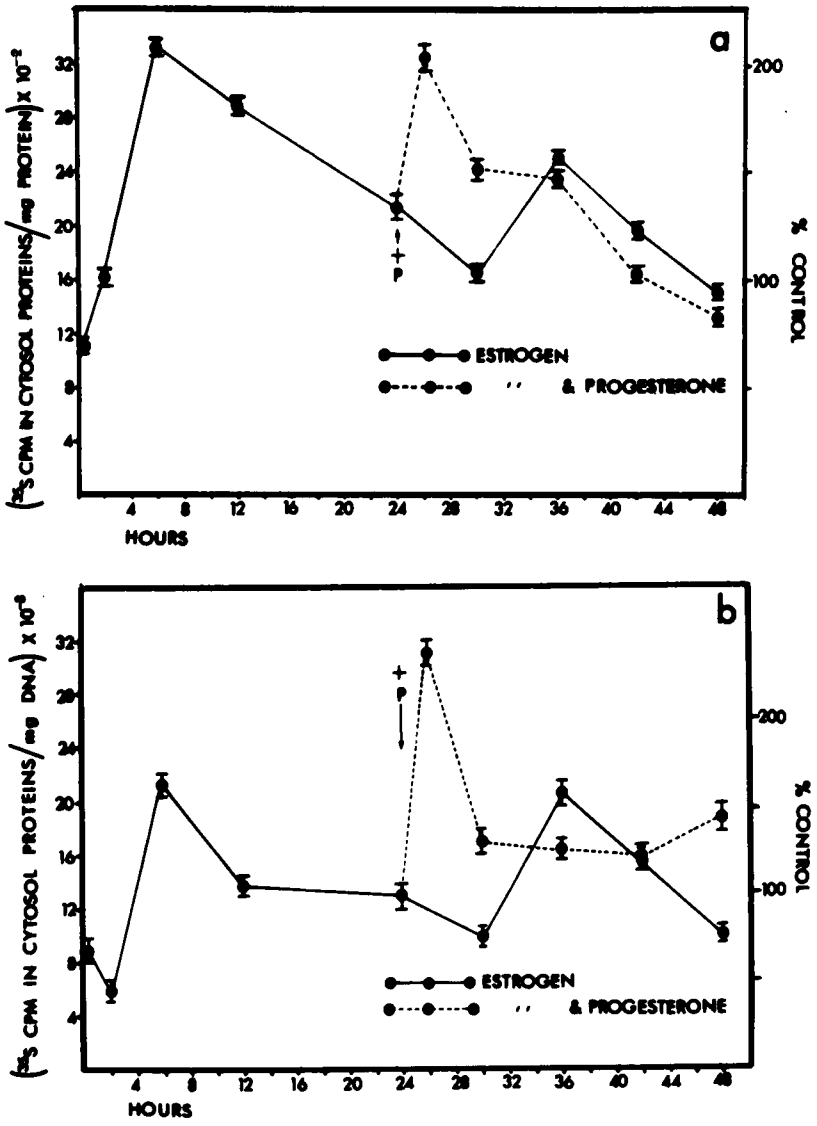

Figure 5

rate of synthesis of uterine "induced protein" reported recently by Katzenellenbogen [18].

Following P_4 treatment at 24 hours, a sharp increase in the rate of incorporation into cytosol proteins occurred within 2 hours. While progesterone decreased E_2R_c concentration, it concomitantly mediated an increase in the rate of incorporation of labeled [^{35}S]-methionine into soluble uterine protein. Hence, decreases in E_2R_c concentration which are mediated by P_4 are not coordinated with decreased rates of protein synthesis.

Incorporation into the "Nuclear" Fraction. The rate of incorporation of [^{35}S]-methionine into the uterine "nuclear" fraction appeared similar to the incorporation into cytosol protein. Increased activity was seen by 8 hours after E_2 treatment at 0 hours and within 2 hours following the P_4 treatment at 24 hours (Fig. 6). This nuclear preparation was not purified to homogeneity, but incorporation can be attributed primarily to proteins associated with the nuclear and membraneous fractions. The effects of E_2 treatment on the rate of nuclear protein synthesis are similar to those reported by Means and Hamilton [16].

As with soluble cytosol proteins, E_2 and P_4 both increase the rates of nuclear protein synthesis.

Figure 6. Rate of incorporation of [^{35}S]-methionine into the "nuclear" fraction.

The *in vivo* animal treatment was described in Fig. 2, p. 54. Results are expressed as [^{35}S] CPM/mg DNA ± S.E.M. (N=4).

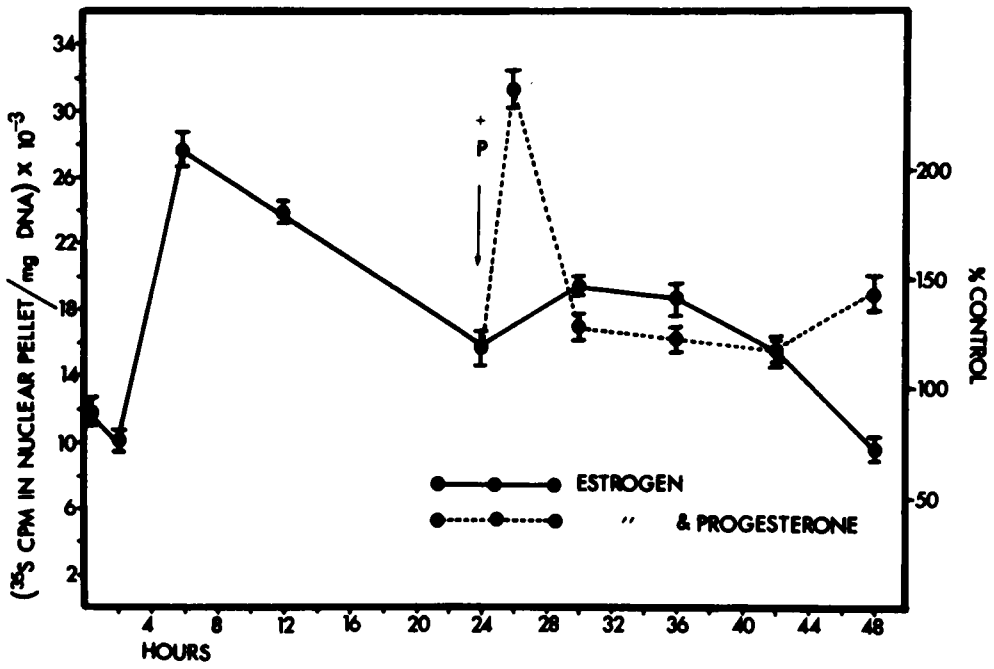
(35S CPM IN NUCLEAR PELLET/mg DNA) X 10-3
% CONTROL
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Figure 6

IV. DISCUSSION

Progesterone (P_4) has been shown to decrease or oppose long-term estrogen (E_2)-mediated increases in E_2R_c concentration in the rat uterus [7, 8, 26]. This opposition could not be attributed to competitive inhibition or to negative cooperative effects on the estrogen binding site [7, 8]. This report describes the temporal sequence of events contributing to "modulation" of E_2R_c compared to changes in the rate of uterine protein synthesis monitored via pulse-labeling.

The total E_2R_c concentration during the early replenishment period showed maximum depletion within 2-6 hours after E_2 treatment. This was followed by a return to control E_2R_c/mg DNA levels by approximately 18-20 hours (Fig. 2B, p. 74) which is consistent with the data reported by Sarff and Gorski [3] and Cidlowski and Muldoon [5]. After 24 hours of E_2 stimulation *in vivo*, the concentration of E_2R_c normalized to either protein or DNA (Fig. 2A and 2B) was well above control values [18]. P_4 opposition to the increased E_2R_c concentration was shown to occur rapidly in time. The effect of P_4 was particularly convincing since it occurred when concentrations of E_2R_c were clearly above saline injected control levels.

The modulation of uterine E_2R_c concentration is apparently comprised of an early restoration phase ("replenishment") as well as a later phase in which E_2R_c concentration is increased above control levels. The replenishment phase, which occurred in the first 0-20 hours after E_2 treatment, has been shown to be sensitive to cyclohexamide [3-6]. While work with inhibitors suggests that E_2R_c synthesis must

occur during early replenishment, it is also conceivable that synthesis of some other protein(s) is required to recycle existing receptor already involved in mediation of estrogenic signals [6]. In addition, little information exists as to what extent excess nonradioactive estradiol from the "priming" treatment dilutes the specific activity of the [^{3}H]-estradiol in the assay during the early phases of replenishment. Considering these complexities it is particularly difficult to inquire about molecular events in the early replenishment phase of E_2R_c modulation. This investigation focused on the later phase of modulation where increased concentrations of E_2R_c were clearly above control levels.

Progesterone might have opposed E_2-mediated increases in E_2R_c by accelerated degradation of receptor. Experiments were designed to measure degradation rates of E_2R_c and also to inquire whether replenishment resulted from activation of existing stores of "receptor precursor." Such a hypothetical activation should not be confused with recycling of existing E_2R_c which was not studied in this experimental design. E_2R_c concentrations reached maximal levels by 36 hours after initial E_2 treatment. Experiments designed to terminate at 36 hours showed no evidence for receptor activation or degradation. This evidence therefore suggests that E_2 mediated increases in E_2R_c concentration may primarily reflect increased synthesis of the binding protein. Since both E_2 and P_4 promote hypertrophy in terms of increased rates of protein synthesis, correlation of P_4 with decreased E_2R_c concentration supports a model where changes in E_2R_c concentration are independent of hypertrophy. Hence, variation in E_2R_c concentrations

is not merely part of a spectrum of synthetic events which occur as a function of changes in total protein synthesis.

Since E_2R_c concentration is specifically increased by estradiol and selectively decreased by progesterone, estrogen mediated modulation may represent a mechanism of molecular amplification which contributes in part to the cascading growth observed in the uterus. Progesterone may mediate its antagonism to E_2 stimulation [2, 20-26] by selectively decreasing the synthesis of the binding protein. It is evident that P_4 does not limit all translational (and/or transcriptional) processes since rates of general protein synthesis increase concomitantly with decreases in E_2R_c concentration.

Pulse-labeling studies were performed with [^{35}S]-methionine since it has a high rate of incorporation into uterine tissue components [23]. Rates of incorporation into the ribosomal fractions support a model in which both E_2 and P_4 can stimulate a maximal utilization of ribosomes. Our data are consistent with the report of increased ribosome formation 4-12 hours after E_2 treatment by Moore and Hamilton [12] as well as with the reports concerning increased polyribosome formation by Teng and Hamilton [13], increased capacity by Greenman and Kenny [14], and increased rate of peptide chain initiation by Suvatte and Hagerman [15] following E_2 treatment.

The fact that synthetic rates in the ribosomal fraction do not mirror synthetic rates in the cytosol or nuclear fraction suggests that ribosomes represent a limited capacity component which is maximally utilized; hence, protein synthesis may be limited more stringently by transcriptional controls.

An alternative speculation to decreased synthesis of E_2R_c mediated by P_4 is that P_4 promotes sequestration of E_2R_c in the nucleus. The present experimental design challenges this hypothesis. Other researchers have shown that nuclear bound estradiol (E_2R_n) is exponentially reduced *in vivo* to approximately 10% of cytoplasmic estrogen receptor (E_2R_c) capacity by 16 hours after treatment with E_2. The present studies treat with P_4 at 24 hours, well after the 16 hour point at which nuclear activity is maximally reduced. In addition [^{3}H]-estradiol is introduced and bound to receptor in the high speed supernate *in vitro* in the absence of nuclei. If progesterone could prolong nuclear binding or sequestration of what little E_2R_n remained in the nucleus, this amount of E_2R_n would not be sufficient to explain the magnitude of decreased cytoplasmic receptor observed. Under the conditions employed, a sequestration hypothesis would be more tenable for the trapping of binding protein which is not associated with estradiol; however, such speculation is clearly unsupported in that it supposes that either P_4 can physically interact with the "unloaded" binding protein promoting nuclear retention, or that P_4 influences nuclear interaction so that "unloaded" binding protein is retained in a nuclear position inaccessible to E_2.

Evidence does exist that P_4 promotes binding of E_2R_n to purified rabbit uterine chromatin [28]. It is emphasized, however, that the experimental design in this dissertation was constructed so that levels of nuclear E_2R_c were well reduced, and that cytoplasmic binding protein was separated from the nuclear fraction prior to its interaction with [^{3}H]-estradiol. While P_4 may enhance nuclear binding parameters for

E_2R_n this enhancement does not appear in the experiments described here.

Both E_2 and P_4 mediated increases in rates of cytosol and nuclear protein synthesis (Figs. 5 and 6, pp. 85 and 88). Incorporation into nuclear protein was consistent with the depression of nuclear protein synthesis (0-2 hours after E_2 treatment) and subsequent increase (after 4 hours) reported by Means and Hamilton [16].

These experiments reconfirmed incorporation into rat cytosolic protein after E_2 treatment as demonstrated by Means and Hamilton [16] and Barnea and Gorski [17]. In addition, synthetic events specifically directed by progesterone were described.

V. SUMMARY

Changes in total E_2R_c concentrations were compared to changes in rates of protein synthesis after steroid treatment in uterine tissue of long-term castrate Sprague Dawley rats. Animals were treated with estradiol-17β (E_2) alone or with progesterone (P_4) after 24 hours of E_2 "priming." E_2R_c concentration was "modulated" by E_2 (0.1 g) in two distinct temporal phases: A. Short-term depletion with restoration to basal E_2R_c concentration occurred after 20 hours of treatment. B. Increases above basal levels were seen after 20-24 hours. Twenty-four hours after E_2 treatment, the administration of P_4 (1 mg) caused the concentration of E_2R_c to decrease within 2 hours. As determined from E_2R_c decay experiments, modulation of E_2R_c by E_2 or P_4 was not caused by activation of some soluble receptor precursor nor by degradation of functional E_2R_c. Uterine protein synthesis (monitored by [^{35}S]-methionine incorporation and measured under the same treatment schedule as used for E_2R_c quantitation) revealed that the progesterone-stimulated incorporation of [^{35}S]-methionine into ribosomal, cytoplasmic and nuclear fractions resembled the E_2-stimulated response. Since the administration of progesterone results in hypertrophy as well as decreased E_2R_c concentration the modulations of E_2R_c concentration following either E_2 or P_4 treatment are both specific events which are distinct from synthetic hypertrophy.

It is proposed that estrogen-mediated increases in E_2R_c concentration represent a mechanism of molecular amplification which contributes in part to the cascading growth observed in the uterus.

Progesterone antagonizes estrogen stimulation by limiting the E_2R_c concentration (in some unknown manner), thereby providing a biochemical "brake" to estrogen stimulation.

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PART IV: SYNTHESIS AND LIMITATIONS

I. THE SCIENCE OF SCIENCE

"The published record, is permanent; it is there for all time as a source of pride or shame as the case may be." Hubert Bradford Vickery in tribute to R. J. Anderson, Editor-in-Chief of the Journal of Biological Chemistry [1].

What we write, we suspect others will read; indeed we write for others to read. What we put down on paper must be carefully constructed to provide a firm dwelling for accuracy and honesty. We provide an unambiguous history of "what," not of "whatever." Construction must be premeditated, its mortar being accuracy, completeness and logic. Speculation has little feeling for accuracy, completeness or logic. In fact, to speculate in the wildest fashion is to fantasize. No rules, no bounds, no limitations exist for the speculation. At best, any rules are improvisational. For the sake of example, a marksman has a much better probability of hitting a target at a given distance if careful attention is paid to the type of weapon, to the type of ammunition, to range, to sighting and to firing position than if the weapon is picked up and fired from the hip. Indeed, under each circumstance the target may be struck; it may even continue to be struck under repeated fire. The endpoint has little to do with the issue because the issue is how *best* to *guarantee* that the endpoint is reachable. Nevertheless, speculation is *not* without function; it is an exercise in connection and plays a major role in generating ideas on what to *try*, *not* on what to *conclude*. The final part of this dissertation presents a precíse of the dissertation, which is followed

by a general statement of interpretation based on inductive and deductive logic. Finally, a section is included which deals with future courses, probabilistic suppositions, and what needs to be tried next.

II. PRECÍSE

A "batch" procedure which utilizes hydroxylapatite for the adsorption of the estradiol-17β receptor complex is described. This assay was characterized with respect to washing efficiency, binding specificity, competition, adsorption time, sensitivity and stability against KCl ionic strength. The technique described has a wide range of operational sensitivity (capable of detecting specific estradiol-17β binding to a cytosol fraction containing from 5 to 500 μg protein per 225 μl) is run entirely at low temperature (0-2 C) and is performed under conditions of buffer and pH which insure maximum stability for the binding protein. This technique was used in subsequent experiments to measure the effect of estrogen and progesterone on estrogen receptor concentration.

The antagonistic effect of progesterone (P_4) on the ability of target tissues to respond to estrogen (E_2) has long been recognized. Progestational processes which are distinct from E_2-dominated events may be mediated through limitations placed on the cytosolic E_2 receptor (E_2R_c). To test the effect of P_4 on E_2R_c availability, mature Sprague Dawley rats ovariectomized for at least four weeks were used. *In vivo* pretreatment with estradiol-17β (25 μg) for 48 hours increased E_2 binding per uterus from 1.86 ± 0.32 to 4.53 ± 0.84 picomoles as determined by intact tissue incubated with varying concentrations of [^{3}H]-estradiol-17β and Scatchard analysis. Specific assay of the E_2R_c revealed that low physiological doses of E_2 (0.1 μg) were able to increase the uterine receptor concentration from 47.92 ± 3.33 to

115.17 ± 7.39 picomoles/mg DNA and the vaginal receptor concentration from 119.12 ± 9.43 to 660.40 ± 95.23 picomoles/mg DNA within 48 hours. Complete reversal to baseline occurred when E_2 was administered for 48 hours and P_4 (1000 µg) for the last 24 hours. Thus, in uterine and vaginal tissues P_4 opposed the ability of E_2 to increase the cytosolic E_2 receptor concentration. Similar assays performed on pituitary and hypothalamic tissues demonstrated high affinity E_2-binding proteins which did not change their cytosolic concentration as a response to estradiol or progesterone. Hill plot analysis of the simultaneous interaction of E_2 and P_4 with the cytosolic E_2 receptor ranged from 1.02 to 1.142, suggesting neither positive nor negative cooperativity. Therefore, P_4 neither competes for binding with E_2 nor acts as a negative heterotropic effector. *In vivo* manipulations led to the suggestion that E_2R_c concentration is directly influenced by estrogen and progesterone.

The rate of E_2-mediated increases in estrogen receptor concentration as well as the rate of decline in E_2R_c activity with time after P_4 was examined. Concomitantly the relative rates of protein synthesis were examined under the same treatment schedule by utilizing pulse labeling with [^{35}S]-methionine. In general, this approach involved *in vivo* treatment of long-term castrate female Sprague Dawley rats, coupled to *in vitro* pulse-labeling (30 min) with [^{35}S]-methionine at appropriate intervals after treatment. E_2R_c levels were determined by the hydroxylapatite assay which absorbs E_2R_c but not free steroid.

"Modulation" of uterine E_2R_c concentration could be divided into three components: (1) an early E_2R_c "replenishment" (return

to baseline following an initial depletion of available receptor), (2) an E_2R_c increase above baseline levels at later time periods, and (3) a decrease in E_2R_c concentration in the presence of P_4. Specifically, *in vivo* treatment with estradiol (0.1 μg, I.P. in saline) showed "replenishment" or return to baseline of E_2R_c (by 16 hours) following an initial depletion to 20% of control between 0-3 hours. Approximately 20 hours after estrogen treatment, E_2R_c per mg protein rose 50% above control levels, thus exhibiting net increases in receptor concentration. The administration of P_4 (1.0 mg, S.C. in propylene glycol) 24 hours after E_2 was able to block increases in E_2R_c as well as decrease E_2R_c per mg protein or DNA.

Considering the complexities involved in E_2R_c control during the first 16 hours after E_2, it is difficult to inquire about specific molecular events in this replenishment phase. In the present study, manipulations have focused on the phase of modulation where increased concentrations of E_2R_c are clearly above control levels (i.e., 24-48 hours after E_2 stimulation).

The role of P_4 on E_2 modulation of E_2R_c could be explained by an increased degradation of E_2R_c. Using an experimental design to analyze E_2R_c decay, it was also possible to see if replenishment resulted from activation of existing stores of "receptor precursor." No evidence was detected implicating any soluble protein factors (possibly soluble degradative enzymes?) in either the P_4-mediated E_2R_c decay or the E_2-mediated receptor activation.

This phase of E_2R_c modulation (24-48 hours after E_2) is characterized by net increases in E_2R_c concentration expressed per mg of protein

or mg DNA, which suggests an E_2-stimulated receptor synthesis. P_4 may simply decrease or block synthesis of E_2R_c. Alternatively, progesterone may act non-specifically to limit all translational and/or transcriptional processes. Hence, general rates of protein synthesis as well as E_2R_c concentration should decrease under the influence of P_4.

Pulse-labeling studies were performed with [^{35}S]-methionine because of its high specific activity and its known high incorporation into uterine tissues. [^{35}S]-Methionine uptake into the ribosomal fraction became maximal in 6-24 hours and dropped to baseline by 48 hours after E_2 stimulation. Treatment with P_4 restored and prolonged the elevated ribosomal incorporation for 6-8 additional hours due to E_2 stimulation. Ribosomal incorporation data support a model in which both E_2 and P_4 can stimulate a maximal utilization of ribosomes. The fact that both E_2 and P_4-mediated increases in rates of synthesis was supported by increased [^{35}S]-methionine uptake into cytosol and nuclear proteins as well as the ribosomal fraction.

Thus, the effects of P_4 on E_2R_c concentration cannot be generalized to a decrease in net protein synthesis. These effects suggest that E_2 and P_4 exert differential effects on transcriptional and translational controls. It is suggested that modulation of estrogen receptors is in part responsible for this differential. These studies are consistent with a hypothesis that (a) the E_2-mediated increases in E_2R_c concentration may present a molecular amplification which, at least in part, contributes to the cascading growth phenomena observed in the uterus, and that (b) P_4 may in part mediate its antagonism to E_2 stimulation of E_2R_c by "selectively" limiting the concentration of E_2R_c and thereby provide a specific biochemical "brake" to estrogen responsiveness in target tissue.

III. LOGICAL INTERPRETATION

Logical interpretation based on formal inductive and deductive constraints is the acknowledged basis of science. Without the discipline of formal logic unique conclusions would be impossible. Even within systems of logic, conclusions may be impossible due to the absence of uniqueness, the incompleteness of information or the occurrence of paradox. Hence, while it is impossible to conclude without some system of logic, realistic situations may militate against all but the most generalized conclusions.

The spirit of the preceding parts to this dissertation have assumed an inductive philosophy. Having obtained evidence that the administration of estrogen and progesterone each results in a unique effect upon estrogen receptor concentration, experiments were designed to examine the contribution of competition, negative cooperativity and receptor turnover. As the contribution of each of these was discounted, it became inductively apparent that control of estrogen receptor *synthesis* was more likely. Such a conclusion is supported by the time course in which modulation occurs in terms of parallel inductive evidence. Unfortunately, the contributions due to recycling of receptor are not testable at this point in time and leave a major piece of inductive evidence to be surmised. Hence, while inductive argument depends upon elimination for its premise, in a realistic sense elimination of all putative components may be impossible.

Experiments which directly test synthesis of receptor in terms of [^{35}S]-methionine incorporation have been performed, but appear

subject to isotope dilution by endogenous methionine pools and have not yielded unequivocal data.

Conclusions themselves are subject to the completeness of the data. The principal thrust of this dissertation is to show that treatment with estrogen and progesterone modulates estrogen receptor concentration; however, no evidence has been presented to eliminate mediation of the response by some subsequent hormone, either released in response to estrogen and progesterone [2] or for which estrogen and progesterone are precursors [3]. Direct information could be obtained better *in vitro* but *in vitro* systems are only now coming into use [4] and generally do not supply all the characteristics of the *in vivo* response.

IV. FUTURE COURSES

The most important single question pertinent to this dissertation concerns the role of the estrogen receptor. Until it is firmly established that this protein has a role which regulates gene activity, all data must remain circumstantial. The serendipitous involvement by the receptor remains an undisproved alternative. Should the estrogen receptor prove to be a regulatory protein, then the effect of modulating the concentration of this regulatory protein becomes mediation of positive and negative control.

In particular, hormonal gene regulation in eucaryotes must be multivalent in contrast to univalent regulation by metabolic inducers in procaryotes. The large number (6,000-20,000) of estrogen receptors [5] contained in a single cell from estrogen responsive tissue strongly suggests that a multivalent complex may exist. Differential affinities by various genomic regions for a regulatory receptor could easily account for regions with different transcriptional sensitivity and could lead to an explanation for preferential accumulation of certain transcriptional products. If certain regions coded for components which were necessary for the functional capacity of other regions, the total hormonal response might appear quantal, and an explanation for the prolonged time to respond in steroid hormone systems could be obtained. Amplification of this regulatory signal could involve a servo loop where a regulatory receptor protein controls regions of the genome coding for itself; hence transcriptional sensitivity would be positively modified by the product being transcribed. Another regulatory protein might

remove this sensitivity and hence de-emphasize regulation by the first regulatory protein. Thus, a system may be developed which involves transcription of several products, some of which have a regulatory role for further transcription and may possess a potential for modifying transcriptional sensitivity to steroid hormones. As a prerequisite to accepting this model, one must view regulatory protein action as both reversible and time dependent such that alteration of the "mass" involved mediates alteration of the hormone action.

A second speculation involves the diversified nature of living systems. Current concepts assume that steroid hormones operate differently than protein hormones and tacitly imply that all steroid hormones operate by the same mode of action. It is already clear that different steroid hormones elicit different biological responses: some enhance growth, others inhibit growth. It is not surprising that the same steroid might operate differently in different systems. Certainly estrogen cannot perform the same function for the crayfish that it does for the laboratory rat! It is possible that both increased gene load and increased complexity have occurred in the evolution of a steroid-influenced genome. It is as easy to imagine that mutation may cause gain or loss of steroid-controlled genes as it is to imagine that mutation in regulatory proteins could influence which parts of the genome are expressed. Thus, regulation by steroid hormones probably has a highly diversified character.

Time or developmental stage is also important. A system may remain non-responsive until some critical developmental stage is reached. Once reached, the hormone via its respective receptor may serve to

maintain this stage to insure differentiation. Differentiation is the result of the proper modification to the genome. Part of this modification allows for a steroid-binding regulatory protein which permits certain parts of the genome to be expressed. Little evidence exists to implicate estrogen in adult cellular differentiation in any role besides maintenance. In addition, removal of hormone may effect the maintained state of the responsive tissue, but does not result in total loss in hormonal responsiveness [5].

Finally, once it is established that the estrogen receptor does physically regulate gene activity, it will be possible to characterize the nature of such genes as to whether they comprise a regular, invariant set or whether the set changes. In addition, such regulation may prove to be the result of an acceptor intermediate. In any event, the challenge at hand is to find the way the estrogen receptor functions.

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VITA

Edward John Pavlik was born the first son to parents John and Mary in Cleveland, Ohio on December 16, 1946. He attended elementary and secondary schools in Cuyahoga County, Ohio. He received the Bachelor of Science degree in Mathematics from the University of Denver in August 1969, and completed studies on the Master of Science degree in Biological Sciences in August 1971. During the summer of 1970, he was a NIH trainee in the Biostatistics and Biometrics Program at the Department of Preventive Medicine, University of Washington, Seattle. In 1971 he enrolled in graduate studies at Princeton University and was awarded a NIH traineeship.

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