1977

Estrogen receptor in mammary tissue: basic characteristics and relation to breast cancer.

Gregory Paul. Bondy
University of Windsor

Follow this and additional works at: https://scholar.uwindsor.ca/etd

Recommended Citation
https://scholar.uwindsor.ca/etd/1964

This online database contains the full-text of PhD dissertations and Masters' theses of University of Windsor students from 1954 forward. These documents are made available for personal study and research purposes only, in accordance with the Canadian Copyright Act and the Creative Commons license—CC BY-NC-ND (Attribution, Non-Commercial, No Derivative Works). Under this license, works must always be attributed to the copyright holder (original author), cannot be used for any commercial purposes, and may not be altered. Any other use would require the permission of the copyright holder. Students may inquire about withdrawing their dissertation and/or thesis from this database. For additional inquiries, please contact the repository administrator via email (scholarship@uwindsor.ca) or by telephone at 519-253-3000 ext. 3208.
The quality of this microfiche is heavily dependent upon
the quality of the original thesis submitted for microfilming.
Every effort has been made to ensure the highest
quality of reproduction possible.

If pages are missing, contact the university which
granted the degree.

Some pages may have indistinct print especially if
the original pages were typed with a poor typewriter
ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles,
published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed
by the Canadian Copyright Act, R.S.C. 1970, c. C-30.
Please read the authorization forms which accompany
this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

La qualité de cette microfiche dépend grandement de la
qualité de la thèse soumise au microfilmage. Nous avons
tout fait pour assurer une qualité supérieure de repro-
duction.

S'il manque des pages, veuillez communiquer avec
l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut
laisser à désirer, surtout si les pages originales ont été
dactylographiées à l'aide d'un ruban usé ou si l'université
nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur
(articles de revue, examens publiés, etc.) ne sont pas
microfilmés.

La reproduction, même partielle, de ce microfilm est
soumise à la Loi canadienne sur le droit d'auteur, SRC
1970, c. C-30. Veuillez prendre connaissance des for-
mules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS RÉCU
ESTROGEN RECEPTOR IN MAMMARY TISSUE:
BASIC CHARACTERISTICS AND RELATION TO BREAST CANCER

by,

Gregory Paul Bondy

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Biology in Partial Fulfillment
of the requirements for the Degree of Master of Science at
The University of Windsor

Windsor, Ontario, Canada
1977
ABSTRACT
ESTROGEN RECEPTOR IN MAMMARY TISSUE:
BASIC CHARACTERISTICS AND RELATION TO BREAST CANCER

by
Gregory Paul Bondy

Characteristics of estrogen receptor from the lactating mammary gland of C3H mice were compared with the well-documented properties of rat uterine estrogen receptors.

Dextran-coated charcoal (DCC)/Scatchard plot analysis demonstrated that estradiol-17β (E₂) binds to mammary estrogen receptor with a very high affinity (Kd = 5 × 10⁻¹¹ M) and the concentration of receptor sites increased from ~18 pmoles/g cytosol protein at 3 days of lactation to ~44 at 20 days. When concentrated mammary cytosol (>5 mg protein/mL) was centrifuged on sucrose density gradients prepared in low ionic strength buffer the receptor sedimented at ~9-10S. Dilution of cytosol protein and/or shortening incubation time (of cytosol with [³H]-E₂) caused the receptor to sediment nearer the "8S" region usually observed in uterine cytosol. On high ionic strength (0.4 M KCl) gradients both the uterine and mammary cytosol receptors sedimented at ~4-5S. Mammary "4S" peaks were difficult to detect unless the [³H]-E₂, used to label receptor was of a very high specific activity. Excessive protein concentration or prolonged incubation caused the appearance of a ~6-8S shoulder on high
salt gradients. Much E₂ binding capacity was lost when cytosol was fractionated with ammonium sulfate. Presence of EDTA in the homogenizing buffer stabilized the receptor in dilute cytosol, whereas EDTA appeared to cause aggregation of receptor when ammonium sulfate fractions were resuspended in EDTA-containing buffer. There was no significant protease activity in mammary cytosol, using conventional cytosol preparation techniques either in the presence or absence of ionic calcium. Receptor extracted from mammary nuclear fraction sedimented in the expected 5S region. When appropriate analytical techniques are used the mammary nuclear and cytosol receptor exhibits characteristics indistinguishable from those of identically analyzed uterine receptor.

Substrains of C3H mice with and without the mouse mammary tumor virus (MMTV) were compared to determine if MMTV altered estrogen action. Uterine weight response to oral diethylstilbestrol (DES) was the same for both substrains (MMTV⁺ and MMTV⁻). The concentration of receptors in lactating mammary tissue did not differ significantly for MMTV⁺ versus MMTV⁻ mice, the affinity was significantly higher, however, in cytosol from MMTV⁻ mice.

The binding of DES to mammary and uterine receptors of C3H mice was assayed directly (with [³H] -DES) and indirectly by competition between DES and [³H] -E₂. On sucrose density gradients, [³H] -DES bound to a component which sedimented in the position as "8S" estrogen receptor labeled with [³H] -E₂. Incubation of the cytosol with 100-fold excess unlabeled DES
or E₂ eliminated the "8S" binding peak. [³H]-DES also sedimented with a component in the 4-5S region; this component bound much larger quantities of [³H]-DES than [³H]-E₂ and was not eliminated by incubation with 100-fold excess of unlabeled estrogen. On high ionic-strength gradients [³H]-DES-labeled receptor dissociated to a 4-5S form as did receptor labeled with [³H]-E₂. A high-affinity binder was extracted from mammary nuclei of mice injected with [³H]-DES in vivo. The general qualitative properties of cytosol receptor appeared to be the same when labeled with [³H]-DES as with [³H]-E₂. By direct assay, the [³H]-DES receptor interaction had a Kₐ of 2.6 x 10⁻¹⁰ M compared with 6.3 x 10⁻¹⁰ M for [³H]-E₂. By competition studies the apparent affinity of receptor for DES was essentially the same as for E₂. The apparent concentration of high-affinity binding sites in mammary cytosol was higher for [³H]-DES than for [³H]-E₂; this probably was due to [³H]-DES binding to non-receptor proteins, possibly serum albumin. Overall, intracellular (receptor) binding of DES does not appear to be fundamentally different from binding of natural estrogens.

Human breast tumor estrogen receptors were measured using DCC/Scatchard plot analysis and SDG analysis. Receptor was detected in approximately 70% of all specimens. There were three common sedimentation patterns; specimens with primarily 8-9S receptor, samples with both 8-9S and 4-5S species, and specimens containing 4-5S only.

Techniques used in mammary receptor analysis were used
to test putative estrogens. In vitro binding assays revealed that $\Delta^9$-THC and cannabis resin did not compete with $E_2$ for estrogen binding proteins.

The detailed account of mammary gland characteristics presented in this study should find application in further investigations concerning mammary gland growth and differentiation in the normal and neoplastic state.
ACKNOWLEDGEMENTS

I am most grateful to Dr. A.B. Okey of the Department of Biology, University of Windsor, for his constant encouragement and guidance throughout my graduate career. Dr. Okey gave selflessly of his time and provided an atmosphere which I can only hope will be matched in my future endeavours. I am looking forward to working further with Dr. Okey, and to the knowledge and experience he will undoubtedly provide.

I am indebted to Dr. A.H. Warner of the Department of Biology and Dr. R.J. Thibert of the Department of Chemistry for their helpful criticism and suggestions in the preparation of this thesis. I would like to especially thank Dr. Warner for his scientific contributions and his generous donation of laboratory facilities.

I am deeply appreciative of the valuable technical assistance that was provided by Michelle Mason.

I gratefully acknowledge scholarships and awards from the National Research Council, the Department of Health and Welfare, and the University of Windsor. Financial support for the research described herein was provided by the National Cancer Institute of Canada and the National Research Council.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES?</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF APPENDICES</td>
<td>xvi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xvii</td>
</tr>
<tr>
<td><strong>CHAPTER I.</strong> RECEPTORS AND THE MECHANISM OF ACTION OF STEROIDS</td>
<td>1</td>
</tr>
<tr>
<td>A. An Overview of the Mechanisms of Steroid Hormone Action</td>
<td>3</td>
</tr>
<tr>
<td>B. Mammary Gland Receptors</td>
<td>7</td>
</tr>
<tr>
<td><strong>CHAPTER II.</strong> METHODS FOR MEASURING ESTROGEN RECEPTORS</td>
<td>10</td>
</tr>
<tr>
<td>A. Introduction</td>
<td>10</td>
</tr>
<tr>
<td>B. Assay Procedures</td>
<td>14</td>
</tr>
<tr>
<td>1) Preparation of &quot;Cytosol&quot; Receptors</td>
<td>14</td>
</tr>
<tr>
<td>2) Preparation of Nuclear Receptors from Animals Injected with Radioligand in vivo</td>
<td>15</td>
</tr>
<tr>
<td>3) Sucrose Gradient Centrifugation Assay</td>
<td>16</td>
</tr>
<tr>
<td>4) Dextran-coated Charcoal Assay</td>
<td>24</td>
</tr>
<tr>
<td>C. Data Analysis</td>
<td>26</td>
</tr>
<tr>
<td>1) Scatchard Plot Analysis</td>
<td>26</td>
</tr>
<tr>
<td>2) RAC Procedure</td>
<td>32</td>
</tr>
</tbody>
</table>
CHAPTER III. CHARACTERISTICS OF ESTROGEN RECEPTOR FROM LACTATING MAMMARY TISSUE OF C3H MICE

A. Materials and Methods
   1) Animals and Tissue Preparation
   2) Cytosol Preparation
   3) Dextran-coated Charcoal Assay/Scatchard Plot Analysis
   4) Sucrose Density Gradient Analysis
   5) Ammonium Sulfate Fractionation
   6) Proteolysis Assay

B. Results
   1) Receptor Affinity and Concentration
   2) Sedimentation Properties of Receptor on Low Ionic-strength Sucrose Gradients
   3) Receptor Sedimentation on High Ionic-strength Sucrose Gradients
   4) Effect of Ammonium Sulfate Fractionation and EDTA on Receptor Sedimentation Profiles
   5) Protease Assay on Mammary Homogenate and Cytosol

C. Discussion

CHAPTER IV. ESTROGEN BINDING IN MAMMARY TISSUE OF C3H MICE WITH OR WITHOUT THE MOUSE MAMMARY TUMOR VIRUS

A. Introduction
B. Materials and Methods .................................. 65
1) Uterine Weight Bioassay for Estrogenic
  Potency of Oral DES .................................. 65
2) Tissue Preparation for Estrogen Binding ....... 65
3) Scatchard Plot Analysis ............................. 66
4) Sucrose Density Gradient Analysis .............. 67
5) Nuclear Uptake in vivo .............................. 67
C. Results ................................................. 67
D. Discussion .............................................. 77

CHAPTER V. DIETHYLSLETBESTROL BINDING TO ESTROGEN
  RECEPTOR IN MAMMARY TISSUE AND UTERUS OF
  C3H MICE ............................................. 82
  A. Introduction ........................................ 82
  B. Materials and Methods ............................ 83
    1) Animals and Tissue Preparation ............... 83
    2) Cytosol Preparation ............................. 83
    3) Sucrose Density Gradient Analysis .......... 84
    4) Nuclear Uptake in vivo ......................... 84
    5) Dextran-coated Charcoal Assay/Scatchard
       Plot Analysis .................................. 85
    6) Indirect Estimation of Binding Affinities
       by Ratio of Association Constants .......... 86
  C. Results ............................................. 86
  D. Discussion ......................................... 94

CHAPTER VI. ESTROGEN RECEPTORS IN HUMAN BREAST
  CANCER ............................................... 106
  A. Introduction ...................................... 106
B. Materials and Methods .......................... 107
  1) Collection and Preservation of Specimens .. 107
  2) Preparation of Cytosols ....................... 109
  3) Assays for Specific Estrogen-binding
     Proteins ........................................ 109
C. Results ........................................... 110
D. Discussion ........................................ 115

CHAPTER VII. TESTING OF THE POTENTIAL ESTROGENIC
ACTIVITY OF $\Delta^9$-THC AND CANNABIS RESIN
USING IN VITRO ESTROGEN-RECEPTOR
BINDING TECHNIQUES ................................ 124

A. Materials and Methods .......................... 125
  1) Animals and Cytosol Preparation ............... 125
  2) Sucrose Density Gradient Analysis .............. 125
B. Results and Discussion .......................... 126

CHAPTER VIII. SUMMARY ............................. 131
APPENDIX .............................................. 132
REFERENCES .......................................... 146
VITA AUCTORIS ........................................ 159
LIST OF TABLES

Table 1. Properties of estrogen receptors in mammary tissue ........................................ 9
Table 2. Relative concentrations of binding sites in mammary cytosol as detected by different assay techniques ......................... 97
Table 3. A practical handling schedule for breast specimens submitted for measurement of estrogen-binding capacity .................. 108
Table 4. Frequency of molecular forms of human breast tumor estrogen receptors ............. 116
Table 5. Detection of receptor using DCC or SDG procedure .............................................. 119
LIST OF FIGURES

Figure 1. General model of steroid hormone action illustrating intracellular events occurring after the initial formation of a steroid-receptor complex ............... 5

Figure 2. Schematic diagram of the sucrose density gradient procedure utilized in the detection of estrogen receptor .......... 13

Figure 3. Apparatus used in the layering of receptor preparations onto sucrose gradients ... 20

Figure 4. The determination of the amount of pmoles radioligand bound to receptor from SDG sedimentation profiles .................. 23

Figure 5. An idealized Scatchard plot ............... 28

Figure 6. A Scatchard plot illustrating the correction procedures utilized in the detection of specific receptor binding .......... 31

Figure 7. Saturation, concentration and dissociation constant for specific estrogen binding sites in mouse mammary tissue ......... 40

Figure 8. Sedimentation pattern of cytosol estrogen binders from mammary tissue or uterus of C3H/HeJ mice ................. 43
Figure 9. Effect of protein concentration of the sedimentation coefficient of mouse mammary estrogen receptor .............................................. 45

Figure 10. Effect of mammary cytosol addition on sedimentation of mouse uterine estrogen receptor .................................................. 48

Figure 11. Effect of high ionic strength buffer on sedimentation of mammary and uterine estrogen receptors which had been incubated with "low" specific activity $[^3H]-E_2$ (91.3 Ci/mmol) ........................................... 50

Figure 12. Effect of protein concentration on the sedimentation of mammary estrogen receptor in the absence or presence of 0.4 M KCl ................................................................. 52

Figure 13. Effect of ammonium sulfate fractionation on receptor sedimentation in the absence or presence of EDTA ................................. 54

Figure 14. Proteolysis assay in crude mammary homogenate and mammary cytosol ................................................................. 57

Figure 15. Uterine weight response to oral DES in MMTV$^+$ or MMTV$^-$ C3H mice ............................................................... 69

Figure 16. Representative Scatchard plots for binding of $[^3H]-E_2$ in mammary cytosol from MMTV$^+$ or MMTV$^-$ C3H mice ........................................... 72
Figure 17. Estrogen-receptor concentration in mammary cytosol of lactating MMTV+ or MMTV- C3H mice ........................................ 74

Figure 18. Sedimentation patterns of cytosol estrogen binder from lactating mammary tissue and uteri of C3H/HeJ or C3H/HeJfH mice .... 76

Figure 19. Sedimentation pattern of estrogen binder extracted from mammary nuclei of a C3H/HeJ mouse (bearing MMTV) ............... 78

Figure 20. Sedimentation of cytosol estrogen binders incubated either with [3H]-E2 or [3H]-DES 88

Figure 21. Effect of [3H]-DES concentration on the sedimentation pattern of mouse mammary cytosol ........................................ 91

Figure 22. Effect of ionic strength on the sedimentation of mouse mammary cytosol receptor incubated either with [3H]-E2 or [3H]-DES 93

Figure 23. Sedimentation pattern of receptor from mouse mammary nuclei after an in vivo injection of 0.2 ug [3H]-DES ............ 96

Figure 24. Scatchard plot analysis of [3H]-E2 and [3H]-DES binding in mouse mammary cytosol 99

Figure 25. Comparative competition of unlabeled DES and unlabeled E2 for specific, high-affinity binding sites in mouse mammary cytosol ........................................ 101
Figure 26. Sedimentation of estrogen receptors from human breast tumors ........................................ 112
Figure 27. Sedimentation of 4-5S form of estrogen receptors from human breast tumors .... 114
Figure 28. Representative Scatchard plot from a human breast tumor ........................................ 118
Figure 29. Comparison of ER binding expressed as binding per unit protein versus binding per unit tissue weight ........................................ 122
Figure 30. Effect of cannabis resin and Δ⁹-THC on [³H]-E₂ binding to rat and mouse uterine estrogen receptor ........................................ 128
Figure 31. Effect of Δ⁹-THC on [³H]-E₂ binding to mouse mammary estrogen receptor .......... 129
# LIST OF APPENDICES

**Appendix A.** Radioactive labeling of marker proteins .................................................. 133

**Appendix B.** Lowry technique for protein measurement .................................................. 135

**Appendix C.** Liquid scintillation counting cocktails ...................................................... 137

**Appendix D.** Preparation of 66% stock sucrose .............................................................. 138

**Appendix E.** Statistics ........................................................................................................ 139

**Appendix F.** Radiochemical specifications ................................................................. 141

**Appendix G.** Chemicals used and suppliers ................................................................. 144
LIST OF ABBREVIATIONS

B : radioligand bound to receptor protein
BSA : bovine serum albumin
Ci : Curie
CV : coefficient of variation
cpm : counts per minute
DCC : dextran coated charcoal
DES : diethylstilbestrol
DMBA : 7,12-dimethylbenz(a)anthracene
DNA : deoxyribonucleic acid
dpm : disintegrations per minute
DTT : dithiothreitol
E2 : estradiol-17β
EDTA : disodium ethylenediamine tetraacetate
ER : estrogen receptor
Folin C : Folin-Cicalteau phenol reagent
[H]-DES : tritium labelled diethylstilbestrol
HED-buffer : 0.01 M N-2-hydroxyethyl-piperazine-N-2-ethane sulfo nic acid/0.0015 M EDTA/0.001 M DTT (pH 7.4)
HEPES : N-2-hydroxyethylpiperazine-N-2-ethane sulfo nic acid
Kd : apparent dissociation constant for the radioligand-receptor interaction
LSC  liquid scintillation counting
M    molar
mM   millimolar (10^-3)
µM   micromolar (10^-6)
nM   nanomolar (10^-9)
pM   picomolar (10^-12)
fM   femtomolar (10^-15)

MMTV refers to the form of mouse mammary
       tumor virus normally transmitted through
       milk.

N_s  number of specific binding sites for
      radioligand
ND   not determined

POPOP 1.4-bis-(5-phenyloxazol-2-yl) benzene
PPO   2,5-diphenyloxazole

r   correlation coefficient
RAC  ratio of association constants

mRNA messenger ribonucleic acid
rRNA ribosomal ribonucleic acid

rpm revolutions per minute
S    Svedberg unit for sedimentation constant
S.A. specific activity
sc   subcutaneous
S.D. standard deviation
SDG  sucrose density gradient
SEM  standard error of the mean

xviii
<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE-buffer</td>
<td>0.01 M Tris/0.0015 M EDTA (pH 7.4)</td>
</tr>
<tr>
<td>TD-buffer</td>
<td>0.01 M Tris/0.001 M DTT (pH 7.4)</td>
</tr>
<tr>
<td>TDE-buffer</td>
<td>0.01 M Tris/0.001 M DTT/0.0015 M EDTA (pH 7.4)</td>
</tr>
<tr>
<td>TDK-buffer</td>
<td>0.01 M Tris/0.001 M DTT/0.4 M KCl (pH 7.4)</td>
</tr>
<tr>
<td>TDEK-buffer</td>
<td>0.01 M Tris/0.001 M DTT/0.0015 M EDTA/0.4 M KCl (pH 7.4)</td>
</tr>
</tbody>
</table>

$\Delta^9$-THC: delta-9-tetrahydrocannabinol

Tris: tris(hydroxymethyl)aminomethane

U: the amount of radioligand either free in solution or bound to proteins with only low affinity

w/v: weight per unit volume
CHAPTER I

RECEPTORS AND THE MECHANISM OF ACTION OF STEROIDS

Mediation of cellular responses through receptors currently is one of the most promising models in molecular biology. Cellular receptors have been discovered for a variety of macromolecules, including opiates (Pert & Snyder, 1973), chemical carcinogens (Poland et al., 1976), and polypeptide hormones such as insulin (Cuatrecasas, 1974). Many of the techniques and concepts used in the identification of cellular receptors were pioneered by researchers in the field of steroid hormone action during the last two decades. Workers in this field have speculated that molecules may exert their effects in manners analogous to steroid hormones, and steroid hormone mechanisms may provide an insight into the function of other cell macromolecules.

Historically, the major technical breakthrough leading to the identification of a steroid hormone receptor was the synthesis of a highly radioactive preparation of estradiol (Jensen & Jacobson, 1972). By injecting female rats with labeled estradiol-17β, Jensen found that estrogen-responsive tissues ("target tissues") could selectively retain the hormone over a relatively long
period of time whereas non-target tissues could not. The original aim of this study had been to determine whether the hormone had been altered in any way once inside the target cell. Previous researchers had always puzzled over the way a small molecule such as a steroid could control DNA-mediated cell processes, such as protein synthesis. Jensen's studies clearly demonstrated that the hormone was not altered in any way at the target tissue site. This finding gave credence to the receptor hypothesis, an hypothesis which predicted that there were "effector" molecules in target cells to which steroids would bind stereospecifically and trigger steroid-specific cellular processes.

The identification of this effector molecule or receptor was predicated on the availability of highly radioactive steroids. Steroids can bind to a wide variety of biological and non-biological molecules. Thus steroids administered in non-physiological amounts would largely bind to molecules not associated with receptor binding and would mask true receptor binding. The use of highly radioactive steroids allowed physiological concentrations (nM) of the hormone to be used, and this allowed the detection of specific receptor binding over non-specific binding.

It is this molecular probe that Toft and Gorski (1966) used to identify estrogen receptors in rat uteri. Using the technique of sucrose gradient centrifugation, Toft and Gorski found that radiolabeled estradiol-17β was selectively
bound to a protein molecule which had been prepared from
the cytoplasm of rat uterine cells. This molecule satis-
fied two important criteria established for a receptor:
first, the receptor was present in target tissues but was
absent in all other tissues; second, the binding of $^3$H-
estradiol to the receptor could be blocked effectively by
adding excess amounts of unlabeled estradiol or the syn-
thetic estrogen, DES. The binding was unaffected by other
classes of steroid hormones such as androgens and progestins.
This implied a stereospecificity between the receptor and
biologically functional estrogens. Ultimately, steroid
receptors were found in a variety of steroid responsive
tissues, notably androgen receptors in the prostate (Liao
& Fang, 1969), progesterone receptors in the chick oviduct
(O'Malley & Means, 1974), and glucocorticoids receptors in
mammary tissue (Wittliff, 1975). The current "dogma" of
steroid hormone action states that all steroid sensitive
tissues (tissues whose growth, i.e. protein synthesis, is
regulated by steroids) contain cytoplasmic receptors to
which the steroid binds with high affinity and specificity.

A. An Overview of the Mechanism of Steroid Hormone Action

The events following the initial formation of a
steroid-receptor complex have been studied extensively in
the immature rat uterus (Jensen et al., 1974) and the chick
oviduct (O'Malley & Means, 1974). An outline of these
events is shown in Figure 1. The steroid enters the cell,
Figure 1. General model of steroid hormone action illustrating intracellular events occurring after the initial formation of a steroid-receptor complex (Edelman, 1975).
STERIOD -> ACTIVEM COMPLEX -> RECEPTOR -> mRNA -> rRNA -> NUCLEUS -> INDUCED PROTEINS

CAPILLARY

TARGET CELL

PHYSIOLOGICAL EFFECTS
possibly by passive diffusion, and combines with the receptor molecule. In rat uterine tissue, the complexes have been shown to sediment at 8-9S on sucrose gradients containing low salt concentrations (Toft & Gorski, 1966) and at 4-5S on sucrose gradients containing 0.4 M KCl (Erdos, 1968; Korenman & Rao, 1968). Earlier researchers had reported a variety of sedimentation values for the uterine estrogen receptor. Initially thought to be different sub-units of the receptor, they were found to be artifacts induced by the ionic strength of the buffers (Chamness & McGuire, 1975a) and the protein concentration of the cytosol preparation (Stancel et al., 1973).

After hormone binding, the steroid-receptor complex then is "activated" at physiological temperatures to a transformed species (Gorski et al., 1968; Jensen et al., 1968). This "activation" step is accompanied by an increase in sedimentation of the complex from ~4S to ~5S. Only the activated complex can enter the nucleus.

O'Malley and co-workers (1974) have demonstrated with chick oviduct progesterone receptors that the hormone-receptor complex, after entry into the nucleus, binds to selective sites ("acceptor sites") on the target tissue chromatin. This binding activates or derepresses transcription of specific RNA synthesis. The production of mRNA and rRNA directs specific protein synthesis, and the end result is the physiological and morphological changes in response to the hormone.
B. Mammary Gland Receptors

The problem of human breast cancer has promoted an interest in the research on the growth and differentiation of the mammary gland. Estrogens, progestogens, and glucocorticoids are steroid hormones which control mammary gland growth. In light of the discovery of steroid receptors in uterine tissue, it became of great interest to determine if similar receptors could be detected in the mammary gland.

Evidence that the mammary gland selectively retained estradiol was first reported by Puca and Bresciani (1969). However, the separation of receptor proteins in the gland was hampered due to the dormant state of the gland during non-pregnancy. The gland in this state is composed primarily of adipose cells and connective tissue (Turkington, 1972). This problem was circumvented by using lactating mammary tissue; the lactating gland is now in its most proliferative state, and the levels of estrogens and progestogens are minimal. By using lactating mammary tissue, cytoplasmic estrogen receptors were detected in the mouse (Shyamala & Nandi, 1972), the rat (Wittliff et al., 1972; Gardner & Wittliff, 1973a), and the vole (Beers & Wittliff, 1973a). Similarly, glucocorticoid receptors were also detected in the mouse (Shyamala, 1973) and the rat (Gardner & Wittliff, 1973b; Goral & Wittliff, 1973). In addition, estrogen receptors were thoroughly characterized in neoplastic mammary tissues of rodents, such as the
DMBA-induced mammary tumor (King et al., 1969; McGuire & Julian, 1971; Boylan & Wittliff, 1975) and the spontaneous tumors of mice which carried the mammary tumor virus (Shyamala, 1972). A summary of the properties of estrogen binding proteins in mammary tissues is listed in Table 1.

It soon became apparent in my studies, however, that little information was known about several physical-chemical aspects of mammary receptors, particularly in mouse mammary tissue. Important receptor properties such as the dissociation of the 8-9S receptor to the 4-5S form, remained unclear (Shyamala, 1975). High concentrations of proteins were known to cause aggregation in uterine receptor (Stancel et al., 1973). Could this aggregation phenomena be present in mammary tissue preparations which are much higher in protein concentration? These were some of the problems I examined and they will be described in the following chapters.
# Table 1

Properties of Estrogen Receptors in Mammary Tissue

<table>
<thead>
<tr>
<th>Source of tissue</th>
<th>$K_d$ (molar)</th>
<th>$N_q$ (pmoles/g protein)</th>
<th>Sedimentation Constant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactating gland (mouse)</td>
<td>$5.0 \times 10^{-11}$</td>
<td>18-44</td>
<td>8-10</td>
<td>4-5</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>6-9$^b$</td>
<td>8</td>
<td>ND</td>
</tr>
<tr>
<td>Lactating gland (rat)</td>
<td>$8.0 \times 10^{10}$</td>
<td>20-30</td>
<td>8.6</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>$10^{-9}-10^{-10}$</td>
<td>15-40</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lactating gland (vole)</td>
<td>$2.0 \times 10^{-10}$</td>
<td>27</td>
<td>8</td>
<td>ND</td>
</tr>
<tr>
<td>Non-lactating gland (mouse) pregnant:</td>
<td>ND</td>
<td>5-11$^b$</td>
<td>8</td>
<td>5.3</td>
</tr>
<tr>
<td>non-pregnant:</td>
<td>ND</td>
<td>3$^b$</td>
<td>4-5</td>
<td>4-5</td>
</tr>
</tbody>
</table>

*a = number of binding sites per cell  
b = mole $\times 10^{-14}$
CHAPTER II

METHODS FOR MEASURING ESTROGEN RECEPTORS

A. Introduction

Estrogen-binding proteins were originally discovered by Talwar et al. (1964), using the technique of gel filtration. Radiolabeled estradiol was found to be associated with low molecular weight products which eluted with the void volume. Specific "receptor" proteins could not be claimed, however, since the void volume contained many different types of proteins. Toft and Gorski (1966) overcame this problem by separating receptor complexes on sucrose density gradients (SDG). This procedure provided the first means of separating estrogen receptors from the non-specific binding proteins also found in target cells. In the SDG method, a linear sucrose gradient (usually 5-20%) is prepared in a centrifuge tube. The mixture of molecules (in this case cellular proteins and receptor-radioligand complexes) is layered on top the gradient. The gradient then is centrifuged which causes each type of macromolecule or complex to migrate down the gradient at its own rate, a rate determined primarily by its particle weight (Martin & Ames, 1961). The bottom of the tube can next be pierced and the fractions collected. The
procedure is shown diagrammatically in Figure 2.

In hormone-receptor studies, SDG allows the separation of cytosol components and the location of high-affinity binding peaks labeled with radioligand of very high specific activity. It usually reveals two peaks of protein-bound radioactivity: one at 3-9S and another at 4-5S. Whereas the 8-9S binding peak always represents the specific receptor-hormone interaction, the 4-5S peak may contain both specific and non-specific binding components.

The dextran-coated charcoal (DCC) was originally used by Nugent and Mayes (1966), and adapted for steroid hormone binding assays by Korenman (1968). The DCC procedure is a simple technique which separates unbound radioligand from receptor-bound radioligand. After a suitable incubation of cytosol with radioligand, a dextran-coated charcoal suspension is added to the incubation mixture. The dextran coating presumably prevents the absorption of large molecules, such as proteins or receptor- ligand complexes, onto the charcoal but allows absorption of "free" radioligand. This permits the separation of free and bound radioligand in the cytosol fraction. If varying amounts of radioligand are incubated with cytosol, the amount bound at different concentrations can be used to construct a Scatchard plot (Scatchard, 1949). A Scatchard plot permits the estimation of the number of hormone binding sites \( (N_s) \) in the cytosol as well as the apparent dissociation constant \( (K_d) \) of the receptor-hormone interaction.
Figure 2. Schematic diagram of the sucrose density gradient procedure utilized in the detection of estrogen receptor.
Macromolecules layered on gradient

Preformed sucrose gradient

\[ \text{initial state} \]

\[ \text{5\% sucrose} \]

\[ \text{centrifuge 20 hr} \]

\[ \text{20\% sucrose} \]

\[ 4S \]

\[ 8S \]

Fractionate

Centrifuge tube

Collect fractions

Dense Sucrose

\[ \text{DPM} \]

\[ \text{TUBE NO.} \]

Count radioactivity
In my research, I have used these techniques to assay cytoplasmic and nuclear receptors in mouse mammary tissue (Chapters III, IV, V, VII), mouse and rat uterine tissue (Chapters III, IV, V, VII), and human breast tumors (Chapter VI).

B. Assay Procedures

1. Preparation of "Cytosol" Receptors

a) Buffers

The standard buffer used was 10 mM Tris, pH = 7.4 containing 1.5 mM EDTA and 1.0 mM DTT (TDE buffer). The DTT was added just prior to use. Depending on the experiment, the basic buffer was supplemented with various amounts of KCl, usually at a concentration of 0.4 M. Receptor preparations were made using buffers having Hepes substituted for Tris, and no differences in receptor properties were observed.

b) Tissue removal and homogenization

Animals were sacrificed by cervical fracture and pinned on a dissection board. Mammary tissue was removed through a midline incision using a blunt dissection probe and forceps. The tissues were placed in ice-cold TDE buffer and rinsed in several volumes of fresh TDE buffer to remove as much milk as possible. Next, the tissue was blotted, weighed, finely minced with a scalp, and homogenized in 3 ml of TDE buffer for every gram of tissue. Homogenization was performed using a Polytron PT-10.
(Brinkmann Instruments, Rexdale, Ontario) at a setting of "4". It was important to maintain the tissue at 0-4°C. This was accomplished by homogenizing in an ice-water bath and by using bursts (15-20 sec) of the Polytron with cooling between bursts.

The crude homogenate was centrifuged in a fixed angle rotor (Sorvall SS-34) for 15 min at 4000 x g. The supernatant was drawn off without disturbing the floating lipid layer. The crude nuclear pellet was discarded or stored frozen for DNA measurement. The supernatant was centrifuged again at 4000 x g for 15 min. This step was repeated until a relatively clear, that is free of lipid material, supernatant was obtained.

c) Preparation of cytosol

The low speed supernatant was centrifuged at 105,000 x g for 1.5 hrs in a Beckman fixed angle rotor (60 Ti) in order to prepare the cytosol. The high speed supernatant was aspirated without disturbing the floating lipid layer. Cytosol prepared in this manner generally contained 8-10 mg protein/ml (mammary tissue), and was diluted to a suitable protein concentration depending on the experiment. Protein concentration was ascertained using the method of Lowry et al. (1951, Appendix).

2. Preparation of Nuclear Receptors

from Animals Injected with Radioligand in vivo

a) Administration of radioligand

Tritium labeled radioligand was dissolved in 20 ul
of absolute alcohol and brought to a predetermined volume (usually 0.5 ml) with 0.9% saline. The sample was loaded into a syringe and delivered in several subcutaneous injections into the scapular region of a lactating mouse. The animals were sacrificed 2 hrs after injection and tissues were removed and homogenized as mentioned beforehand.

b) Preparation of nuclear extracts

The nuclear pellet obtained from the previous step was resuspended and washed three times in 4-5 volumes of TDE buffer; it was then centrifuged at 4000 x g for 15 min and the supernatants were discarded. The washed pellet was homogenized using the Polytron PT-10 (setting = "6") in TDE buffer (1:1 w/v) at pH = 8.5 containing 0.5 KCl. The nuclear homogenate was allowed to stand for 1 hr at 4°C to ensure maximum extraction of nuclear receptor after which the homogenate was centrifuged at 105,000 x g for 1.5 hrs to obtain a nuclear supernatant. Unbound radioligand was removed by dextran-coated charcoal (see next section) and the supernatant analyzed by sucrose gradient centrifugation.

3. Sucrose Gradient Centrifugation Assay

a) Labeling of cytosol with radioligand in vitro

One milliter of cytosol (usually diluted to ~4 mg protein/ml for mammary tissue) was labeled by adding 20 ul of radioligand in ethanol. Potential competitors were added in 10 ul of ethanol. The samples were incubated
for 3 hrs in a cold room (4-8°C) with constant shaking. After incubation, unbound radioligand was removed by pipetting the sample into 10 x 75 mm culture tubes containing a charcoal pellet. The pellet was derived from a 2-ml volume of DCC suspension (0.5% Norit A charcoal, 0.05% dextran in TE buffer, pH = 7.4) spun at 4000 x g for 20 min. The sample and charcoal were incubated for 15 min. The charcoal was re-pelleted by centrifugation at 4000 x g for 20 min. The supernatant was carefully aspirated using a pasteur pipet. If KCl pretreatments were to be performed, enough crystalline KCl to bring the solution to 0.4 M was added and the sample vortexed vigorously.

b) Gradient preparation

Sucrose solutions (usually 5 and 20%) were prepared in the desired buffer from a 66% stock sucrose solution (Appendix). Linear 5-20% gradients were formed using an automatic gradient former (Isco Model 570, Instrument Specialties Co., Lincoln, Neb.) set at a relative delivery speed of "3.75". The gradients were made in cellulose nitrate tubes, which are essentially non-wettable, and this allowed the gradients to be formed by running sucrose down the sides of the centrifuge tube. The total volume of the tube was 13 ml for the Beckman SW 41 rotor. Gradients were allowed to chill at 4°C for at least 1 hr before use.

c) Sample layering and centrifugation

A 0.5 ml sample was loaded into a pre-chilled 1-ml syringe mounted with a 1½" 22 gauge needle (A schematic
diagram of the apparatus used in the layering procedure is shown in Figure 3). The syringe needle was positioned so that it allowed the sample to run down the centrifuge tube and then onto the top layer of the sucrose gradient. The amount of sample placed on the gradient was 0.5 ml. If $^{14}$C-marker proteins (Appendix) were to be added, they would be added in 10-μl amounts using a micropipet.

All centrifugation was done with a Beckman SW 41 rotor, generally at 40,000 rpm. The length of centrifugation was variable and depended on the degree of separation desired. If detection of receptor in the 8-9S region was desired, 16-18 hrs of centrifugation would be sufficient whereas excellent resolution of 4-5S and 8-9S components could be obtained with 20-24 hrs of centrifugation. The rotor was allowed to accelerate and decelerate with the brake in operation.

d) **Gradient Fractionation**

After centrifugation, the rotor buckets were placed in an ice bath until they were fractionated in order to minimize diffusion. The gradient tubes were fractionated using an Isco Model 640 gradient fractionator set at a flow rate of 0.5-1.0 ml/min and 300-μl fraction size. In this system the tube was positioned and the bottom of the tube was pierced with a hollow needle. By means of a motor-driven syringe, dense sucrose (the solution was generally 10% higher in concentration than the highest concentration of sucrose in the gradient, i.e. 30% for a
Figure 3. Apparatus used in the layering of receptor preparations onto sucrose gradients.
5-20% gradient) was pumped into the bottom of the tube, and thereby forcing out the contents through the top of the tube. Sample fractions were then collected into scintillation vials which were loaded in the automatic fraction collector.

The samples were counted using either a Beckman LS 3100 or a Nuclear Chicago Mark II liquid scintillation counter. "Vialette" scintillation vials (Amersham/Searle) were used, and 5 ml of either Brays solution (Appendix) or a toluene-triton X-100 cocktail (Appendix) was added to the 300-μl fractions. Sample quench was corrected by means of external standardization.

e) Expression of data

(1) Calculation of sedimentation coefficient

The sedimentation coefficient of the receptor was calculated by comparing the migration of known protein standards using the following relationship (Martin & Ames, 1961):

\[
\frac{S_{\text{unknown}}}{S_{\text{marker}}} = \frac{\text{distance}_{\text{unknown}}}{\text{distance}_{\text{marker}}}
\]

Marker proteins routinely used were BSA (4.6S) and α-globulin (7.1S).

(2) Determination of the number of pmoles radioligand bound to receptor

A typical sedimentation profile of a mammary estrogen receptor incubated with and without competitor is shown in Figure 4. In the tube which did not contain
Figure 4. The determination of the amount of pmoles radioligand bound to receptor from SDG sedimentation profiles. The samples illustrated were from a mouse mammary cytosol incubated either with competitor (Panel B) or without competitor (Panel A). The exact location of the receptor binding peaks in the sucrose gradient was dependent on the cytosol protein concentration. The shaded areas (area under the 8-10S receptor binding peak) indicate the regions were total and nonspecific radioactivity were determined. Details of the calculation are in the text.
competitor, radioactivity in the fractions under the 8-9S peak was totalled, and this total represented total ligand binding. Radioactivity in the same 8-9S region of the tube containing competitor was added and this value represented non-specific binding.

It should be noted that the competitor concentration is critical in the detection of non-specific binding. Very high concentrations of competitor may compete for binding even to components of low affinity, leading to an underestimation of non-specific binding (Chamness & McGuire, 1975b). These authors recommend as a general rule that the competitor concentration not exceed 50 times the radioligand concentration.

Specific receptor binding was determined by subtracting the non-specific binding from the total binding.

\[
\text{Specific Binding} = \frac{\text{Total Binding} - \text{Non-Specific Binding}}{(cpm) - (cpm)}
\]

The specific binding was expressed as dpm and converted to pmoles radioligand using the following equation:

\[
\frac{\text{Specific Binding (pmoles)}}{2200} \text{ (S.A. of ligand in Ci/mmol)}
\]

4. Dextran-coated Charcoal Assay

a) Details of assay

Aliquots of 250 ul cytosol (~4 mg protein/ml) were incubated with 250 ul of radioligand prepared in TE buffer. Each full assay consisted of sixteen tubes, the radioligand
concentrations ranging from 8 nM to 160 pM. Competitors or test solutions were added in 10 ul of ethanol. The incubations were conveniently carried out in 10 x 75 mm culture tubes kept cold in culture tube racks (Kryorac, IP Associates). The samples were vortexed and incubated overnight (12-14 hrs) in a cold room at 0-4°C with constant shaking.

At the end of the incubation period, a 100-ul aliquot was taken from each sample and placed in a scintillation vial. This sample represented the "total" amount of radioligand added. A 400-ul aliquot of a DCC suspension (0.5% Norit A charcoal, 0.05% dextran in TE buffer, pH = 7.4) was added to each tube. The samples were vortexed vigorously and incubated for 15 min. The charcoal was pelleted by centrifugation at 4000 x g for 20 min. This process removed most of the radioligand which was not bound to the receptor. Then a 200-ul aliquot was removed from the charcoal-treated supernatant and placed in a scintillation vial. This sample represented the amount of radioligand bound to the "receptor". A 5-ml aliquot of scintillation fluid (Appendix) was added and the samples were counted using LSC procedures.

b) Expression of data

The radioactivity data for the bound and total samples were converted to pmoles using the following equation:

$$\text{pmoles/ml} = \frac{\text{sample (dpm/ml)}}{2200 \text{ (S.A. of ligand in Ci/mmole)}}$$
The data were normalized for protein concentration as follows:

\[
\text{pmoles/g protein} = \frac{\text{pmoles/ml}}{\text{Protein Concentration (g/ml)}}
\]

The final calculation involved the determination of the amount of unbound radioligand.

\[
\text{Unbound} = \text{Total} - \text{Bound}
\]

The bound and unbound data were analyzed using a Scatchard plot.

C. Data Analysis

1. Scatchard Plot Analysis

The interaction between a radioligand and a receptor can be analyzed mathematically using a Scatchard plot. The salient features of an idealized Scatchard plot are shown in Figure 5. The x-intercept represents the number of receptor molecules present in the cytosol. The slope of the line indicates the affinity of the receptor-ligand interaction. The shape of the plot also will reveal information of the ligand-receptor interaction. A straight line indicates an homogeneous system, that is, one class of binding sites whereas a non-linear plot indicates a heterogeneous system. Present information indicates that steroid receptors are a homogeneous system (Gorski et al., 1973).

Steroid receptor proteins are characterized by their high-affinity for the hormone but also for their very
Figure 5. An idealized Scatchard plot where:

B = bound estradiol (pmole /g cytosol protein)

U = unbound estradiol (pmole /g cytosol protein)

N_s = number of receptor sites (pmole/g cytosol protein)

K_d = apparent dissociation constant (molar)
\[ K_d = \frac{-1}{\text{slope}} \]
limited capacity. There is a limit by which the amount of radioligand will bind to receptor. Addition of more radioligand will result in binding to lower-affinity components such as serum albumin and other cellular proteins. A Scatchard plot, if suitably constructed should be able to differentiate between specific receptor binding and non-specific binding by means of the slopes of the binding curve. Figure 6 illustrates a typical Scatchard plot for the binding of $^3$H-estradiol with mouse mammary cytosol receptor. In curve A it can be noted that there are two distinct regions of binding: the vertical portion of the line represents high affinity binding to specific receptor proteins; the horizontal portion of the line is characteristic of low affinity binding to non-specific binding proteins. The extrapolation of the vertical portion of the binding curve to the x-intercept is a simple, but incorrect, procedure many investigators perform to calculate the $N_s$ value. The procedure can be applied to systems where the levels of contaminating proteins are low, such as immature rat uterus, but is inappropriate for binding systems such as mouse mammary tissue with high levels of proteins. This point will be dramatically illustrated in $[^3H]$-DES binding to mammary receptor (Chapter V). To obtain an accurate determination of both $K_d$ and $N_s$, the correction procedure of Chamness and McGuire (1975b) must be utilized.

The correction procedure is outlined as follows: From the uncorrected binding curve (Figure 6, Curve A) a tangent is drawn to the horizontal portion of the curve.
Figure 6. A Scatchard plot illustrating the correction procedures utilized in the determination of specific receptor binding. Data shown was obtained from a study where mouse mammary cytosol was incubated with several concentrations of $^{3}H\text{-E}_{2}$. Details of the correction procedures are listed in the text.

Curve A = total binding which has not been corrected for non-specific binding.

Curve B = linear extrapolation of the vertical portion of the total binding curve (Curve A) to determine $N_{s}$.

Curve C = plot corrected for non-specific binding using the method recommended by Chamness and McGuire (1975b).
The y-intercept of this tangent represents the B/U limiting ratio \( (B/U_{\text{limit}}) \). The B/U limiting ratio is multiplied by the unbound ligand concentration \((U)\) at each point to determine non-specific binding at that point. The value is the subtracted from the measured bound ligand \((B)\) to find the specific bound ligand \(B_{sp}\).

\[
B_{sp} = B - (U \times B/U_{\text{limit}} B/U_{\infty})
\]

The \(B_{sp}\) is plotted against the \(B_{sp}/U\) ratio to produce the corrected binding line (Curve C). The line for the corrected points was fitted by least squares linear regression (Appendix).

2. PAC Procedure

The method used was adapted from Korenman (1969) and consisted essentially of using DCC assay/Scatchard plot analysis to determine the concentration of a test compound (i.e. DES, Chapter V) required to reduce cytosol binding to 50% of the control value (no competitor). This was conveniently done by plotting the \(N_{p}^{0}\) value against the logarithm of the competitor concentration. The dissociation constant \(K_{C}\) of the test compound was calculated using the following relationship (Wittliff, 1975):

\[
K_{C} = K_{S} \left[ C_{C} \right]^{50} \frac{[S_{t}]}{[S_{t}]}^{50}
\]

where:
\( [C_{50}] \) = the concentration of competitor (test compound) required to reduce receptor binding by 50%.

\( [S_{50}] \) = the concentration of non-radioactive ligand required to reduce receptor binding by 50%.

\( K_C \) = the dissociation constant for competitor-receptor complexes.

\( K_S \) = the dissociation constant of \([^3H]\)-labeled ligand-receptor complexes.

The affinity by which an estrogen binds to the receptor generally parallels the potency of the hormone in the whole animal (Korenman, 1969). A powerful estrogen such as DES has a dissociation constant in the order of \(2.6 \times 10^{-10} M\) (Okey & Bondy, in preparation) whereas weak and non-estrogenic compounds have a much lower affinity.
CHAPTER III

CHARACTERISTICS OF ESTROGEN RECEPTOR FROM LACTATING MAMMARY TISSUE OF C3H MICE

Is there a universal receptor, identical in all tissues, that will respond to a particular hormone? This is a fundamental question that has been asked about hormone function. The uterine estrogen-receptor has been studied intensively, but much less is known about the properties of estrogen receptor in mammary tissue. The classical uterine cytosol receptor has been described as a protein which selectively binds physiologically active estrogens with very high affinity and limited capacity. It sediments at about 8S on sucrose density gradients prepared in low ionic strength buffer, and dissociates to an approximate 4S form in the presence of 0.4 M KCl (Stancel et al., 1973). Sica et al. (1976) recently presented a model which hypothesizes that the uterine 4S receptor is a dimer of equivalent 2.8S subunits and that the 8S form may be a tetrameric self-association of 2.8S subunits. In the few reported studies on estrogen binding in rodents' mammary tissues, the receptor characteristics generally have not been notably different from uterine receptor (Shyamala & Nandi, 1972; Gardner & Wittliff, 1973a; Beers and Wittliff, 1973a; Bondy & Okey, 1977). However, Shyamala (1975) recently
suggested that the estrogen receptor of mammary glands may contain a bound metal ion which is necessary for maintenance of the proper subunit structure to permit hormone binding. Characterization of mammary receptor is essential to understanding basic mechanisms of estrogen action and may have great practical value in illuminating the role of estrogens in mammary carcinogenesis. Thus I studied the characteristics of estrogen receptor in mammary tissue of C3H mice because this strain is highly susceptible to estrogen-induced mammary tumors (Gass et al., 1974). Apparent properties of the receptor varied with the analytical technique used. This study re-emphasizes the importance of controlling simple, but critical variables in characterization of steroid receptors.

A. Materials and Methods

1. Animals and Tissue Preparation

Mice used were 4-6 month old C3H/HeJ females purchased from The Jackson Laboratory, Bar Harbor, Maine, or reared in our laboratory from Jackson stock. Mice were killed by cervical fracture 10 days (unless otherwise noted) after they had delivered a litter; lactating mammary tissue was removed by blunt dissection, rinsed with iced TDE buffer (to remove excess milk), dried on filter paper and minced with a scalpel before homogenization. Uteri were prepared in a similar fashion. All procedures were done at 0-4°C unless otherwise indicated.
2. Cytosol Preparation

Minced tissues were homogenized in 3 ml TDE buffer (buffer modified as noted in particular experiments) per g tissue using a Polytron PT-10 at a setting of "4". The homogenate was centrifuged three times at 4000 x g for 15 min and the resulting supernatant was centrifuged at 105,000 x g for 90 min to obtain a supernatant (cytosol) fraction. Cytosol was carefully removed from beneath the surface lipid layer with a disposable pipet, then diluted with appropriate buffer to the protein concentration desired for particular experiments. Final protein concentration was determined by the method of Lowry et al. (1951).

3. Dextran-coated Charcoal Assay/Scatchard Plot Analysis

The concentration of receptor sites and the $K_d$ for the estrogen-receptor interaction were determined by incubating cytosol (~2 mg protein/ml) with $[^3H]E_2$ (138.1 Ci/m mole) at concentrations between 8 nM and 160 pM for 12-14 hrs at 0-4°C. After incubation an aliquot was removed for determination of total $[^3H]E_2$ in the incubation tube, then "unbound" $[^3H]E_2$ was removed with DCC. Total $[^3H]E_2$ and "bound" $[^3H]E_2$ were determined by liquid scintillation counting and the data were analysed and corrected for non-specific binding by use of the Scatchard plot as described by Chamness and McGuire (1975b).

4. Sucrose Density Gradient Analysis

The following general procedures was used with modifications for particular experiments as described in
the Figure legends. Cytosol aliquots were incubated either for 3 hrs or 12-14 hrs with $[^3\text{H}]-\text{E}_2$ at a concentration of 2 nM. After incubation unbound $[^3\text{H}]-\text{E}_2$ was removed by adding 1 ml of cytosol to a pellet of DCC. DCC was resuspended on a vortex mixer, then removed by centrifugation. One-half ml of this supernatant was layered onto a linear 5-20% sucrose gradient prepared in the appropriate buffer. Gradients were centrifuged at 40,000 rpm for 22-24 hrs in a Beckman SW 41 rotor at 4°C. Forty 300-ul fractions were collected from each gradient tube on an automatic fractionator. Radioactivity in each fraction was determined by LSC and corrected for counting efficiency. Bovine serum albumin (4.6S) and human gamma-globulin (7.1S) were used as marker proteins to determine the approximate sedimentation coefficients of the radioactive peaks by the method of Martin and Ames (1961). Only the BSA marker position is shown on gradient profiles in the Figures.

5. Ammonium Sulfate Fractionation

Mammary cytosol (7.8 mg protein/ml) pooled from 4 mice was incubated for 3 hrs with 2 nM $[^3\text{H}]-\text{E}_2$. After incubation the labeled cytosol was placed in a test-tube immersed in ice on a magnetic stirrer. Ammonium sulfate crystals were added with constant stirring over a 20 min period until the final concentration was 20%. The sample was allowed to stand in ice for 10 min, then was centrifuged at 10,000 x g for 20 min; the supernatant was discarded and the precipitate resuspended either in TDE or TD buffer.
Unbound $[^3H] - E_2$ was removed by DCC treatment and the resulting cytosol was analyzed on sucrose gradients prepared in various buffers.

6. Proteolysis Assay

Proteolytic activity was measured using a sensitive method for detecting amino groups (Fields, 1971) liberated by hydrolysis of endogenous proteins. The specific application of this procedure for protease measurement was developed by P. Na{\c{s}}ainis and A.H. Warner of this department (personal communication). Aliquots (0.05 ml) of the sample to be measured were diluted with 0.95 ml of a solution containing 0.053 M Na$_2$B$_4$O$_7$ and 0.53 M NaOH; then 0.025 ml of 1.1 M 2,4,6-trinitrobenzene sulfonic acid was added and the sample incubated at 25°C for 15 min. The reaction was stopped by addition of 2 ml of a solution containing 0.1 M NaH$_2$PO$_4$ and 1.5 mM Na$_2$SO$_3$. The absorbance of the sample was immediately measured at 420 nm. Validity of the assay parameters was checked using trypsin digestion of denatured BSA as a standard.

B. Results

1. Receptor Affinity and Concentration

Total binding of $[^3H] - E_2$ in mammary cytosol was not saturated at concentrations up to 3 nM (Figure 7A). Specific, high-affinity binding is of limited capacity, however, as shown by incubation in the presence of competitor (Figure 7A) or by Scatchard plot analysis (Figure 7B). The concentration of high-affinity binding sites (receptor) increased from 18 pmoles/g cytosol protein at 3 days of lactation to 25 pmoles at 10 days and 44 pmoles at 20 days (data not shown).
Figure 7. Saturation, concentration and dissociation constant for specific cytoplasmic estrogen binding sites in mouse mammary tissue. Data shown are from a DCC assay on an individual C3H/HeJ mouse which had been lactating for 10 days. Detailed techniques are described in the text. A) Cytosol (2 mg protein/ml) was incubated for 12 hrs at 4°C either with $[^3H]E_2$ alone (○○○, Series A) or with $[^3H]E_2$ plus 100 nM non-radioactive $E_2$ (●●●, Series B). The specific (receptor) binding (△△△) was obtained by subtracting binding in Series B from Series A. B) Scatchard plot analysis of saturation data shown in Figure 1A. (○●○●), total binding which has not been corrected for non-specific binding; (○○○), plot corrected for non-specific binding using the method recommended by Chamness and McGuire (1975b). The line shown for corrected data points was calculated by least squares linear regression. The number of high affinity binding sites ($N_s$, pmoles/g cytosol protein) was obtained from the intercept of the calculated line on the abscissa; the dissociation constant ($K_d$, pmoles/L) was derived from the slope of the calculated line.
Scatchard plot analysis reveals essentially one class of high-affinity binding sites in mammary cytosol (Figure 7B); the affinity of the estradiol-receptor interaction is very high throughout lactation ($K_d = 5 \times 10^{-11}$ M).

High-affinity binding is specific for estrogenic substances; binding is eliminated by incubation in the presence of 100 nM non-radioactive $E_2$ (Figure 7A) or by 100 nM DES, a potent synthetic non-steroidal estrogen (Figure 8A). Other steroids (androgens, progestins, corticoids) have little effect on binding of [$^3$H]-$E_2$ to receptor unless extremely high concentrations of competitor are used (data not shown).

2. Sedimentation Properties of Receptor of Low Ionic-strength Sucrose Gradients

Specific binding peaks for [$^3$H]-$E_2$ appear at -5S and -10S when concentrated mammary cytosol prepared in TDE buffer is centrifuged on 5-20% sucrose in the same buffer (Figure 8A,B). Most specific binding is in the "10S" peak; specific binding in the "5S" region is difficult to detect unless [$^3$H]-$E_2$ used to label receptor is of very high specific activity. Uterine estrogen receptor sedimented at -8S while mammary receptor from the same animal sedimented at -10S (Figure 8B). Dilution of mammary cytosol to lower protein concentrations shifts the binding peak nearer "8S" (Figure 9). Incubation of uterine cytosol with concentrated mammary cytosol causes a shift in sedimentation of the uterine receptor to -9S and broadens the binding peak.
Figure 8. Sedimentation pattern of cytosol estrogen binders from mammary tissue or uterus of C3H/HeJ mice. Cytosols in TDE buffer were incubated with $[^3H]-E_2$ (138.1 Ci/mmole) for 12 hours, then layered onto 5-20% sucrose gradients prepared in TDE buffer. A) Mammary cytosol (5 mg protein/ml) from a mouse which had been lactating for 10 days, incubated in the absence (○--○) or presence (■■■) of 100 nM non-radioactive DES, then centrifuged for 22 hrs. b) Mammary cytosol (○--○), 6 mg protein/ml) from a mouse which had been lactating for 20 days; (▲▲▲) uterine cytosol (2 mg protein/ml) from the same animal. These gradients were centrifuged for 24 hrs. Note the difference in ordinate scales between panels A and B.
Figure 9. Effect of protein concentration on the sedimentation coefficient of mouse mammary estrogen receptor. Cytosol from a mouse which had been lactating for 20 days was initially prepared in TDE buffer at a concentration of 6 mg protein/ml, then incubated for 12 hrs with 2 nM $[^3H]_E_2$ (138.1 Ci/m mole) either undiluted (○—○) or diluted with TDE buffer 1:2 (●—●) or 1:3 (■—■). Gradients were centrifuged for 22 hrs.
The increase in S value for the uterine receptor is directly proportional to the total protein concentration in the incubation medium (Figure 10B).

3. Receptor Sedimentation on High Ionic-strength Sucrose Gradients

Uterine estrogen receptor, which sediments ~8S on low ionic-strength gradients, dissociates to a form sedimenting ~4S on gradients containing 0.4 M KCl (Figure 11A). Initial attempts to dissociate mammary receptor did not produce any clear 4-5S peaks (Figure 11B). However, incubation of mammary cytosol with \( ^3H \)-E\(_2\) of higher specific activity increased the binding peak height and allowed resolution of 4-5S binding near the top of the gradients (Figure 12, A-D). Gradients in Figure 12 also demonstrate that long incubation (12 hrs) of highly-concentrated cytosols leads to formation of 6-8S aggregates which do not dissociate to 4-5S in the presence of 0.4 M KCl. Dilution of cytosol protein (Figure 12, C & D) eliminates intermediate sedimentation peaks on 0.4 M KCl gradients; the resulting "5S" peaks remain broad however. Sharp, well-defined peaks can be obtained on both low-salt and high-salt gradients by reducing the incubation time of cytosol with \( ^3H \)-E\(_2\) (Figure 13A). Even in cytosol with a high protein concentration (7.8 mg/ml), sedimentation peaks are sharp if the incubation time is reduced to ~3 hrs (Figure 13A). The conversion of receptor from ~8-10S to ~4-5S by 0.4 M KCl is quantitative and occurs without loss of binding. The agreement in specific
Figure 10. Effect of mammary cytosol addition on sedimentation of mouse uterine estrogen receptor.

A) Mammary and uterine cytosols were prepared in TDE buffer from a mouse which had been lactating for 10 days. One aliquot of uterine cytosol (2 mg protein/ml) was diluted 1:2 with TDE buffer (▲▲) while a second aliquot was diluted 1:2 with "receptor-depleted" mammary cytosol which had a protein concentration of 6 mg/ml (○○). Samples were then incubated for 12 hrs with 2 nM $[^3H]E_2$ (91.3 Ci/mmole) and centrifuged on 5-20% sucrose gradients in TDE buffer for 22 hrs. Binding sites were eliminated from mammary cytosol before mixing with uterine cytosol by incubating the mammary sample with 100 nM non-radioactive DES for 3 hrs; unbound DES was removed from mammary cytosol by DCC treatment before mixing with uterine cytosol. B) Mammary and uterine cytosols were prepared as in "A". The gradient profiles represent binding to a constant amount of uterine receptor mixed with varying dilutions of depleted mammary cytosol: (○○), total protein 4 mg/ml, 9.0S; (●●●), total protein 2.5 mg/ml, 8.5S; (■■■), total protein 1.3 mg/ml, 8.1S.
Figure 11. Effect of high ionic strength buffer on sedimentation of mammary and uterine estrogen receptors which had been incubated with "low" specific activity $[^3H]-E_2$ (91.3 Ci/mmol). A) Uterine cytosol (1.8 mg protein/ml) prepared in TDE buffer was incubated with 3 nM $[^3H]-E_2$ for 12 hrs. One aliquot of the cytosol was layered onto a 5-20% sucrose gradient prepared in TDE buffer (O—O); to a second aliquot, crystals of KCl were added to give a final concentration of 0.4 M. This sample was allowed to stand for 15 minutes, then layered onto a 5-20% sucrose gradient prepared in 0.4 M KCl (O—O). Gradients were centrifuged at 40,000 rpm for 22 hrs. B) Mammary cytosol (5.4 mg protein/ml) was prepared and centrifuged as described for uterus. (O—O), sedimentation in TDE buffer; (O—O), sedimentation in TDEK buffer.
Figure 12. Effect of protein concentration on the sedimentation of mammary estrogen receptor in the absence or presence of 0.4 M KCl. Cytosol was prepared in TDE buffer at varying protein concentrations, then incubated with 2 nM [\(^{3}\)H]-E\(_2\) of high specific activity (138.1 Ci/m mole) for 12 hours. Aliquots were layered onto 5-20% sucrose gradients prepared either in TDE buffer (○-○) or in TDEK buffer (●-●) and centrifuged for 24 hrs at 40,000 rpm. Samples layered on TDEK gradients were brought to 0.4 M with KCl crystals before layering. A) Cytosol from a mouse which had been lactating for 20 days, 8 mg protein/ml. B) Cytosol from a mouse which had been lactating for 10 days, 6 mg protein/ml. C) 1:2 dilution of "B" cytosol (3 mg protein/ml). D) 1:3 dilution of "B" cytosol (2 mg protein/ml).
Figure 13. Effect of ammonium sulfate fractionation on receptor sedimentation in the absence or presence of EDTA. Mammary cytosol was prepared in TDE buffer from pooled tissue of 4 mice which had been lactating 10-12 days. Cytosol then was incubated with 2 nM [³²P]-E₂ (136.1 Ci/mmol) for 3 hrs at 4°C. One portion of cytosol was analyzed on low (O-O) and high salt (●-●) gradients without further treatment (Panel A) while the remainder of the cytosol was partially "purified" by ammonium sulfate fractionation as described in the text. All gradients were centrifuged at 40,000 rpm for 22 hrs. A) Unfractionated cytosol (7.8 mg protein/ml) B) Ammonium sulfate fraction resuspended in TDE buffer and centrifuged on gradients prepared in TDE buffer (final concentration: 2 mg protein/ml obtained from 10 ml of original unfractionated cytosol resuspended in 3 ml of TDE buffer) C) Ammonium sulfate fraction resuspended in TD buffer and centrifuged on gradients prepared in TD buffer (final concentration: 2.3 mg protein/ml obtained from 10 ml of original unfractionated cytosol resuspended in 3 ml of TD buffer).
binding between low-salt and high-salt assays on the same
cytosol is 96% (Figure 12A), 95% (Figure 12B), 99%
(Figure 13A) and 86% (Figure 13C).

4. Effect of Ammonium Sulfate Fractionation and
EDTA on Receptor Sedimentation Properties

Ammonium sulfate-fractionated receptor resuspended
in buffers containing EDTA sediments as broad aggregated
bands both on low-salt and high-salt gradients (Figure 13B).
Resuspension of the same cytosol fraction in EDTA-free
(TDE) buffer produces a sharp ~9S peak on low-salt gradients
and a broad ~5S peak on high-salt gradients (Figure 13C).
Ammonium sulfate fractionation increased the specific
activity of label in cytosol from 3270 dpm/mg cytosol
protein before fractionation to 7030 dpm/mg protein in the
resuspended ammonium sulfate precipitate; however, only
19% of the original binding protein was recovered in the
final fractionated cytosol. No further purification was
attempted. Tissue homogenized in EDTA-free buffers yields
much less receptor than is obtained with TDE buffer (data
not shown); thus, protease assays were performed on crude
mammary homogenate and on cytosol to determine if they
contain calcium-dependent proteolytic enzymes which might
be inhibited by EDTA.

5. Protease Assay on Mammary Homogenate and Cytosol

Crude mammary tissue homogenate contained prote-
olytic enzyme activity equivalent to 12 ug crystalline
trypsin per ml when incubated at 25°C (Figure 14).
Addition of excess Ca$$^{++}$$ did not produce detectable activity.
Figure 14. Proteolysis assay in crude mammary homogenate and mammary cytosol. Mammary tissue from a mouse which had been lactating for 10 days was prepared by the same method used in receptor analysis except that HED buffer was substituted for TDE buffer to avoid interference and high background in the assay. (○—○) crude homogenate suspension incubated at 25°C; (●—●) crude homogenate suspension incubated at 4°C; (□—□) cytosol incubated at 25°C.
in cytosol prepared in EDTA-containing buffer.

D. Discussion

Special care is needed in characterization of mammary estrogen binding because of the very low concentration of receptor and the high level of other macromolecules secreted by lactating tissue. The factors which affect sedimentation characteristics of uterine receptor have been thoroughly described in Stancel et al. (1973); they have emphasized the importance of protein concentration, incubation time, and ionic strength as critical variables in characterization of uterine estrogen receptor by sucrose gradient analysis. My experiments have shown that these same factors significantly alter sedimentation of mammary estrogen receptor.

Initially I attempted to overcome the problem of low receptor levels in mammary cytosol by keeping the cytosol protein concentrated. This allows detection of specific binding peaks on low-salt or high-salt gradients; the peaks typically are broad, however, indicating aggregation and lack of homogeneity in the binding molecule(s). Sharpness of the peaks can be considerably enhanced, even with high protein concentrations, by reducing the incubation time from ~12 hrs to ~3 hrs. I also have found it very helpful to use \( ^3 \text{H} \)-E\(_2 \) of exceptionally high specific activity; this has been necessary in separating binding peaks from background radioactivity in the 4-5S range near the top of the gradient.

Although short incubation times produce the clearest
SDG patterns, I have found that longer incubations (12-14 hrs) consistently yield better quantitative results in the DCC assay; i.e. Scatchard plots are more linear and the $N_s$ values are slightly increased by longer incubation. It is probable that binding association rates do not allow receptor to approach saturation in 3 hrs at the very dilute $[^3H]-E_2$ concentrations used in the DCC assay. Receptor may aggregate during long incubations, but aggregates still retain the ability to bind hormone quantitatively as shown by the conservation of binding when 9-10S aggregates are dissociated to 4-5S by high ionic-strength buffers. Zava et al. (1976) also have shown that even protamine-precipitated estrogen receptor retains the ability to bind hormone, and to quantitatively exchange that hormone for radio-labeled hormone when temperature is elevated to facilitate dissociation. Sedimentation coefficients reported for my experiments should be regarded as approximate. The BSA and gamma-globulin marker proteins were centrifuged in tubes separate from cytosol samples; $S$ values can be determined more accurately by including $^{14}C$-labeled marker proteins in the same tubes with samples (Stancel et al., 1973).

Taking the technical considerations into account, the properties of mouse mammary estrogen receptor appear to be essentially the same as the well-studied receptor of rat uterus (reviewed by Jensen et al., 1974). The most notable difference is in the concentration of receptor sites; uteri of lactating rats exhibit an increase in receptor concentration
from -100 pmoles/g cytosol protein on the first day of lactation to -300 pmoles/g at day 20 (Keightley & Okey, 1974). Mammary cytosol receptor concentrations are only about 10-30% of uterine levels, as reported in this study and in studies on rat mammary (Beers & Wittliff, 1973b; Esueh et al., 1973; Gardner & Wittliff, 1973a; Keightley & Okey, 1974), mouse mammary (Shyamala & Nandi, 1972; Bondy & Okey, 1977), and vole mammary (Beers & Wittliff, 1973a).

The dissociation constant in the present study ($K_d = 5 \times 10^{-11} \text{ M}$) would seem to indicate that the affinity of the estradiol-receptor interaction is higher in mouse mammary tissue than previously reported for uterus. It should be noted, however, that the Scatchard plot correction procedure employed (Chamness & McGuire, 1975b) tends to decrease the calculated number of binding sites and to increase the calculated affinity. I have applied this correction procedure to assays on rat uterine cytosol and have calculated $K_d$ values of $5-8 \times 10^{-11} \text{ M}$.

As previously discussed under technical considerations, the sedimentation characteristics of mouse mammary cytosol receptor vary considerably depending upon the procedure used. The mammary receptor does not appear to have any unique properties which would indicate that it is fundamentally different from uterine receptor. The high sedimentation coefficient of mammary receptor on low-salt gradients is a product of high protein concentration, prolonged incubation or both. Similar changes in sedimentation of uterine receptor can be induced by incubating it in the presence of
concentrated mammary cytosol. The low concentration of receptor in mammary cytosol makes it unlikely that the change induced in uterine receptor sedimentation is due to specific association of mammary receptor (or its subunits) with uterine receptor. It is more likely that the increased $S$ values are due to non-specific association of uterine receptor with other macromolecules in mammary cytosol. Stancel et al. (1973) have reported that uterine receptor, diluted with ovalbumin or lactic dehydrogenase, sediments on high-salt gradients in the same position as receptor diluted with plain buffer; thus, increased $S$ values might not be produced simply by increasing the total protein concentration with any protein, but may depend upon the type of macromolecule to which the receptor is exposed.

In my experiments with high ionic-strength gradients (0.4 M KCl) mammary cytosol receptor sedimented nearer "5S" than the usual "4S" form considered typical of uterine receptor (Jensen et al., 1974); this shift, too, probably is an artifact due to high protein concentration as has been described for uterine receptor by Stancel et al. (1973) and Chamness and McGuire (1972). Rat mammary cytosol receptor on high-salt gradients also sediments nearer "5S" than "4S" (Gardner & Wittliff, 1973). Because of the low receptor concentration in mammary cytosol, it is difficult to perform dilution experiments to determine if receptor might sediment nearer "4S".

A key event in estrogen action is a temperature-
dependent transformation of cytosol receptor from "4S" to 
"5S" prior to nuclear uptake (Jensen et al., 1974). For 
reasons stated above, the form of receptor I detected in 
cytosol incubated at 0-4°C probably is a non-specifically 
aggregated form of the "4S" receptor rather than receptor 
which has undergone transformation. I have been able to 
extract "5S" receptor from mammary nuclei of C3H mice 
injected in vivo with [3H]-E2 (Chapter IV).

Homogenization of mammary tissue in EDTA-free buffer 
yields much less receptor than homogenization in TDE buffer. 
Although there is significant protease activity in crude 
mammary homogenate incubated at 25°C, it seems unlikely that 
the very low levels of proteolysis at 0-4°C could account 
for loss of receptor during sample preparation. Mammary 
cytosol also has little proteolytic activity, with or 
without Ca++, even at 25°C. Resuspension of ammonium 
sulfate-precipitated receptor in buffers containing EDTA 
leads to production of large aggregates on low-salt or 
high-salt gradients. Shyamala (1975) reported a similar 
phenomenon in mammary cytosol from lactating BALB/cCrCl 
mice; she interpreted this result (along with the effects 
of other chelating agents and addition of metal ions) to 
mean that mammary estrogen receptor contains a bound metal 
ion which is essential for maintaining a receptor confor-
mation which can bind steroid. She also reported, however, 
that unfractonated cytosol in EDTA-containing buffer 
undergoes marked aggregation when centrifuged on gradients
containing 0.4 M KCl. I find that sharp 5S peaks can be obtained in the presence of EDTA if the incubation time or protein concentrations are not excessive. It is likely that EDTA-induced aggregation of ammonium sulfate-fractionated receptor is due primarily to enhanced non-specific interactions of receptor with other macromolecules in the concentrated samples.

Overall, the mammary estrogen receptor does not appear to have any unusual properties which would distinguish it from uterine receptor nor would suggest a unique function which might explain the high sensitivity of mammary tissue to estrogen-supported carcinogenesis. Considering the technical difficulties which exist in attempting to understand estrogen receptor structure and function in uterine tissue, it is not surprising that so little is known about the properties of mammary receptor.
CHAPTER IV

ESTROGEN BINDING IN MAMMARY TISSUE OF C3H MICE
WITH OR WITHOUT THE MOUSE MAMMARY TUMOR VIRUS

A. Introduction

Estrogen treatment of C3H mice having the milk-borne mouse mammary tumor virus (MMTV) results in a very high incidence of mammary tumors at an early age (Gass et al., 1974). Mice with the milk-transmitted virus but free of estrogen do not develop tumors, nor is estrogen an effective carcinogen in the absence of MMTV (Gass et al., 1974). The mechanism(s) of interaction between estrogen and MMTV in mammary tumor induction is unknown. Various steroids have been demonstrated to stimulate production of RNA-containing tumor viruses by infected cells (Parks et al., 1974; Fowler et al., 1975) and it is possible that the action of estrogen is primarily to condition mammary cells for virus production/infection.

It has been demonstrated, however, that the presence of MMTV alters the sensitivity of mammary tissue to somatotropic hormone (Nandi, 1966). Thus, I measured estrogen binding to cytosol estrogen-receptor of MMTV-bearing and MMTV-free C3H mice to determine if this key initial step in estrogen action might be changed by the presence of MMTV.
Also, uterine weight response to oral diethylstibestrol (DES) was assayed in the two substrains of mice to determine if MMTV had any generalized effect on responsiveness of estrogen target tissues.

B. Materials and Methods

1. Uterine Weight Bioassay for Estrogenic Potency of Oral DES

Adult female C3H/An and C3H/Anf mice (Cumberland View Farms, Clinton, Tennessee) were ovariectomized under ether anesthesia. Two weeks later the mice began diets containing 0, 12.5, 50, 100 or 250 parts per billion DES, and one week thereafter they were killed and their uteri removed, cleaned of extraneous tissue and weighed.

2. Tissue Preparation for Estrogen Binding

Mice used in estrogen-binding studies were C3H/HeJ (with MMTV) or C3H/HeJ$^{+}$ (MMTV-free) purchased from the Jackson Laboratory, Bar Harbor, Maine, or reared in our laboratory from Jackson stock. Mice were killed by cervical fracture 10 days after they had delivered a litter. Mammary tissue was removed by blunt dissection, cut into small pieces, rinsed with iced TDE buffer (to remove excess milk), dried on filter paper, and finely minced for homogenization. All procedures were done at 0-4°C unless otherwise indicated. Minced tissues were homogenized in 3 ml TDE buffer per g tissue using a Polytron PT-10 at a setting of "4". The homogenate was centrifuged at 1400 x g for 15 minutes and the
resulting supernatant fluid was centrifuged at 105,000 x g for 90 min to obtain a cytosol (supernatant) fraction. Cytosol was diluted to about 2 mg protein/ml with TDE buffer and the protein concentration was determined by the method of Lowry et al. (1951).

3. Scatchard Plot Analysis

The concentration of high-affinity estrogen binding sites ("receptors") was determined as previously described (Keightley & Okey, 1973). Briefly, this involved incubation of cytosol with $^{3}$H-E$_{2}$ in the incubation tube and "unbound" $^{3}$H-E$_{2}$ was removed with dextran-coated charcoal. Total $^{3}$H-E$_{2}$ and "bound" $^{3}$H-E$_{2}$ were determined by liquid scintillation counting and the data were analyzed and corrected for non-specific binding by use of Scatchard plot as described by Chamness and McGuire (1975b). Saturability of binding was checked by using unlabeled E$_{2}$ (0.1 uM) as a competitor.

4. Sucrose Density Gradient Analysis

Cytosol aliquots were incubated overnight at 4°C with $^{3}$H-E$_{2}$ at a concentration of 2nM. In competition experiments unlabeled DES (0.1 uM) was added before $^{3}$H-E$_{2}$. After incubation, unbound $^{3}$H-E$_{2}$ was removed by adding 1 ml of cytosol to a charcoal pellet made by centrifuging 2 ml of a suspension containing 0.5% charcoal/0.05% dextran in TE buffer at 4000 x g for 20 min. Charcoal was resuspended on a vortex mixer, and then removed by centrifugation. One-half ml of this supernatant was layered onto a linear 5-20% sucrose gradient prepared in TDE buffer.
Gradients were centrifuged at 40,000 rpm for 20-24 hrs in a Beckman SW 41 rotor at 4°C. Forty 300-µl fractions were collected from each gradient on an ISCO Model 640 gradient fractionator. Radioactivity in each fraction was measured by liquid scintillation counting and corrected for counting efficiency. Bovine serum albumin (4.6 S) and human gamma-globulin (7.1 S) were used and marker proteins to determine approximate sedimentation coefficients of the radioactive peaks using the method of Martin and Ames (1961).

5. Nuclear Uptake in vivo

Tritiated E₂ (0.05 µg; 136 Ci/m mole, in 0.9% NaCl) was injected sc into the scapular region of a C3H/HeJ mouse which had been lactating for 10 days. Two hours later the animal was killed and the mammary tissue removed, rinsed in TDE buffer, and homogenized as described above. The homogenate was centrifuged at 1600 x g for 15 min; then the nuclear pellet was washed three times with TDE buffer. The pellet was homogenized in TDE buffer containing 0.5 M KCl (pH 8.5), and the homogenate was allowed to stand at 4°C for 1 hr to extract nuclear receptor. After centrifuging the homogenate at 105,000 x g for 1.5 hr, the supernatant was treated with DCC to remove unbound steroid, then 0.5 ml was layered onto a 5-20% sucrose gradient prepared in TDE buffer containing 0.5 M KCl (pH 8.5). The gradients were centrifuged and fractionated as described above for cytosol SDG analysis.

C. Results

Uterine weight response to oral DES was not significantly different in MMTV⁺ vs. MMTV⁻ C3H/An mice (Figure 15).
Figure 15. Uterine weight response to oral DES in MMTV\(^+\) or MMTV\(^-\) C3H mice. Ovariectomized mice with the milk-borne MMTV (C3H/An) or without the virus (C3H/An\(\_f\)), 5 per dose, were fed DES for 1 week at the dose levels indicated. Dose-response lines were calculated by least-squares linear regression, omitting the 250 ppb dose. Individual response lines shown have a significant slope and linearity, but do not significantly differ between C3H/An and C3H/An\(\_f\).
The response was linear over the range 0-100 ppb DES for each substrain, but 250 ppb DES (an effective carcinogenic dose (Cass et al., 1964)) was outside the linear-response range of the assay.

Scatchard plot analysis of mammary cytosol reveals high-affinity $E_2$ binding typical of the classical estrogen receptor (Figure 16). The concentration of estrogen-receptor sites in mammary cytosol was not significantly different between MMTV$^+$ vs. MMTV$^-$ groups (Figure 17); however, the dissociation constants ($K_d$) for the $E_2$-receptor interaction were significantly different ($p < 0.02$), indicating a higher affinity of binding in MMTV$^-$ mice.

On sucrose density gradients the uterine receptor sedimented in the expected "6S" region (Figure 18). Sedimentation in mammary cytosol, however, was nearer "10S". This was true both for MMTV$^+$ mice (Figure 18, Lower panel) and for MMTV$^-$ C3H mice; the sedimentation coefficient was consistently larger in mammary cytosol than in uterine cytosol from the same animal. The large S value for the mammary receptor is not unique to C3H mice; SDG analysis of mammary cytosol from a lactating C57BL/6 mouse also revealed a higher S value than the uterine receptor in the same animal (data not shown).

Specificity and limited capacity of both uterine and mammary cytosol binders are illustrated by the extinction of binding peaks when cytosols were incubated with $[^3H]E_2$ in the presence of 0.1 µM non-radioactive DES (Figure 18).
Figure 16. Representative Scatchard plots (the ratio of bound $E_2$ [(pmoles/g cytosol protein)] to unbound $E_2$ [(pmoles/g cytosol protein)] for binding of $[^3H]E_2$ in mammary cytosol from MMTV+ or MMTV- C3H mice. The binding assay described in the text was done on mammary cytosol from lactating C3H/HeJ (with milk-borne MMTV) and C3H/HeJ_fh mice (without the virus). Lines are calculated by least squares linear regression using the correction for non-specific binding recommended by Chamness and McGuire (1975b). The number of high-affinity $E_2$ binding sites ($N_s$: pmoles/g cytosol protein) is obtained from the intercept of calculated lines on the abscissa. Dissociation constants ($K_d$: pmoles/L) are derived from the slopes of the calculated lines.

- = uncorrected data;  O = plot corrected for non-specific binding using the method of Chamness and McGuire (1975b). Overall assay precision was assessed by computing the coefficient of variation (CV) for all animals in which sufficient cytosol was available to do multiple determinations ($N = 16$). For $N_s$ the CV was 7%; for $K_d$ the CV was 17.5%. 
C3H HeJ
$N_S = 30.0 \text{ pmole/g}$
$K_d = 1.1 \times 10^{-10} \text{ M}$
$r = 0.9018$

C3H He-Jh
$N_S = 38.1 \text{ pmole/g}$
$K_d = 7.9 \times 10^{-11} \text{ M}$
$r = 0.893$
Figure 17. Estrogen-receptor concentration in mammary cytosol of lactating MMTV+ C3H mice.

Scatchard plot analysis (Chamness & McGuire, 1975b) of $E_2$ binding was done on mammary cytosol from C3H/HeJ mice (with milk-borne MMTV) and C3H/HeJ$_{fr}$ mice (without the virus). Large bars indicate the mean number of binding sites ($N_s$: pmoles/g cytosol protein): small vertical bars give the standard error of the mean for each group. (○) = $N_s$ values for individual mice. The difference between group means is not statistically significant by "t"-test ($p > 0.05$).

Dissociation constants ($K_d$: pmoles/L) derived from Scatchard plot analysis for each individual animal were statistically analyzed: for MMTV+ mice the mean ($\pm$SEM) $K_d$ value was $128 \pm 20.7$ ($N = 13$); for MMTV- mice the mean $K_d$ value was $66 \pm 9.0$ ($N = 14$). By "t"-test the means are significantly different ($p < 0.02$).
Figure 18. Sedimentation patterns of cytosol estrogen binder from lactating mammary tissue and uteri of C3H/HeJ or C3H/HeJ_{fh} mice. Mammary or uterine cytosols (6 mg protein/ml) were incubated with 2 nM $[^3H]E_2$ in TDE buffer and separated on 5-20% linear sucrose gradients as described in the text. Sedimentation coefficients were calculated from BSA and human gamma globulin marker proteins run in separate tubes.

Upper panel: C3H/HeJ_{fh} mouse (MMTV$^{-}$)

Lower panel: C3H/HeJ mouse (MMTV$^{+}$)

(○) = uterine cytosol; (■) = mammary cytosol; (▲) = mammary cytosol incubated in the presence of 0.1 uM DES
Testosterone or dihydrotestosterone (1.0 μM) were ineffective in reducing binding of \(^{3}H\)-E\(_2\) to cytosol receptor (data not shown).

Receptor extracted from the nucleus of a C3H/HeJ (MMTV\(^{+}\)) mouse injected in vivo with \(^{3}H\)-E\(_2\) sedimented at 5S on a gradient containing 0.5 M KCl (Figure 19).

D. Discussion

Past experiments on mice from Cumberland View Farms have shown that C3H/An have high mammary tumor incidence when treated with 250 ppb DES, while similarly treated C3H/An\(_{f}\) mice rarely develop mammary tumors (Gass et al., 1974). C3H mice freed of milk-transmitted MMTV have a very low mammary tumor incidence even when allowed to breed; the tumor incidence rises near 100%, however, when these mice are reinoculated with MMTV (Ihle et al., 1976). At the time estrogen-receptors in mammary cytosol were to be studied, C3H mice were not available from Cumberland View Farms; hence, comparisons were made on C3H/HeJ versus C3H/HeJ\(_{fr}\) mice from The Jackson Laboratory. It previously was reported that breeding/stressed C3H/He Jackson female mice (with milk-borne MMTV) had a mammary tumor incidence near 100% by 18 months of age; MMTV-free C3H/HeJ\(_{fr}\) mice developed only about a 7% tumor incidence late in life (Riley, 1975).

The uterine weight bioassay does not reveal any significant difference between the two substrains; hence the presence of MMTV does not seem to cause any generalized
Figure 19. Sedimentation pattern of estrogen binder extracted from mammary nuclei of a C3H/HeJ mouse (bearing MMTV). $[^3H] - E_2$ (0.05 ug) was injected sc in the scapular region; the mouse was killed two hours later and the receptor extracted from mammary nuclei as described in the text. The nuclear extract was centrifuged on a 5-20% sucrose gradient containing 0.5 M KCl. The sedimentation coefficient is calculated from a BSA marker run in a separate tube.
change in sensitivity of mice to exogenous carcinogenic doses of estrogen.

Only a few reports exist on specific estrogen-binding in mouse mammary tissue (Fuca & Brecciani, 1969; Shyamala, 1972; Shyamala, 1975; Shyamala & Nandi, 1972) to quantitate estrogen-receptor in high-tumor versus low-tumor strains.

As reported in this chapter, the presence of MMTV in mice of similar genetic background does not substantially alter the concentration of cytosol receptor sites for estrogen. The affinity of the estrogen-receptor interaction was lower in C3H mice bearing MMTV. Shyamala previously reported (Shyamala & Nandi, 1972) that estrogen-independent mammary tumors from strains GRS/A mice (with MMTV) have a defective nuclear uptake of estrogen-receptor complex. In my experiments receptor could be extracted from mammary nuclei of MMTV-bearing mice and sedimented in the 5S region as does the classical rat uterine receptor (Jensen & Desombre, 1973). Whether nuclear uptake might become defective during the process of malignant transformation in mouse mammary awaits further study.

Previous reports on the characteristics of cytosol estrogen receptor in rodent lactating mammary tissues indicated that it sediments near 8-9S for the Fischer rat (Gardner & Wittliff, 1973a), vole (Beers & Wittliff, 1973a) and BALB/c mouse (Shyamala, 1975). Specific binding in the 8-9S region also has been reported to exist in DES-induced
mammary tumors of C3H mice (Lewko et al., 1976). The "10S" form reported in this chapter is partly due to non-specific aggregation caused by the high protein concentrations necessary to detect mammary cytosol binding peaks. In experiments discussed earlier (Chapter III), the mammary estrogen receptor was found to sediment nearer to 8-9S if a higher specific-activity \(^{3}\text{H}\)-E\(_2\) and diluted cytosol was used. There do not appear to be any dramatic differences in cytosol estrogen binding which can be attributed to the presence of MMTV.
CHAPTER V

DIETHYLSTILBESTROL BINDING TO ESTROGEN RECEPTOR
IN MAMMARY TISSUE AND UTERUS OF C3H MICE

A. Introduction

Diethylstilbestrol (DES), a synthetic non-steroidal estrogen, is an effective mammary tumor inducer in C3H mice having the mouse mammary tumor virus (Gass et al., 1974). DES also has been implicated in human vaginal adenocarcinoma (Herbst et al., 1971). The mechanism of tumorigenesis by DES is not known, but it generally is believed that the tumorigenic potential of DES is associated with its estrogenic properties. Mechanisms of estrogen action have been greatly clarified by discovery of specific, high-affinity binding sites ("receptors") for estradiol-17β (E₂) in such classical estrogen target tissues as rat uterus (Jensen & DeSombre, 1972). Receptors with high affinity for E₂ are also found in mammary tissue of C3H mice (Bondy & Okey, 1977). Although DES is a biologically potent estrogen and has been shown in several studies to compete with E₂ for estrogen binding sites (Korenman, 1969; Geynet et al., 1972), there is little direct evidence available for the nature of possible DES binding in estrogen target tissues. It also has been reported that certain synthetic hormones may bind to
different cellular receptors than do equivalent "natural" hormones (Agarwal, 1976). Thus, I used $[^3H]$-DES of high specific-activity to determine directly if DES binds to estrogen receptor sites and whether binding might differ in any fundamental way from $[^3H]$-E$_2$ binding.

B. Materials and Methods

1. Animals and Tissue Preparation

Mice used were 4-6 month old C3H/HeJ females reared in our laboratory from stock originally purchased from the Jackson Laboratory, Bar Harbor, Maine. Mice were killed by cervical fracture 10 days after they had delivered a litter and the lactating mammary tissue was removed by blunt dissection. Tissues were rinsed with several volumes of iced TDE buffer (to remove excess milk), dried on filter paper, and minced with a scalpel before homogenization. Uteri were obtained from non-pregnant, non-lactating females and prepared in a similar fashion. All procedures were done at 0-4ºC unless otherwise indicated.

2. Cytosol Preparation

Minced tissues were homogenized in 3 ml TDE buffer per g tissue using a Polytron PT-10 at a setting of "4". The homogenate was centrifuged twice at 4000 x g for 15 min and the resulting supernatant was centrifuged at 105,000 x g for 90 min to obtain a supernatant (cytosol) fraction. Cytosol was carefully removed from beneath the surface lipid layer with a disposable pipet, and then diluted with TDE buffer to a concentration ~4 mg protein/ml. Final protein concentration
was determined by the method of Lowry et al. (1951).

3. Sucrose Density Gradient Analysis

Samples for gradient analysis were prepared by incubating 1 ml of cytosol for 3 hrs at 0-4°C with radioligand, usually at a concentration of 2 nM. In competition studies cytosols were pre-incubated with competitors for 15 min before addition of radioligand. After incubation, unbound radioligand was removed by adding the cytosol to a pellet of DCC. DCC was resuspended on a vortex mixer, then removed by centrifugation. One-half ml of this supernatant was layered onto a linear 5-20% sucrose gradient prepared in the appropriate high- or low-salt buffer. Gradients were centrifuged at 40,000 rpm in a Beckman SW 41 rotor at 4°C. Forty 300-μl fractions were collected from each gradient tube on an automatic gradient fractionator. Radioactivity in each fraction was determined by LSC and corrected for counting efficiency. Bovine serum albumin (4.6S) and human gamma globulin (7.1S) were used as marker proteins to determine the approximate sedimentation coefficients of the radioactive peaks by the method of Martin and Ames (1961).

4. Nuclear Uptake in vivo

$[^3H] - DES$ (0.2 μg; 62.4 Ci/mmol in 0.9% NaCl) was injected subcutaneously into the scapular region of a C3H mouse, which had been lactating for 10 days. The mouse was sacrificed 2 hrs after injection and its mammary tissue was removed, rinsed in TDE buffer, and homogenized as
described above. The homogenate was centrifuged at 1600 x g for 15 min; the supernatant was decanted, and the nuclear pellet washed three times with TDE buffer. The washed pellet was homogenized in TDE buffer containing 0.5 M KCl (pH 8.5) and the homogenate was allowed to stand for 1 hr at 4°C to extract nuclear receptor. After centrifuging at 105,000 x g for 1.5 hr, the supernatant was mixed with DCC pellet to remove unbound DES, and then layered onto a 5-20% sucrose gradient prepared in TDE buffer containing 0.5 M KCl (pH 8.5). The gradients were centrifuged and fractionated as described for cytosol SDC analysis.

5. Dextran-coated Charcoal Assay/Scatchard Plot Analysis

The estrogen-binding capacity and the $K_d$'s for the estrogen-receptor interaction were measured by the dextran-coated charcoal assay (DCC) using either [$^3$H]$E_2$ (152 Ci/mmole) or [$^3$H]DES (62.4 Ci/mmole) as radioligands. Aliquots of cytosol (~2 mg protein/ml) were incubated with radioligand at 15 concentrations between 10 nM and 70 pM. After incubation at 0-4°C overnight, an aliquot was removed for determination of total radioligand in the incubation tube, and then "unbound" radioligand was removed with DCC. The radioactivity in the samples were measured by LSC and the data were analyzed and corrected for non-specific binding using the Scatchard plot as described by Chamness and McGuire (1975b).
6. Indirect Estimation of Binding Affinities by Ratio of Association Constants

The method used was adapted from that of Korenman (1969) and consists essentially of using the DCC-assay/Scatchard-plot analysis to determine the concentration of competitor (i.e., DES) required to reduce cytosol binding of \( [^3\text{H}]\)-E\(_2\) to 50% of the control (no competitor) value. Cytosol was prepared as described above except that tissues from several animals were pooled. Full DCC/Scatchard analysis of \( [^3\text{H}]\)-E\(_2\) binding was performed in the presence of unlabeled DES or unlabeled E\(_2\) at concentrations between 0.1 \( \mu \text{M} \) and 1 \( \text{pM} \). The \( N_s \) and \( K_d \) for \( [^3\text{H}]\)-E\(_2\) binding at various competitor concentrations were computed by corrected Scatchard plot analysis as described above. \( N_s \) values were plotted against competitor concentrations and the \( K_c \) (dissociation constant for the competitor-receptor interaction) was computed using the ratio-of-association-constants method as described by Wittliff (1975).

C. Results

As shown in Figure 20, \( [^3\text{H}]\)-DES-labeled mammary cytosol shows a sedimentation profile nearly identical to cytosol labeled with \( [^3\text{H}]\)-E\(_2\). Binding of \( [^3\text{H}]\)-DES in the "8S" region is extinguished by incubation in the presence of unlabeled estrogens (100 nM DES or 100 nM E\(_2\)) androgens (testosterone or dihydrotestosterone) did not inhibit \( [^3\text{H}]\)-DES or \( [^3\text{H}]\)-E\(_2\) binding unless androgen concentrations were higher than 10 \( \mu \text{M} \) (data not shown). The small peak of \( [^3\text{H}]\)-DES bound in the "4-5S" region in mammary cytosol is not altered by incubation.
Figure 20. Sedimentation of cytosol estrogen binders incubated either with $[^3H]$-DES or $[^3H]$-E$_2$. Cytosols from the mammary gland and uterus of a C3H/HeJ mouse which had been lactating for 10 days were prepared in TDE buffer. Aliquots of the cytosols were incubated either with $[^3H]$-E$_2$ (138.1 Ci/m mole) or $[^3H]$-DES (62.4 Ci/m mole) for 3 hrs at 4°C; then 500 ul aliquots were layered onto 5-20% sucrose gradients prepared in TDE buffer. Gradients were centrifuged for 20 hrs at 40,000 rpm.

(A) Mammary cytosol (4.0 mg protein/ml) incubated with 1.3 nM $[^3H]$-E$_2$.

(B) The same cytosol incubated with 1.0 nM $[^3H]$-DES in the absence of competitors (○○) or in the presence of either 100 nM unlabeled DES (■■■) or 100 nM unlabeled E$_2$ (▲▲▲).

(C) Uterine cytosol (1.0 mg protein/ml) incubated with 1.0 nM $[^3H]$-DES in the absence (○○○) or presence (■■■) of 100 nM DES.
in the presence of competing estrogens indicating that this peak is not specific, saturable "receptor". Uterine cytosol incubated with $[^3H]$-DES exhibits binding in the "8S" region and binding in the "4-5S" region; binding in the "4-5S" peak is partially inhibited by incubation in the presence of 100 nM unlabeled DES indicating that this peak may be a mixture of high-affinity, low-capacity binding ("receptor") and a high-capacity binder which is not saturable by 100 nM DES.

Binding in the "8S" region of mammary cytosol is saturated between 5-10 nM $[^3H]$-DES (Figure 21.) $[^3H]$-DES binding in the "4-5S" region increases in proportion to the incubating concentration of $[^3H]$-DES used and radioactivity in the "4-5S" region remains bound even after charcoal treatment. The radioactivity in this region is not displaced by incubation in the presence of 100 nM unlabeled DES, whereas the "8S" peak is extinguished. Binding of $[^3H]$-E$_2$ in the "4-5S" region does not exhibit this very high capacity (data not shown).

On high ionic-strength (0.4 M KCl) gradients $[^3H]$-E$_2$ labeled receptor sediments at 4-5S as does receptor labeled with $[^3H]$-DES (Figure 22). The quantity of receptor detected using either $[^3H]$-DES or $[^3H]$-E$_2$ on high ionic-strength gradients is equivalent to the amount detected using low ionic-strength gradient analysis on the same cytosols.

After in vivo injection of lactating mice with $[^3H]$-DES a "4-5S" high-affinity binder can be extracted from
Figure 21. Effect of $[^3]H$-DES concentration on the sedimentation pattern of mouse mammary cytosol. Cytosol (4.5 mg protein/ml) from the mammary gland of a mouse which had been lactating for 10 days was prepared in TDE buffer. Aliquots of cytosol were incubated at 4°C for 3 hrs with 2.0 nM (○—○), 5.0 nM (□—□) or 10.0 nM (△—△) $[^3]H$-DES. An additional aliquot was incubated with 10 nM $[^3]H$-DES plus 100 nM unlabeled DES (■—■). Samples (500 ul) were layered onto 5-20% sucrose gradients prepared in TDE buffer and were centrifuged for 24 hrs at 40,000 rpm.
Figure 22. Effect of ionic strength on the sedimentation of mouse mammary cytosol receptor incubated either with $[^{3}\text{H}]$-E$_2$ or $[^{3}\text{H}]$-DES. Cytosol (4.0 mg protein/ml) was prepared in TDE buffer from the mammary gland of a mouse which had been lactating for 10 days. Aliquots were incubated for 3 hrs either with 2.0 nM $[^{3}\text{H}]$-DES (Panel A) or 2.0 nM $[^{3}\text{H}]$-E$_2$ (Panel B). Samples of 500 ul were layered on a 5-20% sucrose gradients prepared in TDE buffer without additional salt (○—○) or TDE buffer containing 0.4 M KCl (●—●); samples centrifuged on high-salt gradients were brought to 0.4 M KCl 15 minutes prior to layering on the gradient. Centrifugation was for 20 hrs at 40,000 rpm.
mammary gland nuclei (Figure 23).

Repeated SDG assays (corrected for the difference in specific activity of the radioligands) show that the quantity of "8S" receptor detected is somewhat higher (1.2X) when $[^3H]$-DES is used than when $[^3H]E_2$ is used on the same cytosols (Table 2). In DCC/Scatchard-plot assays (Figure 24) $[^3H]$:DES consistently yields a much higher (1.6X) apparent concentration of binding sites than does $[^3H]E_2$ (Table 2). By direct DCC/Scatchard-plot analysis $[^3H]$:DES is bound to mammary cytosol with an affinity about 4 times lower than that for $[^3H]E_2$ binding. In determinations on cytosols from six different mice the $K_d$ for the DES-receptor interaction was $2.6 \pm 0.39 \times 10^{-10}$ M (mean $\pm$ standard deviation) compared with $8.3 \pm 2.9 \times 10^{-11}$ M for $E_2$. Indirect measurement by RAC indicated a $K_d$ of $8 \times 10^{-11}$ M for DES binding in mammary cytosol (Figure 25). The reasons for the apparently lower affinity and higher capacity of binding using $[^3H]$:DES as ligand are considered in the Discussion.

**B. Results**

DES long has been recognized as a very potent estrogenic compound. It is used widely as an effective *in vitro* competitor for estrogen receptor sites in assays on animal tissues (Toft & Gorski, 1966) and human breast tumors (Wittliff, 1975). Although there is extensive literature which indicates indirectly (through competition experiments) that DES binds to estrogen receptor, surprisingly little information exists on direct assessment of binding, i.e., by
Figure 23. Sedimentation pattern of receptor from mouse mammary nuclei after an in vivo injection of 0.2 μg [3H]-DES. Nuclear extract, prepared as described in the text, was layered on a 5-20% sucrose gradient containing 0.4 M KCl. Centrifugation was for 20 hrs at 40,000 rpm.
Table 2. Relative concentration of binding sites in mammary cytosol as detected by different assay techniques. Sucrose density gradient analyses (SDG) and dextran-coated charcoal/Scatchard-plot analyses (DCC) were performed on several mammary cytosols using either $[^3H]_{-}^{\text{DES}}$ or $[^3H]_{-}^{\text{E}_2}$ as radioligands. Values in the table indicate the concentration of binding sites detected relative to $[^3H]_{-}^{\text{E}_2}$ by SDG as the standard ($=100$).

<table>
<thead>
<tr>
<th>Technique</th>
<th>Ligand</th>
<th>SDG</th>
<th>DCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$[^3H]_{-}^{\text{E}_2}$</td>
<td>100</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>$[^3H]_{-}^{\text{DES}}$</td>
<td>120</td>
<td>210</td>
</tr>
</tbody>
</table>
Figure 24. Scatchard plot analysis of $[^3H]E_2$
(Panel A) and $[^3H]DES$ (Panel B) binding in mouse mammary
cytosol. The samples illustrated were from mammary
cytosol (2.0 mg protein/ml) prepared in TDE buffer from
a C3H mouse which had been lactating for 10 days.
Aliquots of cytosol were incubated for 16 hrs at 4°C
with concentrations of $[^3H]E_2$ or $[^3H]DES$ between 10nM
and 70 pM. Specific, high-affinity binding (○—○)
was computed using the method of Chamness and McGuire
(1975b).

(●—●) = uncorrected data; (○—○) = plot corrected
for non-specific binding using the method of Chamness
and McGuire. $r =$ correlation coefficients for corrected
plots.
Figure 25. Comparative competition of unlabeled DES and unlabeled $E_2$ for specific, high-affinity binding sites in mouse mammary cytosol. The number of available receptor sites ($N_s$, pmoles/g cytosol protein) was determined in the presence of various concentrations of unlabeled DES or $E_2$ using Scatchard plot analysis as previously described. Dotted lines indicate the competitor concentration required to reduce specific receptor binding to 50% of the control (no competitor) level.
using \([^{3}H]\)-DES.

The experiments reported in this chapter indicate that \([^{3}H]\)-DES binding in mammary or uterine tissue is very similar to binding of \([^{3}H]\)-E\(_{2}\). The qualitative properties of cytosol estrogen receptor using \([^{3}H]\)-DES as the ligand appear to be the same as when receptor is labeled with \([^{3}H]\)-E\(_{2}\). On low ionic-strength sucrose gradients \(^{3}\)H-DES-labeled receptor sediments in the same position as receptor labeled with \([^{3}H]\)-E\(_{2}\). Both \([^{3}H]\)-E\(_{2}\) and \([^{3}H]\)-DES-labeled receptors dissociate to \(-4-5\)S when centrifuged on gradients containing 0.4 M KCl. DES also induces receptor transformation and nuclear uptake as evidenced by recovery of \(-4-5\)S receptor from mammary nuclei of mice injected in vivo with \([^{3}H]\)-DES.

The concentration of high-affinity binding sites indicated by DCC/Scatchard-plot analysis consistently is higher when \([^{3}H]\)-DES is used as ligand than when \([^{3}H]\)-E\(_{2}\) is used with the same cytosol. DCC/Scatchard-plot analysis with \([^{3}H]\)-DES may overestimate the concentration of receptor sites and underestimate the affinity of receptor-DES interaction. Although Scatchard plots using \([^{3}H]\)-DES are highly linear, DCC assays yield higher \(N_{S}\) values than are obtained by SDG analysis with \([^{3}H]\)-DES (or with \([^{3}H]\)-E\(_{2}\), either by DCC or SDG techniques). The increase in apparent concentration of "receptor" sites may be due to relatively high-affinity binding of \([^{3}H]\)-DES to components in cytosol other than estrogen receptor. Large quantities of \([^{3}H]\)-DES
sediment with a 4-5S component in mammary cytosol; binding in this peak is not removed by charcoal treatment and is not inhibited by incubation in the presence of 100 nM unlabeled DES. These counts not removed by charcoal treatment would tend to increase the amount of $[^3H] $-DES remaining "bound" at high incubating concentrations in DCC/Scatchard-plot assays. The effect would be to increase the apparent concentration of "receptor" sites while lowering the affinity of the "receptor"-DES interaction. The nature of the 4-5S binding component has not been determined; its affinity and capacity for DES are substantially higher than for $E_2$. Sheehan (1977) has reported that DES binds to BSA 30X better than does $E_2$. Thus, serum contamination in the preparation of cytosol may furnish an additional albumin component which binds DES more effectively than $E_2$. The large apparent $N_s$ values obtained by DCC/Scatchard-plot analysis with $[^3H] $-DES emphasize the importance of correcting for "non-specific" binding at each incubating concentration as recommended by Chamness and McGuire (1975b). If $N_s$ is estimated by extrapolation of the steep portion of the uncorrected Scatchard plot, the apparent concentration of "receptor" obtained is 4-6 times higher than is obtained with $[^3H] $-DES by SDG or $[^3H] $-$E_2$ either by SDG or DCC. The correction procedure substantially reduces the calculated $N_s$ value, but the values are still significantly greater than assays performed with $[^3H] $-$E_2$.

Previous estimates of the affinity of DES-receptor
interaction in rat uterus have been made using competitive binding techniques with unlabeled DES. By these methods DES has been reported to interact with receptor with an estimated affinity ranging from 0.25X (Van Beurden-Lamers et al., 1974) to 13X (Ellis & Ringold, 1971) that of E₂. The majority of competition experiments indicate that DES and E₂ bind with about equal affinity (Korenman, 1972; Geynet et al., 1972; Wittliff, 1975). My competition studies with mouse mammary cytosol show that DES is slightly more effective than E₂ in inhibiting binding of [³H]-E₂ to receptor. The Kₐ for DES binding as derived from FAC methods is 8 × 10⁻¹¹ M compared with 2.6 × 10⁻¹⁰ M using [³H]-DES in a direct DCC/Scatchard-plot assay. By either direct or indirect measurement, the affinity of mouse mammary cytosol receptor for DES is not greatly different than for E₂.

DES has been the focus of a long-standing concern about risks associated with human exposure to exogenous estrogens. Considerable effort has been expended in attempting to determine, through chronic animal studies, whether a "carcinogenic dose-threshold" exists for DES and, if so, what the implications are for humans exposed to chronic low-level residues of DES in food (Cole et al., 1975). The risks of short-term prenatal exposure to large doses of DES also have received wide attention (Herbst et al., 1971). At present it is impossible to state with confidence the degree of carcinogenic risk associated with human exposure either to short or long-term DES. Ultimately, the evaluation
of "safety" must involve knowing the mechanism(s) by which potentially toxic substances act. The important question with regard to DES is whether it has any unique properties which make it fundamentally different from other estrogenic substances (reviewed by Lipsett, 1977).

The experiments reported in this chapter indicate that DES-binding to mammary cytosol receptor and subsequent uptake into the nucleus are essentially the same as for E₂. Previous workers also have reported that DES promotes receptor transformation (Jensen & DeSombre, 1972) and nuclear uptake (Ruh & Bandendistel, 1977) in a fashion very similar to E₂. Based on uterotrophic response and nuclear retention time, Clark et al. (1977) have classified DES with E₂ as a long-acting estrogen.

Thus, it appears that the receptor-mediated intracellular actions of DES in target tissues are not unusual or different from those of "natural" estrogens. DES binding differs from E₂ in that DES does not bind to transport globulins (Sheehan, 1977) but does bind to serum albumin with higher affinity than that of E₂. Recent studies show that E₂, as well as DES, induces mammary tumors in C3H mice having the mouse mammary tumor virus (Norvell et al., 1977). It is likely that the higher potency of DES as a carcinogen, when taken orally, is more related to overall uptake, transport metabolism than to any fundamental difference in binding to intracellular receptors in target tissues.
CHAPTER VI

ESTROGEN RECEPTORS IN HUMAN BREAST CANCER

A. Introduction

The subject of hormone-dependent tumors has come to assume a great clinical importance in relation to the treatment of human breast cancer. Approximately one-third of all breast tumors will respond to endocrine therapy (Dao, 1972). In the remainder of breast cancer cases, which are classified as hormone independent, chemotherapy is primarily the choice of treatment. The problem that confronts a physician is the choice between endocrine therapy and chemotherapy. Since hormone-independent and hormone-dependent breast cancer cannot be distinguished histologically, there has been no way to predict in advance which patients will respond to endocrine therapy. Thus, for every patient benefitted, two must undergo unnecessary surgery.

Researchers began to look for biochemical markers of hormone dependence. One approach has been the assay of estrogen receptor (ER) in tumor tissue. When a steroid hormone (i.e. estrogen) acts on its target tissue, it must bind to a biologically specific, high affinity receptor in the cytoplasm. The hormone cannot enter the nucleus and exert its physiological effects without first binding to the cytosol receptor (Chapter I).
In 1961, a research group (Folca et al., 1961) determined that patients who responded to endocrine treatment (adrenalectomy) generally had a much higher uptake of radiolabeled estrogen in the tumor than those patients who did not respond to hormonal treatment. This led to the finding of specific estrogen binding proteins in the cytosols of tumors obtained at biopsy (Jensen et al., 1967). It was determined that human tumors which regress following endocrine therapy usually contain high concentrations of ER while non-responsive tumors are deficient in estrogen receptors.

At the Department of Biology, I have assisted in the assay of 70 human tumors for the Ontario Cancer Foundation Windsor Clinic. Procedures and illustrative data will be described.

B. Materials and Methods

1. Collection and Preservation of Specimens

Biopsied breast tumors and/or tumor metastases were obtained from the various hospitals in Essex County, Ontario. Following surgery and examination by the hospital pathologist, the tissues were placed in plastic specimen containers, kept on ice and brought to the laboratory. The handling schedule is summarized in Table 3. Proper coordination with the hospital staff is imperative if a biologically active sample is to be obtained.

The tumor tissue was trimmed of excess fat or
TABLE 3

A PRACTICAL HANDLING SCHEDULE FOR BREAST SPECIMENS
SUBMITTED FOR MEASUREMENT OF ESTROGEN-BINDING CAPACITY

1. Notify Dr. Okey's office (phone number below) that a specimen is being sent. Preferred notice: 1-2 days before surgery.

Within the requirements of the surgeon and pathologist, treat the specimen as follows:

2. Immediately after surgical removal, place the specimen in a glass container which should be kept on ice. It is important to keep the specimen chilled throughout its handling, but it should not be frozen.

3. After the pathologist has obtained his samples and released the specimen, have it trimmed of necrotic and connective tissues, fat, and skin.

4. It is necessary to have at least 0.5 g of non-necrotic tumor tissue to perform the estrogen-binding assay. Any additional amount of tumor tissue (not required by the pathologist, etc.) should also be sent — even up to 20 g if available.

5. Place the trimmed tissue in a closed glass container, pack in ice and transport as quickly as possible after surgery to:

Dr. A.B. Okey
Room 20
Biology Building
University of Windsor
Phone: 253-4232 ext. 445
connective tissue and was cut into small pieces of approximately 4 mm². After being weighed, the tissue was used fresh or placed in polyethylene vials and quickly frozen using liquid nitrogen. The samples were stored either in liquid nitrogen or at (-) 70°C in a Revco freezer. Storage at (-) 20°C or 4°C is not effective and results in ER breakdown (Ratajczak & Hahnel, 1974).

2. Preparation of Cytosols

The preweighed frozen tissues were shattered using a piston-driven pulverizer (Thermovac Industries). The frozen powder was homogenized in cold TDE buffer utilizing a mechanical homogenizer (Polytron PT-10) for short pulses (15 to 20 sec). The tissue was kept continuously on ice and one minute cooling intervals were allowed between homogenizing intervals. The homogenate was centrifuged at 4000 x g for 15 min, and the supernatant removed and centrifuged at 105,000 x g for 1.5 hr to obtain a cytosol fraction. The cytosol fraction was used directly for the SDG technique or diluted to 2.0 mg protein/ml for use in the DCC procedure. Protein was measured using the method of Lowry et al. (1951). All previous steps and subsequent procedures were carried out at 0-4°C.

3. Assays for Specific Estrogen-binding Proteins.

a) Sucrose density gradient analysis

Linear 5-20% sucrose gradients were prepared in TDE buffer as described in Chapter II. Samples for gradient
analysis were prepared by incubating 1.0 ml of cytosol with 2.0 pmoles of $^3$H-estradiol-17β for 3 hrs at 0-4°C. Control cytosols were incubated with 100 pmoles nonradioactive DES prior to the addition of the labeled estradiol. After incubation, the nonbound estradiol was removed by treatment with dextran-coated charcoal and the treated cytosol was applied to a sucrose gradient.

b) Dextran-coated charcoal assay

Aliquots of cytosol (2 mg protein/ml) in TDE buffer were added to a series of tubes that contained [3H]-estradiol at concentrations that ranged from 1 nM to 12 pM. Control cytosols were preincubated for 15 min with $10^{-7}$ M non-labeled estradiol-17β prior to the addition of [3H]-estradiol. After a 12-14 hr incubation, a DCC suspension was added to each tube; the sample mixed and incubated for an additional 15 min. The tubes were centrifuged at 4000 x g for 20 min and a supernatant fraction was removed and counted using LSC procedures.

C. Results

The common gradient profiles that were obtained from human breast tumors are shown in Figures 26 and 27. In Figure 26A, there is a major peak of radioactivity found in the region which corresponds to 8-9S. In Figure 26B, there are two major peaks of bound radioactivity, one at 8-9S and another at 4-5S. Whereas the 8-9S binding peak always represents the specific estrogen interaction, the 4-5S peak contain both specific and non-specific components.
Figure 26. Sedimentation of estrogen receptors from human breast tumors. Panel A illustrates the sedimentation pattern of a breast tumor specimen that contained mainly 8-9S receptor. Panel B represents the sedimentation profile of a specimen that contained both a 4-5S and 8-9S receptor species. Cytosols were prepared in TDE buffer and 0.5 ml aliquots incubated at 0-4°C for 3 hrs with 1.0 nM [³H]-E₂ in the absence (O--O) or presence of 100 nM unlabeled DES (●●●). After pretreatment with DCC, the samples were layered onto 5-20% sucrose gradients prepared in TDE buffer and centrifuged in a Beckman SW 41 rotor at 40,000 rpm for 20-22 hrs.
Figure 27. Sedimentation of 4-5S form of estrogen receptor from human breast tumors. Panel A represents a specimen that contained a 4-5S binding peak which was eliminated upon incubation with unlabeled DES. Panel B illustrates a sample that contained a non-specific 4-5S binding peak which was not obliterated upon incubation with unlabeled DES. Cytosols were prepared and centrifuged as described in the legend for Figure 26.

(○—○) = receptor binding with [3H]-E2
(●—●) = non-specific binding measured in the presence of 100 nM unlabeled DES
Figure 27, Panel A, illustrates a gradient profile where only 4-5S binding is present but which is interpreted as specific binding since incubation with unlabeled DES obliterate the peak. However, the majority of 4-5S binding peaks generally represent non-specific binding (Panel B). The prevalence of each molecular form is summarized in Table 4.

A representative Scatchard plot from a human tumor is illustrated in Figure 28. Analysis reveals a $K_d$ of the order of $2.0 \times 10^{-10}$ M and a binding site measurement of 14.6 pmoles/g cytosol protein.

D. Discussion

Whenever possible, tumors are assayed using both DCC and SDG techniques. The DCC procedure is valuable because it is impossible to saturate and detect all receptor sites in the solution (Schrader, 1975) and the DCC procedure accurately measures the $K_d$ value. The DCC assay also has the added advantage of detecting lower levels of ER (Table 5). However, since tumor samples are sometimes small (less than 0.5 g), not enough cytosol can be obtained for both assays. The SDG is given priority since a much smaller amount of cytosol is required for assay and it yields the most information. Only the SDG allows the detection of the 8-9S binding component which specifically identifies ER. The DCC is limited since it cannot detect the differences between the 4-5S and 8-9S forms of the receptor. Wittliff and Savlov (1975) have reported the presence of high-
TABLE 4
FREQUENCY OF MOLECULAR FORMS OF HUMAN
BREAST TUMOR ESTROGEN RECEPTORS

<table>
<thead>
<tr>
<th></th>
<th>No. with 8-9S receptors only</th>
<th>No. with 8-9S and 4-5S receptors</th>
<th>No. with 4-5S receptor only</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5/26</td>
<td>14/26</td>
<td>7/26</td>
</tr>
<tr>
<td></td>
<td>= 19%</td>
<td>= 54%</td>
<td>= 27%</td>
</tr>
</tbody>
</table>
Figure 28. Representative Scatchard plot from a human breast tumor. Cytosol was prepared in TDE buffer and incubated for 14 hrs at 0-4°C with concentrations of [³H]-E₂ between 1 mM and 12 pM. Specific, high-affinity binding (O-O) was computed using the method of Chamness and McGuire (1975b).

(O-O) = uncorrected data

(O-O) = plot corrected for non-specific binding
\[ N_s = 14.6 \text{ pmoles/g} \]
\[ K_d = 1.9 \times 10^{-10} \text{ M} \]
\[ r = .70 \]
### TABLE 5
DETECTION OF RECEPTOR USING
DCC OR SDG PROCEDURE

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Estrogen receptor</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Detected</td>
<td>Not detected</td>
</tr>
<tr>
<td>DCC</td>
<td>31/45 = 69%</td>
<td>14/45 = 31%</td>
</tr>
<tr>
<td>SDG</td>
<td>25/49 = 51%</td>
<td>24/49 = 49%</td>
</tr>
</tbody>
</table>
affinity 4-5S receptor in tumors which do not respond to hormone treatment which the DCC procedure would have indicated as a positive (high) ER value.

There are several requirements which affect the reliability of assaying tumor tissue ER. These can be summarized as follows:

1. Proper collection and preparation of the tissue is critical.

   The tumor must be trimmed of any excess fat or connective tissue since binding will be eventually expressed as fmoles estrogen per gram of tumor. A high correlation was also noted between data expression in fmoles/g tissue and pmoles/mg protein (Figure 29). The need for cold conditions during the assay has been previously shown (McGuire & De La Garza, 1973) and this demonstrates clearly that elevated temperatures cause rapid degeneration of the receptor protein. Prolonged storage can only be effective at (-) 70°C or greater (Ratazetzak & Hahnel, 1974).

2. Homogenization should be thorough.

   The receptor is a constituent of the cytoplasm of target cells. Since breast tumors are very tough, tissue pulverization followed by mechanical homogenization using a rotating-knife must be used.

3. Requirement for thiols.

   The receptor stability is considerably enhanced by DTT or mercaptoethanol (McGuire & De La Garza, 1973; Braunsberg et al., 1972). This indicates a requirement for
Figure 29. Comparison of ER binding expressed as binding per unit protein versus binding per unit tissue weight.
$r = 0.889$
4. Interferences from lipids

The final cytosol must not contain lipid. Estradiol will be selectively partitioned by the presence of fat and cause a decrease in specific ER binding (Johnson et al., 1975).

5. Counting errors

Since the concentrations of labeled estrogen are low, counting errors must be minimized. Therefore, quench correction is done for each sample and long counts are employed to ensure that the counting error is less than 1.0%.

The SDG and DCC assays are the overwhelming procedures of choice as exemplified by the number of research groups using them (McGuire et al., 1975). Gel filtration can be used to separate bound and unbound forms of $[^3H]-E_2$ (Chamness et al., 1972). However, it offers no substantial advantage over the DCC method and it is not nearly as convenient. Electrophoresis has been applied to detect the protein to which $[^3H]-E_2$ is binding (Korstein & Persijn, 1972), but electrophoresis has been found to be relatively insensitive and subject to interferences from serum proteins (McGuire et al., 1975). The search continues for the development of hormone binding techniques which are simple enough to be part of a routine clinical laboratory and yet can also ensure proper detection of receptor.
CHAPTER VII

TESTING OF THE POTENTIAL ESTROGENIC ACTIVITY OF $\Delta^9$-THC AND CANNABIS RESIN USING IN VITRO ESTROGEN-RECEPTOR BINDING TECHNIQUES

Numerous reports have indicated that cannabis preparations may demasculinize male rats (Dixit et al., 1974; Okey & Truant, 1975; Thompson et al., 1973; Collu et al., 1975), depress testosterone levels in men (Kolodny et al., 1975), and may lead to abnormal breast growth in men (Harmon & Aliapoulios, 1972, 1974). Solomon et al. (1976, 1977) claimed that $\Delta^9$-THC had estrogenic activity, based on uterine bioassays and vaginal smear techniques. Although this claim would be an attractive and simple explanation for the mechanism by which cannabinoids demasculinize or feminize (or both), several objections were raised concerning the technical validity of the study (Okey & Bondy, 1977). In addition, earlier reports had indicated that $\Delta^9$-THC and other cannabinoids were devoid of any estrogenic activity (Thompson et al., 1973; Okey & Truant, 1975).

In this chapter, additional evidence demonstrating the lack of estrogenic activity by cannabinoids will be presented. These findings are based on competition for estrogen-receptor sites. It is well known that estrogens (both natural and
synthetic) stimulate true uterine or mammary gland growth by first binding to specific cytoplasmic receptor proteins (Chapter I). Thus, a sensitive in vitro test for potential estrogens can be done by determining whether the substance competes with $[^3H]$-estradiol for binding to estrogen receptors.

A. Materials and Methods

1. Animals and Cytosol Preparation

Mouse mammary tissue was obtained from C3H/HeJ females which had been lactating for 10-12 days. Uterine sources were sexually mature C3H mice or 21-day-old Wistar-strain rats. The tissues were homogenized in three times their volume of TDE buffer. All procedures were done at 0-4°C. The homogenates were centrifuged three times at 4000 x g for 15 min, and then the supernatant was removed and centrifuged at 105,000 x g for 1.5 hrs in order to obtain a supernatant (cytosol) fraction.

2. Sucrose Density Gradient Assay

Cytosol aliquots were incubated overnight (in later experiments, the incubation period was shortened to 3 hrs) with 2 nM $[^3H]$E$_2$ in the presence or absence of potential competitors for estrogen receptor sites. The $\Delta^9$-THC (95.6 percent pure, NIMH lot SC75518) and cannabis resin were added in 10 ul aliquots of 95% ethanol. After incubation, unbound steroid was removed by adding the sample to a pellet of DCC. Charcoal was sedimented by centrifugation at 4000 x g for 15 min; samples (0.5 ml) of the supernatants
were then layered onto linear sucrose gradients (5 to 20 percent) prepared in TDE buffer. Gradients were centrifuged at 40,000 rpm for 20-22 hrs in a Beckman SW 41 rotor at 4°C. Forty 300 ul fractions were collected from each gradient and the radioactivity in each was measured by LSC.

B. Results and Discussion

Sedimentation peaks for the two uterine samples illustrated in Figure 30 correspond to previous reports (King et al., 1969; Jensen & DeSombré, 1973) that uterine cytosol sediments predominately at the 8-9S region in immature rats (bottom panel) and nearer the 4-5S region in sexually mature animals (top panel). The addition of enormous amounts of either cannabis resin or Δ⁹-THC had no effect on the binding of [³H]-estradiol-17β. Additional experiments with lactating mouse mammary tissue (Figure 31) also show that Δ⁹-THC does not bind to the active site of the estrogen receptor. Cannabinoid metabolites (Δ⁸-THC, 11-OH-Δ⁹-THC) were also tested and found to have no effect on receptor binding (data not shown).

The data presented do not rule out the possibility that cannabis or Δ⁹-THC may be metabolized to an estrogenic form. However, uterine bioassay data (Thompson et al., 1973; Okey & Truant, 1975) give no indication that significant amounts of estrogen metabolites might be formed in vivo. These findings, in addition to the in-vitro binding data, provide convincing evidence that cannabinoids have no estrogen-like activity.
Figure 30. Effect of cannabis resin and $\Delta^9$-THC on $[^3H]E_2$ binding to rat and mouse uterine estrogen receptor. Cytosols from mature C3H mice and 21-day-old Wistar rats were prepared in TDE buffer. Aliquots of the cytosols were incubated with 2.0 nM $[^3H]E_2$ (91.3 Ci/mmol) for 12 hrs at 0-4°C in the presence or absence of potential competitors. Samples were layered onto 5-20% sucrose gradients prepared in TDE buffer and centrifuged for 22 hrs at 40,000 rpm.

(■) = control (no competitor)
(■) = 10^{-7} M DES
(■) = 10^{-5} M $\Delta^9$-THC
( ) = cannabis resin containing 19% cannabidiol, 14% cannabinol and 10^{-5} M $\Delta^9$-THC
Figure 31. Effect of $\Delta^9$-THC on $[^3H]E_2$

binding to mouse mammary estrogen receptor. Cytosol
(4.0 mg protein/ml) was prepared in TDE buffer from
the mammary gland of a mouse which had been lactating
for 10 days. Aliquots were incubated 3 hrs with
2.0 nM $[^3H]E_2$ (138 Ci/mmol) in the presence or
absence of $\Delta^9$-THC; 500 ul samples were layered onto
5-20% sucrose gradients prepared in TDE buffer.
Centrifugation was for 20 hrs at 40,000 rpm.

(○) = control (no competitor)

(●) = $10^{-5}$ M $\Delta^9$-THC

(▲) = $10^{-3}$ M $\Delta^9$-THC
CHAPTER VIII

SUMMARY

1. The kinetic and sedimentation properties of estrogen binding proteins ("receptors") from lactating mouse mammary tissue are the same as estrogen receptors in rat uterus.

2. Radiolabeled diethylstilbestrol binds to C3H mouse uterine and mammary gland estrogen receptor in a manner similar to estradiol.

3. The concentration of mammary gland estrogen receptors in C3H mice with and without the mouse mammary tumor virus is not significantly different; the affinity of the estradiol receptor interaction is significantly higher in mice without MMTV.

4. Sucrose density gradient analysis and the dextrancoated charcoal assay are the preferred methods for measuring specific estrogen receptors in the cytosols of human breast tumors.

5. Cannabis resin and \( \Delta^9 \)-THC do not compete with estradiol in vitro for specific estrogen binding proteins.
APPENDIX A

Radioactive Labeling of Marker Proteins

$^{14}$C-labeled marker proteins were prepared using a method originally developed by Rice and Means (1971) and later adapted by Stancel and Gorski (1975). Proteins labeled by this procedure were BSA (4.6S) and $\alpha$-Globulin (7.1S).

A. Reagents

1. Borate buffer (0.2 M). Dissolve 38.1 g of Na$_2$B$_4$O$_7$.10H$_2$O in about 400 ml of warm (~40°C) distilled water; transfer to a 500 ml volumetric and dilute to volume with distilled water. The solution is brought to pH = 9.0 with concentrated HCl. Buffer is made fresh and is kept at room temperature (cooling will precipitate sodium borate).

2. Marker proteins (10 mg/ml). Dissolve 100 mg of protein in approximately 8.0 ml of borate buffer; transfer to a 10 ml volumetric and make to volume with borate buffer.

3. $^{14}$C-formaldehyde (4 mM). A 4 mM stock is prepared by diluting 16 ul $^{14}$C-formaldehyde (NEN, S.A. = 46 Ci/mole) with 1.33 ml of distilled water.

4. Sodium borohydride (0.5 mg/ml). Dissolve 10 mg NaBH$_4$ in approximately 15 ml of water; transfer to a 20 ml volumetric and make to volume with distilled water. The solution is prepared just prior to use.
B. Procedure

1. Pipet 1.0 ml of marker protein into a test tube and place in an ice bath.
2. Add 10 ul of $^{14}$C-formaldehyde.
3. Swirl gently and allow to react for 1 min.
4. Sequentially, add two 5-ul aliquots of sodium borohydride.
5. The procedure (Steps 2-4) is repeated four-five times.
6. After the last sequence of additions, add an additional 10 ul of sodium borohydride to reduce any remaining formaldehyde.
7. Unreacted components are removed by dialysis. The reaction mixture is pipeted into a cellulose casing and the ends of the bag carefully tied off. The bag is suspended in distilled water and dialysis carried out at room temperature.
8. The $^{14}$C-protein stocks may be stored at (-) 20°C.

C. Technical Notes

$^{14}$C-BSA prepared in this manner had a very high level of radioactivity (10 ul = 100,000 dpm). Working $^{14}$C-markers are prepared by diluting the original stock 1:100 with distilled water.
APPENDIX B

Lowry Technique for Protein Measurement

A. Reagents

1. Reagent A. Dissolve 20 g Na₂CO₃ and 4 g NaOH in 900 ml of distilled water before adding 0.2 g of sodium potassium tartrate. Transfer to a liter volumetric and make up to volume with water. Store the reagent in the refrigerator in a stoppered polyethylene bottle.

2. Reagent B. Dissolve 0.5 g CuSO₄·5H₂O in 100 ml of distilled water. Store as reagent A.

3. Reagent C. Alkaline copper solution. Mix 50 ml of reagent A with 1 ml of reagent B just prior to use. Discard after one day.

4. Folin reagent. Dilute 50 ml of Folin-Ciocalteau reagent (2N) with 70 ml of distilled water. Store as reagent A.

5. TE buffer.

6. TDE buffer.

7. Standard protein stock (5 mg/ml). Dissolve 500 mg BSA in about 70 ml of TE buffer; transfer to a 100 ml volumetric and dilute to volume with TE buffer. Store in refrigerator.

B. Procedure

1. Pipet 100 ul of cytosol into a numbered test tube.

2. Add 900 ul of distilled water and mix.

3. Prepare blanks and standards in an identical manner, using TE, TDE, 2.0 mg/ml BSA, and 4.0 mg/ml BSA as starting
materials.

4. Pipet 5 ml of reagent C to the tubes, mix and let stand for 10 min.

5. After 10 min, add 0.5 ml of Folin reagent, mix immediately and allow color development at room temperature for 30 min.

6. After 30 min, measure absorbance at 660 nm against the TE blank. Subtract the TDE blank from the unknown and read the concentration from a standard protein curve, established using BSA solutions at various concentrations.

C. Technical Notes

The cytosol buffer (TDE) contains Tris and DTT which interfere with the Lowry reaction by producing color (Vallejo & Lagunas, 1970). The DTT interference can be removed by peroxide treatment (Geiger & Bessman, 1972). Use of proper blanks and suitable sample dilution eliminate the need for the peroxide treatment. The Millar (1959) modification of the Lowry procedure can also be used if a large number of samples is to be analyzed.
APPENDIX C

Liquid Scintillation Counting Cocktails

A. Toluene-Triton X-100 Cocktail

A toluene-triton X-100 cocktail was used in the majority of the experiments, with a counting efficiency of 36-38 per cent. The cocktail was prepared according to the following formula:

1 liter toluene
500 ml Triton X-100
8.25 g PPO
0.18 g POPOP

B. Bray's Solution

The counting cocktail used in some early experiments was Bray's solution (Bray, 1960). Counting efficiency with this cocktail was 30-33 per cent. The solution was purchased commercially (British Drug Houses) or prepared from the following formula:

PPO 4 g
POPOP 0.2 g
Naphthalene 60 g
Methanol 100 ml
Ethylene glycol 20 ml
1,4 dioxane to 1 liter
APPENDIX D

Preparation of 66% Stock Sucrose

Sucrose solutions used in sucrose gradient procedures were prepared from a 66% stock sucrose solution as suggested by Cline and Ryel (1971).

A. Reagents

1. TE buffer (0.01 M Tris-HCl, 0.0015 M EDTA, pH = 7.4).
2. Sucrose (Ribonuclease-free, Swartz-Mann).

B. Procedure

Dissolve 1710 g of sucrose in 990 ml of TE buffer. This is accomplished by adding the sucrose slowly to the buffer with constant mechanical stirring. Store at 4°C.
APPENDIX E

Statistics

All statistical tests and repetative calculations were performed on a Monroe model 326 programable calculator. The majority of the programs were obtained from the Wang 300 series program library.

A. Least Square Regression Line

The linear least-squares fit of input data points \((X, Y)\) were calculated using the following:

\[
\text{slope} = a_1 = \frac{N \langle XY \rangle - \langle X \rangle \langle Y \rangle}{N \langle X^2 \rangle - \langle X \rangle^2}
\]

\[
\text{intercept} = a_0 = \langle Y \rangle - a_1 \langle X \rangle
\]

The least square line of \(Y\) on \(X\) has the equation:

\[
Y = a_0 + a_1 X
\]

B. Correlation Coefficient

The correlation coefficient of input data points \((X, Y)\) was calculated as follows:

\[
\text{Correlation coefficient} = r = \frac{\langle XY \rangle - \langle X \rangle \langle Y \rangle}{\sqrt{\left( \langle X^2 \rangle - \frac{\langle X \rangle^2}{N} \right) \left( \langle Y^2 \rangle - \frac{\langle Y \rangle^2}{N} \right)}}
\]
C. Student's "t" Test

The value of t was determined from the equation:

\[ t_{inc} = \frac{\bar{X} - \bar{Y}}{\sqrt{\left(\frac{\left(N_X - 1\right) - SD_X^2 + (N_Y - 1) SD_Y^2}{N_X + N_Y - 2}\right) \left(\frac{1}{N_X} + \frac{1}{N_Y}\right)}} \]

where: \( \bar{X} = \frac{\Sigma X}{N_X} \)  \( \bar{Y} = \frac{\Sigma Y}{N_Y} \)

\( SD_X = \) Standard deviation of X
\( SD_Y = \) Standard deviation of Y

D. Standard Deviation and Standard Error of the Mean

Standard deviation of X by the N method was computed as follows:

\[ SD_X = \sqrt{\frac{X^2 - N \bar{X}^2}{N}} \quad \text{where} \quad \bar{X} = \frac{\Sigma X}{N} \]

Standard error of the mean of X by the N method was computed as follows:

\[ SEM_X = SD_X \sqrt{\frac{1}{N}} \]
APPENDIX F

Radiochemical Specifications

A. Estradiol-17β

Radiolabeled estradiol-17β was obtained from two suppliers: Amersham/Searle (Arlington Heights, Illinois) and New England Nuclear (Boston, Mass.) at a variety of specific activities.

1) [2,4,6,7(α)-H] estradiol
Supplier: Amersham/Searle, Batch #28
S.A. = 85 Ci/mmol
Concentration = 1.0 mCi/ml
M.W. = 272
Position of label:

2) [2,4,6,7-3H(N)] estradiol
Supplier: New England Nuclear, Lot no. 853-130
S.A. = 91.3 Ci/mmol
Concentration = 1.0 mCi/ml; 0.003 mg/ml
M.W. = 272.4
Position of label:

141
3) \([2,4,6,7,16,17-^3\text{H}(N)]\) estradiol

Supplier: New England Nuclear, Lot no. 690-172

S.A. = 138.1 Ci/mmole

Concentration = 1.0 mCi/ml; 0.002 mg/ml

M.W. = 272.4

Position of label:

4) \([2,4,6,7,16,17-^3\text{H}(N)]\) estradiol

Supplier: New England Nuclear, Lot no. 690-164 & 1004-036

S.A. = 152.0 Ci/mmole

Concentration = 1.0 mCi/ml; 0.0018 mg/ml

M.W. = 272.4

Position of label:
B. Diethylstilbestrol (DES)

$[^3H]$ Diethylstilboestrol

Supplier: Amersham/Searle, Batch #5

S.A. = 62.4 Ci/m mole

Concentration = 1.0 mCi/ml; 0.0044 mg/ml

M.W. = 272

Position of label:

Label at one of the ethyl groups

C. Formaldehyde

$[^14C]$ Formaldehyde

Supplier: New England Nuclear, Lot no. 930-235

S.A. = 46 mCi/m mole

Concentration = 0.25 mCi/0.016 ml; 0.16 mg/0.016 ml

M.W. = 30

Position of label:

EC*HO
Appendix G

Chemicals Used and Suppliers

Albumin, bovine serum (Sigma)
Ammonium sulfate, enzyme grade (Swartz/Mann)
Bray's solution (BDH)
Charcoal, Norit A, pharmaceutical neutral (BDH)
Dextran, M.W. = 60,000-90,000 (ave 86,000) clinical grade (Sigma)
DES, diethylstilbestrol (Sigma)
Methoxy-\textsuperscript{3}H-diethylstilbestrol; S.A. = 62.4 \textsuperscript{Ci}/mmole (A/S)
Dihydrotestosterone, 5\textalpha-androstan-17\beta-ol-3-one (Sigma).
1,4-dioxane, scintanalyzed (Fisher)
DTT, dithiothreitol (Sigma)
EDTA, disodium ethylenediamine tetraacetate (Fisher)
Estradiol-17\beta, estradiol-1,2,5(10)-triene-3, 17\beta-diol (Sigma)
  2,3,6,7-\textsuperscript{3}H-estradiol-17\beta; S.A. = 85 \textsuperscript{Ci}/mmole (A/S)
  2,4,6,7-\textsuperscript{3}H-estradiol-17\beta; S.A. = 91.3 \textsuperscript{Ci}/mmole (NEN)
  2,4,6,7,16,17-\textsuperscript{3}H-estradiol-17\beta; S.A. = 138.1 \textsuperscript{Ci}/mmole (NEN)
  2,4,6,7,16,17-\textsuperscript{3}H-estradiol-17\beta; S.A. = 152.0 \textsuperscript{Ci}/mmole (NEN)
Folin\textsuperscript{C}, Folin-Ciocalteau phenol reagent 2N solution (Fisher)
Formaldehyde-\textsuperscript{14}C; S.A. = 46 m\textsuperscript{Ci}/mmole (NEN)
Gamma-globulin, human; Cohn fraction II (Sigma)
Hepes, N-2-hydroxyethylpiperazine-N-2 ethanesulfonic acid (A/S)
Naphthalene (Fisher)
POPOP, 1,4-bis-(5-phenyloxazol-2-yl) benzene (A/S)
PPO, 2,5-diphenyloxazole (A/S).
Sodium borohydride (Fisher)
Sucrose, ribonuclease-free (Swartz/Mann)
Toluene (Fisher)
Triton X-100 (A/S)
2,4,6-trinitrobenzene sulfonic acid (Sigma)
Tris, Tris(hydroxymethyl)aminomethane (Fisher)

a = Sigma Chemicals, St. Louis, Missouri
b = Swartz/Mann, Orangeburg, New York
c = British Drug Houses (BDH), Toronto, Ontario
d = Amersham/Searle (A/S), Arlington Heights, Illinois
e = Fisher Scientific Co., Don Mills, Ontario
f = New England Nuclear (NEN), Boston, Mass.
REFERENCES


Kolodny, R.C., Masters, W.H., Kolodner, R.M. & Toro, G.
Depression of plasma testosterone levels after chronic
intensive marihuana use. N. Engl. J. Med. 290:872-874,
1974.

Korenman, S.G. Radioligand binding assay of specific
estrogens using a soluble uterine macromolecule.

Korenman, S.G. Comparative binding affinity of estrogens
and its relation to estrogenic potency. Steroids 13:
163-177, 1969.

Korenman, S.G. & Rao, B.R. Reversible disaggregation of the
cytosol-estrogen binding protein of uterine cytosol.

Korstein, C.B. & Persijn, J.P. Simple assay for specific
estrogen binding capacity in human mammary tumors.

Lewko, W.M., Norvell, M.J. & Shellenberger, T.E. Estrogen-
binding proteins in mouse mammary tumors induced by
17:197, 1976 (abstr.).

Liao, S. & Fang, S. Receptor-proteins for androgens and
the model of action of androgens on gene transcription
in ventral prostate. Vitam. Horm. (New York) 27:17-
90, 1969.

Nugent, G.A. & Mayes, D.M. Determination of plasma testos-
sterone by the use of competitive protein binding.


Norvell, M.J., Highman, B., Farmer, J.H. & Shellenberger, T.E. Mammary tumorigenesis and pathological changes in female mice fed diets containing diethylstilbestrol


VITA AUCTORIS

Name:

Gregory Paul Bondy

Born:

November 6, 1952, Windsor, Ontario

Elementary Education:

St. Joseph Grade School, River Canard, Ontario

Secondary Education:

General Amherst High School, Amherstburg, Ontario 1966-1970

University Education:

University of Windsor, Windsor, Ontario B.Sc. (Hon.) in Biology. 1971-1975.

Awards and Scholarships:

RODA Summer Scholarship. Summer/1975.
Board of Governors Gold Medal (Biology). 1975.