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THE ESTROGEN RECEPTOR
AND ITS ROLE IN MAMMARY TUMOUR INDUCTION IN RATS
TREATED WITH DIMETHYLBENZ(a)ANTHRACENE

BY

DAVID D. KEIGHTLEY

A Dissertation Submitted to the Faculty of Graduate Studies through the Department of Biology in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy at the University of Windsor

WINDSOR, ONTARIO, CANADA
1973
ABSTRACT

The estrogen receptor and its role in the induction of mammary tumours was investigated in rats following dimethylbenz(a)anthracene (DMBA) administration.

The cytosol estrogen receptor of rat mammary gland and uterus was shown to bind estradiol with high affinity ($K_s = 10^9$ to $10^{10}$ M$^{-1}$), although the number of binding sites in these tissues is limited. The receptor sedimented at 8S in a sucrose density gradient.

Using dextran-coated charcoal as a means of separating bound from unbound $^3$H-estradiol-17β it was found that an excess of unlabeled estradiol-17β displaced labeled hormone from the receptor sites. Another steroid, dihydrotestosterone, in physiological concentrations did not compete with $^3$H-estradiol-17β for binding to the receptor sites.

Although DMBA and estradiol interact in tumourigenesis following DMBA administration to rats, this was shown not to involve competition between these two molecules for the cytosol estrogen receptor sites in vitro. In vivo, however, DMBA administration to rats increased the number of receptor sites in mammary gland but not in uterus. DMBA also enhanced metabolism of estradiol, lowering endogenous levels of this steroid in mammary gland and uterus of rats treated with the hydrocarbon. It is suggested that in mammary gland DMBA has the effect of stimulating early cell division; this probably represents the early stages of neoplasia in that tissue.
PREFACE

The first major aim of this study was to characterize the estrogen receptor which is present in estrogen target tissues and to determine some of the properties of association and dissociation of this receptor with estradiol-17β. With this information it was possible to determine, for any estrogen target tissue, both the number of binding sites for the hormone and the intrinsic association constant for the estradiol-receptor interaction.

The second part of the study was concerned with the role of the estrogen receptor in DMBA-induced tumourigenesis in the mammary glands of female rats, since it has been suggested that the DMBA molecules bind to proteins within mammary gland epithelial cells, and that such a step is essential for the induction of tumours (Dao, 1971). Estrogen receptors are good candidates for such binding. The in vitro effects of DMBA on the effective number of estrogen receptor sites and on the intrinsic association constant were investigated. Further, the effects of DMBA administration on the number of binding sites and on the intrinsic association constant were studied over 20 days in vivo in lactating rats. To assess the influence of the hydrocarbon on endogenous estradiol levels, a radioreceptor assay for estradiol, utilizing the estrogen receptor of rat uterus as the binding protein was developed, and estradiol levels measured in mammary gland and uterus following DMBA administration. It was possible, then, to distinguish between an effect of DMBA on the estrogen receptor and an effect on metabolism.
ACKNOWLEDGEMENTS

I am deeply indebted to Dr. A. B. Okey of the Department of Biology, University of Windsor, not only for the valuable guidance and advice given to me throughout this study, but also for the generous friendship he has shown. He has provided considerably more than research guidance in that his strong sense of moral values has provided a model to emulate, and his sense of humour has made working with him a delight.

I should also like to thank Dr. T. K. S. Mukkur for his persistent advice on my research, and for the amount of time he has spent thinking about the problems that have arisen in the course of my work. Dr. A. H. Warner also has given generously of his time, and for this I am most grateful.

I should also like to express gratitude to my wife, Janet, for her patience, and for her moral and financial support during the preparation of this thesis.
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ABBREVIATIONS USED

B  estradiol bound to the receptor protein
BSA  bovine serum albumin
Ci  Curie
cpm  counts per minute
DHT  dihydrotestosterone; 5α-androstane-17β-ol-3-one
DMBA  7,12-dimethylbenz[a]anthracene
dpm  disintegrations per minute
E₂  estradiol-17β; estra-1,3,5(10)-triene-3,17β-diol
EDTA  disodium ethylenediamine tetraacetate
Folin-C  Folin-Ciocalteau phenol reagent
³H-E₂  tritiated estradiol-17β
hr  hour(s)
Kₛ  intrinsic association constant for the estradiol-receptor interaction
l  litre
µl  microlitre (10⁻⁶l)
M  molar
mM  millimolar (10⁻³M)
µM  micromolar (10⁻⁶M)
νM  nanomolar (10⁻⁹M)
pM  picomolar (10⁻¹²M)
fM  femtomolar (10⁻¹⁵M)
min  minute(s)
Nₛ  number of specific binding sites for estradiol-17β
POPOP  1,4-bis-(5-phenyloxazol-2-yl) benzene
PPO  2,5-diphenyloxazole
S  Svedberg unit for sedimentation constant
S.A.  specific activity
Tris  Tris(hydroxymethyl)aminomethane
U  the amount of estradiol either free in solution or bound to proteins with only low affinity
CHAPTER I

THE MECHANISM OF ACTION OF ESTROGEN

That low concentrations of estradiol are capable of stimulating growth of the uterus has been known for some time, and has even been used in a bioassay of estradiol. The steroid also has a stimulatory action on mammary tissue and vagina. However it was not until recently that the basis of the tissue specificity of the hormone and the mechanism of action of the hormone were understood.

When Jensen & Jacobson (1962) administered radiolabeled estrogen in "physiological" doses to rats they found that some tissues rapidly took up, and, in particular, retained estrogen while other tissues did not. Thus these workers classified organs and tissues as being "target" or "non-target" for the particular hormone. Target tissues retained estrogen and generally showed some change in response upon being stimulated by the hormone. Such studies must be examined critically however, since the results depend upon the nature of the estrogen, the age and type of animal used, the level and sequence of hormone administration, and the route of administration (Emmens & Miller, 1969).

The estrogen target tissue most often used in the laboratory is the rat uterus. This tissue has been shown to take up estrogen from the circulation and concentrate it in the endometrium, and, to a lesser extent, in the myometrium (Evans & Hahnel, 1971). Uptake in the rat uterus requires only 15 minutes and the hormone is retained in the
uterus for 6 hours or more (Jensen & Jacobson, 1962).

Similar results for uptake and retention have since been found for ovary (Saduddin, 1971), vagina (Stone & Baggett, 1965), hypothalamus (Kahwanago et al., 1969; Kimmel & Leavitt, 1972), mammary gland (Puca & Bresciani, 1969a) and pituitary gland (Anderson et al., 1973b; Kimmel & Leavitt, 1972). Certain tumours such as those of mammary tissue (Hahnel & Twaddle, 1973; Hahnel et al., 1971; Shyamala, 1972) and vagina (Terenius et al., 1971) have also been found to take up and retain estradiol, as have uterine eosinophils (Tchernitchin et al., 1972; Tchernitchin & Chandross, 1973). Non-target tissues such as liver, kidney and skeletal muscle do not show prolonged retention of estrogens (Jensen & Jacobson, 1962).

1) Estrogen in the cytoplasm of target cells

Estradiol-17β is able to influence the activity of a target cell without itself being metabolized (Jensen & Jacobson, 1962). For example, estradiol-17β is retrieved in an unaltered form from a tissue in which it has exerted its estrogenic effects (Puca & Bresciani, 1969b). But it was not until the mid 1960s when it was realized that within target tissue cells estradiol was bound to proteins, and that an estrogen-protein interaction is essential for estrogen action.

Recent work has shown that two types of estrogen binding are apparent in the cytoplasm of target cells. One estradiol binding protein shows high specificity for this estrogen, low capacity for it, and a slow rate of dissociation of the estradiol-protein complex. The second type of protein shows low specificity, high capacity, and a high rate of dissociation. The former binding protein is
referred to as a specific "receptor" for estradiol, and the latter as a non-specific binder of the hormone (Erdos et al., 1969).

(a) Non-specific binding

Non-specific binding of estradiol has been found to be higher in target tissues than in non-target tissues (Rochefort & Beaulieu, 1969), and the non-specific binder is most likely a protein, or a number of proteins. The binding of estradiol to such proteins appears to be unimportant in eliciting the effects of the hormone, but may be functional in preventing metabolism of the hormone within the cytoplasm. In this way the complex of estradiol and non-specific binder may act as a store for the hormone within the cell.

(b) Specific cytosol binding

The specific cytoplasmic receptor for estradiol was originally isolated from uterine tissue, where it was found that estradiol bound with high affinity to a protein in the 100,000 x g supernatant, a fraction referred to as cytosol. Thermodynamic studies reveal that each estrogen-binding protein may bind only one estradiol molecule (Giannopoulou & Gorski, 1971a). The high specificity and the high affinity of the cytosol receptor (Best-Belpomme et al., 1970) allows concentration of the hormone from the blood into the target cells (Toft et al., 1967). A two- to four-fold concentration of the hormone from the blood into uterine cytoplasm, and a three-fold concentration of the hormone from the cytoplasm to the nucleus has been found (Maurer & Chalkley, 1967; Puca & Bresciani, 1969b).

Sucrose gradient analysis of the uterine cytosol fraction shows that the hormone is concentrated in a fraction which sediments at 8S,
although different methods of isolation and identification have shown that this peak may sometimes sediment at different positions (Clark & Gorski, 1969; Gorski et al., 1968; Jensen et al., 1968; Jungblut et al., 1971; Moriyama & Siiteri, 1972; Reti & Erdos, 1971; Rochefort & Baulieu, 1969). At high estradiol concentrations (Toft & Gorski, 1966) or after extraction with KCl at concentrations of 0.3M or higher (Erdos et al., 1968; Jensen et al., 1971; Mueller, 1971; Toft et al., 1967) estradiol is found in association with a 4S particle. Conversion from the 8S to the 4S form is reversible upon reduction of the high KCl concentrations (Rochefort & Baulieu, 1968).

Addition of 0.001M CaCl₂ to the incubation medium for the cytosol fraction causes irreversible conversion of the 8S unit into the 4S form (Rochefort & Baulieu, 1971).

The 8S-estradiol complex may be a composite of at least two subunits, although a greater number of subunits has been proposed (Mueller, 1971). Part of the evidence for further subunits lies in the fact that in cells homogenized in 0.15M KCl, a 6S estradiol receptor has been isolated (Baulieu et al., 1971; Giannopoulos & Gorski, 1971b). The 4S and 6S particles are capable of binding estradiol with an affinity close to that of the 8S molecule (Giannopoulos & Gorski, 1971b).

Since the 8S cytosol receptor for estrogen can be prepared consistently from target tissue cells its concentration in the target cells under various physiological conditions can be assessed. It has been found, for instance, that there is an increase in the concentration of the cytosol receptor just prior to estrus, and a
decrease by metestrus in the rat (Iacobelli, 1973; Shain & Barnea, 1972). It has also been found that the concentration of the receptor decreases with age of the animal, and certainly decreases after puberty (Lee & Jacobson, 1971; Clark & Gorski, 1970). Throughout development the level of the 8S cytosol receptor appears not to be dependent upon ovarian secretion, but may be an autonomous property of uterine cells (Clark & Gorski, 1970).

The functional levels of 8S cytosol receptor in the cytoplasm of target tissue cells may be under hormonal control when considered on a short term basis. Estrogens tend to increase the levels of receptor in the cytoplasm, while androgens and anti-estrogens decrease its level.

The 8S cytosol receptor is at least part protein. Some of the evidence for this is that the receptor molecule of the cytosol fraction is able to recognize stereospecific differences between estrogens (Noteboom & Gorski, 1965). The extremely high free energy for the estradiol-receptor interaction (-12.7 Kcal / mole) and the high association constant for the reaction ($10^9$ to $10^{10}$ M$^{-1}$) suggest a highly stereospecific interaction between the receptor site and the steroid (Ellis & Ringold, 1971). In addition it has been found by competition studies that the receptor can identify differences between estradiol-17β and estradiol-17α, estrone, estriol, diethylstilbestrol, and other estrogens (Rochefort & Baulieu, 1969; Rochefort & Capony, 1972; Stone, 1971; Toft & Gorski, 1966; Wenzel, 1971). Such differences are reflected in the different degrees of binding between the receptor and the particular estrogen. The
integrity of the 17β-hydroxy group appears to be necessary for the binding (Baulieu et al., 1967b; Baulieu et al., 1971; Hahnel et al., 1973), and there has been found a parallelism between receptor attachment and biological activity.

Jensen et al. (1967) have found that blocking of sulphhydryl groups on the cytosol estrogen receptor prevents uptake and retention of estrogens by the receptors. And the estrogen-receptor complex may be disrupted by proteases, but not by nucleases or lipases (Notides et al., 1973; Toft & Gorski, 1966). This further indicates that the receptor is at least part protein.

Since its initial isolation from the rat uterus, the 8S cytosol receptor has also been found in human uterus (Limpaphayom et al., 1971; McGuire et al., 1972), bovine uterus (Reti & Erdos, 1971; Rochefort & Baulieu, 1971; Yamamoto, 1972), sow (Baulieu et al., 1967a), sheep (Shutt & Cox, 1972), rabbit (Mester et al., 1970) and guinea pig uterus (Pasqualini & Palmada, 1972), and may be general for mammalian uteri (Baulieu et al., 1971).

The 8S-estradiol complex has also been found in hypothalamus and pituitary gland (Kahwanago et al., 1969), vagina (Jensen et al., 1966; Stone, 1971), mammary gland (Puca & Bresciani, 1969a) and mammary tumours (Shyamala, 1972).

The 8S estradiol receptor from the cytosol fraction may be the universal estrogen receptor for estrogen target tissues in mammals. As yet, however, no one study has looked at the relative concentration and properties of the 8S receptor in different target organs in any
one species. A further interesting study from an evolutionary viewpoint would be to attempt to isolate the 8S receptor from estrogen-sensitive tissues of lower vertebrates such as amphibians and reptiles.

(2) Estrogen in the nucleus of target cells

It appears that the 8S receptor for estradiol is functional in transporting that hormone from the cytoplasm to the nucleus of target cells (Figure 1). Consequently at body temperature the largest proportion of estradiol within the cell is found in the nuclear fraction (DeHertogh et al., 1973; Maurer & Chalkley, 1967; Noteboom & Gorski, 1965; Puca & Bresciani, 1968).

Sucrose density gradient analysis of 0.4M KCl extracts of uterine nuclei after labeled estradiol administration show association of the label with a fraction sedimenting at 5S. There is a simultaneous disappearance of activity from the 8S peak of the cytosol fraction (Jensen et al., 1968; Musliner et al., 1970). With increasing time the proportion of labeled estradiol bound to the 5S particle increases while that bound to the cytosol receptor decreases (Moriyama & Siiteri, 1972), a transformation which is temperature dependent.

The appearance of the 5S fraction at the nucleus may be due to proteolysis of the 8S receptor since it is an irreversible change, even after the KCl is dialysed out (Baulieu et al., 1971). Cytosol 8S receptor protein is necessary for the formation of the nuclear 5S component (Brecher et al., 1967).

Accumulation of labeled estradiol in a 5S peak extracted from the nuclear pellet has been found for a number of estrogen target
Figure 1. Diagrammatic representation of some events occurring after entry of estradiol (E\textsubscript{2}) into a uterine cell. Details are in text. 

R\textsubscript{c}, cytosol receptor; R\textsubscript{n}, nuclear binding protein for estradiol. The cytosol receptor may be extracted from the cytoplasm either complexed with estradiol or with no hormone bound to it.
tissues, including rat uterus (Baulieu et al., 1971) rat anterior pituitary gland (Friend & Leavitt, 1972; King et al., 1965), and rat vagina (Anderson et al., 1972a).

In a fashion similar to the estradiol-stimulated increase in the cytosol estrogen receptors there has also been found an increase in the nuclear 5S compound after estrogen treatment (Anderson et al., 1972a; 1972b). Perhaps the 5S complex of the nucleus is also under some sort of hormonal control or, more likely, the changes may only reflect fluctuations in the 8S complex in the cytoplasm.

Certainly the 5S-estradiol complex is necessary for the elaboration of estrogenic actions at the nucleus. This has been found for the estradiol-stimulated incorporation of $^3$H-uridine triphosphate into calf uterine RNA, and in the stimulation of "total" RNA polymerase (Arnaud et al., 1971a; 1971b). For these responses the cytosol receptor alone is inadequate.

Within the nucleus estrogen acts to increase the template activity of uterine chromatin both generally (Musliner & Chader, 1972) and for DNA-dependent RNA polymerase in particular (Barker, 1971; Barker & Warren, 1966). It was suspected that this was initiated by removal of histones from the DNA, but estradiol itself does not appear to attach to histones (Maurer & Chalkley, 1967; Means & O'Malley, 1972; Sluyser, 1971), and neither does estradiol attach to nucleic acids or nucleotides (Shyamala & Gorski, 1969). Estradiol seems to act through non-histone proteins which have been identified in the nucleus. For example, within 15 minutes after estradiol administration to rat uterus there is an increase in the specific
activity of a nuclear protein as revealed by $^{14}$C-amino acid incorporation studies (Barker, 1970). The protein is found in association with the arginine-rich $F_3$ histone fraction, and its increase in specific activity is correlated with a decrease in concentration of the $F_3$ histone (Barker, 1970; 1971; Sluyser, 1971). Since this step is not inhibited by actinomycin-D (Barker, 1970; 1971), the estradiol-5$\beta$ complex may operate by activating or releasing a pre-existing protein or protein-synthetic machinery. Certainly estradiol administration to uterine tissue decreases the histone concentration in the chromatin.

The reduction in the histone seen at 15 minutes after estradiol administration could account for a derepression of part of the genome (Barker, 1970; Sluyser, 1971), allowing the synthesis of small amounts of a new species of high molecular weight RNA, possibly messenger RNA (Hamilton, 1968; 1971; Knowler & Smellie, 1971; Means & Hamilton, 1966; Means & O'Malley, 1972). This stage is inhibited by actinomycin-D (Jensen & DeSombre, 1972).

Following the synthesis of the messenger RNA a new protein is apparent at 30 to 40 minutes, with its maximum levels detected at 1 to 2 hours (Barnea & Gorski, 1970; Katzenellenbogen & Goński, 1972; Notides & Gorski, 1966). This protein detected both in the nucleus and cytoplasm has been referred to as an "induced protein" (Barnea & Gorski, 1970). It may be responsible for further and more extensive derepression of the genome of target tissue cells (Means & O'Malley, 1972).

Three events have been observed following the estradiol-induced
increase in the induced protein. They are not mutually exclusive, and all may be involved in the extensive derepression of the genome. All three effects have been observed 1 to 4 hours after the initial estradiol administration.

Firstly, some workers have reported an activation of RNA polymerase (Andress et al., 1972; Gorski, 1964; Mendelson & Anderson, 1973) whose increase is dependent on continued synthesis of protein (Gorski, 1964; Mueller, 1971). There has also been reported a stimulated transport of nuclear RNA into the cytoplasm following estradiol stimulation of uterine cells (Hamilton, 1968).

Thirdly, it has been hypothesized that the induced protein induces further derepression of the genome, stimulating ribosome (Eilon & Gorski, 1972) and RNA synthesis (Hamilton, 1968; Rosenfeld et al., 1972), and subsequently the synthesis of a nuclear acidic protein (Knowler & Smellie, 1971). This latter has been shown in vitro to be effective in histone removal, and is suspected to increase template activity in vivo (Johns, 1972; Liarakos et al., 1972). The result of these activities, and of the increased RNA polymerase activity and nuclear RNA transport into the cytoplasm is a synthesis of measurable quantities of a new species of RNA (Knowler & Smellie, 1971; Munns & Katzman, 1971a; Nicolette & Babler, 1972; Raynaud-Jammet & Baulieu, 1969), and a detectable rise in the total RNA of the target tissue cells by 6 hours (Segal et al., 1965). Much of the RNA is tRNA, rRNA, and 45S-rRNA precursor (Knowler & Smellie, 1971; Luck & Hamilton, 1972; Munns & Katzman, 1971b). The efficiency
of conversion of 45S-rRNA precursor into 18S and 28S rRNA is increased (Hamilton et al., 1971), and an increase in polyribosomes to a maximum at 12 hours has been reported (Eilon & Gorski, 1972; Hamilton, 1968; 1971).

Amino acid incorporation studies have also revealed an increase in target cell protein synthesis, with a rise in total protein levels at 12 hours (Means & O'Malley, 1972). It is possibly some of this protein which is enzymic in nature (Lang, 1971), and which influences the metabolism of the target cell, such as increasing glycogen metabolism (Kofoed et al., 1972) or by increasing phospholipid metabolism (Rinard, 1972).

An increased tissue content of DNA is found at 20 to 24 hours after estradiol administration. This appears to require the continued presence of estradiol (Kaye et al., 1972).

Also influencing nuclear control of uterine cell function is the estradiol-stimulated increase in ribonuclease (Schrader & Greenman, 1970). This enzyme may alter uterine responses to estradiol by altering RNA metabolism.

(3) **The actions of estradiol on the uterus**

The effects of estradiol on the chromatin of target tissue cells have been outlined above. But one of the most easily observed responses of the uterus to estradiol is an increase in wet weight, due mainly to water uptake, an effect which is seen 4 to 6 hours after estrogen treatment (Billing et al., 1969; Hisaw, 1959; Munns & Katzman, 1971a). This is most likely a result of an increased blood
supply to, and an increased permeability of the capillaries within the uterus, a change which is found 1 hour after estradiol (Szegö & Sloan, 1961). There is also increased uptake of electrolytes into uterine cells as a result of the uterine hyperemia. These effects may be the result of the estrogen-stimulated action of histamine on the uterus (Szegö, 1965), although it has recently been shown that there is a good correlation between the water uptake by the uterus and the amount of estradiol specifically bound in the nucleus (Anderson et al., 1973a). A second wave of water uptake occurs at around 10 to 20 hours (Munns & Katzman, 1971a).

The increased uptake of water and electrolytes into uterine cells may be facilitated by increased permeability to water of the cell membrane. This is indicated by the slight decrease in membrane potential which has been observed in the myometrium after estradiol treatment (Csapo, 1961). The increased permeability of the membranes also permits increased rates of entry for a number of metabolic precursors (Oliver, 1971).

Certain enzymes are also found to increase in activity following estradiol treatment (Lang, 1971; Singhal et al., 1972). Certainly the enzymes of glycolysis (Kofoed et al., 1972) and carbohydrate metabolism (Rinard, 1972; Singhal & Lafrenier, 1972) are stimulated within 4 to 6 hours after estrogen administration. The effects of estradiol on uterine cytoplasmic enzymes may be mediated through cyclic AMP (Singhal & Lafrenier, 1972).
In the uterus, then, estrogens may act at at least three levels. Firstly there may be a direct rapid action to increase the permeability of the cell membrane. Secondly there may be a stimulation of adenyl cyclase with subsequent changes in the activity of certain enzyme systems. And thirdly, and probably more slowly, estrogen may stimulate RNA synthesis, with a consequent alteration in the rate, and in the nature of protein synthesis within the cell (Figure 1).
CHAPTER II

DMBA AND MAMMARY TUMOUR INDUCTION IN RATS

Estrogen binding proteins have been found in some mammary gland tumours in both rats and humans. Estradiol is essential for the maintenance of such tumours; in some cases it is thought that estradiol is essential also for the induction of mammary gland tumours. For example, 7,12-dimethylbenz(a)anthracene (DMBA) induction of mammary tumours in rats requires estrogen, and perhaps also a functional estrogen receptor.

1) Normal mammary gland development

In normal rat mammary gland estradiol is known to bind to an 8S receptor protein which can be isolated from the cytoplasm, and to a 5S protein extractable from the nucleus (Shyamala & Nandi, 1972). It is most probable that estradiol then exerts its effects on mammary gland parenchymal cells in a manner similar to that described above for uterine cells (Chapter I). Within mammary parenchymal cells, therefore, estradiol may have the three-fold action of stimulating protein synthesis, increasing membrane permeability, and of stimulating certain enzyme systems. The results of this activity consist of development of ducts from the epithelial cells, and to a much lesser extent the formation of alveoli. This latter requires prolonged estrogen treatment. Progesterone is more efficient at promoting alveolar development; it also has been found to induce proliferation
of mammary epithelium (Huggins et al., 1962). However hypophysectomized rats require prolactin, an anterior pituitary gland hormone, as well as estrogen and progesterone for normal mammary gland development. Adrenal corticoids and STH (growth hormone) have also been shown to promote duct development in immature rats.

Towards late pregnancy in the rat, prolactin, progesterone, estrogen, STH and adrenal corticoids all are required for maintenance of the gland.

Lactogenesis increases greatly following parturition, the process being stimulated at this time by the fall in circulating levels of estrogen and progesterone, whose levels remain low throughout lactation (Shyamala & Nandi, 1972). But there are also positive factors influencing lactogenesis in that prolactin and adrenal corticoids both stimulate the process. It appears that other anterior pituitary hormones such as STH and TSH (thyroid stimulating hormone) also could stimulate lactogenesis. Insulin may be necessary also (Topper, 1970; Turkington, 1971).

(2) DMBA in the mammary gland

How carcinogens interact with hormones in mammary tissue is still obscure, although some information is available concerning one carcinogen, the polycyclic hydrocarbon DMBA. A single oral administration of 15 to 20 mg of this hydrocarbon to 50-day-old female Sprague-Dawley rats is sufficient to induce the formation of mammary tumours in all of the animals so treated within 30 to 60 days (Huggins et al., 1961).
Following an oral administration of 20 mg DMBA to the rat, some of the hydrocarbon in its parent form is found in the mammary tissue, although DMBA does tend to accumulate in fat deposits (Flesher, 1967; Grubbs & Moon, 1973b). Much of the hydrocarbon is converted to other forms by the liver microsomal enzymes, and by the cytochrome P-450 system in particular (Kinoshita & Gelboin, 1972). DMBA has been shown to be a substrate for this enzyme system (Nebert & Gelboin, 1968) and stimulation of the enzyme system by DDT pretreatment enhances DMBA metabolism (Okey, 1972).

The main active metabolite of DMBA is a "K-region" epoxide, and it has recently been suggested that this compound may be the ultimate carcinogen (Kuroki et al., 1972; Selkirk et al., 1971). It is uncertain whether DMBA alone is capable of inducing tumours or whether it must first be converted to an epoxide, a transformation which need not necessarily take place in the liver, but which could occur in the mammary gland itself. This has been supported by transplant experiments performed by Dao (Dao et al., 1968).

Once in the cell, DMBA or its epoxide may bind to proteins within the cytoplasm (Heizmann & Wyss, 1972), as has been reported for another hydrocarbon 3-methylcholanthrene (Slaga et al., 1973; Toft & Spelsberg, 1972). The similarity in structure between estradiol-17β and DMBA (Huggins & Yang, 1962) has led to speculation that the hydrocarbon is capable of binding to the 8S estradiol receptor of estrogen target tissues (Dao, 1971). Hierowski & Madon (1968) demonstrated that 20-methylcholanthrene, a potent carcinogen, inhibits the binding of estradiol to its receptor protein.
and this could occur through competition for the same binding sites in the cytoplasm.

Following an oral administration of radiolabeled DMBA to rats, much of the activity is bound to nucleic acids in mammary parenchymal cells (Flesher, 1967), and the activity bound in this form accounts for much of the hydrocarbon found within such cells (Janss et al., 1972). Although it was thought that there was a positive correlation between the extent of binding of a hydrocarbon to DNA and the carcinogenic properties of the hydrocarbon (Brookes & Lawley, 1964), this is now recognized not to be applicable in most cases (Kuroki et al., 1972). But presumably binding of DMBA or of some metabolite of the hydrocarbon to either a cytoplasmic protein molecule and/or to nucleic acids within mammary parenchymal cells represents the "initiation" step in mammary tumourigenesis resulting in the formation of neoplastic cells (Beuving & Bern, 1972). Following this an "activation" step is essential for the production of a palpable tumour (Dao & Sunderland, 1959), and it is this latter step which probably is regulated by hormones.

One hypothesis concerning the action of DMBA in inducing tumour formation is that the planar DMBA molecule (or more likely its epoxide) is capable of intercalating between the bases of the DNA strand. In such a position it is possible that it may cause a frame-shift in the read-out from the DNA which would be transmitted not only to messenger RNA but also to the DNA of daughter cells (Ames et al., 1973; Grover, 1973). Tominaga et al. (1973) have shown that RNA synthesis is necessary for DMBA tumourigenesis.
Hormones and DMBA tumourogenesis

Ovariectomy immediately following DMBA administration to rats (Heiman et al., 1968) or 30 days prior to DMBA treatment (Welsch et al., 1968), or DMBA administration to male rats (Dao & Sunderland, 1959) allows only a low incidence of mammary tumours. Following the administration of estrogen to such animals tumour incidence is increased (Dao, 1971; Geyer et al., 1953). A further observation is that tumour incidence following DMBA treatment depends upon the stage of the estrous cycle when the DMBA is administered (Young et al., 1970). Thus estradiol appears to be essential for the induction and/or development of tumours following DMBA treatment (Dao, 1967; Libby & Dao, 1966).

In the induction stages of tumourigenesis it is possible that estradiol is essential as a modifier of mitotic activity in mammary epithelial cells, and that a certain rate of mitosis is essential for such cells to be susceptible to tumour induction by DMBA (Dao, 1971). Perhaps the intercalation of the DMBA molecule into the DNA strand can occur at only one part of the cell cycle. Also the estradiol-stimulated histone removal from the chromatin of epithelial cell nuclei could facilitate such an intercalation.

Later stages of tumourigenesis could also require estrogen. Beuving & Bern (1972) found that ovariectomy following DMBA treatment inhibits multiplication of "carcinogen-altered" cells, and some later stages of tumours may regress following ovariectomy (Boylan & Wittliff, 1973). Most workers seem to agree that estrogen is essential for the growth and development of mammary tumours arising as a result of
DMBA ingestion by rats (Beuving & Bern, 1972; Meites, 1972a; Sinha & Dao, 1972). However it is uncertain whether estrogen itself is the co-carcinogen or whether it is estrogen-stimulated prolactin (Dao, 1967). It is known that in the rat increased estrogen levels tend to increase blood prolactin levels (Meites, 1972b), possibly through inhibition of prolactin-inhibiting hormone from the hypothalamus (Klaiber et al., 1969). Further, increased prolactin levels following treatment with certain drugs or by pituitary grafts can increase the growth rate of mammary tumours (Dao, 1971; Meites, 1972a). Conversely, reduction of serum prolactin levels is associated with declining growth rate of mammary tumours following DMBA ingestion (Meites, 1972b). The actions of the two hormones could therefore be synergistic in the process of tumourigenesis, although it has been reported that prolactin alone is adequate to allow growth of the tumours (Sinha et al., 1973). However estradiol, and not prolactin is the essential hormone for tumour induction, and prolactin may even be inactive in this process (Klaiber et al., 1969).

A second anterior pituitary gland hormone, STH, has also been found to be stimulatory to tumour growth and development in rats (Meites, 1972a). But during tumourigenesis both STH and prolactin remain relatively stable with respect to their concentration in the blood (Meites, 1972a).

While an excess of estrogen alone is capable of causing mammary tumour development in rats (Cutts & Noble, 1964), an excess of estrogen administered to rats following treatment with DMBA inhibits the growth and development of tumours (Huggins et al., 1962), although total inhibition is not achieved. In such a case it is thought that
the excessive amounts of estradiol may interfere with the peripheral actions of prolactin in mammary tissue (Meites, 1972b), demonstrating further an interrelationship between estrogen and prolactin.

Most tumours resulting from DMBA ingestion are estrogen-dependent (Sander & Attramadal, 1968). They regress following ovariectomy (Beuving & Bern, 1972; McGuire et al., 1972) and within the cytoplasm of such tumours there is a protein which has been identified as the estrogen receptor (Jensen et al., 1972; King et al., 1965; Mobbs, 1966; Wittliff et al., 1972). Thus the DMBA-induced mammary tumour may be classified as an estrogen target tissue, although some 10 to 12% of such tumours do not show estrogen dependency, and do not contain the estrogen receptor (Jensen et al., 1972).

It is not only estrogen which plays a role in tumourigenesis in mammary tissue following DMBA ingestion. Adrenal steroids such as cortisol and cortisone, necessary for normal mammary development, are also essential for development of neoplastic cells, since adrenalectomy inhibits tumour development (Sterental et al., 1963).

Progesterone generally appears to be inhibitory to tumourigenesis following DMBA treatment (Jul1, 1966; Welsch et al., 1968). Thus tumour induction by DMBA does not occur in pregnant rats where circulating progesterone levels are high (Huggins et al., 1961), or in rats treated with progesterone prior to DMBA ingestion (Jabara et al., 1973). Perhaps hyperfunctioning mammary epithelial cells found in mammary gland during pregnancy are refractory to the carcinogenic effects of DMBA (Dao, 1971). But once formed, mammary tumours are stimulated by progesterone (Huggins et al., 1962; Jabara et al., 1973).
Androgens appear to have an inhibitory effect on mammary tumour growth and development (Griswold & Green, 1970; Talley et al., 1973), although any inhibitory effects appear to diminish during the latter stages of tumour growth. Androgens may act in this manner through an inhibition of estrogenic actions at the level of the receptor protein (Rochefort et al., 1972).

(4) The estrogen receptor and DMBA tumorigenesis

The estrogen receptor has been implicated in DMBA tumorigenesis in rats. Since estrogen is essential for the induction of mammary tumours by DMBA, this implies that estrogen binding to the cytosol receptor, and subsequently to the nuclear acceptor are also necessary. Perhaps anti-estrogens administered prior to DMBA administration may inhibit DMBA tumorigenesis, in some cases by competition with the estrogen for receptor binding. In the present study I tested the hypothesis that androgen acts as an anti-estrogen by competition with estradiol for the estrogen binding sites (Chapter IV).

It has also been suggested that the cytosol estradiol receptor is involved in the action of DMBA in the cell. I tested the hypothesis that DMBA binds to the estrogen receptor of rat uterus and mammary gland, competing with estradiol for binding to the receptor sites. This study was carried out in vitro (Chapter IV). I also tested the hypothesis that DMBA in vivo causes persistent changes in estrogen receptor levels in mammary gland and uterus (Chapter V). Simultaneously the effects of DMBA on estrogen metabolism were determined (Chapter V).
CHAPTER III

THE CYTOSOL ESTROGEN RECEPTOR AND
TECHNIQUES USED IN ITS MEASUREMENT

Measurement of the number of binding sites for estradiol-17β
(\(N_s\)) and of the intrinsic association constant for the estradiol-
receptor interaction (\(K_s\)) was carried out by means of dextran-
coated charcoal as described below. This technique involved the
preparation of a 105,000 x g supernatant (cytosol) fraction of the
tissue, incubation of this with labeled estradiol-17β, and finally
addition of dextran-coated charcoal to determine the amount of
labeled steroid bound with high affinity in the cytosol fraction.
This procedure, carried out at different concentrations of the
radioactive estrogen, allowed the construction of a Scatchard plot
from which values of \(N_s\) and \(K_s\) could be determined. This procedure
is outlined in a flow-chart (Figure 2).

(1) Cytosol preparation

The tissue to be investigated was isolated from a rat which had
been killed either by cervical fracture or by ether overdose. It was
washed in cold Tris buffer (Tris-HCl, 0.01M; EDTA, 0.0015M; pH 7.4),
patted dry on a filter paper, weighed, and cut into 2 mm lengths
with scissors. The slices were ground at 4°C in a Ten Broeck glass-
glass homogenizer with three times their volume of the cold Tris
buffer. The resultant homogenate was centrifuged for 15 min at
3,000 x g and the supernatant spun at 105,000 x g for 90 min in an MSE ultracentrifuge.

The ultracentrifuge supernatant was measured for protein by the method of Lowry et al. (1951; Appendix), diluted to around 2 mg protein per ml with the Tris buffer and again measured for protein. This cytosol fraction was kept at 4°C and all subsequent steps were carried out at this temperature. The cytosol fraction was stored for no more than 12 hr before use.

(2) Estradiol and dextran-charcoal incubations

Aliquots of 0.5 ml of the cytosol fraction were incubated in 11 mm x 77 mm polypropylene tubes, or in 10 mm x 75 mm glass tubes with concentrations between 1 x 10^{-10} M and 5 x 10^{-8} M of either 6,7-3H-estradiol-17β (Amersham/Searle; specific activity 40 Ci / mmole; Appendix) or 2,4,6,7-3H-estradiol-17β (Amersham/Searle; specific activity 85 Ci / mmole; Appendix). The purity of the steroids was checked by thin layer chromatography and found to be in excess of 95% (Appendix). In the incubations the labeled estradiol was added in a volume of ethanol not exceeding 25 μl. Where necessary, other chemicals such as DMBA or unlabeled estradiol were added at this stage in 5 to 10 μl of solvent. A 12 hr incubation at 4°C ensured equilibration of estradiol with those protein molecules which bound the steroid with high affinity (see below).

At the end of the incubation period a 100 μl aliquot was removed from each tube and placed in 10 ml Bray's solution (Appendix) in a glass scintillation vial.
Following this, 0.4 ml dextran-coated charcoal in the Tris buffer (500 mg charcoal; 50 mg dextran; 100 ml Tris buffer) was added to each tube and the mixture incubated with periodic shaking over 3 hr at 4°C. This time interval permitted the dextran-charcoal to remove most of the non-specifically bound, as well as the unbound labeled steroid, from solution (see below). To measure residual unbound estradiol, a blank series of tubes was set up using the Tris buffer as the incubating medium instead of the cytosol fraction.

At the end of the 3 hr period the tubes were centrifuged at 4000 x g for 20 min to sediment the dextran-charcoal with adsorbed estradiol. A 100 µl aliquot of the supernatant was placed in 10 ml Bray's solution in a glass scintillation vial, and the samples counted for 1 min in a Nuclear Chicago Mark II liquid scintillation counter. Channels ratio of the external standard was used as the means of quench correction. Counting efficiency was around 40%.

The difference between the cytosol aliquot counts and the buffer aliquot counts after the dextran-charcoal treatment allowed an estimate of the estradiol specifically bound to protein in the cytosol fraction at zero time. The aliquot removed from the estradiol-cytosol incubation mixture measured the "total" estradiol in the medium. Thus the "unbound" steroid at that incubating concentration of estradiol could be calculated, and the ratio of bound to unbound estradiol determined. This procedure carried out at a variety of incubating concentrations of ³H-estradiol-17β provided data for either a Scatchard plot (Scatchard, 1949), or a double reciprocal plot (see below).
Before the number of binding sites for estradiol and the intrinsic association constant could be measured it was important to know the time required for equilibration of estradiol with its receptor sites. Also the time required by dextran charcoal to remove free and non-specifically bound estradiol from the equilibrated mixture had to be determined. Once these values were ascertained some normal values for $N_S$ and $K_S$ could be obtained for rat tissues for comparison with values in the literature.

(a) Equilibration of receptor and estradiol-17β

Aliquots of 0.5 ml of rat uterine cytosol preparation (1.33 mg protein per ml) were incubated at 4°C in polypropylene tubes with $5.8 \times 10^{-10}M$, $2.5 \times 10^{-9}M$, or $1.5 \times 10^{-8}M$ $^3H$-estradiol-17β for various times between 1 and 25 hr. At the end of the incubation period the incubation mixtures were treated with dextran-charcoal as described above, and the supernatant measured for radioactivity. The amount of $^3H$-estradiol-17β bound with high affinity to proteins at the given times of incubation was determined.

The curves obtained using the two lower concentrations of estradiol (Figure 3) indicated that the cytosol fractions binding the steroid reached equilibrium within 12 hr. A 4-fold increase in estrogen concentration to $1.5 \times 10^{-8}M$ demonstrated that equilibrium required a longer time at such high steroid concentrations. Moreover there was a steady increase in steroid binding with time possibly indicating that the specific receptor was saturated, and that the excess free estradiol was being taken up by molecules with lower affinity and higher capacity than the receptor. Certainly
Figure 3. Equilibration curve for 6,7-\(^3\)H-estradiol-17\(\beta\) (E\(_2\)) and cytosol fraction from rat uterus. Three concentrations of estradiol were used (5.8 \times 10^{-10} M; 2.5 \times 10^{-9} M; 1.5 \times 10^{-8} M) and the amount of label specifically bound in the cytosol fraction (dpm / ml) plotted against time of incubation. The reaction was stopped at 1, 3, 5, 7, 10, 12 or 24 hr by incubation with dextran-charcoal for 3 hr, and radioactivity measured in the supernatant following centrifugation. Values for blank tubes (Tris buffer instead of cytosol fraction) were subtracted from the cytosol values.
concentrations of estradiol greater than $10^{-8} M$ are in excess of those required to saturate the receptor sites (Williams & Gorski, 1971).

Thus for the range of $^3H$-estradiol-17β concentrations used in the experiments discussed in this dissertation, equilibration of estradiol and receptor was reached within 12 hr at 4°C.

(b) Dextran-charcoal incubations

Aliquots of 3.5 ml of rat uterine cytosol-fraction (1.8 mg protein per ml) were placed in 25 ml Erlenmeyer flasks in a Dubnoff apparatus at 4°C. These flasks were paired with similar flasks containing 3.5 ml of the Tris buffer to act as blanks. To pairs of flasks was added 6,7-$^3H$-estradiol-17β in ethanol to concentrations of 32,000 dpm / ml, 64,000 dpm / ml, 288,000 dpm / ml, 579,000 dpm / ml, and 1,237,000 dpm / ml. This was measured by removing 0.2 ml from each flask and counting the activity after it was placed in 10 ml scintillation fluid (PPO, 0.45%; POPOP, 0.05%; toluene, 99.5%).

Following this 3.3 ml dextran-charcoal was added to each flask, the mixture shaken at 120 oscillations per min, and 0.5 ml aliquants centrifuged for 15 min at 4,000 x g at 1, 3, 5, 10, 12 and 20 hr after the charcoal addition. A 100 µl aliquot of the supernatant was counted in 10 ml of the scintillation fluid to determine the amount of label not bound to the charcoal. The difference in counts between the buffer and cytosol tubes gave the amount of estradiol remaining on the specific receptor proteins within the cytosol fraction, as well as that which was free and which the charcoal did not have sufficient time to remove. Results are shown in Figure 4.
Figure 4. Time course of dissociation of estradiol binding macromolecules by means of dextran-coated charcoal at 5 concentrations of $^3$H-estradiol-17β ($3 \times 10^{-10}$ M, △; $6 \times 10^{-10}$ M, ◦; $3 \times 10^{-9}$ M, X; $6 \times 10^{-9}$ M, ○; $1 \times 10^{-8}$ M, ▲). The bound estradiol is expressed as dpm/ml remaining in the supernatant after centrifugation of the charcoal and estradiol-cytosol mixture at the times indicated. Blank values were subtracted from the bound dpm to allow for that free $^3$H-estradiol not taken up by the dextran-charcoal.
The results illustrated two main features concerning the estradiol binding proteins of rat uterus cytosol fraction. Firstly there were two types of proteins present in the fraction as far as estradiol binding was concerned. One appeared to have high capacity to bind estradiol and only low affinity for the steroid. This binding moiety dissociated rapidly when dextran-charcoal was added to an estradiol-cytosol mixture, a dissociation represented by the initial steep decline of the curves of Figure 4. Also contributing to this part of the curve was the removal of free $^3$H-estradiol-17β from solution.

A second estradiol binding component had limited capacity to bind the hormone, and a high affinity for it. The latter was demonstrated by the very slow rate of dissociation of the hormone from the binding protein after addition of the dextran-charcoal. For the low affinity binding macromolecule the half time for dissociation by means of dextran-charcoal was around 0.25 hr, while that for the high affinity binding was around 30 hr.

The second feature of the graph was the time required to remove most of the free estradiol and the non-specifically bound estradiol from solution by means of the dextran-charcoal technique. Under the conditions used ($4^\circ$C; Tris buffer, pH 7.4) a good estimate of zero-time specifically bound estradiol could be obtained from the 3 hr value. The labeled estradiol in the supernatant after a 3 hr incubation with dextran-charcoal gave measurements within 5% of the extrapolated zero-time value of the high affinity binding sections of the curves of Figure 4.
(3) Sucrose density gradient analysis

A 5% to 20% gradient of sucrose in the Tris buffer was made in a volume of 10.5 ml in a cellulose nitrate tube (Beckman) using an ISCO model 570 gradient former. The gradients were kept chilled in ice water, and 0.3 ml of either uterine cytosol fraction, equilibrated for 12 hr at 4°C with 16,000 dpm of \(^3\)H-estradiol-17β, or bovine serum albumin (BSA) standard (5 mg per ml) layered on top of the gradient. Also layered on a gradient was the 4,000 x g supernatant after a 3 hr incubation of equilibrated estradiol-cytosol mixture with dextran-charcoal. The tubes were centrifuged at 38,000 rpm for 24 hr at 4°C in a SW41 rotor on a Beckman model L2-65B ultracentrifuge.

Using 30% sucrose solution in the Tris buffer as a chaser in an ISCO model 640 density gradient fractionator, 40 fractions of 0.3 ml volume were collected from each tube at the rate of 0.5 ml per min. Sample number 1 was obtained from the top (5%) end of the gradient.

For the tubes containing cytosol fraction, an 0.1 ml aliquot of the fraction was placed in a scintillation vial with 10 ml Bray's solution and counted for 1 min. A radioactivity profile could then be drawn (Figure 5).

For the tubes containing BSA the 0.3 ml fractions were diluted with 0.7 ml distilled water and the absorbance of the sample at 280 nm read on a Beckman DB spectrophotometer.

BSA sedimented in one peak at 4.6S in the gradient. Radioactivity was found in a second peak further down the tube. The sedimentation
Figure 5. Sedimentation of estradiol-receptor complex from rat uterus in a 5% to 20% gradient of sucrose in Tris buffer. Cytosol fraction from rat uterus was incubated for 12 hr at 4°C with 30 pM $^3$H-estradiol-17β and the equilibrated mixture layered on the gradient and centrifuged for 24 hr at 38,000 rpm. Following fractionation the radioactivity profile (△) was determined and a peak found at 8S.

Following the equilibration of estradiol with the receptor, a similar cytosol fraction was incubated for 3 hr with dextran-charcoal. The supernatant was layered on a 5% to 20% sucrose gradient and centrifuged as described above. The radioactivity profile (●) demonstrates removal of all activity but that bound in the 8S peak.

The BSA standard run on a separate gradient was detected by absorbance at 280 nm. It sedimented at 4.6S in the gradient (○).
constant of the radioactivity peak was calculated according to the method of Martin & Ames (1961) from the equation
\[
\frac{S_1}{S_2} = \frac{d_1}{d_2}
\]
where \( S_1 \) and \( S_2 \) are the sedimentation constants for molecules 1 and 2, respectively, and \( d_1 \) and \( d_2 \) the distances the molecules move through the gradient. From the equation, the radioactivity sediments in a peak at 8.15.

The discrete peak of radioactivity indicated that binding most likely was to one species of macromolecule, although it is possible that many different molecules binding estradiol could sediment at the same position. However, the Scatchard plots indicate an homogeneous binding system at such low concentrations of the hormone (see below). Also, the peak persisted following a 3 hr incubation with dextran-charcoal (Figure 5) indicating that the estradiol was bound with high affinity to a macromolecule in the peak. The radioactivity profile confirmed that dextran-charcoal is an efficient adsorbant of estradiol-17\( \beta \) (Binoux & Odell, 1973). The macromolecule binding \(^3\)H-estradiol-17\( \beta \) was the estrogen receptor which has been identified by other workers (Baulieu et al., 1967b; Giannopoulos & Gorski, 1971a; Jensen et al., 1971).

Martin & Ames also found that in a linear sucrose density gradient the molecular weight of macromolecules may be calculated according to the formula
\[
\frac{S_1}{S_2} = \left[ \frac{M_{W1}}{M_{W2}} \right]^{\frac{2}{3}}
\]
where \(\text{MW}_{1}\) and \(\text{MW}_{2}\) are the molecular weights of molecules 1 and 2, respectively. Thus the molecular weight of the 8S receptor for estradiol was calculated to be 160,000, a crude measurement which approximates that of 200,000 reported by Jensen & DeSombre (1972). The estimate of Puca and co-workers (Puca et al., 1971) is a little more precise and gives the molecular weight of the 8S receptor as 236,000, while that of Gardner & Wittliff (1973) places the molecular weight at 250,000. Use of the above formulae following sucrose density gradient centrifugation is not a good technique for molecular weight determination since it assumes that molecules in the gradient are spherical. This need not be so in the case of the estrogen receptor. Also the formula assumes a partial specific volume of 0.725 cc/g and while this may be a close approximation for most proteins it still may introduce a further error into the calculations.

(4) The Scatchard plot

The interaction of estradiol-17\(\beta\) with the high affinity receptor sites may be described by a Scatchard plot (Scatchard, 1949). This involves the determination of the amount of estradiol which is bound (B) and that which is not bound (U) to the receptor following equilibration of the two molecules at any given concentration of estradiol. A plot of the ratio of bound to unbound estradiol against bound estradiol over a range of incubating estradiol concentrations (Figure 6) permits the calculation of the number of binding sites for estradiol \(N_s\) and of the intrinsic association constant \(K_s\). Since it has been assumed that one molecule of estradiol binds to
one molecule of receptor protein (Ellis & Ringold, 1971), the number of estradiol molecules bound is equal to the number of available receptor sites.

This technique is valid only in an homogeneous system. A test for homogeneity is in the nature of the plot: a straight line indicates homogeneity, while a curve indicates heterogeneity in the binding sites. As indicated by Scatchard plots in Chapters IV and V the binding of estradiol to its high affinity receptor sites is homogeneous at concentrations of estradiol-17β less than $5 \times 10^{-9}$ M.

The equation of the line shown in Figure 6 is

$$\frac{B}{U} = N_s K_s - K_s B,$$

where $B =$ bound estradiol (pmole / g cytosol protein)  
$U =$ unbound estradiol (pmole)  
$K_s =$ intrinsic association constant (litres / pmole)

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

![Figure 6](image)

Figure 6. A Scatchard plot illustrating the specific binding of a molecule to a ligand. Details are in the text.
The slope of the line is \(-K_S\) and the intercept of the line with the abscissa is the value \(N_S\). The intercept on the ordinate is the value \(N_SK_S\).

With high concentrations of estradiol-17\(\beta\) (in excess of \(10^{-8}\) M) in the incubating mixture not all of the estradiol is bound to the high affinity sites, leaving some bound to proteins having lower affinity for the steroid. The Scatchard plot obtained under these conditions is indicated in Figure 7 and consists of two main sections.

![Figure 7](image)

Figure 7. Scatchard plot showing both a component with high specificity and limited capacity (a), and one with lower affinity and unlimited capacity (b).

The initial linear component (a) represents the high affinity binding of the steroid, and the linearity indicates that the system is acting as if it were homogeneous when such low concentrations of the steroid are involved. As the concentration of the estradiol increases, much more of the hormone is bound to proteins having lower affinity for the molecule. Such proteins also show unlimited capacity for
binding of the steroid. This latter phenomenon, shown in part (b) of Figure 7 was demonstrated following addition of a 100-fold excess of unlabeled estradiol. In this case part (a) of the curve disappeared, and only a near-horizontal line remained (Chapter IV, Chart 1).

Thus in a Scatchard plot where low affinity binding of estradiol occurs at high concentrations of the steroid, it is necessary only to extrapolate part (a) of the plot to the abscissa to determine \( N_s \), and to determine the slope of that part of the plot to gain an estimate of \( K_S \) (Hahnel & Twaddle, 1973). Such extrapolation and fitting a line to a series of points was done by the "least squares" method of linear regression analysis (Appendix).

(5) The double reciprocal plot

Sometimes used as an alternative to the Scatchard plot, the double reciprocal plot (Lineweaver & Burk, 1934) can also be used to determine \( N_s \) and \( K_S \) (Baulieu et al., 1971). A plot is shown in Figure 8.

As for the Scatchard plot, both the bound estradiol (B) and that which is not bound to the receptor protein (U) are determined for a variety of incubating concentrations of estradiol-17\( \beta \). In an homogeneous system such as that found for estradiol and cytosol fraction with concentrations of the steroid less than \( 5 \times 10^{-9} \text{M} \), a linear relationship is seen. The intercept on the ordinate is the reciprocal of \( N_S \), and the slope is the reciprocal of \( N_S K_S \). The line was fitted to the points by the "least squares" method of linear regression analysis.
Figure 8. A double reciprocal plot such as that obtained by incubation of low concentrations of estradiol-17β with cytosol.

(6) Concentration independence of \( K_s \)

In most of the work carried out in these studies care was taken to use uterine or mammary homogenates with protein concentrations of 2.0 (± 0.2) mg per ml cytosol fraction, but in some instances the protein concentration fell out of this range. Therefore it was necessary to determine whether or not the intrinsic association constant for the estradiol-receptor interaction \( (K_s) \) was independent of the cytosol protein concentration over the range of concentrations used (1 to 3 mg protein per ml).

Cytosol fraction was prepared from 3 uteri freshly excised from mature Sprague-Dawley rats. Using Tris buffer, the cytosol was diluted to 1.25, 2.37, or 3.02 mg protein per ml. Both \( K_s \) and \( N_s \) were measured for each dilution using the techniques described above. Points for the Scatchard plots of the results (Figure 9) demonstrated
Figure 9. Scatchard plots (ratio of bound estradiol [B; pmoles per g cytosol protein] to unbound estradiol [U; pmoles] against bound estradiol [B]) showing the lack of effect of change in cytosol protein concentration on $N_S$ and $K_S$ for estradiol-17β. Rat uterine cytosol fraction at protein concentrations of 1.25 mg per ml (●), 2.37 mg per ml (□), and 3.02 mg per ml (X) were prepared from a common pool of uterine cytosol. Aliquots of each concentration were incubated with varying amounts of $^3$H-estradiol-17β prior to addition of dextran-charcoal. Counting aliquots of the supernatant of the charcoal incubation mixture gave the bound concentration of estradiol (B).
that there were no significant differences between the 3 dilutions of the cytosol fraction with respect to $K_S$ or $N_S$. Values for these parameters are shown in Table 1. Thus $K_S$ is independent of protein concentration, at least over the range of 1 to 3 mg protein per ml.

Table 1. Values for $K_S$ and $N_S$ obtained at 3 concentrations of protein in rat uterus cytosol fraction. $N_S$ and $K_S$ were determined from linear regression analyses of the points of Figure 9.

<table>
<thead>
<tr>
<th>Protein concn. (mg / ml)</th>
<th>$N_S$ (pmole / g protein)</th>
<th>$K_S$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>676</td>
<td>$2.0 \times 10^9$</td>
</tr>
<tr>
<td>2.37</td>
<td>691</td>
<td>$2.0 \times 10^9$</td>
</tr>
<tr>
<td>3.02</td>
<td>692</td>
<td>$2.0 \times 10^9$</td>
</tr>
</tbody>
</table>

(7) Estradiol binding in non-target tissues

Cytosol fractions from female rat skeletal muscle and from female rat cerebrum were obtained as described above and the ratio of bound to unbound estradiol determined over a range of incubating estradiol concentrations, as was done for target tissues described above. Results are presented in Figure 10. Simultaneously binding studies were performed on cytosol fraction from rat uterus, and the results are presented in Figure 10 for comparison with the non-target tissues.

Following incubation with dextran-charcoal only a small proportion of added $^3$H-estradiol was bound to proteins within cytosol fractions from skeletal muscle or cerebrum. This is indicative that the estrogen receptor is not present in these tissues. Also the binding
Figure 10. Estradiol binding in skeletal muscle (X), cerebrum (O), and uterus (■) of the rat. Cytosol fractions were obtained from each of the 3 tissues and incubated with a range of concentrations of $^3$H-estradiol-17β. The data are presented in the form of a Scatchard plot.
seen in uterine or mammary cytosol fractions is not due to plasma proteins such as albumin or globulin which could be present in the homogenates.

(8) Normal values for $K_s$ and $N_s$ in rat tissues

Using the techniques described above, $N_s$ and $K_s$ were measured for rat uterus and rat lactating mammary gland. The values obtained were then compared with those obtained by other workers (Table 2).

Table 2. Values for $N_s$ and $K_s$ obtained for rat uterus and rat lactating mammary gland at $4^\circ$C. Values of $N_s$ are expressed as pmole per g cytosol protein, while those of $K_s$ are M$^{-1}$.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>$N_s$</th>
<th>$K_s$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>uterus</td>
<td>85</td>
<td>$8.3 \times 10^9$</td>
<td>Keightley &amp; Okey, 1973</td>
</tr>
<tr>
<td>uterus</td>
<td>28 - 222</td>
<td>$3.55 \times 10^9$</td>
<td>Mester et al., 1970</td>
</tr>
<tr>
<td>uterus</td>
<td>150</td>
<td>$1.7 \times 10^9$</td>
<td>Steggle &amp; King, 1970</td>
</tr>
<tr>
<td>mammary: week 1*</td>
<td>15</td>
<td>$8.3 \times 10^9$</td>
<td>Keightley &amp; Okey, 1973</td>
</tr>
<tr>
<td>mammary: week 1</td>
<td>15</td>
<td></td>
<td>Beers &amp; Wittliff, 1973</td>
</tr>
<tr>
<td>mammary: week 2</td>
<td>21</td>
<td>$3.6 \times 10^9$</td>
<td>Keightley &amp; Okey, 1973</td>
</tr>
<tr>
<td>mammary: week 2</td>
<td>20</td>
<td>$1.3 \times 10^9$</td>
<td>Wittliff et al., 1972</td>
</tr>
<tr>
<td>mammary: week 3</td>
<td>39</td>
<td>$1.5 \times 10^{10}$</td>
<td>Keightley &amp; Okey, 1973</td>
</tr>
<tr>
<td>mammary: week 3</td>
<td>40</td>
<td></td>
<td>Beers &amp; Wittliff, 1973</td>
</tr>
</tbody>
</table>

*Week after commencement of lactation.

The excellent agreement between the results obtained in the present study and those obtained by other workers further demonstrates the reliability of the techniques used.
CHAPTER IV

EFFECTS OF DIMETHYLBENZ(a)ANTHRACENE AND QHYDROTESTOSTERONE
ON ESTRADIOL-17β BINDING IN RAT MAMMARY CYTOSOL FRACTION

The role of estradiol in the induction of mammary tumours is unknown, although binding of this hormone to its cytosol receptor most likely occurs (Sinha & Dao, 1972). In the past it has been tempting to speculate that the similarity in structure between the hydrocarbon and steroid molecules leads to competition between the two for the same binding sites on macromolecules in target cells (Dao, 1971). Such speculation is reinforced by studies that show that some actions of estradiol-17β are inhibited by DMBA. For example, Libby & Dao (1966) found an inhibition of estradiol-stimulated incorporation of 14C-formate and 3H-adenosine into the mammary gland RNA of female rats following a single p.o. dose of 20 mg DMBA. Inhibition was observed only in female rats with functional ovaries, or in castrate rats receiving daily doses of estradiol-17β.

It was hypothesized that these actions may result from an inhibition by DMBA of estradiol-17β binding to its specific cytosol receptor protein. The investigations included both in vivo and in vitro additions of DMBA in a study of the number of binding sites and of their intrinsic association constants both in uterine and mammary cytosol fractions from rats. Non-radioactive estradiol-17β
was used to determine the influence of a specific competitor with
$^3$H-estradiol-17$\beta$ for its high affinity binding sites in mammary
cytosol preparations.

Androgens are effective in inhibiting the development of
mammary tumours following DMBA administration (Beuving & Bern, 1972).
Because of their structural similarity to estrogens, androgens also
may be effective in inhibiting the binding of estradiol-17$\beta$ to its
receptor sites. Therefore I tested the hypothesis that DHT is able
to prevent growth of tumours induced by DMBA by competing with
estradiol for binding sites in mammary cytosol fraction.

(1) Animals and tissues

To study the effects of DMBA on uterine cytosol binding of
estradiol, 10 female Sprague-Dawley rats were used. At age 50 days,
5 rats were given a single p.o. dose of 20 mg DMBA in 1 ml corn
oil. Three days later, all 10 animals were sacrificed by cervical
section, and their uteri excised and placed in the Tris buffer at
4$^\circ$C. In the studies on mammary cytosol fractions, 7 female Sprague-
Dawley rats aged 80 days were mated: when the litter was 2 days old,
3 animals were given DMBA as described above. The animals were
sacrificed by cervical section 3 days later, and the lactating
mammary glands were removed and placed in cold Tris buffer. Lactating
mammary tissue was used to ensure a high proportion of parenchymal
cells in the tissue sections.

For the non-radioactive estradiol-17$\beta$ and DHT competition
experiments, pooled mammary tissue from 5 Sprague-Dawley rats that
had been lactating for 15 to 20 days was used.
Cytosol fractions were prepared and incubated with $^3$H-estradiol-17β as described in Chapter III. Scatchard plots and double reciprocal plots were then used to obtain values for $N_S$ and $K_S$.

(2) Results

Both the Scatchard plot and the double reciprocal plot gave similar values for the number of binding sites for estradiol-17β and for the intrinsic association constant. Such agreement is demonstrated in the tables of results. In 120-day-old Sprague-Dawley rats that had been lactating 15 to 20 days, a Scatchard plot (Figure 11) revealed that there were 39 pmoles of estradiol-17β bound per g cytosol protein. The intrinsic association constant was high at $1.5 \times 10^{-10} \text{ M}^{-1}$. These results are similar to those obtained by Wittliff et al. (1972). Non-radioactive estradiol-17β at a concentration of $10^{-11} \text{M}$ had no effect on these parameters, whereas $10^{-9} \text{ M}$ estradiol-17β caused a decrease in both $N_S$ and $K_S$. Estradiol-17β at $10^{-7} \text{ M}$ concentration abolished binding of the tritiated steroid (Table 3). A double reciprocal plot of the same data used in plotting Figure 11 (Figure 12) further reveals the competitive nature of the binding of labeled and unlabeled estradiol-17β to the receptor sites.

In rats of a similar age and state of lactation, concentrations of DHT as high as $10^{-5} \text{ M}$ had no effect on the number of receptors for estradiol-17β (Figure 13). There was, however, a decrease in $K_S$ with concentrations of DHT up to $10^{-7} \text{ M}$ (Table 4). At the $10^{-5} \text{ M}$ concentration of DHT there was a 10-fold reduction in $K_S$. 
Figure 11. A Scatchard plot [the ratio of bound estradiol (B; pmol) to unbound estradiol (U; pmol) against bound estradiol (B; pmol per g protein)] showing the effects of addition of increasing amounts of unlabeled estradiol-17β to mammary cytosol fraction (2.96 mg protein per ml). For the control values (X), incubations were performed with concentrations of $^3$H-estradiol-17β between $3 \times 10^{-11}$M and $3 \times 10^{-8}$ M. The experiment was repeated with unlabeled estradiol-17β added to concentrations of $10^{-11}$ M (O), $10^{-9}$ M (△), or $10^{-7}$ M (□). Values of $K_s$ were obtained from the slope of the plot following correction of the B:U ratio for protein concentration. $N_s$ and $K_s$ values are summarized in Table 3.
Figure 12. A double reciprocal plot [the reciprocal of the concentration of estradiol bound (1/B; pmoles per g protein) against the reciprocal of that unbound (1/U; pmoles)] following incubation of rat mammary cytosol fraction (2.96 mg protein per ml) with $^3$H-estradiol-17β alone (X) or with final concentrations of unlabeled estradiol-17β of $10^{-11}$ M (O), $10^{-9}$ M (Δ), or $10^{-7}$ M (●). The plot shown is from the same data as used to obtain the Scatchard plot of Figure 11. $N_S$ was determined from the intercept on the ordinate, and $K_S$ from the intercept of the extrapolated plot on the abscissa. $N_S$ and $K_S$ values are summarized in Table 3.
Figure 13. A Scatchard plot showing the effects of addition of increasing amounts of DHT to mammary gland cytosol fraction (2.66 mg protein per ml). For the control values (X), concentrations of $^3$H-estradiol-17B between $3 \times 10^{-11}$ M and $3 \times 10^{-8}$ M were incubated with the cytosol fraction. The experiment was repeated in tubes containing DHT at concentrations of $10^{-9}$ M (O), $10^{-7}$ M (△), and $10^{-5}$ M (●). Values of $N_s$ and $K_s$ are summarized in Table 4.
Table 3. The effects of non-radioactive estradiol-17β ($E_2$) on
$^3$H-estradiol-17β binding in rat lactating mammary cytosol fraction.

Incubating concentrations of estradiol-17β ranged between
$3 \times 10^{-11}$ M and $3 \times 10^{-8}$ M. From Scatchard plots and double
reciprocal plots values for $N_S$ and $K_S$ were determined. $E_2$ in
ethanol was added to tubes of the same cytosol fraction. The final
concentration of the unlabeled $E_2$ in each tube is indicated in the
table. Ethanol alone was added to control tubes.

<table>
<thead>
<tr>
<th></th>
<th>$N_S$ at plot (pmoles/g protein)</th>
<th>$K_S$ at plot (M$^{-1}$)</th>
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<tbody>
<tr>
<td></td>
<td>Scatchard double reciprocal</td>
<td>Scatchard double reciprocal</td>
</tr>
<tr>
<td>Control</td>
<td>39</td>
<td>1.5 x $10^{-10}$</td>
</tr>
<tr>
<td>+ $10^{-11}$ M $E_2$</td>
<td>41</td>
<td>1.9 x $10^{-10}$</td>
</tr>
<tr>
<td>+ $10^{-9}$ M $E_2$</td>
<td>31</td>
<td>1.0 x $10^{-10}$</td>
</tr>
<tr>
<td>+ $10^{-7}$ M $E_2$</td>
<td>0</td>
<td>1.2 x $10^{-10}$</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.0 x $10^{-9}$</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.6 x $10^{-9}$</td>
</tr>
</tbody>
</table>

Mammary cytosol fraction that had been obtained from rats
which had been lactating only 5 days revealed fewer estradiol-17β
binding sites (approx 15 pmoles estradiol-17β bound per g protein)
than those rats that had been lactating for 15 to 20 days (approx.
40 pmoles estradiol bound per g protein). These results are similar
to those of Beers & Wittliff (1973), and could possibly be accounted
for by an increase in proportion of parenchymal cells in the
isolated mammary tissue of rats that had been lactating longer.
Alternatively there could either be an increase in the number of
Table 4. The effects of DHT on $^3$H-estradiol-17β binding in rat lactating mammary cytosol fraction. Results for the control lactating mammary cytosol fraction were obtained in a manner similar to that described in Table 3. In this case, however, DHT in ethanol was added at the concentrations indicated.

<table>
<thead>
<tr>
<th></th>
<th>$N_s$ at plot</th>
<th>$K_s$ at plot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Scatchard</td>
<td>Double</td>
</tr>
<tr>
<td></td>
<td>reciprocal</td>
<td>reciprocal</td>
</tr>
<tr>
<td>Control</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>+ 10^{-9} M DHT</td>
<td>38</td>
<td>39</td>
</tr>
<tr>
<td>+ 10^{-7} M DHT</td>
<td>40</td>
<td>41</td>
</tr>
<tr>
<td>+ 10^{-5} M DHT</td>
<td>38</td>
<td>35</td>
</tr>
</tbody>
</table>

Table 5. The effects of DMBA both in vivo and in vitro on $^3$H-estradiol-17β binding in rat lactating mammary cytosol. Data were obtained as described in Table 3. DMBA in 5 µl acetone was added to cytosol fraction just prior to $^3$H-estradiol. Final concentrations of DMBA are indicated. Control tubes were treated with 5 µl acetone. In vivo animals were treated as described in text.

<table>
<thead>
<tr>
<th></th>
<th>$N_s$ at plot</th>
<th>$K_s$ at plot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Scatchard</td>
<td>Double</td>
</tr>
<tr>
<td></td>
<td>reciprocal</td>
<td>reciprocal</td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>14</td>
</tr>
<tr>
<td>10^{-5} M DMBA</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td>10^{-3} M DMBA</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>20 mg DMBA</td>
<td>39</td>
<td>38</td>
</tr>
</tbody>
</table>
receptor sites in each cell or a decrease in competitive (endogenous) estradiol as lactation progresses. The experiments did not allow a determination of which of these possibilities or combination of possibilities gave rise to our results.

Table 6. The effects of DMBA both in vivo and in vitro on $^3$H-estradiol-17β binding in rat uterine cytosol fraction. Uterine cytosol fraction from Sprague-Dawley rats was treated in the same manner as the mammary cytosol of Table 5.

<table>
<thead>
<tr>
<th></th>
<th>$N_s$ at plot (pmoles/g protein)</th>
<th>$K_s$ at plot ($M^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Scatchard</td>
<td>Double reciprocal</td>
</tr>
<tr>
<td>Control</td>
<td>85</td>
<td>89</td>
</tr>
<tr>
<td>$10^{-5}$ M DMBA</td>
<td>70</td>
<td>70</td>
</tr>
<tr>
<td>$10^{-3}$ M DMBA</td>
<td>43</td>
<td>44</td>
</tr>
<tr>
<td>20 mg DMBA</td>
<td>103</td>
<td>103</td>
</tr>
</tbody>
</table>

DMBA in vitro at concentrations of $10^{-3}$ M or $10^{-5}$ M did not alter the number of specific binding sites: the association constant decreased slightly (Figure 14). The latter concentration of DMBA was chosen since it is close to the concentration reported for DMBA in mammary tissue of the mature rat following a carcinogenic DMBA dose (20 mg; Flesher, 1967). DMBA administered p.o. to the rat did not influence the association constant, but increased $N_s$ in the cytosol fraction of these animals (Table 5).
Figure 14. A Scatchard plot showing the effects of addition of DMBA to mammary gland cytosol fraction (2.65 mg protein per ml). Control values (X) were obtained as described in Figure 13. Scatchard plots were also obtained following the in vitro addition of DMBA to concentrations of $10^{-5}$ M (○) or $10^{-3}$ M (△). Cytosol fraction was isolated from rats of the same age and state of lactation, and that had been treated with 20 mg DMBA p.o. 3 days previously. The number of binding sites was determined in a manner similar to that of the in vitro experiments (●). Results are summarized in Table 5.
Uterine cytosol fractions were prepared for comparison with mammary cytosol fractions. In the uterine cytosol preparations DMBA tested in vitro reduced the number of apparent binding sites for estradiol-17β. Also, the higher the concentration of DMBA used, the lower the number of binding sites measured (Table 6). When the cytosol preparation was incubated with $10^{-3}$ M DMBA there was a decrease in $K_s$. In uterine tissue from rats administered DMBA p.o. the number of apparent binding sites increased, but the DMBA treatment had no effect on $K_s$ (Table 6). The relative increase in binding sites from 85 to 103 pmoles per g protein was not as great as that seen in mammary tissue (from 15 to 39 pmoles per g protein) following the same p.o. dose of DMBA.

(3) Discussion

A number of compounds are capable of decreasing the $K_s$ value for the reaction between estradiol-17β and its specific receptor sites. Such reduction may not be due to competition for binding sites but rather may be the result of non-specific interaction of the added compound with other parts of the binding macromolecule inducing changes in conformation. In such a case, $N_s$ need not be altered, but $K_s$ is changed. In the case of true competition for binding sites, there is seen a reduction in $N_s$. This implies that certain of the sites have been occupied by the competitor with such affinity that estradiol can not dislodge the competitor.

Non-radioactive estradiol-17β did compete with the labeled steroid for binding to the protein receptor of the lactating mammary cytosol fraction, reducing the number of available binding sites.
and also reducing the intrinsic association constant. These results 
were seen at low concentrations (10^{-9} M) of estradiol-17\beta and reflect 
the low capacity of the receptor macromolecules for estradiol-17\beta.

The high specificity of the receptor sites for the estrogen 
was shown by the lack of change in the number of binding sites 
with concentrations of DHT as high as 10^{-5} M in the cytosol fraction. 
DHT did not compete significantly with ^3H-estradiol-17\beta for binding 
to the receptor in lactating mammary cytosol fraction. DHT did 
cause a slight reduction in K_s, but this value was still high 
(1.7 \times 10^{-9} M^{-1}), even at the greatest concentration of DHT tested. 
These results are similar to those obtained by Jungblut et al. (1971) 
with testosterone in the uterus. Thus although DHT is capable of 
inhibiting tumour development in DMBA-treated rats, it probably 
does not act by competition with estradiol-17\beta for its specific 
receptor sites.

DMBA given p.o. to rats 3 days prior to sacrifice increased 
the number of binding sites for ^3H-estradiol-17\beta in the cytosol 
fraction. There was no change in K_s. Such an increase in N_s may 
have been due to a DMBA-stimulated increase in general metabolism 
(Dao, 1971) and of steroid metabolism in particular, thereby 
reducing the amount of endogenous estradiol available to bind to 
the receptor sites. The result was an increase in the number of 
sites available to bind estradiol-17\beta.

In the in vitro situation high concentrations of DMBA decreased 
the amount of ^3H-estradiol-17\beta bound to the specific receptor in 
uterine cytosol fraction, whereas the mammary cytosol fraction
DMBA caused no change in $N_s$. The reason for the difference between the 2 types of tissue is unclear. The concentration of DMBA ($10^{-5}$ M) found in the mammary gland of rats following a carcinogenic (20 mg) dose of the hydrocarbon (Flesher, 1967) did not significantly affect the extent of estrogen binding, and only a slight reduction in $K_s$ was found. It was concluded that there was no significant competition between estradiol-17β and DMBA for the estrogen binding sites, at least at the concentrations of DMBA used for the induction of tumours. Although the inhibitory effects of tumour induction by high levels of estradiol (Dao, 1971) do not appear to be due to DMBA and estradiol competition for estrogen binding sites, there is the possibility that estradiol could alter DMBA binding to a cytosol macromolecule different from that which binds estradiol (Heizmann & Wyss, 1972). A similar result appears to obtain in uterus for 3-methylcholanthrene (Toft & Spelsberg, 1972). Thus DMBA may bind with high affinity to cytosol macromolecules in mammary tissue, but these macromolecules do not appear to be the estrogen receptors.
CHAPTER V

EFFECTS OF DMBA ON RECEPTOR BINDING OF ESTRADIOL-17β IN UTERUS AND MAMMARY TISSUE THROUGHOUT LACTATION IN THE RAT

It has been suggested that DMBA, because of its similarity in structure to estradiol, is capable of binding to the cytoplasmic estrogen receptor (Dao, 1971). However, Kightley & Okey (1973; Chapter IV) have shown that DMBA in vitro does not compete with estradiol-17β for binding to the receptor proteins in rat mammary gland or uterus. DMBA in vivo does not inhibit uptake of estradiol-17β into rat mammary gland (Torgersen & Sander, 1970). But DMBA in vivo does increase the number of measurable binding sites for estradiol in the mammary gland (Kightley & Okey, 1973) and enhances uptake of estradiol-17β in the mammary glands of rats pretreated with DMBA (Torgersen & Sander, 1970).

The present study was undertaken to determine if a single carcinogenic dose of DMBA might cause persistent changes in estrogen or estrogen receptor levels in mammary tissue.

Estradiol concentrations and the number of estrogen receptor sites were followed through lactation both in control rats and in DMBA-treated rats to determine the effects of a carcinogenic p.o. dose of DMBA on these parameters. The study was carried out on

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1. The substance of this Chapter has been submitted for publication in Cancer Research.
lactating rats to ensure an adequate supply of mammary epithelial cells for binding studies (Shyamala & Nandi, 1972).

Estradiol binding in uterine cytosol was also studied to determine whether or not the effects of the hydrocarbon were general for estrogen target tissues, or were restricted to mammary gland.

(1) Animals and tissues

Female Sprague-Dawley rats aged 90 days were mated and the day on which the litter was dropped recorded. The following day at 0800 hr the animals were given either a p.o. dose of 15 mg DMBA in 0.5 ml corn oil, or a p.o. dose of 0.5 ml corn oil alone. At 6 hr, 18 hr, 2, 5, 10, or 20 days following treatment, 3 control animals and 3 DMBA-treated animals were sacrificed by ether overdose. Mammary tissue and uteri were removed from each animal and pooled within treatment groups for each sample time. Preparation of cytosol fractions and construction of Scatchard plots following incubation of $^3$H-estradiol-17β with the cytosol fraction has been outlined above (Chapter III).

(2) Radioreceptor assay for estradiol-17β

Following storage for up to 4 weeks at -5°C samples of either uterine cytosol fraction or mammary cytosol fraction (0.2 ml sample with 0.3 ml distilled water) were heated at 60°C for 30 min to release protein-bound estradiol. Estradiol then was extracted 3 times with 3 ml ethyl ether. The ether fractions were pooled and evaporated to dryness under a stream of air in 10- x 75-mm tubes prior to the addition of 16,000 dpm of 2,4,6,7-$^3$H-estradiol-17β.
and 0.2 ml of freshly prepared rat uterus cytosol fraction (approx. 2 mg protein per ml). Standards for the cytosol fraction assays consisted of known amounts of unlabeled estradiol-17β in ethanol (20, 50, 100, 200, 500, 1000 or 2000 pg) which had been evaporated to dryness and to which was added 0.2 ml of the 7000 x g supernatant of male rat skeletal muscle homogenate (2 mg protein per ml) and 0.3 ml distilled water. The standards were then subjected to the same extraction process as were the unknowns. Extraction of standards from male rat skeletal muscle homogenate provided a close approximation of the extraction losses of estradiol from the uterine and mammary cytosol fractions.

Following incubation of the evaporated ether extract, 3H-estradiol, and uterine cytosol fraction for 1.75 hr at 21°C, 0.2 ml dextran-coated charcoal was added and the mixture incubated for a further 3 hr at 4°C. Centrifugation at 4,000 x g for 15 min sedimented the dextran-charcoal, allowing 0.2 ml of the supernatant to be counted in 10 ml Bray's solution for an estimation of the bound estradiol. A standard curve of bound dpm against estradiol concentration was constructed over the range of 20 to 2000 pg estradiol-17β.

3 Results

Figure 15 is typical of the Scatchard plots obtained and indicates that estradiol-17β was bound with high affinity (10⁹ to 10¹⁰ M⁻¹) to a protein in the cytosol fraction, suggesting that this protein most likely was the estrogen receptor. For the control animals the average association constant measured over the 6
Figure 15. A Scatchard plot for the binding of estradiol to receptor proteins from the uteri of DMBA-treated lactating rats (X), or from control animals (O). Individual points were obtained by incubation of $^3$H-estradiol-17β at 8 concentrations between $9 \times 10^{-10}$ M and $1.1 \times 10^{-8}$ M with uterine cytosol fraction (2.0 mg protein per ml). The values for $K_s$ and $N_s$ for each sample time are found in Table 7.
sample times was slightly higher for lactating mammary tissue
($10^{10} \text{ M}^{-1}$) than for uterine tissue ($4.0 \times 10^9 \text{ M}^{-1}$). The association
constant was consistently higher for control lactating mammary tissue
(average: $10^{10} \text{ M}^{-1}$) than for lactating mammary tissue of DMBA-treated
rats (average: $6.3 \times 10^9 \text{ M}^{-1}$).

At 6 hr and 18 hr following DMBA administration to experimental
rats, or oil administration to control rats the number of estrogen
binding sites in uterine cytosol of both groups was about 70 to
100 pmoles per g cytosol protein (Table 7). The number of binding
sites trebled by day 5 and reached a maximum of 300 to 500 pmoles per
g cytosol protein by day 10 after treatment. There were no
significant differences between control and DMBA-treated rats with
respect to the number of estrogen receptor sites over the 20 day
period of investigation (Figure 16).

Up to 18 hr following treatment there were no differences
between control and DMBA-treated rats in the concentration of
estrogen binding sites in lactating mammary cytosol fraction (16 to
17 pmoles per g cytosol protein; Table 7). However, by day 2 after
treatment the number of binding sites for estradiol in mammary
cytosol fraction was higher for DMBA-treated animals than for the
control group (Figure 17). The difference was still measurable at
day 10, although by day 20 following treatment the number of
binding sites in the mammary cytosol of the control group had
reached the level of that in the DMBA-treated rats.

Utilization of the estrogen receptor of uterine cytosol fraction
in a competitive protein binding assay for estradiol (a "radioreceptor
Table 7. Values for $K_s$ and $N_s$ for uterus and mammary gland of control, and DMBA-treated lactating rats.

The number of binding sites for estradiol-17\(\beta\) ($N_s$; pmole per g cytosol protein) and the intrinsic association constant for the estradiol-receptor interaction ($K_s$; nM\(^{-1}\)) were derived from Scatchard plots. These values were determined in both uterine and mammary cytosol fractions in lactating rats which had been treated the day following parturition with either 0.5 ml corn oil alone (control rats) or 15 mg DMBA in 0.5 ml corn oil (DMBA rats). Each value obtained represents that for the pooled uteri or pooled mammary gland of 3 rats of each of the control and DMBA-treated groups.

<table>
<thead>
<tr>
<th>Time after treatment</th>
<th>Uterus</th>
<th>Mammary gland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>DMBA</td>
</tr>
<tr>
<td>6 hr</td>
<td>$N_s$</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>$K_s$</td>
<td>5.6</td>
</tr>
<tr>
<td>18 hr</td>
<td>$N_s$</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>$K_s$</td>
<td>5.0</td>
</tr>
<tr>
<td>2 days</td>
<td>$N_s$</td>
<td>269</td>
</tr>
<tr>
<td></td>
<td>$K_s$</td>
<td>2.7</td>
</tr>
<tr>
<td>5 days</td>
<td>$N_s$</td>
<td>252</td>
</tr>
<tr>
<td></td>
<td>$K_s$</td>
<td>3.8</td>
</tr>
<tr>
<td>10 days</td>
<td>$N_s$</td>
<td>358</td>
</tr>
<tr>
<td></td>
<td>$K_s$</td>
<td>2.9</td>
</tr>
<tr>
<td>20 days</td>
<td>$N_s$</td>
<td>298</td>
</tr>
<tr>
<td></td>
<td>$K_s$</td>
<td>4.0</td>
</tr>
</tbody>
</table>
Figure 16. Estrogen receptor levels in the uteri of control and DMBA-treated rats through lactation. Lactating Sprague-Dawley rats were treated with either 0.5 ml corn oil (control group; O), or 15 mg DMBA in 0.5 ml corn oil (X) and their uteri isolated at 6 hr, 18 hr, 2, 5, 10, or 20 days following treatment. The uterine cytosol fractions were incubated with varying amounts of $^3$H-estradiol-17$\beta$ to obtain Scatchard plots from which values for the number of available binding sites for estradiol ($N_s$; pmoles per g cytosol protein) could be determined. Day 0 of treatment occurred approximately 1 day after parturition.
Figure 17. Changes in the number of available binding sites for eastradiol-17β ($N_s$; pmole per g cytosol protein) in mammary cytosol fractions prepared from the same lactating rats of Figure 16. The animals had been treated with either 0.5 ml corn oil (O) or with 15 mg DMBA in 0.5 ml corn oil (X). Values of $N_s$ were determined as described in the caption to Figure 16.
assay") is not new (Korenman, 1968; Shutt, 1969). It has the advantage that the binding protein is specific for estradiol-17β. Consequently it is not necessary to purify samples beyond a simple extraction since estriol, estrone, estradiol-17α, corticoids and androgens interfere only insignificantly with the estradiol-receptor interaction (Ismail, 1972; Shutt, 1969). The standard curve for the assay was linear over the range 20 to 500 pg estradiol-17β (Figure 18) and the technique provided a reliable method for measurement of the estrogen. Levels of estradiol-17β were generally lower in the mammary cytosol fractions of DMBA-treated animals compared with control animals (Figure 19). This effect first was seen at 18 hr following treatment and persisted throughout lactation. A similar result was found for uterine cytosol (Figure 20).

(4) Discussion

Concentrations of DMBA in the order of $10^{-5}$ M have been found in the mammary glands of female Sprague-Dawley rats treated p.o. with 20 mg of the hydrocarbon (Flesher, 1967). Such levels of the hydrocarbon in vitro are capable of reducing slightly $K_s$ for the estradiol-receptor interaction in the cytosol fraction of rat mammary gland (Keightley & Okey, 1973), although no change is seen in the number of measurable binding sites for estradiol at these concentrations of the hydrocarbon. These results were supported by the present in vivo experiment where the $K_s$ values for the estrogen-receptor interaction in the mammary glands of rats treated with DMBA were consistently lower than the $K_s$ values for the control
Figure 18. Standard curve for radioreceptor assay for estradiol-17β. Unlabeled estradiol-17β in ethanol (20, 50, 100, 200, 500, 1000 or 2000 pg) was evaporated to dryness prior to the addition of 0.2 ml of the 7000 x g supernatant of male rat skeletal muscle homogenate, and 0.3 ml of distilled water. This was extracted 3 times with 3 ml ethyl ether and the pooled ether fraction evaporated to dryness in glass tubes. Following the addition of 0.2 ml of freshly prepared rat uterus cytosol fraction and 16000 dpm of $^3$H-estradiol-17β the mixture was incubated for 1.75 hr at 21°C. After dextran-charcoal treatment, the bound dpm were plotted against the quantity of unlabeled estradiol initially added.
Figure 19. Levels of estradiol-17ß in uterine cytosol fraction as measured by radioreceptor assay. Uterine cytosol fractions from either control lactating rats (○) or from DMBA-treated rats (X) were frozen for up to 4 weeks prior to the assay. The samples were thawed, and 0.2 ml fractions added to 0.3 ml distilled water in 15 ml test tubes. This was done in triplicate for each sample. Following extraction 3 times with 3 ml ether the pooled ether fraction was assayed for estradiol-17ß as outlined in the caption to Figure 18.

On the figure are shown the standard deviation of the 3 determinations on each sample, and the mean value.
Figure 20. Levels of estradiol-17β in mammary cytosol fraction as measured by radioreceptor assay. Mammary cytosol fractions were either from control lactating rats (O) or from DMBA-treated rats (X). The day of treatment was 1 day after parturition. Each determination was made in triplicate, and both the standard deviation and the mean values for the determinations are shown.
rats, suggesting concentration of the hydrocarbon in mammary tissue. A reduction in $K$ with DMBA was not seen in uterus probably because in that tissue DMBA had not been concentrated to an extent where it could interfere with the estrogen-receptor interaction. Only low concentrations of DMBA have been found in the uteri of rats given p.o. 20 mg DMBA (Flesher, 1967).

An increase in the number of estrogen binding sites in the mammary glands of DMBA-treated rats compared with control animals was first obvious at 2 days after treatment, and was not seen in the uterus. One hypothesis to account for these results is that endogenous estradiol levels were lower in the DMBA-treated rats compared with the control rats, resulting in less competition for binding sites when these were measured in vitro. Certainly estrogen levels were lower both in uterine and mammary tissue of the DMBA-treated rats, probably because of enhanced metabolism of the hormone following hydrocarbon stimulation of liver enzyme systems (Lu et al., 1973). But while this could contribute to the increased estrogen receptor levels which were seen in mammary tissue it is not an adequate explanation since any general enhancement of estrogen metabolism should also be reflected in enhanced binding site numbers in the uterus. This was not seen. Therefore despite direct measurements indicating lower estradiol levels in the mammary glands and uteri of DMBA-treated rats, the similarity in the number of binding sites in the control and treated uteri suggests that a general reduction in estrogen levels can not account for the mammary gland results.
Alternative hypotheses for the increase in receptor sites seen in mammary gland include either an actual increase in the number of receptor sites in each epithelial cell, or an increase in the proportion of epithelial cells in the mammary gland, or perhaps both of these events occur simultaneously in the mammary gland following DMBA treatment.

The rate of mitoses in normal mammary gland epithelial cells is generally low, but a steady rise in the number of epithelial cells occurs throughout lactation (Munford, 1963). This probably is the cause of the gradual increase in estrogen receptor sites seen in control rats in this study and in that of Beers & Wittliff (1973). An increased rate of cell division occurs in DMBA-induced tumours (Grahame & Bertalanffy, 1972), and in normal mammary tissue as soon as 2 weeks following DMBA treatment (Janss et al., 1972). DMBA-induced tumours are detectable histologically within 11 days of DMBA administration (Huggins, 1967). Thus DMBA probably is capable of bringing about an increase in cell numbers in the mammary gland within 2 days of administration of the hydrocarbon. These effects, which may be the initial stages of neoplasia, could be effected either directly by DMBA, or indirectly through an effect of DMBA on hormone levels. For example, the reduction in estrogen concentration seen following DMBA administration to rats could stimulate cell division in the mammary gland if the initial estradiol levels were low (Turkington, 1971). But to determine whether or not this was so would require precise measurements of the estradiol concentration within the epithelial cells of the mammary gland, rather than a
measurement of whole-gland estradiol levels.

While the possibility of an increase in the number of estrogen receptor sites in each cell following DMBA treatment can not be discarded, it appears likely that DMBA stimulates an early division of epithelial cells in the mammary gland of lactating rats. The hydrocarbon could exert this effect directly, or through a reduction in endogenous estradiol levels through enhancement of metabolism of the steroid.

Whatever the reason for the increase in receptors in the mammary gland, the result could be an increase in the extent of histone removal from chromatin since this is dependent upon the estrogen-receptor interaction (Hamilton et al., 1971). In this way an interaction between DMBA and DNA may be facilitated, and the carcinogenic actions of the hydrocarbon enhanced, for with the polycyclic hydrocarbons the carcinogenic effects may be dependent upon binding of "activated" hydrocarbon to DNA (Grover, 1973).
CHAPTER VI

GENERAL CONCLUSIONS

(1) The estrogen receptor

The 100,000 x g supernatant (cytosol) fraction isolated from uterus or mammary gland of the rat contains a number of molecules which bind estradiol. Many of these molecules bind the steroid with only low affinity ($K_s < 10^8 \text{ M}^{-1}$), but there is one macromolecule, the estrogen receptor which has an association constant in the order of $10^9$ to $10^{10} \text{ M}^{-1}$ for estrogen binding (Jensen & DeSombre, 1973; Keightley & Okey, 1973).

Some properties of the estrogen receptor may be studied by incubating different concentrations of the labeled steroid with fixed amounts of cytosol fraction and determining the proportion of label bound to the receptor at each concentration. Separation of bound and unbound hormone may be achieved by incubation with dextran-coated charcoal which acts as an adsorbant for the steroid (Binoux & Odell, 1973). Construction of a Scatchard plot (Scatchard, 1949) then permits calculation of both the intrinsic association constant for the estradiol-receptor interaction, and of the number of binding sites for the steroid. Using the above techniques it was found that an excess of unlabeled estradiol, when added to cytosol fraction equilibrated with $^3$H-estradiol-17β completely dislodges the bound label (Keightley & Okey, 1973). This implies reversible binding to the cytosol receptor. At physiological concentrations,
a second steroid hormone, dihydrotestosterone, does not compete with estradiol for binding to receptor sites (Keightley & Okey, 1973). This emphasizes the specificity of the receptor sites.

(2) DMBA and the estrogen receptor

DMBA is a potent inducer of mammary tumours in rats, but estrogen is required for this action (Dao, 1971). This implies that a functional estrogen receptor is also necessary for the action of DMBA, and the receptor has been hypothesized to play a dual role in DMBA tumourigenesis in rats.

Firstly, estrogen binds to the estrogen receptor in rats treated with DMBA. This has been shown in estrogen uptake experiments (Torgersen & Sander, 1970) and in in vitro experiments where the animals were treated with DMBA and at various times after this the mammary glands were tested in vitro for estrogen binding capacity (Chapter V). In fact, DMBA enhances estrogen binding capacity of the mammary glands of rats treated with the hydrocarbon. Thus the estrogen receptor of the cytosol fraction of rat mammary gland and uterus is capable of binding estradiol. Since estrogen is necessary for DMBA tumourigenesis, as has been shown by ovariectomy and replacement therapy experiments (Heimann et al., 1968; Welsch et al., 1968) it seems likely that the receptor is functional in transferring the estrogen to the nucleus where a derepression of the genome follows. In some tumours, however, nuclear uptake of estradiol appears to be reduced (Shyamala, 1972).

A per os administration of 20 mg DMBA to 50-day-old rats gives rise to levels of DMBA in the mammary gland in the order of $10^{-5}$ M (Flesher, 1967). Such levels in vitro were shown not to
compete with estradiol-17β for estrogen receptor sites (Keightley & Okey, 1973).

A second possible role of the estrogen receptor in the target tissue of DMBA-treated rats is in the binding of DMBA. It has been postulated that the similarity in structure between the hydrocarbon and steroid molecules could allow binding of the hydrocarbon to the estrogen receptor (Dao, 1971). In this manner the DMBA may be transferred to the nucleus where it interacts with the genome to induce tumour formation. However, this study has shown that $10^{-5}$M DMBA, the level found in the mammary gland of rats treated p.o. with 20 mg of the hydrocarbon (Flesher, 1967), does not compete with estradiol-17β for binding to the receptor sites in vitro (Keightley & Okey, 1973). Nor does estrogen priming alter uptake of DMBA into mammary gland in vivo (Torgersen & Sander, 1970). Thus DMBA does not appear to bind to the estrogen receptor in the rat mammary gland or uterus.

Further studies by Heizmann & Wyss (1972) have revealed that polycyclic hydrocarbons are bound to cytoplasmic macromolecules which are not the estrogen receptors. For example, 20-methylcholanthrene, a potent carcinogenic hydrocarbon binds to a cytoplasmic macromolecule which sediments at 8S in a sucrose density gradient, and which is not the estrogen receptor (Toft & Spelsberg, 1972). As yet it is unknown whether such binding is necessary for the action of the carcinogen in tumourigenesis, or whether the binding is only of low affinity, and non-specific. It could be fruitful to determine the nature of the cytoplasmic binding of polycyclic
hydrocarbons and their metabolites in that the discovery of an inhibitor of the binding of the hydrocarbon to the cytosol macromolecule could provide a means for inhibition of tumourigenesis by polycyclic hydrocarbons, assuming that such binding is an essential step in tumourigenesis.

This study also showed that DMBA administration reduces circulating estradiol levels. This finding was not unexpected since it is known that DMBA stimulates the cytochrome P-450 system and other steroid metabolizing enzyme systems of the rat liver (Lu et al., 1973). It is possible that a reduced level of estradiol in the tissues allows increased binding of labeled estradiol to the receptor in vitro because of reduced competition for the sites. But the change in endogenous estradiol levels had no effect on the number of binding sites measured in the uterus of control and DMBA-treated rats (Chapter V). Therefore it is unlikely that the DMBA-induced changes in endogenous estradiol levels can account for significant changes in the number of measurable binding sites.

Administration of 20 mg DMBA p.o. to 1 day post partum lactating rats increased the estrogen receptor levels in the mammary gland but not in the uterus (Chapter V). This effect cannot be accounted for by changes in endogenous estradiol levels. Most likely the increased number of estrogen receptor sites is indicative of increased parenchymal cell numbers in the mammary gland of the DMBA-treated rats. This may represent the initial stages of neoplasia following DMBA treatment.
(3) **Proposals on the mechanism of action of DMBA**

It seems clear that a functional estrogen receptor is necessary in target tissue cells for DMBA tumourigenesis. But the role of the receptor is to bind estradiol and not DMBA. This hydrocarbon, or the more active "K-region" epoxide may bind to other macromolecules in the cytoplasm and such binding may be necessary for the transfer of the hydrocarbon to the nucleus. It is in the nucleus where the hydrocarbon exerts its effects. Grover (1973) has suggested that the DMBA molecule or its epoxide intercalates into the DNA strand causing a frame-shift in read-out that could be transmitted to daughter cells. Alternatively the active metabolite of DMBA could itself cause a general derepression of the genome, perhaps through histone removal. In this way neoplasia could be stimulated.

Estradiol causes a derepression of the genome (Barker, 1970). Such a derepression could facilitate intercalation of the hydrocarbon molecule into the DNA strand. Also estradiol is known to stimulate mitosis (Kaye et al., 1972) and it could be that DMBA most readily interacts with DNA only at certain stages of the cell cycle. Hence estradiol treatment could increase the chance of a potentially carcinogenic interaction between the hydrocarbon and DNA.

That DMBA-induced tumours are specific for mammary tissue may be due to the presence in mammary tissue, and not in other estrogen target tissues, of enzyme systems capable of converting DMBA to an active epoxide. This theory of local metabolism of DMBA does have some experimental evidence to support it (Dao et al., 1968).
The studies reported above, then, have yielded some information on the actions of a carcinogenic polycyclic hydrocarbon at the cellular level. While the mechanism of action of the hydrocarbon remains unknown, a gradual accumulation of knowledge of the various interactions of the DMBA molecules within the cell brings us closer to an understanding of carcinogenesis.
CHAPTER VII

APPENDICES.

(1) Lowry technique for protein measurement

This procedure was adapted from that described by Lowry et al. (1951).

Solutions used:

solution A: \( \text{Na}_2\text{CO}_3 \) 20g; \( \text{NaOH} \) 4g make up in 1 litre of distilled water before adding 0.2g of sodium-potassium tartrate.

solution B: \( \text{CuSO}_4 \cdot 5\text{H}_2\text{O} \) (0.5% solution).

solution C: 50 ml solution A + 1 ml solution B

This was made up just prior to the determination.

Folin solution: 5 ml Folin C (Folin-Ciocalteu phenol reagent, 2N solution) + 7 ml distilled water.

Technique:

A 100 \( \mu \)l aliquant of the cytosol fraction (Tris buffer in the case of the blank) and 0.9 ml of the Tris buffer constituted the 1 ml sample. To this was added 5.0 ml solution C. After 10 min 0.5 ml of Folin solution was added and the mixture shaken immediately and well. After 30 min at room temperature the absorbance at 660 nm was measured.

A standard curve was established using bovine serum albumin solutions of various concentrations.

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(2) **Chemicals used and suppliers**

Albumin, bovine (Sigma*)

Charcoal; Norit A, pharmaceutical neutral (BDH, Toronto)

Dextran; M.Wt. 60,000 - 90,000 (ave. 86,900); clinical grade (Sigma)

Dihydrotestosterone; 5α-androstan-17β-ol-3-one (Sigma)

9,10-dimethyl-1,2-benzanthracene; 7,12-dimethylbenz(a)anthracene (Sigma)

1,4-dioxane; scintanalyzed (Fisher**)

EDTA; disodium ethylenediamine tetraacetate (Fisher)

Estradiol-17β; estra-1,3,5(10)-triene-3,17β-diol (Sigma)

6,7-3H-estradiol-17β; S.A. 40,040 mCi / mmole (Amersham/Searle***)

2,4,6,7-3H-estradiol-17β; S.A. 85,000 mCi / mmole (Amersham/Searle)

Ether; ethyl ether (Mallinckrodt, St. Louis, Missouri)

Ethylene glycol; (Fisher)

Folin C; Folin-Ciocalteau phenol reagent; 2N solution (Fisher)

Naphthalene; (Fisher)

POPOP; 1,4-bis-(5-phenyloxazol-2-yl) benzene (Amersham/Searle)

PPO; 2,5-diphenyloxazole-(Amersham/Searle)

Tris; Tris(hydroxymethyl)aminomethane; 0.1M solution (Fisher)

*Sigma Chemicals, St. Louis, Missouri

**Fisher Scientific Co., Don Mills, Ontario

***Amersham/Searle, Arlington Heights, Illinois
(3) **Purity check for labeled estradiol**

As soon as the labeled steroid was received it was checked for purity by thin layer chromatography using an Eastman Chromagram kit (#6078). Firstly the steroid (dissolved in benzene by the supplier) was evaporated to dryness under nitrogen and redissolved in 95% ethanol. A $10^{-3}$ dilution of the stock solution was used for chromatography.

The thin layer used was silica gel 100µ in thickness and supported on a 200µ thick polyethylene sheet with a polyvinyl alcohol binder. A known volume of diluted stock was spotted at the origin of the chromatogram and the sheet sealed in a glass container. It was allowed to run for 90 min using a mobile phase of chloroform : acetone (70 : 30). Unlabeled estradiol-17β was run as a check. At the end of the running time the gel was dried in air and the unlabeled estradiol-17β detected as a blue spot by means of a Na$_2$CO$_3$/ Folin C reagent spray (sprayed firstly with 20% Na$_2$CO$_3$ and then with 25% Folin C solution). The columns spotted with labeled steroid were then marked off in 6 equal sections from origin to front and the gel in each section scraped into a scintillation vial and counted in 10 ml Bray's solution.

It was found for both the 6,7-$^3$H-estradiol-17β and the 2,4,6,7-$^3$H-estradiol-17β that 95% of the activity was found in the section of the chromatogram adjacent to the spot of unlabeled estradiol-17β.
(4) Labeled estradiol-17β

Labeled estradiol-17β was obtained from Amersham/Searle (Arlington Heights, Illinois) at two specific activities.

(a) 6,7-³H-estradiol-17β

\[
\text{Batch 25 = 40,040 mCi / mmole}
\]

Thus 1 mCi / ml = 6.802 μg/ml = 24.97 μM \( E_2 = 2.22 \times 10^9 \) dpm

(b) 2,4,6,7-³H-estradiol-17β

\[
\text{Batch 17 = 85,000 mCi / mmole}
\]

Thus 1 mCi / ml = 3.165 μg/ml = 11.63 μM \( E_2 = 2.22 \times 10^9 \) dpm

(5) Bray's solution

This solution used for counting aqueous samples was adapted from Bray (1960).

PPO 4g
POPOP 0.2g
naphthalene 60g
methanol 100 ml
ethylene glycol 20 ml
1,4-dioxane to 1 litre
6. Least square regression line and standard error of the estimate

(From Wang 300 series program library) (CAL 360 - STAT - 4)

This program solves the equation of the regression line, which provides the "best fit" to a set of data points given by \((X_1, Y_1), (X_2, Y_2), \ldots, (X_n, Y_n)\). For a given value of \(X\) (say, \(X_1\)) there will be a difference between the value \(Y_1\) and the corresponding value as determined from the line \(L\). This difference is referred to as a deviation, or error.

\[
Y = a_0 + a_1X
\]

When the sum of the squares of the deviations is a minimum, we have obtained the line of best fit, called a least square line. This least square line of \(Y\) on \(X\) has the equation:

where the constants \(a_0\) and \(a_1\) are determined by solving, simultaneously, the normal equations for the least square line

\[
X = a_0N + a_1\Sigma X
\]

\[
XY = a_0X + a_1\Sigma X^2
\]

The constants \(a_0\) and \(a_1\) were determined in our program from the following formulae:
\[
a_1 = \frac{N \Sigma XY - (\Sigma X)(\Sigma Y)}{N \Sigma X^2 - (\Sigma X)^2}
\]
\[
a_0 = \frac{\Sigma Y - a_1 \Sigma X}{N}
\]

Once we have determined the constants \(a_0\) and \(a_1\), we have then obtained the least square regression line of \(Y\) on \(X\). Now, a measure of the scatter about the regression line is supplied by the quantity:

\[
S_{y.x} = \sqrt{\frac{\Sigma Y^2 - a_0 \Sigma Y - a_1 \Sigma XY}{N}}
\]

which is called the standard error of the estimate of \(Y\) on \(X\).

The standard error estimate has properties analogous to those of the standard deviation. For example, if we constructed lines parallel to the regression line of \(Y\) on \(X\) at respective vertical distances \(S_{y.x}, 2S_{y.x}, 3S_{y.x}\) from it, we should find, if \(N\) is large enough, that there would be included between these lines about 68%, 95%, and 99.7% of the sample points.


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