THE AUTORADIOGRAPHIC LOCALIZATION OF ESTROGEN BINDING SITES IN HUMAN MAMMARY LESIONS

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SHORT TITLE OF THESIS

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ABSTRACT

The biochemical assay of human mammary carcinomas for estrogen receptors is of proven clinical utility, but the cellular localization of estrogen binding sites within these lesions is less certain. This thesis describes the identification of estrogen binding sites as visualized by thaw-mount autoradiography after *in vitro* incubation in a series of 17 benign and 40 malignant human female mammary lesions. The results of the *in vitro* incubation method compared favorably with data from *in vivo* studies in mouse uterus, a well-characterized estrogen target organ. In noncancerous breast biopsies a variable proportion of epithelial cells contained specific estrogen binding sites. Histologically identifiable myoepithelial and stromal cells were, in general, unlabeled. In human mammary carcinomas, biochemically estrogen receptor-positive, labeled and unlabeled neoplastic epithelial cells were identified by autoradiography. Quantitative results from the autoradiographic method compared favorably with biochemical data.

RÉSUMÉ

Le dosage biochimique des récepteurs oestrogéniques dans les adénocarcinomes mammaires humains a un intérêt clinique bien établi, cependant la localisation cellulaire des sites de liaison aux oestrogènes dans ces tumeurs demeure incertaine. Les travaux décrits dans cette thèse ont permis d'identifier les sites de liaison de l'oestradiol au moyen de l'autoradiographie après incubation in vitro dans une série de 57 cas de lésions mammaires dont 17 étaient bénignes et 40 étaient des adénocarcinomes. Les résultats obtenus avec la méthode d'incubation in vitro se comparent favorablement avec ceux obtenus dans des études in vivo portant sur l'utérus de souris, un organe cible des oestrogènes bien caracterisé. Dans les lésions mammaires bénignes une proportion variable des cellules épithéliales contenaient des sites spécifiques de liaison oestrogénique. Les cellules myoépithéliales lorsqu'elles pouvaient être identifiées et les cellules stromales étaient en général négatives. Les adénocarcinomes mammaires avec un dosage de récepteur oestrogénique positif contenaient à la fois des cellules épithéliales marquées et des cellules négatives. Les résultats quantitatifs obtenus avec la méthode autoradiographique se comparent favorablement avec ceux obtenus avec les méthodes biochimiques.

PUBLICATIONS

Included as part of this thesis are the texts, appropriately modified, and figures of original papers published in or submitted to the following learned journals and book:

1. Buell RH, Tremblay G. Autoradiographic demonstration of uptake and retention of [3H]-estradiol after in vitro incubation. J Histochem Cytochem 1981; 29:1316-1321. (Chapter II of thesis)

2. Buell RH, Tremblay G. Autoradiographic demonstration of [3H]-estradiol incorporation in benign human mammary lesions. Am J Clin Pathol 1984; 81:30-34. (Chapter III of thesis)

3. Buell RH, Tremblay G. The localization of [3H]-estradiol in estrogen receptor-positive human mammary carcinoma as visualized by thaw-mount autoradiography. Cancer 1983; 51:1625-1630. (Chapter IV of thesis)

4. Buell RH, Tremblay G. The application of thaw-mount autoradiography for the localization of putative estrogen target cells in human mammary lesions. In: Pertschuk LP, Lee SH, eds. Morphologic Localization of Putative Steroid Receptors, vol. II. Boca Raton, CRC Press Inc. in press. (Chapters II-IV of thesis)

5. Buell RH, Tremblay G. Autoradiographic demonstration

of estrogen binding in human breast cancer after *in vitro* incubation. Cancer Res. submitted. (Chapter IV of thesis)

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DEDICATION

This thesis is dedicated with love to Angelika, Christina, and Thomas.

"...I have been actuated solely by the motives that guide all of us in the exercise of our profession-primarily, the interests of those who place themselves under our care, and secondarily, the progress and advancement of the healing art." -George Thomas Beatson (8)

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I. REVIEW OF THE LITERATURE AND INTRODUCTION

A. Historical background (56,57,59,103)

The rationale for the study of estrogen receptors (ER) in human mammary carcinoma has as its foundation the early clinical observations that some patients with advanced breast cancer would respond to hormonal manipulations while others would not. In 1896 Sir George Beatson (8) first reported that ovariectomy resulted in tumor regression in some young women with advanced breast cancer. Jensen (56) has pointed out that even prior to that study, Cooper, in 1836, had noted changes in tumor growth during different phases of the menstrual cycle. It was not until the early 1940's, however, when surgeons, encouraged by the results of orchiectomy in the treatment of prostatic cancer, utilized ovarian resection as a recognized therapeutic modality for premenopausal breast cancer patients (56).

In this same period radiotherapists began administering exogenous estrogens to patients with primary inoperable breast cancer. They believed that by stimulating the growth of the tumor, it would be more radiosensitive. They found, however, that some tumors had regressed with estrogen administration alone (1,11). It was subsequently determined that about one out of three postmenopausal patients responds to hormonal

therapy. In 1952 when it became feasible to remove the adrenal glands, Huggins and Bergenstal (52) reported that bilateral adrenalectomy in postmenopausal patients with advanced breast cancer resulted in regression of the tumor in some patients. Others obtained similar results with hypophysectomy (78,109).

Several conclusions were evident from these early clinical studies (56,57). It was apparent that the growth of some breast cancers was influenced by their hormonal milieu. Only a minority of patients with metastatic breast cancer, however, would respond to endocrine manipulations. For these, hormonal therapy, additive or ablative, provided the best form of treatment. It became obvious that there was a great need to identify which patients would be helped by such therapy and thus avoid unnecessary procedures such as adrenalectomy on the majority of patients where hormonal manipulations would be of no value. From clinical observations, the relative indications for each therapeutic modality were developed empirically. Bulbrook and his colleagues (5) attempted to develop a more objective means of predicting remissions by analyzing the urinary steroid hormone metabolites in patients with breast cancer. Their approach was, unfortunately, not sufficiently accurate to be of

clinical use (56).

In 1959 and 1960 a breakthrough in basic research provided a major impetus to the clinical investigation of hormonal responsiveness in breast cancer when it became possible to synthesize radiolabeled estrogens and to demonstrate the uptake and retention of these radiolabeled compounds in target tissues of experimental animals (41,60). Prior to this time, conventional analytical chemical techniques were unable to detect accurately the very low concentrations of steroids in the bloodstream and in target tissues (103). In target tissues specifically bound steroids are generally in the range of femtomoles per milligram of cytosol protein. (Indeed, as O'Malley and Schrader (103) have pointed out, were the human palate as sensitive to flavor as a target cell to steroid hormones, one would be able to taste a pinch of sugar in a swimming pool.) Once radioactive compounds were synthesized the door was open to investigate the biochemistry and physiology of steroids. Ultimately this knowledge was applied to the clinical investigation of hormone responsiveness of mammary carcinoma.

These early studies demonstrated that after <u>in vivo</u> injection of the radioactive compound, estrogen responsive tissues accumulated and retained radioactivity

after it had been cleared from nontarget organs (41,58,60). This observation suggested that target cells contained estrogen binding components. In 1961 Folca, Glascock and Irvine (39) gave [3H]-hexestrol to women with advanced breast cancer prior to adrenalectomy. After two hours, a greater amount of radioactivity was found in the tumors of four patients who responded to treatment than in tumors of six who did not respond. In 1965 and 1966 it was found that mammary tumors in experimental animals accumulated radioactive estrogens (58,67,97). At this time Jensen and his colleagues developed a means of examining the uptake of [3H]-estradiol with an <u>in vitro</u> incubation system (58). In 1966 they began the first investigation of human breast cancers.

In 1968 Gorski et al (43) and Jensen et al (62) reported their studies on the intracellular mechanism of action of estradiol. It was apparent that, in the absence of circulating estrogens, estradiol binds to a substance isolated from the cytosol (using standard homogenization techniques). After exposure to estrogens, this substance, the estrogen receptor, with bound estradiol was localized predominantly in the nucleus. Based on these observations, methods were devised to detect the cytosolic estrogen receptor in homogenates of

frozen breast cancer tissue avoiding the need for incubating fresh tissue slices. These techniques were adopted by various laboratories, and in 1974 the first meeting on estrogen receptors in human breast cancer was held under the sponsorship of the Breast Cancer Task Force of the National Cancer Institute of the United States. It was shown that biochemical assays of human breast carcinomas for estrogen receptors are of value in predicting the clinical response to endocrine manipulations (56).

In subsequent years the use of estrogen receptor assays to identify hormonally responsive tumors was adopted by most cancer treatment centers. It was apparent, however, that not all patients with estrogen receptor-positive tumors responded to hormonal manipulations (31,88,89,167). Horwitz et al (50) in 1975 suggested that the biochemical assay of breast tumors for progesterone receptors as well as estrogen receptors might provide a more accurate means of predicting response. This hypothesis was based on a knowledge that in experimental animals and MCF-7 cells the synthesis of progesterone receptors is dependent on an intact pathway for estrogen action (31,89). The presence of progesterone receptors in a tumor would therefore imply a functional estrogen-receptor complex. Research in this

field was greatly facilitated by the synthesis of the radioactive ligand, R 5020, a progestin which does not bind to nonspecific proteins such as corticosteroid binding globulin (125).

In 1979 a second conference was held at Bethaseda, Maryland under the auspices of the Breast Cancer Task Force and the National Cancer Institute. From data presented at that conference it was apparent that routine assay of breast cancer tissue for steroid hormone receptors had become widely adopted in most cancer institutions. The results presented confirmed the previous observation that not all patients with estrogen receptor-positive tumors responded to hormonal therapy. Moreover, a certain percentage of patients with estrogen and progesterone receptor-positive tumors did not respond (29).

B. <u>The Problem of Nonresponsive Estrogen Receptor-Positive Carcinoma</u> (77,79,88)

The problem of patients with estrogen receptor-positive tumors failing to regress after hormonal therapy has gained more attention from investigators in recent times. In those cases where a tumor is negative for hormonal receptors, the situation is relatively unambiguous, and many oncologists treat with chemotherapeutic agents since there is little chance

of obtaining a remission with hormonal therapy (less than 10%) (79). The situation is more uncertain for ER-positive tumors where only about 50% of patients respond (167). Even when the tumor contains both estrogen and progesterone receptors, about 20% do not respond as expected (167). The theoretical reasons for this failure of hormonal therapy are obscure. Several authors have discussed this problem in detail (77,79,88). In essence the explanations suggested can be summarized in four general categories:

1. A defect in the biochemical pathway of estrogen action after the initial necessary binding to the receptor:

Estrogen receptor-positive tumors may fail to respond to hormonal therapy because of a defect in the pathway of estrogen action. The binding of estradiol to its cytosolic receptor is only the first, albeit necessary, step for estrogen action. The lack of progesterone receptors in some estrogen receptor-positive tumors clearly suggest that there is a defect in some tumors (31).

2. The possible effects of hormones other than estrogen:

Other hormones, both steroidal and nonsteroidal, may also have an effect on breast cancer growth. Indeed, a

variety of hormonal receptors including those for progesterone, androgen, glucocorticoid (4), and prolactin (107) have been identified in human tumors. The role of these receptors in modulating tumor growth in humans is not fully established.

3. Possible methodologic or therapeutic problems:

The borderline between an ER-positive and -negative tumor may have been improperly determined (79). The use of quantitative rather than qualitative data seems to improve the ability to predict patient response. The likelihood of a response increases as the absolute ER level increases (29).

In assessing a response to hormonal therapy it has also been pointed out that the criteria are very rigid. The criteria for "objective" remission often require a decrease in lesion size of at least 50% and if one accepts Stoll's analysis of this criterion (139), it would imply that a remission required the killing of at least 99% of the tumor cells. He has questioned whether one could reasonably expect any better results than are presently obtained.

In addition to these problems it is possible that the therapeutic modalities used are inadequate for potentially responding tissue (77). For instance, it is known that visceral metastases (e.g. to the liver) often

do not respond well to hormonal therapy. The reasons for this failure are unknown since these metastases may be as likely to contain ER as elsewhere (76). The therapy itself may be inadequate (77). It is known, for instance, that 10-15% of patients who do not respond to prior oophorectomy do respond to adrenalectomy. If only oophorectomy had been done, they would be recorded as a "false-positive."

4. The possibility of tumor heterogeneity with subpopulations of estrogen receptor-positive and -negative tumor cells (77,79):

This reason is, perhaps, the most frequently proposed explanation for why estrogen receptor-positive tumors fail to respond to hormonal manipulations. It has been speculated by many authors that estrogen receptor-positive tumors actually are composed of both estrogen receptor-positive and -negative cells. There is some evidence that suggests this possibility. Much of it comes from assays of multiple biopsy specimens from individual patients. Rosen et al (129) noted discordant results in 24% of the cases he studied with either simultaneous biopsies of different metastases or in temporally different biopsies. Leung et al (76) noted in 12 of 43 patients where multiple biopsies were assayed there was a different result. Jensen et al (61), noted

differences in 4 of 214 patients, Kiang and Kennedy (66) in 3 of 15 (although two of these contained few viable tumor cells), and Webster et al (162) in 28% of multiple simultaneous biopsies. Allegra et al (2) have reported differences in the ER content in multiple or sequential biopsies from individual patients. In their study there were differing results in 15% of patients with multiple simultaneous biopsies. In patients receiving hormonal therapy the median ER level fell from 66 fmols/mg protein prior to therapy to 8 fmols. This change was interpreted to indicate that hormonal therapy selectively eliminated ER-positive cells (as expected) but ER-negative cells remained.

Additional information in this regard has been obtained from other biochemical studies. Børjesson and Sarfaty (12) studied the ER content of breast cancer cells from individual tumors which were separated into subpopulations by velocity centrifugation, buoyant density centrifugation and density gradient electrophoresis. From their results they concluded that breast cancers can be composed of a heterogeneous population of ER-positive and -negative cells with the large cells containing estrogen receptor.

From these observations it would be reasonable to conclude that one possible explanation for partial

response or early recurrence in estrogen receptor-positive cases is tumor cell heterogeneity. While some information supporting this hypothesis has been obtained from biochemical studies, considerable knowledge could be derived from studies employing morphological methods to identify ER in tissue sections.

C. Biochemical Aspects of the Estrogen Receptor System Relevant to Morphologic Localization (7,18,19,64,167)

As discussed by Baulieu (7), the estrogen receptor is the protein within the cell which is responsible "for the interpretation of the signal received (here the hormone) so that a cellular response is initiated." From biochemical and physiologic studies it is apparent that the functional receptor is present in appreciable amounts only in target tissues and demonstrates a high affinity for estradiol (Kd of the order of 0.1 nM) (167). Receptors are found in limited amounts within cells (around 10,000 per normal cell) and thus can be saturated with increasing amounts of hormone (167). Receptors are, for the most part, specific for a class of hormones, and, thus, estradiol binding to ER can be inhibited by hormones such as diethylstilbesterol but not progesterone. In classic theory (43,62) the free (unliganded) receptor is cytoplasmic and, when bound to estradiol, undergoes a temperature dependent activation

(transformation) and translocates to the nucleus. There the activated estrogen-receptor complex binds to nuclear "acceptor" sites producing an effect characteristic of the tissue such as synthesis of progesterone receptors in the uterus (167).

It should be noted that recent autoradiographic (84,133), immunohistochemical (68,118) and biochemical data (164) have suggested that the majority of ER, liganded and unliganded, are nuclear in location. The biochemical implications of these hypotheses may be more "cosmetic than substantive" (131). If these hypotheses are substantiated, however, the interpretation of histochemical and immunohistochemical methods localizing putative ER predominantly in the cytoplasm will require reexamination.

Of the characteristics of the estrogen receptor system noted above, Morrow et al (98) have pointed out that in routine biochemical studies a major criterion for establishing the presence of ER in a tissue is the demonstration of high affinity binding. This affinity is estimated by Scatchard analysis of data from saturation binding studies (167). One cannot readily assess the binding affinity by most morphologic methods, however, and must rely on other properties such as steroid specificity and appropriate tissue distribution.

In both biochemical and morphologic methods of assaying for ER it is well known that estradiol can bind to nonspecific sites, i.e. for purposes of this discussion non-receptor sites. In most instances such sites can be identified by the inability of appropriate radioinert ligands to displace the radioactive ligand from these binding sites. In the case of type II sites, however, this property is not true.

Type II binding sites, first described by Clark and his coworkers, have been identified in both the cytoplasm and nucleus of the rat uterus and other tissues including human mammary carcinomas (21,32,33,81,151). This class of binding sites is distinct from the estrogen receptor (type I sites). The cytoplasmic and nuclear forms appear to be unrelated. Unlike other nonspecific binding sites (low affinity type III sites), these sites can be saturated, and binding to these sites can be inhibited by estradiol and diethylstilbesterol. In the cytoplasm these sites have been detected by saturation analyses for ER by including relatively high concentrations of [3H]-estradiol from 20 nM to 80 nM (21). They have been found to sediment as a 4S peak in postlabeled sucrose density gradient analysis while nontransformed ER sediment as an 8S peak (21). These type II sites are distinct from the estrogen receptor in that they have a

lower estimated affinity for estradiol (30 nM versus 0.8 nM for ER in the rat uterus), are present in a fourfold greater amount, and are not depleted from the cytoplasm when the tissue is exposed to estradiol (in contrast to the estrogen receptor) (21). Cytoplasmic type II sites are found predominantly in target tissues but are present in lesser amounts in nontarget tissues (21).

Nuclear type II sites (32,33,81,151), which appear to be unrelated to the cytoplasmic form, have an approximate Kd of 16-20 nM in rat uterine tissue (32) and 4 nM in human tumors (151). They are rapidly increased in amounts by estradiol administration to mature ovariectomized rats and have a higher binding capacity than type I sites (estrogen receptor). Binding to these sites, like the cytoplasmic sites, can be inhibited by estradiol and diethylstilbesterol. These sites appear to be tissue specific and are found in appreciably lesser amounts in nontarget tissues such as spleen (32).

The physiologic significance of cytoplasmic type II binding sites is unknown although they may aid in concentrating estrogen in target tissues such as uterus (32). On the other hand, the levels of nuclear type II sites can be correlated with long term uterotrophic response to estrogenic hormones (32,81). Antagonism of uterotrophic response to estrogens is associated with a

decrease in measurable levels of nuclear type II sites. More recently an endogenous inhibitor of [3H]-estradiol binding to nuclear type II sites has been described (80). Although the exact nature and significance of this substance remains to be clarified, it has been speculated that it may modulate estrogen induced DNA synthesis by inhibiting estrogen stimulation of nuclear type II sites. In human breast carcinomas, the presence of nuclear type II sites has been shown to be correlated strongly with the presence of progesterone receptors (151).

In essence, therefore, type II sites do share some features of the estrogen receptor such as relatively high affinity, limited capacity and steroid specificity. There is much evidence to suggest that nuclear type II sites have an important physiologic function, but the true estrogen receptor (the type I site) is essential for estrogen action. As pointed out by Chamness et al (19), however, knowledge of type II estrogen binding is necessary for the proper understanding and intrepretation of morphologic methods of localizing estrogen receptors.

D. Immunohistochemical and Fluorescent Cytochemical Methods for Localizing Estrogen Receptors (19,100,114,157)

Autoradiographic methods were the first techniques to be applied extensively for the localization of

estrogen binding sites in experimental animals. This avenue of investigation has largely been overlooked by those studying estrogen receptors in human breast cancer. It its place, efforts have been directed toward developing a relatively facile and inexpensive technique which could be applied to the routine study of ER in breast cancer in most clinical pathology laboratories. Since 1976 when the first immunofluorescent methods were described (101,112) until the present a variety of techniques have been developed and correlations with biochemical assay reported (6,10,24,26,34, 36,46,54,68-70,73-75,90,91,99,101,102,105,111-118,120,121, 152,159-161). To date, however, none of these methods have been validated (29) and, therefore, their results must be interpreted with caution. Moreover, in the recent literature the accuracy of these methods for localizing estrogen receptors, as opposed to other estrogen binding sites, has been questioned (18,19,86,98,146,157).

The morphologic methods for investigating ER (excluding autoradiography) may be divided into two general groups: immunohistochemical and fluorescent cytochemical (19,114,157). In general the accuracy of these methods can be assessed with a knowledge of 1) the affinity of the ligand for ER as determined biochemically, 2) the ability of appropriate steroids

(e.g. DES) to suppress the observed staining while others (e.g. progesterone) are without effect, 3) the tissue distribution observed with the method in biochemically characterized experimental animals, 4) the concentration of ligand necessary to visualize receptor (i.e. is it high enough to bind to type II sites?), 5) the lack of nonspecific binding to other substances, and 6) the correlation with biochemical assays and, ultimately, with biologic behavior in the case of tumors.

Of the immunohistochemical methods, the first to be described were those of Pertschuk and coworkers (112,115) and Nenci et al (101). The former technique utilizes polyestradiol phosphate as a ligand which is localized with immunofluorescent methods using anti-estradiol antibody as the primary antiserum. The method was found to provide almost 90% correlation with biochemical assay in initial studies (115). Taylor et al (152) more recently used a modified technique with paraffin sections and found it to be quite promising although they obtained only 60% correlation with biochemical assays. Other investigators examining this method have raised several serious questions concerning its validity (86,98). In particular, the ligand used in this assay is a polymerized estradiol, polyestradiol phosphate, which contains phosphate groups in the 3 and 17 position of the

estradiol molecule. It is known that substitution in these positions of estradiol reduces the affinity of estradiol for the receptor (45). Morrow et al (98) studied the relative binding affinity of polyestradiol phosphate for ER and concluded it was unable to compete with [3H]-estradiol for the receptor. McCarty et al (86) found a relative binding affinity of less than 0.001 (estradiol=1.00). Studies of the tissue distribution of staining by this method were done by Morrow et al (98) who observed staining in nuclei of muscle in the rat diaphragm, a nontarget organ, and dextran-coated charcoal assay of the diaphragm confirmed the absence of receptor. They (98) and McCarty et al (86) concluded that this technique was unacceptable. Taylor et al (152) felt the method was promising. However, these investigators used paraffin embedded tissue, and it is known that the estrogen receptor is extremely temperature sensitive. It would seem unlikely that during impregnation and embedding of the tissue the estrogen receptor would retain its ability to bind estrogens if indeed any ER survived the fixation. Both Pertschuk et al (115) and Taylor et al (152) obtained varying degrees of correlation with biochemical assays, but Morrow et al (98) and McCarty et al (86) were unable to obtain acceptable agreement. For these reasons, especially that

polyestradiol phosphate apparently does not bind appreciably to ER, it is difficult to place much credence on the results of investigations using this method. In fact, Pertschuk et al have apparently discontinued its use in favor of a histochemical method (113).

Other immunohistochemical techniques have been described (69,70,91,99,101,161). These methods utilize estradiol as the ligand, and thus the affinity of the probe for ER is established. Many were able to suppress the staining observed by using DES, nafoxidine, and/or tamoxifen and could demonstrate appropriate tissue distribution. However, as pointed out by Chamness et al (19), all used relatively high concentrations of estradiol. These authors have suggested that the staining observed may be due primarily to cytoplasmic type II binding of estradiol. It had been known, and was emphasized by Chamness et al (19) and Morrow et al (98), that in <u>in vitro</u> systems it is very unlikely that an anti-estradiol antibody (which distinguishes the 3 and 17 position of estradiol) can bind to estradiol already bound to receptor (which also distinguishes the 3 and 17 position). This concept has been confirmed in studies by Castañeda and Liao (17) and Fishman et al (38). It would seem more likely, therefore, that the staining observed by these methods which can be inhibited represents type

II sites, a possibility suggested by Mercer et al (91) from their studies. While some of these methods do correlate with biochemical assays, this relation may simply reflect a correlation of type II sites with ER as suggested by Chamness et al (19).

More recently immunohistochemical methods utilizing anti-receptor antibody have been developed (68,118,120). These methods avoid many of the problems discussed above. In general, their validity depends in great measure on the specificity and sensitivity of the primary antisera used. The methods require further investigation and confirmation before any conclusions can be drawn, but they are without a doubt promising and probably represent the direction of future research.

The second general group of methods are those which utilize a fluorescent probe, either estradiol attached to fluoresceinated bovine serum albumin (BSA) or estradiol linked to fluorescein isothiocyanate (for review see 114). Here, as with the immunohistochemical methods, each can be assessed using the same general criteria. As was the case with polyestradiol phosphate, however, a primary concern is a knowledge of the relative affinity of the ligand for the estrogen receptor. The affinity of steroid-BSA conjugates for receptors has been studied by Rao et al (124). These workers found that the affinity

depends on the steroid:albumin ratio and the site on the steroid ring used for conjugation. In their studies they used a ligand similar to that developed by Lee but with a steroid:albumin ratio of 8:1 (rather than the 24:1 for Lee's probe (73)). While that ligand did show an affinity for ER, whether the same would be true for other ligands is not established. McCarty et al (86), using a ligand similar to Lee's, demonstrated a low relative affinity but the steroid:albumin ratio was not stated. Pertschuk et al later described a probe utilizing estradiol linked to BSA via a hemisuccinate bridge in the 17 position and a steroid:albumin ratio of 4:1 (113). Others have utilized estradiol linked to fluorescein isothiocyanate (for reviews see 19,114,157). The affinities of the fluoresceinated ligands have also been studied and have been reported to be in the micromolar to nanomolar range (19,25,27,86). Chamness et al (19) have pointed out, however, that in some preparations there may be sufficient free estradiol present to account for the competition of [3H]-estradiol binding observed in such studies. It would appear, therefore, that the true affinities remain to be established for many fluorescent ligands. In considering the other general criteria for these methods most investigators were able to suppress fluorescence by exposure to inhibitors. Pertschuk et al

have been able to demonstrate apparent competitive inhibition (114). Some have demonstrated appropriate tissue distribution. The concentration of ligand used in almost all methods is relatively high, however, and according to Chamness et al (19) would be sufficiently high to detect type II binding. Attempts to correlate results with biochemical assays have been made with Lee and Pertschuk examining the largest numbers of cases. Lee has been unable to obtain correlation with biochemical assay (73). Pertschuk et al, on the other hand, have obtained a 91% correlation with biochemical assay (113) and, moreover, have demonstrated a reasonable correlation with biologic behavior (116).

Thus while several relatively inexpensive and uncomplicated methods exist for localizing estrogen binding sites in tissue sections it is clear from the preceeding discussion that the nature of the binding sites is not established. Moreover, it is not known if any of the existing methods, fluorescent or immunohistochemical, are sufficiently sensitive to detect the limited number of receptors present in target cells (18,19,86,157). It should also be noted that in many instances the methods detect mainly cytoplasmic rather than nuclear binding sites. In view of recent proposals that ER are predominantly nuclear (68,84,118,133,164),

caution must be exercised in interpreting data from these methods. If the new hypotheses prove to be correct, those methods demonstrating cytoplasmic staining would not be accurately localizing ER. This would not, however, detract from their utility as a clinical tool if they correlate well with biologic behavior of mammary carcinoma.

E. Autoradiographic Techniques for Localizing [3H]-Estradiol

As a practical technique, microscopic autoradiography was first introduced about 30 years ago with the development of methods for preparing histologic sections of tissues overlaid by photographic emulsion (145). During the ensuing years it was evident that the methods were, in many instances, accurate and sensitive. In the majority of these experiments substances such as [3H]-thymidine were used which became incorporated into tissues and were resistant to leaching after appropriate fixation and preparation. Diffusible molecules such as steroids, however, are not covalently bound to substances within the tissues and, as a result, are susceptible to translocation or loss during routine autoradiographic procedures (144,148).

Attempts to identify steroids in tissue sections by autoradiography were being made as early as 1951 when

Leblond described the localization of an impure [14C]-progesterone preparation in the endometrium (71). In 1960 de Paepe (28) studied the distribution of [3H]-estradiol in the uterus by using 20 micrometer frozen sections of tissue previously fixed in 10% formaldehyde, washed in water, and overlaid with photographic emulsion. He reported that the grains observed appeared over endometrial cells and in the lumina adjacent to the apical regions of the cells. In 1963 Mobbs (96) attempted to identify [3H]-estradiol in the rat uterus after <u>in vitro</u> incubation at 37°C for one hour in Medium 199 containing 0.12 micromolar estradiol. The tissue was then fixed in 10% formol saline and embedded in paraffin. Six micrometer sections were prepared and overlaid with Kodak AR 10 stripping film. The distribution of grains was somewhat variable with occasional relatively heavy labeling of the luminal epithelium. At the cellular level the grains appeared predominantly cytoplasmic. He did acknowledge, however, that radioactivity may have been lost during histologic processing. In 1963 Ullberg and Bengtsson (156) reported on their whole animal and specific organ autoradiographic study of the distribution of [3H]-estradiol. In these experiments tissues were frozen, and freeze-dried sections were apposed against stripping film. They found

localization of grains over cells of the endometrial glands with the nuclei containing more radioactivity. In 1965 Michael (94) used frozen sections 5-7 micrometers to identify [3H]-hexestrol at the apical border of uterine glands four hours after injection. In the same year Inman et al (53) reported their results of a study of the autoradiographic localization of [3H]-estradiol in rat uterus. Three different embedding methods were used with tissue fixed in either 6.5% glutaraldehyde or 1% osmium tetroxide. The sections were dipped in liquid emulsion. They found grains at the apical and basal regions of epithelial cells. There was labeling of white blood cells and strong labeling of red blood cells with one of the methods. Uriel et al (158) have also described a method for localizing [3H]-estradiol where paraffin embedded tissue is exposed to the radiolabeled ligand. This method reveals grains over endometrial stromal cells, but other target cells such as endometrial glands and myometrium were unlabeled.

As knowledge of the biochemistry of the estrogen receptor system evolved, it became apparent that, in many instances, these techniques produced various artifacts, especially dislocation or loss of the radioactive ligand. In cell fractionation studies investigators had identified [3H]-estradiol in the nuclear fraction (62)

after injection while in some of the autoradiographic studies described above it was localized over the cytoplasm. Stumpf and Roth (147) in 1966, however, published the first detailed study of methods to localize [3H]-estradiol in tissue sections. These authors investigated the results of six different techniques of localizing two diffusible compounds [3H]-estradiol and [3H]-mesobilirubinogen. Of the various methods, the first two described, dry- and thaw-mount autoradiography were judged to be the most accurate and subsequently have been widely adopted. Unlike most previous methods these techniques avoid the steps in processing that are responsible for potential artifacts. Thus they avoid tissue fixation, dehydration, embedding and exposure to liquid photographic emulsion. In general, previous studies included some or all of these steps. The dry-mount and thaw-mount methods, moreover, demonstrated a nuclear localization of [3H]-estradiol in tissue sections consistent with biochemical studies. This agreement provided evidence supporting the superiority of these methods over the other autoradiographic techniques, and, as noted by Stumpf (145), in recent years the technique of thaw-mount autoradiography has been widely used by many investigators.

F. Introduction to the Experimental Section

As discussed above, the biochemical assay of human mammary carcinomas for estrogen receptors is of proven clinical utility in predicting patient response to hormonal therapy (29). Less is known, however, about the distribution of specific estrogen binding sites in human mammary lesions, both benign and malignant. It is also not entirely clear why almost 50% of estrogen receptor-positive mammary carcinomas do not respond favorably to endocrine manipulations. One possible reason suggested is that ER-positive mammary carcinomas may be composed of subpopulations of estrogen receptor-positive and -negative cells. Valid morphologic methods would be necessary to investigate these aspects of the pathology of the human breast.

Although immunohistochemical and histochemical methods have been developed for identifying specific estrogen binding sites, the accuracy of these techniques for demonstrating ER have not yet been completely validated (29). Autoradiography is another means of identifying estrogen binding sites in human breast lesions. The technique of thaw-mount autoradiography is established in its ability to localize accurately bound radioactive steroids in tissue sections but has been overlooked in the investigation of human mammary lesions. An autoradiographic investigation of human tissue

requires *in vitro* incubation, however, and the incubation method may have associated problems (143). It is necessary, therefore, to determine the accuracy of the incubation method.

The purpose of these studies is to investigate the distribution of estrogen binding sites in human mammary lesions using autoradiography rather than other more commonly employed methods. In Chapter II the accuracy of the *in vitro* incubation method is assessed using for study the mouse uterus, a well-characterized target organ. In Chapter III the distribution of estrogen binding sites in benign human mammary lesions is described. In Chapter IV the findings in a series of human breast carcinomas are presented, and the results of the autoradiographic method are correlated with data from biochemical assays.

EXPERIMENTAL SECTION

II. THE AUTORADIOGRAPHIC DEMONSTRATION OF UPTAKE AND RETENTION OF [3H]-ESTRADIOL IN MOUSE UTERUS AFTER IN VITRO INCUBATION

A. Introduction

As has been discussed in the preceeding chapter a variety of methods have been described for the localization of estrogen receptors in target cells. While the immunohistochemical and histochemical techniques have been popularized, their validity has been questioned, and relatively little work has been done using autoradiographic techniques for the examination of human breast tissue. Autoradiography has been used extensively, however, for the study of estrogen target cells in animal tissues, but in the majority of instances the ligand was injected (for review see ref. 149). Such *in vivo* methods would not be feasible for an investigation of human tissue where an accurate *in vitro* incubation method would be required. There are several reports demonstrating steroids in tissues using *in vitro* incubation and autoradiography (13,22,23,62,84,132,133, 138,143,153,154,163). Some of these describe results obtained with methods other than thaw- or dry-mount autoradiography, and the accuracy of the data must be questioned. Moreover, since *in vitro* methods for localizing [3H]-estradiol do have associated problems (143), prior to the study of an unknown tissue, the accuracy of the incubation method must be examined in a well-characterized target organ.

With the intention of using a thaw-mount autoradiographic technique for demonstrating estradiol target cells in human mammary lesions, it was decided, therefore, to investigate first the accuracy and limitations of an *in vitro* incubation method in the uterus of experimental animals where the biochemical properties are well studied and the distribution of target cells has been described. The results from *in vivo* injection studies could also be compared with data from *in vitro* incubation experiments.

B. Materials and Methods

Tissue: Female Balb/c mice, 7-8 weeks of age, were used for these experiments. For the in vivo studies and for a few of the initial in vitro experiments the mice were obtained from a breeding colony maintained by Dr. G. Shyamala of the Lady Davis Insitute for Medical Research of the Sir Mortimer B. Davis-Jewish General Hospital. In all subsequent experiments the mice were purchased (Canadian Breeding Farm, St. Constant, Quebec). The animals were kept under routine conditions in the animal facilities at the Lyman Duff Medical Sciences Building and were given food and water ad libitum. On the day of the experiment the mice were sacrificed in the morning by cervical dislocation. The uterus was removed as soon as possible via a midline abdominal incision and stripped of fat and connective tissues. The lower portion of the uterus and cervix were removed. Each uterine horn was placed on dental wax with several drops of medium to avoid drying and diced into sections approximately 2 mm in length. For in vivo studies the slices were frozen and processed as described below. For in vitro studies the slices were placed in the appropriate medium. This entire procedure required about 10 minutes.

In Vivo Injections: All in vivo injection studies were done in collaboration with Dr. G. Shyamala and her

coworkers who performed the injections. For the injection, an ethanolic stock solution of [3H]-estradiol (S.A. 90-115 Ci/mM, New England Nuclear, Lachine, Quebec) was diluted with normal saline and a dose of 0.1 micrograms per 20 gram body weight was given subcutaneously. Two hours after the injection the tissue was removed as described and frozen.

In Vitro Incubations: For each experiment, a series of simultaneous incubations was conducted using one or two pieces of tissue. The tissue was placed in Medium 199 (Gibco, Grand Island, N.Y.) at 37°C and equilibrated with 95% oxygen and 5% carbon dioxide at pH 7.3. To assess the total amount of [3H]-estradiol uptake, the tissue was first incubated for one-half hour in 10 ml of medium only. It was then transferred to 10 ml of medium containing 5 nM [2,4,6,7]-[3H]-estradiol (S.A. 90 or 102 Ci/mM, New England Nuclear, Lachine, Quebec). The [3H]-estradiol was purified by thin layer chromatography prior to use. To determine the amount of nonspecifically bound [3H]-estradiol, sections were preincubated for one-half hour in medium containing 500 nM nonradioactive estradiol or diethylstilbesterol (DES) (Sigma Chemical Co., St. Louis, Mo.) to obtain an effective block of ER (65). The sections were then transferred to 10 ml of medium containing 5 nM [3H]-estradiol plus 500 nM

unlabeled steroid. In several cases, to assess binding specificity, additional sections were preincubated and coincubated as above with 500 nM radioinert progesterone or hydrocortisone (Sigma Chemical Co., St. Louis, Mo.). In all cases, after one-half hour incubation in [3H]-estradiol with or without excess unlabeled steroid, the tissue was transferred to 10 ml of medium containing 3.5 gm/100 ml bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) and incubated for two hours with shaking but without the oxygen-carbon dioxide gas. The incubations were carried out in a Dubnoff Metabolic Shaking Incubator (Precision Scientific, Chicago, Ill.) at 37°C. In several cases tissue was incubated as above omitting all steroids as a control for the mounting procedure. In other instances pieces of diaphragm were included in the incubations for comparison. After the incubation, the tissue was briefly blotted with tissue paper, frozen, and stored at -76°C until use.

Tissue Freezing and Storage: To freeze the tissue, a small amount of OCT compound (Lab-Tek Products, Naperville, Ill.) was placed on the specimen holder of the cryostat (Harris-Cryostat Model CTD, International Equipment Co., Needham Heights, Mass.), and the bottom surface of the compound was allowed to freeze while the rest remained liquid. The specimen was then placed in

the liquid compound and oriented to allow for cutting cross sections of the uterine horn. The specimen was then frozen using liquid Freon 12 (Dupont, Canada). This procedure was accomplished by adjusting the flow rate from the Freon storage tank in such a way that a continuous stream of liquid drops resulted rather than a fine spray. The frozen tissue was removed from the specimen holder and placed in either plastic or glass specimen vials. The vials had been precooled by placing them in the cryostat at -25°C for about one hour prior to use. The tissue was stored at -76° C until use.

Dip-coating Procedure: The darkroom used for dip-coating was equipped with a Kodak safelight (Wratten Series OA 2) placed on the ceiling (about 6 feet from slides and emulsion). The darkroom was not temperature or humidity controlled, but for dip-coating a relative humidity of about 75% was maintained with a portable humidifier. Microscopic slides (Fisher Scientific Ltd., Montreal, Quebec) were cleaned in absolute alcohol, dried, and placed in black slide boxes. On the day of dip-coating, the photographic emulsion, Kodak NTB 2 (Eastman Kodak, Montreal, Quebec) was removed from storage in the refrigerator and allowed to come to room temperature. It was then transferred to the darkroom to a waterbath which had previously been heated to 40-45°C.

The emulsion was allowed to liquify for 30 minutes to 1 hour in the dark. At this time a cylindrical flask, also in the waterbath, was filled with liquid emulsion and allowed to remain for around one-half hour while any air bubbles escaped. At this time the safelight was turned on, and individual slides were dipped vertically into the liquid emulsion. They remained in the emulsion for about 2 seconds and were removed and placed in a vertical position on slide racks. The racks had wet tissue paper at the base to absorb excess emulsion. The slides were allowed to dry with the safelight off for 2 to 4 hours and were then placed in black microscope slide boxes containing Drierite (W.A. Hammond Drierite Co., Xenia, Ohio) wrapped in gauze. The slide boxes were sealed with black tape and placed in the refrigerator at about 4°C for storage.

Preparation of Frozen Sections: The frozen tissue was transferred to the cryostat and secured on a specimen holder using a small amount of liquid OCT compound which was allowed to freeze. Care was taken to prevent any of the liquid compound from touching the tissue (which was encased in frozen compound). The specimen holder was secured to the microtome tightly, and the cryostat used was adjusted at -25° C and set to cut 4 micron sections. The thickness of the sections was not actually

determined.

To produce sections, the block was first faced down to give a complete section of the tissue. Effort was made, however, to avoid cutting deeply into the block. Sections were cut with the cryostat top closed. The microtome knife and roll plate surfaces were briefly cooled prior to sectioning with several drops of liquid nitrogen or Freon 12. The sections were cut and allowed to remain on the blade until several had been cut. An effort was made to utilize only serial sections in order to obtain some degree of uniformity of section thickness.

Dry-mount Autoradiographic Procedure: The dry-mount autoradiographic procedure was similar to that described by Stumpf and colleagues (147,148). When several frozen sections had been cut, the cryostat cover was opened, and the tissue sections were transferred to the wells of a Lab-Tek tissue culture chamber/slide (Canlab, Montreal, Quebec) by sliding them off the knife into the chambers with a camel hair brush. The culture chamber and brush had been previously cooled and were dipped in liquid nitrogen prior to use.

When a sufficient number of sections had been cut, the plastic top of the slide tissue culture chamber, which had been previously perforated and dipped in liquid nitrogen, was put in place. By using metal forceps,

dipped in liquid nitrogen, the covered slide with sections was transferred to the precooled specimen chamber of the Cryopump (see below) for freeze-drying. If obvious thawing occurred during the procedure, the tissue became adherent to the plastic chamber, and those sections in that slide were not used.

The apparatus used for freeze-drying was the Cryopump designed by W.E. Stumpf and purchased from Thermovac Industries Corp., Copiague, N.Y. (148). It consists of a sample chamber and a compartment for the molecular sieve. The procedure used was essentially as described by Stumpf and Sar (148) for freeze-drying tissue outside the cryostat which was not sufficiently large to hold the assembled apparatus. The tissue sections were allowed to freeze-dry in this apparatus for 12 to 16 hours. The apparatus was then connected to a tank of extra dry nitrogen gas and the vacuum was broken. The specimen holders were removed and placed in a dessicator until mounting. The dry-mounting was done in the darkroom essentially as described (148).

Thaw-mount Autoradiographic Technique: This procedure was used for all *in vitro* experiments and is similar to the method described by Stumpf and Roth (147). It was performed with the cryostat in the darkroom with the safelight on. With the aid of a high-intensity light

(Tensor Corp., Brooklyn, N.Y.), frozen sections were cut as previously described. The high-intensity light was turned off. With the safelight on, a dip-coated slide was removed from the box and gently pressed against the microtome knife where the sections were lying. Care was taken to avoid any movement of the slide while it was in contact with the sections. The slide was then examined to ensure that the sections had been mounted and returned to a storage box. The microtome knife was then carefully cleaned with cold Kleenex tissue paper kept in the cryostat, and the procedure repeated. After the appropriate number of sections had been cut, generally five slides for each block, the slide box was sealed and stored at -15°C for exposure. The exposure times for mouse uterus after <u>in vitro</u> incubation varied from approximately one to two months.

<u>Photographic Processing and Histologic Staining:</u> Prior to development, the slide box was allowed to come to room temperature. The chemicals for developing were all purchased at local Montreal camera stores and were Kodak D-19 developer and Kodafix. The developer was weighed out into 40 gram aliquots and stored in plastic covered containers. For developing, one 40 gram quantity was diluted with 254 milliliters of distilled water and stirred until completely dissolved. The stock solution

of Kodafix was diluted one part with three parts distilled water to make 200 milliliters. The reagents were then placed in the darkroom in glass containers and allowed to reach temperature equilibration, 18 to 20°C. All experimental and control slides for a given experiment and exposure time were developed simultaneously. In the darkroom, with the safelight on, the appropriate slides were removed and placed in a glass slide rack. For dry-mounted slides the tissue was first breathed on several times to improve adherence of the sections to the emulsion. This procedure was not always successful, and often sections were lost during development. The slides were immersed in developer for one minute with occasional agitation. They were then transferred to distilled water for 10 seconds with agitation and placed in the Kodafix solution for five minutes with slight initial agitation. After the development was completed, the slides were transferred to distilled water (mouse uterus or breast) or phosphate buffered saline (human tissue) at room temperature and washed with three 15-minute changes. After washing, the wet emulsion was removed from the back of the slides with a razor blade and wet Kleenex tissue. The slides were placed in slide racks to air dry. After drying, sections of mouse tissue were stained with methyl green pyronin.

Human tissues were fixed for one minute in 1% paraformaldehyde (15) and stained with hematoxylin and eosin. The fixation seemed to improve staining but did not appear to alter the localization of the grains. All slides were mounted with Permount.

Quantification of Results: For the quantitative analysis of selected results from mouse uterine tissue, a method similar to that employed by Bergeron et al (9) was used. The number of grains was counted in frames overlying only endometrial glandular cells in selected areas. Frames overlying both glandular cells and lumen or interstitial space were excluded. The areas selected for quantitation were judged to be representative and included the regions illustrated and other areas on the same slides. The counting was done using a 9.5 X 9.5 micron frame. Statistical analyses of the results were done using a Student t-test.

C. Results

The data from the initial studies using dry-mount or thaw-mount autoradiography after in vivo injection of [3H]-estradiol agreed with the results of Stumpf and Sar (149). For the mouse uterus, the appearances of the autoradiographs were similar to those after in vitro incubation and will be described below. In one instance the lactating breast of the mouse was examined after in vivo injection. The results were as described by Sar and Stumpf (130). Uptake was identified in some but not all alveolar cells (fig. II-1) and in some connective tissue cells (not illustrated).

In sections of mouse uterus incubated only with [3H]-estradiol (fig. II-2A) silver grains were localized predominantly over presumptive target cells. On closer examination these grains appeared mainly over the nuclear region. Some cytoplasmic labeling was evident, but represented a relatively minor contribution to the total. In the interstitial spaces and glandular lumina, a few randomly scattered silver grains were present.

In sections incubated with unlabeled estradiol (fig. II-2B) or DES (fig. II-2C) the total number of grains was greatly reduced. On higher magnification no nuclear grains were identified in many cells, while in others only a few grains were seen. Some cytoplasmic grains

remained, and in the interstitial areas and glandular lumina the scant labeling was relatively unaffected. In sections coincubated with progesterone (fig. II-2D) or hydrocortisone, the distribution of the silver grains was not noticeably different from that seen in incubation with [3H]-estradiol only. A quantitative comparison of these sections is given in Table II-1 where it can be seen that the difference in average number of grains per frame between the section incubated with only [3H]-estradiol and those with excess unlabeled estradiol or DES is significant. Progesterone appears to have little effect on the labeling present. In sections of diaphragm incubated with [3H]-estradiol only, scattered grains were observed when compared to uterus, and sections of uterus incubated with medium only were essentially devoid of grains (fig. II-3).

Examination of the individual cells in the uterus revealed the presence of labeling in most but not all cell types (fig. II-4). The surface luminal epithelium, glandular epithelium, and stromal cells of the endometrium almost always contained radioactivity. Blood vessel endothelium was negative, while muscle of the arteries was either negative or equivocal. In the myometrium, the smooth muscle cells displayed a fairly uniform distribution of grains throughout the thickness.

In evaluating the overall distribution of labeling seen in sections incubated with [3H]-estradiol with or without excess unlabeled steroid there was some variability in degree of labeling from area to area over the slide in some cases. For example, as illustrated in Figure II-5, in the section incubated with [3H]-estradiol only, glandular cells in one region showed obviously more grains per cell when compared to glandular cells in another area. Quantitative data from these areas is presented in Table II-2, where it can be seen that this difference is significant. Within individual glands, however, this difference appeared less marked, and the great majority of cells tended to be labeled similarily.

D. Discussion

The demonstration of [3H]-estradiol uptake and retention in tissues from experimental animals has been accomplished by thaw-mount and dry-mount autoradiography following *in vivo* injections (144,147-149). The results of the initial injection studies described here agree well with data from those previous investigations. Although there are several reports demonstrating steroids in tissues or cell cultures using *in vitro* incubation with autoradiography (13,22,23,62,84,132,133, 138,143,153,154,163), this avenue of investigation has largely been overlooked in the study of the localization of estrogen receptors in human mammary lesions, where a knowledge of the distribution of estrogen target cells would be desirable both for practical and theoretical reasons.

Autoradiography has the advantage of employing the same ligand, [3H]-estradiol, used for biochemical assays. The affinity of this molecule for ER is well-documented, and [3H]-estradiol can be detected in tissues incubated in nanomolar concentrations of the ligand. A potential disadvantage of an autoradiographic method for the examination of human tissue, however, is the necessity of *in vitro* incubation. Biochemical studies have indicated that in *in vitro* incubations of uteri perhaps only 50% of

[3H]-estradiol is specifically bound (110,165,166). Consistent with these observations, in early experiments in this laboratory, incubation methods without chase or with chase in medium only were relatively ineffective for identifying target cells due to a high degree of nonspecific binding of [3H]-estradiol. Strobl and Lippman (140), however, have shown that washing cancer cells in medium containing 3.5 gm% bovine serum albumin reduced total, nonspecific, and specific [3H]-estradiol binding more effectively than medium alone. Tchernitchin and coworkers (153,154) in their autoradiographic studies of uterine tissue noted that perfusion of tissue with albumin resulted in a decrease in the number of grains over the extracellular spaces. Inclusion of albumin in the chase would also more closely simulate physiological conditions where the tissue is bathed with serum containing albumin. On this basis, to decrease the background, 3.5 gm% bovine serum albumin was added to the chase medium. The effect was to reduce total and nonspecific binding when compared to preliminary experiments where the chase medium was devoid of bovine serum albumin. The reduction of silver grains in sections incubated with excess unlabeled estradiol or DES was dramatic, allowing easier differentiation of specific uptake in sections incubated with [3H]-estradiol only.

In addition to the problem of nonspecific binding of estradiol there are other difficulties associated with in vitro incubation methods as discussed in detail by Stumpf (143). As he has illustrated, the most restricting problems are focal tissue damage and penetration artifacts. With the present incubation method, occasional [3H]-estradiol target cells in mouse uterus failed to reveal any label; however, the number of unlabeled cells is comparable to that described by Stumpf for in vivo studies (142). On the other hand, although structures such as endometrial glands showed relatively uniform labeling within a given area, there was regional variation in the amount of labeling observed after these incubations not seen in in vivo studies (142). This difference is believed to be an artifact of the technique and may be due to poor penetration of [3H]-estradiol or to a variable efficiency of the albumin chase. The observed distribution of [3H]-estradiol among the various cell types in the uterus after in vitro incubation, however, compares favorably with that described by others after in vivo injection. The apparent nuclear localization of [3H]-estradiol within target cells seen with this method also is consistent with biochemical (43,62,108,164) and previous autoradiographic (84,133) studies. Thus, this method appears capable of localizing

accurately target cells in the mouse uterus. While there are artifacts associated with the technique, as long as they are recognized they do not appear so severe as to preclude this method from use in a qualitative study of the distribution of estrogen target cells in human material.

It is believed, moreover, that the labeling observed with this method is physiologically significant. Although the relative contribution to the number of silver grains by [3H]-estradiol bound to type II binding sites (21,32,33) cannot be assessed from these experiments, it should be noted that nuclear labeling is predominant with this method, and nuclear type II sites appear to have an important physiologic function as discussed previously. Moreover, the ability of target organs to take up and retain physiologic amounts of estradiol that can be inhibited by anti-estrogens, first indicated the possibility of estrogen-binding components in target cells (60), and this property remains an important characteristic of those cells (64). In the present experiments, uterine cells have been shown to take up and retain estrogen during a two-hour chase with bovine serum albumin simulating <u>in vivo</u> conditions. This retention suggests that the cells identified as specifically labeled are, in fact, target cells.

Table II-1. Quantitative Comparison of Sections Illustrated in Figure II-2 (a)

Slide(b)	No. of glands counted	No. of frames	Total No. of grains	Average grains per frame ± SEM
[3H]E (Fig. II-2A)	8	52	793	15.25 ± 0.65
[3H]E+E (Fig. II-2B)	9	52	244	4.69 ± 0.36*
[3H]E+DES (Fig. II-2C)	5	59	336	5.69 ± 0.40*
[3H]E+Pg (Fig. II-2D)	8	52	823	15.83 ± 0.97#

(a) The number of grains in frames overlying only endometrial glandular cells in selected areas was counted. The areas selected were those glands illustrated in Fig. II-2 and other representative regions on the same slides. The counting was done using a 9.5 X 9.5 micron frame. The sections were exposed 36 days.

(b) [3H]E:[3H]-estradiol; E:estradiol; DES:diethylstilbesterol; Pg:progesterone.

* Significantly different from the average observed for tissue incubated with [3H]-estradiol only ($p<0.001$; t test)

\# No significant difference from the average observed

for tissue incubated with [3H]-estradiol only ($p>0.6$; t test).

Table II-2. Quantitative Comparison of Endometrial Glands in Areas Illustrated in Figure II-5 (a)

Area	No. of glands counted	No. of frames	Total No. of grains	Average grains per frame ± SEM
High density (Fig. II-5A)	3	17	778	45.76 ± 1.84
Low density (Fig. II-5B)	3	10	275	27.50 ± 2.45*

(a) The number of grains in frames overlying the endometrial glandular cells illustrated in Fig. II-5 was counted in the same manner as for Table II-1. The section was exposed 68 days.

* The difference observed in the two areas is significant ($p<0.001$; t test).

Figure II-1. Thaw-mount autoradiograph of mouse lactating mammary gland after *in vivo* injection of [3H]-estradiol. The figure demonstrates the nuclear retention of radioactivity in some but not all alveolar cells. (680 X, methyl green pyronin, 5 months exposure)

Figure II-1

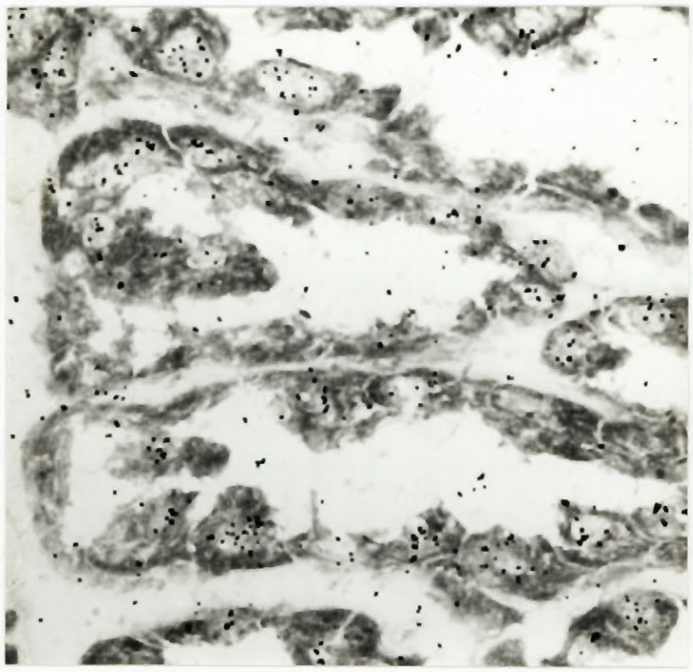


Figure II-2A. Uterus incubated for one-half hour in 5 nM [3H]-estradiol and chased for 2 hours. The section shows apparent nuclear localization of silver grains in cells of the endometrial glands and stroma.

Figure II-2B. Another piece from the same uterus preincubated and coincubated with 500 nM unlabeled estradiol. There is a marked reduction in total number of silver grains, and apparent nuclear labeling has been almost eliminated when compared with the section shown in A.

Figure II-2C. Another piece from the same uterus preincubated and coincubated with 500 nM diethylstilbesterol. The appearances are essentially the same as for B.

Figure II-2D. Another piece from the same uterus preincubated and coincubated with 500 nM progesterone. The uptake and retention of [3H]-estradiol has been relatively unaffected by this nonestrogen steroid when compared with A. (all 680 X, methyl green pyronin, 36 days exposure)

Figure II-2

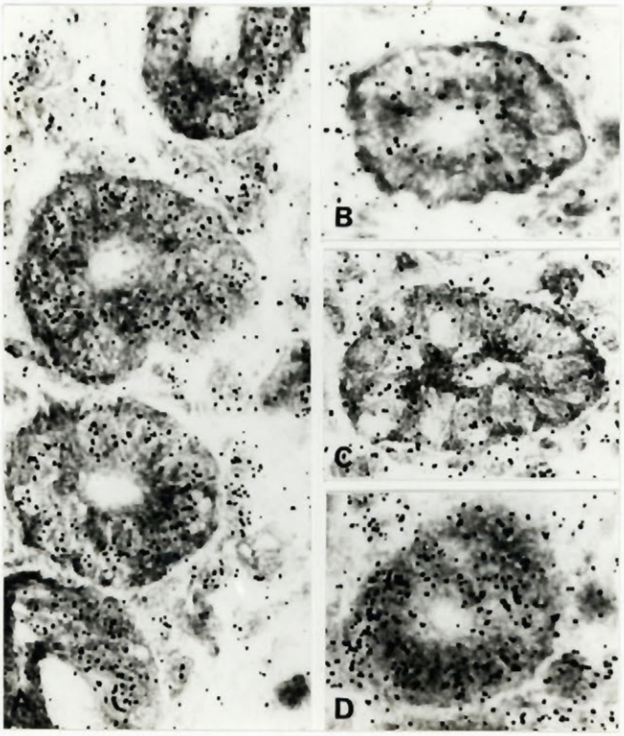


Figure II-3A. Section of diaphragm incubated in 5 nM [3H]-estradiol and chased for 2 hours. There is random scattering of silver grains with no apparent nuclear localization. (425 X)

Figure II-3B. Section of uterus incubated with diaphragm illustrated in A. There is labeling of endometrial glandular and stromal cells. The silver grains appear to be located predominantly over the nuclei. (375 X)

Figure II-3C. Section of uterus incubated in medium without [3H]-estradiol. The section is essentially devoid of silver grains. (425 X) (all sections methyl green pyronin, 34 days exposure)

Figure II-3

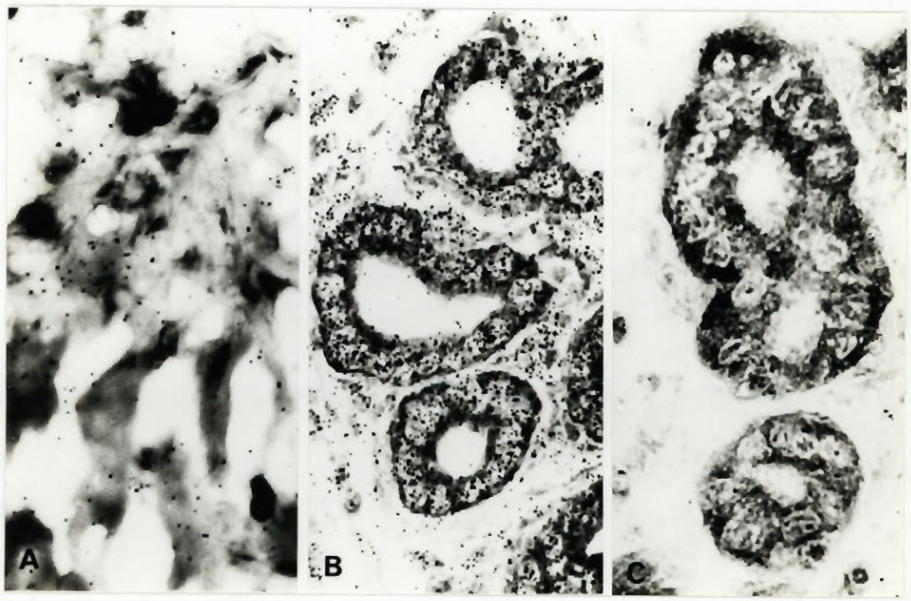


Figure II-4A. Section of uterus incubated in 5 nM [3H]-estradiol and chased for 2 hours. There is localization of silver grains in endometrial glands, stroma (top) and myometrium (below). (600 X, 34 days exposure)

Figure II-4B. Section showing localization of grains in surface epithelium of uterus. (680 X, 36 days exposure)

Figure II-4C. Section showing labeling of endometrial stromal cells while adjacent blood vessel endothelium is unlabeled (arrows). (760 X, 36 days exposure) (all sections methyl green pyronin)

Figure II-4

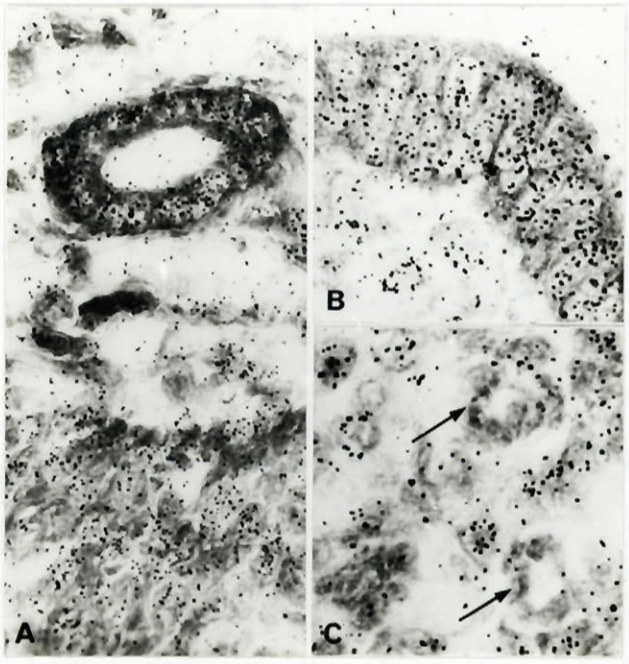
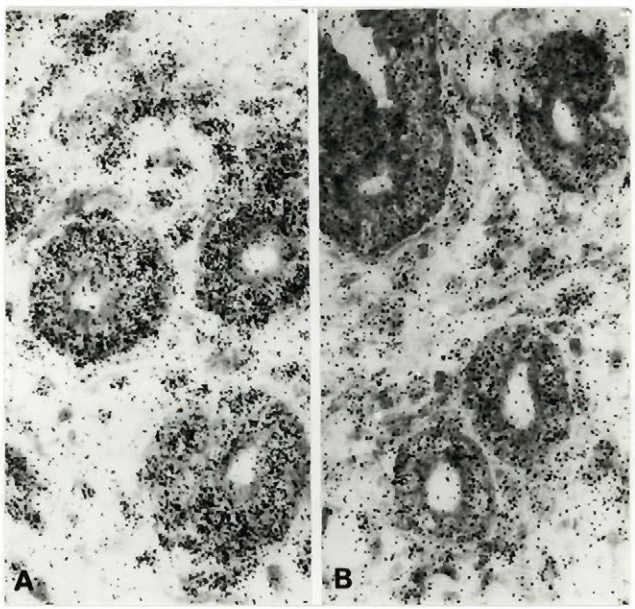


Figure II-5A. Section of mouse uterus incubated in 5 nM [3H]-estradiol and chased for 2 hours. The glands shown reveal marked labeling, which is relatively uniform within each gland in this area.

Figure II-5B. Another area of the same slide where the amount of label is obviously less than in A. Despite the regional variation in the slide, within this area, the glands appear similarily labeled. (425 X, methyl green pyronin, 68 days exposure)

Figure II-5



III. THE AUTORADIOGRAPHIC DEMONSTRATION OF [3H]-ESTRADIOL INCORPORATION IN BENIGN HUMAN MAMMARY LESIONS

A. Introduction

The normal human breast, as well as mammary lesions, are influenced by their hormonal milieu. A remarkable example is the response of certain patients with advanced breast cancer to endocrine manipulations. In the past decade the biochemical basis for these observations has been investigated extensively, especially with respect to the steroid hormone receptor levels in mammary carcinoma (31). Reports detailing data on the levels of estrogen receptor (ER) in benign mammary conditions are, however, more limited, although these lesions are considered to have, at least in part, a hormonal basis (42,44,82). Available biochemical data suggest that nonmalignant mammary lesions are, in general, less frequently estrogen receptor-positive and contain on average quantitatively less ER than malignancies (3,48,55,63,72,76,82, 128,137,155).

Biochemical assays, however, depend upon the homogenization of tissue, and the data reported represent an average for all cells homogenized. Thus ER-positive cells may be undetected in a sample where the majority of cells are ER-negative. Accurate morphologic methods for localizing ER in tissue sections would have a potential advantage over biochemical assays in their ability to discriminate a few ER-positive cells in an otherwise

ER-negative tissue and to identify the histologic types of cells containing receptor.

To date, the ability of recently reported histochemical and immunohistochemical methods to demonstrate the estrogen receptor as assayed biochemically is controversial (18,19,86,98,146). Another method potentially applicable to the investigation of estrogen binding proteins in tissue sections is autoradiography. The previous chapter described investigations on the use of thaw-mount autoradiography after in vitro incubation as a means of localizing estrogen binding proteins in a well-characterized target organ, the normal mouse uterus. In this chapter, the distribution of estrogen binding sites, as determined by a slightly modified technique, is described in a series of benign human mammary lesions.

B. Materials and Methods

The 17 patients were all female and ranged in age from 18 to 77 with a mean of 33.9 years. In all cases, biopsy material was obtained at the time of diagnostic frozen section. Blocks of tissue 1-to-2 mm thick were placed on ice in Medium 199 with Hepes buffer (Gibco, Burlington, Ontario) within 15 minutes of the biopsy and were transported to the laboratory. To determine total and nonspecific estrogen binding a series of simultaneous incubations was carried out. Tissue was placed in 5 ml of Medium 199 containing 5 nM [3H]-estradiol (S.A. 102 or 111 Ci/mmol, New England Nuclear, Lachine, Quebec) with or without a 100-fold or 200-fold excess of unlabeled estradiol or diethylstilbesterol (DES) (Sigma Chemical Co., St. Louis, Mo.). The tissue was incubated for 45 minutes at 30°C with shaking. It was then transfered to 5 ml of medium containing 3.5 gm% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) and incubated at 30° C with shaking for 2 or 3 hours. The tissue then was placed in OCT compound, frozen with liquid Freon 12 and stored at -76°C as described in the previous chapter.

The thaw-mount autoradiographic procedures used were as described in the previous chapter. The slides were allowed to expose at -15°C for generally 2-6 months. No case was considered negative until at least 6 months of

exposure. The slides, after developing as described previously, were stained with hematoxylin and eosin.

In evaluating the autoradiographs, putative estrogen target cells were identified on the basis of a greater density of cellular labeling localized over the nuclear region in sections incubated in [3H]-estradiol alone, as compared with that observed following exposure to an excess of unlabeled estradiol or DES. In the study of human breast carcinomas, to be described in the next chapter, it was found that this subjective analysis could, in general, be confirmed by quantitative analysis.

C. Results

In 9 cases a diagnosis of fibroadenoma was made, while the remaining 8 revealed evidence of varying degrees of fibrocystic disease. Histologically, the fibroadenomas tended to be relatively cellular with moderate to abundant numbers of stromal cells, although in one instance the lesion appeared somewhat senescent with a fibrotic stroma and compressed atrophic ducts. In the cases of fibrocystic disease a variety of changes were identified including adenosis, duct dilatation, and sclerosis.

Examination of the autoradiographs of sections incubated in only [3H]-estradiol revealed areas considered to be positive for estrogen binding proteins in 4 out of the 9 cases of fibroadenoma; the remainder failed to display any specific uptake. These positive areas were confined to ductal structures (Fig. III-1), which were scattered randomly throughout the sections and often juxtaposed to negative ducts. At higher magnification (Fig. III-2) specific labeling was localized mainly over the epithelial cell layer with many, but not all, of the epithelial cells positive. On closer examination, the grains appeared predominantly over the nuclear region of these cells, with relatively little labeling over the cytoplasm. Adjacent

histologically identifiable myoepithelial cells were negative. Stromal cells also tended to be unlabeled. On occasion the lumen of a duct displayed many silver grains, while most adjacent epithelial cells were not labeled. The acellular stroma revealed only randomly scattered grains. In contrast, comparable sections of these 4 positive fibroadenomas when incubated in [3H]-estradiol with excess unlabeled estradiol or DES revealed far fewer grains which tended to be scattered randomly with no preferential nuclear localization (Fig. III-3).

In the cases of fibrocystic disease, specific labeling was identified in 3. In these areas, the histologic changes included those of adenosis with epithelial hyperplasia (Fig. III-4). The silver grains appeared to be localized predominantly over epithelial cells, which again appeared heterogeneous with some positive and some negative cells. Myoepithelial cells, where they could be identified, were negative, as were the majority of stromal cells. In the positive epithelial cells, the grains were located mainly over the nuclear region of the cell, and in comparable sections incubated in the presence of excess radioinert estradiol or DES, the grains were far fewer and randomly scattered.

D. Discussion

The normal mammary gland of experimental animals contains both estrogen and progesterone receptors, and the levels of these receptors undergo modulations with different developmental states (47,126,134,168). Truly normal human breast tissue is not, however, readily available for assay, but in studies of adjacent "normal" tissue from mastectomies for carcinoma the levels of ER have been reported to be low (63,155). In carcinomas, however, the levels of ER may vary from insignificant amounts to markedly elevated values, and the majority of carcinomas are estrogen receptor-positive. While the steroid hormone receptor status has been investigated extensively in human breast malignancies, much less is known about benign lesions. One limitation of biochemical assays of benign lesions is that such assays of necessity use homogenates of tissue. For example, in fibrocystic disease, a very polymorphous condition with abundant fibrous tissue, it is not possible to discern which, if any, components of the lesion are ER-positive.

Morphologic methods for localizing ER would obviate this problem; however, the recently described histochemical and immunohistochemical methods have not been proven conclusively yet in their ability to demonstrate ER (18,19,86,98,146,157). Thaw-mount

autoradiography provides another potential means for localization of estrogen binding proteins in human tissue sections. This technique also requires further documentation of its ability to identify accurately ER in human tissue because of various possible factors such as metabolism of the ligand, chemographic artifacts, and latent image fading (143). Moreover, the possible contribution of nuclear type II sites to the labeling observed cannot be determined in these studies. Despite these possible limitations, thaw-mount autoradiography does utilize a ligand with a known affinity for ER and offers a potentially sensitive means for localizing bound radioactivity. With this method putative estrogen target cells have been identified in normal mouse uterus (14) as has previously been discussed. In this chapter the results of a study of benign human mammary lesions have been presented.

In the cases studied, the most specifically bound radioactivity was found in epithelial cells, while stromal cells tended to be negative. Biochemical studies have documented that the normal lactating breast of experimental animals contains significant levels of ER (47,126,168), but the relative distribution between the epithelial and stromal components is less well established. Using dry-mount autoradiography Sar and

Stumpf (130) have reported the uptake of injected [3H]-estradiol in some connective tissue cells as well as epithelial cells in the lactating mammary gland of mice and rats. Haslam and Shyamala (47) have documented that in the virgin mouse both the epithelial component and the cleared fat pad contain estrogen receptors. In contrast, Rao et al (123) have suggested that the stromal cells of human fibroadenomas tend to be ER-negative. The present investigation supports this suggestion. The differences between these results and those of Sar and Stumpf (130) and of Haslam and Shyamala (47) may reflect different functional states or a species difference. It is also possible that the levels of ER present in stromal cells may be so low as to be beyond the limits of detection of the thaw-mount autoradiographic method used.

In this investigation, all identifiable myoepithelial cells appeared to be unlabeled which is in agreement with the findings of Sar and Stumpf (130) in the lactating mammary gland of mice and rats. If one considers the means by which mammary ductal epithelial cells derive their nutrients and hormones from the blood supply, it may not be surprising that myoepithelial cells would be ER-negative. Ozello (104) has postulated the existence of an epithelial-stromal junction in the human breast that may regulate the exchange of substances such

as trophic hormones between the circulation and the mammary epithelium. Thus, if the myoepithelial cells, interposed between the epithelial and vascular elements, were to contain significant levels of estrogen binding proteins, their presence might reduce greatly the amount of free steroid available to epithelial cells. It should be noted, however, that in the current study, these lesions were not evaluated by electron microscopy or by histochemical or immunohistochemical methods for identifying myoepithelial cells, and it is possible that some labeled cells with evidence of myoepithelial differentiation were not appreciated as myoepithelial at the light microscopic level.

The percentage of positive cases observed in the present study agrees, in general, with the findings of others using biochemical assay. Thus for fibroadenomas, Allegra et al (3) found 55% to be ER-positive. Martin et al (82) found 42% positive while in this study 44% were positive. For fibrocystic disease, 38% were found to be positive in this series, while Allegra et al (3) found 25% and Jacquemier et al 63% (55). Biochemical assay was not done on the tissues in the present study, and direct comparison is therefore not possible.

It is of interest that both in this present study of benign lesions and in the investigation of human mammary

carcinomas (to be discussed in the next chapter) the epithelial cell population was heterogeneous with respect to ER-positivity. In contrast, such heterogeneity was not observed in the uteri of 7-to-8-week-old mice (14). Whether such differences reflect technical factors such as endogenously bound steroid in human material is not known. Shannon et al (132) have shown, however, that with a similar autoradiographic method, exchange of radioactive ligand for endogenously bound unlabeled steroid does occur. The present findings would suggest, therefore, that, unlike myoepithelial and stromal cells, which in general fail to contain detectable levels of ER in all states examined, the levels of ER in the human mammary epithelium undergo modulations. The factors responsible for this variation may reside at least in part at the cellular level, since all cells presumably have been exposed to the same hormonal milieu. In this regard Shyamala and Haslam (134) have demonstrated that the mouse mammary gland epithelium undergoes modulations of steroid hormone receptor levels related to different functional states and that this tissue-specific change appeared in part unrelated to the hormonal milieu. Studies such as these suggest that in addition to consideration of the hormonal milieu, factors operative within the individual cells also must be involved in

controlling the levels of steroid hormone receptors. Since both the benign and malignant mammary epithelium may be potentially responsive to hormonal therapy, there is clearly a need for additional investigation, including morphologic techniques, to elucidate the control mechanisms responsible for determining ER levels in human breast tissue.

Figure III-1. Section of a fibroadenoma incubated only in [3H]-estradiol demonstrating silver grains localized over the epithelial cells of ducts (arrows). It can be seen that not all epithelial cells are positive and not all ducts are labeled to the same degree. The stromal cells are negative, and the acellular stroma reveals only randomly scattered grains. (375 X, hematoxylin and eosin, 127 days exposure)

Figure III-1.

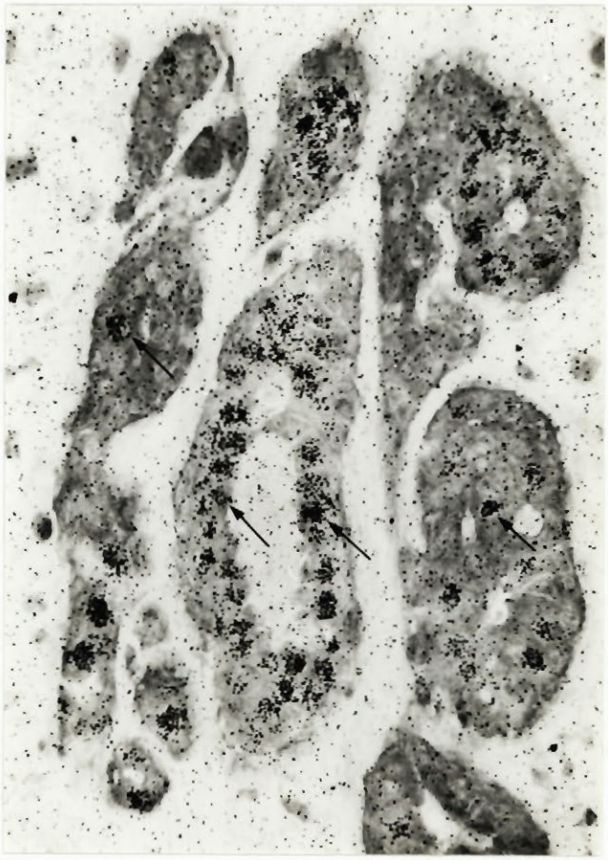


Figure III-2. Another area of the fibroadenoma illustrated in Figure III-1 where it is apparent that the specific labeling observed is over the epithelial component, while adjacent myoepithelial cells (arrowheads) are unlabeled. (600 X, hematoxylin and eosin, 127 days exposure)

Figure III-2.

Figure III-3. A section of the fibroadenoma illustrated in Figures III-1 and 2, which has been incubated with [3H]-estradiol with excess unlabeled estradiol. It can be seen that the degree of labeling is reduced, and the grains present appear randomly scattered. This appearance is representative of the entire section. (600 X, hematoxylin and eosin, 127 days exposure)

Figure III-3.

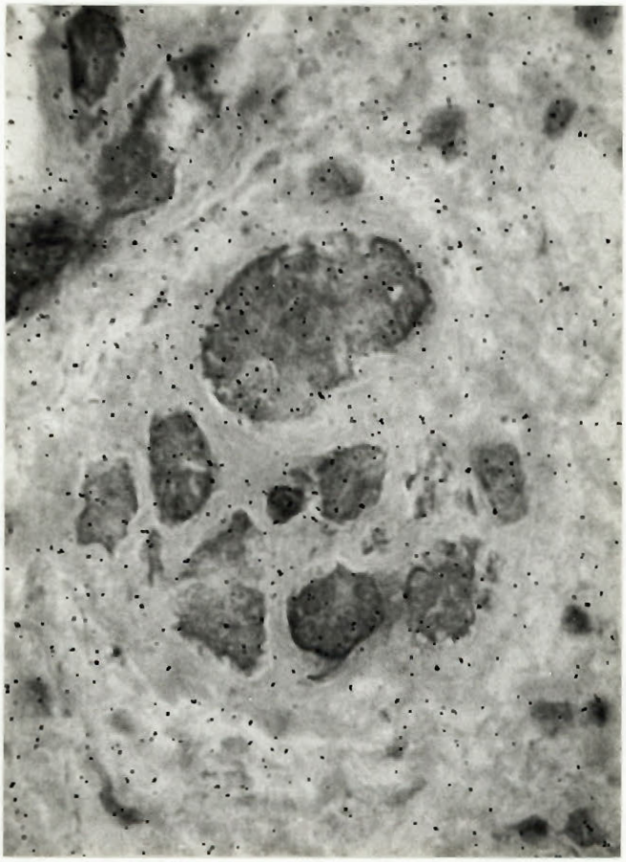
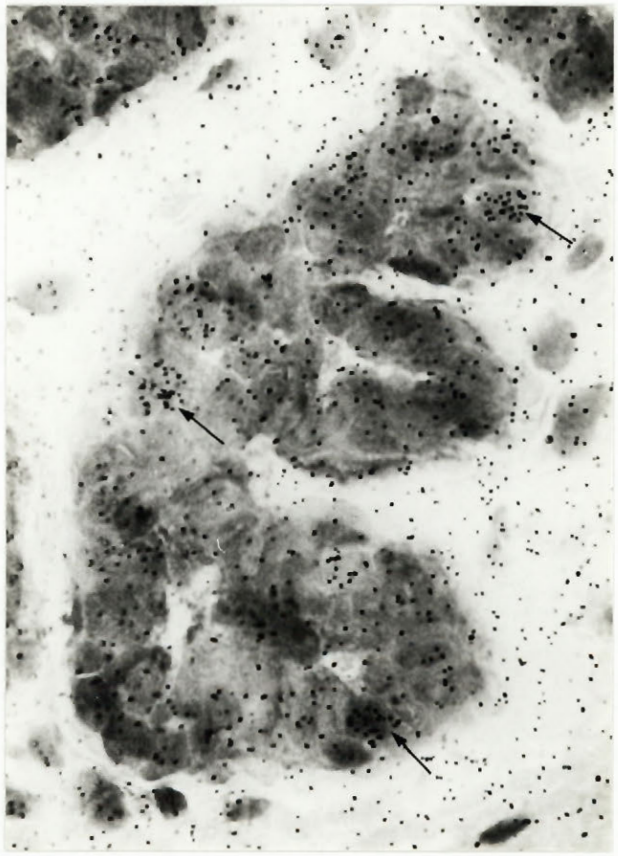


Figure III-4. A section incubated only in [3H]-estradiol showing some adenosis with epithelial hyperplasia. It can be seen that some, but not all, epithelial cells (arrows) show a concentration of silver grains, which, on higher magnification, is over the nuclear region. Another piece of this tissue, incubated with excess unlabeled estradiol, failed to demonstrate this nuclear concentration of grains and showed only random scattering of grains throughout the entire section. (800 X, hematoxylin and eosin, 208 days exposure)

Figure III-4.



IV. THE AUTORADIOGRAPHIC DEMONSTRATION OF ESTROGEN BINDING IN HUMAN BREAST CANCER AFTER IN VITRO INCUBATION

A. Introduction

Knowledge that the biochemical assay of human breast cancers for steroid hormone receptors is useful for predicting patient response to hormonal therapy has stimulated interest in developing a morphologic method for assessing tissue content of these receptors. To date, the major emphasis of research in this field has been on immunohistochemical and histochemical methods. While these methods offer promise for future routine application, their validity in demonstrating steroid hormone receptors as assayed biochemically remains to be established (29). Thaw-mount and dry-mount autoradiography are also applicable to the problem and were among the first methods to be applied successfully for localization of bound radioactive steroids in tissue sections of experimental animals (144,149). Indeed, in early investigations of the biochemical parameters of the estrogen receptor, dry-mount autoradiography was used to provide morphologic evidence on the intracellular localization of those receptors (62). Despite recent questions concerning the interpretation of some of this data (84,133), the methods described by Stumpf and Roth remain established in their ability to demonstrate bound steroid hormones in target tissues in part because these methods avoid procedures that might produce artifactual

dislocation of the steroid (147). Autoradiographic techniques also offer the advantage that, under appropriate conditions, they can be quantitated (22,23,150).

The application of thaw-mount autoradiography to human tissue, however, has been more or less overlooked as a means of localizing specifically bound steroids in material like human breast cancers. Since autoradiographic methods with human tissue require in vitro incubation of fresh tissue, a number of potential artifacts must first be evaluated. It must also be ascertained that data derived from these methods do indeed correlate well with biochemical assays. Previous chapters presented data assessing the accuracy of an in vitro incubation method using the mouse uterus (14) and localizing putative target cells in a series of benign breast biopsies (16). In this chapter the findings are presented from 40 cases of human breast cancer, and the autoradiographic data is correlated with results of biochemical assays.

B. Materials and Methods

Tissues: Tissues from primary, recurrent, and metastatic breast carcinomas were obtained at the time of frozen section, diced into sections 1-2 mm thick, and transported to the laboratory in Medium 199 with 25 mM Hepes buffer (Gibco, Burlington, Ontario), pH 7.3. All patients were female and ranged in age from 23 to 74 with a mean of 54.6 years.

Chemicals: [2,4,6,7]-[3H]-estradiol (S.A. 102 or 111 Ci/mmol) was purchased from New England Nuclear, Lachine, Quebec and purified by thin layer chromatography prior to use. Unlabeled estradiol and diethylstilbesterol and bovine serum albumin were from Sigma Chemical Co., St. Louis, Mo. Liquid Freon 12 was from Dupont, Canada. Other miscellaneous reagents were purchased from Fisher Scientific Ltd., Montreal, Quebec. For thaw-mount autoradiography Kodak NTB 2 emulsion and developing reagents were from Eastman Kodak, Montreal, Quebec.

Incubation of tissue: To determine total and nonspecific binding of [3H]-estradiol, blocks of tissue were incubated for 45 minutes at 30°C in Medium 199 containing 5 nM [3H]-estradiol with and without a 100-fold or 200-fold excess of unlabeled estradiol or DES. The tissues were then transferred to a medium containing 3.5 gm% bovine serum albumin and incubated,

usually for 4 hours. After these incubations the tissue was embedded in OCT compound, frozen with liquid Freon 12, and stored at -76°C. Sections were cut for autoradiography, generally within 2 weeks.

Thaw-mount autoradiography: The thaw-mount autoradiographic method used has been described in previous chapters. The slides were exposed at -15°C for periods generally from 2-6 months. They were then developed and stained with hematoxylin and eosin. In several cases, as a control for negative chemography, the emulsion was fogged by light prior to mounting, and the sections were then mounted and exposed as usual (127).

Quantitation of autoradiographs (15): The number of nuclear grains was counted for 50 cells in tissues incubated in [3H]-estradiol both with and without an excess radioinert steroid. At the same time the approximate nuclear cross-sectional area was determined for each of these cells using the formula $A=\pi ab/4$. The data was expressed as number of nuclear grains per 10 square microns nuclear cross-sectional area. For tissues incubated in [3H]-estradiol only an area subjectively judged to be positive was selected for quantitation. For tissues exposed to excess unlabeled steroid, the appearances of the slides were the same for all areas. Positive cells were defined as those demonstrating a

threefold or greater number of grains per 10 square microns nuclear cross-sectional area in slides of tissue exposed only to [3H]-estradiol than the average for tissue exposed to excess radioinert estradiol. A case was considered to be positive by autoradiographic criteria if: 1) the mean number of grains per 10 square microns nuclear cross-sectional area for tissue exposed only to [3H]-estradiol was greater than the mean exposed to excess unlabeled competitor and 2) at least 25% of cells quantified were considered to be target cells. If a case did not meet these criteria it was classified as negative. Criteria were not established for borderline cases.

In five cases the amount of label over the acellular stroma was determined by counting the grains observed within 5.8 X 5.8 micron frames overlying only acellular areas. One hundred frames in each of four randomly selected areas were counted, and the average obtained was expressed as the number of grains per 10 square microns.

Biochemical assays: Biochemical assays were performed in the clinical laboratories of the Royal Victoria Hospital by established methods (87). A case was considered biochemically positive if there were specific binding of greater that 12 fmols/mg protein, borderline if there were more that 6 but less than 12

fmols/mg protein, and negative if there were less than 6 fmols/mg protein.

C. Results

Thirty-one cases (78%) were considered to be estrogen receptor-positive by autoradiographic criteria. These cases revealed that in tissues exposed only to [3H]-estradiol the radioactivity was incorporated primarily by the neoplastic epithelial cells and could be suppressed by exposure to unlabeled estradiol (Figure IV-1). The vast majority of stromal cells, inflammatory cells and endothelial cells were negative. The acellular stroma revealed only randomly scattered grains. Quantitation of this data demonstrated that the number of grains over the acellular stroma was insignificant when compared with the labeling seen over nuclei of putative target cells (Table IV-1). Within nests of infiltrating carcinoma, positive cells could be identified admixed with negative cells (Figure IV-2). Grains were apparent mainly over the nuclear region, with comparatively little cytoplasmic labeling. Among the positive cells the density of the nuclear labeling was variable as can be seen in the histogram in Figure IV-3. It was also apparent that there were differing numbers of positive cells within nests of tumor in any given case. In some of these nests most cells were labeled, in some a heterogeneous population was observed, and in others most cells were unlabeled. Several foci of intraductal

carcinoma were identified. In some of these a heterogeneous population of positive and negative cells could be seen (Figure IV-4). In other places most of the intraductal component was unlabeled. In some cases, atypical hyperplastic lesions were seen incorporated within the infiltrating carcinoma. Some of these lesions showed specific labeling (Figure IV-5).

In nine cases (22%) considered to be negative by autoradiographic criteria the appearance of the tissue exposed only to [3H]-estradiol resembled that of the tissue incubated with excess unlabeled estradiol (Figure IV-6). The grains were randomly scattered over the section. They were few in number, when compared with positive cases, and there was no evidence of a nuclear localization.

Biochemical assay was done on 37 cases. Of these, 76% were estrogen receptor-positive, 16% borderline, and 8% negative (Table IV-2). Of the 28 cases biochemically positive 26 were positive by autoradiographic assessment, resulting in 93% agreement. In those cases where there was disagreement the tissues contained 15 and 14 fmols/mg cytosolic protein. Of the biochemically borderline cases, four were considered negative by autoradiography while two were positive, including one (case 32) which was markedly positive. The mean number of grains in the

other cases tended to be lower than usually seen for positive cases. There was uniform agreement between the autoradiographic and biochemical evaluation of all three biochemically estrogen receptor-negative cases. Therefore, if one excludes borderline cases for which there were no autoradiographic criteria established in this investigation, the overall agreement between this method and biochemical assay in the 31 cases is 94%. If the borderline cases are included, the overall agreement drops to 78%.

D. Discussion

This investigation has demonstrated that estrogen receptor-positive human mammary carcinomas are often composed of a heterogeneous population of "target" and "nontarget" cells as assessed by the present autoradiographic criteria. Similar data have been obtained with immunohistochemical (68,115,120) and histochemical (6,36,46,73,74,113) methods, and the present investigation thus supports the earlier findings. This possible heterogeneity has also been suggested from biochemical (12,122,135) and (2,129) clinical studies. The significance of this tumor cell heterogeneity in relation to patient response to hormonal therapy has not been clearly established. Clinical studies have shown that not all estrogen receptor-positive tumors respond to hormonal therapy (31). Since the binding of estrogen to its receptor represents only the first step in the biochemical pathway of estrogen action, measurements of progesterone receptors which assess the functional integrity of the estrogen receptor system, are done and correlate well with the clinical behavior of mammary carcinoma (20,31). Data from these studies suggest that failure of ER-positive tumors to respond to endocrine therapy may be the result of a defect in the pathway of estrogen action subsequent to the initial binding. It has

also been suggested, however, that tumor cell heterogeneity for ER may also be a factor for lack of response or early recurrence (77,79,88).

While there are studies available reporting a correlation between histochemical assays of steroid hormone binding sites and patient response (116), there is little known about the percent of ER-containing cells required for a tumor to respond. Stoll (139) has suggested, on a theoretical basis, that for an ER-positive tumor to respond, as defined by most criteria, the 50% decrease in measurable diameter would require a kill of 99% of the tumor cells. It is of interest, however, that in the present series of cases none of the tumors examined contained 99% target cells. If one can assume that about 50% of ER-positive cases examined in this series would respond to endocrine manipuation, it would appear that Stoll's theoretical analysis may require further investigation to determine experimentally the relationship between the percent of target cells in a tumor and clinical response.

Some caution must be taken, however, in the interpretation of data from any histochemical assay of this type since the tissue is being examined only at one point in time. The identification of putative target cells with this autoradiographic method is dependent on

the presence of suppressible nuclear labeling. A requisite for localizing specifically bound [3H]-estradiol would, therefore, be that the nuclear binding sites be in an active state capable of binding the radioactive ligand. It may be that, at the time of examination, a certain percent of target cells escaped detection as the receptor was in a non-binding state for [3H]-estradiol or depleted possibly as a result of nuclear processing of ER (51). It may also be that ER were present but at levels below the limits of detection of this method.

The variability in uptake and retention of [3H]-estradiol observed is not considered to be a technical artifact such as poor penetration of the ligand into the tissue blocks during incubation, since cells with relatively few or no nuclear grains could be identified adjacent to strongly labeled cells indicating adequate diffusion of [3H]-estradiol to the areas evaluated. Furthermore, in studies using normal mouse uterus incubated with similar methods the vast majority of target cells were labeled and cellular heterogeneity was not as evident (14). It should be noted, however, that the observed tumor cell heterogeneity may be in part due to random cell death during the incubation or to failure of exchange of the radioactive ligand with

endogenously bound hormone. Shannon et al (132) have shown, however, that with a similar method using 17 nM [3H]-estradiol exchange can occur.

Among estrogen target cells there also appeared to be a variability in estrogen uptake. This difference in labeling suggests that the target cells contain variable amounts of specific estrogen binding sites. A similar finding was observed with other techniques (91,113). The significance of this observation is not clear since, as has been pointed out (123), little is known about the minimum number of cytosolic receptors necessary for estrogen action.

In this investigation several foci of intraductal carcinoma were examined with some found to contain positive cells while others were essentially negative. Based on his histochemical observations of intraductal carcinoma, Lee (74) has suggested that these lesions are most often ER-negative. Although the finding of ER-positive intraductal areas in the present series would not support this suggestion, there are too few numbers of cases to draw any definite conclusion. Parl and Wagner (106) noted that predominantly intraductal lesions were less often biochemically ER-positive than infiltrating lesions. On the other hand, in his series of cases McCarty et al (85) found 67% of intraductal carcinomas

contained greater than 10 fmol/mg protein, a slightly greater percentage than for infiltrating duct carcinomas. The biochemical assessment of intraductal carcinomas could be hindered by the possible inclusion in tissue homogenates of adjacent hyperplastic lesions which themselves may contain estrogen binding sites (15,55) thus yielding a false value for the intraductal component. It would appear, therefore, that morphologic methods could be valuable to assess more thoroughly such lesions to determine if there are indeed modulations in the cellular ER content during the progression of lesions from atypical hyperplasia through a phase of intraductal carcinoma to frankly invasive ductal carcinoma.

This study also demonstrated that in the breast cancers examined, stromal cells rarely incorporated [3H]-estradiol while some nonneoplastic ducts retained significant amounts of radioactivity. The number of stromal cells varies markedly in the various histologic types of breast cancer and can represent a substantial proportion of the cellular population of the tumor. If these cells contained estrogen binding proteins in appreciable amounts, biochemical analysis might yield a false positive ER assay result. The data from this investigation suggest, however, that this possibility is unlikely and support the findings of Rao et al (123) who

proposed that, in fibroadenomas and cystosarcoma phyllodes, stromal cells tend to be ER-negative.

In this report we have demonstrated that the autoradiographic findings correlate well with data from biochemical assays. Others, using immunohistochemical or histochemical methods have also reported a good correlation (90,113,115,120). It should be noted, however, that, unlike other techniques, the present autoradiographic method assesses only nuclear binding sites which are believed to be of physiological significance and are consistent with current theories on the intracellular localization of ER, regardless of whether one accepts the classical theory (43,62) or more recently proposed hypotheses (68,84,133,164). Other methods, although correlating well with biochemical assays, demonstrate often only cytoplasmic binding sites. The nature of those cytoplasmic sites would be less clear and would require further investigation if the recent hypotheses suggesting a nuclear localization of both the liganded and free estrogen receptor prove to be correct.

From the comparison with biochemical assays, it is apparent that the autoradiographic method fails to detect the presence of specific estrogen binding in tumors with an ER content of less than about 15 fmols/mg protein (see Table IV-2). It may be that longer exposure times would

improve the sensitivity of the autoradiographic method, thereby allowing detection of these lower levels. It is also possible that this apparent loss of sensitivity is the result of potential artifacts of the autoradiographic method including latent image fading or negative chemography. In several controls done to assess negative chemography, however, there was no evidence of significant interaction between the tissue and emulsion resulting in loss of grains, suggesting that this phenomenon is not the explanation for the loss of sensitivity.

Another possible explanation for this relative lack of sensitivity may be related to the use of the four-hour wash with bovine serum albumin during the incubation procedure. It was previously found during studies with mouse uterus, that a wash of this nature including albumin was very effective for decreasing the nonspecific binding, thus permitting detection of putative target cells (14). Tchernitchin and coworkers (153,154) did not note in their autoradiographic studies a loss in total number of nuclear grains after a wash with albumin for one hour. Strobl et al (140,141), however, have shown that in the cell system they studied, an albumin wash while being effective in decreasing nonspecific binding, also resulted in a loss of some specifically bound

estradiol. Such a loss of specific binding, if it is occuring in the present method, might account for the relative lack of sensitivity.

The observation of one case (case 32) which was markedly positive by autoradiography and only borderline by biochemical analysis requires consideration. The biochemical assays measured cytosolic ER only, while the autoradiographic method assessed nuclear bound radioactivity, and this difference might account for the discrepancy. Positive chemography in the autoradiographic method seems an unlikely explanation, since the labeling observed could be suppressed by radioinert estradiol and DES. The label observed may be associated with nuclear type II (151) sites rather than nuclear ER, accounting for the discrepancy. Other, and more likely, possibilities include a difference in the area of the tumor examined (135) or inadvertent errors in tissue handling prior to biochemical assay.

In these experiments the tissue was incubated with [3H]-estradiol for 45 minutes at 30° C only with a subsequent four-hour wash. Under these conditions one would expect the predominant labeling observed in target cells to be nuclear (14). According to classical theory (43,62), the ligand is assumed to have bound the cytosolic receptor. The resulting complex has then

undergone a temperature-dependent activation and translocated to the nucleus. According to recent theories (84,133,164), the functional estrogen-receptor complex is also considered to be in the nucleus. For this reason the number of nuclear grains was used to differentiate target and nontarget cells. While the presence of significant nuclear labeling does not necessarily guarantee an entirely functional ER system, its absence suggests either a relative paucity of ER or a defect in the estrogen receptor system. Such a defect might include an abnormal estrogen receptor, an inability of receptor transformation, or perhaps an abnormality of nuclear acceptor sites. In the cases studied, there was no subjective evidence of significant cytoplasmic labeling in the absence of nuclear labeling (i.e. failure of translocation as has been reported (35)) although quantitation to support this impression has not been done. Incubation for differing times and temperatures to determine the temperature dependence of the presumed activation and translocation or to identify unbound nuclear receptor was not done. It is, therefore, not possible from the present data to determine if unbound receptor is predominantly cytoplasmic or nuclear. The contribution to the labeling observed from nuclear type II sites has not been assessed, and, in determining

putative target cells, it has been assumed that if any labeling were from such type II sites these sites would have physiological significance as previously discussed.

From these studies it can be concluded, therefore, that on the basis of an autoradiographic method such as the present one, estrogen receptor-positive human mammary carcinomas appear to be composed of target and nontarget cells at the time of biopsy. Subject to the potential experimental artifacts and limitations in interpretation discussed above, an autoradiographic method could be feasible as an investigative tool in studying the biology of human breast cancer as it has already proved to be in the study of the physiology of experimental animals. It is unlikely, however, that such a method would prove practical in the assessment of human tumors in a clinical setting because of the need to incubate fresh tissue and the lengthy exposure times required. It is, nevertheless, an important method of localizing estrogen binding sites, and, since it employs the same radioactive ligand employed in biochemical assays, direct comparative studies including "saturation" studies and demonstration of high affinity binding (22,23) would be feasible. Correlative autoradiographic and biochemical investigations could provide important information in the study of the biology of human breast cancer.

Table IV-1. Quantitative Comparison of Labeling over Tumor Cell Nuclei and Acellular Stroma

No	ER(a)	[3H]E+E or DES(b)		[3H]E(b)	
		Stroma	Nucleus	Stroma	Nucleus
8	112	0.22 ± 0.28	0.67 ± 0.77	0.32 ± 0.34	3.77 ± 1.92
11	100	0.16 ± 0.23	0.97 ± 0.80	0.22 ± 0.31	6.13 ± 3.54
12	80	0.27 ± 0.36	0.55 ± 0.50	0.21 ± 0.32	5.26 ± 3.09
15	71	0.26 ± 0.30	0.63 ± 0.43	0.40 ± 0.41	3.29 ± 2.12
22	40	0.10 ± 0.19	0.31 ± 0.32	0.14 ± 0.23	2.10 ± 1.27

(a) Femptomoles per milligram of cytosolic protein

(b) Mean number of grains per 10 square microns ± standard deviation observed over acellular stroma or nuclei of tumor cells incubated in medium indicated. [3H]E: [3H]-estradiol; E: radioinert estradiol; DES: radioinert diethylstilbesterol.

Table IV-2. Quantitative Comparison of Autoradiographic and Biochemical Data

No.	Age (a)	Histologic type (b)	ER (c)	Exposure time (d)	[3H]E+E or DES(e)	[3H]E (e)	Result (f)
				BIOCHEMICAL ASSAY POSITIVE			
1.	47	IDC	519	75	0.62 ± 0.51	3.74 ± 2.35	74+
2.	66	IDC	478	42	0.24 ± 0.21	3.14 ± 2.62	88+
3.	52	IDC	348	68	0.39 ± 0.32	4.99 ± 2.99	84+
4.	66	ILC	296	168	0.78 ± 0.50	4.59 ± 2.44	88+
5.	56	IDC	205	41	0.51 ± 0.47	5.40 ± 2.58	98+
6.	64	IDC	176	295	0.13 ± 0.20	0.53 ± 0.27	72+
7.	68	IDC	129	63	0.67 ± 0.57	2.79 ± 1.96	64+
8.	49	IDC	112	63	0.67 ± 0.77	3.77 ± 1.92	80+
9.	65	IDC	107	78	0.70 ± 0.44	3.53 ± 2.94	68+
10.	53	IDC	101	107	0.47 ± 0.37	4.45 ± 3.35	88+
11.	73	IDC	100	84	0.97 ± 0.80	6.13 ± 3.54	80+
12.	43	IDC	80	76	0.55 ± 0.50	5.26 ± 3.09	96+
13.	52	IDC	79	71	1.30 ± 0.85	3.34 ± 1.83	34+
14.	52	IDC	74	188	0.16 ± 0.23	0.80 ± 1.30	46+
15.	68	ILC	71	67	0.63 ± 0.43	3.29 ± 2.12	72+
16.	51	ILC	70	75	0.62 ± 0.45	3.55 ± 2.56	72+
17.	50	IDC	51	48	0.23 ± 0.29	2.51 ± 1.58	94+
18.	66	IDC	48	113	0.36 ± 0.36	1.99 ± 1.21	72+
19.	43	IDC	48	129	0.47 ± 0.36	2.02 ± 1.64	66+
20.	46	IDC	48	76	0.80 ± 0.46	3.30 ± 1.68	76+
21.	74	ILC	47	38	0.32 ± 0.32	4.00 ± 2.99	88+
22.	39	IDC	40	81	0.31 ± 0.32	2.10 ± 1.27	76+
23.	55	IDC	39	57	0.60 ± 0.42	5.01 ± 2.67	90+
24.	66	ILC	35	77	0.96 ± 0.53	4.54 ± 1.76	84+
25.	50	IDC	32	48	0.84 ± 1.09	4.30 ± 2.61	80+
26.	60	IDC	16	251	0.017 ± 0.062	0.10 ± 0.15	38+
27.	57	IDC	15	238	0.40 ± 0.32	0.46 ± 0.44	10-
28.	49	IDC	14	191	0.29 ± 0.55	0.16 ± 0.26	0-

				BIOCHEMICAL ASSAY BORDERLINE			
29.	34	IDC	11	123	0.34 ± 0.33	0.47 ± 0.36	4-
30.	45	IDC	10	291	0.15 ± 0.24	0.08 ± 0.14	4-
31.	39	IDC	10	188	0.26 ± 0.37	0.50 ± 0.50	20-
32.	65	ILC	8	168	0.81 ± 0.59	4.99 ± 3.11	78+
33.	46	IDC	7	273	0.04 ± 0.10	0.06 ± 0.12	26+
34.	65	IDC	7	249	0.14 ± 0.28	0.027 ± 0.11	4-
				BIOCHEMICAL ASSAY NEGATIVE			
35.	42	IDC	5	203	0.74 ± 0.40	0.76 ± 0.49	0-
36.	23	IDC	4	62	0.54 ± 0.45	0.60 ± 0.59	4-
37.	48	IDC	0	146	0.66 ± 0.45	0.48 ± 0.60	2-
				BIOCHEMICAL ASSAY NOT DONE			
38.	56	ILC	--	110	0.74 ± 0.59	4.05 ± 3.24	72+
39.	58	IDC	--	66	0.46 ± 0.35	5.57 ± 2.52	96+
40.	61	ILC	--	44	0.34 ± 0.36	3.31 ± 2.20	88+

(a) years

(b) IDC=infiltrating duct carcinoma; ILC=infiltrating lobular carcinoma

(c) femptomoles per milligram of cytosolic protein

(d) days

(e) mean ± standard deviation of number of nuclear grains per 10 square microns nuclear cross-sectional area for 50 cells evaluated from tissue incubated in indicated medium. [3H]E: [3H]-estradiol; E: unlabeled estradiol; DES: unlabeled diethylstilbesterol.

(f) autoradiographic evaluation indicating % positive cells identified and assessment of case as ER-positive or ER-negative.

Figure IV-1A. Autoradiograph of an estrogen receptor-positive human mammary carcinoma after <u>in vitro</u> incubation with [3H]-estradiol only. Grains can be seen associated with many of the neoplastic epithelial cells, and, on closer examination, this labeling is located mainly over the nuclear region. Data from the biochemical assay of this lesion revealed it to be estrogen receptor-positive with 348 fmols/mg protein.
Figure IV-1B. Breast cancer tissue adjacent to that shown in figure IV-1A after <u>in vitro</u> incubation with [3H]-estradiol plus a hundredfold excess of radioinert estradiol. The majority of labeling seen in figure IV-1A has been supressed, and there is no evidence of a nuclear concentration of the few remaining grains. (both figures 400 X, hematoxylin and eosin, 68 days exposure)

Figure IV-1.

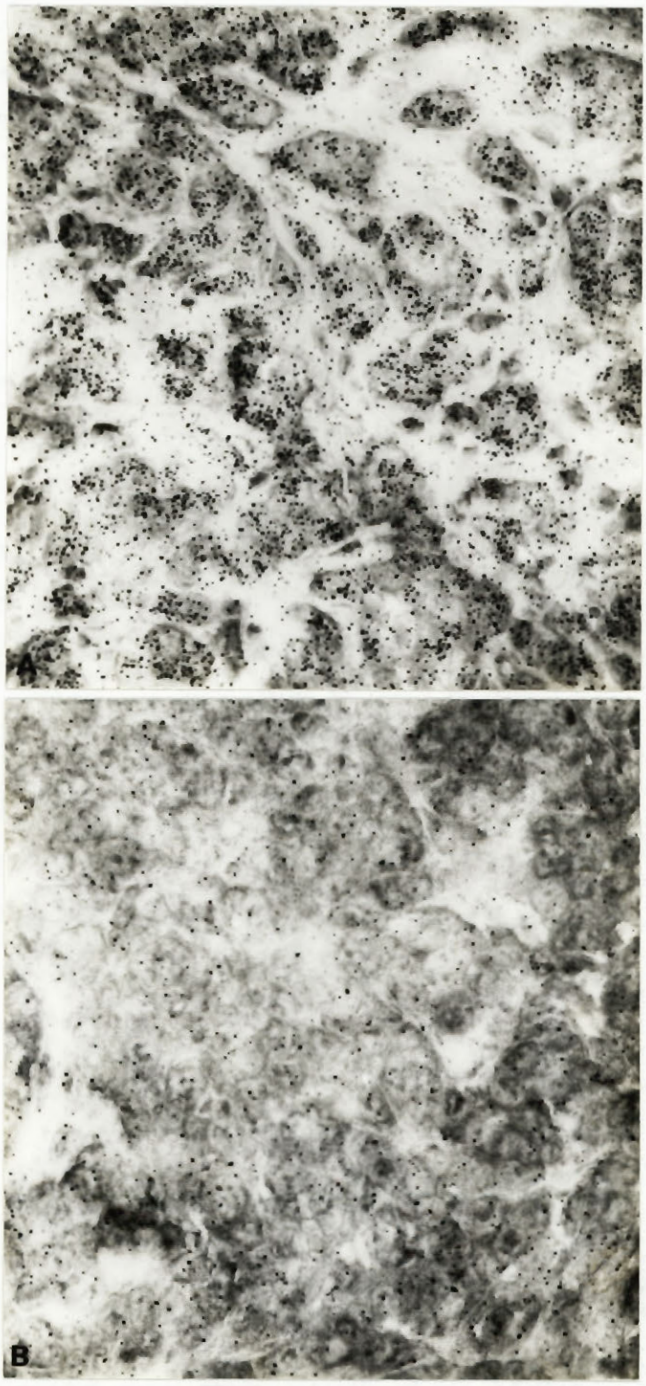


Figure IV-2. High power view of an estrogen receptor-positive infiltrating duct carcinoma (containing 107 fmols/mg protein) incubated in [3H]-estradiol only. In this nest of tumor cells one can appreciate the predominantly nuclear localization of radioactivity in putative target cells admixed with unlabeled cells. The variability of degree of labeling in apparent target cells is also evident. (800 X, hematoxylin and eosin, 78 days exposure)

Figure IV-2.

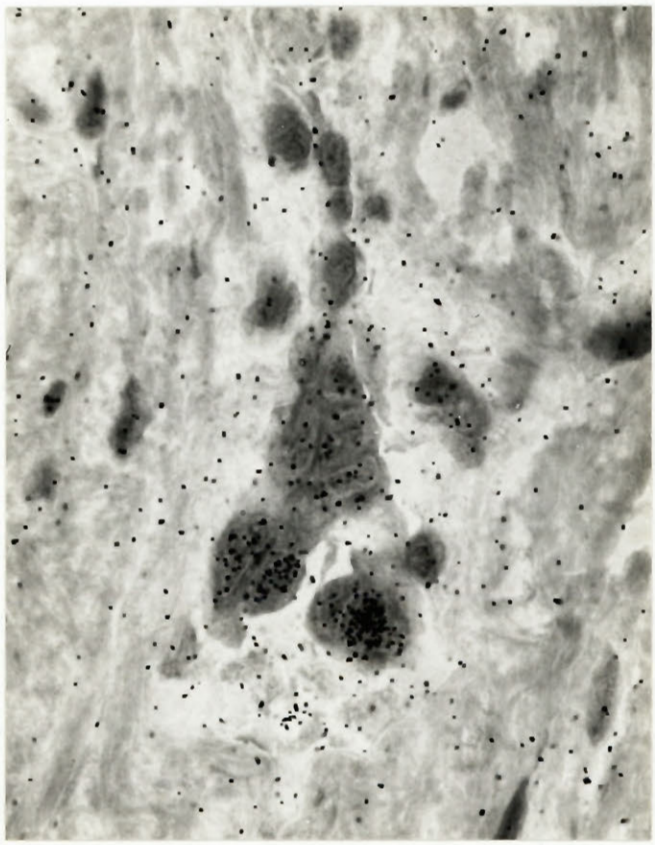
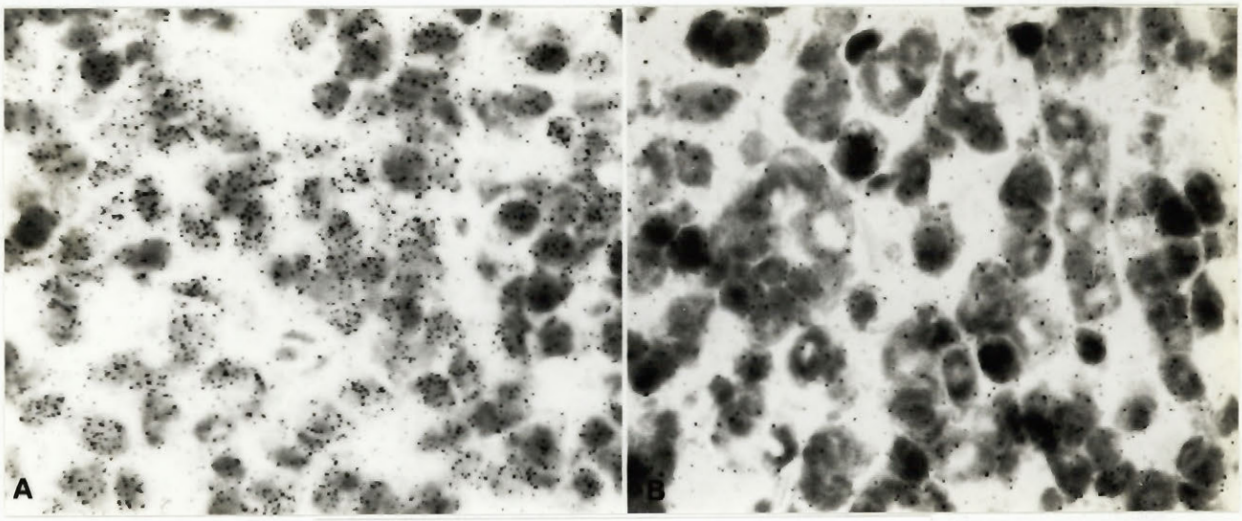


Figure IV-3A. Section of an estrogen receptor-positive breast carcinoma incubated in [3H]-estradiol only. Many, but not all, tumor cells reveal uptake and retention of [3H]-estradiol.

Figure IV-3B. Piece of tissue adjacent to the section illustrated in figure IV-3A. This tissue was incubated in 5 nM [3H]-estradiol with two-hundredfold excess of nonradioactive estradiol. The number of grains is greatly reduced when compared to figure IV-3A. (both figures 500 X, hematoxylin and eosin, 67 days exposure)

Figure IV-3C. Histogram depicting the variability among 50 tumor cells in number of nuclear grains per 10 square microns nuclear cross-sectional area for tumor illustrated in figures IV-3A and B. The mean number of grains for tissue incubated in [3H]-estradiol plus excess unlabeled estradiol was 0.63 grains per 10 square microns. In tissue incubated with only [3H]-estradiol, all cells with 1.9 or more grains were considered target cells. Some cells demonstrated more than ten times the mean observed for tissue exposed to nonradioactive estradiol.

Figure IV-3.


A
B

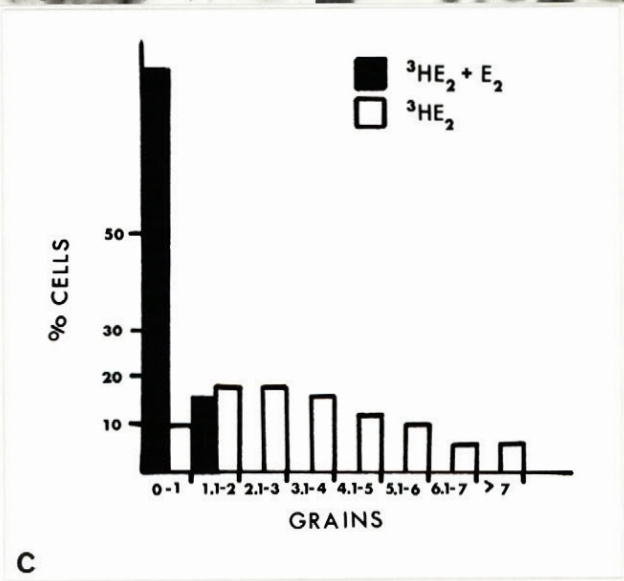

$^3HE_2 + E_2$
$^3HE_2$
% CELLS
50
30
20
10
0-1
1.1-2
2.1-3
3.1-4
4.1-5
5.1-6
6.1-7
> 7
GRAINS
C

Figure IV-4. A focus of intraductal carcinoma with central necrosis from an infiltrating lesion. Some, but not all, neoplastic cells within this area reveal specific labeling. Biochemical assay was not done on this lesion. (600 X, hematoxylin and eosin, 66 days exposure)

Figure IV-4.

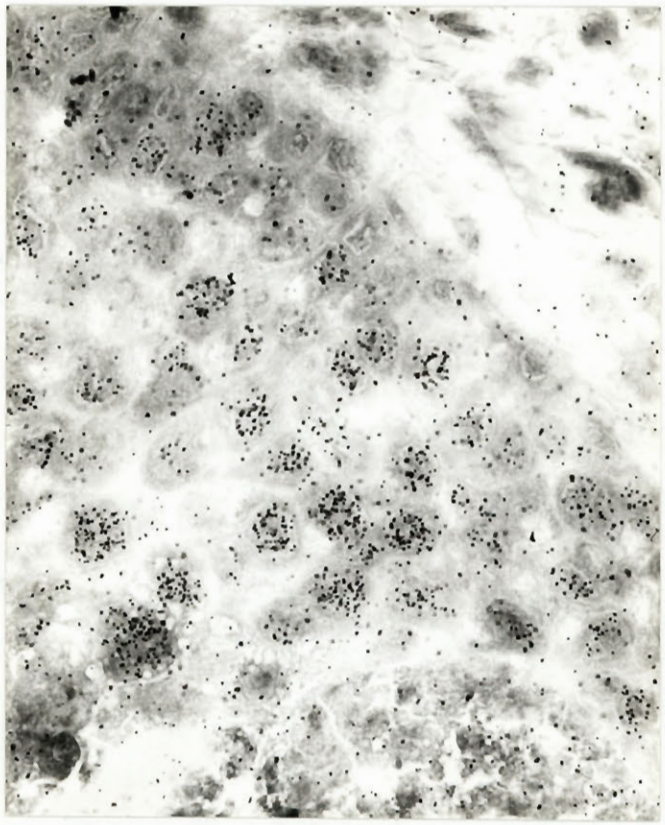


Figure IV-5. Section of estrogen receptor-positive carcinoma incubated in [3H]-estradiol demonstrating labeling of epithelial cells of nonneoplastic mammary ducts (arrows). An adjacent nest of malignant cells (arrowhead) is also labeled. This uptake of [3H]-estradiol was greatly reduced in an adjacent piece of tissue exposed to excess DES. (500 X, hematoxylin and eosin, 84 days exposure)

Figure IV-5.

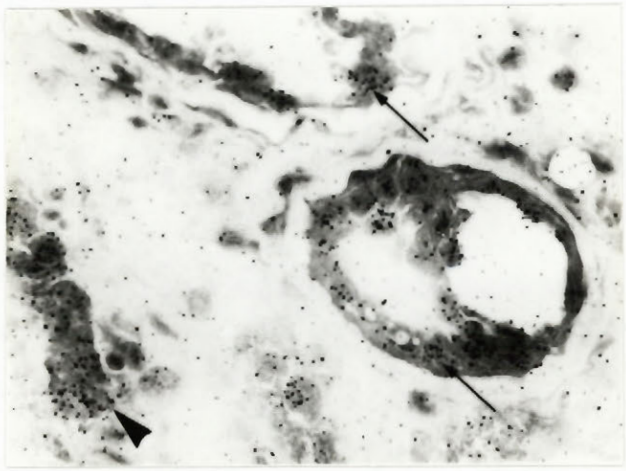
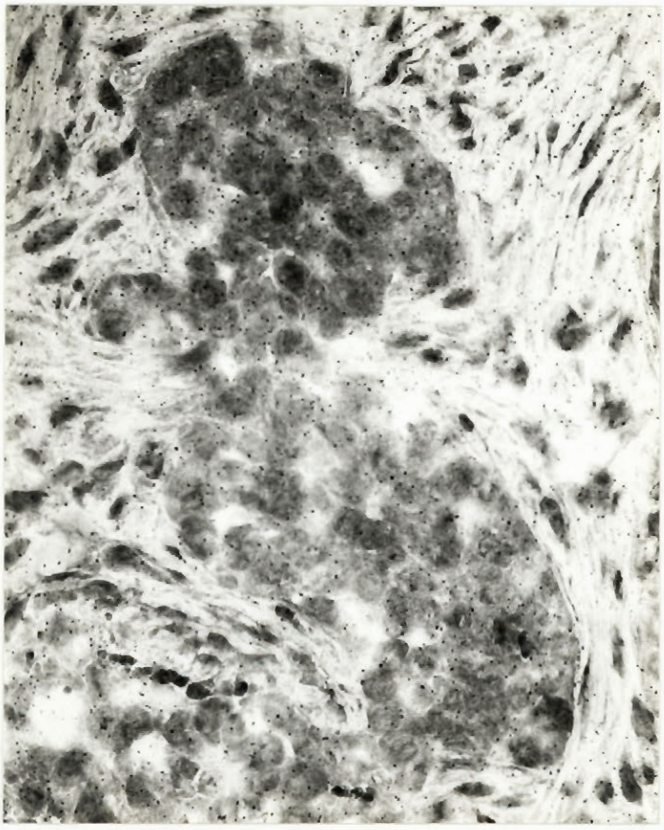


Figure IV-6. Section from an estrogen receptor-negative carcinoma (5 fmols/mg protein). The appearance of this autoradiograph is similar to that of adjacent tissue exposed to excess unlabeled estradiol. The grains are sparse and randomly scattered with no evidence of a nuclear localization. (400 X, hematoxylin and eosin, 203 days exposure)

Figure IV-6.



V. GENERAL DISCUSSION

Autoradiography has been used extensively for the localization of steroid binding sites in tissues from experimental animals after *in vivo* injection of radioactive ligands (for review see 149). The accuracy of the autoradiographic method has been thoroughly assessed (147,148). Those studies have provided important information on the identification and distribution of putative target cells. When combined with relevant biochemical data, the autoradiographic observations have aided in clarifying the physiologic role of steroid hormones. The application of autoradiographic studies to human tissues, however, would require *in vitro* incubation of fresh tissue since *in vivo* injections of radioactive ligands are not feasible.

In vitro studies are complicated by possible technical problems not encountered in *in vivo* work (143). As discussed in chapter II, the potential artifacts were investigated using the normal mouse uterus, a well-characterized estrogen target organ. The use of a wash was found to be necessary to decrease nonspecific binding of the ligand, one of the problems associated with *in vitro* studies. Such a procedure is, of course, not necessary with *in vivo* studies since, in most instances, the tissue is removed 1-2 hours after injection of the steroid when free and loosely bound

steroids have been cleared, and significant radioactivity remains only in target organs (119). With *in vitro* studies it appears that a wash is necessary to simulate this *in vivo* clearance mechanism. The use of albumin in that wash was justified by its ability to greatly decrease the nonspecific background labeling. As was noted in chapter IV, however, inclusion of albumin in the wash may have resulted in some loss of sensitivity of the method. Whether perfusion of the tissue with medium only, as used by Shannon et al (132), would result in an improved sensitivity is unknown in the absence of correlative biochemical studies. It should be noted, moreover, that, *in vivo,* target tissues are perfused with serum containing albumin. The present method, therefore, seems more analogous to physiological conditions than a method using a wash in medium only.

In considering this *in vitro* autoradiographic method, it is important to determine if specifically labeled cells do indeed represent estrogen target cells. The close agreement between the autoradiographic and biochemical assessment of breast carcinomas (chapter IV) would certainly support the validity of the method. In chapter II it was shown that specific labeling was present in the appropriate cell types of the normal mouse uterus, an estrogen target organ. Proper tissue

distribution was demonstrated by the absence of labeling in the diaphragm, a nontarget organ. Although formal competitive inhibition studies were not done, proper steroid specificity was demonstrated by suppression of labeling by radioinert estradiol and DES (in concentrations one-hundredfold to two-hundredfold greater than [3H]-estradiol) and lack of suppression by progesterone and by hydrocortisone in similar excess. This data is consistent with that expected for binding to estrogen receptors. In these experiments saturation analysis and demonstration of high affinity were not done. Such studies have recently been carried out by others using a similar in vitro autoradiographic method (22,23) thus lending credence to the assertion that specifically labeled cells identified in the present investigation represent potential estrogen target cells. Further support comes from the observation that the bound radioactivity demonstrated has remained after the wash with bovine serum albumin. As previously noted (chapter II), this ability of cells to take up and retain estrogen first suggested the presence of high affinity binding substances (60) and remains an important characteristic of target cells (64).

Additional support for the validity of the method comes from the finding of predominantly nuclear labeling

in the putative target cells. Indeed, in the studies with human tissue (chapters III and IV), the presence or absence of specific nuclear labeling was used to identify target and nontarget cells. The nuclear localization of specific labeling is consistent with current theories on the intracellular localization of estrogen receptors whether one accepts the classical theory or more recently proposed hypotheses. According to classical theory (43,62), it has been assumed that, during the course of the incubation (at 30°C), the ligand has bound the cytosolic receptor, and the resulting complex has undergone a temperature-dependent transformation and translocated to the nucleus. The observed nuclear localization would also be consistent with more recently proposed hypotheses (68,84,133,164) suggesting that even the unliganded (as well as liganded) ER are nuclear. Because the number of specific nuclear grains has been used to identify target cells, however, there is the possibility that some labeling represents nuclear type II sites. Since these sites correlate well with true uterine growth (81) and the presence of progesterone receptors in human mammary carcinomas (151), even this possibility should not diminish the physiologic importance of the nuclear labeling observed. In summary, there seems to be ample justification for considering

specifically labeled cells as potential estrogen target cells. Whether these cells are capable of responding to estrogens would require further investigations demonstrating an estrogenic effect characteristic of the tissue (e.g. synthesis of progesterone receptors). Such studies are beyond the scope of this project.

Conversely, the absence of specific labeling in a cell would imply that it is not an estrogen target cell. Thus, as noted, cells in the mouse diaphragm, a classic nontarget tissue, failed to incorporate [3H]-estradiol after in vitro incubation. Since, when possible, areas with positive cells were selected for assessment, inadequate diffusion of the ligand could not explain an absence of label. As was noted in chapter IV, however, target cells may have failed to demonstrate significant label as a result of cell death, endogenously bound estrogen, lack of sensitivity of the method, or low levels of ER due to nuclear processing. Such factors would presumably have been apparent to some extent in studies on the mouse uterus, however, where cellular heterogeneity for ER was not as apparent. It would seem, therefore, that an absence of specific labeling indicates in most instances a nontarget cell.

This study has demonstrated that in 41 percent of noncancerous human female breast biopsies a variable

proportion of epithelial cells contained specific estrogen binding sites. In 78 percent of human mammary carcinomas greater than 25 percent of the malignant epithelial cells demonstrated specific labeling. The presence of estrogen binding sites in these cells suggests they are potentially subject to the regulatory control mechanisms of estrogen action and, therefore, may represent a better differentiated cell population. Indeed, there are many studies suggesting that for human mammary carcinoma, estrogen receptor-positive tumors are, in fact, better differentiated histologically and clinically.

In studies relating various pathologic features of breast cancer with the presence of ER, better differentiated tumors more often contain ER than poorly differentiated lesions (37,83,85,95,106,157). Histologically better differentiated lesions (lesions with tubular or adenoid cystic components) are more likely to contain significant levels of ER. Tumors with marked necrosis, a feature often associated with poor differentiation, tend to be ER-negative. The nuclear grade seems to relate even more closely with the presence of steroid hormone receptors. Tumors with "better differentiated" nuclei tend to be ER-positive. A correlation is evident at the ultrastructural level as

well where tumors with features of differentiation such as intracellular canaliculi, junctional complexes, and gland formation with secretion often contain ER and PgR (85). (It is interesting to note that many of these ultrastructural features can be interpreted as representing differentiation in an epithelial rather than myoepithelial direction. It was shown (chapter III) that histologically identifiable myoepithelial cells fail to contain specific estrogen binding sites.)

A similar concept has emerged from studies on the thymidine labeling index in relation to ER status in mammary carcinoma (40,92,93,136). Meyer et al (92,93) have demonstrated a good correlation between the presence of estrogen receptor and low thymidine labeling indices in human mammary carcinoma. They have also shown that high thymidine labeling indices were associated with histologic features of poor differentiation such as nuclear anaplasia and necrosis. High thymidine labeling indices were also noted to be associated with inoperability or early recurrence of the lesion. Meyer and Hixon (92) have suggested that the lack of ER may be associated with early recurrence because of high rates of cellular replication.

Clinical studies have demonstrated that estrogen receptor-positive lesions tend to behave in a

biologically less aggressive fashion than negative tumors (29,31). Thus there is a longer time to recurrence in receptor-positive lesions and these are, as expected, more amenable to hormonal therapy. Whether they respond better to chemotherapy is as yet not established (29). It is evident from these pathological and clinical studies, therefore, that the presence of ER in malignant mammary epithelial cells is suggestive of a better degree of cellular differentiation than when ER is lacking.

In this investigation ER-positive tumors were found to contain a heterogeneous population of target and nontarget cells. The significance of this heterogeneity in terms of patient response to hormonal therapy is largely unknown. It is not surprising, however, that breast cancers would be heterogeneous for ER since tumor cell heterogeneity seems to be a general property of many neoplasms (49). In mammary carcinoma the heterogeneity for ER may indicate different subpopulations of tumor cells. It may also be that the individual tumor cells are undergoing modulations of ER levels with time. It has been shown that in MCF-7 cells the estrogen receptor has a half-life of 3 to 5 hours and receptor synthesis is rapid (30). These rates suggest that tumor cells can rapidly regulate ER levels. Thus, while estrogen receptor-positive mammary carcinomas contain a

heterogeneous population of ER-positive and negative cells, it is not established if the ER-negative cells remain as such or if they are able to regain ER and thus the capability of responding to estrogenic stimulation. The effect of therapy on the degree of tumor cell heterogeneity for ER in ER-positive mammary carcinomas is also largely unknown. Additional work is needed both to assess the potential clinical utility of any histochemical assay for ER and the significance of tumor cell heterogeneity for ER in terms of clinical response to hormonal therapy.

VI. CONTRIBUTIONS TO KNOWLEDGE

1. A systematic autoradiographic study of the localization of estrogen binding sites in benign and malignant human mammary tissue.
2. A comparison of this autoradiographic data in human breast cancer with biochemical assays.
3. Use of this autoradiographic technique to demonstrate:

    a. A heterogeneous population of putative estrogen target and nontarget cells in human mammary carcinoma.

    b. Localization of estrogen binding sites in a variable percent of epithelial cells in some benign human mammary lesions.

    c. The absence of estrogen binding sites in human mammary myoepithelial and stromal cells.

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