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Cancer Res 1984;44:415-421.

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Comparison of Histochemical and Biochemical Assays for Estrogen Receptor in Human Breast Cancer Cell Lines¹

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ABSTRACT

Two human breast cancer lines, MCF-7 and T47D cells, were investigated for the presence of estrogen receptor (ER) by biochemical and histochemical techniques. Using the dextran-coated charcoal technique and isoelectric focusing, MCF-7 cells were ER positive, and T47D cells were ER negative. Fluorescein conjugates to 17 β -estradiol by the sixth carbon (17 β -estradiol-6-carboxymethyloxime:bovine serum albumin:fluorescein isothiocyanate and 17 β -estradiol-6-iminoxyacetylfluoresceinamine) and by the 17th carbon {N-fluoresceino-N'-[17 β -(estradiol hemisuccinamide)ethyl]thiourea, 17-FE} were prepared for cytochemical evaluation of the ER status of the two cell lines. The binding affinity of the estradiol conjugates for ER varied, the 17-FE conjugate having the highest affinity of 0.08 relative to 17 β -estradiol. Following incubation with 10 nM 17-FE, both MCF-7 and T47D cells displayed cytoplasmic and nuclear fluorescent staining. Isoelectric focusing of MCF-7 cytosol incubated in the presence of 10 nM 17-FE revealed binding of the fluorescein conjugate to a protein species which did not bind 17 β -[³H]-estradiol. Isoelectric focusing of T47D cytosol revealed binding of 17-FE to two protein components, neither one of which showed specific binding of 17 β -[³H]estradiol. The results suggest different protein binding species for fluoresceinated estradiol conjugates and [³H]estradiol and help to explain reported differences in histochemical and biochemical ER analyses.

INTRODUCTION

When estradiol enters a target cell, it rapidly complexes to a specific cytoplasmic estrogen-binding protein, commonly referred to as ER.² The estradiol:receptor complex translocates into the nucleus, where it binds to chromatin and induces gene activation and an acceleration of biosynthetic processes (36). Although all 3 steps (binding to cytoplasmic receptor, translocation to the nucleus, and association with chromatin) are essential for attaining the biological response of the estrogen-responsive tissue, in the majority of breast cancer patients, only the hormone binding to the cytoplasmic receptor is currently being evaluated.

While biochemical assays for ER may indicate the presence of steroid binding capacity per unit weight of tumor tissue protein, they cannot identify the cellular origin of the receptors. In addition, the presence of protein-containing connective tissue provides a source of error not easily corrected for in the receptor assay. Another drawback of the biochemical assay is the amount of tissue (0.2 to 0.5 g) needed for accurate determination of

binding. With newer mammographic techniques, breast tumors of less than 10 mm can be detected which would not allow a biochemical assay because of lack of tissue. Finally, since the tissue for analysis is consumed in the assay, the presence of malignant tumor cannot be completely verified by histological examination.

Because of these disadvantages inherent in the biochemical assay, several laboratories have undertaken the development of immunofluorescent and cytochemical techniques for the morphological demonstration of ER. The correlation between morphological techniques and the standard biochemical assays for ER has been variable. Pertschuk *et al.* (40, 41), for example, found a good correlation of their immunofluorescent method with the dextran-coated charcoal method, while Lee (24, 25) noted a lack of correlation between his cytochemical method and the biochemical assay. A comparison of the dextran-coated charcoal method with the immunohistochemical polyestradiol phosphate technique yielded a 89% correlation in one laboratory (40) but no correlation at all in another laboratory (32). These disparate results of biochemical and morphological techniques are further confounded by an apparent lack of agreement between the individual morphological methods. For example, in the fluorescent steroid histochemical techniques (25), myometrium is used as a positive control, while the peroxidase:antiperoxidase method fails to stain myometrial cells (23). 6-FE may be applied directly to formalin-fixed tissue (12), while the immunocytochemical methods require incubation with estradiol prior to fixation to stabilize receptor activity (23). In one study, the intensity of fluorescence of the normal mammary glandular epithelium was chosen as an intrinsic standard for grading the cancer cells which were considered ER positive when they fluoresced as intensely or stronger than the normal epithelial cell and classified as ER negative when they showed weaker fluorescence (24). In contrast, none of the other investigators indicated positive staining of normal mammary gland epithelium. In conclusion, there is a surprising lack of agreement between the individual morphological assays on the one hand and between the morphological and biochemical on the other.

To resolve some of the discrepancies between morphological and biochemical techniques of ER determinations, we chose 2 human breast cancer cell lines: MCF-7 cells which are ER positive and T47D cells which are ER negative by biochemical assay. By eliminating the interference of stromal nonepithelial tissue elements present in surgical breast cancer specimens, we could directly assess the receptor content of the malignant cells using both morphological and biochemical techniques.

MATERIALS AND METHODS

Specific Chemicals

The following compounds were purchased from the sources indicated: 17 β -[2,4,6,7-³H]estradiol (specific activity, 101.7 Ci/mmol) and Omnifluor

¹ This work was supported by Biomedical Research Support Grant RR05424 and American Cancer Society Institutional Research Grant IN-25T.

² The abbreviations used are: ER, estrogen receptor; 6-FE, 17 β -estradiol-6-iminoxyacetylfluoresceinamine; 6-FE-BSA, 17 β -estradiol-6-carboxymethyloxime:bovine serum albumin:fluorescein isothiocyanate; 17-FE, N-fluoresceino-N'-[17 β -(estradiol hemisuccinamide)ethyl]thiourea; PBS, phosphate-buffered saline.

Received December 20, 1982; accepted October 3, 1983.

(New England Nuclear, Boston, Mass.); Dextran T-70 and Sephadex G-75 superfine resin (Pharmacia Fine Chemicals, Piscataway, N. J.); Ampholine (pH 3.5 to 10; LKB-Produktor, Stockholm, Sweden); and Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

All other chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Fisher Scientific Co. (Pittsburgh, Pa.).

Tritiated estradiol was purified in a reverse-phase C₁₈ Sep-Pak cartridge (Waters Associates, Milford, Mass.).³ Stock solutions of tritiated estradiol were prepared in ethanol and stored at -20° in the dark.

Cell Culture

MCF-7 cells are a human breast cancer cell line which was kindly supplied to us in living culture at passage 143 by Dr. Charles McGrath of the Michigan Cancer Foundation. T47D cells are a human breast cancer cell line grown in continuous culture, which was provided at passage 88 by the Breast Cancer Task Force Cell Culture Bank, EG & G/Mason Research Institute, Worcester, Mass. Both cell lines were grown in Iscove's modified Dulbecco's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with insulin (10 µg/ml) and 10% calf serum at pH 7.2 to 7.4 in a humidified atmosphere with 7.5% CO₂ at 37°. Fluorochrome Hoechst 33258 (9) was used routinely to screen cells for *Mycoplasma*. Experimental cells were grown in insulin-supplemented Iscove's modified Dulbecco's medium containing 5% calf serum stripped of endogenous hormones by 30-min incubation at 45° with a dextran-coated charcoal pellet (0.25% activated charcoal:Norit A:0.0025% dextran in 0.01 M Tris-HCl, pH 8.0, at 4°, 1 ml/ml serum).

Cell Harvest

When cells had grown to approximately 90% confluency, they were removed by standard trypsinization or by a 10-min incubation at 37°C with 1 mM EDTA in Ca²⁺-, Mg²⁺-free Hanks' balanced salt solution. The cells were washed twice in Tris buffer (10 mM Tris-HCl:1.5 mM EDTA:10 mM monothioglycerol:10% glycerol, pH 7.5).

Preparation of Cytosol

All experiments were performed at 4°. Cells were resuspended in Tris buffer (3 ml buffer per ml packed cells) and homogenized in a glass-Teflon homogenizer until they were more than 90% disrupted as estimated by phase-contrast microscopy or trypan blue exclusion. The homogenate was centrifuged for 30 min at 10⁵ × g in a Beckman SW 60 Ti rotor. The supernatant cytosol was separated from the pellet and immediately used in the appropriate experiment.

Isoelectric Focusing

Isoelectric focusing of the ER was performed by adapting the method of Boyd and Spelsberg (3) for the analysis of the progesterone receptor. Sephadex G-75 superfine resin was prewashed with distilled water, followed by washes with absolute ethanol on a Pyrex glass filter funnel (porosity, 40 to 60 µm). The resin was dried on the funnel overnight using continuous suction. Five g of the resin were mixed with 95 ml of the Tris buffer, pH 7.5, and 5 ml of LKB (pH 3.5 to 10) Ampholine solution (40% w/v). The final concentration of Ampholine was 2% (w/v). The slurry was poured onto an LKB isoelectric-focusing glass plate rimmed by silicone rubber (23 x 11 cm). Electrode strips soaked in Tris buffer:Ampholine solution had been placed at the ends of the isoelectric-focusing plate beforehand. The slurry was dried with a light stream of cool air until the weight (*i.e.*, water content) decreased by 25%. The plate was then placed on the cooling unit (0°) of an LKB 2117 Multiphor. An electrode strip was soaked in 1 M H₃PO₄ and placed at the anodal end of the plate on top of the other strips. Similarly, a second electrode strip was soaked in 1 M NaOH and placed at the cathodal end of the plate. The cytosol sample was mixed with the slurry, applied in pH 7 to

³ H. Meyer, personal communication.

8 region of the plate, and focused for 8 hr at 8 watts/plate at 0°. Following focusing, the resin was sectioned into 30 fractions, and the pH gradient was determined with a flat membrane electrode (Ingold Electronics, Lexington, Mass.) placed on the surface of alternate fractions. Each section was eluted through plastic columns with 9 ml of Tris buffer, pH 7.5, at 4°. The eluants were analyzed for specifically bound 17β-[³H]estradiol by the dextran-coated charcoal method and for fluorescein by spectrophotometric analysis at 495 nm.

Analysis of Specifically Bound [³H]Estradiol

Bound 17β-[³H]estradiol was measured by means of a multiple-point dextran-coated charcoal assay modified from the method of Korenman and Dukes (22). All steps were performed at 4° unless otherwise specified.

The concentration of bound 17β-[³H]estradiol in tumor cell cytosol was determined by incubating a constant volume of cytosol (150 µl) for 20 hr at 4° with 5 different concentrations of labeled steroid, ranging from 0.15 to 3.0 nM (final assay concentration). Nonspecific binding was determined in a parallel assay by including a 200-fold molar excess of diethylstilbestrol in 3 of the 5 assay points. At the end of the incubation period, 0.6 ml dextran-coated charcoal solution (0.25% activated charcoal:Norit A:0.0025% dextran in 10 mM Tris-HCl:1.5 mM EDTA:10% glycerol, pH 7.5) was added to each reaction tube. The tubes were vortex mixed, incubated for 20 min, and then centrifuged at 2400 × g for 10 min to pellet the charcoal. An aliquot of the supernatant was added to 8 ml scintillation fluid (0.7 liter toluene, 0.3 liter Triton X-100, and 4.0 g Omnifluor). The radioactivity was counted for 10 min in a Beckman LS-233 liquid scintillation counter at a counting efficiency of 36%. All counts were corrected to 100% efficiency by external standardization.

The concentration of specifically bound 17β-[³H]estradiol present in the eluants of the isoelectric-focusing fractions was determined indirectly as the difference between total and nonspecific binding. Tubes containing 2 ml of each fraction eluant were incubated either alone at 4° to assess the total bound radioactivity or in the presence of a 200-fold excess of unlabeled estradiol at 37° for 60 min to exchange the labeled steroid:receptor complexes with unlabeled steroid (3, 6). Following incubation, the heated tubes were recooled to 4°, and dextran-coated charcoal pellets were prepared by precipitating 1.5 ml of charcoal suspension at 2400 × g for 10 min. The tubes containing the charcoal pellets were placed on ice, and 1.5 ml from the incubation tubes was added, followed by vortex mixing, incubation for 20 min, and centrifugation at 2400 × g for 10 min. A 1.0-ml aliquot of each supernatant was counted in scintillation fluid as described above.

The protein concentration of the cytosol and of the isoelectric focusing eluant fractions was determined by the Bio-Rad protein assay, a modification of the method of Bradford (4), using recrystallized bovine serum albumin as a standard.

Fluorescent-labeled Steroid Derivatives

6-FE:BSA. 6-Keto-17β-estradiol was synthesized from 17β-estradiol diacetate by the method of Longwell and Wintersteiner (28). Synthesis of the corresponding 17β-estradiol-6-carboxymethyloxime was by a modification of the method of Erlanger *et al.* (15) described by Lee (25). Identification of 6-keto-17β-estradiol and 17β-estradiol-6-carboxymethyloxime was carried out by melting point determination and thin-layer chromatography on silica gel plates (solvent system, ethyl acetate:benzene:ethanol:acetic acid, 30:30:10:0.3).

The highly reactive carboxyl group of the oxime was coupled to bovine serum albumin:fluorescein isothiocyanate as described by Dean *et al.* (13). Bovine serum albumin:fluorescein isothiocyanate was either purchased (Sigma) or synthesized according to the method of Rinderknecht (43). The bovine serum albumin:fluorescein isothiocyanate complex was passed through a 2.8- x 18-cm Sephadex G-25 column equilibrated with

0.02 M sodium phosphate buffer, pH 6.5, and dialyzed against the same buffer to assure complete removal of any loosely bound fluorescein isothiocyanate. The ratio of fluorescein to bovine serum albumin was calculated from absorbance measurements at 280 and 495 nm. The molar ratio of steroid to fluorescein was determined by absorbance measurement at 340 and 495 nm, respectively, using the unconjugated oxime and fluorescein isothiocyanate as standards.

6-FE. This was obtained by reacting the *O*-carboxymethylhydroxylamine derivative of 6-keto-17 β -estradiol with fluoresceinamine in the presence of dicyclohexylcarbodiimide as described by Dandliker *et al.* (12). The product moved as one spot on thin-layer chromatography plates using chloroform:ethanol:water, 70:28.5:1.5, as a solvent system.

17-FE. A 17 β -estradiol:fluorescein derivative with fluorescein linked to position 17 of the steroid was prepared by attaching fluorescein isothiocyanate to 17 β -estradiol via a succinamide:ethylamine bridge, forming 17-FE (1). Briefly, 3-acetyl-17 β -estradiol hemisuccinate was obtained from 17 β -estradiol via 17 β -estradiol:hemisuccinate. In the presence of dicyclohexylcarbodiimide and ethylenediamine, 3-acetyl-17 β -estradiol:succinamide:ethylamine is formed. Fluorescein isothiocyanate is then introduced, the acetyl group is cleaved, and the final product is purified by thin-layer chromatography in 2 solvent systems, ethylacetate:methanol, 80:20, and chloroform:methanol, 95:5 (1). The fluorescein concentration of the purified fluoresceinated estradiol derivatives, 6-FE and 17-FE, was determined by absorbance measurement at 495 nm, using fluorescein isothiocyanate as standard. The steroid concentration was determined by a modified Kober reaction using 17 β -estradiol as standard (2).

Competition Experiments

To determine the relative binding affinity of the fluoresceinated ligands to ER, cytosol was incubated with increasing concentrations of FE (10^{-10} to 10^{-6} M) in the presence of a fixed, saturating dose of 17 β -[3 H]estradiol (5 nM). Bound and unbound fractions were separated using dextran-coated charcoal. Results are expressed as the percentage of reduction in 17 β -[3 H]estradiol binding produced by FE as compared with the reduction produced by a 200-fold molar excess of diethylstilbestrol (21).

Cytochemical Localization of Estrogen Binding

MCF-7 and T47D cells were stained for ER in 2 ways, either attached to coverslips or as a cell suspension.

The cell suspension was obtained by harvesting the cells, washing with Tris buffer, and resuspending in the same buffer at a concentration of approximately 1.0×10^6 cells/ml. The cells were incubated with 17-FE, the ligand with the highest affinity for ER. The stock solution of 17-FE (1×10^{-3} M) was diluted with Tris buffer just prior to use to achieve a final ligand concentration of 10^{-7} to 5×10^{-9} M. The time of incubation was 60 min, and the temperature was 4, 22, or 37 $^\circ$. Following the incubation, the cells were washed twice with cold PBS 7.74 g NaCl:0.55 g KCl:14.0 g Na $_2$ HPO $_4$:5.46 g NaH $_2$ PO $_4$ per liter H $_2$ O, pH 7.3) and spread on slides with a cytocentrifuge. The slides were mounted in 50% buffered glycerol in PBS.

MCF-7 and T47D cells were also grown on coverslips. Before reaching confluence, they were washed carefully with cold PBS, fixed for 30 min in 8% formalin:2.5% picric acid in 0.1 M phosphate buffer, pH 7.5, and washed again in PBS. The cells were incubated for 60 min at 4 and 37 $^\circ$ with 17-FE at a final concentration of 10^{-8} M in PBS. Following the incubation, the cells were washed carefully twice with cold PBS and mounted in 50% buffered glycerol in PBS, pH 7.3.

The slides were examined with a Leitz Orthoplan microscope equipped with an Osram HBO 100-watt superpressure mercury lamp and a Leitz Ploemopak 2 vertical illuminator. The appropriate combination of exciting filters (KP 490), dichroic beam-splitting mirror (TK 510), and barrier filter (K 510) allowed the selective visualization of fluorescein. The fluorescent microscopic findings were recorded on Ilford PAN F black and white film.

RESULTS

Typical dose competition curves obtained with the fluorescein-labeled estradiol derivatives are illustrated in Chart 1. Comparing the inhibition curves with that of unlabeled estradiol itself shows that all the substituted estradiol derivatives have weaker binding affinity for ER than native estradiol. The ligand with the highest affinity, 17-FE, has a binding affinity of 0.08 relative to 17 β -estradiol. The other ligands have even weaker binding affinities.

Demonstration of Fluorescence in Tumor Cells. MCF-7 and T47D cells were stained for ER in either a fixed or unfixed state. Following fixation on coverslips, both cell lines yielded similar staining patterns (Figs. 1 and 2). When the tumor cells were incubated at 4 $^\circ$ for 1 hr with 1×10^{-8} M 17-FE, a diffuse or lace-like pattern of fluorescent staining was observed in the cytoplasm. The nuclei were spared or showed hazy and faint fluorescence. Frequently, the nucleoli displayed conspicuous fluorescent staining. Variation of the temperature to 37 $^\circ$ failed to reliably alter the cellular distribution of staining. At both temperatures, the intensity of the fluorescence varied only slightly between cells on the same slide. While the MCF-7 and T47D cells each displayed their typical growth pattern, no significant difference in distribution or intensity of fluorescence staining was noted upon close examination of the 2 cell populations.

To rule out the possibility of artificially increasing the cellular permeability to 17-FE by fixation, cells were also investigated as a cell suspension spread on slides with a cytocentrifuge. Due to the different treatment of the cells, their morphological appearance in the cytocentrifuge preparation differed from that of the coverslip culture.

The fluorescence staining pattern, however, again failed to show a reproducible difference between the 2 cell lines. Both MCF-7 and T47D cells displayed cytoplasmic and/or nuclear fluorescence when 1×10^{-8} M 17-FE was utilized as a ligand (Figs. 3 and 4). Variation of temperature (4, 22, 37 $^\circ$) did not lead

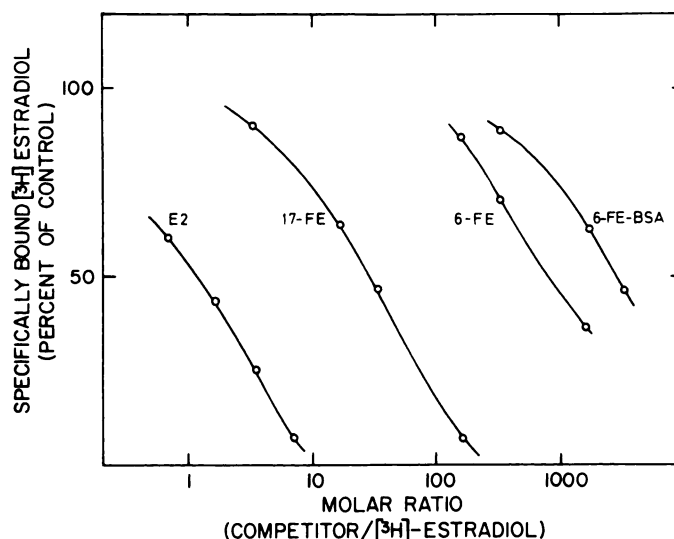


Chart 1. Relative competition of unlabeled 17 β -estradiol and fluorescent-labeled estradiol for 17 β -[3 H]estradiol binding sites in MCF-7 cytosol. Cytosol was incubated with 5 nM 17 β -[3 H]estradiol and various amounts of excess competitors. Results are expressed as the percentage of reduction produced by the competing ligand as compared with the reduction produced by a 200-fold molar excess of diethylstilbestrol. Each curve depicts a summary of 3 experiments; variability between experiments was less than 10%. E2, unlabeled 17 β -estradiol.

to a discernible alteration in staining distribution but caused an apparent increase in staining intensity with temperature.

Dextran-coated Charcoal Assay and Isoelectric Focusing of Cytosol from MCF-7 Cells. The dextran-coated charcoal assay of MCF-7 cytosol revealed the presence of ER at a concentration of 87 fmol/mg cytosol protein. Isoelectric focusing of the cytosol incubated with 10 nM 17β -[3 H]estradiol displayed 2 species of ER. One species focused at a pI of 7.3, and the other focused at a pI of 6.8 (Chart 2). On the second peak, an

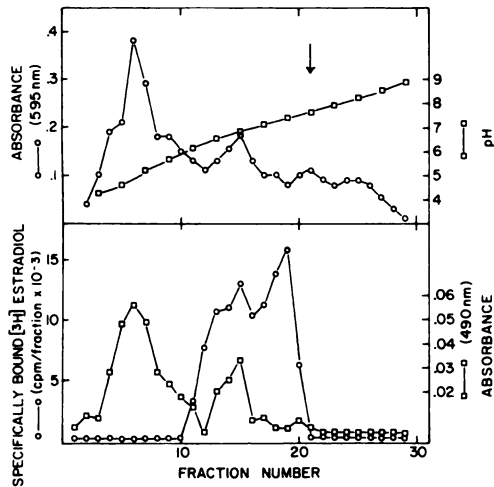


Chart 2. Isoelectric focusing of MCF-7 cytosol incubated with 5 nM 17β -[3 H]-estradiol or with 10 nM 17-FE. The cytosol was applied to the pH 7 to 8 region of the isoelectric-focusing plate (\square). The plates were focused for 8 hr at 8 watts/plate at 0°. Following the focusing, the gel was fractionated. The fractions were eluted, and the protein concentration of each eluate was assayed at 595 nm (\circ). Aliquots of each of the eluates were assayed for bound 17-FE by using the dextran-coated charcoal procedure and by measuring the absorbance at 490 nm (\square). Specifically bound 17β -[3 H]estradiol was measured as described in "Materials and Methods." Points, one of 4 experiments that gave essentially the same results.

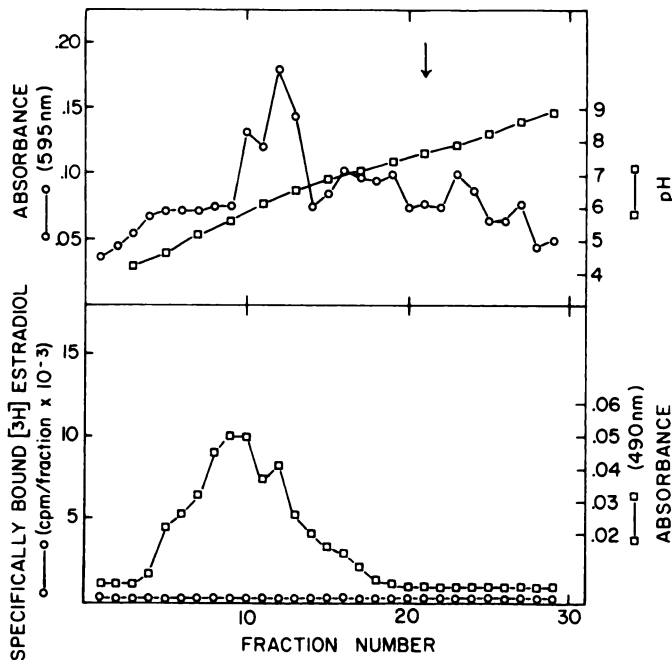


Chart 3. Isoelectric focusing of T47D cytosol incubated with 5 nM 17β -[3 H]-estradiol or with 10 nM 17-FE. Top, \square , pH of the fractions; \circ , protein concentration measured at 595 nm. Bottom, \square , 17-FE concentration; \circ , specifically bound 17β -[3 H]estradiol. Points, one of 5 experiments that gave essentially the same results.

anodal shoulder was observed, which could not be resolved by using crude cytosol. To verify that the isoelectric-focusing method was reflecting the true receptor, focusing was performed in the absence and presence of a 200-fold excess of unlabeled estradiol. Competition for specific binding of the 17β -[3 H]estradiol at both pIs occurred.

The 17-FE binding protein species of the MCF-7 cytosol focused primarily at a pI of 5.5 (Chart 2). A second, smaller peak was observed at a pI of 6.8, coinciding with one of the 17β -[3 H]estradiol binding species. No discrete focusing of 17-FE was noted at a pI of 7.3, pointing to a difference in ligand binding of 17-FE versus 17β -[3 H]estradiol.

Isoelectric Focusing of Cytosol from T47D Cells. Isoelectric focusing of T47D cytosol failed to show specific binding of 17β -[3 H]estradiol corresponding to the absence of detectable ER in the dextran-coated charcoal assay (Chart 3).

When the cytosol was focused in the presence of 17-FE, ligand binding to 2 protein components was observed at an acid pI of 6.0 to 6.5 (Chart 3).

DISCUSSION

The biochemical methods of ER determination fail to distinguish tumor cells from normal tissue components and do not provide information concerning the proportion of ER-positive malignant cells. These limitations have prompted the development of morphological methods for determining the ER status. The techniques for *in situ* localization of ER may be broadly classified into immunological methods using antibodies against estradiol and cytochemical methods using estradiol coupled to a fluorescein marker (Table 1).

The immunological methods are based on the assumption that the estradiol antibody is stereochemically capable of attaching to the steroid situated in the ER binding site. However, immunocytochemical studies using sucrose gradient analysis failed to detect the presence of antibody:estradiol:receptor complexes (31). In addition, radioimmunoassay methods to detect competition between cytosol ER and antibody for binding of estradiol also suggest that a complex of antibody:estradiol:receptor was not formed (7, 16, 17). These experiments involved the use of immobilized estradiol antibody, which was coupled to an insoluble polymer matrix and incubated with 17β -[3 H]estradiol. Upon addition of tumor cytosol, the ER would compete successfully with the antibody for any available hormone and decrease the amount of 17β -[3 H]estradiol bound to the polymer matrix. The migration of 17β -[3 H]estradiol into the cytosol in proportion to

Table 1
Proposed methods for the *in situ* localization of ER

Methods	
Immunological	
Immunofluorescent	
Estradiol:antiestradiol (33, 34)	
Polyestradiolphosphate:antiestradiol (40)	
Peroxidase:antiperoxidase	
Estradiol:antiestradiol (23)	
Cytochemical	
6-FE (12)	
6-FE-BSA (25)	
17β -Estradiol-6-carboxymethyloxime:bovine serum albumin:horseradish peroxidase (46)	
1-(N)-Fluoresceinyl:estrone:thiosemicarbazone (35, 42)	

the ER presumably reflects a lack of immune complex formation of antibody:estradiol:receptor (7, 17).

In view of these reservations concerning the validity of immunological methods of ER localization, this study concentrated on the cytochemical methods which offer the theoretical advantage of direct binding of the fluorescence-coupled ligand to the receptor. Since estradiol becomes chemically modified in the process of introducing the fluorescence marker, the affinity of the coupled steroid derivative for the receptor needs to be compared to that of native estradiol. The competition experiments performed in this study indicate that the affinity for the receptor of all fluoresceinated ligands is orders of magnitude lower than that of 17 β -estradiol. Even the affinity of the most avid ligand, 17-FE, is still one order of magnitude less than that of 17 β -[³H]estradiol. Similar results were obtained by McCarty *et al.* (29), who analyzed the relative binding affinity of several fluorescent-labeled ligands substituted at either the 17th or the sixth carbon.

Once a suitable fluorescein:conjugate of 17 β -estradiol has been synthesized, several fundamental questions need to be answered before the results of the morphological receptor examination can be equated with the biochemical ER determination. Are there limitations in standard tissue-processing techniques which may influence microscopic ER evaluation? Are the fluoresceinated ligands with their decreased binding affinity capable of binding to ER? Are they specific for ER, or do they also label other protein species? In conventional frozen sections, many cells are transected, permitting unrestricted diffusion of soluble proteins into the supernatant. Since the receptor is a soluble protein, an undetermined amount of ER will "leach out" of the cell of origin and distribute itself in the supernatant aqueous layer (45). Fixation of fresh tissue in aldehyde or alcohol fixatives will restrict the tendency of the receptor to diffuse but may lead to denaturation of ER and an increase in nonspecific binding (39, 45). In view of these factors, caution must be exercised in the interpretation of the histochemical findings. In this study, we utilized cell cultures uncontaminated by protein constituents from connective tissue, blood, and other cellular elements which may interfere with the interpretation of breast biopsy specimens stained for ER. To rule out the possibility of artificially increasing the cellular permeability to 17-FE by fixation, the cells were stained for ER in either a fixed or unfixed state. Although the shape of the fixed cells differed considerably from the appearance of the cells dispersed by the cytocentrifuge, there was no reproducible difference in staining intensity of 17-FE. Thus, at least under the experimental conditions of a uniform cell culture system, mild fixation does not contribute to artifactual staining.

ERs are characterized as high-affinity, limited-capacity binding sites with an equilibrium dissociation constant of approximately 10⁻¹⁰ M for 17 β -estradiol. At this concentration, nearly all the binding sites are occupied by hormone. At concentrations greater than 10⁻⁹ M, the limited capacity of the receptor sites is exceeded, and no matter how much more estradiol is added, the receptor molecules are saturated. Other types of cellular binding sites exist, however, which have lower affinity but greater capacity for estradiol (8, 37, 38).

Such lower-affinity, high-capacity binding sites become evident upon isoelectric focusing of cytosol from MCF-7 cells which was incubated with 17-FE at a concentration of 10⁻⁸ M. The fluorescein-labeled hormone binds to 2 protein species at pI 5.5 and 6.8, which would account for the positive cytochemical staining of the MCF-7 cell cultures with 17-FE. Only one of the 2 binding

proteins, the pI 6.8 species, can be characterized as ER with high-affinity binding for 17 β -[³H]estradiol at a concentration of 2 \times 10⁻⁹ M. At this concentration, 17 β -[³H]estradiol does not bind to the pI 5.5 protein, indicating a lower affinity of this binding species.

The MCF-7 human breast cancer cell line was derived from the pleural effusion of a woman with metastatic adenocarcinoma of the breast (44). These cells have been well characterized as containing ER (5, 10). In this study, the MCF-7 cytosol was ER positive by both the dextran-coated charcoal technique and isoelectric focusing. The T47D cell line has also been established from the pleural effusion of a metastasizing infiltrating ductal carcinoma of the breast (20). The cell line has been characterized biochemically as ER negative (20). In this study, the T47D cytosol was ER negative by both the dextran-coated charcoal technique and isoelectric focusing following incubation with 17 β -[³H]estradiol. When the T47D cytosol was incubated with 17-FE, isoelectric focusing revealed binding of the ligand to 2 protein components between pH 6.0 and 6.5, corresponding to the positive cytochemical staining of the T47D cells visualized under the fluorescent microscope.

The covalent attachment of fluorescent dyes to steroid hormones lowers the binding affinity to the receptor protein and necessitates the use of relatively higher ligand:conjugate concentrations than are ordinarily utilized for radiolabeled ligands in conventional biochemical systems (26). Because of the lowered binding affinity, the fluoresceinated estradiol binds to ER as well as other cellular proteins. Estrogen binding proteins, which have at least a 10-fold-lower affinity for estradiol than the classical ER, have been described by Panko and Clark (37) as so-called type II estrogen binding sites. The importance of the type II sites for hormone-dependent growth in breast cancer is as yet undetermined (38). Unlike the classical ER, type II binding proteins do not undergo translocation to the nucleus (11). In summary, the biochemical and histochemical experiments carried out in parallel in this study do not support the claim that the existing cytochemical methods of *in situ* ER localization are equivalent to established biochemical methods of ER determinations. The binding of fluoresceinated estradiol derivatives to binding proteins other than ER may help explain the discrepancies observed between the biochemical and morphological ER assays in breast cancer tissue. The difference in affinity for ER of the various fluoresceinated estradiol derivatives, in turn, appears to be responsible for the reported discrepancies among existing morphological methods.

The recent preparation of monoclonal antibodies to ER provides a novel approach to the biochemical and morphological detection of ER in human breast cancer (19). The monoclonal antibody reacts with ER without interfering with the ability of the receptor protein to bind or retain estradiol (18). Since the antibody appears to recognize antigenic determinants on the receptor molecule that are different from the hormone binding site, the determination of ER using monoclonal antibody needs to be correlated with current reference methods of biochemical ER determination (14, 30).

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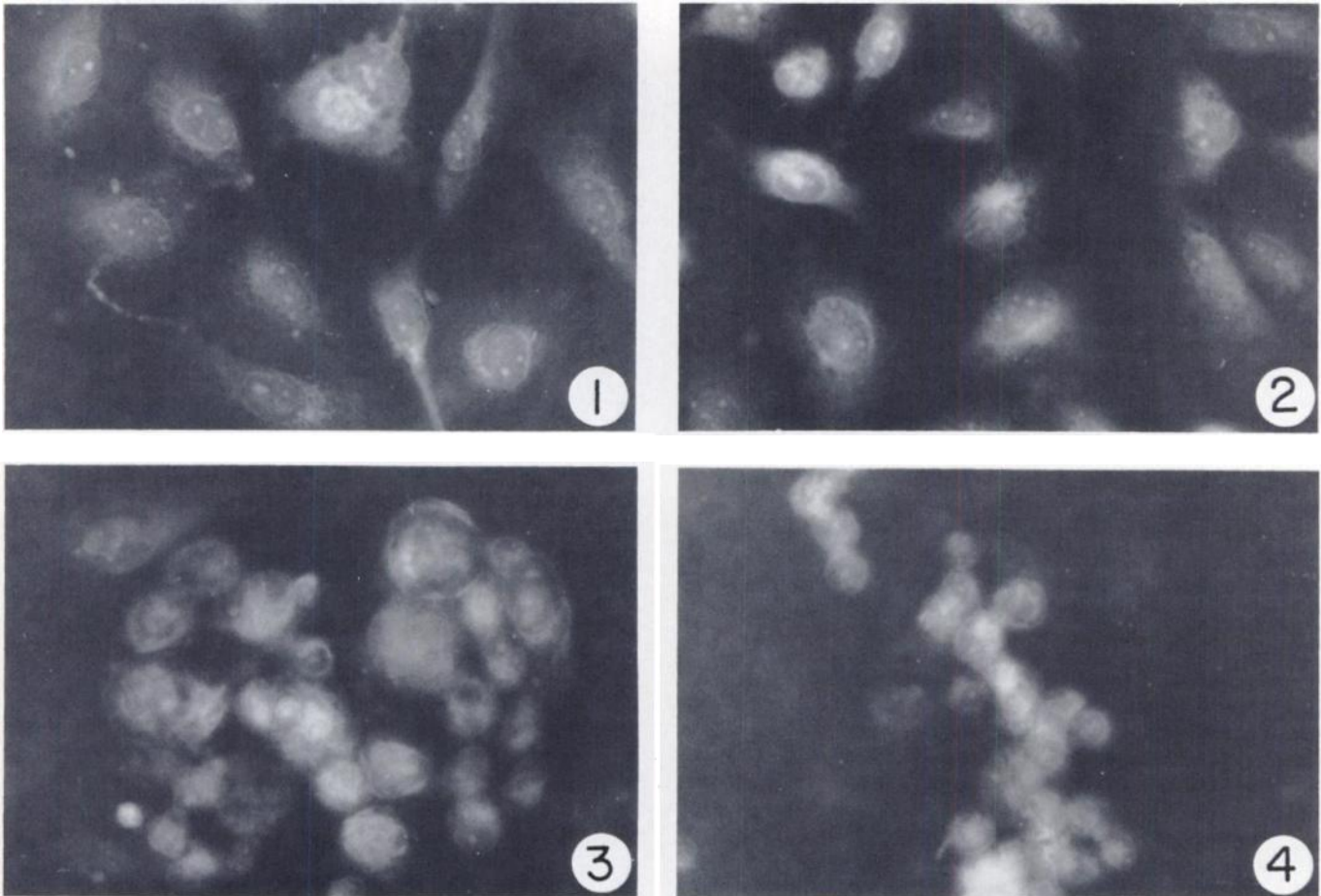


Fig. 1. Cultured MCF-7 tumor cells were incubated with 17-FE for 60 min at 37°. Fluorescent staining was observed in the cytoplasm, frequently accentuated in the perinuclear region. Most nuclei showed a hazy fluorescence with conspicuous staining of the nucleoli. In a few cells, 17-FE was concentrated in the nucleus. $\times 400$.

Fig. 2. Cultured T47D tumor cells were incubated with 17-FE for 60 min at 37°. $\times 400$.

Fig. 3. Dispersed MCF-7 tumor cells were incubated with 17-FE for 60 min at 37°. Most cells displayed cytoplasmic staining. In a few cells, the fluorescent estrogen is concentrated in the nucleus. $\times 250$.

Fig. 4. A cluster of T47D tumor cells was incubated with 17-FE for 60 min at 37°. Fluorescent estrogen was visualized in both the cytoplasm and the nuclei. $\times 250$.