A DEMONSTRATION OF ANDROGEN AND ESTROGEN RECEPTORS IN A HUMAN BREAST CANCER USING A NEW PROTAMINE SULFATE ASSAY

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Separate receptors for estrogen and androgen are demonstrated in cells from metastic human breast cancer. By criteria of binding affinity, number of binding sites, and specificity of the receptor for different steroids, the receptors are shown to be distinguishable. The protamine sulfate receptor assay technique employed allows both kinds of receptor to be quantified conveniently and reproducibly without interference by plasma steroid-binding components.
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SOME PATIENTS WITH METASTATIC BREAST CANcer respond dramatically to a variety of steroid hormonal manipulations, whereas many other tumors appear to be hormone-independent.[24] A better understanding of the nature of the interaction between steroid and target tissue clearly is essential to efforts aimed at improving results of hormonal therapy. Recent investigations have drawn attention to the early events in steroid hormone action.[10] It has been demonstrated that the first step in steroid hormone action appears to be the binding of steroid to specific receptor molecules found in the cytoplasm of target cells. An appreciation of this fact has led to a search for estrogen receptors in human breast cancer specimens in the hope that their presence or absence might be correlated with response or lack of response to hormonal therapy. A summary of recently reported results has been encouraging.[14] As would be predicted, lack of detectable estrogen receptor activity almost invariably means that the patient will not respond to endocrine manipulation. Although patients whose tumor samples contained estrogen receptor responded about twice as frequently to estrogen administration or castration as unselected cases, many patients who were estrogen-receptor positive failed to respond to therapy objectively. Although many hypothetical possibilities suggest themselves, one reasonable explanation for the lack of clinical response in the presence of estrogen receptor is that the tumor may be stimulated by other trophic hormones that minimize the effects of estrogen therapy. Support for this concept may be inferred from recent work demonstrating receptors for androgens[13,18,27] or progesterone[8,9] in some human breast tumor samples. In the present work we report the simultaneous existence of steroid receptors for estrogens and androgens in the same breast tumor sample. By the multiple criteria of quantity of receptor, binding affinity data, and specificity for different steroids, these receptors are unequivocally shown to be different and coexistent. Furthermore, one of the assay techniques we employ, based on protamine sulfate precipitation of receptor, allows convenient and complete differentiation of intracytoplasmic androgen and estrogen-receptor sites from their plasma binding component, sex steroid-binding globulin, a major difficulty in previously available methods for assessment of androgen receptors.[13,27]

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MATERIALS AND METHODS

Preparation of Malignant Breast Cancer for Assay

A 62-year-old postmenopausal woman with metastatic breast cancer developed ascites, which on histologic examination contained ~30,000 cells per mm,[3] of which more than 95% were malignant. Fresh material collected by aseptic paracentesis was concentrated by centrifugation at 800*g* for 10 minutes and washed

three times in ice-cold Dulbecco's phosphate-buffered saline (PBS). Following hypotonic lysis of erythrocytes the cells were washed again in PBS and stored frozen at −70°C until assayed.

Steroid Receptor Assays

Dextran-coated charcoal competitive protein-binding assays[16] and sucrose density gradients[26] were performed as described elsewhere. Protein was determined by the Lowry technique.[12]

The protamine sulfate assay we employ is a modification of the technique of Chamness et al.[3] based on an observation of the precipitation of steroid receptor by protamine sulfate noted by Steggles and King.[23]

Cytosols were prepared for assay as for charcoal assays.[16] Two-tenths milliliter of cytosol was pipetted into cold 12 × 75-mm plastic or glass tubes. Two-tenths milliliter of protamine sulfate (Sigma) solution, 1.5 mg/ml, in a buffer consisting of 10 mM Tris-HCl_2, pH 7.40, 1.5 mM EDTA and 0.5 mM dithiothreitol (buffer A) was added to the tubes and mixed briefly. After standing for 5 minutes at 0°C, the tubes were centrifuged at 800*g* for 10 minutes and the supernates removed by suction. The precipitated receptor is in the form of a tightly adherent film on the bottom of the tube. Radioactive steroid solutions, [^{3}H] estradiol* (Amersham Searle), 100 Ci/mM, or [^{3}H] 5 α dihydrotestosterone, 146 Ci/mM (Amersham Searle), with or without unlabeled competitor were prepared in buffer A and 0.2 ml was added to each tube. After incubation at 0°C for 18 hours, the tubes were washed with three 2-ml portions of buffer A by rapidly adding the buffer and removing it with suction. Bound steroid is counted by either cutting off the bottoms of the plastic tubes and dropping them into liquid scintillation vials or eluting the steroid with two 1-ml portions of absolute ethanol from the glass tubes and counting in Aquasol (New England Nuclear) in a Packard liquid scintillation counter (efficiency for [^{3}H] ≅40%).

Steroid Receptor Exchange Assays

Cytosol preparations were divided in half. One portion was incubated with 10^{-8}M unlabeled estradiol for 1 hour, after which the cytosol was mixed with 0.1 volume of dextran-coated charcoal solution (100 mg activated charcoal plus 1 mg dextran/ml in 10mM Tris-HCl, pH 8.0) to remove unbound steroid, and centrifuged for 10 minutes at 800*g*. This cytosol is termed "presaturated." The control and presaturated cytosol were protamine sulfate precipitated as before, and then incubated for varying times at either 0°C or 22°C in 10^{-8}M [^{3}H] estradiol with or without 10^{-6}M unlabeled estradiol. At the end of the incubation time, bound counts were assessed as noted above.

RESULTS

Estradiol receptors are readily demonstrable in these breast cancer cells by either dextran-coated charcoal, sucrose density gradients, or protamine sulfate assay techniques. A typical binding curve obtained by the charcoal technique for cytoplasmic estradiol receptor is shown in Figure 1. The expected properties of a limited capacity high-affinity receptor are demonstrated. In the inset of this figure, the data are replotted according to the method Scatchard.[20] The straight line which is obtained (r = .983) is consistent with this assay technique recognizing a single class of receptor molecules for estradiol of uniform affinity. The dissociation constant of 1.15×10^{-9}M is in agreement with the range of literature values reported for estradiol receptor.[15,28] Assuming one molecule of steroid bound per receptor, we find 113.5 fmoles of binding sites per milligram of cytoplasmic protein. Using the protamine sulfate assay, we found 107.5 fmoles of binding per milligram of cytoplasmic protein.

Estrogen receptors show characteristic properties on sucrose density gradients. In Figure 2 the sedimentation properties of the receptor from this patient are shown. The peak of bound radioactivity that sediments under low salt conditions at about 8S is totally competable by the addition of a large molar excess of unlabeled estradiol or the antiestrogen Tamoxifen, (ICI 46,474) a triphenylethylene derivative.

In order to establish the binding specificity of the estrogen receptor (as well as differentiate it from the androgen receptor described below), the ability of various unlabeled steroids to compete with [^{3}H] estradiol for the receptor was examined. As shown in Figure 3, estradiol and the antiestrogen Tamoxifen and PD-CI 628 were able to displace [^{3}H] estradiol completely from the receptor. Androgens, such as tes-

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* Trivial names used are: estradiol, 3, 17β dihydroxy-estra-1,3,5 triene; 5α dihydrotestosterone (DHT), 17β hydroxy—5α androstane-3-one dexamethasone, 9α-fluoro-11β, 17α, 21-trihydroxy-16α-methylpregna-1,4-diene-3,20 dione; progesterone, pregn-4-ene-3,20 dione; testosterone, 17β hydroxy- androst-4-ene-3-0one; Androstenedione, androst-4-ene-3,17 dione; dehydroepiandrosterone (DHEA), 3β hydroxy-androst-5-ene-17-one.

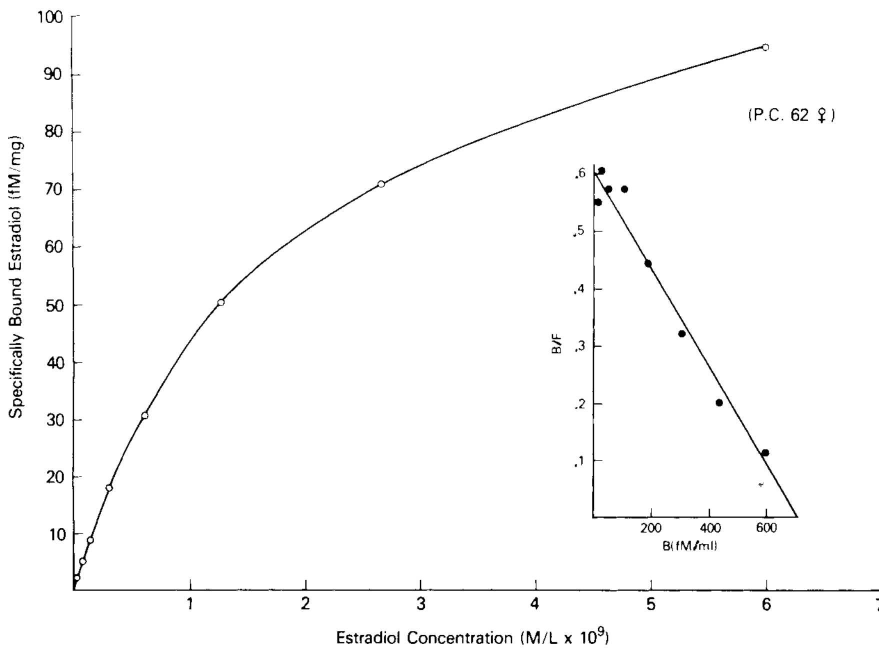

FIG. 1. Binding of [³H] estradiol to receptor in human breast cancer. The dextran-coated charcoal assay described in Methods was used. The data shown are replotted according to Scatchard[20] in the inset.

tosterone, and dihydrotestosterone and the antiandrogen cyproterone acetate were unable to compete significantly with [³H] estradiol, even when present in 2000-fold molar excess. Thus, the receptor showed relatively restricted binding specificities for estrogen and antiestrogens.

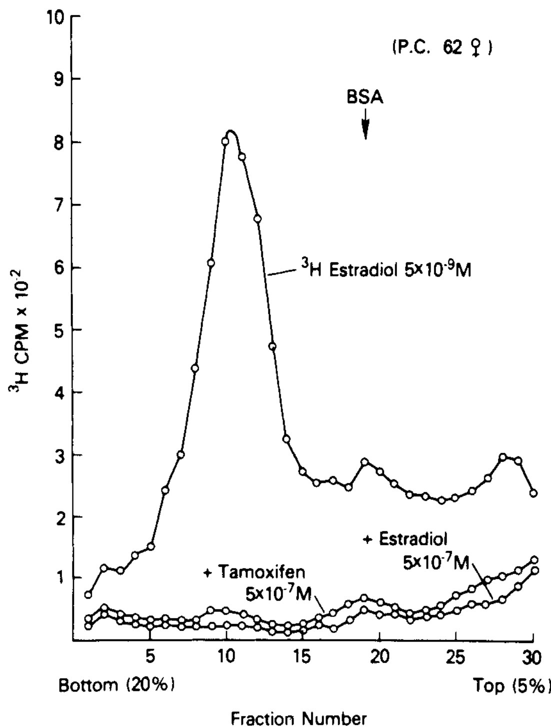

FIG. 2. Sucrose density gradients of estrogen receptor in human breast cancer. Gradients contain [³H] estradiol 5 × 10^{-9}M, plus either unlabeled Tamoxifen 5 × 10^{-7}M or Estradiol 5 × 10^{-7}M.

Tumor cytosols were also shown to have receptors capable of binding [³H] 5 α dihydrotestosterone (DHT). In Figure 4 the binding of [³H] DHT to receptor again shows the properties of limited capacity and high affinity. The Scatchard analysis shown in the inset reveals that DHT binds to a single class of receptor sites (r = .957). The Kd of 2.69 × 10^{-10} is nearly four-fold lower than that of [³H] estradiol to its receptor, but the quantity of receptor, by the dextran-coated charcoal technique, 23.2 fmoles per mg of protein, is only a fourth of that for estradiol receptor. Using the protamine sulfate technique we estimate that there is 26.4 fmoles of binding per milligram of protein, a value in excellent agreement with that obtained by charcoal assay.

Aside from the above differences in binding capacity and affinity, the most compelling evidence that DHT receptor is distinct from the estradiol receptor is provided by Figure 5, in which the ability of various steroids to compete with [³H] DHT for receptor is shown. As compared with Figure 3, the differences in binding specificities between DHT and estrogen receptors are striking. Androgens, such as unlabeled DHT, testosterone and Δ^4 androstenedione, effectively and completely compete with [³H] DHT. Estradiol has some ability to compete with [³H] DHT for binding, but clearly less than any of the unlabeled androgens. The antiandrogen cyproterone acetate displaces some [³H] DHT,

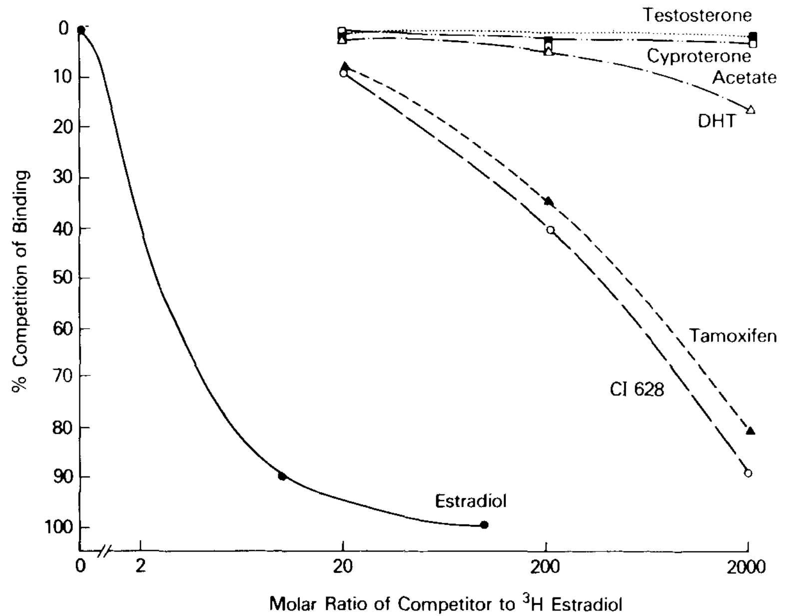

FIG. 3. Ability of various steroids to compete with [^{3}H] estradiol for receptor sites. Estradiol (●——●), CI628 (O----O), Tamoxifen (Δ----Δ), Dihydrotestosterone (DHT) (Δ-··-Δ), Cyproterone (□-··-□) or Testosterone (■·······■).

whereas the antiestrogens Tamoxifen and Parke–Davis CI 628 are totally unable to inhibit [^{3}H] DHT binding, even when present in 2000-fold molar excess. Clearly these cells contain distinct receptors for androgen and estrogen. Since washed ascites cells are used in this case, there is no interference with sex steroid-binding globulin, as there would be in solid tumor sample.

Progesterone receptors have been recently reported in some tumor specimens of patients with breast cancer.[8,9] By sucrose density gradient analysis and competitive binding assay we were unable to demonstrate significant binding of either [^{3}H] dexamethasone or [^{3}H] progesterone to receptor sites in the present case. We have found progesterone and, very rarely, glucocorticoid receptor activity in both primary and metastatic human breast cancer samples from other patients.

A unique advantage of the protamine sulfate assay technique is that binding of steroids to plasma transport proteins does not interfere with the quantification of receptor. In Table 1,

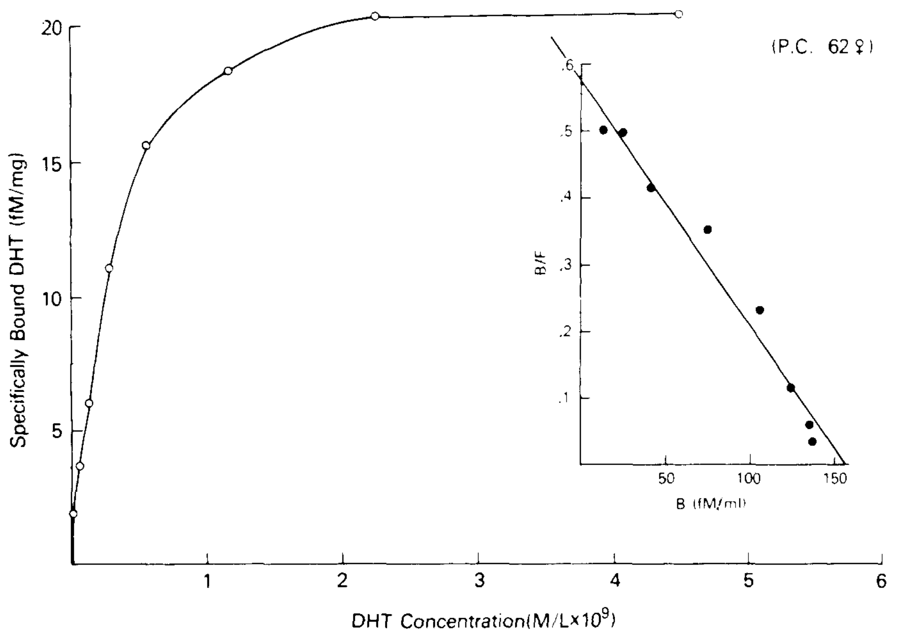

FIG. 4. Binding of [^{3}H] 5 α dihydrotestosterone to receptor in human breast cancer. The data shown are replotted according to Scathard[14] in the inset.

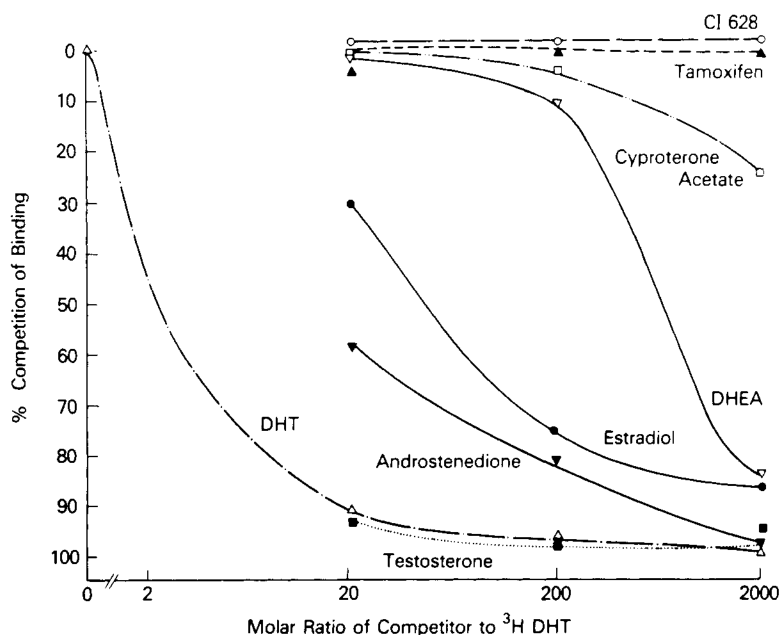

FIG. 5. Ability of various steroids to compete with [^{3}H] 5 α dihydrotestosterone (DHT) for receptor sites Estradiol (●——●), CI628 (O----O), Tamoxifen (△----△), Dihydrotestosterone (△-··-△), Cyproterone acetate (□-···-□), Testosterone (■····■), Androstenedione (▽——▽), Dehydroepiandrosterone (DHEA) (▽——▽).

comparison of binding of tritiated DHT, estradiol, and progesterone to a diluted normal human serum sample is shown by contrasting the protamine sulfate technique with the dextran-coated charcoal technique. As shown, there is insignificant binding to plasma proteins of estradiol, DHT, or progesterone when the protamine sulfate technique is used, whereas plasma proteins markedly interfere with the charcoal assay.

Another advantage of the protamine sulfate assay is its sensitivity at low levels of cytosol protein. In Figure 6 a plot is shown of specific [^{3}H] estradiol binding as a function of cytosol protein concentration in serially diluted samples. The straight line obtained suggests that the assay is quantitatively valid for very low protein concentrations and that sensitivity is limited primarily by counting time.

TABLE 1. Comparison of Specific Binding of Tritiated DHT, Estradiol and Progesterone to Serum Proteins Using Either Dextran-Coated Charcoal or Protamine Sulfate Assays

	Dextran-coated charcoal	Protamine sulfate
[^{3}H] DHT 5×10^{-9}M	13,000*	222*
[^{3}H] Estradiol 5×10^{-9}M	150	0
[^{3}H] Progesterone 5×10^{-9}M	2,800	0
[^{3}H] Progesterone 5×10^{-7}M	14,000	0

* Binding is in CPM/200λ of cytosol for both protamine sulfate and dextran-coated charcoal assays. Counts shown are the difference between the average of tubes incubated with labeled steroid alone minus the average of tubes incubated with an excess of unlabeled competitor.

An additional advantage of the protamine sulfate assay lies in the stabilization of the protamine sulfate receptor against heat denaturation; because of this, endogenous unlabeled steroid bound to receptor can be exchanged with [^{3}H] estradiol and the previously occupied cytoplasmic sites can be assessed. Results are shown in Figure 7. The "presaturated" cytosol had been previously incubated for 1 hour with saturating amounts of unlabeled estradiol as described in Methods. As shown, [^{3}H] estradiol is maximally bound to unoccupied receptors after about 1 hour at 22°C, but 3 hours are required to reach equilibrium at 0°C. There is no detectable loss of receptor after 18 hours' incubation at either temperature. Presaturated cytosol receptor slowly exchanges nonradioactivity equal to the amount of receptor present. At 22°C the exchange is virtually complete at 18 hours and the same number of cytoplasmic sites are identified, whether or not unlabeled estradiol was previously bound to the receptor.

DISCUSSION

The evidence for estrogen dependence of some animal mammary carcinomas,[6,21] human breast cancers in vivo,[7] and human breast cancers in continuous tissue culture[11] is generally con-

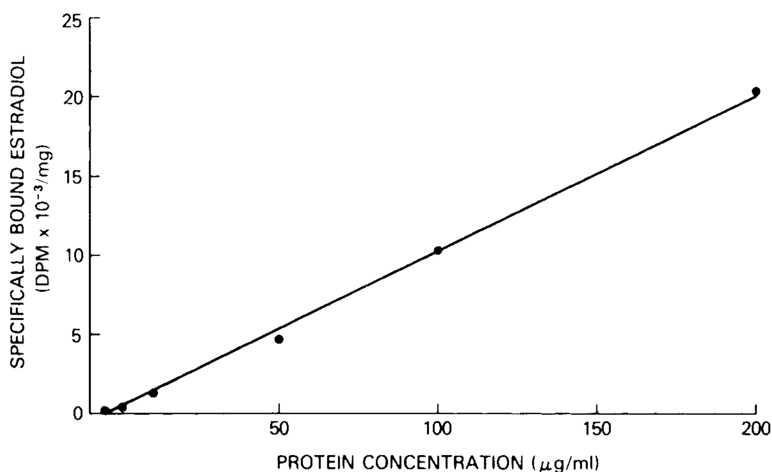

FIG. 6. Specific estradiol binding using the protamine sulfate technique compared with protein concentration of serially diluted cytosol. The straight line obtained emphasizes the validity and sensitivity of this procedure for low protein concentrations.

vincing. In all three systems, it has been possible to demonstrate high affinity estradiol receptors for some tumor tissues. While androgen,[25] insulin,[4] and prolactin[19] dependencies of animal carcinomas are well established, evidence for a critical role for these and other trophic hormones in human breast cancer in vivo is far less satisfactory. Part of the problem no doubt lies in the difficulty of ablating potentially trophic hormones one at a time. Nonetheless, the response of male breast cancer patients to castration,[5] the response of females with breast cancer to hypophysectomy after a previous response to castration and adrenalectomy, as well as some in vitro data with short term organ explants,[1,22] have all suggested that other hormones aside from estrogen may alter the growth of human breast cancer significantly. Clearly, a better understanding of mechanisms whereby some tumors respond to certain hormones, whereas others do not, would aid in the selection of specific endocrine manipulations in selected patients. Because of the established primary role of receptors in the initial interaction of hormones with target tissue,[17] it would seem reasonable to examine human breast cancer not only for estrogen receptor, but for receptors for other hormones as well, in the hope that their presence or absence might be correlated with response to specific hormonal manipulations.

In the present work we present data partially characterizing different receptors for estrogen and androgen in a patient with metastatic breast cancer. The difficulty in examining human tu-

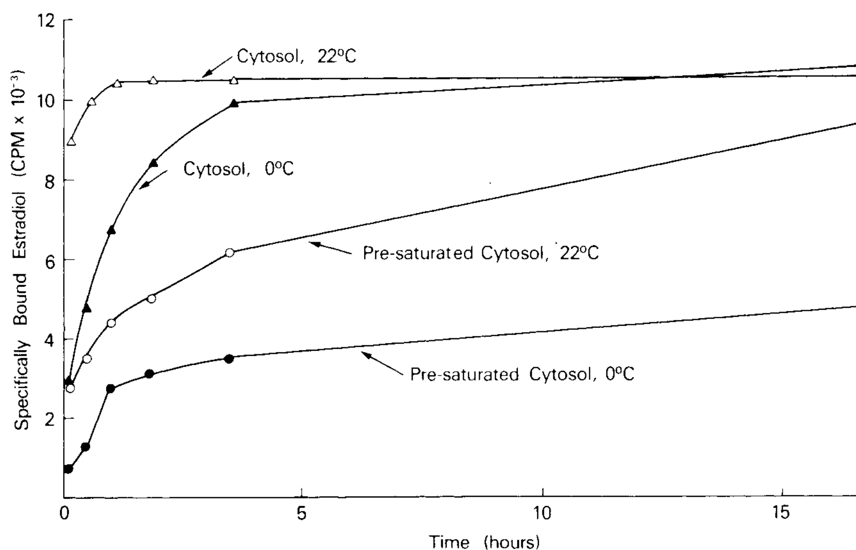

FIG. 7. Kinetics of binding of [^{3}H] estradiol to protamine sulfate precipitated receptor as a function of temperature and receptor occupancy.

mor samples for androgen receptor lies in separating receptor binding from association of androgen with sex steroid-binding globulin in the serum, which almost invariably contaminates such specimens. Recently an electrophoretic technique has been developed in which plasma and cellular binding may be separated by differences in charge,[13,27] but this technique is laborious and not well suited to multiple determinations. Liao and Castañeda have also reported a technique for receptor assay which uses steroid antibodies to separate steroid bound to receptor from the free fraction and the plasma protein-bound fraction.[2] With the protamine sulfate technique for receptor precipitation, binding of steroid to sex steroid-binding globulin is not seen. Binding of glucocorticoids or progesterone to transcortin is not seen with this assay procedure.

Using the protamine sulfate technique, we obtain values in excellent agreement for both estrogen and androgen receptors with those determined by dextran-coated charcoal assay. We are now employing this assay for the routine quantification of multiple steroid hormone receptors in human breast cancer samples in the hope that, taken in concert, patterns of receptor activity may prove more useful in guiding patient management than the determination of estradiol receptor alone. We also emphasize that this assay is suitable for examining receptor sites occupied by endogenous steroid because of the stabilizing properties of the protamine sulfate precipitation step.

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