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Batch Assay Method Evaluation of Cytoplasmic Estrogen Receptor. Relative Immunity of Hydroxylapatite Method from Errors of Measurement*

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Abstract—*Utilizing a refined hydroxylapatite assay technique for the measurement of cytoplasmic estradiol-17β binding in both immature rat uterine tissue and human breast cancer biopsies, we compare the specificity and sensitivity of our method to those capabilities of previously reported batch techniques which prescribe either (i) adsorption of free estradiol to dextran-coated charcoal; or (ii) precipitation of cytosol by protamine sulfate, followed by the incubation of the residue with ligand. Compared to alternative methods, the hydroxylapatite assay yields (a) no losses of specific estrogen-binding activities over a wide range of concentrations of cytosol protein, (b) saturation plots which suggest more uniformity in the specific estradiol-binding sites being measured, (c) no alteration in the recovery of estradiol-binding activity with variations of the time of adsorption, and (d) specific binding values that are not significantly influenced by the presence or absence of bovine serum albumin, a nonspecific, nonsaturable estradiol-binding protein.*

INTRODUCTION

An acknowledged contribution to the understanding of estrogen action in target tissue has been the isolation and study of specific, soluble estradiol-binding proteins [1].

With the multiplicity of batch assay techniques for such estrogen receptors that have been reported, one immediately becomes concerned about the equivalence of binding values thus obtained from laboratory to laboratory [2]. It is not immediately apparent, for example, whether such commonly employed methods as the use of dextran-coated charcoal (DCC) [3, 4], the application of hydroxylapatite (HA) [2, 5], or the precipitation of the estrogen binding protein by protamine sulfate (PS) [6–8] yield matching values of specific cytoplasmic estrogen binding activity from different biological sources. Furthermore, there is considerable diversity in the reported conditions for the application of DCC: respective charcoal and dextran concentrations have varied from 5% and 0.5% [9] to 1.0% and 0.01% [10], 0.5% and 0.005% [11–13], or 0.25% and 0.0025% [4], while adsorption times to charcoal have varied from 15 min [10, 11] to overnight [12]. In addition, the presence of bovine serum albumin (BSA) as a nonspecific binding protein has been shown to affect the amount of estradiol apparently bound to cytoplasmic estrogen receptor, depending upon the concentration of charcoal utilized [14]. Finally, other metabolites such as ATP, which have been previously suggested to influence the binding of steroid to receptor [15, 16], are known to be readily adsorbed to charcoal [17].

An alternative to the charcoal assay for large numbers of samples is to apply hydroxylapatite (HA) to adsorb the weakly acidic estrogen binding protein. By using filters under suction to partition the bound steroid from the original equilibrium mixture [18], we depart from previously reported hydroxylapatite techniques [2, 5]. We find that the HA method in comparison to the DCC and PS techniques is unique in yielding specific

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binding values that are not appreciably influenced by variations of either the adsorption time at the end of the binding reaction or the amounts of nonspecific, nonsaturable estradiol-binding proteins that may be present in different cytosol preparations. As the assay method most sensitive to a broad range of cytosol protein concentrations and relatively free from interference from BSA, the HA method appears advantageous in its clinical application, in which wide variations of specimens (some of extremely limited quantity) may be anticipated.

MATERIALS AND METHODS

Chemicals

[2, 4, 6, 7]-^{3}H-estradiol-17β (100–110 Ci/mmole) and Aquasol-2 were obtained from New England Nuclear Corp., Boston, Mass.; salmon sperm protamine sulfate, diethylstilbestrol, 2-mercaptoethanol, dithiothreitol, Norit A and Dextran C clinical grade (mol.wt ~ 70,000) from the Sigma Chemical Co., St. Louis, Mo.; fatty acid-poor bovine serum albumin (Pentex Fraction V) from the Miles Laboratories, Elkhart, Ind.; human serum albumin from the Massachusetts Public Health Biologic Laboratories, Boston, Mass.; Bio-Gel HTP hydroxylapatite from Bio-Rad, Richmond, Calif.; sucrose from Schwarz-Mann, Orangeburg, N.Y.; and Earle's solution (Ca^{2+}-and Mg^{2+}-free) from Gibco, Grand Island, N.Y.

Rat uteri

Rat uteri were obtained from 21-day old female Sprague–Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), as described previously [18].

Human breast cancer specimens

Fresh human tumor was obtained in the operating room, placed in ice, trimmed of fat and normal breast tissue, and minced into small pieces which were immediately frozen in liquid nitrogen. Histological confirmation of each tumor sample was obtained. Of the human breast (hBr) carcinoma specimens assayed, no significant loss of binding activity could be observed over at least a month in samples stored at −90°C.

Buffers

Buffers were adjusted to pH 7.4 at 4°C. TEM buffer consisted of 10 mM Tris, 1.5 mM EDTA, and 2 mM 2-mercaptoethanol; TED buffer contained 10 mM Tris, 1.5 mM EDTA and 0.5 mM dithiothreitol; and TP buffer was comprised of 50 mM Tris and 5 mM potassium phosphate. T buffer, containing 10 mM Tris, was adjusted to pH 8.0 with HCl.

Preparation of cytoplasmic extracts

All operations were carried out at 4°C. Frozen rat uterine pellets or hBr carcinoma specimens approximately 1 cm^3 were processed as described previously [18].

Protein determination

Protein was measured spectrophotometrically at a wavelength of 330 nm by the microbiuret method [19].

Protamine sulfate precipitation and assay of cytosol receptor

All operations were carried out at 4°C. Cytoplasmic estradiol binding protein was precipitated by protamine sulfate prior to its incubation with 1 nM [^{3}H] E_2 *in vitro*, according to the method of Lippman and Huff [7]. Bound estradiol was measured in the bottoms of polystyrene tubes, which had been severed from the remaining tube mass with a heated scalpel blade and dropped into liquid scintillation vials, to which 10 ml of Aquasol-2 was added before counting was performed at an efficiency of 36% in a Packard Model 3330 Tri-Carb spectrometer (Packard Instrument Co., Downers Grove, Ill.). Specific binding was determined by subtracting the activity observed in incubations containing a 100-fold excess of DES from the binding values observed in the fractions lacking DES.

Charcoal–dextran assay of cytosol receptor

Activated Norit A was washed with 1N HCl, filtered under suction, and rinsed several times with T buffer until the pH of the filtrate was 8.0. The residue was dried at 50°C for 4 hr before use.

Cytosol was incubated with 1 nM [^{3}H] E_2 for 20–24 hr at 0°C in the presence or absence of 100-fold excess of DES, in a final volume of 0.2–0.5 ml. To 0.2 ml portions of the equilibrated mixtures, 0.5 ml of DCC suspension (1.0, 0.5 or 0.25% activated Norit A; 0.01, 0.005 or 0.0025% Dextran C in T buffer) was added with mixing. Cytosol–DCC suspensions were incubated for 30 min with vigorous mixing every 5 min before they were centri-

fuged at 2000 ***g*** for 10 min. A portion (0.5 ml) of the supernatant was subsequently removed for the measurement of radioactivity under the conditions for scintillation counting described above. Our technique, originally described by McGuire and De La Garza [4], included variations of either the concentrations of DCC or the incubation period with DCC in order to reproduce the conditions of other investigators employing the DCC assay method [9–13].

Hydroxylapatite assay of cytosol receptor

After equilibration of the cytosol with 1 nM [^{3}H] E_2 in the presence or absence of 100 nM DES, adsorption to HA was allowed to occur at 4°C for at least 25 min before the residue was washed and recovered on a filter manifold under suction, as previously described [18].

Sucrose gradient evaluation of estrogen receptor

Binding of [^{3}H] E_2 to receptor was determined in linear 10–30% sucrose gradients, as described previously [18].

RESULTS

Rat uterine studies

Comparison of hydroxylapatite and sucrose gradient assay results. Essentially all total [^{3}H] E_2-binding activity of rat uterine cytosol measured by the HA technique sedimented as an 8S peak in a 10–30% sucrose gradient, while less than 7% of the estradiol-binding activity produced by a non-specific estrogen-binding protein (human serum albumin) in the 4S region of the gradient appeared as total [^{3}H] E_2-binding activity in the HA technique (Fig. 1). Because DES inhibited over 92% of the [^{3}H] E_2-binding activity of rat uterine cytosol in both the HA and SG assay methods (data not shown), "total" and "specific" binding activities were essentially synonymous terms with regard to the data shown.

Saturation plot and Scatchard analysis of different batch assay results. The immature rat uterine cytosol estrogen receptor became saturated at ligand concentrations exceeding 3 nM in all the assay regimens followed (Fig. 2).

Once saturation was achieved in the HA assay (Fig. 2A), further increases in both the total and nonspecific binding values with increasing ligand concentrations were nearly

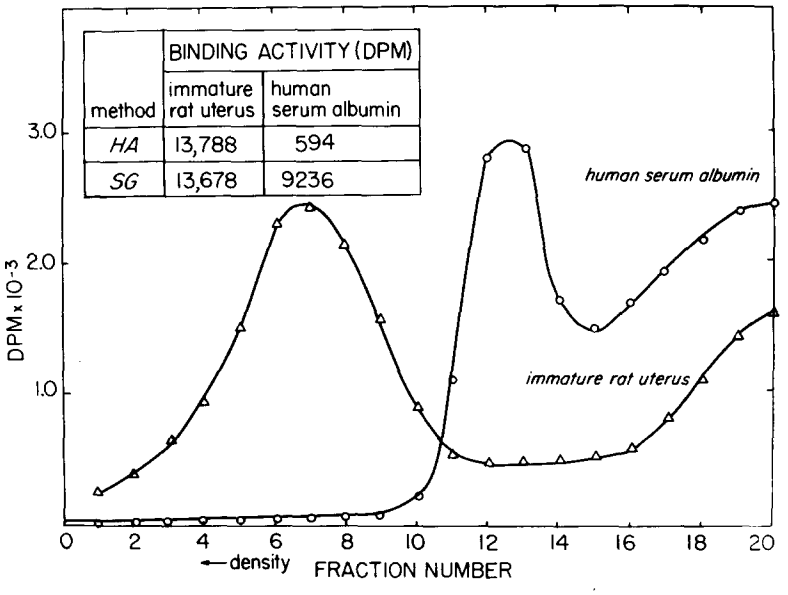

Fig. 1. Recovery of total [^{3}H]E_2 *binding activity of immature rat uterine cytosol and of human serum albumin by both HA and SG techniques. Aliquots of equilibrated solutions containing* 1 nM [^{3}H]E_2 *and either rat uterine cytosol* (1.12 mg/ml) *or human serum albumin* (11.78 mg/ml) *were each assayed by the HA or SG method. The profile indicates the location of* [^{3}H]E_2 *binding fractions of either rat uterine cytosol* (△) *or human serum albumin* (○) *subjected to SG analysis, with fraction* 1 *representing the bottom of the gradient. Inset shows total binding activities obtained by the SG or HA assay techniques per* 0.2 ml *of incubation mixture, with* dis/min (dpm) *obtained by the SG method calculated from the peak areas illustrated.*

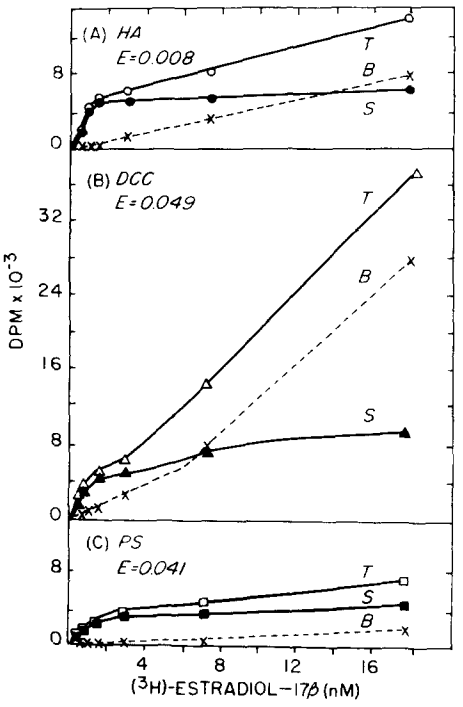

Fig. 2. Saturation of $[^3H]E_2$ *binding of immature rat uterine cytosol, obtained by different batch assay techniques. Background binding values were determined in the presence of DES at concentrations 100 times each indicated amount of radioactive ligand. Total (○, △ or □), specific (●, ▲ or ■) and background (×)* $[^3H]E_2$ *binding values were obtained with immature rat uterine cytosol (0.4 mg/ml) incubated with* $[^3H]E_2$ *for 22 hr and subjected to (A) HA, (B) DCC and (C) PS assay methods. Points represent the means of duplicate determinations. Inset shows E values obtained in the saturation plots of each assay method, E representing the ratio of the slope obtained at the highest two concentrations of* $[^3H]E_2$ *to the slope derived at the lowest two concentrations of radioactive ligand.*

parallel, resulting in a virtually horizontal curve ($E=0.008$) denoting specific estradiol binding. In addition, the transformation from the linear to the saturated (horizontal) portions of the specific binding curve was relatively abrupt, occurring within a narrow range of added $[^3H]E_2$. This suggested that the filling of only a single class of binding sites available in limited amounts was measured.

Saturation of binding sites in the DCC method was evident at ligand concentrations of 1–2 nM (Fig. 2B). However, an abrupt upward deflection of the curve denoting background estrogen binding occurred at radioactive ligand concentrations exceeding 4 nM. The specific binding curve, indicating the difference between total binding and that observed in the presence of 100-fold excess concentrations of DES, followed an erratic course from 4–16 nM of radioactive ligand and did not indicate the absolute saturation of specific binding sites for $[^3H]E_2$ suggested in the HA technique (E values were increased 6-fold).

Saturation in the PS method occurred at significantly higher ligand concentrations (2–3 nM) and displayed a broad region of curvature in the saturation plot over a relatively wide range of ligand concentrations. This suggested either the inefficient filling of binding sites by increasing amounts of $[^3H]E_2$ or the saturation of more than a single class of binding sites (Fig. 2C). Saturation of specific estradiol-binding sites was not absolute ($E=0.041$).

These differences in the saturation plots were amplified if the curves were redrawn according to the method of Scatchard [20]. The affinity of receptor for ligand was greatest in the HA method (Table 1), suggesting a minimum of contribution from cytoplasmic proteins other than the estrogen receptor which have been shown to bind estradiol [21].

*Table 1. Ligand affinities and binding site concentrations of immature rat uterine estrogen receptor measured by various batch assay methods**

Assay method	K_d ($M\times10^{-10}$)	Specific estrogen binding site concentration ($M\times10^{-10}$)
HA	1.6±0.3	22.4±3.3
DCC†	2.0±0.1	18.3±0.8
PS	9.7±0.7	20.4±0.9

*Determined by least squares analysis ($R^2>0.96$) of linear portions of Scatchard plots derived from saturation data (Fig. 2). Results are shown with standard errors.

†0.25% Norit A charcoal and 0.0025% Dextran C.

Performance of different batch assays at various cytosol protein concentrations. Figure 3 demonstrates a loss of the recovery of bound receptor in both the DCC and PS techniques at lower cytosol concentrations. This deficiency was offset in the DCC method but accentuated in the PS assay by the presence of 10 mg/ml of a nonspecific protein (BSA) in the cytosol.

These results were essentially unchanged if the radioligand concentration was increased to 3 nM. Raising the dextran concentration to 1/10 the amount of charcoal (1.0% charcoal and 0.1% dextran) eliminated only half the loss of specific estradiol-binding activity.

The loss of recovery of estrogen receptor in the DCC method was only partially alleviated

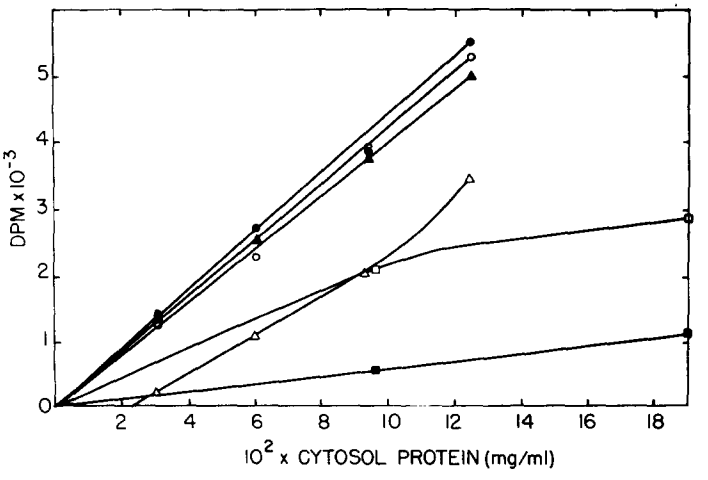

Fig. 3. Comparative recoveries of specific [^{3}H]E_2 *binding activities observed at different protein concentrations of immature rat uterine cytosol, assayed by the HA, DCC or PS techniques. One portion of rat uterine cytosol was equilibrated with* 1 nM [^{3}H]E_2 *at* 4°C *for* 22 hr *with or without* 100 nM *DES at a final cytosol protein concentration of* 0.12 mg/ml *and then diluted in the absence or presence of BSA* (10 mg/ml) *with TEM buffer containing* 1 nM [^{3}H]E_2 *and either lacking or containing DES,* 100 nM, *to several concentrations of protein. Solutions were then subjected to either the HA or DCC regimens. It was then possible to evaluate the specific binding activities obtained by either the DCC method of assay of cytosol dilutions performed in either the absence* (△) *or presence* (▲) *of BSA or the HA assay of cytosol diluted in either the absence* (○) *or presence* (●) *of BSA. A separate portion of cytosol* (0.19 mg/ml) *was divided into two portions, one of which was supplemented with BSA* (10 mg/ml). *Dilutions in TEM buffer either lacking or containing BSA were carried out before all cytosol solutions were precipitated with PS and then incubated with* 1 nM [^{3}H]E_2 *in the absence or presence of* 100 nM *DES at* 4°C *for* 22 hr *in solutions either lacking or containing BSA. Specific binding activities were ultimately obtained from cytosol solutions in the absence* (□) *or presence* (■) *of BSA. All results are the means of duplicate determinations.*

if the adsorption time to the dextran-coated charcoal was reduced to 10 min (Fig. 4). Recovery of rat uterine cytosol receptor by the HA method was constant over the time period observed and remained unchanged for at least 2 hr (data not shown).

Human breast carcinoma studies

Affinity of receptor for ligand. As evaluated by Scatchard plots, the scope of the dissociation constants measured by the HA method of all our hBr cancer cytoplasmic extracts which exhibited specific ligand binding activity of over 10 fmole/mg protein was 0.7–3 $\times 10^{-10}$M, within the range of ligand affinities of estrogen receptor that have been reported for hBr carcinoma cytosol extracts [22, 23].

Measurement of multiple molecular forms of estrogen receptor by the HA method. In all hBr carcinoma cytosol extracts subjected to sucrose gradients, virtually the entire 8S peak area plus the DES-inhibitable portion of the 4S region (if present) was quantitatively reflected by the specific binding values obtained from our HA technique (data not shown).

Recovery of specific binding activities at different cytosol protein concentrations by the HA, DCC and PS methods. Table 2 indicates how differences of recovery of specific cytoplasmic estradiol-binding activity appeared among the three assay methods compared, depending upon whether a low (0.2 mg/ml) or higher (0.8 mg/ml) concentration of cytosol protein in equilibrium with 3 nM ^{3}H-estradiol was used. Binding activities of rat uterus were significantly lower in the DCC assay at lower concentrations of cytosol protein, the depletion becoming even more pronounced at greater concentrations of charcoal. At the maximum concentration of charcoal–dextran (1.0%/0.01%), a loss of specific binding occurred at even the higher amount of cytosol protein. Similar results were seen in the three hBr tumor specimens shown, although for one (hBr Tumor A), the loss of binding activity was less severe. Specific binding values obtained in the PS assay were occasionally depressed, as shown by hBr Tumor C. Twenty other specimens that were found to contain 3 fmole/mg protein or less of binding activity in the HA method did not reveal higher

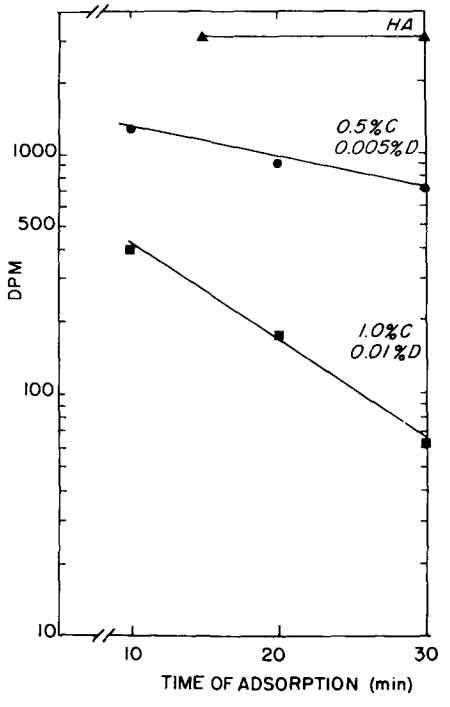

Fig. 4. Time course of recoveries of specific [^{3}H]E_2 binding activities observed at different protein concentrations of immature rat uterine cytosol, assayed by either the HA or DCC technique. Incubations were followed by the dilution of rat uterine cytosol to a final protein concentration of 0.2 mg/ml, *according to the technique described in the legend to Fig. 3, with the following modifications: BSA was omitted, and the specific binding was measured after the indicated adsorption times to either HA* (▲), 0.5% *charcoal and* 0.005% *dextran* (●), *or* 1.0% *charcoal and* 0.01% *dextran* (■).

of specifically bound [^{3}H]E_2 by the HA method, one must ask whether this technique measures components in addition to the actual estrogen receptor, as has been suggested elsewhere [24]. Figure 1 demonstrates that greater than 93% of at least one estrogen-binding component (human serum albumin) of the steroid target cell, whose affinity for estradiol ($10^5 M^{-1}$) is approximately 10^{-4} that of the estrogen receptor [25], escaped detection by the HA method but not by sucrose gradients. Thus, it would appear that our utilization of the HA technique measures estrogen-binding proteins whose affinity for steroid is greater than $10^5 M^{-1}$. Only two estrogen-binding proteins in addition to actual estrogen receptors are known which fit into this category. One is the sex steroid binding globulin, which is not inhibited by DES [26], eliminating it from further consideration in this study. The other is α-fetoprotein, which is also refractory to inhibition of estrogen binding by DES [24] and has a relatively low affinity for steroid [27], making its detection in our HA assay unlikely. In addition, we have shown that the estrogen-binding activity in the SG assay that is sensitive to DES inhibition is quantitatively detected in the HA assay.

Compared to the HA method, the DCC and PS techniques in this investigation would

Table 2. Specific ^{3}H-*estradiol-17β binding activity obtained by HA, PS and DCC assay techniques in cytosol preparations of rat uterine and human breast cancer tissue at different concentrations of cytosol protein**

Method of separation of bound and free ligand	Specific binding of ^{3}H-estradiol-17β (fmole/mg protein)							
	Immature rat uterus		hBr Tumor A†		hBr Tumor B		hBr Tumor C	
	0.2 mg/ml	0.8 mg/ml	0.2 mg/ml	0.8 mg/ml	0.2 mg/ml	0.8 mg/ml	0.2 mg/ml	0.8 mg/ml
HA	850±17	1189±6	119±3	107±2	38±2	37±2	74±4	66±1
PS	863±27	956±14	121±2	117±3	34±2	36±1	8±1	4±0
DCC: 1.0% C/0.01% D†	91±6	827±4	14±1	46±1	2±0	4±0	12±0	14±0
0.5% C/0.005% D	453±5	1150±20	39±0	80±0	12±0	17±1	18±1	37±2
0.25% C/0.0025% D	714±1	1171±4	69±2	92±1	23±1	17±2	38±2	39±1

*Cytoplasmic extracts incubated at the indicated final protein concentrations with 3 nM ^{3}H-estradiol-17β, either in the absence or presence of 300 nM diethylstilbestrol at 4°C for 22 hr. Separation of bound and free steroid was performed by the designated methods, and binding activity obtained in the presence of diethylstilbestrol was subtracted to yield the specific values (means of duplicate determinations) shown.

†hBr = human breast; C = charcoal; D = dextran.

amounts of estrogen-binding activity in either the DCC or PS method.

DISCUSSION

Given the tendency to recover higher values seem to be less consistent, despite our apparent reconstruction of the DCC or PS assay conditions reported elsewhere to yield quantitative results [3, 4, 6, 7, 11, 13]. There are three reasons why this variance with other laboratories may have occurred: (i) As shown

above, the presence or absence of nonspecific estrogen-binding proteins will alter the results of the DCC and PS assays while not affecting the HA technique. The amounts of nonspecific estrogen-binding proteins in rat uterine cytosol preparations will vary among laboratories, according to the extent and method of trimming, washing and exsanguination of the uteri as well as the means of sacrifice of the experimental animals. Further variations can be anticipated among different target tissues or their sources. The amounts of nonspecific estrogen-binding proteins in biopsied material from human breast carcinomas can be expected to vary even more widely, possibly reflecting why deficiencies of cytosol receptor determined by the DCC or PS assays may or may not be present (Table 2). The relative lack of certain nonspecific estrogen-binding proteins (e.g., serum albumin) in our rat uterine preparations may be responsible for the pronounced loss of specific estrogen-binding activity in the DCC assay in this investigation. (ii) The mol. wt of the dextran and the degree of activation of the charcoal may vary among laboratories not sharing these reagents. (iii) The previously described PS method followed in our investigations utilized sources of cytoplasmic estrogen receptor other than the immature rat uterus [6, 7]. It is possible that α-fetoprotein or some other component unique to immature rat uterine cytosol interferes with the performance of the PS assay.

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