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# AN ANALYSIS OF THE DECREASE IN THE ASSAYED LEVEL OF CHARGED BOVINE ESTROGEN RECEPTOR OBSERVED AT PHYSIOLOGICAL IONIC STRENGTH

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**Summary**—Lower assayed levels of heifer uterine estrogen receptor (ER) occur at physiologic ionic strength when ER is separated from [$^3$H]estradiol by Dextran-coated charcoal treatments, or by gel filtration on Sephadex or polyacrylamide resins. The assayed level of charged ER in buffers containing 150–200 mM ionic strength is approximately one-half that of ER levels assayed in buffers either at 0–50 or 400–450 mM ionic strength. Treatment of ER with trypsin or molybdate eliminates this observed reduction. Evidence is presented that the decrease results from a preferential adsorption of ER to the assay resins at 150–200 mM ionic strength. This adsorption is likely to be mediated by a hydrophobic region of the ER, which is removed by trypsin cleavage.

## INTRODUCTION

The Dextran-coated charcoal (DCC) assay for estrogen receptors (ER) is widely acknowledged to underestimate receptor levels in buffers of high ionic strength (e.g. 400 mM KCl). As originally described [1], such underestimation was attributed to charcoal-catalyzed dissociation of estradiol from the receptor, termed "stripping." In low ionic strength buffers, underestimation of receptor levels results from adsorption of ER to DCC when the protein concentration of receptor samples is low [2, 3].

It has been suggested that adsorption of ER to DCC also occurs at high ionic strength. Katzenellenbogen *et al.*[4] noted that underestimation of ER levels does not occur in high ionic strength buffers if receptor preparations are first exposed to low levels of trypsin. These authors suggested that DCC can no longer adsorb the estradiol-binding receptor fragment. In addition, one report has shown that ER levels measured when proteolysis is inhibited are underestimated at intermediate ionic strengths (200–300 mM KCl) more than at lower or higher ionic strengths [5].

The study reported here compares the DCC assay with two gel filtration assays at various ionic strengths. For each assay method, underestimation of ER is most significant in buffers of 150–200 mM ionic strength. Demonstrable adsorption of receptors to gel filtration columns accounts for the observed underestimation. The similar response to ionic strength of receptor in both DCC and gel filtration assays supports the conclusion that DCC adsorbs ER.

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## EXPERIMENTAL

### *Materials*

[2,4,6,7,16,17-$^3$H]Estradiol 17$\beta$ ([$^3$H]$E_2$) (140–142 Ci/mmol) was supplied by Amersham Corp. Dithiothreitol was purchased from Boehringer Mannheim Biochemicals. Bio-Gel P-10 was purchased from Bio-Rad Laboratories. Activated charcoal, dextran (clinical grade, avg. Mr = 81,600) and soybean trypsin inhibitor type 1-S were from Sigma. Scintillation-grade toluene, Triton X-100 and 2a70 preblended scintillant (2,5 diphenyloxazole, *p-bis*(*o*-methylstyryl) benzene, 49:1 w/w) were from Research Products International. All other chemicals used were reagent-grade quality.

### *Cytosol preparation*

Heifer uteri obtained at a local slaughterhouse were kept on ice during collection and transportation. All subsequent steps were done at 4°C unless otherwise noted. The procedure is that of Weichman and Notides[6] with minor modifications. Uteri were stripped of connective tissue, cut into pieces, and frozen in liquid nitrogen. Tissues were stored at −60°C until needed. Frozen tissue was pulverized with a liquid nitrogen-cooled steel mortar and pestle, homogenized for 30 s in 4 vol of DT buffer (40 mM Tris pH 7.5 at 20°C, 1 mM dithiothreitol) with a Polytron P-10 (Brinkman Inst.) homogenizer at setting 4–5. Some tissue samples were homogenized in DT buffer plus 0.5 mM phenylmethylsulfonyl fluoride (PMSF) to inhibit proteolysis. Homogenization was repeated 4 times at 90-s intervals. The homogenate was centrifuged at 20,000 *g* for 15 min.

The resulting low-speed supernatant was then spun at 220,000 *g* for 45 min. The floating lipid layer was discarded and the collected cytosol was adjusted to 30% ammonium sulfate by adding, over a 15-min period with stirring, a saturated solution of $(NH_4)_2SO_4$ in 40 mM Tris, pH 7.5. This mixture was stirred for 30 min more, then centrifuged for 15 min at 17,600 *g*. The supernatant was discarded, and the ammonium sulfate precipitate was frozen at −60°C for later use. When needed, frozen protein samples were resuspended in DT buffer equal to 5–6% of the original cytosol volume, allowed to dissolve for 1 h, then centrifuged at 17,600 *g* to clarify the solution. The supernatant obtained was desalted on a 1.5 × 18 cm column of Sephadex G-25 equilibrated in DT buffer. Alternatively, resuspended and clarified cytosol was desalted using the centrifuge column technique (*vide infra*) with columns equilibrated in DT buffer.

*Preparation of molybdate treated ER*

All steps were performed as above except as follows: ten millimolar $Na_2MoO_4$ was included in the homogenization buffer; solid $(NH_4)_2SO_4$ rather than a saturated solution was used for protein precipitation; a 30–50% $(NH_4)_2SO_4$ fraction was collected because, in the presence of molybdate, ER is most abundant in this fraction [7]; precipitated receptor was resuspended in TD buffer containing 10 mM $Na_2MoO_4$ and desalted by dialysis for 15 h against 1000 vol of the same buffer.

*Estradiol binding*

[$^3$H]Estradiol, supplied in a toluene–ethanol solution, was evaporated to dryness then resuspended in 40 mM Tris pH 7.5, 10% ethanol. In all experiments, nonspecific binding was assayed in the presence of a 200-fold excess of unlabeled estradiol, or a 500- to 1,000-fold excess of unlabeled diethylstilbestrol (DES). Specific binding was assessed by subtracting counts bound in the presence of competing ligand from those bound in the absence of competing ligand. Data are expressed as means plus or minus 1 standard deviation of the samples unless noted otherwise.

*DCC Assay method*

Dextran-coated charcoal (1% w/v activated charcoal, 0.1% Dextran) was prepared by stirring overnight in DT buffer. When required, the salt concentration of the suspension was adjusted by pelleting the DCC (2,000 *g* for 30 min), then replacing the supernatant with DT buffer containing KCl. Two variations of the DCC assay were employed. In one, 200 μl receptor samples containing various concentrations of KCl were mixed with a suspension of DCC in DT buffer (133 μl) resulting in a 60% dilution of the initial KCl concentration. The combined mixture was swirled briefly, incubated for the times noted in the figure legends, then centrifuged at 2,000 *g* for 5 min. Aliquots of the supernatant (250 μl) were counted in 3.4 ml of scintillation fluid. In the second variation, exactly the same procedure was followed except that the aliquots of DCC contained KCl concentrations identical to those of the ER samples with which they were mixed. In this way, no dilution of KCl resulted from addition of DCC.

*Gel filtration procedure*

Plastic 3 cc syringes were fitted with 70 μm pore size polyethylene filter discs cut from sheet stock (Bolab, Inc.), and packed with 2.5 ml of Sephadex G-25 resin or 2.0 ml of Bio-Gel P-10 resin. Columns were equilibrated with buffer by washing with a minimum of 10 column volumes immediately before use. Routinely, 16 drop fractions (~400 μl) were collected at flow rates of 15–25 ml/h.

*Centrifuge column gel filtration technique*

The method of Penefsky[8] was used with little modification. Tuberculin syringes were fitted with 70 μm pore size filter discs. Some assemblies were siliconized with no significant change in results. Sephadex G-50 resin was packed to the 1.0 or 1.2 ml mark, drained by gravity, then partially dehydrated by spinning at 630 rpm in an IEC 6000 centrifuge (100 *g* at tip of column). When multiple buffers were required for equilibration of these columns, truncated 20 ml plastic syringe barrels were press-fitted into the top of the tuberculin syringes. The junctions were sealed with the aid of short lengths of plastic tubing placed over the tip of the 20 cc syringes. Buffer was then placed in the upper reservoirs to the 10 ml mark, and the assemblies were spun in 50 ml plastic centrifuge tubes. Five minutes at 630 rpm was sufficient to elute 8–9 ml of buffer through freshly prepared columns. Washing was facilitated by trimming the finer syringe tip to a 45° bevel, thus allowing buffer to escape to the side. The reservoirs were then removed from the tuberculin syringes and the resin in these was resuspended gently with a Pasteur pipette, allowed to drain, then dehydrated as before. For analysis, samples of 100 μl were applied to the columns at room temperature. After 2–4 min, these were spun for 2 min at 630 rpm and the eluate collected directly into scintillation vials for counting. Routinely, columns were washed with 10–15 ml of buffer by the centrifuge method described above, then reused. No radioactivity was noted to remain in the columns following this procedure.

*Trypsin treatment*

Receptor samples were incubated with 20 μg/ml trypsin for 30 min at 4°C. Soybean tryspin inhibitor was then added to a final concentration of 40 μg/ml.

*Scintillation counting*

Triton X-100 (330 ml) was mixed with toluene (670 ml) and 6.3 g/l of preblended scintillant

(PPO/*bis*-MSB). Counting efficiency was 30–40% for tritium and was assayed by internal standards where necessary.

*Protein determination*

Protein concentrations were determined by the method of Bradford[9] using BSA as a standard. Receptor preparations containing 1–7 mg/ml of protein were used without apparent variations in results.

## RESULTS

*Ionic strength dependence of ER determination by dextran coated charcoal*

The DCC assay has been reported to progressively "strip" estradiol from 4S receptors at salt concentrations above 150 mM KCl as the concentration of DCC and duration of exposure increase [1]. Data obtained in this study with 5S receptors do not indicate a comparable salt- or time-dependence. In Fig. 1, receptor samples were incubated in various DTK buffers (DT plus 0–667 mM KCl) and measured using DCC suspended in DT buffer. At 400 mM final KCl concentration (i.e. 667 mM initial KCl concentration), underestimation of ER levels did occur (81 ± 12% of control in DT buffer). However, lowest ER levels (48 ± 2%) occurred in those samples with 120 mM final KCl concentration (200 mM initial KCl concentration).

Correlation of the minimum receptor level with a particular ionic strength is not possible if the ionic strength at which the receptors are labeled with estradiol (incubation conditions) differs from the ionic strength present during exposure to DCC (assay conditions). Accordingly, experiments of the type described in Fig. 1 were performed using DCC in buffers of the same ionic strength as the receptor samples. Using this method, no change in the incubation ionic strength occurred during the assay. In

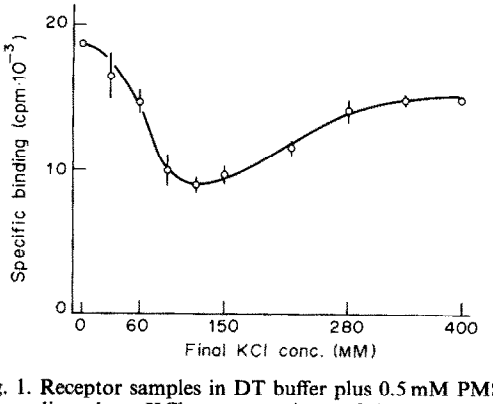


Fig. 1. Receptor samples in DT buffer plus 0.5 mM PMSF were adjusted to KCl concentrations of 0–667 mM and incubated with 4 nM [$^3$H]$E_2$ for 20–23 h. DCC in DT buffer was added for 10–20 min, diluting the KCl in each sample 40% to the indicated final concentration. Non-specific binding was determined in the presence of a 200-fold excess of unlabeled estradiol. Samples were assayed in triplicate.

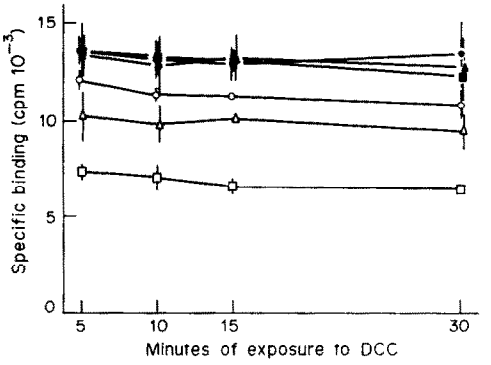


Fig. 2. Receptor samples were adjusted to 0 (○, ●), 150 (□, ■) or 400 (△, ▲) mM KCl prior to incubation with 4 nM estradiol (2 nM [$^3$H]$E_2$, 2 nM unlabeled $E_2$) for 14.5–18.5 h. DCC in buffer of appropriate ionic strength was incubated with samples for the indicated times. Trypsinized samples (●, ■, ▲) were treated during the first 30 min of charging with $E_2$. Non-specific binding was assayed in the presence of a 1000-fold excess of diethylstilbestrol. Each point is the mean ±2 SD of duplicate measurements.

Fig. 2, ER values were again lower in samples assayed in DTK 150 buffer (60 ± 2%) in contrast to those in DT alone (control = 100%) or in DTK 400 (87 ± 2%). The reduction in ER levels in Fig. 2 corresponds closely to those in Fig. 1 when, in the latter, the ionic strength during the DCC assay, rather than during the incubation with estradiol, is considered. The degree of receptor underestimation is therefore determined by the ionic strength present at the time ER is exposed to DCC.

Two additional characteristics of the receptor are evident from Fig. 2. The process responsible for reduction of ER levels was essentially complete after 5 min exposure; ER values decrease only slightly from 5 to 30 min after addition of DCC. In contrast to native 5S receptors, trypsin-treated receptors (4S in sucrose gradient sedimentation, data not shown) were not affected by alterations in ionic strength. Relative to native 5S ER assayed in DT buffer (control = 100%), trypsinized receptor samples assayed in DT, DTK 150 or DTK 400 displayed uniformly higher ER levels (115 ± 4%). The salt-dependent variations in measurement of ER, therefore, only occur in samples of unmodified (5S) receptors.

*Ionic strength dependence of ER determination by gel filtration*

Similar observations were made in measurements using gel filtration techniques. These were expected to quantitate ER levels giving results independent of ionic strength (except at very low ionic strengths, where some column resin-ER binding could be expected [10]). Using one method (zonal gel filtration), samples in DTK 150 displayed lower ER levels (52 ± 6%) compared to those in DT buffer (control = 100%) or those in DTK 400 (90 ± 13%) [Fig. 3].

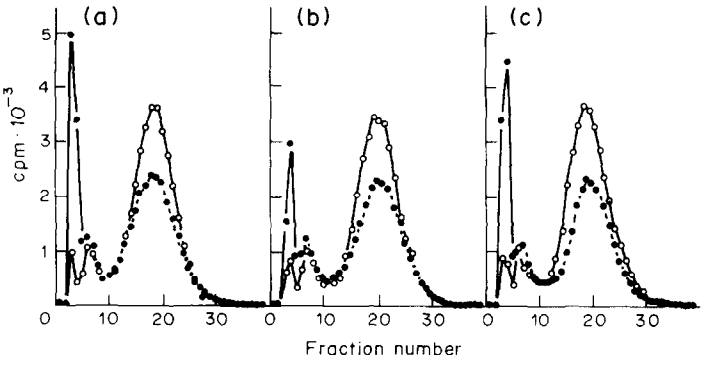


Fig. 3. Receptor samples in DT (Panel A), DTK 150 (Panel B) or DTK 400 (Panel C) were incubated with 4 nM [$^3$H]$E_2$ for 16–23 h. Samples were applied to separate Sephadex gel filtration columns equilibrated in corresponding buffers. Counts in fractions 1–10 were summed for calculation of specific binding. Duplicate assays were done without (●) and with (○) a 200-fold excess of unlabeled $E_2$. Only one example of each duplicate is illustrated.

In other experiments, using the centrifuge column gel filtration technique [8], native or trypsinized receptor preparations were assayed to determine the effects of ionic environment. Untreated ER samples in DTK 150 demonstrated lower ER levels (55 ± 7%) than those in DT (control = 100%), or those in DTK 400 (113 ± 9%). As shown earlier for DCC assays, trypsin-treated receptor preparations displayed equal or higher assayed ER levels (101 ± 15%, 116 ± 14% and 150 ± 9% for samples in DT, DTK 150 and DTK 400, respectively) compared to untreated receptors in DT buffer (control = 100%). Therefore, two different gel filtration techniques yielded results that confirmed those obtained with the DCC assay.

*Testing alternative explanations for low ER assays at 150 mM KCl*

We considered three possibilities that could account for the lower apparent ER measured in buffer containing 150 mM KCl:

(1) changes in the equilibrium binding capacity of receptors could have occurred because of alterations in ligand binding sites; (2) estradiol could have rapidly dissociated from some receptors during the assay; or (3) receptor–estradiol complexes could have adsorbed to both the DCC and Sephadex resin. Each of the above was tested in a series of experiments.

(1) That a reversible alteration of ligand binding sites might be occurring was tested by first inducing "dissociation" of bound estradiol in DTK 150, and then allowing ligand "rebinding" to the receptor in DTK 400. In order that the presumed dissociation of labeled estrogen in DTK 150 could be detected, excess unlabeled ligand was added to the receptor samples prior to shifting from DTK 150 to DTK 400. The unlabeled ligand should have prevented rebinding of [$^3$H]estradiol. Assays were performed by the centrifuge column technique in DTK 400. The results (not shown) indicate that the receptor samples retained quantitatively the initially bound labeled estradiol despite the addition of excess unlabeled ligand. Therefore, dissociation of ligand followed by rebinding did not occur.

If the apparent decreased binding at 150 mM KCl resulted from irreversible inactivation of ligand binding sites, this could be measured by initially incubating receptor samples in DTK 150 for increasing periods of time before raising the KCl concentration to 400 mM to stop the process. Inactivated receptor would then be unable to bind estradiol when assayed in DTK 400. In an experiment of this type, receptor preparations in DT buffer plus 1 μM pepstatin A (to inhibit proteolysis) were charged with [$^3$H]estradiol, in the absence or presence of a 200-fold excess of unlabeled estradiol, for 20 h at 4°C. Each sample was then divided: two thirds of the sample was adjusted to 150 mM KCl, while one-third remained in buffer without KCl (control). The incubations were continued for 1, 2, 4, 8, 16 or 36 additional hours. At each time point, aliquots of samples incubated in DTK 150 were readjusted to 400 mM KCl for 15 min, then assayed for bound estradiol by the centrifuge column gel filtration procedure. An aliquot of receptor incubated in DTK 150 was assayed in DTK 150; a parallel aliquot of receptor incubated in DT buffer was assayed in DT buffer (control). Under these conditions, receptor samples incubated in DTK 150, then assayed in DTK 400 demonstrated as much ER (101 ± 5% at 36 h) as samples incubated for the entire period in DT buffer (control = 100%). Samples maintained throughout (incubated and assayed) in DTK 150 demonstrated reduced ER levels (72 ± 2%) compared to controls. Taken together, these experiments lead us to conclude that the lower ER values observed in DTK 150 were not caused by inactivation of ER in solution.

(2) To test the second possibility, rapid receptor-estradiol dissociation during the assay, two experimental approaches were undertaken. Following gel filtration, vacant receptors resulting from dissociation of bound estradiol could, in theory, be recharged. However, this result has never been real-

ized (data not shown). Alternatively, if estradiol were dissociating from the receptor during gel filtration, free estradiol should be recoverable, in an amount corresponding to the apparent loss of receptor-bound ligand. To test this, ER preparations, in which free estradiol had been removed by preliminary gel filtration in DT buffer, were analyzed by zonal gel filtration in either DT or DTK 150 buffer. When compared to control samples in DT buffer, samples in DTK 150 demonstrated only $50 \pm 7\%$ as much receptor-bound [$^3$H]estradiol. But in neither treatment was a significant amount of estradiol eluted as free ligand (Fig. 4). These results suggested that the third possibility is most likely, i.e. lower levels of ER in samples analyzed in DTK 150 resulted from ER complexes adsorbing to the gel filtration columns.

This conclusion is also supported by the following results. When the columns in the above experiment were eluted with DTK 400 after initial elution in DT or DTK 150, additional ER was recovered. Total recovery of ER from columns initially loaded in DTK 150 was still $22 \pm 6\%$ less than that from columns initially loaded in DT buffer, indicating that a proportion of receptors remained bound.

To confirm that receptors were binding to gel filtration columns equilibrated in DTK 150 to a greater extent than to columns equilibrated in DT, the following experiment was performed. Receptor samples were loaded onto Sephadex G-25 columns in either DT or DTK 150 and washed extensively with starting buffer, then with DTK 400, and finally with 6 M guanidine HCl (Gu HCl). Specifically bound [$^3$H]estradiol recovered at each stage is listed in Table 1. Consistent with previous results, ER eluting from the columns was lower in samples applied in DTK 150 ($19{,}403 \pm 1{,}779$ dpm) vs those in DT ($35{,}898 \pm 852$ dpm). The additional ER recovered after elution with DTK 400 was comparable from samples originally loaded in DTK 150 ($13{,}551 \pm 672$ dpm) and those loaded in DT ($12{,}214 \pm 528$ dpm). When the columns were subsequently eluted with 6 M Gu HCl, significantly more ER was recovered from columns loaded in DTK 150 ($15{,}659 \pm 2{,}967$ dpm) than from columns loaded in DT buffer ($5{,}419 \pm 211$).

Table 1. Adsorption of ER during Sephadex gel filtration

| Washes | Specifically bound DT | [$^3$H]-$E_2$ recovered (dpm) DTK 150 |
|---|---|---|
| Loading buffer | $35{,}898 + 852$ | $19{,}403 + 1{,}779$ |
| DTK 400 | $12{,}214 \pm 528$ | $13{,}551 \pm 672$ |
| 6 M Gu HCl | $5{,}419 \pm 211$ | $15{,}659 \pm 2{,}967$ |
| Total | $53{,}531 \pm 1{,}024$ | $48{,}613 \pm 3{,}524$ |

Receptor samples were incubated in DT or DTK 150 with 1 $\mu$M leupeptin and 4 nM [$^3$H]$E_2$ for 18–30 h at 4°C. Nonspecific binding was assayed in parallel incubations with a 200-fold excess of unlabeled estradiol. Aliquots of 100 $\mu$l were applied to separate columns equilibrated in DT or DTK 150 and eluted with starting buffer. Forty fractions were collected. Columns were then developed sequentially with DTK 400 and 6 M guanidine HCl (Gu HCl). Twenty fractions were collected with each wash. Radioactivity eluting from the columns returned to background levels before each successive wash. Counts eluting in fractions 1–10, 41–60 and 61–80 were summed for measurement of specific binding. Assays were done in duplicate.

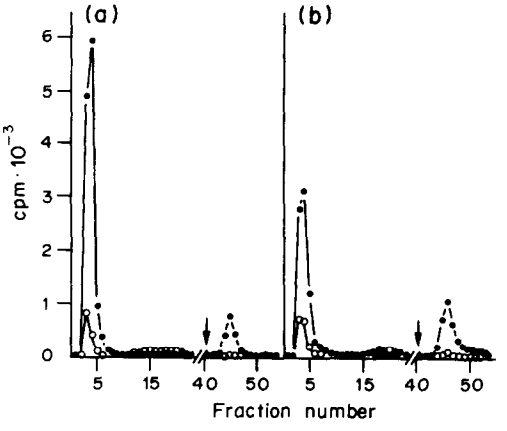


Fig. 4. Receptor samples were incubated in DT buffer plus 1 $\mu$M leupeptin with 4 nM [$^3$H]$E_2$ for 6 h at 4°C, then 15 min at 29°C, then cooled to 4°C. Non-specific binding was assayed by parallel incubations in the absence (●) and presence (○) of a 200-fold excess of unlabeled $E_2$. Individual samples were analyzed as follows. To remove unbound estradiol, 100 $\mu$l aliquots were applied to centrifuge columns equilibrated in DT buffer. Of the sample eluted from the column, 85 $\mu$l were immediately adjusted to a final volume of 140 $\mu$l in DT or DTK 150 buffer. Of this, 20 $\mu$l were counted directly to control for recovery of ER, and 100 $\mu$l were assayed immediately by zonal gel filtration on Sephadex columns equilibrated in DT (Panel A) or DTK 150 (Panel B). After 40 fractions were collected, each column was then eluted with DTK 400 (arrows). Assays were done in duplicate. Single examples of each are shown.

Although this experiment demonstrated that the reduction of ER in DTK 150 resulted from binding of receptor complexes to Sephadex resin, we have not conducted comparable experiments with DCC and centrifuge column gel filtration assays. However, the similarity of results seen with each assay technique support the conclusion that adsorption of ER is occurring in each case.

*ER Assays with a polyacrylamide resin*

Because dextran is a component in both DCC particles and Sephadex resin, we questioned whether the similar results observed were caused by the presence of dextran. If so, substitution of a polyacrylamide gel resin (Bio-Gel P-10) should eliminate those changes in ER levels peculiar to dextran. Still, a reduction in receptor complexes measured in DTK 150 relative to DT buffer was also seen with the polyacrylamide resin (Table 2). These results suggest that ER complexes can bind to several commonly used "inert" substrates.

*Binding of unoccupied estrogen receptors to resins*

Unoccupied receptors also appeared to bind to Sephadex resin to a greater extent in DTK 150 than DTK 400, based on the following results. Unlabeled receptor preparations (130 $\mu$l) in DTK 150 or DTK

Table 2. Adsorption of ER during polyacrylamide gel filtration

| Washes | Specifically bound DT | [$^3$H]-$E_2$ recovered (dpm) DTK 150 |
|---|---|---|
| Loading buffer | 146,910 ± 1,469 | 86,683 ± 14,270 |
| DTK 400 | 8,121 ± 832 | 29,842 ± 9,947 |
| 6 M Gu HCl | −124 ± 258 | 32,866 ± 771 |
| Total | 154,907 ± 1,708 | 149,391 ± 17,412 |

Receptor samples were incubated in DT buffer with 0.5 mM phenylmethylsulfonyl fluoride and 10 nM [$^3$H]$E_2$ for 24–61 h at 4°C. Nonspecific binding was assayed in parallel incubations with a 200-fold excess of unlabeled estradiol. A gel filtration column was prepared (see Experimental section) using 2 ml of Bio-Gel P-10 polyacrylamide resin. Immediately prior to gel filtration, receptor samples (90 μl) were adjusted to 0 mM or 150 mM KCl by addition of DT or DTK 1034 buffer (15.3 μl). Aliquots of 100 μl were applied to the column and eluted as described for Table 1. Counts eluting in fraction 1–6, 41–60 and 61–80 were summed for measurement of specific binding. Assays were done in duplicate.

400 buffer were applied to centrifuge columns equilibrated in corresponding buffers, and 100 μl aliquots of the eluate were incubated for 16 h at 4°C with 4 nM [$^3$H]$E_2$. All samples were then assayed by the centrifuge column technique using columns equilibrated in DTK 400. Samples initially filtered through DTK 150 columns demonstrated lower ER levels than those from DTK 400 columns. Under these conditions, different levels of ER must result from adsorption of uncharged receptors to centrifuge columns equilibrated in DTK 150.

*Effect of molybdate on receptor adsorption*

Molybdate has been reported to reversibly interact with ER to prevent dimerization and to interfere with nuclear binding [7, 11]. We examined the effect of molybdate on the adsorption of ER to Sephadex (Table 3). Molybdate-treated ER did not bind to Sephadex resin at any salt concentration tested. When molybdate was removed by dialysis prior to the receptor assay, underestimation of ER was greater in buffers of 20 mM ionic strength compared to buffers of 150 or 400 mM ionic strength. Separate sucrose gradient analysis indicated that the 4S molybdate-treated ER species had been converted to 5S ER species after removal of molybdate by dialysis (data not shown).

Table 3. Molybdate inhibits adsorption of ER to Sephadex

| Sample | Incubation/Filtration buffer | Relative binding |
|---|---|---|
| 1 | 10 mM $Na_2$ $MoO_4$ | 104 ± 11% |
| 2 | 10 mM $Na_2MoO_4$ + 130 mM KCl | 101 ± 16% |
| 3 | 10 mM $Na_2MoO_4$ + 380 mM KCl | 101 ± 18% |
| 4 | 20 mM NaCl | 54 ± 11% |
| 5 | 20 mM NaCl + 130 mM KCl | 86 ± 8% |
| 6 | 20 mM NaCl + 380 mM KCl | 100% |

Molybdate-treated ER was dialyzed against 1000 vol of DT containing 10 mM $Na_2MoO_4$ or 20 mM NaCl for 15 h. Samples were then incubated with 4 nM [$^3$H]$E_2$ in the absence or presence of a 200-fold excess of unlabeled estradiol for 14–15 h at 4°C. KCl was present at the final concentrations indicated. Samples were analyzed on freshly prepared centrifuge columns equilibrated in the indicated buffers. ER levels measured in sample 6 were used as controls for comparison to other samples. Assays were done in triplicate.

## DISCUSSION

The results presented here clearly illustrate the limitation of two common techniques for quantifying ER levels. The DCC assay is often cited, as detailed by Peck and Clark [1], for underestimating receptor levels when used in a high salt environment. In their report, measured ER levels fell progressively as the salt concentration was increased from 150 mM to 300 mM KCl. Lukola *et al.*[5] noted a minimum ER level at approx 300 mM KCl, with values increasing at higher or lower ionic strengths. Results reported here (Figs 1 and 2) demonstrate that the ionic strength of the ER/DCC assay mixture, rather than the ionic strength of the buffer in which the receptors were incubated with estradiol, determines the degree of receptor underestimation. In addition, the maximum effect was observed not at 400 mM added KCl, but at 120–150 mM added KCl (150–180 mM total ionic strength).

The maximum reduction in assayed ER levels was approx 50%. Because trypsin treatment prevents the salt-induced underestimation of ER, endogenous proteases [12–14] may act in a similar way in untrypsinized receptor samples. Samples of ER prepared in the absence of protease inhibitors (leupeptin, phenylmethylsulfonyl fluoride) often demonstrated only slightly decreased ER values in DTK 150 buffer.

Reduced ER levels are not caused by salt-dependent receptor inactivation, or receptor–ligand dissociation either in solution or during gel filtration. Receptor complexes are adsorbed to Sephadex and polyacrylamide gel filtration matrices in DTK 120–150. By inference, adsorption of ER to DCC is the most likely explanation for receptor underestimation in that assay.

The results obtained with each assay were substantially similar, though some differences were consistently observed. The DCC assay measured lower ER levels in DTK 400 than in DT buffer. By contrast, similar or slightly higher ER values were measured in DTK 400 relative to DT buffer with the gel filtration assays. Receptor underestimation was generally greater in the DCC and zonal gel filtration assays than in the centrifuge column assay. Finally, the intra-assay variability for the centrifuge column technique was much greater than for the DCC or zonal gel filtration assays.

Adsorption of ER to DCC, Sephadex, and polyacrylamide occurs despite little structural similarity among these materials. Hydrophobic interactions are likely to be involved, based on the nonionic character of the resins and the tendency of the receptor, once adsorbed, to resist elution in DTK 400. For comparison, reported interactions between the receptor and polyanions, such as DNA [15] or oligo(dT)-cellulose [16, 17], are disrupted more by 400 mM KCl than 150 mM KCl. Underestimation of ER is prevented both in the DCC assay and centrifuge column assay by trypsin treatment. Our data therefore support the suggestion by Katzen-

ellenbogen *et al.*[4] that trypsin treatment cleaves the estrogen binding domain of the receptor from the DCC-binding domain. It has been reported that the porcine ER possesses a strongly hydrophobic binding region which is cleaved from the estrogen binding region by trypsin [18]. Whether the surmised hydrophobic interactions described here and elsewhere are only indicative of undegraded receptor, or are in fact necessary for the role of ER in gene expression, is not clear. It is intriguing that the extent of ER adsorption is most pronounced at ionic strengths putatively corresponding to intracellular conditions. However, our observation that uncharged receptors also are adsorbed to Sephadex does not add support to the implied physiologic significance of this type of interaction.

Activated charcoal, Sephadex and polyacrylamide used in the DCC and gel filtration assays strongly adsorb up to 50% of the ER being measured. We have suggested that the adsorption process, which is prevented by treatment with trypsin or molybdate, involves a hydrophobic portion of the receptor. The greatest underestimation of ER levels by the DCC and gel filtration assays occurs not at 400 mM added KCl but at 120–150 mM added KCl.

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