Pergamon

0960-0760(94)00099-9

J. Steroid Biochem. Molec. Biol. Vol. 50, No. 5/6, pp. 313–318, 1994

0960-0760/94 $7.00 + 0.00

Equilibrium Hormone Binding to Human Estrogen Receptors in Highly Diluted Cell Extracts is Non-cooperative and has a K_d of Approximately 10 pM

Marie Salomonsson, Bo Carlsson and Johan Häggblad*

KaroBio AB, PO Box 4032, 141 04 Huddinge, Sweden

It is generally accepted that the K_d for hormone binding to estrogen receptors in extracts ranges between 0.1–1 nM and that binding displays positive cooperativity due to formation of homodimers. After carefully optimizing assay procedures, to diminish ligand depletion phenomena and to fully control recoveries, we find a single class of non-interacting high affinity hormone binding sites with a K_d of approx. 10 pM. Ligand depletion was avoided by decreasing receptor concentrations to 5–8 pM. We were therefore obliged to employ radioiodinated estradiol as a probe as the specific radioactivity of tritiated estradiol was too low to maintain the accuracy of the binding assay. Human estrogen receptor extracted from the MCF7 cell line and recombinantly produced (in yeast) wild-type human receptor have identical equilibrium hormone binding characteristics.

J. Steroid Biochem. Molec. Biol., Vol. 50, No. 5/6, pp. 313–318, 1994

INTRODUCTION

Regardless of the salt concentration used during extraction, the magnitude of reported equilibrium dissociation constants in cell-free extracts, determined at or below room temperature, for estradiol binding to estrogen receptors (ER) is generally in the 0.1 to 1 nM range [1–6]. Scatchard transforms of estradiol binding curves are in some reports convex curvilinear, suggesting positive cooperativity of estradiol binding [7, 8].

Regular radioligand binding experiments require a step where bound radioligand is separated from free. There are various ways the separation can be accomplished. For the steroid and thyroid hormone receptors, bound radioligand is traditionally separated from free by ion exchange chromatography [9]; adsorption to dextran coated charcoal [10], hydroxylapatite [6] or porous glassbeads [11]; removing whole cells or nuclei from the incubation [4]; or, finally, by gel permeation chromatography on Sephadex gels [12].

In order to accurately evaluate equilibrium binding experiments, free radioligand must be determined in a precise way. As free radioligand in most cases is calculated by subtracting bound radioligand from total added radioligand, it is of importance that the recovery of the separation step is accounted for. Most separation steps employed for hormone binding to intracellular hormone receptors "extract" bound radioligand, i.e. receptors, from the reaction mixture, free radioligand concentration may thus be overestimated in cases where the recovery of the separation step is less than 100% and unaccounted for.

In this report we present results on binding of 17β-[2,4,6,7-^{3}H]estradiol ([^{3}H]E2) and of 17β-[16α-^{125}I]iodo-estradiol ([^{125}I]E2) to human estrogen receptors, in cell-free systems, with a method in which the recovery of receptors, when separating bound from free radioligand, is almost 100%. In order to obtain accurate conditions for the determination of the equilibrium dissociation constant, we were obliged to exchange [^{3}H]E2 for [^{125}I]E2, as receptor concentrations had to be drastically decreased. The results presented in this report strongly deviate from traditionally obtained equilibrium dissociation constants for hormone binding to human estrogen receptors as we obtained equilibrium dissociation constants in the 5–15 pM range.

*Correspondence to J. Häggblad.
Received 10 Nov. 1993; accepted 9 May 1994.

Abbreviations: DCC, dextan coated charcoal; G25, Sephadex G25 gel; HAP, hydroxyl apatite; hER(MCF7), human estrogen receptor from MCF7 breast tumour cells; hER(y), human estrogen receptor expressed in a yeast system; [^{3}H]E2, 17β-[2,4,6,7-^{3}H]estradiol ([^{3}H]E2); [^{125}I]E2, 17β-[16α-^{125}I]iodo-estradiol.

EXPERIMENTAL

Materials

[^{3}H]E2 (85–115 Ci/mmol) and [^{125}I]E2 (2200 Ci/mmol) where obtained from New England Nuclear (MA, U.S.A.). Diethylstilbestrol and monothioglycerol where purchased from Sigma (MO, U.S.A.). Sephadex G25 columns (QS-2A) was obtained from Isolab Inc. (OH, U.S.A.). Hydroxyl apatite (Bio-Gel-HTP) was purchased from BIO-RAD (CA, U.S.A.). Dextran T70 was purchased from Pharmacia (Uppsala, Sweden). Cell culturing media, non-essential amino acids and fetal calf serum where obtained from GIBCO (NY, U.S.A.).

Buffers

$TEGK_{50}$, $TEGK_{400}$; 20 mM Tris, 1 mM EDTA; 10% glycerol, 6 mM monothioglycerol with 50 *or* 400 mM KCl; pH = 7.8. Buffer A; 10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM dithiothreitol; pH = 7.9.

Preparation of native ER

MCF-7 cells (human breast carcinoma) where cultured in RPMI 1640 supplemented with L-glutamine, non-essential amino acids and 10% foetal calf serum. Medium was changed, 48 h before harvesting, to Dulbecco's medium (without phenol red) supplemented with L-glutamine, non-essential amino acids and 10% charcoal dextran-treated foetal calf serum. Near-confluence cells were harvested and washed in PBS (without Ca^{2+} and Mg^{2+}). The cells were pelleted and kept in a freezer (−70°C) for 2 days. Homogenization was done in buffer A, using the B pestle of a Dounce homogenizer. NaCl was added (0.7 M) after homogenization and the homogenate was kept on ice for 1 h before centrifugation. The homogenate was centrifuged for 45 min at 130,000 *g* at 4°C. The supernatant, containing hER(MCF7), was collected and stored (−70°C) for ligand binding studies.

Expression of the human ER in Saccharomyces cerevisiae

The wild-type hER(y) was expressed and extracted from mechanically lysed yeast cells according to Harris Wooge *et al.* [2]. The yeast cells (strain BJ 5460) used for the carrier yeast extract (see below) were grown and lysed under the same conditions as the yeast cells which were expressing hER. The carrier yeast extract was tested for ligand binding; it contained no specific estradiol binding sites.

Receptor binding assays

Receptor preparations were diluted in $TEGK_{50}$ or in $TEGK_{400}$. The hER containing extracts were highly diluted and 0.5% (v/v) carrier yeast extract was added to the diluent to prevent adsorption of the receptor protein to vials. Diluted receptor preparations where incubated with radioactive labelled estradiol in the presence or absence of a 300-fold excess of diethylstilbestrol. All incubations were done in polypropylene test tubes overnight at 4°C in a total volume of 200 μl. Binding of radioligand to polypropylene vials is minimal under the described conditions (results not shown). The dextran coated charcoal (DCC) assay was performed as described previously [10] and the hydroxyl apatite (HAP) assay as described previously [13]. The G25-column method was carried out essentially as previously described for the thyroid hormone receptor [14] except that the buffer was changed to $TEGK_{50}$ or to $TEGK_{400}$ and no histones were used. Bound radioactivity was measured with standard techniques in Wallac (Turku, Finland) analytical instruments.

Data evaluation

Specific binding was determined by subtracting non-specific binding (determined with an excess of unlabelled diethylstilbestrol) from total binding. Free ligand concentration was estimated by subtracting total bound ligand from total added ligand. Equilibrium dissociation constants (K_d) were calculated as free concentration of radioligand at half maximal binding, by fitting data to the Hill equation $\{LR = [(B_{max} \times Ln_H)/(Ln_H + K_d n_H)]$; where n_H denotes the Hill coefficient; LR, bound radioligand; B_{max}, maximally bound radioligand; and L, the free concentration of radioligand$\}$ and to the Scatchard equation $\{LR/L = B_{max}/K_d - LR/K_d)\}$. Curve fitting was done in KaleidaGraph 2.1.3 (Abelbeck Software, PA, U.S.A.) using a Macintosh II (4/40).

RESULTS

Comparison of separation methods

In order to evaluate the determination of the K_d, a number of separation methods were tested. In Fig. 1(A), results from separation of free from bound radioligand accomplished by DCC, G25 and HAP are shown. Aliquots from one reaction mixture per concentration of radioligand [[^{3}H]E2 and hER(y)-extract in $TEGK_{50}$ buffer] were used with all three methods of separation. The order of recovery of maximally bound radioligand, from high to low, was G25 > HAP > DCC. The HAP- and DCC methods used were standard assay procedures described in handbooks [e.g. 10] and in scientific reports [e.g. 13] for [^{3}H]E2 binding to hER in extracts. The G25 method was originally described for triiodotyronine (T3) binding to nuclear T3-receptors [12, 14]. As the G25 method had a very high recovery of un-denatured receptor protein, we decided to optimize this procedure.

Receptor recovery of the G25-based assay

To estimate the receptor recovery of the G25-based assay, one incubation mixture was repeatedly passed over a G25 column. We observed that there was no significant decrease in eluted (bound) radioactivity

between separation runs (run 1, 100% vs run 2, 96 ± 5%; triplicates). Also, in situations where incubation mixtures contained an excess receptor to radioligand, all added radioactivity was eluted from the columns (results not shown). These observations show that the receptor recovery in the G25-separation step is close to 100%; and that the recoveries need not to be taken into account when calculating the concentration of free radioligand.

Results at high and low receptor concentrations with the G25-based assay

To investigate the risk of ligand depletion, binding assays with low receptor concentrations were performed with [^{3}H]E2, the G25 assay and hER(y)-extract in $TEGK_{400}$ buffer. During the course of this series of experiments a high variability in the shape of the binding curves was observed at low salt concentrations in the original $TEGK_{50}$ buffer (results not shown). Therefore, the high salt concentration $TEGK_{400}$ buffer was chosen in the continued experiments as it minimized the observed variation at low salt concentration. In the [^{3}H]E2/G25/hER(y)-configuration, the lowest receptor concentration used with maintained precision in the determination of binding affinity was around 0.2–0.3 nM. In Fig. 1(B) a typical result with [^{3}H]E2, the G25 assay and hER(y) is presented. At 0.5 nM receptor concentration, the binding data displayed non-linear Scatchard plots [Fig. 3(A)].

To be able to further decrease receptor levels, in order to minimize the risk of ligand depletion we changed [^{3}H]E2 to [^{125}I]E2; the latter possessing a 22-fold higher specific radioactivity. When employing [^{125}I]E2 in binding experiments, where bound radioligand was separated from free by the G25 assay, we were able to perform experiments below 10 pM in B_{max}. At these low receptor concentrations, the K_d for [^{125}I]E2 binding to hER(y) or to hER(MCF7) was in the 5–15 pM range [Fig. 2(A and B)]. At 5–10 pM hER concentration the binding data transformed into linear Scatchard plots [Fig. 3(A and B)].

DISCUSSION

The effects of unknown receptor recovery and of ligand depletion on the estimation of equilibrium hormone binding affinity

Taken together, our results show that when receptor concentrations are carefully considered, in binding experiments designed to determine the equilibrium dissociation constant for hormone binding to the hER, the obtained K_d is considerably lower than previously reported. As judged from our results with the non-optimized DCC- and the HAP separation methods, receptor recoveries must be considered when free radioligand is calculated. Preliminary tests with ion-exchange membranes and porous glass beads were also performed (not shown). The receptor recoveries in all these methods were typically 50% of the chosen G25-based assay.

We propose furthermore that the previously reported high K_d-values, in the 0.1 to 1 nM range of hormone, and the previously reported positive cooperativity of hormone binding may be results of insufficient specific radioactivity of the radiolabelled probe and of ligand depletion at high receptor concentrations. This is exemplified by the binding curves in Fig. 1(A) which all become convex curvilinear when transformed into Scatchard plots (transforms not shown). Ligand depletion [15, 16] may occur at low radioligand concen-

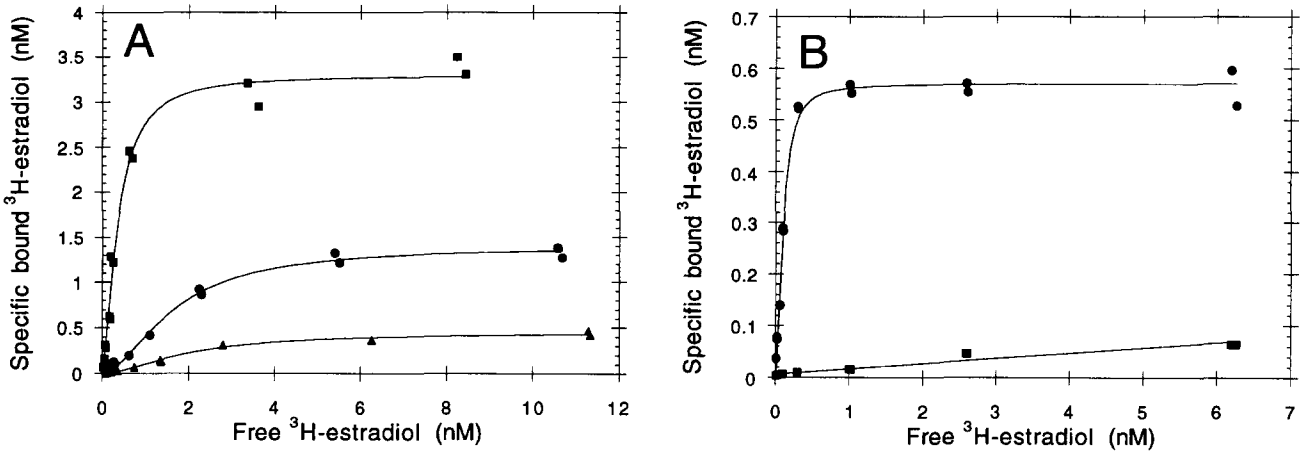

Fig. 1. (A) Comparison of effects from using HAP, G25 or DCC as medium to separate bound from free radioligand following binding of [^{3}H]E2 to hER(y) in $TEGK_{50}$. Results presented are specific binding from a typical experiment performed in duplicates. ■–■, G25; ●—●, HAP; ▲—▲, DCC. (B) Typical equilibrium binding isotherm for binding of [^{3}H]E2 to hER(y) in $TEGK_{400}$ at the lower hER(y)-concentration range allowed for the *tritiated* radioligand. Results presented are from an experiment performed in duplicates. Curve fitting was accomplished by the Hill equation ($K_d = 0.097$ nM; $n_H = 1.77$) A convex curvilinear Scatchard transformation of these data is displayed in Fig. 3(A). ●—●, Specific binding; ■—■, non-specific binding.

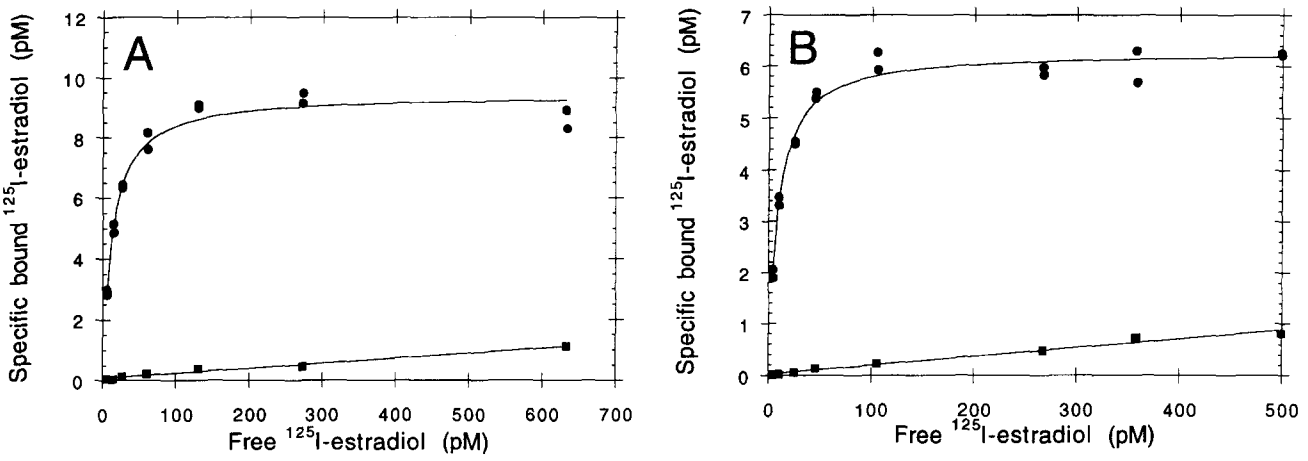

Fig. 2. (A) Typical equilibrium binding isotherm for binding of [^{125}I]E2 to hER(y) in TEGK$_{400}$. (B) Typical equilibrium binding isotherm for binding of [^{125}I]E2 to hER(MCF7) in TEGK$_{400}$. Both data sets were obtained at the lower receptor concentration range allowed for the *iodinated* radioligand. Curve fitting was accomplished by the Hill equation: A; hER(y) $K_d = 12$ pM; $n_H = 0.93$: B; hER(MCF7) $K_d = 8.2$ pM; $n_H = 0.96$. Linear Scatchard transformations of hER(y)- and hER(MCF7) data are displayed in Fig. 3(B and C), respectively. ●—●, Specific binding; ■—■, non-specific binding.

trations in Fig. 1(A) as the B_{max} is considerably higher than the observed free concentration of radioligand at half maximal binding (EC_{50}). This is also evident in Fig. 1(B) where the EC_{50} (≈ 0.1 nM) is lower than the B_{max} (≈ 0.5 nM). Again the Scatchard-transform is convex curvilinear [Fig. 3(A)], i.e. the binding data

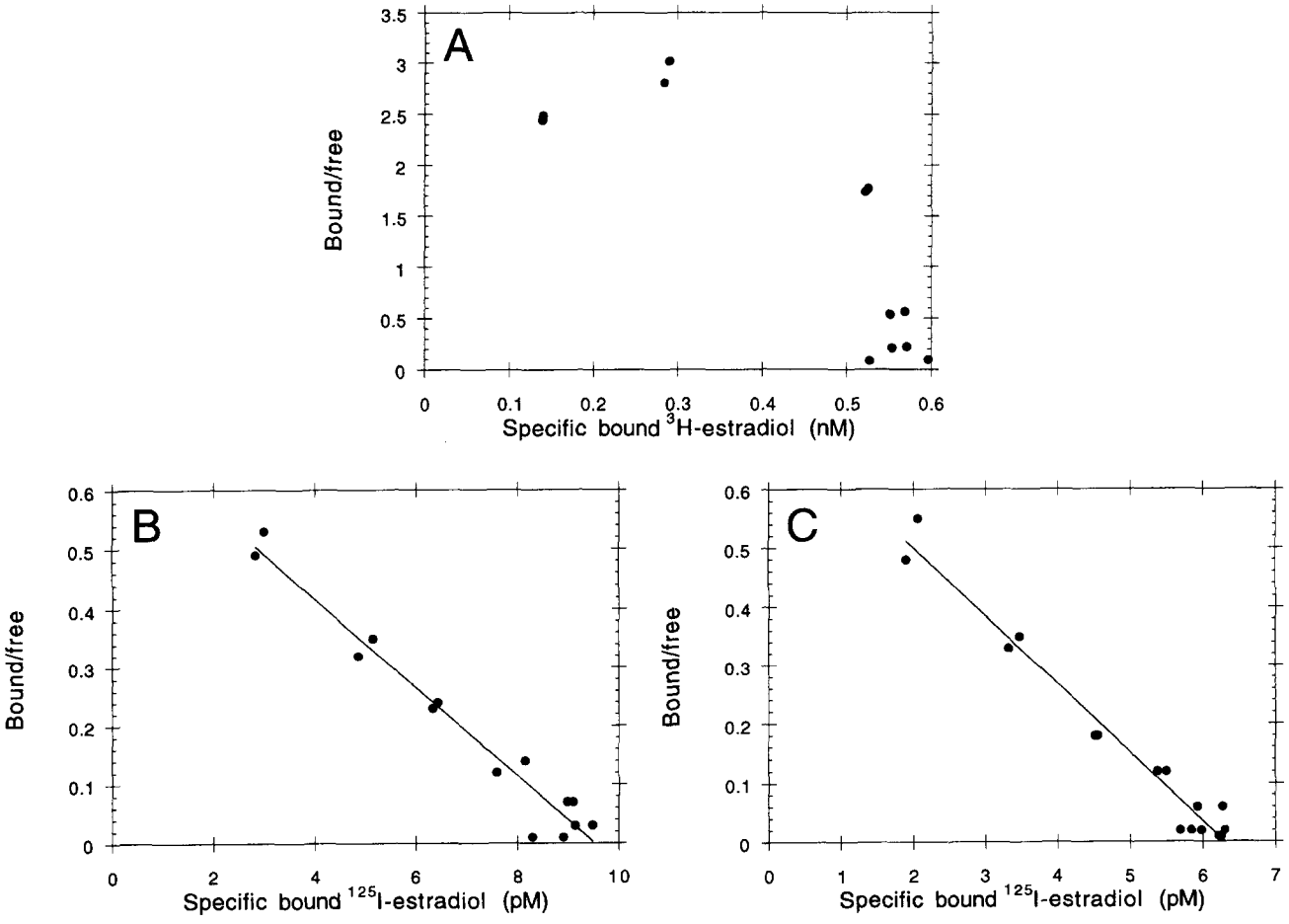

Fig. 3. Scatchard transformations of data sets from Fig. 1(B) in A; from Fig. 2(A and B) in B ($K_d = 13$ pM) and C ($K_d = 9.0$ pM), respectively.

deviates from the "simple case" Michaelis Menten equation (the Hill equation where $n_H = 1$).

Further support for radioligand depletion in the [^{3}H]E2/G25/hER(y)-configuration was obtained during titration of the B_{max} while measuring the "K_d". In the 0.3 to 3.5 nM B_{max}-range, we observed that the lower the B_{max} the lower the "K_d" (results not shown). As a consequence of radioligand depletion, the EC_{50} does not equal the true K_d.

No results were obtained at pre-equilibrium conditions as incubation time-periods up to 72 h did not change obtained results, regardless of separation method employed (results not shown). No significant receptor degradation was observed during the prolonged incubations.

Avoiding ligand depletion by diluting receptor concentration and by using a probe with high specific radioactivity

In order to perform experiments that fulfill the basic assumptions of the adsorption isotherm approach to determination of equilibrium binding affinity, receptor extracts were highly diluted in a carrier-containing vehicle. Furthermore, radioiodinated estradiol was used instead of tritiated. Previously reported determinations of relative or absolute binding affinities suggest almost indistinguishable values for the tritiated and the iodinated hormone [3, 17]. In the present experiments, indirect evidence pointing to equal affinities were obtained as no apparent decrease in affinity was observed when [^{3}H]E2 was changed to [^{125}I]E2. The 16α-iodinated hormone displays estrogenic activity in human breast cancer cells [18]. Thus, despite the difference in structure between the compounds, both are potent estrogens with similar binding affinities.

By continuously decreasing B_{max} we obtained a "stable" K_d; i.e. below a breaking-point (approx. 50 pM) the K_d did no longer decrease with decreasing receptor concentration (not shown). At very low receptor concentrations (<8 pM) binding of [^{125}I]E2 to hER fulfills the Michaelis Menten equation for simple bimolecular binding (n_H equals 1). Thus, Scatchard plots were linear [Fig. 3(B and C)]. In the receptor concentration region below 8 pM, the K_d for binding of radioiodinated hormone is approx. 10 pM. Our findings do not contradict events that may lead to positive cooperativity of estradiol binding to full-length hER, as the cooperativity may be a result of the transition from a low-affinity to a high-affinity form upon activation (dissociation of heat shock proteins/homodimerization). In the set of experiments described in the present report, low concentrations of high-salt extracted receptor was used. High salt extractions yield activated, high affinity (slow dissociation-rate) ERs. In order to achieve accurate conditions ($B_{max} < K_d$) for the determination equilibrium dissociation constants, low concentrations of receptor had to be used. As a consequence of the "$B_{max} < K_d$"-condition, the study of the receptor concentration-dependence of cooperativity phenomena with high-affinity hERs is difficult to perform with equilibrium binding experiments. A better choice may be kinetic binding experiments investigating shifts in dissociation-*rate* constants.

The biological significance of the findings presented in this report may only be speculative at this time. If the activated human ER homodimer displays a hormone binding affinity in the 10 pM range in its transcription-control compartment and the free concentration of hormone inside the cell approximates the total concentration in serum (range: ≈0.2–1.5 nM), the transcriptional control by the ER would be constantly triggered. Thus, there may, along with the extracellular globulin binding mechanisms, exist intracellular estradiol-binders that moderate estradiol levels in various intracellular compartments. Interestingly, the equilibrium binding affinity presented here closely matches functional response constants (EC_{50} values; 7–8 pM) obtained in dose–response experiments with hER and reporter gene-transfected COS-1 and CHO cells [19]. Furthermore, previously obtained kinetic results [20] describing ratios between dissociation- and association rates (≈40 pM) support the equilibrium binding affinity obtained by us. Also equilibrium hormone binding characteristics by ER in intact rat uterine cells [21] support our *in vitro* findings as the cells were shown to possess a single class of high affinity estradiol binding sites. We propose that the K_d for hormone binding to high salt-extracted human ER is considerably lower than previously anticipated and that the ultra-high affinity has functional significance.

Acknowledgements—We wish to express our gratitude to Dr Tom Lavin and to Professor Geoffrey Greene for sharing with us the history of hormone binding to intracellular hormone receptors. We also want to thank Professor Bert O'Malley for providing the yeast expression system for human ER.

REFERENCES

1. Wittliff J. L., Wenz J. D., Dong J., Nawaz Z. and Butt T. R.: Expression and characterization of an active human estrogen receptor as a ubiquitin fusion protein from Escherichia coli. *J. Biol. Chem.* 265 (1990) 22,016–22,022.
2. Harris Wooge C. H., Nilsson G. M., Heierson A., McDonnell D. P. and Katzenellenbogen B. S.: Structural requirements for high affinity ligand binding by estrogen receptors: A comparative analysis of truncated and full length estrogen receptors expressed in bacteria, yeast, and mammalian cells. *Molec. Endocr.* 6 (1992) 861–869.
3. Grill H.-J., Manz B., Belovsky O., Krawielitzki B. and Pollow K.: Comparison of [^{3}H]oestradiol and [^{125}I]oestradiol as ligands for oestrogen receptor determination. *J. Clin. Chem. Biochem.* 21 (1983) 175–179.
4. Reese J. C., Wooge C. H. and Katzenellenbogen B. S.: Identification of two cysteines closely positioned in the ligand-binding pocket of the human estrogen receptor: Roles in ligand binding and transcriptional activation. *Molec. Endocr.* 6 (1992) 2160–2166.
5. Longcope C., Arunachalam T. and Rafkind I.: Biologic activity of the iodoestrogens and their use in breast cancer. *Adv. Exp. Med. Biol.* 138 (1981) 191–210.

6. Pavlik E. J. and Coulson P. B.: Hydroxylapatite "batch" assay for estrogen receptors: increased sensitivity over present receptor assays. *J. Steroid Biochem.* 7, (1976) 357–368.
7. Notides A. C., Lerner N. and Hamilton D. E.: Positive cooperativity of the estrogen rèceptor. *Proc. Natn. Acad. Sci. U.S.A.* 78 (1981) 4926–4930.
8. Sasson S.: Equilibrium Binding analysis of estrogen agonists and antagonists: relation to the activation of the estrogen receptor. *Path. Biol. (Paris)* 39 (1991) 59–69.
9. Santi D. V., Sibley C. H., Perriard E. R., Tomkins G. M. and Baxter J. D.: A filter assay for steroid hormone receptors. *Biochemistry* 12 (1973) 2412–2416.
10. Leake R. E. and Habib F.: Steroid hormone receptors: assay and characterization. In *Steroid Hormones a Practical Approach* (Edited by B. Green and R. E. Leake). IRL Press Ltd, Oxford (1987) pp. 67–92.
11. Greene G., Harris K., Bora R., Kinders R., Moore B. and Nolan C.: Uterine estrogen receptor assayed by the controlled pore glass (CPG) method in adult rat after steroid administration. *Molec. Endocr.* 2 (1988) 714–726.
12. Samuels H. H., Tsai J. S., Casanova J. and Stanley F.: Thyroid hormone action: in vitro characterization of solubilized nuclear receptors from rat liver and cultured GH1 cells. *J. Clin. Invest.* 54 (1974) 853–865.
13. Berkenstam A., Glauman H., Martin M., Gustafsson J-Å. and Nordstedt G.: Hormonal regulation of estrogen receptor messenger ribonucleic acid in T47D and MCF-7 breast cancer cells. *Molec. Endocr.* 3 (1989) 22–28.
14. Appriletti J. W., Baxter J. D. and Lavin T. N.: Large scale purification of the nuclear thyroid hormone receptor from rat liver and sequence-specific binding of the receptor to DNA. *J. Biol. Chem.* 263 (1988) 9409–9417.
15. Seeman P., Ulpian C., Wreggett K. A. and Wells J. W.: Dopamine receptor parameters detected by [^{3}H]spiperone depend on tissue concentration: Analysis and examples. *J. Neurochem.* 43 (1984) 221–235.
16. Hulme E. C. and Birdsall N. J. M.: Strategy and tactics in receptor-binding studies. In *Receptor-ligand Interactions: A Practical Approach* (Edited by E. C. Hulme). IRL Press Ltd, Oxford University Press, New York (1992) 64–176.
17. Hochberg R. B.: Interaction of 16α-^{125}I-estradiol with estrogen receptor and other steroid-binding proteins. *Proc. Natn. Acad. Sci. U.S.A.* 77 (1980) 328–332.
18. Lippman M. E., Do H. M. and Hochberg R. B.: Specific estrogen receptor binding and biological effects of 16α-iodoestradiol on human breastcancer cells. *Cancer Res.* 41 (1981) 3150–3154.
19. Reese J. C. and Katzenellenbogen B. S.: Characterization of a temperature-sensitive mutation in the hormone binding domain of the human estrogen receptor. *J. Biol. Chem.* 267 (1992) 9868–9873.
20. Notides A. C. and Weichman B. M.: Estradiol-binding kinetics of the activated and nonactivated estrogen receptor. *J. Biol. Chem.* 252 (1977) 8856–8862.
21. Walent J. H. and Gorski J.: Estrogen binding is a noncooperative process in primary rat uterine cells. *Endocrinology* 126 (1990) 2383–2391.