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Oestrogen Receptor Assay—Limitation of the Method*

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Abstract—*The variability of estimates of* K_d *and binding capacity* (fmol *oestrogen bound/*mg *cytosol protein*) *of oestrogen receptors* (*RC*) *which may arise from different cytosol protein concentrations* (0.25–5 mg/ml), *different charcoal concentrations* (0.25–5 mg/ml) *and cytosols from different, histologically comparable portions of the same tumour has been studied using a dextran-coated charcoal* (*DCC*) *assay.* K_d *value correlated positively to protein concentration and negatively to charcoal concentration, whereas the binding capacity remained constant over a wide range of protein concentrations. In receptor-poor tumours, low cytosol protein contents precluded a distinction between specific and non-specific oestrogen binding. Both in receptor-rich and receptor-poor tumours, increasing charcoal concentrations added to the incubation mixture decreased the cytosol protein concentration and thus removed RC complexes as well as excess of oestrogen. Both in receptor-rich and receptor-poor tumours, a wide variation in recptor content was observed within histologically comparable portions of the same tumour* (*from* 0 *to* 300 fmol/mg *protein*).

INTRODUCTION

OESTROGEN receptor determination (RC) on breast cancer tissue is performed on particle-free extracts of the tumour tissue, the cytosol. The dextran–charcoal assay (DCC) is often used as a routine assay to separate free and bound oestrogen because a relatively large number of samples can be assayed simultaneously [1–11].

By this method the affinity as well as the total binding capacity of the RC can be estimated from a Scatchard analysis [12, 13]. Binding studies are performed differently from laboratory to laboratory, and the portion of breast tumour tissue used for RC determination is only a section of the total tumour.

It was therefore investigated whether different cytosol protein concentrations, different charcoal concentrations and tissue samples obtained from different parts of the same tumour would influence the estimation of binding capacity and binding affinity as observed with the DCC assay.

MATERIALS AND METHODS

Materials

1. Pooled tissue from rat uterus was used. Rats were killed by ether anaesthesia, and their uteri were removed quickly. Uteri were stripped of fat and mesentery and homogenized as described below (see Methods).

2. Breast cancer tissue as well as benign breast tumour tissue was studied. All but a few biopsy specimens were obtained from primary tumours. The patients comprised both pre-menopausal and post-menopausal subjects. The tissue was received without delay from the pathologist, and damaged and fatty tissues were immediately removed. A representative portion from the remainder was examined histologically and the observations compared with the pathologist's report. The specimen was then stored in a deep freeze (−80°C) until assayed for oestrogen receptors. This was done within one week after receipt of the tissue. For comparison, pooled rat uterus tissue was studied together with the breast tumours and served as a control of the assay.

Methods

(A) *Preparation of cytosols from surgically removed biopsy specimens and rat uteri.* The tissue was minced with a pair of scissors, cooled in liquid nitrogen and homogenized in a Schwingmühle (Retch, Federal Republic of Germany). The homogenate was weighed and suspended in a three-fold volume of TE buffer (Tris 10 mM, EDTA 1.5 mM, pH 7.4).

The homogenate was centrifuged at

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100,000 *g* at 4°C (Beckman; Spinco Ultracentrifuge L50) for 1 hr, and the supernatant, the cytosol, assayed for the amount of high affinity oestrogen receptors.

(B) *The oestrogen receptor assay.* The method used was originally described by Mester *et al.* [14] and Feherty *et al.* [15], and later modified by Daehnfeldt [16] and Poulsen *et al.* [17].

The assay is a dextran-coated charcoal assay which allows calculation of the total binding capacity of high affinity receptors as well as the dissociation constant.

Briefly, 50 μl cytosol was incubated at 2–4°C with ^{3}H-17β-oestradiol (90 Ci/mmol, The Radiochemical Centre, Amersham, U.K.), and at least five different concentrations of 17β-oestradiol (5 μl in ethanol/TKE buffer, Tris 10 mM, KCl 50 mM, EDTA 1 mM, pH 7.4 + 25 μl TKE buffer, ethanol concentration ≦0.5%) ranging from 1.0×10^{-10} to 1.0×10^{-8} M for 2 hr were used. All these assays were performed in duplicate.

The incubation was terminated by addition of 250 μl dextran-coated charcoal suspension [5.0 mg Dextran T-70, 500 mg charcoal (Norit A), 200 ml TKE buffer]. After adsorption for 30 min at 2–4°C the charcoal was spun down (800 *g*, 10 min, 4°C), and the radioactivity in an aliquot of the supernatant was determined by liquid scintillation counting (Packard Tricarb 3003 spectrophotometer, efficiency for tritium approximately 40%). Quench correction was carried out by the channel ratio method. To correct for unspecific binding, controls were used containing 50 μl cytosol, ^{3}H-17β-oestradiol, and 100-fold excess DES (diethylstilboestrol), and the radioactivity was subtracted from the experimental values.

The binding capacities and K_d values were estimated from a Scatchard plot (Fig. 1) [12, 13].

When the results were scattered along a sloping straight line, the biopsies were defined as '*receptor-positive*' (Fig. 1). When the binding data were scattered along a straight line with no slope, when no straight line could be drawn with any confidence, or when no significant binding of oestradiol was recorded, the biopsies were defined as "*receptor-negative*".

Protein measurements were carried out by the method described by Lowry *et al.* [18], and the binding capacities are presented as fmol oestrogen bound/mg cytosol protein.

RESULTS

Effect of increasing cytosol protein concentration on the estimation of binding capacity and apparent dissociation constant within the same tumour

Pooled rat uterus cytosol and cytosol from two different human breast cancers was diluted as indicated in Figs. 2–4. Duplicates of each diluted sample were assayed for oestrogen receptors as mentioned under Materials and Methods.

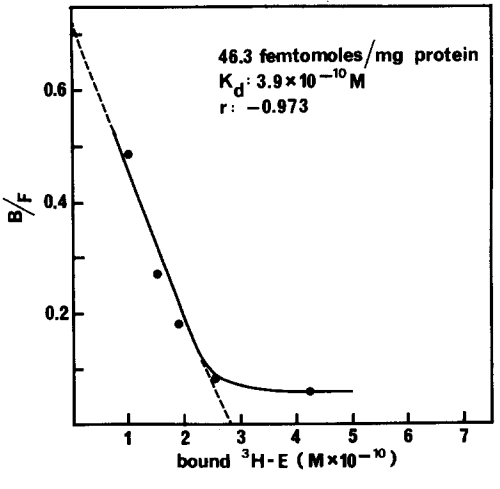

Fig. 1. Binding of oestradiol by carcinoma cytosol. The results are presented in a Scatchard plot with molar concentrations on the abscissa and the ratios bound/free oestradiol on the ordinate. Regression line is calculated by the least square fit from the results in the steepest part of the curve. The interception of this line with the abscissa gives the binding capacity, whereas the slope of this line gives an approximate value of the association constants of the oestradiol receptor complex.

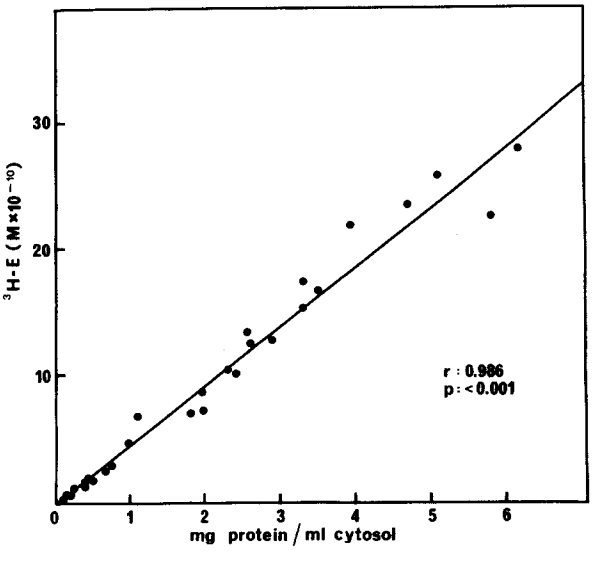

Fig. 2. Binding capacity in relation to protein concentration in cytosol from rat uterus. The cytosol was diluted in TE buffer. Five different concentrations of ^{3}H-17β-oestradiol ranging from 1×10^{-10} to 1×10^{-8} M were incubated with 50 μl cytosol (see Methods). The binding capacities were estimated from a Scatchard plot. Each point represents the estimated total binding capacity (expressed as bound oestrogen $M \times 10^{-10}$ in relation to the cytosol protein concentration).

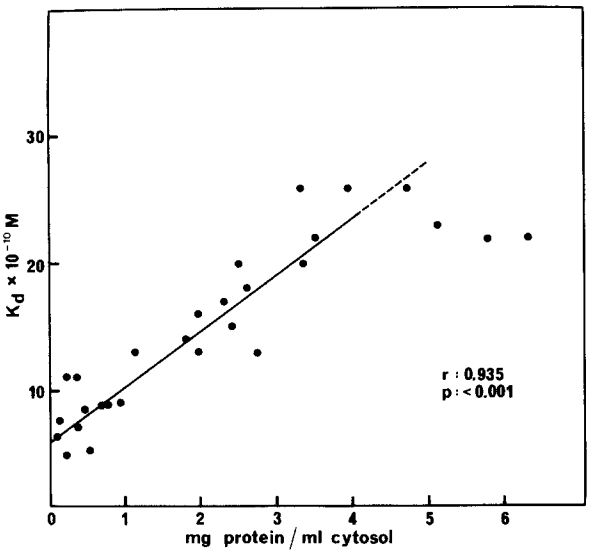

Fig. 3. K_d *values in relation to protein concentration in cytosol from rat uterus. For procedure see legend of Fig. 2. Each point represents the estimation of* K_d *value in relation to the cytosol protein concentration.*

It is seen from Figs. 2–4 that both in rat uterus (Figs. 2 and 3) and in cytosols from breast cancers (Fig. 4) the specific binding was approximately doubled when the protein concentration in the cytosol was doubled. But it was also observed (Figs. 3 and 4) that the estimation of K_d-values based on the analysis of Scatchard plots showed a highly significant positive correlation to protein concentrations.

Variation in binding capacities as a function of cytosol protein concentration in different tumours

Figure 5 shows the influences of protein concentration on the estimation of binding capacities.

It is seen both in cytosols from rat uteri with different RC activity (a–c), and in breast cancer cytosols (1–3) that when the receptor concentrations are low, a higher total cytosol protein concentration is necessary to reveal any receptor binding. It is also seen from this

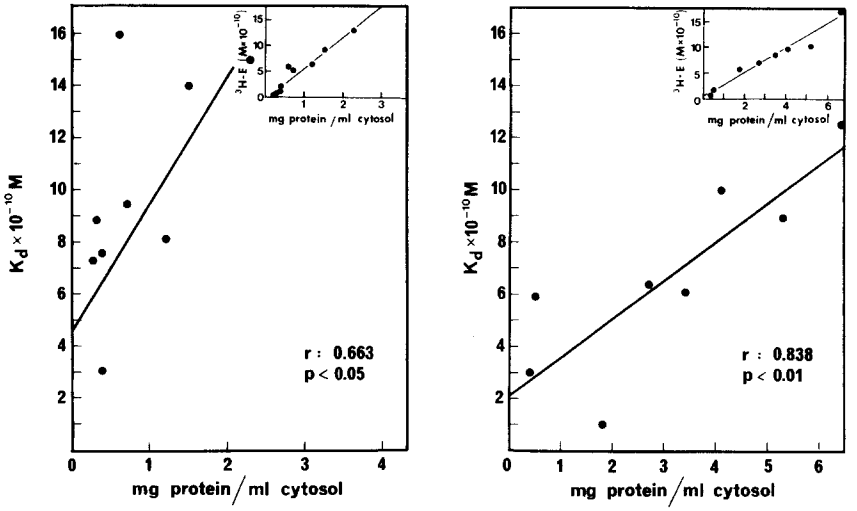

Fig. 4. K_d *value and binding capacity in relation to protein concentration in cytosol obtained from two breast cancers. (Handling of the cytosol described in legend of Fig. 2.) The estimation of binding capacity is illustrated in the upper right corner and the relation between* K_d *values and different cytosol protein concentrations is illustrated by the main figure.*

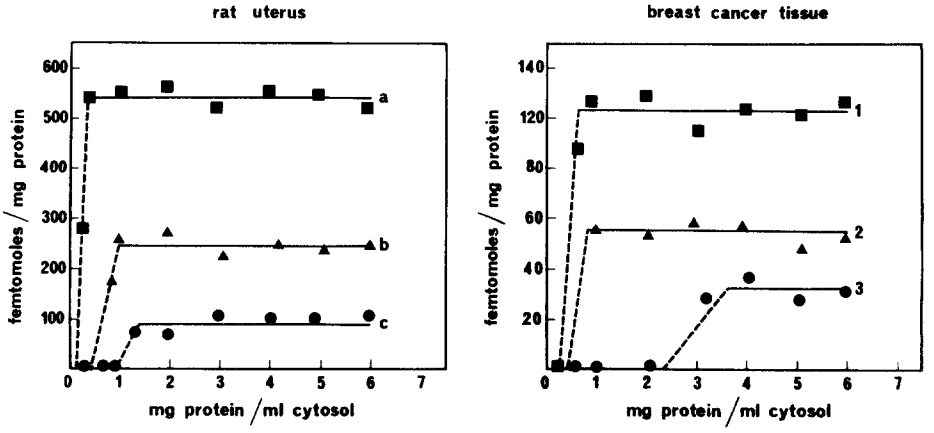

Fig. 5. Cytosols were obtained from rat uterus (a–c), and three different breast cancers (1–3). For preparation of cytosols see legend of Fig. 2. The points represent the estimated binding capacity (fmol *oestrogen/*mg *cytosol protein) in relation to cytosol protein concentration.*

experiment that at low total cytosol protein concentration some receptor activity is lost (a, b, and 1, Fig. 5) even in cytosols from tissue with high receptor activity.

However, it also appears that when the critical amount of binding proteins was obtained, the estimation of RC activity was almost constant within a wide range. It was observed that if cytosol protein concentration approximated 3–6 mg/ml, possible RC activity would be recorded. However, if lower concentrations were used, cytosols with low RC activity would be considered to be RC-negative.

Furthermore, in cytosols with high RC activity, low protein concentration would probably predispose to a falsely low estimation of the binding capacity because some receptors are lost.

From Figs. 6 and 7 it is observed that the proportion of receptor-positive cytosols varied considerably depending upon the cytosol protein concentrations. Only 9% (2/22) were receptor-positive for protein concentrations lower than 1 mg/ml whereas 49% (76/156) were positive for protein concentrations of at least 1 mg/ml (Fig. 6).

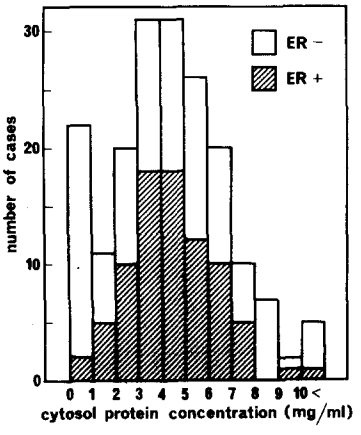

Fig. 6. Occurrence of RC positive (ER+) and RC negative (ER−) breast tumours in relation to protein concentration in breast cancer cytosol.

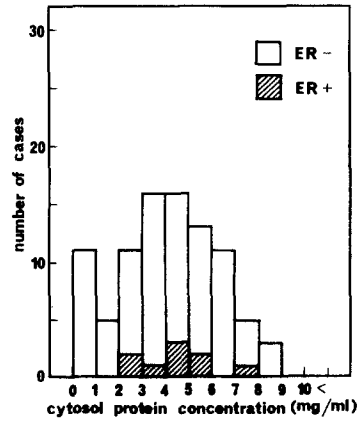

Fig. 7. Occurrence of RC positive (ER+) and RC negative (ER−) breast tumours in relation to protein concentration in benign breast tumour cytosol.

It appears from Fig. 7 that in benign breast tumour cytosols no RC activity was observed with protein contents less than 2 mg/ml. The results from Figs. 5, 6 and 7 indicate that at low protein concentrations, false negative results are likely to occur.

Effect of various charcoal concentrations on the cytosol protein concentration

The results of a representative investigation are shown in Fig. 8.

The cytosol was prepared in TE buffer. After 2 hr of incubation with ^{3}H-17β oestradiol (see Materials and Methods) increasing charcoal amounts (suspended in TKE buffer) were added to the incubation mixture for 30 min. Protein concentration was measured before and after charcoal was added to the cytosol mixture.

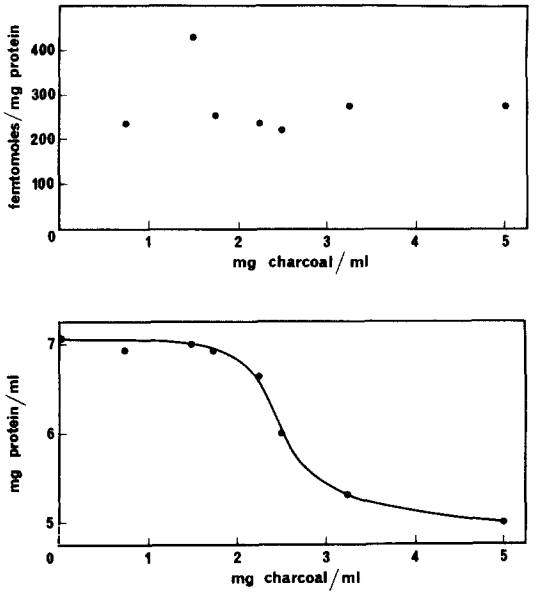

Fig. 8. The cytosol was obtained from rat uterus. The figure shows the influence of increasing amounts of charcoal added to the cytosol on the cytosol protein concentration (bottom). Top shows the relation of estimated binding capacities (fmol/mg protein) to the corresponding charcoal concentrations.

It was observed that lower estimates of protein concentrations were associated with higher charcoal concentrations. Furthermore, it was consistently observed that the decrease was especially pronounced when more than 2 mg/ml charcoal was added.

On the other hand, it was also observed that the estimates of binding capacity per mg protein were not influenced by increasing charcoal concentrations added to the incubation mixture.

The loss of protein varied between 0 and 28%.

Variability of RC-activity in portions from the same breast cancer

In the total material, tissue was received from 199 patients assumed to have malignant breast tumours. Although malignancy was diagnosed in all these patients, in the samples from 21 patients only benign breast tissue was received from the pathologist. Different histologically comparable portions of the same tumour were assayed for RC. The cytosols contained 3 mg protein/ml in each portion. The results are shown in Table 1.

Table 1. *Variability in RC activity from the same breast cancer*

	fmol/mg protein/$K_d \times 10^{-10}$ M				
Tumour No.	Portion No. I	Portion No. II	Portion No. III	Portion No. IV	Portion No. V
1	246/1.4	80/3.1	—		
2	—	13/9.0	11/1.1	—	
3	—	—			
4	—	—	—	—	
5	—	—	—	—	
6	5/4.4	—	—	—	—
7	—	—	—	—	—
8	17/1.4	15/1.2	10/8.3	—	
9	38/4.8	94/3.9	69/1.4	277/6.9	—
10	—	—	—	5/2.0	
11	—	—	—	—	—
12	—	10/4.9	—	—	—
13	—	—	—	—	—
14	83/1.1	—	55/7.9		
15	—	—	—	—	—
16	—	—	108/9.4	—	—
17	49/0.4	54/0.5			

It is seen that both the RC activity and the apparent dissociation constant varied considerably within the same tumour, ranging from portions with apparently no RC activity to portions with very high RC activities.

DISCUSSION

In the present investigation, a DCC assay for RC determination has been used. It has been demonstrated that oestrogen binding can be recorded only when a critical amount of binding proteins is present in the cytosol. This limit seems to vary from 1 mg/ml in tumour cytosols with high RC activity to 3 mg/ml in cytosols with low RC activity. Therefore, if this assay is used for routine determinations, the cytosol protein concentration should not be lower than 3 mg/ml, otherwise tumours with low RC activity could falsely be considered to be RC-negative. The results presented here are consistent with those of others [5]. This limits the usefulness of this technique since either accuracy may be lost because fewer ligand concentrations have to be used or small tissue samples cannot be analysed. This assay seems therefore to be less sensitive than the hydroxylapatite method published by Garola and McGuire [19].

It was observed in the present investigation that a significant decrease in protein content of the cytosols occurred when increasing charcoal concentrations were added to the incubation mixture (Fig. 8). This might indicate that RC oestrogen complexes were adsorbed to the charcoal. That charcoal adsorbs some receptor proteins has also been shown by others [19–22].

Peck and Clark [23] have demonstrated that in high salt cytosols (e.g., presence of 0.2–0.4 M KCl in the incubation buffer) charcoal would strip oestrogen from protein binding sites, and the extent of stripping was positively correlated to the amount of charcoal added. On the other hand, they also demonstrated that in low salt cytosols almost no stripping was observed. In the present investigation, the cytosols were incubated under low salt conditions, and no pronounced stripping was observed since the estimation of binding capacities was almost constant in the presence of different charcoal concentrations added to the cytosol (Fig. 8). Therefore, our

results confirm those reported by Peck and Clark [23].

The charcoal concentration used in the present investigation was 0.25 g%, which is comparable to that of others [4, 9, 10, 19] but lower than the concentrations used by some investigators [5–8, 11, 24]. In these papers a higher proportion of RC-positive tumours was reported than observed in the present study, even when the protein concentrations were below 3 mg/ml [5–11, 19, 24]. The total amount of protein in the incubation mixtures was not significantly higher than ours. Therefore, this discrepancy indicates that differences in charcoal concentrations could not be of major importance. Other factors must be involved.

In this paper it is demonstrated that the estimation of dissociation constant K_d, based on the analysis of Scatchard plots, showed a significantly positive correlation to the protein concentration in the cytosols from the same tumour (Figs. 3 and 4) whereas the estimates of binding capacity per mg protein were independent of the cytosol protein concentration. No data in the present investigation explain conclusively this observation. Others have shown [25, 26] that in cytosols from rat uteri as well as tumours, two distinct types of oestrogen binding sites were found: one with high affinity comparable with some of those found in the present investigation and one with lower affinity, so-called type II oestrogen receptors. These are not supposed to be measured by the DCC assay used in the present investigation, primarily because the range of ligand concentrations is not broad enough. However, it was often observed that the Scatchard plots turned out to be curved (see Fig. 1), especially when the highest concentration of ligand was used, which could indicate that the cytosols contained two specific sites with different affinities. However, a resolution of this two-component system was not possible. This leads to overestimation of the K_d due to increased amount of low affinity sites when the cytosol protein concentration was increased.

Rosenthal [27] has proposed methods for resolving the various binding components which depend on graphic analysis of curvilinear Scatchard plots. This analysis is useful if a very detailed Scatchard plot can be obtained. In routine assay for oestrogen receptors this is not possible because of limited quantities of tissue. In the present paper this correction has not been made routinely because of the reasons mentioned above. However, this correction would not change the conclusion that a positive correlation exists between increasing cytosol protein concentrations and the estimated dissociation constants.

The observation suggests that the cytosol fraction of a tumour is only an operational fraction. The data consequently show that it is important to standardize this fraction as much as possible if comparable studies are to be carried out.

In the present investigation, it was demonstrated that the RC activity varied considerably in different portions of the same tumour, which is consistent with observations by other investigators [1, 28]. It was also observed that non-representative portions, e.g., portions of benign tissue, occurred in malignant tumours. It is therefore concluded that the quantitation of RC activity based on a single tumour sample is imprecise, and furthermore the importance of a careful histological examination of the portions received cannot be stressed enough.

REFERENCES

1. Braunsberg H. Factors influencing estimation of oestrogen receptors in human malignant breast tumours. *Eur J Cancer* 1975; **11:** 499.
2. Feherty P, Farrer-Brown G, Kellie AE. Oestradiol receptors in carcinoma and benign disease of the breast: An *in vitro* assay. *Br J Cancer* 1971; **25:** 697.
3. Hawkins RA, Hill A, Freedman B. A simple method for the determination of oestrogen receptor concentrations in breast tumours and other tissues. *Clin chim Acta* 1975; **64:** 203.
4. Korenman SG, Dukes BA. Specific estrogen binding by the cytoplasm of human breast carcinoma. *J Clin Endocrinol Metab* 1970; **30:** 639.
5. Leclercq G, Heuson JC, Schoenfeld R, Mattheiem WH, Tagnon HJ. Estrogen receptors in human breast cancer. *Eur J Cancer* 1973; **9:** 665.
6. Leclercq G, Heuson JC, Deboel C, Mattheiem WH. Oestrogen receptors in breast cancer: A changing concept. *Br Med J* 1975; **1:** 185.

7. Leung BS, Moseley HS, Davenport CE, Krippaehne WW, Fletcher WS. Estrogen receptor in prediction of clinical responses to endocrine ablation. In: McGuire WL, Carbone PP, Vollmer EP, eds. *Estrogen Receptors in Human Breast Cancer.* New York: Raven Press, 1975: 107.
8. Maass H, Engel B, Nowakowski H, Stolzenbach G, Trams G. Estrogen receptors in human breast cancer and clinical correlations. In: McGuire WL, Carbone PP, Vollmer EP, eds. *Estrogen Receptors in Human Breast Cancer.* New York: Raven Press, 1975: 175.
9. McGuire WL, Pearson OH, Egaloff A. Predicting hormone responsiveness in human breast cancer. In: McGuire WL, Carbone PP, Vollmer EP, eds. *Estrogen Receptors in Human Breast Cancer.* New York: Raven Press, 1975: 17.
10. McGuire WL, de la Garza M, Chamness GC. Evaluation of estrogen receptor assays in human breast cancer tissue. *Cancer Res* 1977; **37:** 637.
11. Barnes DM, Ribeiro GG, Skinner LG. Two methods for measurement of oestradiol-17β and progesterone receptors in human breast cancer and correlation with response to treatment. *Eur J Cancer* 1977; **13:** 1133.
12. Hähnel R, Twaddle E. Estimation of the association constant of the estrogen–receptor complex in human breast cancer. *Cancer Res* 1973; **33:** 559.
13. Scatchard G. The attractions of proteins for small molecules and ions. *Ann NY Acad Sci* 1949; **51:** 660.
14. Mester J, Robertson DM, Feherty P, Kellie AE. Determination of high-affinity oestrogen receptor sites in uterine supernatant preparations. *Biochem J* 1970; **120:** 831.
15. Feherty P, Robertson DM, Waynforth HB, Kellie AE. Changes in the concentration of high-affinity oestradiol receptors in rat uterine supernatant preparations during the oestrous cycle, pseudopregnancy, pregnancy, maturation and after ovariectomy. *Biochem J* 1970; **120:** 837.
16. Daehnfeldt JL. Endogenously blocked high affinity estradiol receptors in the immature and mature rat uterus. *Proc Soc Exp Biol (NY)* 1974; **146:** 159.
17. Poulsen HS, Schultz H, Bichel P. Oestrogenreceptor determinations on fine-needle aspirations from malignant tumours of the breast. *Eur J Cancer* 1979; **15:** 1431.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951; **193:** 265.
19. Garola RE, McGuire WL. A hydroxylapatite micromethod for measuring estrogen receptor in human breast cancer. *Cancer Res* 1978; **38:** 2216.
20. Clark JH, Peck EJ Jr., Schrader WT, O'Malley BW. Estrogen and progesterone receptors: methods for characterization, quantification, and purification. In: Busch H, ed. *Methods in Cancer Research.* New York: Academic Press, 1976: Vol. 12, p. 367.
21. Jungblut PW, Hughes S, Hughes A, Wagner RK. Evaluation of various methods for the assay of cytoplasmic oestrogen receptors in extracts of calf uteri and human breast cancers. *Acta Endocrinol (Kbh)* 1972; **70:** 185.
22. Hähnel R, Twaddle E. Factors that may influence the estradiol receptor assay in human tissues: sex hormone binding globulin and endogenous steroids. *J Steroid Biochem* 1979; **10:** 95.
23. Peck EJ, Jr., Clark JH. Effect of ionic strength on charcoal absorption assays of receptor-estradiol complexes. *Endocrinology* 1977; **101:** 1034.
24. Wittliff JL, Savlor ED. Estrogen-binding capacity of cytoplasmic forms of the estrogen receptors in human breast cancer. In: McGuire EL, Carbone PP, Vollmer EP, eds. *Estrogen Receptors in Human Breast Cancer.* New York: Raven Press, 1975: 73.
25. Clark JH, Hardin JW, Upchurch S. Heterogeneity of estrogen binding sites in the cytosol of the rat uterus. *J Biol Chem* (In press.)
26. Panko WB, Watson C, Clark JH. Human breast cancer: Heterogeneity of estrogen binding sites. (In press).
27. Rosenthal HE. A graphic method for the determination and presentation of binding parameters in a complex system. *Anal Biochem* 1967; **20:** 525.
28. Hawkins RA, Hill A, Freedman B, Gore SM, Roberts MM, Forrest APM. Reproducibility of measurements of oestrogen-receptor concentration in breast cancer. *Br J Cancer* 1977; **36:** 355.