J. steroid Biochem. Vol. 22, No. 1, pp. 39–45, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

PITFALLS IN THE DEXTRAN-COATED CHARCOAL ASSAY OF ESTROGEN RECEPTORS IN BREAST CANCER TISSUE

KIM S. I. PETTERSSON†, RIITTA M. VANHARANTA*
and JUANITA R.-M. SÖDERHOLM
Wallac Biochemical Laboratory in Co-operation with the University of Turku, P.O. Box 10, SF-20101 Turku and *Department of Pathology, Turku University Central Hospital, SF-20520 Turku, Finland

(*Received* 14 *March* 1984)

Summary—This study investigated the influence of the degree of concentration of breast tumor cytosols on the apparent estrogen receptor content as measured by the Dextran-charcoal assay. It was found that the dilution of cytosols to 1–2 mg protein/ml frequently but not always causes highly underestimated receptor concentrations. This could not be explained by the protein loss through adsorption to the charcoal. The effect was also studied in the presence of gelatin, sodium molybdate or with limited trypsinization of the incubation mixture. Addition of 1 mg/ml gelatin in the Dextran-charcoal suspension was very useful in most cases in preventing dilution induced losses in receptor sites. Both trypsinization and addition of sodium molybdate produced increases in receptor concentrations that were not as susceptible to inactivation through dilution of the cytosol. These data suggest that the observed high variability in the dilution induced receptor losses can be explained by receptor heterogeneity: some receptor form(s) are either readily absorbed to or "stripped" by the charcoal particles. As a conclusion we recommend that in order to optimize the estrogen receptor assay as regards both binding sites and affinities the cytosol concentrations should be maintained as high as possible and a protein expander be included in the Dextran-charcoal suspension. Though sodium molybdate frequently gives considerable increases in estrogen binding sites it occasionally has an opposite effect. For this reason we hesitate to recommend its use in routine assays of estrogen receptors.

INTRODUCTION

About one third of patients with recurring metastasizing breast cancer responds with some degree of regression of the tumor when treated with hormonal therapy [1, 2, 3]. With the availability of assay methods for detection of intracellular estrogen receptors it has become possible to select the patients whose tumors are more likely to respond favourably to hormonal manipulations. Within the receptor positive group higher amounts of estrogen receptor enhances the likelihood that the hormone therapy will be successful [3, 4, 5]. Instead of a mere division of tumors in positive and negative specimens it has been recommended that graded categories should be used [5]. Arbitrary threshold levels, usually 3 fmol/mg protein, have been used to exclude a category for which the likelihood of a hormonal response is low. But even among these patients about 10% profit from hormonal therapy [2, 4]. For these reasons it is very important that the estrogen receptor proteins be accurately quantified. This task demands careful handling of the tumour specimens from the time of excision to the final assaying of the receptor content in the cytosol made from it. The most widely used estrogen receptor assay technique, the Dextran-charcoal assay, is based on a multipoint saturation analysis where a fixed amount of the tumor cytosol is incubated with increasing concentrations of labelled hormone. Dextran-coated charcoal (DCC) is used to separate bound from free hormone. Yet, results obtained with this assay principle show high interlaboratory variations [16]. There are remarkable differences of the reported conditions for the application of DCC: Both the amount of charcoal and Dextran as well as the ratio of these two have varied from one study to another over a 10-fold range or more [7]. Low protein concentrations of cytosols have been reported to cause underestimations of estrogen receptor content [7, 8, 9]. Inclusion of sodium molybdate in the cytosol of various tissues has been shown to increase the apparent concentration of most steroid receptors [10, 11, 12].

It was the objective of this study to more systematically investigate the influence of protein content of cytosol on estrogen receptor concentrations as measured by the DCC method and to compare it to the stabilizing effect of sodium molybdate in the cytosol.

†To whom correspondence should be addressed.

Abbreviations: TEDG-buffer, 40 mM Tris–HCl-2 mM EDTA-1 mM dithiothreitol-10% (w/v) glycerol pH 7.4 (25°C); DES, diethylstilbestrol; ODU, optical density unit for the amount of protein in cytosol determined as the difference between the absorbances measured at 280 and 310 nm; SSD, single saturating method; MRM, modified routine method; DM 1, dilution method 1; DM 2, dilution method 2.

EXPERIMENTAL

Materials

[^{3}H]2,4,6,7-Estradiol (115.0 Ci/mmol) was purchased from New England Nuclear. Trypsin (bovine pancreas, type III) was obtained from Sigma, sodium molybdate from Merck and gelatin from Difco. Other reagents used were of analytical purity grade.

Sample preparation

Tissue samples from breast cancer tumors were frozen in liquid nitrogen immediately after excision. The samples were stored at −70°C for a few days up to 2 weeks. Both primary and metastatic tumors were included in the study.

The cancer tissues were thawed at +4°C and homogenized in cold TEDG-buffer with 2–5 ten second bursts with an Ultra-Turrax 18/10 Shaft 10N. The ratio of homogenization buffer to tissue weight varied from 1–4 ml/g in order to obtain as concentrated cytosols as possible.

In the experiments where sodium molybdate was used the homogenate was centrifuged for 5 min at 10,000 *g* in a Beckman model JA-20 centrifuge at 0–4°C. Part of the supernatant was made 20 mM sodium molybdate by addition of a 200 mM stock solution in TEDG-buffer. The cytosol were obtained after centrifugation at 40,000 *g* at 0–4°C for 1 h. The dilution of cytosols used in some experiments is described in detail in the figure legends. All subsequent procedures were performed in an ice-water bath.

Quantitation of estrogen-binding

Routine method. Aliquots of cytosol (0.1 ml) were incubated overnight (16–20 h) at 0°C with six different concentrations of tritiated estradiol (5–0.16 nM) in 0.1 ml of TEDG-buffer with or without a 200-fold excess of unlabelled diethylstilbestrol (DES). Bound and free hormone were separated by incubation with 0.5 ml of a charcoal (0.5%)-Dextran (0.05%) suspension (DCC) in TEDG-buffer for 30 min. After centrifugation 0.5 ml of the supernatant was used for measurement of the radioactivity.

Single saturating dose-method (SSD). 0.1 ml of the cytosol was incubated with 0.1 ml of 5 nM [^{3}H]estradiol in TEDG-buffer overnight at 0°C in triplicate. The non-specific binding was assessed by parallel incubations with a 200-fold excess of DES over radioactive hormone. The methods for removal of unbound [^{3}H]estradiol and the counting of the bound [^{3}H]estradiol were identical to those used in the routine method.

Based on the results from experiments described in the text we decided to compare estrogen receptor concentrations in 21 tumor specimens using three different modifications of the routine DCC assay.

Modified routine method (MRM). The cytosols were assayed from estrogen receptor content as in the routine method except that gelatin (1 mg/ml) was added to the DCC suspension.

Dilution method 1 (DM 1). Cytosol obtained as described above were diluted to 2 ODU/ml with TEDG-buffer. Aliquots (0.1 ml) of the diluted cytosol were incubated overnight at 0°C with three different concentrations of [^{3}H]estradiol (5.0, 2.5, 1.25 nM hormone in 0.1 ml TEDG-buffer). The measurement of the non-specific binding and the separation of the bound and free estradiol were made in the same way as in the routine method.

Dilution method 2 (DM 2). Cytosol containing 20 mM sodium molybdate were diluted as in DM 1 but with 20 mM sodium molybdate in the dilution buffer. Incubation with radioactive hormone was done as in DM 1. For separation of bound and free hormone the DCC suspension of the routine method was modified by addition of 1 mg/ml gelatin and 5 mM sodium molybdate.

Calculation of the binding sites and dissociation constants were done according to the method of Scatchard[13]. Protein content was assayed by the method of Bradford[14]. The receptor content was expressed as fmol/mg protein. A receptor content lower than 3 fmol/mg protein was defined as negative, 3–10 fmol/mg protein as borderline and over 10 fmol/mg protein as positive. Trypsin treatment was performed as described earlier [15].

RESULTS

The estrogen content of a number of cytosols at different dilutions were measured using our routine method. Figure 1 illustrates some typical cases. In all samples dilution decreased the estrogen binding capacity of the breast cancer cytosol. Yet, the decrease varied significantly from one cytosol to another (Fig. 1). In some cytosols 8-fold dilutions (protein concentration 0.5 to 1 mg/ml) caused inactivation of 80–90% of the receptors. On the other hand, in some cytosols only 30% of the binding sites were inactivated by even a 16-fold dilution (~0.5 mg protein/ml).

In agreement with our previous results [15] limited proteolysis with trypsin significantly increased the estrogen binding capacity in undiluted cytosol (Fig. 2). Furthermore, it counteracted to a considerable extent the dilution induced loss in estrogen binding sites.

The addition of 0.5 ml Dextran-charcoal suspension introduced a substantial dilution of the protein content of the incubation mixture (0.2 ml). We therefore decided to study the effect of 1 mg/ml gelatin in the DCC suspension. This caused a clear increase in the binding capacity. The range of augmentation of binding sites was very wide (14–308%), with the most dramatic increases seen in cytosols with low protein content (~3 mg/ml). Mean values from 6 cytosols were 76.2 ± 117.1 (SD) fmol/mg (−gelatin) and 102.9 ± 135.7 (SD) fmol/mg

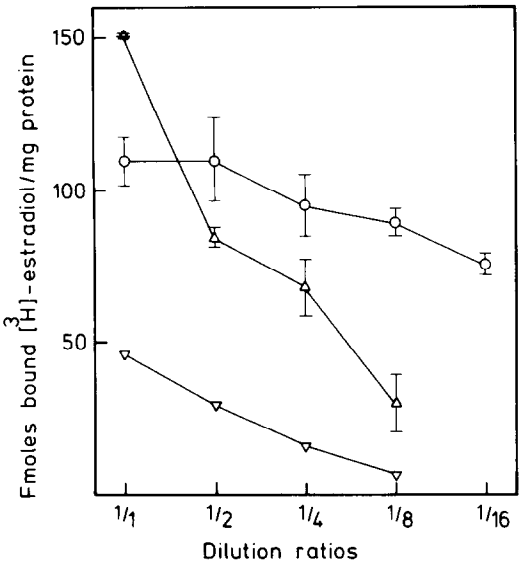

Fig. 1. Effect of dilution of breast cancer cytosols on the amount of [^{3}H]estradiol binding sites. 0.1 ml aliquots of undiluted or diluted cytosol were incubated overnight with a single saturating dose (SSD) of 5 nM [^{3}H]estradiol (0.1 ml) or 6 different concentrations (5–0.16 nM) of [^{3}H]estradiol (0.1 ml) ± 200 DES at 0°C. 0.5 ml of a Dextran (0.05%)–charcoal (0.5%) suspension in TEDG-buffer was used to separate free and bound hormone. The values in the figure represent the means of triplicates ±SD (○, △) for the [^{3}H]estradiol binding measured with SSD or binding capacities calculated from Scatchard plots (▽). All values are corrected for non-specific binding. Protein contents of undiluted cytosols were 4.8 mg/ml (△), 6.6 mg/ml (○) and 9.0 mg/ml (▽), respectively.

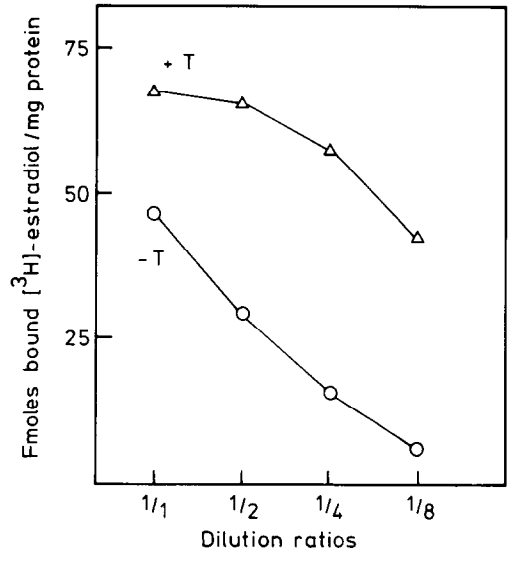

Fig. 2. Effect of trypsin on the quantitation of estrogen receptors in dilutions of breast cancer cytosols. Cytosols were obtained as described in the Experimental Section. Binding capacities of undiluted and diluted cytosols were determined in two parallel sets with the saturation analysis described in the legend of Fig. 1. After overnight incubation at 0°C 5 μl TEDG-buffer with (△) or without (○) 5 μg trypsin/ODU was added to the tubes and incubation at 0°C was continued for 3 h prior to DCC-treatment. The undiluted cytosols contained 9.0 mg protein/ml.

(+gelatin). The results obtained with gelatin were significantly higher ($P = 0.025$) than those obtained without gelatin in the DCC suspension using Wilcoxon's test for paired differences. The affinity remained unaltered or was somewhat increased; mean values for K_d: 0.12 ± 0.07 (SD) nM without gelatin, 0.05 ± 0.04 (SD) nM with gelatin. Mean protein concentration was 6.0 ± 3.2 (SD) mg/ml. Gelatin itself did not possess any saturable estrogen binding sites of high affinity (data not shown).

We also studied the effect of gelatin and/or sodium molybdate in the DCC suspension on cytosols with or without sodium molybdate (Fig. 3). The 12-fold increase in the estrogen binding of the cytosol without molybdate by the mere inclusion of gelatin in the DCC suspension confirms our findings above. Inclusion of molybdate shortly after homogenization, however, still gives a further increase (70%) in estrogen binding.

The apparent additivity of the effects of gelatin in the DCC suspension and sodium molybdate in the cytosol led us to investigate the effects of these two additives separately and together on the dilution induced decrease in apparent receptor concentration of several cytosols. Figure 4 illustrates the results for one cytosol representative for four similar experiments performed on cytosols from different tumors.

Inclusion of gelatin to the DCC suspension retained the estrogen binding capacity in the diluted cytosol at the same level as in the undiluted cytosol. Addition of 20 mM sodium molybdate to the cytosols increased the estrogen binding capacity with an equal amount in both diluted and undiluted cytosol, but it

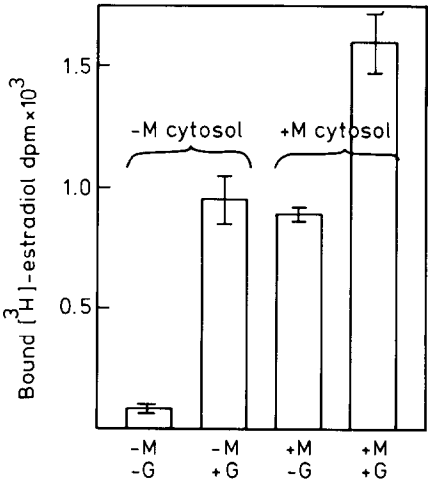

Fig. 3. The effect of sodium molybdate and/or gelatin on the [^{3}H]estradiol binding of a breast cancer cytosol. Cytosols with and without 20 mM sodium molybdate were obtained as described in the Experimental Section. Binding of [^{3}H]estradiol was measured with SSD analysis as described in the legend of Fig. 1 with or without 5 mM sodium molybdate and 1 mg/ml gelatin in the DCC suspension (indexes below). The values in the figure represent the means of triplicates ± SD and have been corrected for non-specific binding. The cytosol contained 3.0 mg/ml protein.

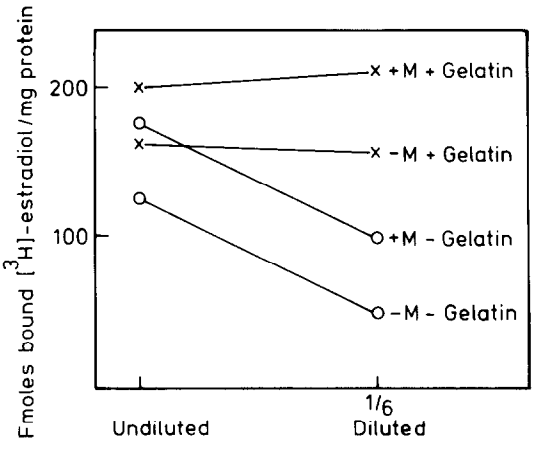

Fig. 4. Effect of dilution of the cytosol on estrogen binding capacity in the presence or absence of 20 mM sodium molybdate in cytosols and/or gelatin (1 mg/ml) in the Dextran-coated charcoal suspension. The binding capacities were calculated from Scatchard plots. Protein content in the undiluted cytosol was 6.0 mg/ml and in the diluted cytosol 0.9 mg/ml.

did not abolish the decrease in estrogen receptor content caused by dilution of the cytosol. The loss of estrogen binding sites was thus roughly equal both in the presence and absence of sodium molybdate.

A protein concentration between 1 and 2 mg/ml, as recommended by McGuire *et al.*[16] for the quantitation of estrogen receptors in cytosol by the DCC assay, seemed to us fairly low considering the extensive loss in estrogen binding sites following dilution of some cytosols. We therefore decided to determine estrogen receptor content simultaneously with our modified routine method (MRM) where 1 mg/ml gelatin [16] was added to the DCC suspension and with two further modifications of the routine method involving dilution of the cytosols to contain 1–2 mg/ml protein. In all other respects dilution method 1 (DM 1) was performed as our routine method. In dilution method 2 (DM) 20 mM sodium molybdate was included in the cytosol and the dilution buffer and 5 mM sodium molybdate and 1 mg/ml gelatin in the DCC suspension. Figure 5 shows the estrogen binding capacities of the cytosols from twenty-one different tumor specimens obtained by these three assay methods. Mean values for the protein concentrations of the diluted and undiluted cytosols were 1.6 ± 0.4 (SD) mg/ml and 4.4 ± 1.9 (SD) mg/ml, respectively. The binding capacities for DM 1, DM 2 and MRM were (mean values $\pm$ SD): 43.0 ± 54.0, 94.0 ± 120 and 110 ± 170, respectively. In four cases assayed with DM 1 no saturable estrogen binding was detected. When assayed with MRM distinct high affinity binding was observed for these cytosols: 1.8, 2.3, 3.6 and 18.5 fmol/mg. Of five cytosols found negative with DM 1, two remained negative, one was borderline and two were positive when assayed with MRM. Of three borderline cases (DM 1) one was found positive with MRM while two remained in the borderline region.

The ratio of binding capacities obtained with the two methods (DM 1/MRM) was 0.47 ± 0.17 (SD); $n = 17$; range 0.18–1.13. Thus by omitting the dilution step and adding 1 mg/ml of gelatin to the DCC suspension substantial increases, up to 6-fold, were seen in fifteen cases whereas six cases were virtually unaltered. The ER values from MRM were significantly higher than those obtained with DM1 ($P < 0.001$, Wilcoxon's test for paired differences).

The results obtained with DM 2 are more complex. Of the four cytosols showing no saturable estrogen binding with DM 1 only one was found to have saturable binding sites (5.9 fmol/mg) of high affinity when assayed with DM 2, despite the presence of gelatin in the DCC suspension.

Compared with the results obtained with MRM about one third of the cases assayed with DM 2 were clearly increased, about one third was unaltered and the remaining third was clearly decreased including the three cytosols with no detectable high affinity

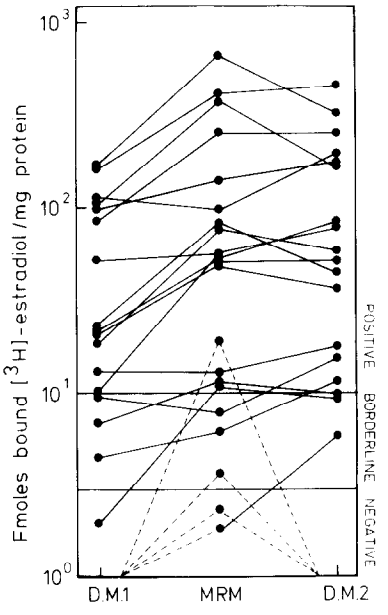

Fig. 5. Comparison of the estrogen receptor binding capacities of 21 breast cancer samples obtained by three different modifications of the DCC assay (described in detail in the Experimental Section). In dilution method 1 (DM 1) cytosols without sodium molybdate were diluted with TEDG-buffer to obtain a protein content of ~2 ODU/ml. In dilution method 2 (DM 2) 20 mM sodium molybdate was included in the cytosol and the dilution buffer. Gelatin (1 mg/ml) and sodium molybdate (5 mM) were added to the DCC suspension. Binding capacities were obtained from a 3-point DCC-Scatchard-plot assay. In the modified routine method (MRM) cytosols without sodium molybdate were used without dilution. Binding capacities were obtained from a 6-point DCC-Scatchard-plot assay. Gelatin (1 mg/ml) was included in the DCC suspensions. The dotted lines indicate the cases where no detectable receptor binding was seen with the respective assay method.

estrogen binding sites. The ratio of binding sites obtained with DM 2 and MRM was 1.29 ± 0.72; $n = 18$; range 0.45–3.28. No significant difference was obtained for the results obtained by DM 2 and MRM using Wilcoxon's test for paired differences.

The dissociation constants for the three groups were [mean values (nM) $\pm$ SD]: DM 1, 0.41 ± 0.42; DM 2, 0.25 ± 0.19; MRM, 0.14 ± 0.24. The mean value of the ratios of K_d's obtained for individual cytosols were: DM 1/MRM, 9.2 ± 11.2 (range 0.5–37) and DM 2/MRM, 5.6 ± 6.0 (range 0.2–24). Diluting the cytosol thus generally had a negative effect on the affinity of the hormone–receptor interaction. The K_d's obtained with MRM were significantly lower than those obtained with either DM 1 or DM 2 ($P < 0.001$ in both cases using Mann–Whitney's U-test).

We also tested the recovery of cytosol proteins after the DCC treatment without gelatin. The loss of protein was $36 \pm 12\ \mu g$ ($n = 27$) from $100\ \mu l$ cytosol and was fairly constant despite wide variations in the initial protein concentration (range 0.4–9.0 mg/l). For the mean protein concentration (1.55 mg/ml) in the diluted cytosols this would correspond to a 20–30% loss of protein.

DISCUSSION

This study confirms and extends previous findings [7, 8, 9] of the importance of protein concentration in the measurement of estrogen receptor content in breast cancer cytosols using the Dextran-coated charcoal assay. In order to minimize losses of estrogen receptor content in cytosols with low protein concentrations the use of a carrier protein has been recommended [17]. It has been assumed that adsorption of protein to the charcoal particles would account for the losses of estrogen receptors [7, 8, 9]. This study shows that the loss of protein due to adsorption to the charcoal in the absence of a protein expander can account for 20–30% decreases in estrogen receptor content when the initial protein amount of the cytosol varies between 1 and 2 mg/ml. In fact, as this study illustrates, up to 80–90% decreases in estrogen binding are frequently seen with diluted cytosols in the absence of a protein expander compared to binding capacities obtained with undiluted cytosols with 1 mg/ml gelatin added to the Dextran-charcoal suspension. Actually, there does not seem to exist a common safe protein concentration within convenient and practical reach of the homogenization procedure used, since even in undiluted cytosols with high protein concentrations considerable increase in estrogen binding are seen when adding 1 mg/ml gelatin to the DCC suspension.

Furthermore, there is much variability in the dilution induced estrogen binding losses between different cytosols. The estrogen binding of some cytosols despite extensive dilution is decreased only to the extent that is expected due to unspecific adsorption of proteins to the charcoal.

Although nonspecific adsorption of proteins from the cytosol to the charcoal cannot explain the present data it is conceivable that preferential adsorption of some receptor form(s) could account for the high variability in estrogen receptor losses. It is widely known that a variety of receptor forms of widely different molecular weights exist in breast cancer cytosols where no precautions have been taken to inhibit endogenous proteolytic acitivity [18].

It is also possible that the radioactive hormone is stripped from its receptor through some factor present in the Dextran-charcoal suspension, which can be neutralized by high protein concentrations of the cytosol or addition of a carrier protein to the DCC suspension. Again, different receptor forms could possibly be more or less prone to this stripping mechanism offering an explanation of the variability between different cytosols. A stripping mechanism by charcoal particles has been described by Peck and Clark[19] for cytoplasmic estrogen receptors at KCl concentrations above 100 mM. A stripping mechanism is also supported by the marked protective effect of high protein concentrations on the affinity of the hormone receptor interaction. The hormone may be more thoroughly stripped from the receptor when total concentration of hormone is low making it less likely to bind another hormone molecule.

Although limited proteolysis with trypsin does not fully protect the estrogen receptor sites against losses upon dilution of the cytosol it gives an increment in estrogen binding sites almost constant throughout the dilution curve. This could be explained by the formation of a meroreceptor [18] like molecule which either is not adsorbed to the charcoal particles or not sensitive to the hypothetical stripping mechanism mentioned above.

The effect of sodium molybdate in the cytosol is very similar in this respect to that of trypsin, i.e. it does not abolish the dilution induced loss in estrogen binding sites but gives an increase in estrogen binding which in absolute amount is constant in both concentrated and diluted cytosols. It has been suggested that the stabilizing effect of sodium molybdate on steroid receptor binding is caused by inhibition of phosphatases [10] or by interaction directly with the receptor thus making it a less desirable object for different inactivating factors [11]. We have previously shown that limited trypsin proteolysis and the use of sodium molybdate in quantitations of estrogen receptor content of breast cancer cytosols using the DCC assay produce roughly the same augmentations in estrogen binding sites [14]. In that study the maximal increase obtained by trypsin or sodium molybdate seemed to be roughly 100%. In view of the present results this apparent limit was probably a result of the particular protein levels used in that study. In this work trypsin and sodium molybdate produced increases in estrogen binding that were substantially higher at lower levels of cytosol protein.

From the 21 breast cancer specimens assayed for estrogen receptor content with the three modifications of our routine DCC assay it is evident that dilution of cytosols to contain between 1 and 2 mg/ml cytosol without carrier protein in the DCC suspension caused highly underestimated estrogen receptor values. It also results in significantly lower affinities making it more difficult to detect small receptor amounts from the background and other possible estrogen binders of low affinity. In this admittedly limited material two tumor specimens found negative with DM 1 (1.9 and 0 fmol/mg) were clearly positive (10.6 and 18.5 fmol/mg) when assayed with MRM. Although it must be emphasized that the higher estimates resulting from the modifications of the method must be reevaluated in terms of clinical response to treatment, it is obvious from the high variability of the dilution effect in different cytosols that there is an apparent chance that some ER-positive tumors specimens may escape detection when using the dilution method without addition of a carrier protein to the DCC suspension.

When the dilution method was modified with the addition of sodium molybdate to the cytosol and molybdate and gelatin to the DCC suspension to avoid losses of estrogen binding sites, expected increases in estrogen binding capacities (up to 3-fold) were seen in one third of the cases whereas one third was more or less unchanged. Surprisingly in seven cases the binding capacities were reduced. This may be accounted for by two factors. The presence of 1 mg/ml of gelatin in the DCC suspension may not be enough to effectively inhibit the loss in binding sites at low protein concentrations of cytosols. This may be the case particularly for the three specimens without detectable saturable estrogen binding, which were also found negative with DM 1. It must also be noted that the affinities in DM 2 despite the use of gelatin were significantly lower than in MRM, although higher than in DM 1. The other possibility is that the use of 20 mM sodium molybdate for some unknown reason reduces the apparent estrogen binding capacity a phenomenon occasionally seen in this laboratory (unpublished data).

CONCLUSION

The protein content of breast tumor cytosols, as this study shows, is an important variable for the quantitation of estrogen receptors using the Dextran-coated charcoal method. Dilution of cytosols to a common protein concentration (1–2 mg/ml) can cause dramatic losses in apparent estrogen receptor content which cannot be explained by a general protein adsorption to the charcoal particles. The high variability in this dilution induced effect between different cytosols is conceivably a consequence of receptor heterogeneity. This view is supported by the facts that formation of mero-receptors through limited trypsin proteolysis and stabilization of a high molecular weight receptor form(s) with sodium molybdate counteracts the dilution induced loss in receptor sites.

Based on the results from this study we recommend that the protein concentration of the cytosol be maintained as high as possible together with the use of expander protein in the Dextran-charcoal suspension.

In this way the ER sites and the affinity of the hormone receptor interaction is best preserved and artifacts from using low protein cytosols with or without protein expander can thus be avoided. Although the use of sodium molybdate can give significantly higher ER content of some cytosols, occasionally it has an opposite effect and we therefore hesitate to recommend it in the routine estimation of ER content of breast cancer cytosols.

Acknowledgement—The financial support of The Cancer Fund of Lempi and Armas Koivurinta is gratefully acknowledged.

REFERENCES

1. Edwards D. P., Chamness G. C. and McGuire W. L.: Estrogen and progesterone receptor proteins in breast cancer. *Biochim. biophys. Acta* **560** (1979) 457–486.
2. Byar D. P., Sears M. E. and McGuire W. L.: Relationship between estrogen receptor values and clinical data in predicting the response to endocrine therapy for patients with advanced breast cancer. *Eur. J. Cancer* **15** (1979) 299–310.
3. Heuson J. C., Longeval E., Mattheim W. H., Deboel M. C., Sylvester R. J. and Leclercq G.: Significance of quantitative assessment of estrogen receptors for endocrine therapy in advanced breast cancer. *Cancer* **39** (1977) 1971–1977.
4. Allegra J. C., Lippman M. E., Thompson E. B., Simon R., Barlock A., Green L., Huff K. K., Do H. M. T., Aitken S. C. and Warren R.: Estrogen receptor status: an important variable in predicting response to endocrine therapy in metastatic breast cancer. *Eur. J. Cancer* **16** (1980) 323–331.
5. McGuire W. L., Zava D. T., Horwitz K. B., Garola R. E. and Chamness G. C.: Receptors and breast cancer: do we know it all? *J. steroid Biochem.* **9** (1978) 461–466.
6. Raam S., Gelman R. and Cohen J. L.: Estrogen receptor assay: interlaboratory and intralaboratory variations in the measurement of receptors using dextran-coated charcoal technique. *Eur. J. Cancer* **17** (1981) 643–649.
7. Rosner A. L., Teman G. H., Bray C. L. and Burstein N. A.: Batch assay method evaluation of cytoplasmic estrogen receptor. Relative immunity of hydroxylapatite method from errors of measurement. *Eur. J. Cancer* **16** (1980) 1495–1502.
8. Skovgaard Poulsen H.: Estrogen receptors in human breast cancer: Comparative features of the hydroxylapatite- and dextran-coated charcoal assay. *Eur. J. Cancer clin. Oncol.* **18** (1982) 1075–1079.
9. Skovgaard Poulsen H.: Estrogen receptor assay—limitation of the method. *Eur. J. Cancer* **17** (1981) 495–501.
10. Sando J. J., Hammond N. D., Stratford C. A. and Pratt W. B.: Activation of thymocyte glucocorticoid receptors to the steroid binding form. *J. biol. Chem.* **254** (1979) 4779–4789.
11. Anderson K. M., Phelan J., Marogil M., Hendrickson C. and Economou S.: Sodium molybdate increases the

amount of progesterone and estrogen receptor detected in certain human breast cancer cytosols. *Steroids* **35** (1980) 273–280.
12. Gaubert C. M., Tremblay R. R. and Dube J. Y.: Effect of sodium molybdate on cytosolic androgen receptors in rat prostate. *J. steroid Biochem.* **13** (1980) 931–937.
13. Scatchard G.: The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad Sci.* **51** (1949) 660–672.
14. Bradford M. M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72** (1976) 248–254.
15. Pettersson K., Vanharanta R., Söderholm J., Punnonen R. and Lövgren T.: Increase in the estrogen binding capacity of breast cancer cytosols following limited proteolysis with trypsin. *J. steroid Biochem.* **16** (1982) 369–372.
16. McGuire W. L., De La Garza M. and Chamness G. C.: Evaluation of estrogen receptor assays in human breast cancer tissue. *Cancer Res.* **37** (1977) 637–639.
17. EORTC Breast Cancer Cooperative Group: Revision of the standards for the assessment of hormone receptors in human breast cancer. *Eur. J. Cancer* **16** (1980) 1513–1515.
18. Sherman M. R., Pickering L. A., Rollwagen F. M. and Miller L. K.: Meroreceptors: proteolytic fragments of receptors containing the steroid-binding site. *Fedn Proc.* **37** (1978) 167–173.
19. Peck E. J. and Clark J. H.: Effect of ionic strength on charcoal adsorption assays of receptor-estradiol complexes. *Endocrinology* **101** (1977) 1034–1043.
20. Miller L. K., Tuazon F. B., Niu E.-M. and Sherman M.: Human breast tumor estrogen receptor: Effects of molybdate and electrophoretic analyses. *Endocrinology* **108** (1981) 1369–1378.
21. Panko W. P., Watson C. S. and Clark J. H.: The presence of a second, specific estrogen binding site in human breast cancer. *J. steroid Biochem.* **14** (1981) 1311–1316.