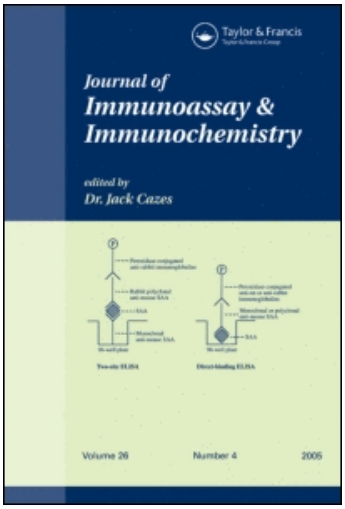


**Journal of Immunoassay and Immunochemistry**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

# Influence of Tissue Homogenization Techniques on Levels of Estrogen and Progesterone Receptors Measured in Calf Uterus and Human Breast Tumors

E. D. Ryan[a]; W. H. C. Walker[a]
[a] Department of Pathology, McMaster University Medical Centre, Hamilton, Ontario, Canada

**To cite this Article** Ryan, E. D. and Walker, W. H. C.(1980) 'Influence of Tissue Homogenization Techniques on Levels of Estrogen and Progesterone Receptors Measured in Calf Uterus and Human Breast Tumors', Journal of Immunoassay and Immunochemistry, 1: 4, 463 — 474
**To link to this Article: DOI:** 10.1080/15321818008056966
**URL:** http://dx.doi.org/10.1080/15321818008056966

JOURNAL OF IMMUNOASSAY, 1(4), 463-474 (1980)

# INFLUENCE OF TISSUE HOMOGENIZATION TECHNIQUES ON LEVELS OF ESTROGEN AND PROGESTERONE RECEPTORS MEASURED IN CALF UTERUS AND HUMAN BREAST TUMORS

E.D. Ryan and W.H.C. Walker

Department of Pathology

McMaster University Medical Centre

Hamilton, Ontario, Canada



## Abstract

We have investigated the solubilization of estrogen and progesterone receptors during homogenization of human breast tumor and calf uterus tissue. With chopped human breast tumor tissue, the maximum yield of binding proteins occurred after mechanical homogenization at 4°C with a polytron PT-10 instrument running at setting 5 for 10-15 seconds. With frozen or lyophilised tissue powders the maximum yield of binding proteins occurred after intermittent vortex mixing with buffer over 60 min. at 4°C. The solubilized receptor proteins were stable for at least 150 min. at 4°C. Mechanical homogenization of the tissue powder suspensions resulted in rapid denaturation of the receptors. No improvement in stability was obtained by including 10 mM monothioglycerol and 10% glycerol in the homogenization buffer.

## INTRODUCTION

Quantitation of intracellular estrogen and progesterone receptors in human breast tumors is now generally accepted as a useful predictor of responsiveness to endocrine therapy (1). The *in vitro* assay procedure most commonly used to detect the receptor proteins uses radioactive steroid to tag the receptor and either dextran-coated charcoal or density gradient centrifugation to separate the unbound steroid from that associated with the receptor. A cell-free solution or "cytosol" is required for analysis of these proteins and this poses unique

problems of tissue homogenization which are not encountered in the analysis of plasma proteins.

To prepare the cytosol fraction, the receptor proteins must first be extracted from the tissue by disrupting the cells. The released tissue proteins are solubilized with excess buffer and the tissue debris is removed by centrifugation. Since both estrogen (2) and progesterone (3) receptor proteins are reported to be extremely labile, it is possible that the mechanical homogenization of the tissue might itself contribute to denaturation of these proteins with artifactual reduction of assayed steroid binding capacities.

The methods used to disrupt the tissue and solubilize the receptor proteins fall into two broad categories. The tissue is either mechanically homogenized directly (4,5) or is first pulverized at a very low temperature (-196$^{o}$) and the resulting powder is mechanically homogenized (6,7). Little attention has been paid to this first step in the analysis of estrogen and progesterone receptors, and we report here a study of the stability of receptors in human breast tumor tissue during the homogenization process.



We have investigated the effect of sulphydryl protecting agents and glycerol which are used by some workers (7,8) in an attempt to stabilize the receptor proteins during homogenization and subsequent incubation. In addition we have extended the study to include the stability of receptors during homogenization of lyophilised powdered calf uterus which has been reported (9,10) to have value as a quality control material because of its favourable stability on long term storage.

## MATERIALS AND METHODS

### Tissue:

Human breast tumors were removed at surgery and were transported to the laboratory on dry ice. Calf uteri were obtained from a local slaughterhouse.

All tissue was stored at -70° before use. Chopped tissue was prepared by cutting the frozen tissue with a dissecting knife into small pieces.

Tissue powders were prepared by freezing the tissue in liquid nitrogen (-196°C) and pulverizing it at that temperature in a stainless steel mortar and pestle. These powders were also stored at -70°C.

Lyophilised powders were obtained by freeze drying the frozen powdered tissue for 16 hours. The condenser was cooled with a dry ice/acetone mixture. These powders were stored in screwtop vials at ambient temp (25-30°).

Chemicals

2, 4, 6, 7 -$^{3}$H-estradiol of specific activity 90 Ci/mmol, 17 -methyl$^{3}$H,21-dimethyl-19-Nor-4,9-pregnadiene-3,20-dione (R5020) of specific activity 85 Ci/mmol and unlabelled R5020 were obtained from New England Nuclear, 549 Albany St, Boston, Mass. 17- β estradiol was obtained from Sigma Chemical Co. St. Louis, Mo. Other chemicals used were of reagent grade quality.

Homogenization:

One gram of tissue (chopped, frozen powder, or lyophilised) was added to 10 ml of 10mM Tris-HCl/1.5mM EDTA buffer (pH7.4) in a 25 x 150 mm glass tube. The tubes were kept on ice and the contents were vortex mixed at 5 minute intervals over a 30 minute period. The tissue was then homogenized using a Polytron PT-10-ST (Brinkmann Instruments, Rexdale, Ont) instrument running at rheostat setting 5. One ml aliquots of suspension were removed just prior to the homogenization (zero time) and at intervals thereafter. The temperature of the suspension did not rise about 4° during homogenization. The homogenates were centrifuged at 30,000 x g for 60 minutes using Brinkmann micro centrifuge tubes in a Sorvall SM-24 rotor.

In other experiments the time-course of receptor solubilization by vortex mixing tissue powder with buffer was investigated. Here, the mechanical

homogenization step was omitted, but the sampling protocol and cytosol preparation was as described above.

Incubation:

Total and non-specific binding of estradiol to receptors was determined by incubating 100 ul of cytosol in 10 x 75 mm glass tubes with 5 pg $^{3}$H-estradiol, without and with 5,000 pg unlabeled estradiol, in a total volume of 650 ul. All solutions contained 10 mM Tris-HCl/1.5 mM EDTA buffer (pH7.4). Incubation time was 18-20 hours at 4$^{o}$C.

For determination of total and non-specific progesterone binding to receptors, 100 ul of cytosol was incubated with 5 pg $^{3}$H R5020, without and with 5,000 pg R5020, in 10 mM Tris HCl/1.5 mM EDTA/buffer (pH7.4) in a total volume of 650 ul. Glycerol was added to the buffer to give a final concentration in the incubation mixture of 34%. All tubes were incubated for 18-20 hours at 4$^{o}$C.

At the end of the incubation period, 200 ul of a suspension of 0.5% charcoal and 0.05% dextran in Tris-EDTA buffer (pH7.4) were added to each tube to give a final charcoal concentration of 0.12%. The mixture was incubated for 30-60 minutes at 4$^{o}$C and then centrifuged at 1,500 x g for 10 min. The supernatant (containing the hormone receptor complex) was poured into scintillation vials containing 5 ml scintillation fluid.

Protein:

Protein was measured by the Lowry (11) method.

Results:

Results are expressed as the proportion of the added labelled ligand bound to the receptor. Incubation without and with a large excess of unlabelled ligand yields, respectively, the proportion of specific plus non-specific and of non-specific binding.

## RESULTS AND DISCUSSION

### Cytosol preparation

After a variety of homogenization or solubilization steps, we prepared the cytosol fraction by centrifugation at 30,000 x g for 60 min in all experiments reported here. In preliminary experiments we compared the binding characteristics of cytosols prepared by centrifugation at 100,000 x g and 30,000 x g and found no difference. McGuire et al (12) have compared the estradiol binding capacities of cytosols prepared by centrifugation at 1600 x g for 10 min with those prepared by centrifugation at 104,000 x g for 40 min and also reported no difference.



Several workers have reported (7,8) inclusion of sulphydryl reagents, either monothioglycerol or dithiothreitol together with glycerol in the homogenization buffer as receptor stabilizers. We have repeated all of the experiments reported here with monothioglycerol (10 mmol/l) plus glycerol (100 g/l) in the homogenizing buffer and have observed no change in receptor stability. Keightley et al (13) have made the same observation with dithiothreitol. We did however include glycerol in the incubation buffer used to measure the progesterone receptor. In this system, glycerol shortens the time required to reach equilibrium but does not affect the final equilibrium position (in preparation).

### Human Breast Tumor Tissue

The binding attributable to estradiol and progesterone receptors of chopped and powdered human breast tumor tissue solubilized by homogenization for increasing times are shown in Figures 1 and 2. The homogenization time dependence of solubilization is similar for both receptors, but a marked difference is seen between the chopped and powdered tissue.

With powdered tissue, the maximum yield of receptors occurs prior to any mechanical homogenization as a result of vortex mixing powder with buffer at 5

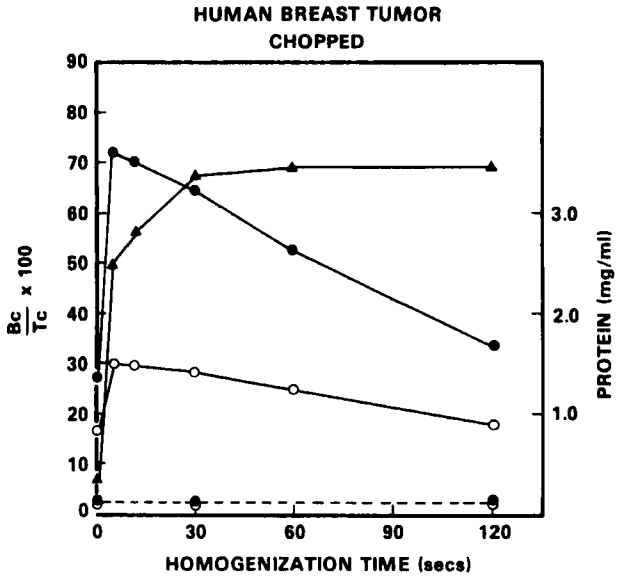


Figure 1. Estrogen (●), progesterone (○), and total protein (▲) solubilized by mechanical homogenization of chopped human breast tumor tissue. The solid line represents specific plus non-specific binding. The dashed line represents non-specific binding. Bc= Bound counts. Tc= Total counts.



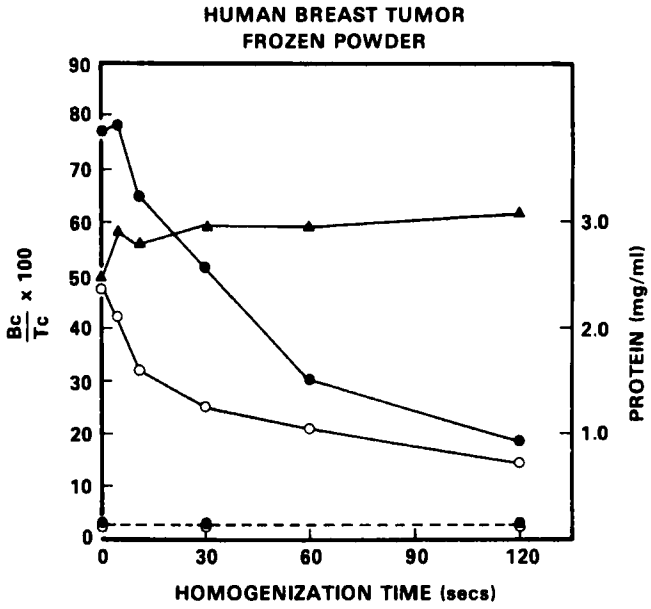


Figure 2. Estrogen (●), progesterone (○) and total protein (▲) solubilized by mechanical homogenization of pulverized human breast tumor tissue. The solid line represents specific plus non-specific binding. The dashed line represents non-specific binding. Bc= Bound counts. Tc= Total counts.

min. intervals over a 30 min. period. Mechanical homogenization of this preparation results in rapid denaturation of the receptors with a 50% loss of binding after homogenization for 30 sec.

By contrast, with chopped tissue, mechanical homogenization was essential for solubilizing the receptors. The maximum yield of binding protein occured after homogenization for 10-15 seconds. Further homogenization resulted in loss of hormone binding capacity but in all eight tumors studied the rate of denaturation was less in the chopped tissue than in the powdered tissue. It is not possible to ascribe this difference between chopped and powdered tissue to freezing in liquid nitrogen and subsequent thawing as both preparations were treated similarly in this respect. The slower denaturation with chopped tissue may be due to a prolongation of receptor solubilization when compared with rapid solubilization from powdered tissue. The data relating to release of the total protein (Figs. 1 and 2) supports this hypothesis.

Progressive destruction of the receptors during homogenization of chopped tumor tissue has practical importance. In many cases, small sections of tumor will adhere to the homogenizer probe and there is a tendency to continue to homogenize until all of these tissue pieces are fragmented. This may take some considerable time, and as a result most of the receptor protein originally present will be denatured. Even with protocols that define homogenization time and conditions, different centres may be expected to produce results that differ as a result of the nature of the mechanical homogenization step.



## Calf Uterus Tissue

Calf uterus has relevance for the laboratory performing routine receptor assays on human breast tumors in that it is a source of positive control material. Homogenous powders are readily prepared and are stable on storage at $-86^{\circ}$ for at least four months (14). An aliquot of this material can be analysed with each batch of breast tumors as part of intra-laboratory quality assurance. An inter-laboratory quality assurance program is also available with

frozen calf uterus powders shipped on dry ice to each participating laboratory (14).

The observation by Koenders et al (9) that both estradiol and progesterone receptors remain stable in lyophilized tissue powder, suggests that such powder might have enhanced convenience as quality control material, especially if it had stability at ambient temperature and could be circulated by mail rather than having to be transported by air-freight on dry ice.

Koenders et al (9) do not specify the conditions used to homogenize their lyophilized powder prior to analysis. Since this initial step can introduce large variability into the receptor assay using frozen powders, we compared the homogenization characteristics of frozen and lyophilized tissue powders. The results are shown in Figures 3 and 4. There was no difference between the frozen and lyophilized tissue powders. The maximum yield of binding protein was obtained after only 2-5 seconds of mechanical homogenization. Further homogenization resulted in considerable loss of binding ability.



## Vortex mixing of tissue powders with buffer

In the experiments reported above, most of the receptors were solubilized from tissue powders (frozen or lyophilised) by vortex mixing with buffer. We have investigated this process in greater detail and find maximum binding activity solubilized after one hour of intermittent vortex mixing with buffer as described in the "methods" section (Figs 5, 6). The yield of binding proteins solubilized is equivalent to that obtained by brief mechanical homogenization. Unlike mechanical homogenization, however, vortex mixing with buffer does not denature the receptors. For monitoring the receptor assay steps subsequent to homogenization of tumor tissue, the present findings suggest that vortex mixing of the quality control tissue powders will yield a reproducible cytosol preparation.

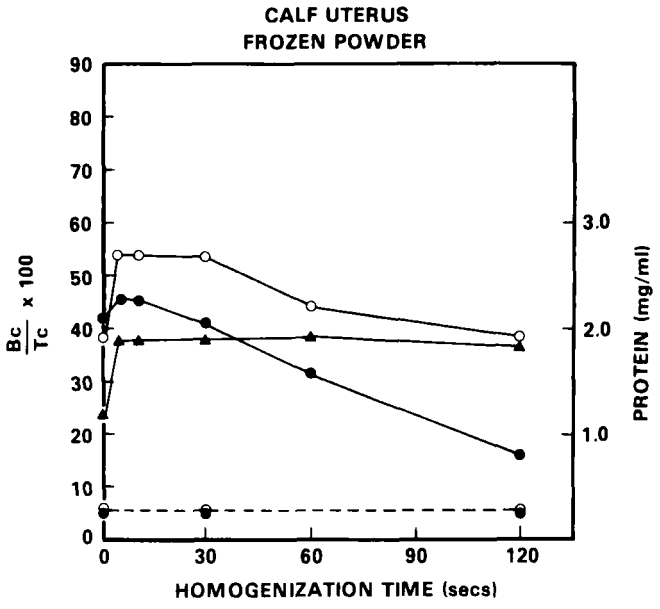


Figure 3. Estrogen (●), progesterone (○) and total protein (▲) solubilized by mechanical homogenization of pulverized calf uterus tissue. The solid line represents specific plus non-specific binding. The dashed line represents non-specific binding. Bc= Bound counts. Tc= Total counts.



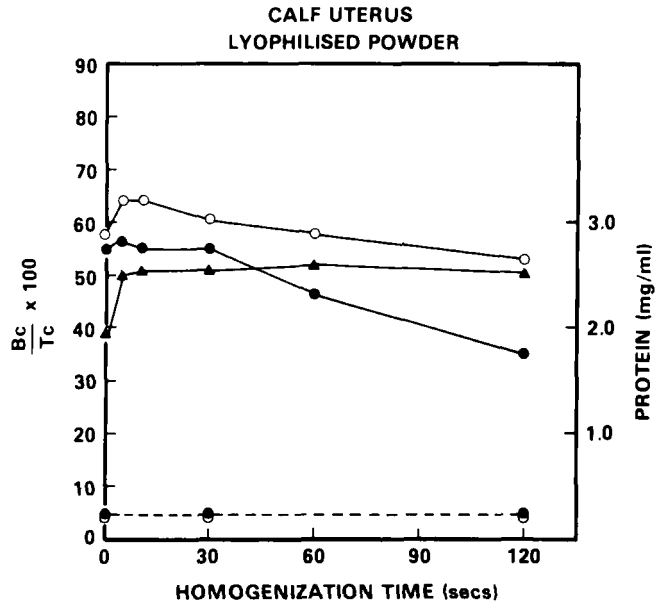


Figure 4. Estrogen (●), progesterone (○) and total protein (▲) solubilized by mechanical homogenization of lyophilised calf uterus tissue. The solid line represents specific plus non-specific binding. The dashed line represents non-specific binding. Bc= Bound counts. Tc= Total counts.

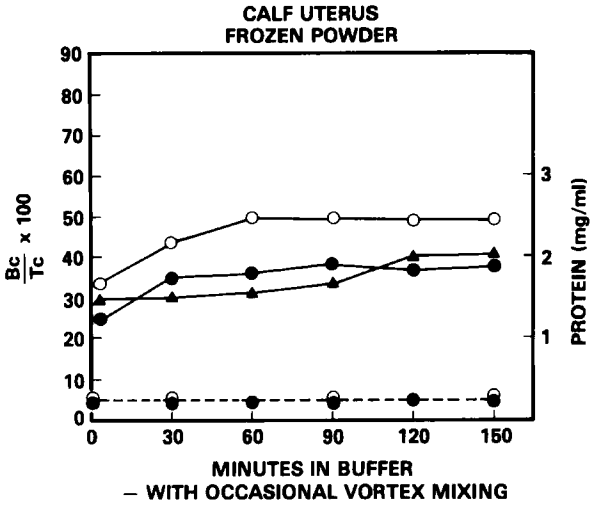


Figure 5. Estrogen (●), progesterone (○) and total protein (▲) solubilized by vortex mixing of pulverized calf uterus tissue with buffer. The solid line represents specific plus non-specific binding. The dashed line represents non-specific binding. Bc= Bound counts. Tc= Total counts.



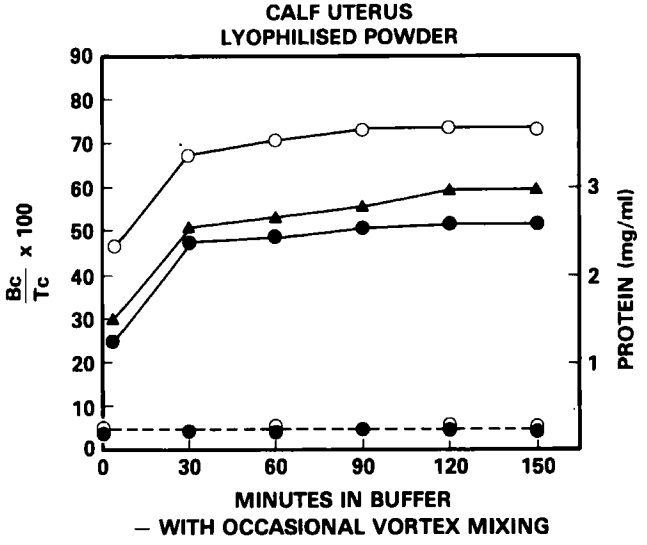


Figure 6. Estrogen (●), progesterone (○) and total protein (▲) solubilized by vortex mixing of lyophilised calf uterus tissue. The solid line represents specific plus non-specific binding. The dashed line represents non-specific binding. Bc= Bound counts. Tc= Total counts.

For laboratories which routinely pulverize breast tumor tissue prior to homogenization our results suggest that vortex mixing with buffer for 60 min will maximally solubilize the receptors and reduce to a minimum the homogenization component of between laboratory variability.

For laboratories that mechanically homogenize chopped tumor without prior pulverization, some measure of the effect of the mechanical homogenization step on receptor stability may be obtained by taking a second tissue powder quality control sample through the mechanical homogenization step. It must be accepted however that such a powder may undergo denaturation at a different rate from the chopped tissue and that there can be no fully adequate control material for such chopped tissue, ranging as it does in particle size and cellular consistency.



## References

1. De Sombre, E.R., et al. Steroid receptors in breast cancer. N. Engl. J. Med. 1979; 301:1011-1012.

2. Woosley, J.T., Muldoon, T.G. The thermolability of rat uterine cytosol estrogen receptors: Basis for a novel cytosol exchange-denaturation assay. J. Steroid Biochem. 1979; 11:1065-1071.

3. Braunsberg, H., Hammond, K.D., MacArthur, J.E. Preservation of progesterone receptors of human endometrium. J. Endocrinol. 1979; 81:143-144p.

4. Liskowski, L., Rose, D.P. Experience with a simple method for estrogen receptor assay in breast cancer. Clinica Chimica Acta 1976; 67:175-182.

5. Hawkins, R.A., Hill, A., Freedman, B. A simple method for the determination of oestrogen receptor concentrations in breast tumors and other tissues. Clinica Chimica Acta 1975; 64:203-210.

6. Allegra, J.C. et al. Distribution, frequency, and quantitative analysis of estrogen, progesterone, androgen, and glucocorticoid receptors in human breast cancer. Cancer Research 1979; 39:1447-1454.

7. Anderson, K.M. et al. Comparison of dextran-coated charcoal and sucrose density gradient analysis of estrogen and progesterone receptors in human breast cancer. Cancer Research 1980; 40:4127-4134.

8. Heidemann, P.H., Wittliff, J.L. Preparation of cytosol for steroid receptor determinations with the Beckman "airfuge". Clin. Chem. 1979; 25:622-625.

9. Koenders, A.J. et al. Estradiol receptor activities in stored lyophilised target tissue. J. Steroid Biochem. 1978; 9:947-950.

10. Godolphin, W., Jacobson, B. Quality control of estrogen receptor assays. J. Immunoassay 1980; 3:363-374.

11. Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 1951; 193:265-275.

12. McGuire, W.L., De La Goroza, M., Chamness, G.G.: Evaluation of estrogen receptor assays in human breast cancer tissue. Cancer Research 1977; 37:637-639.

13. Keightley, D.D. Tilley, W.D., McKant, E.L. Effect of snap freezing, dithiothreitol and storage on estimations of estrogen receptor sites. Clinica. Chimica. Acta 1978; 88:337-343.

14. Wittliff, J.L., Wiehle, S.A., Sandoz, J.P., Fisher, B., Durant, J.R. Establishment of uniformity in steroid receptor determinations used in clinical studies and drug screening program. In Raus, J., Martins, H., Leclercq, G., eds. Cytotoxic estrogens in hormone receptive tumors. New York: Academic Press, 1980; 147-164.