Accurate quantification of estrogen receptor (ER) is essential for optimal clinical characterization of individual cases of breast cancer. If breast tumors are mishandled, the relatively labile ER protein may lose its steroid-binding capacity (become inactivated) and not be measurable by the routine steroid-binding assay. We tested whether the commercial enzyme immunoassay of Abbott Laboratories could quantify inactivated ER. Samples of powdered breast tumors from humans were exposed to various temperature and homogenization conditions known to inactivate ER, and any remaining ER was quantified by both the immunoassay and the steroid-binding assay. For all inactivation conditions tested, the two assay methods detected the same proportions of remaining ER. We conclude that the inactivation reaction for ER also alters one or both of the antigenic site(s) necessary for the immunoassay. Hence, for breast tumors mishandled to the extent of inactivating ER, the immunoassay offers no advantage over the more conventional steroid-binding assay for quantifying any remaining ER.

**Additional Keyphrases:** breast cancer • progestin receptor • receptor inactivation

The usefulness of assays of estrogen receptor (ER) and progesterin receptor (PgR) for the clinical characterization of breast cancer is now well established (1-4). Therefore, accurate quantification of ER and PgR is essential to the complete and reliable clinical evaluation of individual cases of breast cancer.

Current assay methods routinely used in the quantification of ER and PgR depend on the binding of labeled hormones to the receptors (2-4). Hence, the steroid-binding sites of the receptors must be intact and functional for accurate quantification of the receptors.

Steroid receptors are relatively labile proteins and can lose their steroid-binding capacity (become inactivated) through a variety of mechanisms. Receptor inactivation can occur within intact tissues, e.g., through prolonged exposure of excised intact tumor tissue to high temperatures (2-6). Alternatively, steroid receptors may be inactivated during the tissue handling procedures before and during the cytosol preparation of the steroid-binding assay itself, again through high temperatures or excess homogenization (7), so laboratory assay conditions must be optimal (2-4). Finally, steroid receptors may be inactivated after they have been partitioned into the cytosol fraction of the tissue during the receptor assay procedure; these cytosolic receptors may be inactivated by such agents as high temperature, high ionic strength, exposure to active proteases or phosphatases, and oxidation of sulfhydryl groups (8-10).

This last type of receptor inactivation, that of cytosolic steroid receptors, can be inhibited by the use of such agents as sodium molybdate, sulfhydryl-reducing compounds, glycerol, and protease inhibitors, and can thereby be controlled within the assay laboratory (2, 8-11). The other causes of ER inactivation occurring during the receptor assay procedure can be largely eliminated through the use of standardized, optimal quantification methods (2-4, 12, 13).

Unfortunately, even with laboratory methods controlled, it is often not possible to avoid receptor inactivation in intact breast tumors when such inactivation occurs before the excised tumors arrive in the assay laboratory.

Consequently, as an alternative method for quantifying steroid receptors, immunoassays have been developed in which the receptors are quantified through their ability to be recognized by specific antibodies directed against antigenic sites on the receptor proteins (14). Theoretically, an immunoassay should be able to detect a receptor that lacks an intact steroid-binding site but retains its specific antigenic sites. Such immunoassays might prove useful in cases where receptor inactivation precludes the use of the steroid-binding assay for accurate quantification.

If so, immunoassays might prove superior to the routine steroid-binding assay for quantifying "inactivated" steroid receptors. We tested this hypothesis for the accurate quantification of ER of human mammary tumors under controlled conditions of receptor inactivation and found that the commercial immunoassay kit investigated offers no advantages over our standard estradiol-binding method.

**Materials and Methods**

**Chemicals:** [2,4,6,7-3H]Estradiol was purchased from Amersham, Oakville, Ontario, Canada; [3H]R5020 and unlabeled R5020 were from New England Nuclear (Dupont Canada Inc.), Mississauga, Ontario, Canada. Diethylstilbestrol (DES), EDTA, charcoal, dextran 500, estradiol, dihydrotestosterone (DHT), dexamethasone (dex), monothiogly-

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3 Inactivated is the process by which steroid-free receptor is converted to a form incapable of binding steroid.

4 "Cytosol" is the supernatant material resulting from the high-speed centrifugation (e.g., 100 000 x g for 60 min) of a tissue homogenate. This term is not synonymous with "cytoplasm."
cerol, and bovine serum albumin (BSA) were from Sigma Chemical Co., St. Louis, MO. All other chemicals, including sodium molybdate, were from Fisher Scientific Co., Ottawa, Ontario. Doubly-distilled water was used to prepare appropriate solutions.

Tissues: The human mammary tumors studied were those submitted to the Hormone Receptor Laboratory of the University of Alberta and the Cross Cancer Institute for routine assay of ER and PgR; this laboratory is funded by the Cancer Board of Alberta and serves, for routine assay of ER and PgR, the relatively large geographic area of the Province of Alberta. Samples were routinely placed on ice as soon as possible after excision and frozen at −70 °C or in liquid nitrogen within approximately 2 h. All tumors were sent to the laboratory (local samples only), in liquid nitrogen, or on solid CO₂ and stored at −70 °C. Methods for ER and PgR quantification are described below; specimens for which excess tissue remained stored at −70 °C were selectively used, usually within two weeks, for certain receptor experiments described herein. To minimize any assay interference by endogenous estrogens, only tumors from postmenopausal patients were used for the receptor inactivation experiments. Rat uteri, a source of control tissue, were excised from four- to five-week-old Sprague–Dawley rats and used after storage at −70 °C.

Cytosol preparation: Human mammary tumors or rat uteri stored at −70 °C were powdered under liquid nitrogen with a mortar and pestle and were warmed to 0 °C on ice. All subsequent procedures were carried out at 0–4 °C. Tissues were homogenized on ice in approximately 20 mL of buffer per gram of tissue, with two 5-s bursts of a Polytron PT-10 (Brinkmann Instruments, Rexdale, Ontario, Canada) homogenizer at a setting of 4, separated by a 60-s cooling interval. The buffer contained, per liter, 10 mmol of Tris, 1.5 mmol of EDTA, 12 mmol of monothioglycollate, 10 mmol of Na₂MoO₄, and 100 mL of glycerol (pH 7.4 at 22 °C). For certain experiments, we omitted the molybdate from the buffer or homogenized for other periods as indicated in the figure legends. In all cases, we centrifuged the homogenate at 200 000 × g for 30 min, using an SW 60 Ti or Ti 50.3 rotor in an L2-65B or L8-70M ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The resulting supernatant material, designated cytosol, was used for ER and PgR assays.

ER and PgR assays: For routine assays, we incubated 0.2-mL samples of cytosol for approximately 18 h with one of six concentrations of tritiated steroid, to quantify total binding, or one of three concentrations of tritiated steroid in the presence of a 100-fold excess of unlabeled steroid, to estimate nonspecific binding. For ER, we used [³H]estradiol (0.1–5 mmol/L), with or without a 100-fold excess of DES; for PgR, we used [³H]R5020 (0.1–5 mmol/L) plus a 10-fold excess of DHT and dex, with or without a 100-fold excess of unlabeled R5020. The DHT and dex would inhibit [³H]R5020 binding to androgen receptor or glucocorticoid receptor (15), respectively.

For the routine cytosol assays, we separated bound and free steroid by using dextran-coated charcoal (DCC) containing 1 g of BSA per liter (16). Specific binding was calculated by subtracting nonspecific binding from total binding; the resulting data were analyzed by the method of Scatchard (17).

The quality of the ER, PgR, and protein assays used in this laboratory is maintained by quantifying ER, PgR, and protein in a rat uterine cytosol standard daily, and in external standards of lyophilized cytosol supplied monthly by Dr. E. D. Ryan, McMaster University, Hamilton, Ontario, Canada (18). We also make periodic use of standard reference powders supplied by Dr. J. L. Wittliff, University of Louisville, Louisville, KY (3). Representative variabilities for assays of standards using our DCC assay for ER and PgR, respectively, were 191 ± 30 and 229 ± 25 fmol per milligram of cytosol protein (mean ± standard deviation, n = 18) for the rat uterine standard, 33 ± 6 and 0 fmol per milligram of cytosol protein for a repeated McMaster standard (n = 3), and 151 ± 22 and 201 ± 20 fmol per milligram of cytosol protein (n = 3) for Louisville standards. All unknowns supplied as negative for the receptors were reported to be negative by our laboratory and our results have compared favorably with those of other participating laboratories. For the immunoassay, we found ER to be 152 ± 16 fmol per milliliter of cytosol (n = 17; expected value, 147 fmol/mL) for the control supplied with the Abbott kit (see below). If data for certain lot numbers of the kit standard were excluded, the variation decreased (147 ± 9 fmol/mL; n = 14), reflecting the problem that Abbott encountered with its kit standards (see below).

Immunoassays: The commercial enzyme immunoassay (EIA) kits of Abbott Laboratories, North Chicago, IL, were purchased and used essentially according to the instructions provided. The only exceptions were minor: the use of our homogenization buffer above (containing 10 mmol of sodium molybdate per liter instead of 5 mmol/L and also containing monothioglycollate and glycerol) and the use of our in-house protocol for the Lowry protein assay (see below). For two lot numbers, we observed that the values for control samples supplied with the kits did not approximate the expected values. We contacted Abbott Laboratories and were instructed to decrease the volume used to reconstitute the kit standard (e.g., from 1.3 mL to 1.1 mL).

Other methods: Protein concentrations were measured by the method of Lowry et al. (19), with BSA as the standard protein. Since sulphydryl-reducing compounds (20) and molybdate (21) individually, and together, may affect the color reaction of this protein assay, the BSA standards included the appropriate dilutions of our homogenization buffer (above).

Results

Correlation of Immunoassay and DCC Assay

To assess the usefulness of the immunoassay for quantifying inactivated ER, we compared the immunoassay with the steroid-binding assay under control conditions (in the absence of any known inactivation of the ER). We performed this comparison twice: first in 1985 and 1986 and again in 1986 and 1987. During the earlier period, the DCC assay (x) and immunoassay (y) gave very similar results (y = 3.085 fmol per milligram of cytosol protein + 1.065x; r = 0.87; n = 26) but in the second series of experiments, the immunoassay apparently detected more ER than the DCC assay (y = 10.604 fmol per milligram of cytosol protein + 1.315x; r = 0.94; n = 37). These results were for cytosols of individual tumors. To avoid bias, we have not included in these data results from multiple assays on single tumors in the inactivation experiments below, although for each time period those results (for proportions of ER remaining after the inactivating conditions) revealed the same relationship between the DCC and EIA assays as expressed in the above regression analyses.

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Ability of Immunoassay to Detect Temperature-Inactivated ER

One of the simplest ways to inactivate ER is through exposing tumors to high temperatures. To test the ability of the immunoassay to detect temperature-inactivated ER, we exposed aliquots of powdered breast tumors to various temperatures known to inactivate ER. As a model for the fate of ER in intact tissues, relatively homogeneous tumor powders were used rather than portions of tumors, because of the known heterogeneity of ER distribution in different portions of human breast tumors (3, 22, 23). We selected temperatures and times to give approximately 50% inactivation of ER, keeping in mind the expected inter-tumor variations. As shown in Figure 1, ER was inactivated at $-10$, 0, 22, and 37 °C, and the immunoassay and DCC assays revealed proportions of remaining receptor that did not differ between the two assays for any of the temperatures and times chosen. The data, expressed as absolute values (fmol per milligram of cytosol protein) instead of proportions, reflected ratios between the immunoassay and DCC assay similar to the correlation data presented above. The fact that ER was relatively stable under the chosen conditions was noteworthy.

Time-Course for Inactivation of ER

If the epitopes required for the immunoassay were destroyed by a reaction independent of the inactivation reaction (affecting the steroid-binding site), the two reactions might be expected to occur at different rates. To test this, we inactivated ER at 37 °C and compared the immunoassay and DCC assay results at different time intervals. As shown in Figure 2, the two assays detected similar proportions of ER remaining at each interval indicated.

Inactivation of ER by Homogenization Conditions

For the above temperature conditions, PgR loss was known to exceed that of ER—for example, exposure to 22 °C for 60 min inactivated 44.5% of ER and 92.4% of PgR ($P < 0.001$, $n = 5$). In contrast, excessive homogenization of breast tumor tissue causes an inactivation of ER with a concomitant but lesser loss of PgR (7). We have previously established the details of this inactivation for our molybdate-containing buffer (unpublished data) and wished to know if the immunoassay could detect ER after inactivation by this method. Shown in Figure 3 are the results of experiments in which the immunoassay and DCC assay quantified similar proportions of ER remaining after excessive homogenization.

Absence of sodium molybdate from the homogenization buffer results in a lower concentration of ER in the resulting cytosol. It is unknown whether the lower ER population results from inactivation of ER or because a distinct population of nuclear ER is not partitioned into the cytosol in the absence of molybdate. However, inactivation would be confirmed if the immunoassay were to detect more ER than the DCC assay in the absence of the molybdate. As seen in Figure 3, omission of molybdate from the homogenization buffer resulted in about 25% less ER being measurable in the resulting cytosols. However, the DCC and immunoassay did not differ in their ability to quantify ER in the absence of molybdate.

Discussion

Given the usefulness of the estrogen receptor assay in selecting therapy and determining the prognosis for breast-cancer patients, it is important that the assay for this receptor be done on all primary breast tumors (I–4, 12). Moreover, the concentration of receptor as well as its mere
presence is important (1), so it is essential that the receptor-assay results be accurate.

Given the relatively labile nature of steroid receptor proteins, and given the range of agents known to inactivate these receptors (3, 5–11), we had hypothesized that the immunoassay might detect ER even in the absence of an intact steroid-binding site. We have shown herein that the immunoassay does not offer an advantage over the more conventional steroid-binding and DCC assay for the quantification of ER remaining after the exposure of powdered breast tumors to the inactivating conditions of elevated temperatures or excess homogenization.

Aliquots of individual powdered human mammary tumors were used as a model for intact tissues, because the powders were relatively homogeneous and thus obviated errors resulting from the usual simple sampling procedure used for heterogeneous (22, 23) intact tissues. We examined inactivation similar to that thought to occur occasionally in intact breast tumors during inappropriate tumor shipment and handling. This inactivation was caused before the ER in the cytosol fractions prepared from the breast tumors was stabilized. The components of the buffer, the usual extent of homogenization, and the incubation and charcoal assay conditions (above) have been selected to minimize receptor inactivation once the cytosol has been prepared for the assay. We cannot tell if partial inactivation of ER had occurred before the routine tumor shipments arrived in our assay laboratory.

We selected four temperatures for the inactivation reaction and used −70 °C as the control. The −10 °C condition would reflect storage in a household-type refrigerator freezer, 0 °C would represent storage on ice or ice-packs (or exposure to slowly increasing temperatures after the sublimation of solid CO₂), 22 °C would reflect exposure to a common ambient temperature, and 37 °C would represent an unusually high ambient temperature or exposure to body temperature after tumor excision. We proposed that any differences in enzymatic activities, ice-crystal formation, ionic effects, or other causes of inactivation of ER in the tissues might be revealed at different temperatures and might give rise to different quantities of ER as detected by the immunoassay or DCC assay. However, regardless of the temperature of inactivation, the immunoassay detected the same proportion of ER remaining as was detected by the DCC assay. While our study was under way, similar results were found for three tumors exposed to room temperature for 1 h (24).

Another inactivation condition that affects ER is excessive homogenization (7). The mechanism of this inactivation is unknown, because the cold (0 °C) conditions and use of the molybdate buffer would inhibit any homogenizer-caused temperature-induced inactivation. Furthermore, temperature-induced inactivation would have affected PgR more than ER, not vice versa. Therefore, this homogenization-induced inactivation was selected as a contrast to the temperature-induced process, but, again, ER quantities were similar for the immunoassay and DCC assay.

One or both of the antigenic sites essential for the immunoassay may be altered during the inactivation of the ER. We have shown previously that, for glucocorticoid receptor, conditions that inactivate steroid-free receptor elicit transformation (conversion to the nuclear form) of steroid-bound receptor and that such conditions do not simply destroy the steroid-binding subunits of the receptor (11). We have suggested that the dissociation of receptor subunits and conformational changes in the receptor protein are probably the same for both inactivation and transformation. Such changes could also modify or mask antigenic sites on steroid receptors and thereby make immunoassays inoperable following exposure to the inactivating conditions. The proximity of the steroid-binding site of ER to the known antigenic sites used in the immunoassay has been suggested previously (25).

Reportedly, the immunoassay detects more ER than does the steroid-binding and DCC assay for human mammary tumor cytosols (26–28), and some controversy has arisen (29–31). One could postulate that the immunoassay detects inactivated ER, which would not be measured by the DCC assay, and that this explains the discrepancy between the two assay methods. Our results suggest that this explanation is unlikely.

Our finding of a good correlation between the immunoassay and DCC assay is consistent with previous studies. Also, the slope of 1.3 that we observed in the more recent series is in keeping with findings from other North American laboratories (27), whereas our earlier observed slope of 1.0 has been found in European laboratories (26, 28). Deficiencies in our methods for the steroid-binding and DCC assay do not explain the discrepancy between the results for the two assays because our methods have been developed previously taking into consideration all of the variety of concerns suggested recently (32). Also, our use of tissues from premenopausal patients should have minimized any interference by endogenous estrogens in these assays (28). Abbott Laboratories has encountered problems with the standards in the kits (see Materials and Methods), and whether or not the differences between the two assay methods in North America will be eliminated when such problems have been eliminated remains to be seen. We found the immunoassay as easy to use as, and less time-consuming than, the DCC assay.

In the future, it may be possible to modify the enzyme immunoassay by using different monoclonal antibodies, which recognize both intact and inactivated ER while still maintaining receptor specificity. Alternatively, denaturation of the ER may re-expose the necessary antigenic sites that may have been masked by conformational changes of the ER during inactivation. In the meantime, carefully controlled steroid-binding assays and the immunoassay provide comparable data for the quantification of ER, and neither assay can be used to quantify ER inactivated during inappropriate handling of human breast tumors.

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A Simplified Strategy for Testing Thyroid Function

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We assessed a new strategy for thyroid-function testing that involves simultaneous measurement of free thyroxin and thyrotropin, both in singletons, with chemiluminescent assays. Using our current strategy of measuring free thyroxin as a first-line test with selected back-up testing, the results show that, of 810 patients without previous thyroid disease, 445 received back-up tests. Of these, 345 were euthyroid, whereas 63 classified as euthyroid and not selected for further testing in fact had abnormal back-up test results. Evidently the simultaneous measurement of free thyroxin and thyrotropin with the “Magic Lite” technology greatly improves diagnostic efficiency compared with this current strategy.

In recent years, measurement of free thyroxin (T4) in serum has been a popular first-line test of thyroid function. The most widely used procedures are those involving 125I-labeled analogs of T4. Although these methods are very convenient for the analytical laboratory, they are subject to

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1 Nonstandard abbreviations: T4, thyroxin; T3, triiodothyronine; TSH, thyrotropin; and IRMA, immunoradiometric assay.