Comparison of $[^3\text{H}]$Progesterone and $[6,7^{-3}\text{H}]-17,21$-Dimethyl-19-norpregna-4,9-diene-3,20-dione for the Measurement of Progesterone Receptors in Human Malignant Tissue


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ABSTRACT

Progesterone receptors of human tumors of breast and gynecological origin were assayed with $[6,7^{-3}\text{H}]-17,21$-dimethyl-19-norpregna-4,9-diene-3,20-dione or $[1,2,6,7^{-3}\text{H}]-$progesterone plus a 100-fold excess of cortisol by the charcoal extraction method. Whereas both ligands gave concordant levels of binding for the high-capacity samples, higher nonspecific binding of $17,21$-dimethyl-19-norpregna-4,9-diene-3,20-dione to serum albumin resulted in less precise estimates of low-binding-capacity samples.

Ammonium sulfate precipitation of receptor and gradient ultracentrifugation analysis demonstrated that the nonspecific binding of $17,21$-dimethyl-19-norpregna-4,9-diene-3,20-dione was nonprecipitable by 30% ($\text{NH}_4\text{SO}_4$, and migrated with serum albumin. That $17,21$-dimethyl-19-norpregna-4,9-diene-3,20-dione binds to human serum albumin was further shown by gradient ultracentrifugation analysis and the charcoal extraction method.

The importance of adding an excess of cortisol to block binding to corticosteroid-binding globulin with $[1,2,6,7^{-3}\text{H}]$progesterone to measure progesterone receptor binding was redemonstrated.

We conclude that presently the most accurate methods described to measure progesterone receptors in crude cytosol are those that use labeled progesterone as ligand, provided that the nonspecific binding to corticosteroid-binding globulin is blocked by an excess of unlabeled cortisol.

INTRODUCTION

Endocrine therapy of carcinoma of the breast was discovered more than 80 years ago by Beatson (3). Few advances in the ability to predict responses in patients were made until 1971 when Jensen et al. (8) in pioneering studies suggested that estrogen receptors might distinguish estrogen-dependent breast tumors and indicate those tumors amenable to endocrine therapy. This hypothesis has been subsequently confirmed (14).

Recently, the attention of investigators has turned to the measurement of progesterone receptors in the hope that such measurements combined with those of estrogen receptor levels will allow a more accurate prediction of tumor response. The original studies of progesterone receptors suggested that in mammals the synthesis of these receptors may be regulated by estrogen (26), and therefore the presence of progesterone receptors may serve as an additional index of the ability of the tissue to respond to estrogen stimulation (10, 15). In addition, endometrial and ovarian tumors as well as renal carcinomas have been reported to respond to progesterone therapy. Whether or not the response of these tumors is correlated with the presence of progesterone receptors is at present not well established; there is, however, preliminary evidence that this correlation exists in endometrial carcinoma (6).

Several reports have appeared describing methods for the measurement of cytoplasmic progesterone receptors by using either labeled progesterone (17, 25, 28) or the synthetic compound R5020$^3$ (15, 19). The latter compound has been thought to be more suitable because it does not bind to CBG. Moreover, it has a higher affinity for the progesterone receptor and dissociates from it more slowly than does progesterone itself (2, 16). Nevertheless, R5020 has considerable affinity for glucocorticoid receptors (12) and appears to bind nonspecifically to serum and tissue proteins with greater avidity than does progesterone.

The purpose of the present studies was to compare the suitability of both ligands, progesterone and R5020, for the measurement of progesterone receptors in human breast and gynecological tumors. We found that R5020 bound tightly to HSA in a way that impeded the measurement of progesterone receptors of low-capacity tissue samples.

MATERIALS AND METHODS

Reagents. Tris-$\text{HCl}$, EDTA, $\text{NH}_4\text{SO}_4$ (all of reagent grade), and progesterone were purchased from Sigma Chemical Co., St. Louis, Mo. Dithiothreitol and HSA (Grade A) were obtained from Calbiochem, San Diego, Calif. The source of glycerol (spectrometric grade) was Mallinckrodt Chemical Works, St. Louis, Mo. Charcoal (Norit A) was obtained from J. T. Baker Chemical Co., Phillipsburg, N. J., and dextran (Dextran radioimmunoassay) was obtained from Schwarz/Mann, Orangeburg, N. Y. $[^3\text{H}]$Progesterone was purchased from New England Nuclear, Boston, Mass., and $[^3\text{H}]$R5020 and R5020 were generous gifts from Dr. J.-P. Raynaud.

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The \([^3]H\)progesterone and \([^3]H\)R5020 were repurified by Celite column partition chromatography every 2 months, according to the method of Siiteri (22). The solvent system used for both radiolabeled steroids was isooctane/ethylene glycol. The purity of \([^3]H\)R5020 upon receipt was approximately 85%.

**Preparation of Cytosol.** Nonnecrotic tissue obtained at biopsy was trimmed free of excess fat and frozen in liquid nitrogen within 15 min after removal. Samples were stored in liquid nitrogen until assayed, usually within 1 week of collection. The tissue was powdered in a Thermovac Auto-pulverizer stainless steel mortar. The powder was weighed and added to ice-cold 0.01 m Tris-HCl/0.0015 m EDTA buffer (pH 7.4, 24°C) containing 0.5 mm dithiothreitol and 10 or 30% glycerol. The final concentration was 100 mg tissue per ml.

The pulverized tissue was homogenized 3 times for 15 sec each time, with alternate 1-min cooling periods, by using a Polytron tissue homogenizer with a rheostat setting of 8. Cytosol was prepared from the homogenate by centrifugation at 105,000 × g for 30 min. The usual soluble protein concentration was 4 to 5 mg/ml.

**Quantitation of Steroid-binding Components by Charcoal Extraction.** Tritiated ligand with or without unlabeled competing steroids dissolved in absolute alcohol were added to 12 × 75-mm disposable test tubes and evaporated to dryness under a stream of nitrogen. Cytosol (0.2 ml) was added and the tubes were incubated at 4°C for at least 3 hr.

Saturation analysis with \([^3]H\)progesterone was carried out in the presence of a 100-fold excess of nonradioactive cortisol to prevent the binding of \([^3]H\)progesterone to CBG, except as indicated in the results.

After incubation an aliquot (0.05 ml) was removed to determine the total amount of radioactive ligand present in each incubation tube. The amount of ligand bound in each tube was determined by a dextran-coated charcoal method as follows: To 0.1 ml of the incubation mixture, an equal volume of dextran-coated charcoal was added (0.25% charcoal/0.025% dextran in 0.01 m Tris-HCl/0.0015 m EDTA buffer with 10 or 30% glycerol). The tubes were gently vortexed and were incubated at 4°C for 12 min for the 10% glycerol samples and 30 min for the 30% samples. After centrifugation at 800 × g for 20 min, 0.1 ml of the supernatant was removed and the amount of bound radioactive ligand was determined. The efficiency of the charcoal extraction remained >98% for both \([^3]H\)progesterone and \([^3]H\)R5020 up to the addition of a 500-fold excess of cold ligand. The concentrations of radioactive ligand used for saturation analysis varied from 0.2 to 30 nm.

**Ultracentrifugation Analysis.** After incubation of the cytosol with steroids as described previously, 0.2 ml of the cytosol was layered on a linear 20 to 40% glycerol gradient in 0.01 m Tris-HCl/0.0015 m EDTA buffer. Samples were centrifuged at 60,000 rpm for 17 hr at 2°C in a Spinco SW 60 rotor. Each sample for analysis also contained \[^{[14C]}\]HSA as a marker substance (23). Fractions (0.1 ml) were collected with an ISCO gradient fractionator, and radioactivity was measured in a Packard Model 3003 scintillation spectrometer with 5 ml of Triton scintillation fluid.

**Ammonium Sulfate Precipitation.** Saturated \((NH_4)_2SO_4\) was added to dryness under a stream of nitrogen. Cytosol (0.2 ml) was added to 12 × 75-mm disposable test tubes and evaporated to dryness under a stream of nitrogen in 0.01 N Tris-HCl/0.0015 N EDTA buffer plus 10% glycerol. The precipitate was allowed to settle for 1 hr at 4°C and was then collected by centrifugation at 9000 × g for 20 min. The pellet was redisolved in 0.01 m Tris-HCl/0.0015 m EDTA buffer containing 10% glycerol and dialyzed against the same buffer for 3 hr prior to saturation analysis.

**Calculations.** Data for saturation analysis was analyzed according to the method of Scatchard (21). The specific binding was corrected graphically by radial subtraction of the nonspecific component of the Scatchard plot according to the method of Rosenthal (20), assuming that the nonspecific binding yields a horizontal line asymptotic to the curve drawn by the experimental points, as was found to be the case in the present experiments.

As nonspecific bound/free (B/F) is constant, the radial subtraction can be mathematically represented as follows:

\[
B / F (\text{specific}) = B / F (\text{observed}) - B / F (\text{nonspecific})
\]

where B is the concentration of ligand bound to the receptor and F is the free ligand. B/F (nonspecific) is the ordinate of the horizontal line asymptotic to the experimental points.

**RESULTS**

**Comparison of Progesterone Receptor Measurements with \([^3]H\)Progesterone and \([^3]H\)R5020.** Charts 1 and 2 illustrate the results obtained with \([^3]H\)R5020 and \([^3]H\)progesterone plus cortisol by using samples of breast tumor having high and low binding capacities, respectively. In both samples, assays with \([^3]H\)R5020 yielded lower dissociation constants than those with \([^3]H\)progesterone (K_d = 0.5 × 10^-9 M to 0.9 × 10^-9 M versus 1.8 × 10^-9 M to 2.3 × 10^-9 M). However, the binding capacities measured were similar for both ligands (0.78 versus 0.65 nm and 0.04 versus 0.052 nm). Note that background nonspecific binding is 3- to 10-fold higher with labeled R5020 than with \([^3]H\)progesterone and cortisol. This higher nonspecific binding of R5020 was in our experience a constant finding. Seven samples were assayed with \([^3]H\)progesterone plus cortisol as well as with \([^3]H\)R5020; the median values of nonspecific bound/free were 0.081 ± 0.044 (S.D.) for R5020.
and 0.018 ± 0.010 for the progesterone plus cortisol assay; the difference was statistically significant at p < 0.01. This higher background was clearly detrimental when assaying samples with low binding (Chart 2).

**Effect of Inclusion of Unlabeled Cortisol in the [3H]Progesterone Assay.** The importance of adding nonradioactive cortisol when measuring progesterone receptors with [3H]progesterone is illustrated in Chart 3. In crude cytosol the binding measured without the cortisol yielded a much higher capacity (2.28 versus 0.71 nM) and a much lower affinity (Kd = 7.6 × 10⁻⁹ M versus 1.05 × 10⁻⁹ M) than the assay that included an excess of unlabeled cortisol (Chart 3A). For a determination of whether the increased binding capacity and lower affinity observed in the absence of unlabeled cortisol was due to the binding of progesterone to contaminating serum proteins, receptors were partially purified by precipitation with ammonium sulfate. This procedure yielded a receptor preparation (18, 24) almost free of contaminating serum CBG and albumin. When analysis of binding was repeated as before with the (NH₄)₂SO₄ precipitate, no significant difference in either binding capacity or affinity was found whether or not an excess of unlabeled cortisol was included in the assay (Chart 3B). These data suggest that cortisol virtually eliminates interference due to CBG without inhibiting the binding of progesterone to its receptor.

**Effect of (NH₄)₂SO₄ Precipitation on the Nonspecific Binding of R5020.** Chart 4 illustrates the results of an experiment carried out at the same time with the same cytosol preparation, comparing the use of [3H]R5020 with [3H]progesterone plus unlabeled cortisol. As can be seen in Chart 4A, the assay of crude cytosol with R5020 yielded an 80% higher binding capacity than did the assay of this material with progesterone. Interestingly, the binding affinities derived from the 2 assays were nearly identical. However, when these assays were performed with (NH₄)₂SO₄ precipitated receptor, no difference in binding capacities between the 2 assays was observed. Furthermore, R5020 exhibited a 2-fold-greater binding affinity than did progesterone for the partially purified receptor (Chart 4B), whereas the affinity of progesterone for crude cytosol and for the (NH₄)₂SO₄ precipitated receptor was nearly identical. These results suggest that the lower-than-expected affinity observed for R5020 binding in crude cytosol preparations may have been caused by the interaction of R5020 with some contaminant present in the crude cytosol not precipitated by 30% (NH₄)₂SO₄. Furthermore, this contamination probably accounted for the discrepancy in binding capacity detected by the 2 assays.

**Density Gradient Analysis of R5020 and Progesterone Binding.** That the binding of R5020 and progesterone might be further characterized, glycerol gradient ultracentrifugation analyses were performed. Chart 5 shows the results of the density gradient analysis of [3H]progesterone binding to a human uterine myoma cytosol preparation. As can be seen, the binding of [3H]progesterone alone was characterized by a large 4S peak and a smaller 8S peak. When a 100-fold molar excess of cortisol was included with [3H]progesterone in the incubation mixture, most of the binding in the 4S region was eliminated with no effect on the 8S binding. This result suggests that most of the binding in the 4S region was due to contaminating CBG. If the sample was incubated with [3H]progesterone plus a 100-fold molar excess of both unlabeled cortisol and progesterone, no binding in either the 4S or 8S region was observed.

Chart 6 shows an analysis of the binding of [3H]R5020 to the same uterine cytosol preparation. Again, 2 peaks of binding activity were observed, 1 in the 4S region and 1 in the 8S region. When the incubation mixture contained a 100-fold excess of unlabeled R5020, the binding in the 8S region was totally eliminated, whereas that in the 4S region actually increased to approximately the same extent.
Measurement of Progesterone Receptor

On the basis of the gradient analysis, it was possible to calculate a binding capacity of 25.8 pmol/g of tissue for the specific binding of [3H]progesterone to myoma cytosol. This value was in agreement with the binding capacities determined for the same cytosol by Scatchard analysis (27.6 pmol/g) and by a single-point assay with 10 nM [3H]progesterone plus excess cortisol and correction for nonspecific binding using an incubation with excess nonlabeled progesterone (25.8 pmol/g). With R5020 a fair agreement was also demonstrated between the binding capacity determined by gradient analysis (20.3 pmol/g) in the 8S peak only and a single-point charcoal assay performed with the same radioligand at 10 nM concentration with and without a 100-fold excess of R5020 (22.9 pmol/g).

These results show that R5020 binds to a high capacity 4S component that takes up much of the [3H]R5020 displaced from the 8S receptor by excess unlabeled ligand. This effect would tend to overestimate the nonspecific binding measured by adding an excess of nonlabeled R5020 and explains why the specific binding capacity measured with R5020 was lower than that measured with progesterone in both assays, ultracentrifugation analysis and charcoal extraction.

R5020 Binding to HSA. Since the results with ammonium sulfate-precipitated receptors suggested that R5020 binds to serum contaminants, R5020 binding to human serum and purified HSA was compared with that of progesterone in the presence of excess cortisol by using the charcoal method and various amounts of glycerol. The binding of R5020 to serum was always higher than the binding of progesterone to serum regardless of the glycerol concentration present (Table 1). Residual binding with progesterone ranged from 1.5 to 1.8%, whereas 6.0 to 14.4% of R5020 remained after charcoal extraction. Pure albumin yielded a high binding of R5020; a 1% solution of pure HSA bound to 50% of the total [3H]R5020, whereas only a 3 to 5% residual binding of [3H]progesterone occurred. A direct demonstration of the binding of R5020 to purified serum albumin as analyzed by glycerol density gradient centrifugation is shown in Chart 6, inset.

DISCUSSION

Accurate measurement of progesterone receptor binding, which is expected to provide additional information for predicting tumor behavior, has been limited because of the binding of progesterone to contaminating CBG, and because of the relatively low affinity between the natural ligand and the receptor. Feil et al. (7), using mouse and rat uteri, and Young and Cleary (28) and MacLaughlin and Richardson (13), using human endometrial cytosol, were able to distinguish between the binding of progesterone to

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receptors and CBG on the basis of competition studies as well as in studies of affinity and thermolability. Physical separation of the progesterone receptor from the contaminating CBG by (NH₄)₂SO₄ precipitation of the receptor was first described by Rao et al. (18). This method allowed us to demonstrate that the high-affinity binding of progesterone to CBG can be overcome by the addition of cortisol (Chart 3).

The addition of glycerol as first used by Wiest and Rao (1970) (26) stabilizes progesterone binding to the receptor (2, 17). Taking advantage of the stabilizing effect of glycerol and using cortisol to block progesterone binding to CBG, Young et al. (29) as well as Pichon and Milgrom (17) have described progesterone assays in which the natural ligand was used successfully.

Recently, R5020 has been promoted for measurement of progesterone receptor binding because it does not bind significantly to CBG and binds to progesterone receptors with a higher affinity than does the natural ligand. Several assays have been described in which R5020 has been used for either ultracentrifugation or charcoal assay analysis (15, 19). Raynaud et al. (19) used the charcoal extraction method to measure progesterone receptor levels in human breast tumors with either R5020 or progesterone plus cortisol and concluded that the R5020 assay gave better results. They found that the progesterone-cortisol assay gave positive values in 18 of 65 samples in which the R5020 assay showed no detectable binding and suggested that this difference was due to the higher nonspecific binding of [3H]progesterone. However, our results show that nonspecific binding of R5020 is considerably higher than that observed with the progesterone-cortisol assay, so that tumor samples containing low levels of receptor binding were difficult to analyze with R5020 (Chart 2). Unlike the progesterone receptor (18, 24), most R5020 nonspecific binder did not precipitate with 30% ammonium sulfate and appeared to sediment in the 4S region on the glycerol density gradient. These findings suggested that the high nonspecific binding is due to the interaction of R5020 with serum albumin, as proposed by McGuire et al. (15). That albumin has a high affinity for R5020 was demonstrated by charcoal assay as well as by gradient ultracentrifugation analysis (Table 1 and Chart 6). Note that the presence of glycerol did not influence binding of R5020 to albumin.

Raynaud et al. (19) applied the widely accepted method of using parallel incubations containing excess nonlabeled ligand to estimate nonspecific binding. This approach, popularized by Williams and Gorski (27), can lead to serious errors in estimates of both binding capacity and affinity constants as pointed out by Blondeau and Robel (4). In the particular case of R5020, the high affinity this ligand has for albumin (Table 1) results in an overestimate of nonspecific binding because some of the labeled hormone that is prevented from interacting with the receptor by the nonlabeled hormone is bound to albumin and measured in the charcoal assay. The true progesterone receptor content is therefore underestimated. This phenomenon could explain the large proportion of receptor-negative tumors observed by Raynaud et al. (19) when R5020 was used as ligand. For the same reasons, this problem leads to errors in estimates of dissociation constants. The magnitude of error depends upon the degree of contamination of the tissue specimen with serum, which may vary from 10 to 20%.

The use of unlabeled R5020 to assess nonspecific binding by ultracentrifugation analysis of [3H]R5020 binding to progesterone receptors is also subject to errors. That steroid hormone receptors may be present in both 8S and 4S forms in the same tumor cytosol is well known. As seen in Chart 6, the amount of the 4S form cannot be determined because all of the [3H]R5020 displaced from the 8S receptor form by nonradioactive R5020 appeared in the 4S region bound to albumin. This observation clearly demonstrates the redistribution of [3H]R5020 to albumin in the presence of unlabeled R5020 and further supports our conclusion that nonspecific binding is overestimated to the extent that albumin-bound [3H]R5020 is resistant to charcoal extraction. This effect may be magnified by other techniques such as gel filtration for separation of free and receptor-bound hormone.

Obviously, the use of unlabeled progesterone to estimate the specific binding of [3H]R5020 would accentuate these problems. To our knowledge assays for progesterone receptor in which nonradioactive competitors that bind only to receptor and not to serum proteins have not been used. However, this approach has been commonly used for measurement of estrogen receptors. Several methods that use labeled estradiol, with and without the addition of an excess of diethylstilbestrol or nafoxidine, that binds only to the estrogen receptor have been described (9, 5, 11). In these cases the concentration of free steroid in the sample with tracer alone is unknown because its binding to high-affinity serum proteins (sex steroid-binding protein or ß-fetoprotein) is not blocked. This approach can lead to serious errors at low

Table 1

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<th>[3H]R5020 and [3H]progesterone plus cortisol binding to human serum and HSA</th>
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| Five nM [3H]progesterone plus a 100-fold excess of cortisol or [3H]R5020 were incubated with or without a 1/25 dilution of human serum or 10 mg HSA per ml in 0.01 M Tris-HCl/0.0015 M EDTA buffer containing the stated amount of glycerol. The results are the means of 2 different experiments and express bound/total after charcoal adsorption as described in "Materials and Methods."
| [3H]Progesterone at a glycerol concentration of 0% 10% 30% | [3H]R5020 at a glycerol concentration of 0% 10% 30% |
|---|---|---|---|---|---|
| Serum | 0.018 | 0.016 | 0.015 | 0.144 | 0.126 | 0.060 |
| HSA | 0.050 | 0.033 | 0.032 | 0.516 | 0.483 | 0.338 |
| 0.01 M Tris-HCl/0.0015 M EDTA buffer | 0.009 | 0.007 | 0.006 | 0.028 | 0.020 | 0.012 |
binding values and may account for the broad range of $K_d$ values reported for the estrogen receptor (1).

Saturation analysis is best performed by using labeled ligand over a range of concentrations, which allows accurate estimation of nonspecific binding from the Scatchard plot (Chats 1 and 2) without nonlabeled ligand. This approach is advantageous when only small tumor specimens with low binding capacities (see Chart 2) are available, since just 1 aliquot of the sample is needed to determine nonspecific binding and therefore more material is left from which to obtain information.

The ligand chosen may be the natural or a synthetic hormone that discriminates between receptor classes, has high affinity for the desired receptor, and has low nonspecific binding to tissue or serum proteins. A nonlabeled steroid that will not bind to the receptor (e.g., cortisol in addition to [3H]progesterone in the progesterone receptor assay or dihydrotestosterone in addition to [3H]estradiol in the estrogen receptor assay) should be added to block interference from competing serum protein binders. Since most of the nonspecific binding is suppressed in this manner, the concentration of free steroid in cytosol is more accurately estimated.

That glycerol does not prevent destruction of the receptor but in fact decreases the dissociation rate between progesterone and the receptor has been demonstrated (6, 2, 17). We performed preliminary studies of the [3H]progesterone receptor complex dissociation rate in 10% glycerol with breast and gynecological tumors and found that the breast samples had a faster dissociation rate than did samples of uterine origin. Breast samples had a median half-life of 48 min (range, 40 to 50 min), whereas myoma samples in the same medium yielded a median half-life of 139 min (range, 82 to 180 min). This phenomenon, which is currently under investigation in our laboratory, may explain why gradient ultracentrifugation analysis with [3H]progesterone can be performed easily with samples of gynecological origin, whereas breast samples rarely allow this kind of analysis with the natural ligand.

When more glycerol is added to the assay, it is expected that less specific binding will be lost during the charcoal incubation. In this regard the method of Pichon and Milgrom (17), who used 30% glycerol, should yield higher values than the method we described in which only 10% glycerol was used; however, in our experience there was no significant difference in the results with 10 or 30% glycerol in the buffer, as described in "Materials and Methods." This finding probably resulted from the longer charcoal incubation required with higher concentrations of glycerol: 12 min for the 10% glycerol method and 30 min for the 30% glycerol method.

The results of these studies demonstrate that R5020 binds significantly to HSA in such a way that the measurement of progesterone receptors in low-capacity samples is hampered in the charcoal extraction assay and that, as McGuire et al. (15) have pointed out, the 4S form of the receptor cannot be analyzed by density gradient ultracentrifugation. Our findings along with recent evidence that R5020 binds significantly to glucocorticoid receptors (12) known to be present in breast and other tumors suggest that presently the assay of progesterone receptors in crude cytosol is most accurately performed with [3H]progesterone and unlabeled cortisol in a buffer that contains glycerol.

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