Methods for Ligand–Receptor Assays in Clinical Chemistry

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This is intended to be a practical review for the clinical chemist of the laboratory procedures most commonly used to quantitate hormone receptors in various cellular fractions. These procedures include use of charcoal adsorption and hydroxylapatite for intracellular receptors and of centrifugation and filtration for membrane receptors. We discuss the use of the Scatchard analysis to establish the steroid–receptor affinity and the quantity of steroid–receptor binding sites. Both pre- and post-labeled sucrose density gradient methods are outlined. One section is devoted to the direct and indirect methods used in nuclear “exchange” assays. Basic theory underlying each assay is presented, but, more importantly, we assess the advantages and disadvantages of each procedure. On the basis of this information, one may decide which assay is best suited for a particular laboratory and (or) specimen.

Introduction

During the last decade, new information has been obtained with regard to understanding how hormones manifest their physiological responses in target cells. In numerous endocrine systems it has been shown that, before a hormone elicits a biological response, it interacts with a specific receptor. These receptors are protein in nature, have a high affinity and specificity for their particular hormone, and are present only in cells that are the target cells for that hormone. These receptors are of two discrete types: intracellular and located in the cytoplasm and nucleus, or extracellular and present on the plasma membrane. The current understanding is that steroid hormones interact primarily with intracellular receptors and that peptide hormones and catecholamines interact with membrane-bound receptors. The intracellular receptor, after combining with a hormone, becomes localized in the cell nucleus as a hormone–receptor complex (1). This nuclear accumulation of hormone–receptor complex results in hormone-specific synthesis of mRNA, which in turn codes for the synthesis of new protein. The best-documented function of the membrane receptor–hormone complexes is that they act through a second messenger by increasing cyclic AMP concentations within the cell (2), an increase that leads to the activation of protein kinases that catalyze the phosphorylation of proteins.

The discovery that the function of hormones is regulated through their receptors has provoked interest in the measurement of receptors in various normal and pathological tissues, especially in breast-tumor specimens. The rationale behind the quantitation of breast-tumor receptors is that if tumor cells are responding to stimulation by circulating estrogen, then estrogen receptors should be present in these cells; if they are not present, the cells would not likely be stimulated by estrogen. Thus, the presence or absence of estrogen receptors enables the clinician to decide whether or not endocrine-ablation therapy is likely to cause the tumor to regress. Similarly, information as to the presence of progesterone receptors may prove to be of value in the course of treatment for endometrial carcinoma. It has been shown that endometrial carcinoma regresses in certain cases after administration of progesterin (3). In those patients who failed to respond, a further proportion responded when estradiol was administered before the progesterin (3). It follows that progestin treatment is effective through interaction with progesterone receptors, because a well-known result of estrogen stimulation is the synthesis of progesterone receptors. The presence of progesterone receptors has also been used as a “marker” for (i.e., an indicator of) estrogen action (4). If a cell contains both estrogen and progesterone receptors, one can conclude that the estrogen receptors are acting normally at the nuclear level (4).

The clinical utility of membrane receptor assays has not yet approached that of the intracellular receptor assays. However, studies of insulin receptors on circulating monocytes suggest that insulin resistance in six patients with acanthosis nigricans was attributable to a marked decrease in insulin binding to its membrane receptors (5). Two types of receptor defect were found: one was ascribable to a reduced number of insulin receptors, the other was manifested as a lower affinity of the receptor for insulin (5). These studies were clinically useful because, in man, circulating monocytes possess membrane receptor for insulin (5). These studies were clinically useful in that tissue biopsies were not required because, in man, circulating monocytes possess membrane receptors for insulin that are indistinguishable from those in liver and fat (6, 7), especially with respect to the utility of membrane receptor assays. It is not the purpose of this review to provide the clinical chemist with a catalog of detailed methods, but rather with a critical overview of the types of methods currently used, to help him understand the methodology involved and the drawbacks of particular methods. From such an understanding, the clinical chemist will be better able to evaluate the literature critically and to select and further develop the most practical method for use in a particular laboratory setting. Currently used methodologies are referenced from the

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original research literature and, for the sake of completeness, references 8 and 8a include more comprehensive texts on hormone receptors.

**Nature of Hormone–Receptor Interactions**

*Equilibria and affinity.* The interaction of a hormone with its receptor may simply be represented as an equilibrium between receptor (R), hormone (H), and the hormone–receptor complex (RH).

\[ R + H \xrightarrow{k_d} RH \]

The rate of complex formation is expressed by the association constant \(k_a\). The complex dissociates into its composite parts at a rate determined by the backward velocity constant \(k_d\). At equilibrium, the rates of both the reactions are constant and the system is described from the law of mass action:

\[ K_d = \frac{k_d}{k_a} = \frac{[R][H]}{[RH]} \]

The equilibrium dissociation constant, \(K_d\), is determined in one of two ways. In the first, one measures the velocity constants \(k_a\) and \(k_d\). The second—and more common—method is to perform binding analyses at equilibrium in which various concentrations of radioactive hormone are selected so that a saturation curve may be constructed (see below, in Scatchard Analysis). The numerical value of the \(K_d\), usually expressed in moles per liter, is important, because it is used as a measure of the affinity of a hormone for its particular receptor. Typically, high-affinity receptor–hormone interactions have \(K_d\)'s of \(10^{-10}\) to \(10^{-9}\) mol/L, whereas the low affinity interactions of hormones with serum binding proteins have \(K_d\)'s of \(10^{-5}\) mol/L. The \(K_d\) is also equal to the concentration of hormone required to half-saturate the available hormone receptor binding sites.

**Specificity.** The interaction of a hormone with its receptor is highly specific. For example, \([3H]\)estradiol can only be displaced from its receptor by radio-inert estrogens, such as estradiol and diethylstilbestrol, which precisely fit the binding sites, and not by radio-inert androgens, progestins, glucocorticoids, or mineralcorticoids. Similarly, receptors for lutropin found on corpora lutea membranes will not bind follicotropin, somatotropin, or prolactin.

**Isolation of Receptors**

*Specimen collection and preparation.* Hormone receptors are extremely heat-labile proteins; as soon as possible after it is excised, a tissue must be chilled and kept at 4°C. Freshly excised tissue should be rinsed in isotonic saline (NaCl, 9 g/L) to remove blood, after the tissue is finely diced but before being homogenized. Because heat is the most important factor to be controlled, the specimen should be homogenized at 4°C in four to 10 volumes of buffer with a Brinkmann Polytron or Tekmar Tissuemizer, with use of 5-s bursts, for the shortest possible time required to homogenize at least 80% of the tissue. Choice of a buffer system to isolate receptors depends on which cellular fraction is to be examined.

If there is to be a delay of more than a few hours before the receptors are isolated, the specimen should be frozen, preferably in liquid nitrogen. For subsequent isolation of receptors, specimens so stored are generally pulverized to a fine powder, which is then suspended in buffer.

Ideally, all tissue preparations and processing for receptor assays (outlined in Figure 1) should be conducted in a cold room (4°C). If a cold room is not available, ice baths must be used.

When a tumor specimen is to be evaluated for receptor content, one further precaution is advised. Before being pro-

![Diagram](https://via.placeholder.com/150)

**Fig. 1. Protocol for isolation of cellular fractions**

cessed and (or) frozen, the specimen should be examined by a pathologist, to ensure that a representative sample of the tumor has been obtained. This minimizes inaccuracies that result if tumor and non-tumor tissues are together in the same specimen.

**Cytosol.** A tris(hydroxymethyl)methylamine · HCl buffer (10 mmol/L, pH 7.4 at 20°C) is most commonly used to measure receptors in cytosol; it also contains disodium ethylenediaminetetraacetate, 1.5 mmol/L, and diethiothreitol; 1 mmol/L. Glycerol (100 mL/L) and thioglycerol (12 mmol/L) are also commonly added to the buffer. Glycerol helps stabilize the receptors and reduces the rate of dissociation of hormone during subsequent binding assays. The thiol helps stabilize the receptors by protecting sulfhydryl groups, which appear to be essential for hormone binding. After the tissue is homogenized in such a buffer, cell nuclei are removed by centrifuging at 800 × g for 10 min, and cytosol is obtained as a clear liquid by centrifuging the supernate at 105 000 × g for 1 h.

**Nuclei.** Crude nuclear pellets, obtained from the 800 × g fraction (Figure 1), are most commonly used in measuring nuclear receptors (see Nuclear Exchange Assays for details). Ideally, however, results obtained by their use should be confirmed by using preparations of “pure” intact nuclei. These may be isolated by one of several methods (9–12), depending upon the nature of the tissue. Basically, in these methods the tissue is homogenized and the nuclei are isolated in the presence of hexylene glycol (9) or sucrose (0.25 to 0.5 mol/L) and salts (potassium chloride, 25 mmol/L; magnesium chloride, 5 mmol/L), which help stabilize the nuclear membrane.

**Plasma membrane.** It is not necessary to purify plasma membranes before measuring their hormone-specific binding sites. The membrane fraction may be isolated by the following method, based on that described by Shiu and Friesen (13). First, the tissue is homogenized in five volumes of buffer [tris(hydroxymethyl)methylamine, 10 mmol/L; calcium chloride, 10 mmol/L; and sucrose, 0.3 mol/L; pH 7.5 at 4°C]. Then the homogenate is centrifuged at 13 000 × g for 10 min to remove cellular debris, including nuclei and mitochondria. The resulting supernatant fluid is centrifuged at 105 000 × g for 90 min, to give a pellet that is essentially a crude membrane preparation.

**Quantitation of Soluble Receptors for Steroids**

**Separation of Receptor–Hormone Complex from Unbound Hormone**

There are many procedures for distinguishing “free” radioactive steroid from that bound by the receptor (14–25), but only two procedures—charcoal adsorption and hydroxyapatite—are discussed here. We selected these procedures
because they can be completed rapidly, have general utility, and may be used for receptor quantitation in cytosol or to quantitate solubilized nuclear receptors. Although sucrose density-gradient centrifugation may be interpreted as a method for separating bound steroid from free, the technique has a more specialized use and is therefore discussed separately below.

Charcoal adsorption. First described by Koremman et al. (26), this procedure is the method of choice for measuring receptors in crude cytosol. Its advantages include speed and ease of application to large numbers of specimens. One disadvantage, however, is that, according to Peck and Clark (27), as high salt concentrations (>100 mmol of KCl per liter) the charcoal tends to strip [3H]estrodiol from cytoplasmic estrogen receptors.

Acid- and alkali-washed charcoal, exclusive of "fines," is used in the charcoal adsorption assay. Charcoal (1.5 g) and Dextran-80 (15 mg) are dissolved in the buffer of choice (300 mL), stirred for 6 h at room temperature, and then, before use, allowed to stand at 4 °C overnight. After incubating the [3H]steroid with cytosol, an equal volume of charcoal suspension is added to each assay tube, vortex-mixed, and, after 5 min, centrifuged at 2000 x g for 5 min. The supernate is decanted and assayed for [3H] incorporation. Incubation with charcoal for >5 min is discouraged because use of extended periods of incubation tends to strip steroid from the receptor sites and results in an underestimation of the number of receptors.

Hydroxylapatite. One advantage to this method is that it can be used in the presence of high salt concentrations (>100 mmol of KCl per liter). The principle of its reaction is the opposite of that of charcoal adsorption. In the hydroxylapatite procedure, it is the steroid--receptor complex, rather than the free steroid, that is adsorbed to the solid matrix. However, it is the more time consuming of the two separation methods, because the hydroxylapatite must be washed free of any unbound [3H]labeled steroid before the hydroxylapatite with the [3H]steroid--receptor complex can be extracted and its radioactivity counted. The following procedure is based on that described by Erdos et al. (28).

Hydroxylapatite (HAP) is washed extensively at 4 °C with Tris-phosphate buffer [Tris(hydroxymethyl)methylamine, 50 mmol/L; potassium phosphate monobasic, 5 mmol/L; pH 7.4 at 4 °C] until the wash buffer reaches a pH of 7.4. The HAP is resuspended in buffer to make a 600 g/L suspension and stored at 4 °C. Portions of the [3H]steroid--receptor preparation (0.5 mL) are mixed with the HAP suspension (0.5 mL) and incubated at 4 °C for 15 min. After incubation, the mixture is centrifuged (2000 x g, 5 min), and the supernatant fluid is discarded. The receptor remains adsorbed to the HAP. The pelleted HAP is resuspended in 1 mL of ice-cold Tris-phosphate buffer, centrifuged at 2000 x g for 10 min, and the supernate discarded. This is repeated four times. After the final wash, the pelleted steroid--receptor complex is extracted with 1.5 mL of absolute ethanol at room temperature. This extract is added to 10 mL of scintillation fluid, its radioactivity is measured, and the specific binding is determined (29).

Adaptations of the hydroxylapatite method were used by Garola and McGuire, who measured estrogen receptors in less than 50 mg of breast-cancer tissue (30). This method is superior to charcoal adsorption when the protein concentration of the receptor solution is <1 g/L.

Scatchard Analysis

In a clinical laboratory it is not advisable routinely to quantitate receptors by using a single dose of radioactive hormone. It is preferable to measure binding at several concentrations of hormone. By using various concentrations of radioactive ligand, one can establish the affinity of the hormone--receptor interaction and quantitate the number of specific steroid receptor binding sites. To determine these, one incubates the cytosol in the presence of various concentrations of [3H]-labeled steroid (0.1 to 10 nmoL/L) for a time sufficient to establish equilibrium of binding (4 to 18 h at 4°C). The exact concentration depends upon the particular ligand--receptor being studied. The amount of [3H]steroid bound to the receptor at each [3H]steroid concentration is determined by either the charcoal adsorption or hydroxylapatite assay. Because steroids bind to other proteins in cytosol in a non-specific or non-saturable manner, one must quantitate the nonspecific binding and subtract it from total binding to measure specific binding. To do this, it is customary to carry out duplicate binding assays in the presence of a 100-fold excess of nonlabeled steroid, which displaces the radioactive steroid that is bound to nonspecific sites. Diethylstilbestrol is used to correct for nonspecific binding when measuring estrogen receptors, because it is more specific for estrogen receptor sites than is estradiol. Nonradiolabeled estradiol is unsatisfactory for this purpose because it will bind to contaminating serum proteins such as sex steroid binding globulin, in addition to receptors.

For measurement of progesterone receptors, nonradiolabeled progesterone is added to correct for nonspecific binding. At the same time, cortisol (0.1 μmol/L) is added to the cytosol, to inhibit the binding of [3H]progesterone to any contaminating transcortin. Alternatively, the synthetic progestin, 17,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione (R5020), may be used because it does not bind to transcortin (31). Although its use has gained universal acceptance, certain caution should be exercised because it has been shown that R5020 can also interact with glucocorticoid receptors (32).

Results of the binding studies are generally analyzed by using Scatchard plots to compute both the receptor's affinity and the quantity of receptor sites.

The Scatchard plot (33) is a mathematical transformation derived from the law of mass action:

\[
\frac{B}{F} = \frac{1}{K_d} (B - n)
\]

where, at steady state, B is the amount of steroid bound to specific sites, F is the concentration of free steroid, K_d is the equilibrium dissociation constant, and n is the number of binding sites contributing to the equilibrium.

When a Scatchard analysis is performed, three assumptions are made: that unbound steroid is equal to free steroid, that the number of binding sites remains constant throughout the incubation, and that the reaction is fully reversible. These assumptions should be tested to validate a particular assay; if they are not justified, the Scatchard plot will not yield meaningful values.

In practice, Scatchard analyses are set up by measuring binding at equilibrium over the concentration range of [3H]steroid from 0.1 to 10 nmoL/L. However, it is not always possible to carry out the binding studies over such a wide concentration range because of limitations in the amount of tissue available, particularly when breast or endometrial cancer specimens are being analyzed. In these situations, a narrower concentration range should be used: for estrogen receptors, 0.1 to 2 nmoL of [3H]estradiol per liter; for progesterone receptors, 0.5 to 10 nmoL of [3H]progesterone per liter should be used. If these concentration ranges are used, a straight line with a negative slope is obtained when the ratio bound/free is plotted vs bound. A single straight line is indicative of a single class of binding sites. The reciprocal of the slope is equal to the receptor's affinity or K_d (~0.1 nmoL/L for estrogen receptors; ~3 nmoL/L for progesterone receptors). Extrapolation of the line to the abscissa gives one a measure of the concen-
Table 1. Scatchard Analysis on Equilibrium Binding of \[^{3}H\] Estradiol (0.1 to 4 nmol/L)

<table>
<thead>
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<th>Total added</th>
<th>Total bound</th>
<th>Non-specific bound</th>
<th>Specific bound</th>
<th>Free</th>
<th>S/F</th>
<th>S = (10^{-11}) mol/L</th>
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<td>10630</td>
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<td>.05</td>
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Sucrose Density-Gradient Analysis

Sucrose density-gradient analysis is used to quantitate steroid–receptor binding, usually at a saturating concentration of steroid (1 nmol of \[^{3}H\]estradiol per liter and 10 nmol of \[^{3}H\]progesterone per liter). Because these are single-point assays, they give no information regarding the affinity of the measured binding. However, information is obtained regarding the sedimentation coefficient of the receptor (4S, 6S, 8S, or a combination of these), an important biophysical property. A simple explanation of the derivation of the S value is given below.

Molecules or particles spinning around an axis are subjected to a centrifugal force. Under the influence of such a force, the particles will sediment at a certain velocity. The velocity of sedimentation per unit force of field may be expressed as a sedimentation coefficient, \(s\):

\[ s = \frac{\phi (\rho_p - \rho_m)}{f} \]

where \(\phi\) is the volume of the particle (cm\(^3\)), \(\rho_p\) is the density of the particle (g/cm\(^3\)), \(\rho_m\) is the density of the medium (g/cm\(^3\)), and \(f\) is the frictional coefficient (g/s).

The units of \(s\) are “seconda,” and most typical biologically important molecules possess sedimentation coefficients \(> 10^{-13}\) s. The quantity \(10^{-13}\) s has been defined as a Svedberg unit (S), in honor of Svedberg, the originator of this type of analysis. The frictional coefficient of a molecule (\(f\)) depends on its shape, size, and the viscosity of the medium through which it is sedimenting; thus the rate of sedimentation is a function of these three properties.

In a preparative ultracentrifuge the migration of a molecule through a homogenous solution may be disturbed by thermal gradients and mechanical vibration. Such disturbances can be greatly alleviated by forming a gradient of a diffusing substance in the centrifuge tube. Sucrose is the most commonly used material for doing so in the analysis of receptors, and the concentration gradient most generally used is 50 to 200 g/L sucrose, as first described by Toft and Gorski (35).

After the gradient is prepared at room temperature, the tubes are equilibrated at \(4^\circ C\) before applying the sample to the gradient. On centrifugation, the receptor moves through the gradient at a rate determined by its sedimentation coefficient. It is important that centrifugation be stopped before the most rapidly sedimenting species reaches the bottom of the tube. A typical centrifugation time, for a 0.2-mL sample applied to a 5-mL sucrose gradient (50 to 200 g/L) in a conventional swinging bucket rotor at 250 000 \(\times g\), is 18 h. The S values of the sedimenting species are obtained by comparison with those of known markers (3.7S ovalbumin, 6.3S bacterial alkaline phosphatase), which are labeled with \(^{14}C\) by the procedure described by Rice and Means (36) and added as internal standards. An ultracentrifuge with either a swinging-bucket rotor or a vertical rotor is essential for sucrose-gradient analysis. If many samples are to be processed, a gradient maker is a useful piece of equipment to have available.

Prelabeled gradients. In this procedure, the receptor preparation is incubated in the presence of \[^{3}H\]steroid (1 nmol/L) for 1 to 2 h before 0.2 mL of it is added to the gradient. Gradients are centrifuged (18 h, 250 000 \(\times g\)) and fractionated by one of two methods. The first—and least troublesome—involves puncturing the bottom of the centrifuge tube with a needle, allowing the contents to drip out from the bottom. In the second method, fractions are displaced from the top of the gradient by inserting a small-diameter tube through the gradient to the bottom of the tube and gently pumping a solution of very high density through it. Both methods permit the contents of the centrifuge tube to be separated equally into about 25 fractions. The radioactivity present in each fraction is quantitated and customarily is plotted as dpm vs fraction number, with fraction number 1 representing the top of the gradient. A duplicate gradient containing a 100-fold excess of nonradio-labeled steroid is also run. This allows for correction of any binding of the \[^{3}H\] steroid to nonspecific sites. (Figure 2 represents a typical gradient
obtained by using this procedure.) A disadvantage of pre-labeling the receptor preparation before centrifuging is that dissociation of the steroid from the receptor binding site can occur during the 18-h centrifugation.

Postlabeled gradients. These receptor preparations are layered onto a sucrose gradient and centrifuged without any prior labeling of the receptor with \[^3H\]steroid. The gradient is first fractionated as described above, and then each fraction is incubated with \[^3H\]steroid for 2 to 4 h at 4 °C. Radioactive steroid bound to each fraction is determined by either the charcoal adsorption or hydroxylapatite assay. Again, a duplicate series of fractions containing 100-fold excess of radio-inert steroid is required to assay for nonspecific binding (see Scatchard Analysis to measure nonspecific binding). An advantage of this method is that receptors are not underestimated because \[^3H\]steroid does not dissociate from receptor during centrifugation. There are, however, two major disadvantages. Firstly, the procedure is more time consuming to perform than pre-labeled gradients. Secondly, because steroid receptors are more labile in the absence of steroid, they tend to degrade during the 18-h centrifugation. The results may represent an underestimation of receptor binding sites.

Disadvantages of both the pre- and the post-labeled sucrose-gradient methods are lessened by using the recently developed "vertical rotors" (Beckman Instruments and Sorvall, Du Pont Instruments) because the duration of centrifugation is shortened from 18 h to 4 h.

Nuclear Exchange Assays

Target cells respond biologically to a steroid hormone by synthesizing specific proteins. This synthesis is initiated by the interaction and coupling of the steroid with its soluble receptor, which then binds to specific sites on chromatin or nuclei (1, 37). One would expect higher proportions of steroid-receptor complexes to locate on the chromatin of mature animals than immature ones, because the concentrations of steroid hormones that interact with receptors are greater in mature animals. In the clinical situation, specimens for receptor quantitations are generally obtained from adults. In those individuals, the relative quantities of soluble receptors (cytoplasmic) and insolubilized receptors (nuclear) vary according to the hormonal milieu of tissue at the time of excision. For example, in a cycling female, one would predict that cytoplasmic estrogen receptors will be lowest and nuclear receptors highest immediately after the estrogen surge before ovulation. The opposite would also be true: that is, tissue from a postmenopausal woman has more cytoplasmic receptors because the amount of estrogen produced by the ovaries is insufficient to translocate all the receptors to chromatin acceptor sites. Both predictions have been confirmed from studies of human endometrium (38). It is important therefore to consider the hormonal state of an individual and if possible to measure both cytoplasmic and nuclear receptors in the same sample.

Nuclear receptors are quantitated by using the "exchange" technique, which has as its basis the exchange of \[^3H\]steroid for non-labeled steroid bound to the nuclear receptor. Because this is a temperature-dependent phenomenon, the optimum time, temperature, and concentrations of radioactive steroid must be established for each biological system studied before the measurement of specifically bound \[^3H\]steroid can be used to quantitate the number of steroid binding sites in the nuclear fraction. Two techniques, one direct and the other indirect, are used to quantitate nuclear receptors. Anderson et al. (39) pioneered the direct method, with the rat uterus as a model, and exchanged \[^3H\]estradiol for non-labeled estrogen bound to nuclear receptors in crude nuclear pellets obtained from homogenized tissue. The indirect method requires solubilization of the nuclear receptors in buffers with a high salt concentration (KCl, 0.3 to 0.6 mol/L) before the endogenous steroid is exchanged with \[^3H\]steroid. Compared with assays for cytoplasmic receptors, nuclear receptor assays present major problems. The nuclear receptor, especially in tumor tissue, is unstable (40) and because the temperature-dependent ease of exchange varies for each species and tissue type, it is impossible to generalize one method that should be used. Assays presented here are applicable to human tissue.

Direct method. Both estrogen and progesterone receptors have been quantitated in crude nuclei from human endometrium by use of the direct method of nuclear exchange (38). Assays are carried out at 4 °C within 1 h after curettage of tissue. When assay must be delayed, the tissue is frozen at −20 °C in a preservation medium: Minimal Essential Medium with Earle's salts, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 25 mmol/L, without L-glutamine (Gibco); di-methyl sulfoxide, 10 g/L, and charcoal-stripped calf serum, 200 mL/L (38). Crude nuclei are obtained as a pellet after the tissue is homogenized in an isotonic sucrose solution with a Teflon–glass homogenizer (five 15-s bursts at 1000 rpm) and centrifuged at 800 × g for 10 min. The optimum time and temperature required for incubation of \[^3H\]steroid with nuclei to measure nuclear estrogen receptors is 3 h at 30 °C. For progesterone receptors, the optimum conditions are 3 h at 4 °C. Complete details of the methodology are given in reference 38. In our hands this procedure has not proven to be applicable to frozen myometrial or breast-tumor specimens.

Indirect method. Rather than measure the receptors in a crude nuclear fraction as in the direct method, in the indirect method receptors are extracted from the nuclear fraction by use of a salt-containing buffer (KCl, 0.6 mol/L). Once extracted, receptor preparations are centrifuged at 105 000 × g and the supernatant fluid is treated with hydroxylapatite. Hydroxylapatite adsorbs and protects receptors from protease activity that causes receptor degradation (40). After hydroxylapatite-bound receptors are pelleted by centrifugation (2000 × g), they are resuspended in buffer, incubated with \[^3H\]steroid and used in subsequent exchange assays. Generally, the pellet is incubated with 5 nmol of \[^3H\]steroid per liter at 30 °C for 3 h. The pellet is washed in buffer and centrifuged to remove \[^3H\]estradiol not bound to the receptor. Bound hormone is then extracted with ethanol at room temperature and the radioactivity counted with a scintillation counter. Correction for nonspecific binding of \[^3H\]estradiol has been discussed (see Scatchard Analysis). The hydroxylapatite exchange assay has recently been proven to be superior to the alternative protamine sulfate procedure (41), and has been used extensively for determination of estrogen receptors in breast-tumor specimens.

Membrane Receptors

Membrane receptors are about 10 times less concentrated than the cytoplasmic steroid hormone receptors discussed previously. For this reason, the radioactive hormones used to measure membrane receptors must have very high specific activities (~1000 kCi/mol). Such high specific activities are achieved by introducing \(^{125}\text{I}\) into a molecule. It should be noted that even though an \(^{125}\text{I}\)-labeled hormone often maintains its immunoreactive property and is used in radioimmunoassays, both its binding properties for membrane receptors and its biological activity may be lost during the introduction of \(^{125}\text{I}\) into a molecule. In general, the iodination of hormones to be used in receptor assays must be more closely controlled than iodination of hormones for radioimmunoassay. These losses of biological activity have two causes: introduction of large atoms of iodine into the hormone, and oxidation of methionine residues, tryptophan residues, and rupture of the peptide bond vicinal to tryptophan or tyrosine residues (42, 43).
Oxidation occurs during the iodination procedure, where oxidizing agents are necessarily added to convert I\(^{-}\) to I\(_2\) with Na\(^{127}\) as the iodine source. The two most commonly used oxidizing procedures involve Chloramine T (44), which decomposes in water with formation of active chlorine, or hydrogen peroxide in combination with the enzyme lactoperoxidase (EC 1.11.1.7) (45). Precise conditions for iodination of hormones vary according to the nature of the hormone and the quality of the reagents. The reader should be aware that well-documented procedures may require modification in one’s own laboratory, especially when the lactoperoxidase procedure is used.

Binding assays for studying characteristics of hormone receptor binding, such as binding specificity and affinity, require a rapid method for the separation of bound from free hormone. Separation methods (adsorption with charcoal or hydroxyapatite) discussed previously for cytoplasmic and nuclear receptors are not applicable to membrane receptors. The two most commonly used methods for quantitating membrane receptors are based on separation by either centrifugation or filtration. Either of these methods separates bound hormone from free, and then by Scatchard analysis one can determine the affinity of binding and the concentration of binding sites. This procedure is similar to that described for cytoplasmic steroid hormone receptors. Again, nonspecific binding is quantitated by performing duplicate assays in the presence of excess nonlabeled hormone.

**Centrifugation.** After equilibration of binding is completed, membrane fractions are pelleted by centrifugation, leaving unbound hormone in the discarded supernate. At 4 °C, the pellet is washed and re-pelleted to remove any loosely adhering free hormone. The washing procedure at 4 °C is repeated and the radioactivity in the hormone bound to the membrane pellet is finally counted in a gamma counter. To minimize losses of membrane particles during the washing procedure, and to eliminate nonspecific binding to glass assay tubes, it is preferable to use a Beckman Microfuge with conical polyethylene tubes.

After the membrane fraction has been pelleted and washed, the tip of the conical centrifuge tube containing the pellet is cut off and the radioactivity counted. An example of this particular method is described by Rodbell et al. (46) for the measurement of binding of labeled glucagon to liver plasma membranes. Advantages of this method are its ease, speed, and reproducibility. Its disadvantages are that it does not allow binding to be determined for times less than 5 min, high blanks are often a problem because of nonspecific trapping of hormone at high hormone concentrations, and if dissociation occurs during centrifugation and/or washing, the number of binding sites will be underestimated.

**Filtration.** Filtration is the most widely used method for separating free hormone from hormone bound to membranes. This procedure has been used to measure insulin binding to liver membranes (47, 48), prolactin binding to membranes (49, 50), angiotensin binding to adrenal cortex membranes (51), and the beta-adrenergic receptors in responsive cells (52, 53).

This method is extremely simple. At the end of the particular incubation time for binding, the incubation mixture is cooled to 4 °C and poured over a membrane filter (Millipore, Gelman, Sartorius cellulose, or Whatman glass fiber) to which suction is applied. Filtration generally takes about 10 s, and the filter is then washed with an appropriate ice-cold buffer. The filter is removed, dried, and the trapped radioactivity bound to the membranes is determined. A control filter should be added to determine the amount of hormone that nonspecifically binds to the filter in the absence of membranes. Such nonspecific binding can usually be minimized by an appropriate choice of filter.

The advantage of this technique is that bound and free hormone are more rapidly separated than in the centrifugation procedure. The disadvantages are that the filter membranes are expensive and that the technique suffers from the same problem as the centrifugation technique; at high concentrations of hormone there is nonspecific trapping of hormone.

**Caveats**

Two proteins that have high affinities for estradiol have been found in cytosol from human uterine tissue (29, 54). These putative receptors differ in their affinity for estrogen by an order of magnitude (10\(^{-10}\) mol/L and 10\(^{-8}\) mol/L). It is thought that the high-affinity component is the classical receptor, but the function of the lower-affinity component is not known. To distinguish between these components it is necessary to perform a Scatchard analysis. To date, the particular affinity of the receptor found in tumor specimens with regard to subsequent endocrine responsiveness has not been critically evaluated. Until such an evaluation has been made, it is preferable to carry out a Scatchard analysis together with sucrose density-gradient analysis whenever the amount of specimen permits. Single-point assays for the concentration of receptors at an assumed saturating concentration of hormone are not reliable unless at least sucrose gradient analysis is performed to give some information regarding the nature of the binding protein (S value). Charcoal absorption assays should not be performed at protein concentrations of <1 mg/mL, because at low protein concentrations the concentration of receptor sites is underestimated. The preferred method for use with protein concentrations of 0.2 to 1 mg/mL is the hydroxylapatite assay (30).

If present, high concentrations of endogenous hormones bound to receptors can lead to underestimation of receptor content. This is caused by dilution of the specific activity of the radioactive hormone used in the assay, and the relatively slow rate of exchange of endogenous hormone for radioactive hormone from occupied receptor sites. These problems are avoided in nuclear exchange assays by using excess [%H]steroids and by assaysing at elevated temperatures (30 °C) so that complete exchange occurs. High concentrations of receptor sites occupied with endogenous hormone are not common when cytosol preparations are used. However, if an individual is receiving pharmacological doses of hormones, one should consider the possibility of cytoplasmic receptor sites being occupied, and, after removing excess free hormone by charcoal adsorption, carry out the assay under exchange conditions.

In the clinical situation, it should be remembered that the surgeon and pathologist must be made aware of the lability of receptors if representative fresh specimens are to be obtained. If a particular specimen is found to be receptor negative, one must be certain that the representative specimen was not allowed to remain at room temperature after it was excised. Only if attention is paid to detail can the results of the assay be used in making a decision regarding subsequent therapy.

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