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Studies on the Uterine, Cytoplasmic Estrogen Binding Protein. Thermal Stability and Ligand Dissociation Rate. An Assay of Empty and Filled Sites by Exchange†

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ABSTRACT: The thermal stability and the rate of estradiol dissociation from the cytoplasmic estrogen binding protein of rat uterus have been determined at 0, 25, 30, and 37°. Unfilled sites are very labile, but estrogen-filled sites do not undergo detectable degradation over a 24-hr period at temperatures up to 30°. At 37°, however, 50% of the sites are lost over an 8-hr period, while the remainder are stable for up to 24 hr. Dissociation of bound estradiol, as determined by exchange, is complete within 24 hr at 25° and within 8 hr at 30°. Comparison of freshly prepared and exchanged cytosol (24 hr, 25°) on high and low salt–sucrose density gradients shows that considerable aggregation of the binding activity occurs during the exchange period. A procedure for assaying soluble, cytoplasmic estrogen binding sites by exchange has been designed, using the optimum conditions for rapid exchange without degradation of binding capacity. The assay is convenient, quantitative, and linear up to 3 uterine equivalents/ml. Using this assay procedure, the concentration of estrogen binding sites can be determined, regardless of whether they are empty at the time of assay or filled with estradiol, a nonsteroidal estrogen (hexestrol), or an antiestrogen (dimethylstilbestrol, U 11,100A, or CI-628). By following the time course of exchange, the dissociation rate of an unlabeled ligand can be determined.

Numerous studies have demonstrated the presence of a binding protein with high affinity for estrogens in uterine tissue (Jensen and DeSombre, 1972). The binding of estradiol to this protein in the cytoplasm, and the subsequent translocation of the complex to the nucleus, are thought to be steps of fundamental importance in the action of estradiol on the uterus (Jensen and DeSombre, 1972).

Most of the methods for determining the concentration of these high affinity estrogen binding proteins involve a direct assay: addition of a saturating concentration of [^{3}H]estradiol is followed by separation of bound from free ligand; correction is usually made for binding due to sites of low affinity (nonspecific binding). However, this direct assay method, normally run at 0°, does not determine the total concentration of high-affinity binding protein, but only those sites that are unoccupied at the time of analysis; the rate of estradiol dissociation from the binding protein is so slow that only minimal exchange occurs during the time of assay. This limitation of the direct assay method is most serious in those situations in which a large fraction of the binding sites is filled with unlabeled ligand. In these cases, an assay which allows such sites to exchange with labeled estradiol would give a more accurate determination of the total concentration of high-affinity binding sites. Anderson *et al.* (1972) have

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recently described an exchange assay for determining estrogen-filled nuclear binding sites.

In this report we describe studies on the thermal stability of the cytoplasmic estrogen binding protein from immature rat uterus and the rate at which various estrogenic or antiestrogenic ligands dissociate from the binding sites. Using this information, we have established optimal conditions for an exchange assay that allows the determination of the total concentration of binding sites, regardless of what fraction of the sites are filled at the time of analysis. We have used the rate of exchange to measure the rates of dissociation of unlabeled ligands from the binding protein and have investigated the state of aggregation of the binding protein after it has undergone complete exchange.

Experimental Section

Materials. The following compounds were obtained from the sources indicated: estradiol[1] (Searle; Steraloids); [6,7-^{3}H]-estradiol (46.5 Ci/mmol; New England Nuclear); *meso*-hexestrol (Mann); dimethylstilbestrol (Gallard Schlesinger); antiestrogens CI-628 (CI; Parke-Davis) and nafoxidine U-11,-100A (UA; Upjohn); charcoal, Norit A (Sigma); dextran, grade C (Schwarz-Mann); Biosolv BBS-3 solubilizer (Beckman); dimethylformamide (Baker); bovine serum albumin, crystallized and lyophilized (Sigma); ovalbumin, twice recrystallized (Worthington); bovine γ-globulin, crystallized and lyophilized, fraction II (Nutritional Biochemicals); ethylenediaminetetraacetic acid (EDTA, Eastman); trishydroxymethylaminomethane (Tris, Nutritional Biochemicals).

Methods. The two buffers used in these studies were Tris–EDTA buffer (TE) (0.01 M Tris–0.0015 M EDTA–0.02% sodium azide (pH 7.4) at 25°) and Tris–KCl buffer (TK) (0.4 M KCl–0.01 M Tris–0.02% sodium azide (pH 7.4) at 25°). TE buffer was used exclusively, except in the experiments described in Figure 5. The charcoal-dextran slurry consisted of 5% acid-washed Norit A and 0.5% dextran C in 0.01 M Tris (pH 7.4) at 25° containing 0.02% sodium azide.

Stock solutions of [^{3}H]estradiol (9×10^{-8} M) were prepared in TE buffer. The storage solvent supplied by New England Nuclear (9:1 benzene–ethanol) was evaporated under a stream of nitrogen and replaced with an equal volume of 100% ethanol before addition of the buffer. The final concentration was checked by scintillation counting. [^{3}H]Estradiol solutions containing a 100-fold excess of unlabeled estradiol (for determination of nonspecific binding, see section below) were prepared directly from these stock solutions by addition of the appropriate small volume of a 10^{-3} M estradiol solution in ethanol.

Because of their generally low water solubility, the unlabeled competitors (hexestrol, dimethylstilbestrol, UA, and CI)[1] were first prepared as 10^{-3} M solutions in dimethylformamide. Since these compounds are bound with different affinities by the cytoplasmic binding protein, the dimethylformamide stock solutions were diluted with TE buffer to different final concentrations. If the concentration of estradiol used in an experiment was 30 nM, the competitor concentrations used were 30/RAC nM, where RAC is the ratio of the association constant of a particular competitor to that of estradiol ($RAC = K_a^{competitor}/K_a^{estradiol}$ (Korenman, 1970)). The RAC values determined in an independent competition assay are: hexestrol, 3.00; dimethylstilbestrol, 0.37; CI, 0.04; and UA, 0.06 (Katzenellenbogen and Katzenellenbogen, 1973; Katzenellenbogen *et al.*, 1973). The dimethylformamide concentration in the final exchange solutions was never greater than 0.1%.

When a large number of small scale incubations (50–100 μl; either exchange incubations or charcoal–dextran adsorptions, see sections below) were conducted simultaneously, it was convenient to use disposable blood microtiter plates (Cooke Engineering; Scientific Products), which are fabricated of polystyrene and have 96 wells with a 200-μl capacity. These plates can be vortexed effectively and can be covered with an adhesive film for sample storage and during centrifugation.

A toluene-based scintillation fluid containing 0.54% 2,5-diphenyloxazole, 0.0039% *p*-bis[2-(5-phenyloxazolyl)]benzene, and 10% Biosolv BBS-3 was used. Counting was done in minivials (Research Products) containing 5 ml of scintillation fluid in a Nuclear-Chicago Isocap 300 at efficiencies for tritium of 43–48% in single labeled and for tritium and ^{14}C of 47 and 53%, respectively, in double labeled samples.

Preparation of Cytosol. Cytosol was prepared from the uteri of immature (21–25-day old) Holtzmann rats. The rats were decapitated, and their uteri excised and stripped of surrounding fat. All succeeding procedures were carried out at 0–4°. The uteri were collected in TE buffer, rinsed with this buffer, and then homogenized with intermittent cooling in a motor-driven all-glass tissue grinder (Kontes, Duall) at 2–5 uteri/ml. Final adjustment to the desired concentration of uterine equivalents per milliliter was made after homogenization. The homogenate was centrifuged at 180,000*g* for 60 min. The resulting supernatant (cytosol) could be cleanly pipeted from beneath a thin film of lipid material.

Removal of Free Steroid by Charcoal–Dextran Adsorption. Estradiol that is not protein bound is rapidly adsorbed by a slurry of dextran-coated charcoal. Independent experiments have shown that adsorption of a 30 nM estradiol solution is essentially complete (>95% free removed) within 2 min at 0°, when a volume of the standard charcoal–dextran slurry equal to 10% the incubation volume (10% v/v) is used. When estrogen binding protein is present, identical values of specific binding capacity are obtained using adsorption times of 5–60 min at 0° and slurry volumes ranging from 5 to 30% v/v. No loss of binding protein by adsorption to the charcoal was noted under these conditions. Routinely, we have used a 10% v/v of the slurry, 15-min adsorption time (with intermittent vortexing) at 0°, followed by centrifugation at 1500*g* for 7 min.

Correction for Nonspecific Binding. In addition to the high-affinity binding ($K_a \simeq 10^{10}$ M^{-1}), two other components contribute to the estradiol binding capacity that is measured in rat uterine cytosol by charcoal–dextran adsorption: a fraction of low-affinity binding, that is not target-tissue specific, and a small per cent of free estradiol that is not adsorbed by the charcoal–dextran treatment. These two latter components are commonly termed nonspecific binding and in some cases they can account for a sizable fraction of the total binding capacity. However, because they display a linear, nonsaturating behavior up to ligand concentrations of 10^{-5} M or greater, correction for these components can easily be made. Binding capacity is measured in an incubation at a certain concentration of [^{3}H]estradiol ("hot" incubation or total binding; generally 30 nM in these studies) and simultaneously in a

[1] Common names (and abbreviations) used in this paper are: estradiol, 1,3,5(10)-estratriene-3,17β-diol; hexestrol, *meso*-3,4-bis(4′-hydroxyphenyl)hexane; dimethylstilbestrol, 2,3-bis(4′-hydroxyphenyl)-*trans*-2-butene; CI-628 (CI), α-[4-pyrrolidinoethoxy]phenyl-4-methoxy-α'-nitrostilbene; U 11,100A (UA), 1-(2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl)pyrrolidine hydrochloride; TE buffer, 0.01 M Tris–0.0015 M EDTA–0.02% sodium azide; TK buffer, 0.4 M KCl–0.01 M Tris–0.02% sodium azide.

parallel incubation in which a 100-fold excess of unlabeled estradiol has been added ("hot plus cold" incubation; 3000 nM; 1% the original specific activity). The difference in the concentration of bound ligand in the "hot" and the "hot plus cold" incubation is taken as a measure of the high affinity binding and is termed "specific" binding. In figures (Figures 1, 2, 4, and 6), where the ordinate is labeled "specifically bound [^{3}H]estradiol," data have been corrected for the nonspecific contribution before plotting. In Figures 3 and 7 both the total and nonspecific binding data are shown.

Exchange Assay. As described under Results, optimum conditions for achieving complete exchange of estrogen-filled sites involve incubation of cytosol at 25–30° for 18–24 hr in the presence of saturating concentrations of estradiol. The protocol given below describes how a sample of cytosol is to be treated in order to determine both the concentration of estrogen binding sites that are filled at the start of the assay and the total concentration of binding sites. All procedures through step 3 are conducted at 0–4°. (1) Cytosol (*ca.* 3 uterine equivalents/ml) is treated with charcoal–dextran (10% v/v; 15 min) to adsorb any free, unlabeled ligand that would interfere with the specific activity correction (see below). (2) After centrifugation (1500*g*; 7 min) the charcoal-stripped cytosol is divided into two portions which are saturated with 30 nM [^{3}H]estradiol ("hot" exchange) or 30 nM [^{3}H]estradiol plus 3000 nM estradiol ("hot plus cold" exchange). (3) The incubations are allowed to reach equilibrium (1 hr), and a portion is assayed by charcoal–dextran adsorption to determine the concentration of specific binding sites that were unoccupied at the start of the assay. (4) The incubations are warmed to 25–30°, and exchange is allowed to proceed for an 18–24-hr period. (5) After recooling to 0° for 30 min, the specific binding capacity is then assayed by charcoal–dextran adsorption. (6) Small corrections for dilution of specific activity are made (as described below). (7) The concentration of binding sites thus obtained is the total binding capacity; the concentration of sites initially filled with estrogen is the difference between this figure and the concentration determined in step 3.

Time Course of Exchange of Sites Filled with Cold Ligand. In those experiments designed to measure the time course of dissociation of an unlabeled ligand from the estrogen binding protein, the cytosol binding preparation was saturated prior to step 1 with either 30 nM estradiol or 30/RAC nM of another ligand (see Methods). Complete saturation was achieved within 30 min, but because the sites are very stable at 0°, this incubation time was adjusted between 1 and 16 hr, according to what was most convenient. In step 4, aliquots were removed periodically to monitor the time course of the exchange. Because dissociation is very slow at 0° (see Figure 2A), successive aliquots can be cooled to 0° and processed together on a single microtiter plate.

Stability Determinations. Since in all cases uteri are from immature (day 21–25) rats, which have low endogenous estradiol levels, the stability of all the sites can be determined in an independent set of incubations. Prior to step 1, two portions of cytosol were brought to 30 nM [^{3}H]estradiol ("hot" preincubation) and 30 nM [^{3}H]estradiol plus 3000 nM estradiol ("hot plus cold" preincubation), respectively, and incubated at 0°, parallel to the incubations with cold ligand described in the preceding section. In the subsequent assay steps 2 and 3, the portion that was preincubated "hot" is treated and exchanged only with "hot" estradiol, and the "hot plus cold" preincubation, only with "hot plus cold." During the exchange, aliquots from these incubations give a measure of the stability of the total specific binding sites throughout the exchange period.

Correction for Change in Specific Activity. Correction can be made for the change in specific activity that results from dilution of the added [^{3}H]estradiol by the unlabeled ligand that dissociates from filled sites during the course of the exchange. In the case where the dissociating ligand is estradiol, eq 5, derived in the Appendix, can be used; eq 6 should be used for ligands other than estradiol. When the exchange is conducted with 30 nM [^{3}H]estradiol, at a final cytosol concentration of 2 uterine equivalents/ml, the correction is always less than 10% (see Table I, for example).

Sucrose Density Gradient Analysis. Linear 5–20% sucrose density gradients (3.6 ml) were prepared in polyallomer tubes in either TE or TK buffer (Martin and Ames, 1961; Stancel *et al.*, 1973) and were kept at 0–4° for 3–6 hr before use. ^{14}C-Labeled marker proteins (bovine serum albumin, ovalbumin, and γ-globulin) were prepared according to the procedure of Rice and Means (1971).

Cytosol preparations were either fresh (prepared on the day of analysis and incubated with 30 nM [^{3}H]estradiol for 3 hr at 0°), aged (prepared 1 day prior to analysis, incubated with 30 nM [^{3}H]estradiol for 24 hr at 0°), or exchanged (prepared 1 day prior to analysis, incubated 1–2 hr at 0° with 30 nM unlabeled estradiol, charcoal–dextran treated, and then exchanged with 30 nM [^{3}H]estradiol for 24 hr at 25°).

Immediately prior to being layered onto the gradients, the cytosol preparations were treated with charcoal–dextran to remove the excess free steroid. The ^{14}C-marker proteins were added, and approximately 0.5 uterine equivalent (generally *ca.* 200 μl) was applied to each gradient. Low salt gradients (prepared in TE buffer) were centrifuged at 246,000*g* for 13 hr (SW Ti 56 rotor in a Beckman L3-50 centrifuge at 50,000 rpm); high salt gradients (prepared in TK buffer) were centrifuged at 308,000*g* for 16 hr (SW Ti 56 rotor in a Beckman L2-65B centrifuge at 56,000 rpm).

Three-drop fractions were collected from the top of the gradient (ISCO, gradient fractionator Model 183). Sedimentation coefficients were determined according to Martin and Ames (1961), relative to the internal γ-globulin marker (7.0 S) (Sober, 1970). On low salt gradients, the other markers sediment at velocities close to those reported in the literature: ovalbumin, 3.6 S, and bovine serum albumin, 4.7 S (Sober, 1970). However, on high salt gradients, the albumins sediment faster, relative to γ-globulin (4.1 and 5.0 S, respectively). Similar effects of salt on the sedimentation velocity of albumins have been noted by Puca *et al.* (1972).

Results

Optimization of Assay Parameters. Optimization of the exchange assay protocol (see Experimental Section) involved establishing the particular conditions of temperature and steroid concentration that would: (a) provide a reasonably high rate of exchange, but would (b) result in a minimum loss of binding sites, (c) minimize interference by nonspecific binding, and (d) minimize correction for dilution of specific activity.

STABILITY OF BINDING SITES. Figures 1A and B illustrate the stability of filled and unfilled sites at 0 and 25°. While essentially no decrease in binding capacity at either temperature is noted over a 24-hr period when the sites are filled, binding capacity of empty sites decreases rapidly at 25°; even at 0°, about one-third of the sites are lost within 24 hr. In order to minimize thermal degradation during the exchange process,

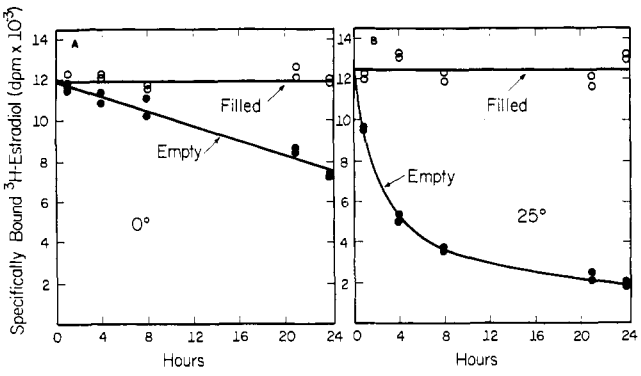

FIGURE 1: Stability of filled and empty high-affinity binding sites at 0 and 25°. Cytosol at 2 uterine equivalents/ml was incubated at 0 or 25°, with or without 30 nM [^{3}H]estradiol. Aliquots from the empty-site assay were resaturated with 30 nM [^{3}H]estradiol; an equivalent volume of buffer was added to the aliquots from the filled-site assay. After 1 hr at 4°, samples were treated with charcoal–dextran and counted. Each sample represents 0.075 uterine equivalent, and data are corrected for nonspecific binding.

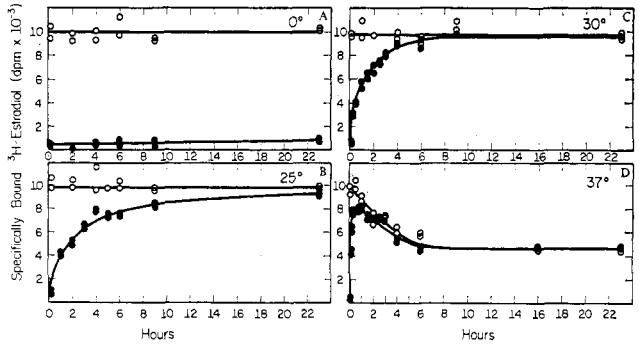

FIGURE 2: Time course of estradiol exchange at various temperatures. Exchange at 1.8 uterine equivalents/ml was conducted as described in the Experimental Section: (●) exchange points; (○) the corresponding stability determinations. Each point represents 0.06 uterine equivalent. All data are corrected for nonspecific binding.

it is clear that saturating concentrations of ligand must be utilized throughout the assay. The results of stability studies at 30 and 37° are given in the next section.

RATE OF EXCHANGE. The rate of exchange of filled sites is limited by the rate of dissociation of cold ligand from these sites. We have determined this rate for estradiol at four temperature (Figures 2A–D). At 0° less than 5% exchange occurs over a 23-hr period; this very slow exchange at 0° helps to simplify the assay procedure involved in running time courses (see Experimental Section). At 25 and 30°, the exchange rate is much higher, essentially reaching completion within 24 or 10 hr, respectively. No instability of sites is noted at either of these temperatures. At 37°, on the other hand, exchange is complete within 1 hr; however, the binding sites are not stable, and *ca.* 15% of the sites are lost within the initial hour. Thus, the number of sites assayed by exchange at 37° is not only low, but is time dependent, making the assay much less reliable and convenient. Temperatures between 25 and 30° were therefore considered optimal for maximum exchange rate consistent with minimal site degradation.

It is interesting that only about 50% of the binding sites are unstable at 37°, undergoing complete degradation within 6–8 hr. The remaining sites are stable throughout the 24-hr incubation at 37°. Rochefort and Baulieu (1971) have previously noted this thermal stability of a fraction of the cytoplasmic estrogen binding sites.

CONCENTRATION OF TRACER LIGAND. There are a number of factors that influenced the choice of 30 nM estradiol as the optimal concentration of tracer ligand. An effective lower limit of 10 nM is established by the requirement that sites be saturated throughout the assay. (The K_a of the rat uterine binding protein is 10^{10} M^{-1} at 0° (Ellis and Ringold, 1971).) This concentration effects saturation of the binding protein at 0°, as well as at 25 or 30°, so that no shift in the position of equilibrium occurs upon raising or lowering the incubation temperature.

Two additional advantages are gained by using estradiol concentrations even higher than the minimal saturating concentration of 10 nM. (1) The rate at which empty sites become filled is more rapid at higher concentrations; with 30 nM estradiol at 0°, saturation is complete within 0.5 hr. (2) The specific activity of the labeled estradiol added is diluted by unlabeled ligand that dissociates from the binding protein during the exchange. However, if the concentration of added estradiol is large relative to the concentration of ligand bound at the start of the assay, the dilution is small and can be corrected for easily (Appendix).

One factor mitigates against using estradiol concentrations higher than 30 nM. Nonspecific binding (see Experimental Section) is a function of both steroid and cytosol concentrations; at 30 nM [^{3}H]estradiol and 2–3 uterine equivalents/ml, the level of nonspecific binding is generally only 10–20% of the total binding capacity (see, for example, Figure 3 and 7). Concentrations of tracer ligand above 30 nM would increase the level of nonspecific binding and would reduce the assay sensitivity (see the following section).

Linearity and Sensitivity. As shown in Figure 3, the exchange assay is linear with respect to cytosol concentration up to at least 3 uterine equivalents/ml. The nonspecific binding is also shown on this figure. Because the adsorption of free estradiol by charcoal–dextran is not 100% complete, the fraction of the total binding that is due to the nonspecific component is larger at lower cytosol concentrations; at 0.3 uterine equivalent/ml, total binding is approximately twice the nonspecific binding. This is considered to be the detection limit of the exchange assay when conducted under these conditions.

Assay of Sites Partially Filled with Unlabeled Estradiol.

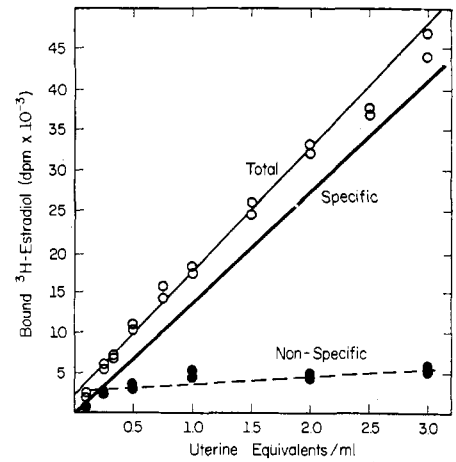

FIGURE 3: Linearity of exchange assay. The different concentrations of cytosol were saturated with 30 nM estradiol (2 hr at 0°), charcoal–dextran treated (10% v/v; 15 min), and then exchanged for 24 hr at 25° against 30 nM [^{3}H]estradiol (specific; (○)) and 30 nM [^{3}H]-estradiol plus 3000 nM estradiol (nonspecific; (●)). Heavy line is specific binding (difference between total and nonspecific lines).

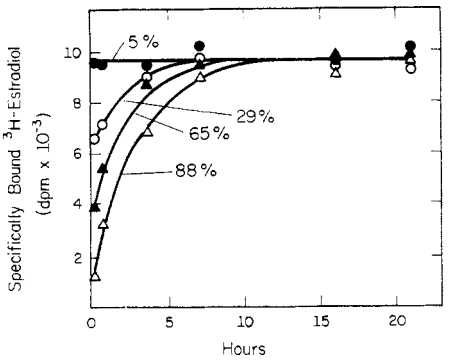

FIGURE 4: Exchange following partial saturation of sites. Cytosol at 3 uterine equivalents/ml was incubated with concentrations of unlabeled estradiol to render the high-affinity binding sites 5, 29, 65, or 88% saturated (see Table I). Exchange was then conducted as usual (see Experimental Section). Each point is the average of two determinations and represents 0.06 uterine equivalent. All data have been corrected for nonspecific binding.

Table I and Figure 4 present the results of binding and exchange measurements on four samples of cytosol, saturated to different degrees with unlabeled estradiol prior to assay. The determination of binding by direct assay (column 3), at 0° as is normally done, provides an accurate measure of the total binding capacity *only* in the case where no cold estradiol had been added prior to the assay, so that all the sites were empty (line 1). In this case the binding measured by exchange (column 4) provides an equally accurate determination. In those cases where a fraction of the sites were filled with unlabeled estradiol prior to assay (lines 2, 3, and 4), the direct assay (column 3) gives a serious underestimate of the actual binding capacity, while assay by exchange (column 4) remains essentially unchanged, and closely approximates the actual total binding in all cases. The difference in the binding capacity determined by the direct assay and the exchange assay is a measure of the sites that were filled with unlabeled estradiol at the start of the assay (columns 5 and 6). The time course of exchange for the four cytosols is shown in Figure 4.

TABLE I: Determination of Binding Capacity in Cytosols Saturated to Different Degrees with Unlabeled Estradiol.[a]

	Total Sites (by Exchange Stability)		Empty Sites (by Direct Assay)	Total Sites (by Exchange)	Filled Sites	
Sample	0 hr (nM)	21 hr (nM)	(nM)	(nM)	nM[b]	%[c]
1	3.26	3.24	2.85	3.01	0.16	5
2	2.81	2.74	1.98	2.77	0.79	29
3	2.82	2.87	1.05	3.00	1.95	65
4	3.10	3.12	0.32	2.65	2.33	88

[a] Cytosol samples 1–4 were brought to 0, 1.7, 3.2, or 30 nM, respectively, in unlabeled estradiol for a 2-hr preincubation at 0°. Assays were performed after the exchange concentrations of "hot" and "hot plus cold" estradiol had been added: direct (column 3; 1 hr at 0°) and exchange (column 4; an additional 21 hr at 25°). Total sites were also determined in a separate set of stability incubations (see Experimental Section, Stability Determination) where "hot" estradiol was used throughout (columns 1 and 2). All concentrations are of specifically bound [^{3}H]estradiol, and are the average of two determinations; in each case the standard error of the mean was less than 10%. Correction for dilution of specific activity in the exchange measurements was made with eq 5 (Appendix) and gave sa_f/sa_i of 0.995, 0.980, 0.931, and 0.924 for assays 1–4, respectively. [b] Difference between direct and exchange assay. [c] Difference in *b* as a per cent of exchange determination.

Status of the Estrogen Binding Protein after Exchange. Stability studies indicate that the number of specific binding sites does not change during the course of the exchange assay; however, since the conditions required to effect complete exchange of estradiol-filled sites involve incubation at 25–30° for periods of up to 24 hr, questions may be raised about other possible alterations in the binding protein.

Little information can be obtained about the association constant for estradiol binding by these sites after exchange, as they are fully saturated at the end of the assay. It is clear, though, that the dissociation constant does not increase to the point that 30 nM estradiol is no longer a saturating concentration.

The sedimentation behavior of the steroid-binding protein complex after exchange at 25° for 24 hr was investigated by sucrose density gradient centrifugaton. This method has been widely used to characterize various associated forms of the rat uterine estrogen binding protein (Giannopoulos and Gorski, 1971; Chamness and McGuire, 1972; Stancel *et al.*, 1973). Sedimentation profiles of fresh (used after 3 hr at 0°), aged (0°, 24 hr), and exchanged cytosol (25°, 24 hr), pictured in Figure 5, show certain differences. When analyzed on low salt gradients (Figure 5A), the peak of binding activity in fresh cytosol is found as a moderately broad peak sedimenting at 7.4 S; a small fraction sediments as heavier aggregates. Aged and exchanged cytosol on low salt gradients show greater degrees of aggregation. Also, a small peak at 4.5 S is found in these gradients.

In high salt gradients (Figure 5B), fresh cytosol (prepared under low salt conditions) shows a double peak of binding activity, at 4.7 and 5.9 S. Again, aggregation of the species involved in the binding is notable in the other two cases. Aged cytosol still shows the same two peaks, although the heavier one has greatly increased in size relative to the lighter. After exchange, the peaks appear only as small shoulders on a rising slope of more rapidly sedimenting aggregates.

Stancel *et al.* (1973) have reported similar time-dependent aggregation of the rat uterine estrogen binding protein, particularly at high cytosol concentrations such as those used here.

Assay of Sites Filled with Nonsteroidal Estrogens and Antiestrogens; Relative Rates of Dissociation. Results described in the preceding sections have shown that the exchange assay can be used to assay binding sites that are filled with estradiol. However, it would be advantageous to be able to assay sites filled with other unlabeled estrogenic or antiestrogenic compounds. Figure 6 demonstrates the validity of the exchange assay as a method for quantitative determination of sites filled either with a nonsteroidal estrogen (hexestrol) or with an antiestrogen (U-11,100A, CI-628, or dimethylstilbestrol).

Competitive binding measurements have shown that these antiestrogens are bound relatively weakly by the cytosol binding protein (Katzenellenbogen and Katzenellenbogen, 1973; Rochefort and Capony, 1972; Korenman, 1970). This is reflected by their rapid exchange with estradiol at 25°. Hexestrol, on the other hand, is known to be bound with even higher

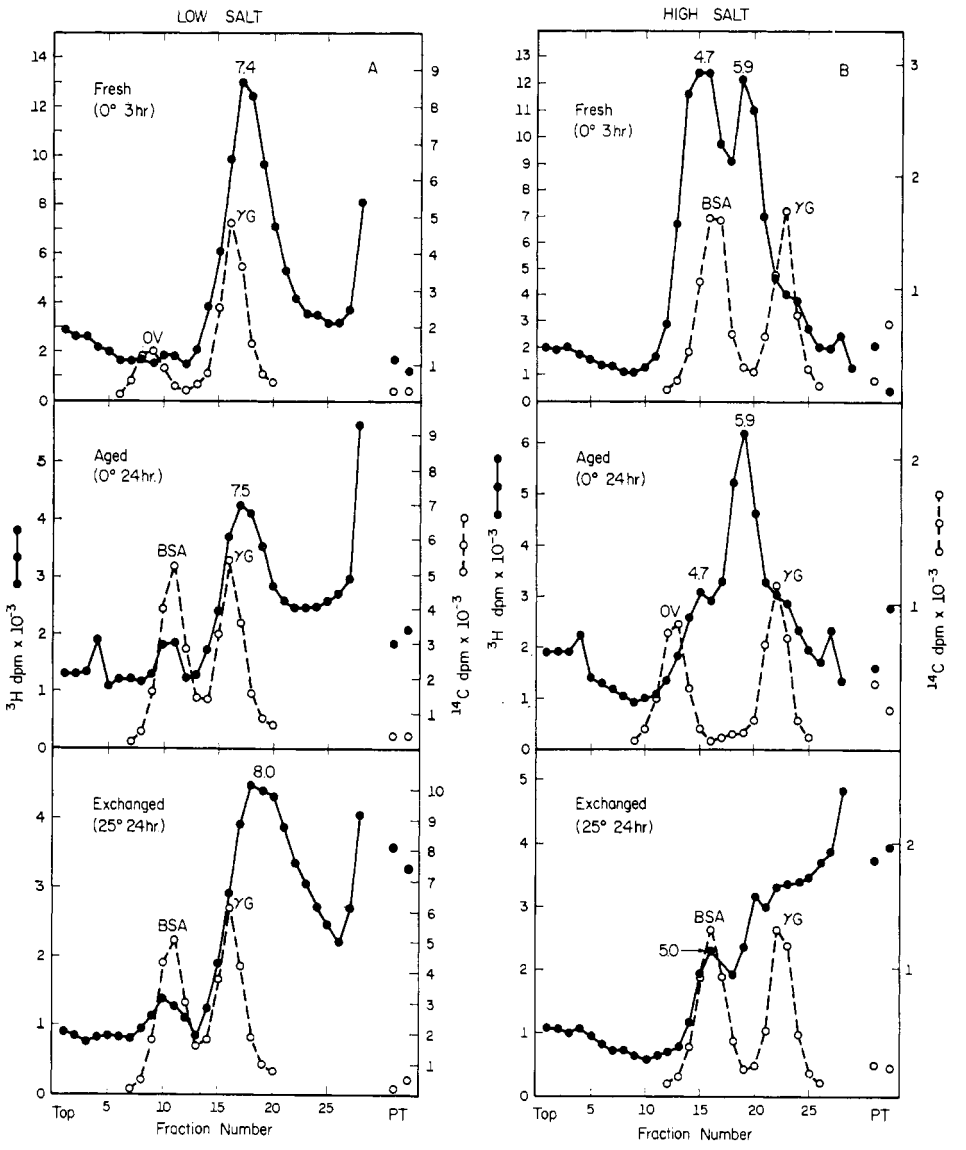

FIGURE 5: Sucrose density gradient profiles of fresh (incubated 0°, 3 hr), aged (0°, 24 hr), or exchanged (25°, 24 hr) cytosol. Cytosol with bound [^{3}H]estradiol (solid line (●–●)) and ^{14}C-labeled marker proteins (dashed line (○- -○); ovalbumin (OV), bovine serum albumin (BSA), γ-globulin (γ-G)) were sedimented in either low salt (TE) gradients (A) or high salt (TK) gradients (B). Three-drop fractions were collected; P represents the bottom of the tube and any pelleted material, and T, the remainder of the tube and any adhering counts. See text for details.

affinity than estradiol (Katzenellenbogen *et al.*, 1973); its dissociation and exchange proceed at a rate somewhat slower than that of estradiol.

As well as validating the exchange assay for determining binding sites filled with unlabeled estrogens and antiestrogens, exchange time courses such as those in Figure 6 permit a qualitative estimate of the relative rates of dissociation of these unlabeled ligands. The more rapidly exchanging species, of course, could be studied more carefully at lower temperatures. A more extensive determination of the dissociation rate of estradiol is shown in Figure 7; the semilogarithmic plot (Figure 7, insert) shows biphasic character, as has been noted in earlier studies (Sanborn *et al.*, 1971; Geynet *et al.*, 1972), and gives dissociation rate constants of 3.9×10^{-4} and 3.9×10^{-5} sec^{-1}.

Discussion

In this paper we describe an exchange assay for the high affinity estrogen binding protein found in rat uterine cytosol. This assay permits the measurement of the total binding capacity, regardless of whether the sites are previously filled with unlabeled estradiol or nonsteroidal estrogens or antiestrogens. The method is convenient, reproducible, and sensitive, and has been miniaturized to permit the assay of a large number of samples with the minimum expenditure of material. Previous methods for assaying cytoplasmic estrogen binding sites have measured only those sites that were unfilled at the time of assay (Mester *et al.*, 1970).

Certain reports in the literature suggested the feasibility of assaying estrogen-filled binding sites by exchange. Kinetic studies by Mester and Robertson (1971), Sanborn *et al.* (1971), and Ellis and Ringold (1971) have shown that the rate of estradiol dissociation from the rat uterine binding protein, while very slow at 0°, becomes reasonably rapid above 20°; however, only Sanborn simultaneously monitored the stability of the binding sites during the course of dissociation. Rochefort *et al.* (1972) have reported the dissociation of nafoxidine (U-11,100 A) from cytoplasmic binding sites by warming to 25° in the presence of charcoal–dextran; however the recovered binding capacity was not quantitated. Our study establishes well-defined and optimized conditions for ac-

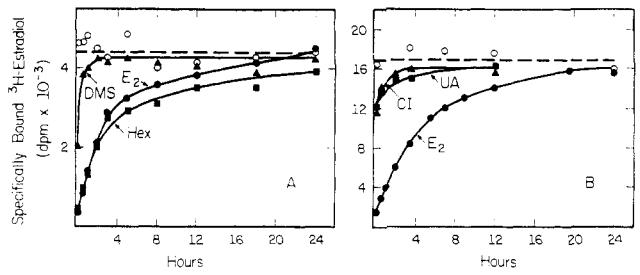

FIGURE 6: Exchange curves of nonsteroidal estrogens and antiestrogens. Each point is the average of two determinations and represents 0.04 uterine equivalent in A or 0.15 uterine equivalent in B. Correction has been made for nonspecific binding. Stability points are shown (O).

complishing a quantitative exchange with the cytoplasmic estrogen binding protein.

Recently Anderson *et al.* (1972) reported that the estrogen binding activity associated with the nuclear–myofibrillar fraction of rat uterus undergoes rapid exchange at 37°. It is notable that the binding activity associated with this particulate nuclear fraction is thermally stable for at least 1 hr at 37°. There have been a few other reports of assay of binding capacity of 0.4 M KCl extracts of calf and rat uterine nuclear pellets by exchange, but in most cases the conditions reported do not appear to be optimal, and recoveries are low and variable (Puca and Bresciani, 1968; Giannopoulos and Gorski, 1971).

There are a number of situations in which this cytoplasmic exchange assay of estrogen binding sites should prove particularly useful. In studies of receptor levels in mature animals, where the endogenous levels of estradiol are high and changing (Feherty *et al.*, 1970; Shain and Barnea, 1971), the exchange assay would allow the determination of both total and empty cytoplasmic binding sites. Direct assay provides information only about sites unfilled at the time of assay and can result in estimates of total binding capacity that are erroneously low (Mester *et al.*, 1970).

In studies of the effectiveness of estrogens and antiestrogens in translocating binding sites from the cytoplasmic to the nuclear compartment, the loss of cytoplasmic binding capacity (both unfilled and total) could be followed with time by the exchange assay. This method is particularly advantageous in those instances where the compounds being studied are not available in radiolabeled form or where their affinity for the

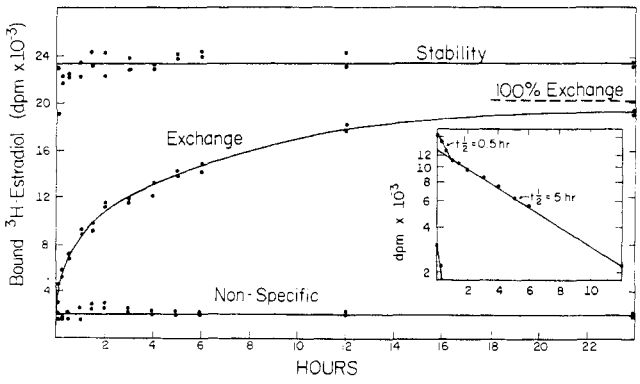

FIGURE 7: Stability and exchange of rat uterine cytosol with 17β-estradiol (25°). Typical time course for estradiol exchange: 100% exchange is the theoretical maximum after correction for dilution of specific activity (see text and Appendix for details). Insert (semilog plot) shows biphasic nature of the dissociation process.

estrogen binding site is so low that high concentrations are needed; serious interference from nonspecific binding would be expected in the latter case.

During the course of an affinity labeling experiment designed to covalently attach a ligand to an amino acid residue within the estrogen binding site, the exchange assay provides a means of measuring the concentration of binding sites that have not undergone covalent blockage. In this sense, it provides a convenient means of documenting a binding site directed labeling process that is analogous to the measurement of v_{max} during affinity labeling studies on enzymes. We are currently utilizing the exchange assay to monitor the course of covalent labeling of the estrogen binding site in rat uterine cytosol with several photoaffinity labeling reagents.

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Appendix: Correction for Change in Specific Activity

If binding sites are filled with unlabeled estradiol at the start of the assay, the unlabeled estradiol that dissociates during the course of the exchange will lower the specific activity of the added, labeled estradiol. While the exchange is proceeding, the specific activities of bound estradiol and free estradiol change as functions of time; treatment of this dynamic situation requires a differential approach (Mester and Robertson, 1971). However, at the end of the assay when the exchange is complete, the two pools of estradiol have equilibrated fully and thus have the same specific activity. In this situation a simple formula can be derived that corrects for the dilution of specific activity, allowing the true binding capacity to be determined from the concentration of labeled estradiol added, its initial specific activity, and the disintegrations per minute of estradiol bound at the start and end of the assay. The dilution of specific activity that occurs is in most cases small (less than 10%) and in some cases can be neglected altogether (see Table I for typical examples), particularly when the concentration of bound estradiol at the start of the assay is small compared to the concentration of labeled estradiol that is added.

The following terms are defined: P_t, total specific binding sites (filled and empty) (picomoles); P_u, specific binding sites unfilled at the start of the assay (picomoles); P_f, specific binding sites filled at the start of the assay (picomoles); dpm_i, disintegrations per minute of specific bound estradiol at the start of the assay; dpm_f, disintegrations per minute of specific bound estradiol after exchange; sa_i, specific activity of the added [^{3}H]estradiol (disintegrations per minute per picomole); sa_f, specific activity after exchange (disintegrations per minute per picomole); E_2*, amount of [^{3}H]estradiol added (picomoles).

At the start of the assay, the cytosol preparation is stripped by charcoal–dextran treatment, so the only unlabeled estradiol present is bound in the high affinity sites. Addition of [^{3}H]estradiol and assay after reaching equilibrium at 0° give

dpm_i (see Experimental Section; Exchange Assay, step 3), which together with sa_i allows the determination of P_u

$$P_u = dpm_i/sa_i \quad (1)$$

After exchange dpm_f is determined (Exchange Assay, step 5). It is related to P_t by the yet unknown sa_f

$$P_t = dpm_f/sa_f \quad (2)$$

Stoichiometry and isotope dilution give the following two relations

$$P_t = P_u + P_f \quad (3)$$

$$sa_f = [E_2^*/(E_2^* + P_f)]sa_i \quad (4)$$

Substituting expressions 1, 3, and 4 into 2 permits P_t to be calculated from the known quantities, dpm_i, E_2^*, dpm_f, and sa_i.

$$P_t = \frac{dpm_f[(E_2^* - dpm_i)/sa_i]}{(sa_i E_2^*) - dpm_f} \quad (5)$$

The largest change in specific activity (sa_f/sa_i) is encountered when all the binding sites are filled with unlabeled estradiol at the start of the assay. Under the conditions we have routinely used (2 uterine equivalents/ml = 3 nM binding protein and 30 nM added estradiol), the correction is less than 10% ($sa_f/sa_i = 0.91$).

In the event that the binding sites at the start of the assay are filled with an unlabeled ligand whose affinity is different from that of estradiol, the more general expression 6 can be utilized. The term RAC is the ratio of association constants ($K_a^{derivative}/K_a^{estradiol}$) and can be determined by competition assay (see the Experimental Section; Korenman, 1970).

$$P_t = \frac{dpm_f(E_2^* - RAC(dpm_i)/sa_i)}{sa_i E_2^* - dpm_f(RAC)} \quad (6)$$

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