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# HYDROXYLAPATITE "BATCH" ASSAY FOR ESTROGEN RECEPTORS: INCREASED SENSITIVITY OVER PRESENT RECEPTOR ASSAYS*

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SUMMARY

A "batch" hydroxylapatite procedure for the adsorption of the uterine estradiol 17$\beta$-receptor complex is described. Characterization with respect to washing efficiency, binding specificity, competition, adsorption time, sensitivity and stability against increasing KCl ionic strength were included. Equilibrium parameters obtained by Scatchard analysis were compared to the range of values found in the literature. $K_a$ and receptor site concentration per uterus obtained by this "batch" technique were found to be well within the range described by these reported values.

This technique is particularly advantageous due to its wide range of operational sensitivity (capable of detecting specific estradiol-17$\beta$ binding to a cytosol fraction containing from 5 to 500 $\mu$g protein per 225 $\mu$l). The assay is run entirely at low temperature (0–2°C). In addition this technique depends on a homogeneous insoluble chemical, hydroxylapatite, which can be obtained in analytical grade quantities of uniform particle size, shows little affinity for free steroid, can be readily packed or resuspended, and appears independent of changes in concentrations of KCl up to 2500 mM.

Additional considerations include the effect of temperature during assay, the importance of empirical correction for non-specific binding, the contributions of binding information on the calculation of equilibrium parameters and statistical evaluation of random error and assay repeatability.

## INTRODUCTION

Steroid hormone receptors are soluble proteins which very likely play key roles in the mechanism of action of steroid hormones (for review see Jensen and DeSombre [1]. Measurement of the steroid–receptor complex requires separation of free steroid from steroid bound to the macromolecular receptor. A wide variety of techniques has been employed to this end including: density gradient centrifugation, gel chromatography, charcoal adsorption of free steroid, dialysis, adsorption to glass and silica mesh, protamine sulfate precipitation, binding to DEAE filters, *in vitro* concentration by free cells and adsorption to hydroxylapatite [1–23, and Table 1]. Disadvantages exist in any procedure and improvement usually implies a gain in sensitivity, in performance time, in reproducibility or in ease of application.

A "batch" adsorption assay is described and characterized for the estradiol-17$\beta$ cytosol receptor ($E_2$–$R_c$) complex using hydroxylapatite (HTP) which is a modification of the column HTP separation technique of Erdos *et al.* [17]. "Batch" adsorption provides an improved method of obtaining large numbers of statistically significant points for quantitative analysis. In addition, this approach is easy to apply and has an operation range capable of detecting specific estrogen binding in uterine cytosol samples containing as little as 5 $\mu$g protein.

In short this technique involves: (a) adsorption of receptor bound [$^3$H]-estradiol complex to hydroxylapatite at an operational temperature of 0–2°C, (b) direct sampling of free steroid in the hydroxylapatite supernatant after centrifugation, (c) removal of unbound steroid by sequential washes of HTP with buffer, and (d) determination of [$^3$H]-$E_2$–$R_c$ bound to the HTP pellet.

## EXPERIMENTAL

*Animals.* Immature female Sprague–Dawley rats were obtained from Sprague Dawley, Madison, Wisconsin. Upon reaching maturity, rats were bilaterally ovariectomized. These animals are referred to as "long-term castrate" females. Experimental animals were used no sooner than 4 weeks postoperatively, and sacrificed by cervical dislocation under light ether anesthesia.

*Cytosol preparation.* Uterine horns were immediately removed, dissected free of fat and connective tissue and placed in Hank's balanced salt solution (HBSS) on ice (Fig. 1). All procedures were performed as outlined in the literature [4, 6, 10] at 0–2°C unless otherwise specified. Homogenization in glass homogenizers was performed in buffer containing Tris (40 mM, pH 7.2), EDTA (1.5 mM), mercaptoethanol (14 mM) and KCl (50 mM). This buffer is referred to

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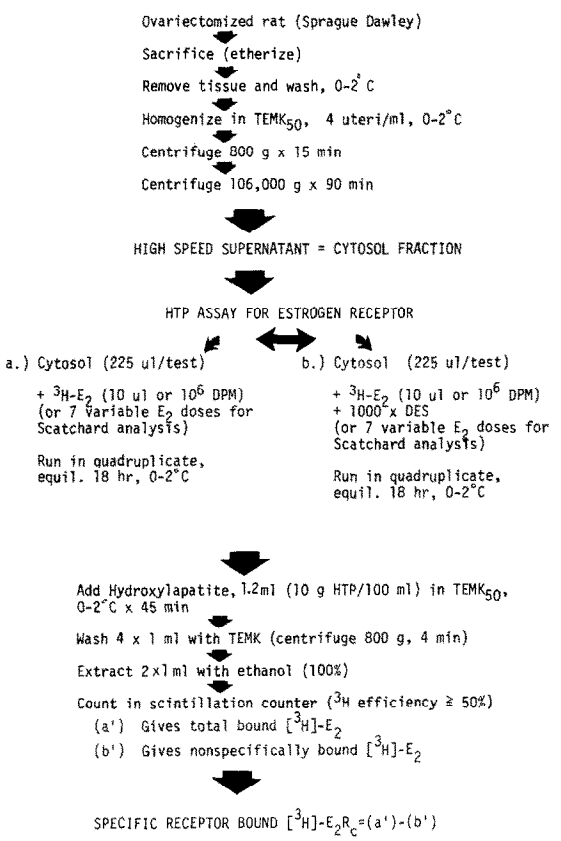


Fig. 1. Hydroxylapatite assay of estradiol 17$\beta$-receptors.

as $TEMK_{50}$ where KCl is the only variable. The homogenate was centrifuged at 800*g* for 15 min to pellet the nuclear-myofibrillar components. This low speed supernatant was then centrifuged at 106,000*g* for 90 min in a titanium (50 Ti, Beckman Industries) fixed angle rotor yielding the high speed supernate or receptor containing cytosol fraction. A homogenization ratio of 4 uterine horns/ml (2 rat uteri/ml) was routinely used unless otherwise specified. The receptor containing "cytosol fraction" was diluted as described in individual experiments. Protein determinations were performed on the cytosol fraction according to the procedure of Lowry *et al.* [24].

*Hydroxylapatite preparation.* Analytical grade hydroxylapatite (HTP or DNA grade HTP, Biorad Inc.) was mixed in $TEMK_{50}$ buffer for 48 h (10g/100 ml). The "fines" were routinely decanted, although comparison with undecanted HTP showed no detectable difference. The HTP suspension was then stored at 0–2°C and used within one week after preparation. Prior to use the HTP was resuspended by stirring for 30–60 min at 0–2°C and divided into 1.2 ml aliquots. Procedures for adsorption and washing HTP–$E_2$–$R_c$ complex free of unbound ligand are detailed in the Results and Discussion section. Bound [$^3$H]-estradiol ([$^3$H]-$E_2$) was removed from the HTP with two ethanol extractions with an extraction efficiency of 95% or greater. Liquid scintillation counting was performed with a PPO-POPOP fluor (2,5 diphenyloxazole and 1,4Bis[2-(5-phenyloxazolyl)]benzene) in a Beckman LS-230 liquid scintillation counter. Counting efficiency for [$^3$H] was 30–40% and external standard quench correction was obtained for each sample.

*Cytosol incubation conditions.* For all studies 225 $\mu$l of cytosol was incubated with 20 $\mu$l ethanol containing either [$^3$H]-estradiol-17$\beta$ (80–100 Ci/mmol; Amersham Searle or New England Nuclear) or [$^3$H]-estradiol-17$\beta$([$^3$H]-$E_2$) and diethylstilbesterol (DES, from Sigma Chemical Corp). For equilibrium studies, incubation times of 18 h at 0–2°C were employed. Purity of the [$^3$H]-$E_2$ was checked by thin-layer chromatography, and conformed to the manufacturer's specification. Incubation was terminated with the introduction of 1.2 ml of HTP solution (0–2°C) followed by vortex mixing for 5 s. The $E_2$–$R_c$ complex adsorbed to HTP was then washed with $TEMK_{50}$ buffer by sequential resuspension and centrifugation. All calculations were performed on a programmable Hewlett–Packard 65 electronic calculator. Specific $E_2$–$R_c$ was determined as the total [$^3$H]-$E_2$ bound minus the nonspecific [$^3$H]-$E_2$. The Ka and uterine $E_2$–$R_c$ concentrations were determined by Scatchard analysis [27].

## RESULTS

A fundamental requirement for any system used to adsorb the $E_2$–$R_c$ complex is that it can be washed clean of free steroid. The degree to which free steroid can be washed free from pure HTP is demonstrated in Fig. 2. There is no difference in "washout" between HTP and finely powdered DNA grade HTP. DNA grade HTP was routinely selected for further use because its fine particle size allows tighter packing by centrifugation. Four washes were sufficient to reduce supernatant wash activity below 1% of the input activity, and to reduce the activity remaining in the HTP (i.e., ethanol extracted activity) to below 1% of the input activity.

Another obligatory requirement placed on a system used to separate the $E_2$–$R_c$ complex is the demonstration of high affinity or specificity for [$^3$H]-$E_2$ binding as distinct from low affinity or non-specific steroid binding. High affinity binding specific for the $E_2$–$R_c$ is reduced (Fig. 3) by the known non-steroidal estrogenic competitor (DES) as well as by cold estradiol-17$\beta$. Increasing concentrations of DES rapidly reduced the total amount of bound activity. Hence, as demonstrated by Williams and Gorski [6] and as shown here, non-specific binding appears to be an unsaturable process. In addition diethylstilbesterol and estradiol at 100- or 1000-fold excess appear as indistinguishable competitors. Specific binding to the $E_2$–$R_c$ can be determined as originally suggested by Williams and Gorski [6] by the difference between the total activity bound in the absence of any com-

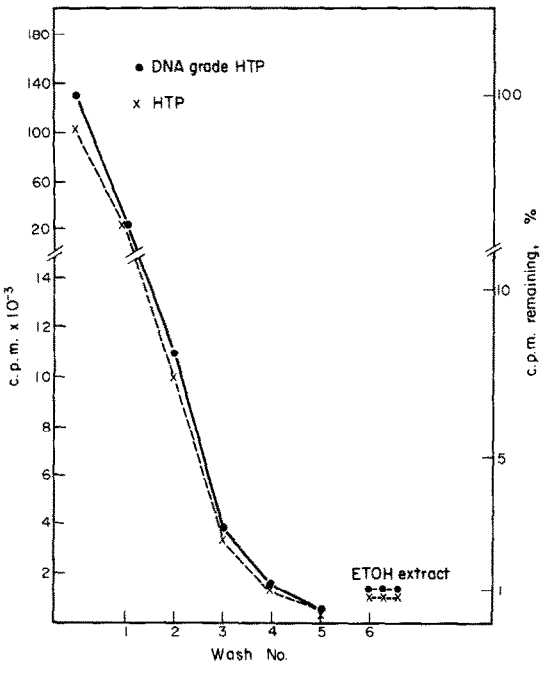


Fig. 2. Retention of free [$^3$H]-estradiol by hydroxylapatite. $TEMK_{50}$ buffer (225 $\mu$l) containing a known concentration of [$^3$H]-estradiol-17$\beta$ was incubated for 45 min in the presence of HTP. Adsorption was terminated by centrifugation at 800*g* for 5 min. The supernatant containing unbound steroid was collected and counted. The pellet was resuspended in 1 ml of $TEMK_{50}$, and successive 1 ml washes with $TEMK_{50}$ were also collected and counted. The activity which had not been removed by washing with $TEMK_{50}$ was determined by extracting the HTP two times with ethanol and counting. Retention of [$^3$H]-$E_2$ by HTP and finely powdered DNA grade HTP were both less than 1% of total c.p.m. Results are expressed as mean c.p.m. ($n = 4$) in the supernatant wash as well as a percentage of the input activity recovered from the extracted HTP pellet after the fifth wash.

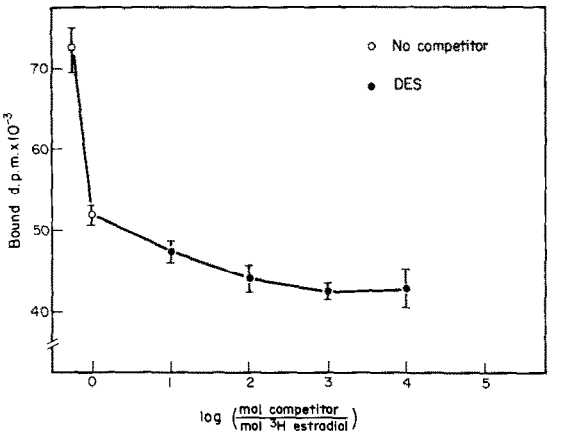


Fig. 3. Adsorption of the $E_2$–$R_c$ to HTP and competition by diethylstilbesterol. Receptor containing cytosol was incubated for 5 h at 0–2°C with a near-saturation amount of [$^3$H]-$E_2$ ($= 450 \times 10^3$ c.p.m./225 $\mu$l cytosol). The tissue to buffer ratio consisted of 14 uterine horns homogenized in 12 ml of $TEMK_{50}$ buffer. Bound [$^3$H]-$E_2$ values ($n = 4$) are expressed as mean d.p.m. $\pm$ the standard error of the mean (S.E.M.). Competition was performed so that there were no effects due to dilution or volume change. The receptor was adsorbed to HTP by incubating for 45 min at 0–2°C. Adsorption was followed by four 1 ml washes with $TEMK_{50}$ and double extraction of the HTP pellet with ethanol. Competition by non-radioactive $E_2$ in 100 and 1000 fold excess yielded: 43,193.8 $\pm$ 2284.7 and 42,690 $\pm$ 778.1 d.p.m. bound respectively. This level of competition was indistinguishable from that achieved with 100- and 1000-fold excess diethylstilbesterol which yielded: 43,834.5 $\pm$ 1772.4 and 42,437.8 $\pm$ 751.8 d.p.m. bound respectively.

petitor and the unsaturable amount of non-specific [$^3$H]-$E_2$ binding which occurs in the presence of excess competitor (Fig. 1), or: specific [$^3$H]-$E_2$–$R_c$ equals [$^3$H]-$E_2$ bound without non-radioactive competitor minus non-specific [$^3$H]-$E_2$ bound in the presence of 1000-fold excess non-radioactive DES.

Therefore, in all determinations of specific [$^3$H]-$E_2$ receptor binding, additional aliquots were run simultaneously containing a 1000-fold excess of non-radioactive competitor in order to accurately estimate non-specific binding. Scatchard or Lineweaver–Burk analysis of $E_2$–$R_c$ gave Ka values of approximately $0.30 \times 10^{10}\ M^{-1}$ (Table 1) and a $E_2$–$R_c$ receptor site concentration of $3.72 \times 10^{-12}$ mol/mg protein ($\pm 0.27$ SEM, $n = 11$).

Optimal and efficient adsorption are also important considerations. The time necessary for maximum adsorption was determined (Fig. 4) and demonstrated a plateau within approximately 30 min. While relatively the same mean amount of specific adsorption occurred after shorter adsorption times, the variability as judged by reduced standard error was seen

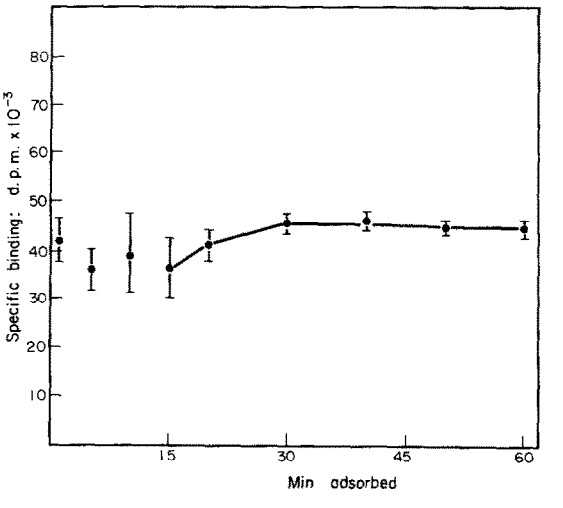


Fig. 4. Adsorption time for binding $E_2$–$R_c$ complex to HTP. Conditions were as described in Fig. 3. The tissue-buffer ratio consisted of 16 uterine horns homogenized in 12 ml of $TEMK_{50}$. Receptor was adsorbed to HTP for varying periods of time. Adsorption was followed by four 1 ml washes with $TEMK_{50}$ and double extraction with ethanol. Specific binding was determined by subtracting the d.p.m. bound in the presence of a 1000 fold excess of DES from the total d.p.m. bound. Results are expressed as mean d.p.m. $\pm$ S.E.M. ($n = 4$).

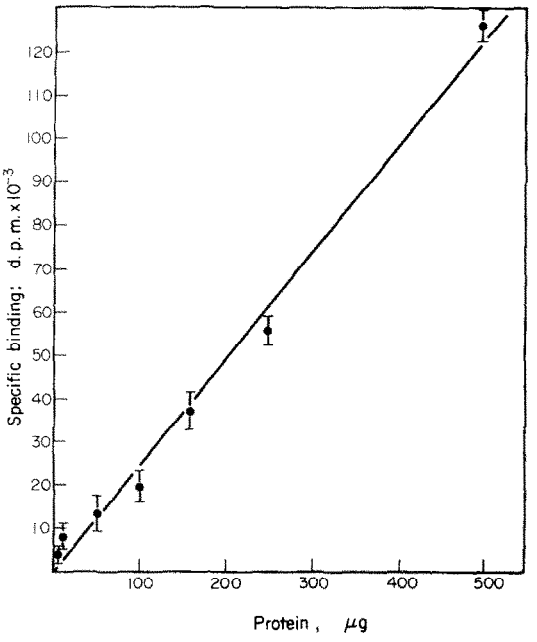


Fig. 5. Adsorption sensitivity of HTP for $E_2$–$R_c$ binding. Conditions were as described in Fig. 3. The tissue-buffer ratio consisted of 22 uterine horns homogenized in 5 ml of $TEMK_{50}$. Adsorption to HTP was performed for 45 min at 0–2°C. Results were expressed as mean d.p.m. ± S.E.M. ($n = 4$). Correction for non-specific binding was made by subtracting [$^3$H]-$E_2$ bound in the presence of a 1000-fold excess of DES from the total d.p.m. bound in the absence of competitor. The soluble protein content in this experiment averaged 525.4 ± 31.3 μg protein per uterine horn. Specific binding is clearly shown over a 50-fold dilution range. Specific binding could be detected down to 1:100 dilution, but clearly disappeared at 1:10,000 dilution. Precision of the assay was judged by comparing experimental mean values to the equivalent value predicted by regression. Values shown were not significantly different ($F \geq 0.950$).

to decrease after 30 min. Thus, all subsequent determinations were routinely adsorbed for 45 min.

The assay sensitivity is described in Fig. 5. Aliquots containing various dilutions of cytosol were assayed. The highest concentration of receptor (1 uterine horn/225 μl) was tested over a dilution range of 1:10,000. The dilution sensitivity was easily demonstrable over a 50–100 dilution range as shown in Fig. 5. Thus, the HTP method is well suited for detecting estrogen receptor in target tissue homogenates which contain 5–500 μg protein.

Other assay techniques are dependent upon low ionic strength conditions for adsorbing the receptor [4, 5, 8]. HTP adsorption as a function of ionic strength is shown in Fig. 6. Buffer concentrations were designed so that only the concentration of KCl was varied. All aliquots were washed five times with $TEMK_{50}$. The first group was double ethanol extracted while the second group was washed with buffers of increasing KCl concentration. After washing the samples with $TEMK_{2500}$ (the final KCl wash of 2500 mmol ionic concentration), a double ethanol extraction was performed. Wash supernatants were collected and counted. No differences in counts retained by HTP or counts recovered in the supernatant could be detected between aliquots subjected to washes of increasing ionic strength vs those washed in $TEMK_{50}$. These data indicate that the [$^3$H]-$E_2$–$R_c$ complex adsorbed to HTP is unaffected by increasing KCl ionic strength. In addition, since the nuclear estradiol receptor [$E_2$–$R_n$] is generally defined as extractable in buffers containing 400–600 mM KCl [21–23], this adsorption technique should prove particularly valuable in quantitating $E_2$–$R_n$ in the presence of high KCl concentration.

Recent studies by Bresciani *et al.* [25] confirm the ability of HTP to bind the $E_2$–$R_c$ complex while not retaining the nuclear $E_2$-acceptor which is apparently a more basic protein. DNA binding to HTP has been well characterized; however, the DNA contamination in these cytosol preparations is very low. Any $E_2$–$R_c$ bound to a DNA–HTP complex would have been eluted off at concentrations of KCl above 300 mM [26] and therefore does not appear to be a contributing factor in this $E_2$–$R_c$ HTP assay, Fig. 6.

In an effort to compare this assay with $E_2$–$R_c$ assays currently in use, a survey of the literature was made (Table 1) to document values for association constants (Ka) and receptor concentrations in the uterus. It is clear that a wide range of $K_a$ values has been reported. This wide range may reflect not only the technique employed and the temperature used, but also the method of correction for non-specific binding.

Heat inactivation of estrogen receptor has been employed as a method of correction for non-specific binding. Some temperature treatments in the literature include 45°C, 60 min [20], 60°C, 30 min [21], 30°C, 30 min [2] and 25°C, 30 min [4]. It is pertinent, therefore, to compare correction by excess competitor with correction by heat inactivation. In addition, such a comparison indicates the relative stability of the receptor at different temperatures.

The effect of pretreatment for 60 min at different temperatures is shown in Fig. 7a in terms of total binding, specific binding and non-specific binding (measured in the presence of 1000× excess DES). It is clear that temperature pretreatment for 60 min in the 30–50°C range reduced total binding to the level of non-specific binding without temperature pretreatment. Hence, pretreatment in this temperature range (30–50°C) for 60 min yielded results identical to correction by unheated excess competitor (0°C).

The effect of pretreatment at 60°C for various times is shown in Fig. 7b and indicates that as pretreatment is prolonged a slight increase in total binding occurs as a result of increasing non-specific binding. Thus, while temperature has been shown to influence the association-dissociation of the steroid-loaded receptor [29], it also has differential labilizing effects on specific and non-specific binding which may also be time dependent. This effect may be of significance in the determination of equilibrium parameters.

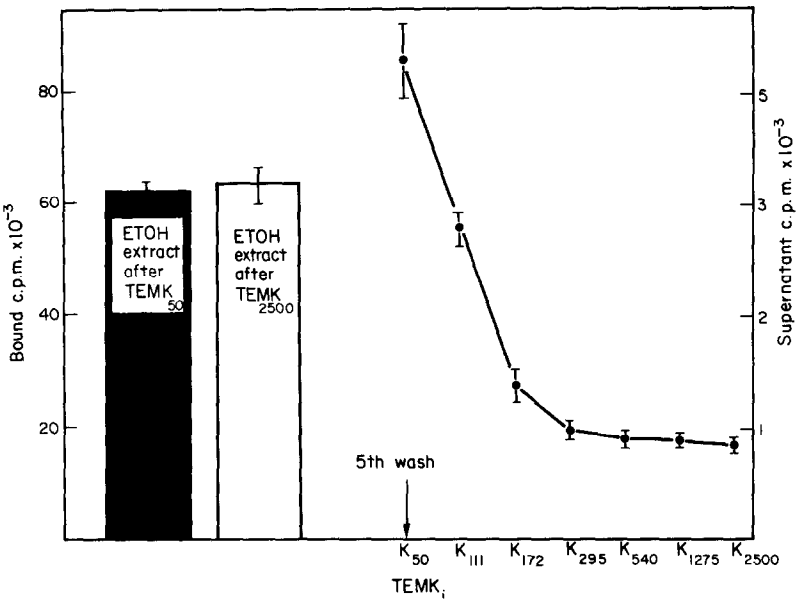


Fig. 6. Adsorption of $E_2$–$R_c$ to HTP: KCl ionic strength insensitivity. Conditions were as described in Fig. 3. Dark bar: Receptor was adsorbed to HTP for 45 min at 0–2°C. Adsorption was followed by five 1 ml washes with $TEMK_{50}$ and a double ethanol extraction. Open bar: Following the 5th wash with $TEMK_{50}$, the receptor containing HTP was washed sequentially with buffer of increasing KCl concentration ($TEMK_{111}$ up to $TEMK_{2500}$) and then double extracted with ethanol. Supernatants were collected after each wash for monitoring [$^3$H]-$E_2$ washed free due to increasing KCl ionic strength. Results were expressed as mean c.p.m.-bound ± S.E.M. ($n$ = 4). Recovery was never less than 95%.

Two anomalies become apparent as a result of competition and temperature. First, pretreatment for 60 min at temperatures above 50°C causes total binding to "rise" as a result of increased non-specific binding. Heat denaturation of protein and exposure of otherwise inaccessible hydrophobic cores may explain this observation. Second, low levels of specific receptor binding persist at temperatures (35–50°C) which reduce total binding to the same level as non-specific binding and at which all receptors should be inactivated. Such an observation may be the result of competition for non-specific binding sites in the true

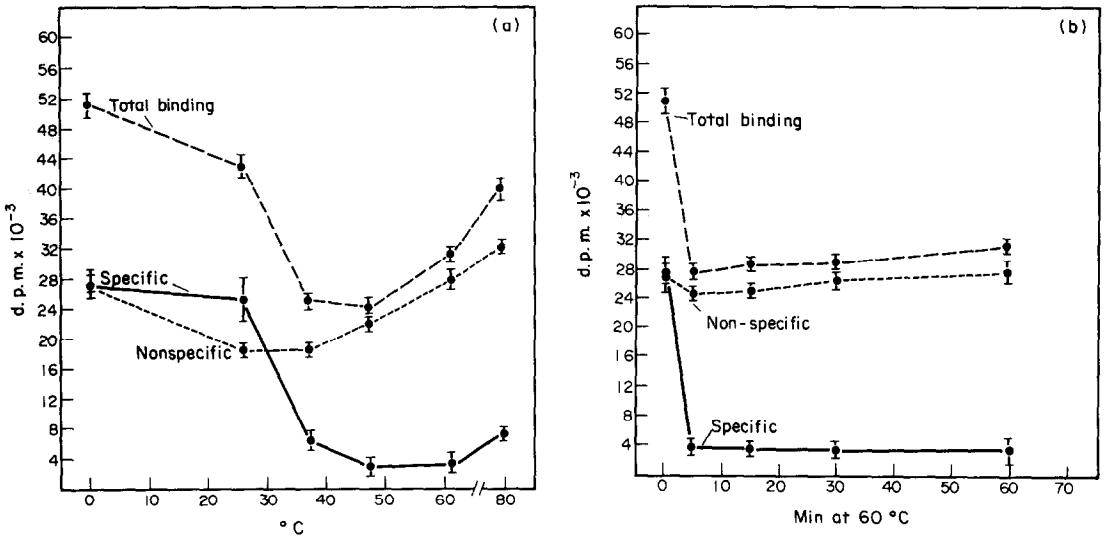


Fig. 7. The effect of temperature on total, specific and non-specific binding. A: Aliquots of cytosol described in Fig. 5 were exposed for 60 min to a series of different temperatures. After rapid cooling to 0–2°C, aliquots were incubated with a saturating concentration of [$^3$H]-estradiol 17$\beta$,(5 × $10^{-12}$ mol/225 $\mu$l) for 14 h at 0–2°C. Non-specific binding was determined in the presence of 1000× excess DES and specific binding was determined as the difference between total and non-specific binding. Results are expressed as mean d.p.m. ± S.E.M. ($n$ = 4). B: Aliquots of cytosol were held at 60°C for varying periods of time. Description of data is as in A.

absence of receptor. Hence, while non-specific binding is a non-saturable phenomenon, it may still display binding parameters which are sensitive to competition by excess non-radioactive DES.

The result of such sensitivity is that in the absence of receptor, competition alone can account for a difference between total and non-specific binding and spuriously indicate the presence of low levels of receptor. Alternatively, the differential sensitivity to temperature between specific receptor binding and non-specific binding may indicate that active receptor still remains after temperature pretreatment, since non-specific binding components may contribute with greater significance to the reduction in total binding activity. The present data does not allow one to distinguish between these alternatives, but does indicate that caution should be exercised when temperature inactivation is attempted.

The HTP assay has been employed in saturation determinations at equilibrium. Evaluation by Scatchard plotting [27] is customarily subjected to simple linear regression analysis. Such analysis is influenced by the amount or "kind" of information as well as by the degree to which the requirements for simple linear regression are met. In the latter case it is assumed that (a) there is no error in the independent variable nor a correlation between errors in the dependent and independent variable, (b) the dependent variable has a uniform variance, (c) error in the dependent variable is random with a normal Gaussian distribution [33].

The implication of these assumptions is directly applicable to the extraction of equilibrium parameters ($K_a$ and total receptor concentration) from Scatchard, Michaelis–Menten, Lineweaver–Burk or Eadie plots [33]. Despite the algebraic equivalency between these plots, each must be regarded as uniquely transforming the error contained in the data points. Thus, as data points lie more closely to some idealized line, the consequences of these transformations are minimized. While appropriate weighting functions can be obtained in order to force any plotting regression to yield the same $K_a$ and total receptor concentration values from the same set of data [33, 34], statistical agreement between different plotting techniques measures minimal deviation from some idealized line. Statistical agreement between different plotting regressions may be used to indicate low variability and low error in the biochemical separation of bound and free steroid.

Statistical comparison utilizing Student's $t$ test is made between regressions obtained by Scatchard and Lineweaver–Burk analysis. In addition both $y$–$x$ and $x$–$y$ regressions were performed since this technique allows inspection of errors in the "independent" variable. In the case of Lineweaver–Burk plots the $y$–$x$ and $x$–$y$ regressions yield the weighted sums of squares of deviations of vertical and horizontal points about the regression line. Hence, averaging the values obtained by both $y$–$x$ and $x$–$y$ regressions minimizes the sums of squares of deviations and yields the "weighted" average function [33].

"Information" depends on derivation and inference such that increasing empirical determination of the data decreases any inferred component. The consideration made now is whether either the inherent properties of the data points or the types of binding parameter information can cause equilibrium parameters (in particular the $K_a$) obtained *via* HTP assay to fluctuate over as wide a range as shown in Table 1. The binding parameter information can be interpreted to cover an expanding data range so that the content becomes more and more empirical. The precise extent of this information is defined for the categories used in Tables 2–5 in the following way:

Category A (total bound activity vs "Δ" free activity): Total bound activity is total bound activity determined by HTP assay while "Δ" free activity is the difference between total activity and total bound activity.

Category B (total bound activity vs empirical free + non-specific bound activity): Activity categorized as "free" activity is totally determined empirically by including free activity determined by HTP assay as well as bound non-specific activity, which in an idealized one component system may be considered as free activity.

Category C (total bound activity vs empirical free activity): Free activity is determined by HTP assay.

Category D (Specific bound activity vs "Δ" free activity): Specific bound activity is determined by the difference between total bound activity and non-specific bound activity. "Δ" free activity is the difference between total activity and specific bound activity. The idealized one component system is generally represented by this category.

Category E (Specific bound activity vs empirical free + non-specific bound activity): Activity categorized as "free" is as defined in category B. All information has components which are totally derived empirically.

Category F (Specific bound vs empirical free activity): Free activity is as defined in category C. This category assumes a realistic philosophy that equilibrium parameters are limited by the amount of free steroid. Thus, it asserts that irrespective of corrections made to specific binding, the empirically determined free activity establishes the amount of steroid bound and hence should be used as the criteria for "free" activity.

Graphical representation of the same "raw" data set utilizing various amounts of information are presented in Scatchard format in Figs. 8a and b and in Lineweaver–Burk format in Fig. 9. It is apparent that correction for non-specific binding influences estimation of total receptor concentration more than estimation of $K_a$. It is also evident that selection of parameters which describe "free" activity has a minimal impact upon estimation of total receptor concentration, but influences the estimation of $K_a$. Figure 9

Table 1. Comparison of published values for association constant ($K_a$) of estrogen receptors

| Association constant $K_a \times 10^{10}$ $M^{-1}$ | Receptor conc. (moles/unit) | Protein conc of cytosol | Uterus/ tissue | Technique | Correction for non-specific binding | |
|---|---|---|---|---|---|---|
| 2.40 | $1–8 \times 10^{-15}$ moles/mg tissue | — | rat | dextran coated charcoal | Analytical[a,b] temp. dissoc. | Mester *et al.* 1970 (21) |
| 0.14 | $0.89 \times 10^{-12}$ moles/uterus | — | immature rat | *in vitro* nuclear binding | temp. dissoc.[b] | Shymala & Gorski 1969 (2) |
| 3.6 | $5.8 \times 10^{-10}$ moles/l | 5.2 mg/ml | adult rat | dialysis, partial purification | analytical[a] | Ellis & Ringold 1971 (3) |
| 4.5 | $4.0 \times 10^{-10}$ moles/l | 15 mg/ml | adult rat | dialysis partial purification | analytical | Ellis & Ringold 1971 (3) |
| 0.8–19.0 | — | — | — | — | — | Ellis & Ringold 1971 (3) |
| 0.03 | $0.9 \times 10^{-12}$ moles/uterus | — | immature rat | glass bead binding | temp. dissoc. | Clark & Gorski 1969 (4) |
| 0.06 | $0.56 \times 10^{-12}$ moles/mg protein | — | adult rat | Silica mesh | temp. dissoc.[b] | Notides 1970 (8) |
| 0.22 | — | — | immature rat, free cells | glass beads, Sephadex G-25 | Competitive saturation: (100 × excess estradiol-17.$\beta$)[c] | Williams & Gorski 1973 (6) |
| 0.04 | — | — | rat | DEAE filters | Competitive saturation (100 × excess estradiol-17,$\beta$) | Santi *et al.* 1973 (7) |
| 4–6.7 | $1.3–2.2 \times 10^{-14}$ moles/mg protein | — | hypothalamus adult rat | Protamine precipitation | Competitive saturation (100 × excess $E_2$)[c] | Korach & Muldoon 1974 (9) |
| 1.0 | — | — | — | dextran coated charcoal | differential dissociation | Alberga *et al.* 1970 (31) |
| 0.30 | $3.2–3.7 \times 10^{-12}$ moles/mg protein | 700 $\mu$g/ml | long term castrate rat | HTP | Competitive saturation (100 × excess $E_2$)[c] | Pavlik & Coulson 1975 |
| 1.60 | $3.1 \times 10^{-10}$ moles/mg | 10 mg/ml | hamster | dextran coated charcoal | — | Talley *et al.* 1975 (36) |
| 0.14 | $1 \times 10^{-12}$ moles/mg protein | 8.6 mg/ml | immature rat | Sucrose gradient | N.C.[d] | Toft *et al.* 1967 (10) |
| 0.77 | — | — | rabbit | dextran coated charcoal | N.C. | Korenman & Rao 1968 (11) |
| 70 | — | — | rat endometrium | *in vitro* incubation | N.C. | Alberga & Baulieu 1968 (12) |
| 0.13–0.35 | $3.2–6.5 \times 10^{-13}$ moles/mg protein | — | calf (partial purif.) | Sephadex G-25 | N.C. | Puca & Bresciani 1969 (13, 28) |
| 0.025–0.5 | — | 5.4 mg/ml | immature rat | Sephadex G-25 | N.C. | Giannopoulos & Gorski 1970 (14) |
| 0.3 | 3.0 moles/kg tissue | — | immature & adult rat | *in vivo* infusion | N.C. | DeHertogh *et al.* 1971 (15, 16) |
| 0.88–1.10 | $0.39–1.54 \times 10^{-9}$ moles/g prot./L | — | calf | HTP | N.C. | Erdos *et al.* 1970 (17) |
| 0.102 | $45 \times 10^{-15}$ moles/mg protein | — | immature | Sephadex G-25 & G-100 | N.C. | Godefroi & Brooks 1973 (18) |
| 0.45 | — | — | mature rat | Biogel P-10 | N.C. | Eisenfeld 1974 (19) |
| 0.77 | $0.3 \times 10^{-12}$ moles/mg protein | — | rat (partial purif.) | protamine precip. | N.C. | Steggles & King 1970 (32) |
| 1.5–1.7 | $0.2–2 \times 10^{-8}$ moles/L | — | calf | HTP | N.C. | Best-Belpomme *et al.* 1970 (29) |

Abbreviations: fm—femtomoles, pm—picomoles.

[a] Analytical correction obtained by least squares solution of two linear equations, one representing high affinity binding, the other low affinity binding.

[b] Elevated temperature is believed to cause more rapid dissociation of ligands at low affinity sites than at high affinity sites. Incubation was at 30°C for 30 min.

[c] Excess non-radioactive ligand cannot saturate low affinity sites. Thus, total activity-activity in the presence of excess unlabelled ligand = high affinity binding.

[d] N.C.—no correction.

[e] Assay conditions include warming to 25°C, 30 min.

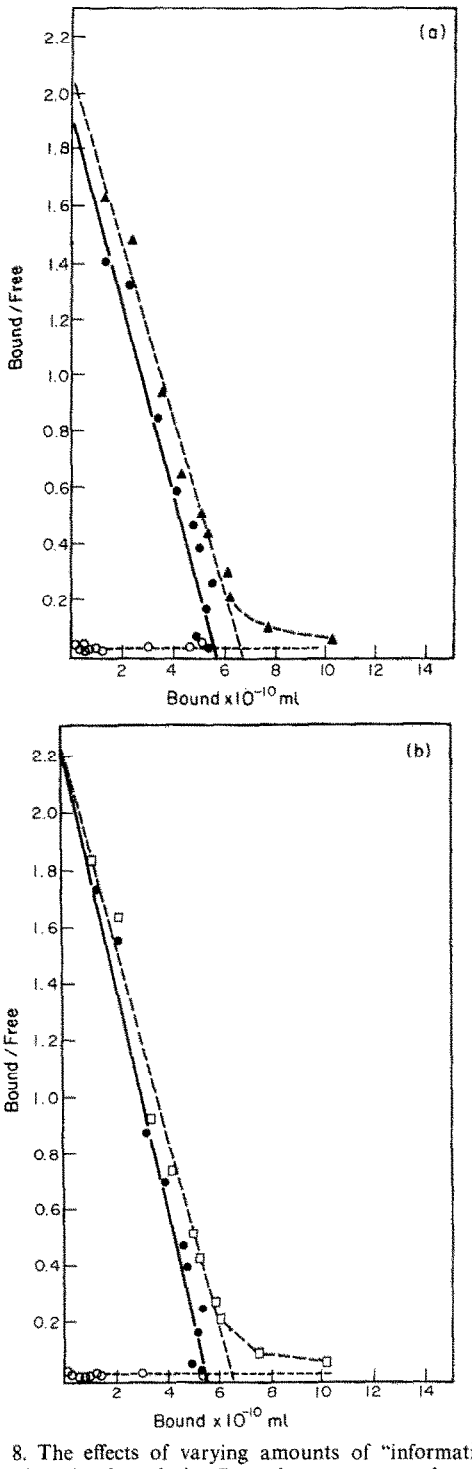


Fig. 8. The effects of varying amounts of "information" upon Scatchard analysis. Cytosols were prepared as described in Methods and Materials and diluted to a protein concentration of 689.3 ± 2.1 μg/ml. Receptor bound activity in 225 μl aliquots was assayed on HTP as described after incubation at 0–2°C for 14 h with varying concentrations of [$^3$H]-estradiol-17β. Total binding, specific binding and non-specific binding are represented by dashed, solid and dotted lines. All lines were obtained by simple linear regression analysis. A: Parameters include "Δ" Free information. B: Parameters include "empirically obtained" Free information. Values for equilibrium parameters are contained in Tables 2–5. Each point is a mean value ($n = 4$).

also shows the effect on the Lineweaver–Burk plot of error in the "independent" variable. The two regression lines shown are the result of $x$–$y$ and $y$–$x$ regressions and demonstrate very close agreement. For total binding activity, the $x$–$y$ and $y$–$x$ regression lines became superimposed on each other.

Comparisons made in Table 2a for $K_a$ values examine the effect of "independent" variable error for each category of information analyzed by Lineweaver–Burk. Comparison of the simple linear regression values with the weighted average value ($\bar{K}_{a_w}$) showed that no statistical difference could be determined at the $F \geq 0.750$ level. Values obtained by $x$–$y$ and $y$–$x$ regressions are remarkably similar; they are characterized by a $\Delta K_a$ which is always much smaller than the least squares standard error and are within 2% of each other as judged by the $[K_a]^{x-y}/[K_a]^{y-x}$ ratio. Similar conclusions can be drawn from data analyzed by Scatchard plotting in Table 2b. Small increases in $\Delta K_a$ and $[K_a]^{x-y}/[K_a]^{y-x}$ values indicate a slightly greater error contribution by the "independent" variables in this analysis and should be expected since error in the amount bound contributes to error in both dependent and independent Scatchard variables. Hence, errors in the "independent" variable do not appear to play significant roles in the determination

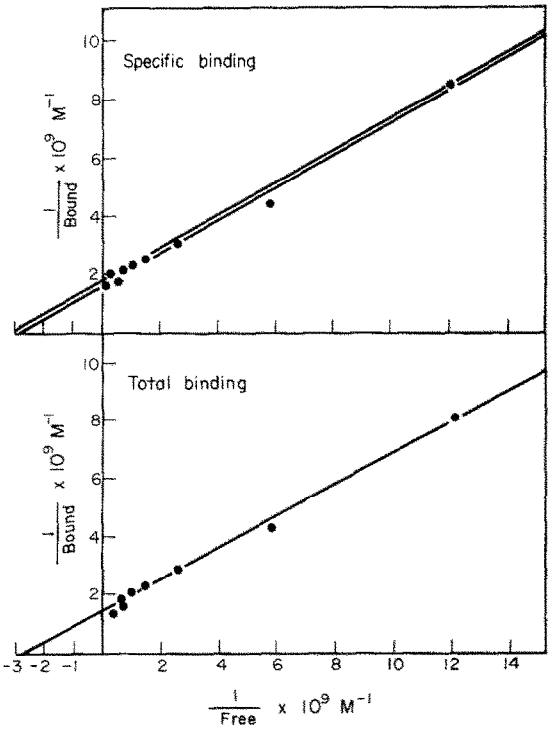


Fig. 9. Lineweaver–Burk representation of the weighted sums of squares of deviations, horizontal and vertical, about the linear equation describing $K_a$. Data described in Fig. 7a was analyzed according to Lineweaver–Burk and subjected to $x$–$y$ as well as $y$–$x$ regression analysis with both regression lines included. Average equilibrium constants ($\bar{K}_{a_w}$) were 3.030 and 2.637 × 10$^9$ M$^{-1}$ for specific and total binding respectively. Each point is a mean value ($n = 4$).

Table 2. Statistical comparison of analytical methods

A. Comparison of "Lineweaver–Burk" values for $K_a$

| Analytical information | | $\times 10^9$ m$^{-1}$ $[K_a]^{x-y}$ | [SEM]$^{x-y}$ | $K_{av}$ | $\Delta K_a$ | $\frac{[K_a]^{y-x}}{[K_a]^{x-y}}$ | $t_{sh}$ | df | $F \geq 0.750$ $P \leq 0.259$ | $r^2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Total bound vs "Δ" Free | A | 2.620 | 0.213 | 2.637 | 0.033 | 1.013 | 0.080 | 7 | N.S.* | 0.994 |
| Total bound vs Empirical free + NS | B | 3.032 | 0.240 | 3.050 | 0.038 | 1.012 | 0.075 | 7 | N.S. | 0.994 |
| Total bound vs Empirical free | C | 3.445 | 0.250 | 3.464 | 0.037 | 1.011 | 0.076 | 7 | N.S. | 0.995 |
| Specific bound vs "Δ" Free | D | 2.996 | 0.258 | 3.030 | 0.068 | 1.023 | 0.132 | 9 | N.S. | 0.988 |
| Specific bound vs Empirical free + NS | E | 3.426 | 0.273 | 3.460 | 0.068 | 1.020 | 0.125 | 9 | N.S. | 0.989 |
| Specific bound vs Empirical free | F | 3.856 | 0.274 | 3.887 | 0.062 | 1.016 | 0.113 | 9 | N.S. | 0.991 |

B. Comparison of "Scatchard" values for $K_a$

| Analytical information | | $\times 10^9$ m$^{-1}$ $[K_a]^{y-x}$ | [SEM]$^{y-x}$ | $K_{av}$ | $\Delta K_a$ | $\frac{[K_a]^{x-y}}{[K_a]^{y-x}}$ | $t_{sh}$ | df | $F \geq 0.750$ $P \leq 0.250$ | $r^2$ |
|---|---|---|---|---|---|---|---|---|---|---|
| Total bound vs "Δ" Free | A | 2.980 | 0.205 | 3.022 | 0.084 | 1.028 | 0.205 | 7 | N.S.* | 0.973 |
| Total bound vs Empirical free + NS | B | 2.943 | 0.213 | 2.989 | 0.093 | 1.032 | 0.216 | 7 | N.S. | 0.969 |
| Total bound vs Empirical free | C | 3.405 | 0.248 | 3.460 | 0.108 | 1.032 | 0.222 | 7 | N.S. | 0.969 |
| Specific bound vs "Δ" Free | D | 3.272 | 0.347 | 3.419 | 0.295 | 1.090 | 0.424 | 9 | N.S. | 0.917 |
| Specific bound vs Empirical free + NS | E | 3.670 | 0.363 | 3.814 | 0.288 | 1.078 | 0.397 | 9 | N.S. | 0.927 |
| Specific bound vs Empirical free | F | 4.044 | 0.377 | 4.184 | 0.281 | 1.070 | 0.371 | 9 | N.S. | 0.935 |

Determinations of total activity, total bound, non-specific bound and free activity were made on cytosols containing 689.3 ± 2.1 μg/ml soluble protein. Equilibrium parameters were calculated for different types of analytical information, subjected to simple linear regression analysis and $t$ test comparisons. The convention $y$–$x$ is used to refer to the regression of variable $y$ on variable $x$ such that the right-most variable is always independent.

* N.S. = not significantly different at $F \geq 0.750$.

of $K_a$. Moreover, values obtained by $x$–$y$ and $y$–$x$ regression are statistically similar. Because of this similarity, simple regression values obtained by Lineweaver–Burk analysis can be compared to values obtained by Scatchard analysis in Table 3. No significant difference ($F \geq 0.900$) could be determined for $K_a$ values obtained by either Lineweaver–Burk or Scatchard analysis. Hence, deviations of the data points are minimal by $K_a$ value criteria, indicating that the HTP assay imparts minimal error in separation of bound and free steroid.

Comparison of amounts of information and $K_a$ values is made in Table 4. It is evident that neither the analytical treatment nor the types of information obtained from HTP assay yields a range of values for $K_a$ as variable as those presented in Table 1. It is not surprising that correction for non-specific binding increases the $K_a$ value; however, $K_a$ values obtained from uncorrected data are not always unconditionally lower. Whenever comparison is made between similar types of free activity (comparisons AD, BE, CF), there is never any significant difference between information categories. Comparison of empirical free categories with "Δ" free categories (comparison AC, DF) *within* the same type of correction for non-specific binding are significantly different. Since these comparisons are independent of the effect of correction for non-specific binding, it is concluded that the amount of information and included in "free activity" influences the $K_a$ graphically (Fig. 8a and b) as well as statistically. This influence should be most pronounced whenever large amounts of non-specific binding components are present to reduce the effective amount of free steroid and hence to limit the amount of steroid which can be bound by the receptor.

Correction for non-specific binding in every case elevates the $K_a$ as is expected. The empirically determined free activity also elevates the $K_a$. Since the empirical category does not ignore the limitations imposed by the levels of free steroid on the amount bound, this category may present a choice of parameter estimation which would not be influenced by large changes in non-specific binding components.

Comparisons made in Table 5 examine the differences in total receptor concentration obtained *via*

Table 3. Comparison of $K_a$ values obtained by Lineweaver–Burk analysis with values obtained by Scatchard analysis

| Analytical information | | $\times 10^9$ M$^{-1}$ Lineweaver–Burk $[K_a]^{x-y}$ | [SEM]$^{x-y}$ | Scatchard $[K_a]^{y-x}$ | [SEM]$^{y-x}$ | $t_{sh}$ | df | $F \geq 0.90$ $P \leq 0.10$ |
|---|---|---|---|---|---|---|---|---|
| Total bound vs "Δ" Free | A | 2.620 | 0.213 | 2.980 | 0.205 | 1.219 | 14 | N.S.* |
| Total bound vs Empirical free + NS | B | 3.032 | 0.240 | 2.943 | 0.213 | 0.276 | 14 | N.S. |
| Total bound vs Empirical free | C | 3.445 | 0.250 | 3.406 | 0.248 | 0.113 | 14 | N.S. |
| Specific bound vs "Δ" Free | D | 2.996 | 0.258 | 3.272 | 0.347 | 0.637 | 18 | N.S. |
| Specific bound vs Empirical free + NS | E | 3.426 | 0.273 | 3.670 | 0.363 | 0.538 | 18 | N.S. |
| Specific bound vs Empirical free | F | 3.856 | 0.274 | 4.044 | 0.377 | 0.404 | 18 | N.S. |

Data and conversion are described in Table 2; $t$ test comparisons were made according to $t_{m+n-2} = |(\bar{x}-\bar{y})/(S(\bar{x}-\bar{y}))|$ where $(\bar{x}-\bar{y})$ = "pooled estimate of standard error" = $\sqrt{(S^2(1/m + 1/n))}$ and $S^2$ = "pooled estimate of variance" = $(m-1)S^2(x) + (n-1)S^2(y)/(m+n-2)$ where $S^2(x)$ and $S^2(y)$ are the variances about $\bar{x}$ and $\bar{y}$ respectively.

* N.S. = not significantly different at $F \geq 0.900$.

Table 4. Comparison of $K_a$ values based on different amounts of "information"

A. Lineweaver–Burk analysis

| Analytical information | | $\times 10^{10}$ M$^{-1}$ $[K_a]^{x,y}$ | $[SEM]^{x,y}$ | $n$ | $t$ Comparison A | B | C | D | E | F |
|---|---|---|---|---|---|---|---|---|---|---|
| Total bound vs "Δ" Free | A | 2.620 | 0.213 | 8 | | | | | | |
| Total bound vs Empirical free + NS | B | 3.032 | 0.240 | 8 | 1.283 | | | | | |
| Total bound vs Empirical free | C | 3.445 | 0.250 | 8 | 2.516† | 1.194 | | | | |
| Specific bound vs "Δ" Free | D | 2.996 | 0.258 | 10 | 1.086 | 0.992 | 1.231 | | | |
| Specific bound vs Empirical free + NS | E | 3.426 | 0.273 | 10 | 2.237† | 1.055 | 0.051 | 1.146 | | |
| Specific bound vs Empirical free | F | 3.856 | 0.274 | 10 | 3.415* | 2.197† | 1.081 | 2.284† | 1.111 | |

B. Scatchard analysis

| | | $\times 10^{10}$ M$^{-1}$ $[K_a]^{x,y}$ | $[SEM]^{x,y}$ | $n$ | $t$ Comparison A | B | C | D | E | F |
|---|---|---|---|---|---|---|---|---|---|---|
| Total bound vs "Δ" Free | A | 2.980 | 0.205 | 8 | | | | | | |
| Total bound vs Empirical free + NS | B | 2.943 | 0.213 | 8 | 0.125 | | | | | |
| Total bound vs Empirical free | C | 3.406 | 0.248 | 8 | 1.324 | 1.414 | | | | |
| Specific bound vs "Δ" Free | D | 3.272 | 0.347 | 10 | 0.677 | 0.757 | 0.299 | | | |
| Specific bound vs Empirical free + NS | E | 3.670 | 0.363 | 10 | 1.543 | 1.614 | 0.577 | 0.793 | | |
| Specific bound vs Empirical free | F | 4.044 | 0.377 | 10 | 2.306† | 2.370† | 1.336 | 1.507 | 0.714 | |

Data and conversion are as described for Table 2; $t$ comparison is based on $t_{m+n-2}|(\bar{x}-\bar{y})/(S(x-y))|$.
* Significantly different, $F \leq 0.995$, $P \geq 0.005$.
† Significantly different, $F \leq 0.975$, $P \geq 0.025$.

Scatchard and Lineweaver–Burk analysis. There is no significant difference between Scatchard values obtained by $x$–$y$ or $y$–$x$ regression ($F \geq 0.900$) nor between values obtained by Scatchard vs Lineweaver–Burk. Hence, there appears to be little difference between equilibrium parameters obtained by Scatchard or Lineweaver–Burk analysis irrespective of the independent variable. Thus, the deviations of the data points are also minimal by total receptor concentration criteria. This supplies sufficient evidence that HTP assay imparts minimal error in separation of bound and free steroid.

Information and total receptor concentration are compared in Table 6. All estimates of total receptor concentration based on total bound activity are significantly different from estimates based on specific binding activity. It is not startling that correction for non-specific binding clearly influences estimation of total receptor concentration.

The previous analyses have dealt exclusively with mean values. A better estimation of random or pure error can be obtained by submitting the individual data points to regression analysis. In so doing the experiment is considered to lack replication so that the residual mean square error is not reduced due to averaging of experimental design points [35]. Comparison of equilibrium parameters obtained with and without replication is made in Table 7. There is no significant difference between $K_a$ values with or without replication analyzed by Lineweaver–Burk or Scatchard; likewise no significant difference exists between estimates of total receptor concentration with or without replication analyzed by Scatchard, or when mean Scatchard values are compared to weighted average Lineweaver–Burk values.

However, comparison of total receptor concentration estimated by Scatchard techniques without replication are significantly different ($F \geq 0.975$) from values estimated by Lineweaver Burk techniques. This difference is clearly the result of non-uniformity of variance, since the difference between compared values is similar to those which are not significantly different with the $t$ value being clearly influenced by variance and degrees of freedom. Since the absolute changes in magnitude are small, averaging appears to be a legitimate means of reducing random error without greatly affecting the estimation process.

## DISCUSSION

The application of hydroxylapatite "batch" adsorption for quantitative studies of the uterine cytosol $E_2$–R has been described. There are several advantages in the "batch" method which are improvements over other established techniques in the literature:

1. Hydroxylapatite $[3Ca_3(PO_4)_2 \cdot Ca(OH)_2]$ is chemically homogeneous and obtainable in highly

Table 5. Comparison of estrogen receptor concentration values obtained by Scatchard and Lineweaver–Burk analysis

| | | Scatchard analysis $\times 10^{-10}$ mol/l $[E_2\ R]^{x,y}$ | $[SEM]^{x,y}$ | $[E_2\ R]_L^{x,y}$ | $t$ | Lineweaver–Burk analysis $\times 10^{-10}$ mol/l $[E_2\ R]_{LB}$ | $t$ | df | $F_{c} 0.90$ $P \leq 0.10$ |
|---|---|---|---|---|---|---|---|---|---|
| Total bound vs "Δ" Free | A | 6.754 | 0.206 | 6.825 | 0.345 | 7.016 | 1.268 | 7 | N.S. |
| Total bound vs Empirical free + NS | B | 6.647 | 0.211 | 6.722 | 0.359 | 6.805 | 0.749 | 7 | N.S. |
| Total bound vs Empirical free | C | 6.607 | 0.210 | 6.682 | 0.359 | 6.641 | 0.162 | 7 | N.S. |
| Specific bound vs "Δ" Free | D | 5.658 | 0.216 | 5.797 | 0.643 | 5.963 | 1.414 | 9 | N.S. |
| Specific bound vs Empirical free + NS | E | 5.604 | 0.250 | 5.722 | 0.469 | 5.845 | 0.960 | 9 | N.S. |
| Specific bound vs Empirical free | F | 5.570 | 0.184 | 5.672 | 0.553 | 5.752 | 0.991 | 9 | N.S. |

Data and conversion are previously described; $t$ comparisons are based on $t_{m-1} = |(x - \mu)/(S(x))|$.

Table 6. Comparison of $E_2$–$R_c$ values based on different amounts of "information" and Scatchard analysis

| | | $\times 10^{-10}$ m/l $[E_2\text{–}R_c]^{x-y}$ | $[SEM]^{x-y}$ | $n$ | $t$ Comparison A | B | C | D | E | F |
|---|---|---|---|---|---|---|---|---|---|---|
| Total bound vs "Δ" Free | A | 6.754 | 0.206 | 8 | — | | | | | |
| Total bound vs Empirical free + NS | B | 6.647 | 0.211 | 8 | 0.364 | — | | | | |
| Total bound vs Empirical free | C | 6.607 | 0.210 | 8 | 0.500 | 0.133 | — | | | |
| Specific bound vs "Δ" Free | D | 5.658 | 0.216 | 10 | 3.607* | 3.226* | 3.105* | — | | |
| Specific bound vs Empirical free + NS | E | 5.604 | 0.250 | 10 | 3.425* | 3.083* | 2.973* | 0.162 | — | |
| Specific bound vs Empirical free | F | 5.570 | 0.184 | 10 | 4.284* | 3.855* | 3.725* | 0.310 | 0.111 | |

Data and conversion are as previously described; $t$ comparisons are based on $t_{m+n-2} = (\bar{x}-\bar{y})/(S(\bar{x}-\bar{y}))$.
* Significantly different, $F \leq 0.990$, $P \geq 0.010$.

purified, analytical grade quantities of uniform particle size.

2. Adsorption of estrogen receptor to HTP can be performed at 0–2°C in contrast to techniques which require warming [4, 5, 8] with concomitant increases in dissociation of the steroid receptor complex.

3. HTP has little affinity for free estradiol, so that free [$^3$H]-$E_2$ can be quickly washed away.

4. DNA grade HTP (Biorad) can be tightly packed by centrifugation at 800*g* for 2 min and is easily resuspended by vortexing.

5. Due to low affinity between free estradiol and HTP, estimation of free steroid in the presence of receptor bound activity can be made by directly sampling the supernatant.

6. The sensitivity of detection was found to exist over a 50–100-fold range and is capable of detecting specific binding in aliquots of rat uterus cytosol containing as little as 5 μg protein.

7. HTP adsorption appears insensitive to changes in KCl ionic strength. Such fortuitous insensitivity should enable the quantitation of nuclear estrogen receptor after high salt extraction from the nuclear fraction.

The procedure and advantages of a modification of Erdo's original column hydroxylapatite technique [17] for extracting $E_2$–$R_c$ complexes have been outlined and discussed. These results further extend a recent report by Williams and Gorski, 1975 [37] on HTP assay. "Batch" processing provides large numbers of points which lend statistical significance to quantitative determinations. The large range of $K_a$ values reported in the literature (Table 1) suggest that the choice of technique, method of correction for nonspecific binding, temperature at which the assay binding is performed, and the receptor yield may all influence these values.

Factors influencing the determination of equilibrium $K_a$ are very complex. For example, ionic strength influences the sedimentation values of the cytosol $E_2$–$R_c$ [11, 14, 23], but does not appear to affect the association constant [14]. Hence, the buffer contribution to the value of the association constant may be minimal. Changes in $K_a$ due to short warming temperature however, may be quite important. For prolonged warming periods there is no change in $K_a$ measured under equilibrium conditions; however, with increasing temperature dissociation is kinetically more rapid than association [24, 28]. The paradoxical result is that association constants calculated by association–dissociation kinetics must decrease with increasing temperature. Hence, provided equilibrium is reached, there may be no measurable effect on $K_a$ values, but increased temperature for short times without complete "re-equilibrium" might cause association constants to behave in a fashion similar to those which are kinetically derived. Moreover, while temperature pretreatment can inactivate the receptor, it also has differential effects on specific and non-specific binding. Hence, caution should be employed when temperature manipulations are made and may explain part of the variability seen in Table 1.

Even more striking is the range of literature values found for receptor site concentration [Table 1 and References 29, 30]. Jensen has postulated variable receptor yield after processing as a possible explana-

Table 7. Comparison of equilibrium parameters obtained with mean and individual values for specific bound vs "Δ" free activity

| | 1. Lineweaver–Burk $\times 10^9$ $M^{-1}$ | | 2. Scatchard $\times 10^9$ $M^{-1}$ | | 3. Scatchard$^{(x-y)}$ $\times 10^{-10}$ mol/l | | 4. Scatchard$^{(y-x)}$ $\times 10^{-10}$ mol/l | | 5. Lineweaver–Burk $\times 10^{-10}$ mol/l | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $[K_a]^{x-y}$ | $[SEM]^{x-y}$ | $[K_a]^{y-x}$ | $[SEM]^{y-x}$ | $[E_2\text{–}R_c]^{x-y}$ | $[SEM]^{x-y}$ | $[E_2\text{–}R_c]^{y-x}$ | $t_{(df)}^{(3\text{ vs }4)}$ | $[E_2\text{–}R_c]_{ss}$ | $t_{(df)}^{(3\text{ vs }5)}$ |
| Mean values ($x$) | 2.996 | 0.258 | 3.272 | 0.347 | 5.658 | 0.216 | 5.797 | 0.643(9) | 5.963 | 1.414(9) |
| Individual values ($x$) | 3.041 | 0.224 | 3.159 | 0.255 | 5.454 | 0.150 | 5.805 | 2.336(39) | 5.802 | 2.316(39)* |
| $t_{48}(x)$ vs. ($x$) | 0.952 | | 0.208 | | 0.635 | | — | — | — | — |
| $F \leq 0.90$ | N.S. | | N.S. | | N.S. | | | | | |
| $P \geq 0.10$ | | | | | | | | | | |

Data and conversion are previously described. $t_{48}$ comparisons are based on $t_{m+n-2} = (\bar{x}-\bar{y})/(S(\bar{x}-\bar{y}))$ while $t_{(df)}$ comparisons are based on $t_{m-1} = (\bar{x}-\mu)/(S(\bar{x}))$.
* Significantly different, $F \leq 0.975$, $P \geq 0.025$.

tion [21] but procedural variability may also be responsible. Statistical analysis of HTP assay data has shown that correction for non-specific binding clearly influences estimation of total receptor concentration, while the amount or "type" of information influences estimation of $K_a$. Neither influence is large enough to explain the spread of values shown in Table 1. All methods for separation of bound from free steroid should not be considered equivalent. As a simple precautionary tactic, temperature changes should be avoided during quantitative determination and an empirical correction should be included for non-specific binding either by excess competitor or by cautious heat inactivation of the receptor [20].

As a criteria for repeatability $K_a$ values were compared from 4 different experiments performed on long-term castrate rats where levels of non-radioactive estrogen were low. None of these values were significantly different (data not shown); however, these *ad hoc* comparisons were *post-facto* and lacked *a priori* statistical discrimination. "Repeatability" can also be determined in terms of the mean square error by making $t$ comparisons. Hence, large errors would cause large deviations from some idealized line which would lie equivalent on both Scatchard and Lineweaver–Burk plots. These deviations would lead to significant differences between data obtained by Scatchard or Lineweaver–Burk analysis when tested by $t$ comparison. This approach is particularly advantageous whenever weighting functions cannot be accurately estimated because a continuous relationship between $S^2(x)$ and $x$ cannot be obtained. Comparisons between Scatchard and Lineweaver–Burk data do not show significant differences, hence the random error in the HTP assay is minimal.

Analysis of Lineweaver–Burk data suggests that the error contained in the independent variable is not significant. In addition, averaging appears to reduce random error or "experimental noise" without affecting the numerical estimates *per se*. Finally, provided that similar protein concentrations are used in each assay, the HTP assay yields similar estimates of $K_a$. Increasing the protein concentration in the cytosol results in decreasing $K_a$ values as reported by Best-Belpomme *et al.* [29] probably as a result of an increased binding potential by non-specific binding components. These $K_a$ values can be elevated by employing an empirical determination of "free" activity.

In summary, this report includes an in depth statistical validation of a batch HTP assay for uterine cytosol $E_2$–$R_c$ which will yield multiple determinations, an increase in sensitivity over current assay methods, and a low background. This assay is easy to handle, insensitive to changes in KCl ionic strength, and very reproducible. It should be especially useful in the future quantitation of either cytosol or nuclear receptors.

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