Characterization of the Interaction between 17β-Estradiol and Its Cytoplasmic Receptor in the Rat Anterior Pituitary Gland†

Kenneth S. Korach‡ and Thomas G. Muldoon*

ABSTRACT: The kinetics of the interaction between estradiol and its specific cytoplasmic receptor in rat anterior pituitary tissue have been investigated. Scatchard plots of saturation analyses, following corrections for nonspecific binding, revealed the presence of a single high-affinity component having a calculated mean equilibrium association constant (K_a) of 3.8×10^{10} M^{-1} and a binding site concentration of 0.2 pmol/mg of cytosol protein. These values were invariant between male and female rats, and among immature, adult, and castrate animals of either sex. The receptor was labile to freezing, necessitating the use of fresh cytosol in each experiment. The association rate obeyed second-order kinetics; the association rate constant had a mean value of 3.5×10^5 M^{-1} sec^{-1}, and was independent of changes in concentration of either reactant. The dissociation reaction, measured by an exchange method, was first order with a calculated concentration-independent rate constant of 1.8×10^{-5} sec^{-1}. As with the equilibrium analyses, no significant differences were observed in these kinetic parameters among groups of animals of different sex or endogenous estrogen titer. Calculated values of K_a as the ratio of the two rate constants were in good agreement with those determined from Scatchard plots. Comparison of cytosol receptor binding among anterior pituitary, hypothalamus, and uterus revealed no measurable differences among these estrogen-responsive tissues with respect to the parameters measured.

The interaction between estradiol and intracellular receptor proteins is an acknowledged primary event presumably associated with elicitation of physiological activities in estrogen-responsive tissues, as has been discussed in several recent reviews (Jensen and DeSombre, 1972; Thomas, 1973; Williams-Ashman and Reddi, 1971). While the nature of this phenomenon in the uterus has been extensively investigated, relatively little work has been devoted to receptor binding in the anterior pituitary and hypothalamus. The presence of estrogen receptors in these latter tissues was demonstrated by several groups (King *et al.*, 1965; Eisenfeld and Axelrod, 1966; Kato and Villee, 1967), and some of the physicochemical properties of the cytoplasmic interactions have been subsequently reported (Notides, 1970; Mowles *et al.*, 1971; Korach and Muldoon, 1973, 1974).

Identification and characterization of estrogen receptors in the anterior pituitary have become an important issue in the area of reproductive physiology. Early studies implicating the role of estrogen feedback on gonadotropin secretion at the pituitary level (Bogdanove, 1963) have been augmented by direct demonstrations of gonadotropin synthesis (Wakabayashi *et al.*, 1968) and release (Piacsek and Meites, 1966; Schneider and McCann, 1970) under the influence of estrogen. Assessment of the role of initial cytosol receptor interactions in ultimate alteration of reproductive function necessitates precognition of the kinetics of binding of free steroid hormone by these receptors. The present studies were undertaken to examine the kinetic and equilibrium parameters of this system, and to determine the influences of sex and endogenous estrogen levels thereupon.

† From the Department of Endocrinology, Medical College of Georgia, Augusta, Georgia 30902. *Received November 13, 1973.* This work was supported by Research Contract No. 70-2149 from the National Institute of Child Health and Human Development, National Institutes of Health.

‡ Present address: Department of Biological Chemistry and the Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, Mass. 02114.

Experimental Section

Tissue Sample Preparation. Male and female Holtzman rats were used in these studies. Adult animals (240–260 g) were castrated at least 2 weeks prior to sacrifice, where applicable; immature animals were sacrificed on day 27 of age (females) or on day 30 (males). Following decapitation, tissues were excised and collected in ice-cold buffer, to be designated in the text as TE (0.01 M Tris–1.5 mM Na_2EDTA (pH 8.0)). Uterine horns were trimmed of fat, slit lengthwise, and gently blotted before collection. The neurohypophysis was removed from the whole pituitary and discarded, and both lobes of the anterior pituitary were utilized. After exposure of the base of the brain, the entire hypothalamus was removed, limited anteriorly by the optic chiasma, posteriorly by the mammillary body, and laterally by the hypothalamic fissures, to a depth of about 3 mm. Homogenization conditions were as previously described (Korach and Muldoon, 1974). Cytosol was prepared as the supernatant fraction from a 60-min, 105,000*g* centrifugation of the homogenate at 4° (Spinco L2-65B ultracentrifuge). Protein content of cytosol preparations was determined on duplicate aliquots according to Lowry *et al.* (1951), using serum albumin standards.

Steroids. Unlabeled 17β-estradiol and cortisol were purchased from Mann and used without further purification. [2,4,6,7-^{3}H]17β-Estradiol (100 Ci/mmol, Amersham Searle) was subjected to descending paper chromatography for purification. The final product, as analyzed with a Packard 7201 radiochromatogram scanner in a single peak corresponding to cochromatographed unlabeled estradiol, was of greater than 98% radiochemical purity.

A Packard TriCarb 3375 liquid scintillation spectrometer (48% efficiency for tritium) was employed for radioactivity measurements. Counting was performed at a level which allowed <2% error at the 95% confidence limit. Conversion to disintegrations per min was by the external standards ratio method. Each sample contained 10 ml of a scintillation mixture composed of 5 g of Permablend II (Packard) dissolved in 1 l. of toluene.

Separation of Bound and Free Estradiol. With the exception of the association rate determinations, all analyses were performed on cytosol samples which were preincubated to equilibrium (18 hr, 4°) in the presence of [^{3}H]estradiol. Receptor binding was quantified by our previously described modification (Korach and Muldoon, 1974) of the protamine precipitation technique of Steggles and King (1970). Both bound and free steroid are determined by this method, since the radioactive content of the precipitated pellet and the supernatant are measured separately.

Removal of free steroid was accomplished by adsorption onto dextran coated charcoal (0.03% Norit A, 0.003% dextran), according to the method of Chamness and McGuire (1972). In standardizing this procedure in our laboratory, protamine precipitation of the final supernatant was utilized to confirm that no significant amount of free estradiol remained after charcoal treatment.

Determination of Equilibrium Association Constant (K_a). This parameter was determined directly by graphical analysis of binding data obtained when cytosol samples were equilibrated in the presence of varying amounts of [^{3}H]estradiol. For each sample, a blank, containing buffer in place of cytosol, was simultaneously processed, and the very low levels of radioactivity found in the blanks were subtracted from the sample binding in each case. Nonspecific binding (*i.e.*, low affinity, high capacity, no demonstrable ligand specificity) was assessed by analysis of samples incubated in the same manner, but with the supplemental addition of a 100-fold molar excess of unlabeled estradiol. Binding values were obtained by protamine precipitation and were plotted in the form of saturation curves. The specific binding at each level of steroid was determined as the difference between those samples containing only [^{3}H]estradiol and those containing excess unlabeled estradiol. Levels of bound steroid thus determined were normalized to that amount of binding corresponding to 1 mg of cytosol protein, and were plotted according to the method of Scatchard (1949) to yield values for the concentration of binding sites (n) and K_a.

Measurement of Association Rate Kinetics. Cytosol was utilized in these experiments within 1 hr of its preparation. This precluded prior determination of the initial receptor concentration ($[R]_0$) of the samples, which can only be quantified accurately by analysis of the cytosol after equilibration with steroid has been achieved, a period of at least 18 hr. Since $[R]_0$ must be known in order to establish the desired concentration of estradiol to be added, the problem was circumvented by preparing cytosol at a concentration which allowed very close approximation of $[R]_0$, as described previously (Korach and Muldoon, 1973). This value was employed only for initial determination of steroid level; the actual value used in the calculation of the association rate constant was obtained from Scatchard analysis of the system at equilibrium for each cytosol preparation used.

Reactions were performed at 0° in ice. The reaction mixture consisted of 1 ml of cytosol to which was added, at time zero, an appropriate concentration of [^{3}H]estradiol in 50 μl of TE buffer. At specific intervals thereafter, 50-μl aliquots were withdrawn into tubes containing 0.01 nM unlabeled estradiol in 0.5 ml of buffer. This excess of unlabeled steroid was employed to prevent, by dilution of tritiated steroid, any further measurable association which would occur during the interim in which the experiment is being conducted. Upon completion of the measured reaction, each sample was treated with protamine to precipitate the receptor complex. In order to assure that steroid adsorption to the tubes was not occurring during the time interval between the first and the final sampling, blank samples, containing buffer in place of cytosol, were analyzed in the same manner. No difference in radioactive content of the supernatant samples was observed between any two blank tubes.

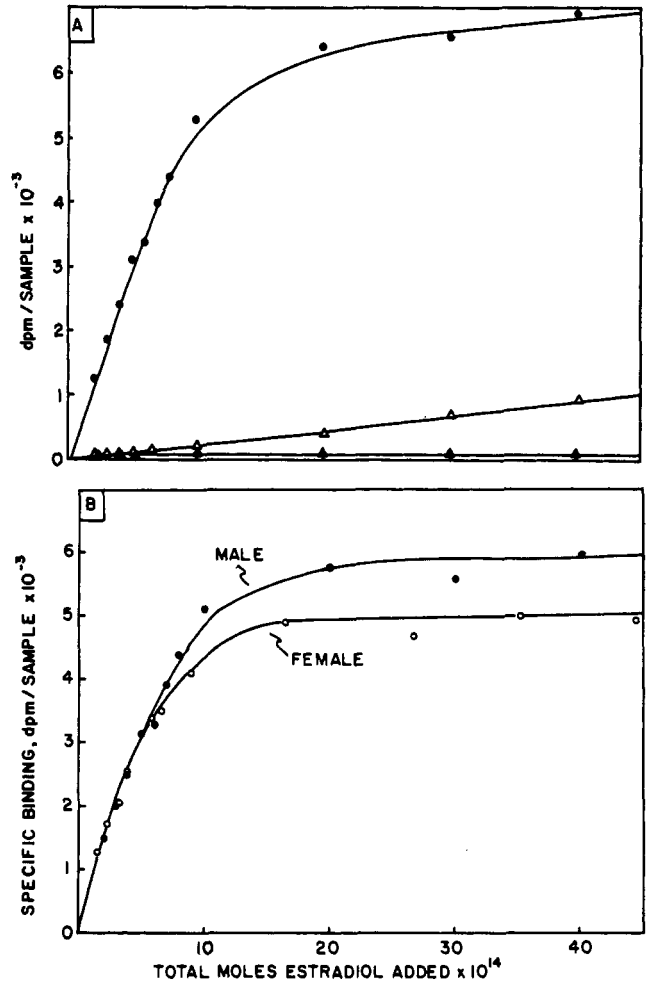

FIGURE 1: Saturation curve of anterior pituitary cytosol binding. (A) 50-μl aliquots of a sample of male pituitary cytosol (●) and cytosol prereacted with protamine (▲), having a protein concentration of 3.25 mg/ml, were incubated with concentrations of [^{3}H]-estradiol ranging from 0.02 to 0.50 nM. A portion of the same cytosol (Δ) was incubated in the presence of a 100-fold molar excess of unlabeled estradiol, in addition to the [^{3}H]estradiol. The incubations were allowed to proceed to equilibrium (18 hr, 4°), following which the bound and unbound fractions were determined directly by protamine precipitation. Blank values have been subtracted from each point. (B) The data shown in (A) (●) and similar data from female cytosol (○) are depicted as specific binding, following elimination of the contribution of nonspecific binding components. The difference between the two curves is due to a lower protein concentration in the female sample (2.78 mg/ml).

Measurement of Dissociation Rate Kinetics. An exchange method, similar to that of Sanborn *et al.* (1971), was used to study the dissociation process of the steroid–receptor complex. Cytosol samples prepared in TE buffer were equilibrated with [^{3}H]estradiol for 18 hr at 4° and then treated with dextran-coated charcoal to remove unbound steroid; an aliquot of the supernatant was analyzed for zero-time concentration of complex. A 100-fold molar excess of unlabeled estradiol was then added to the remainder of the charcoal-treated cytosol sample in order to prevent, by isotope dilution, reassociation of liberated [^{3}H]estradiol with newly available receptor binding sites. At appropriate time intervals, aliquots were taken, diluted with 0.5 ml of buffer, and immediately analyzed by the

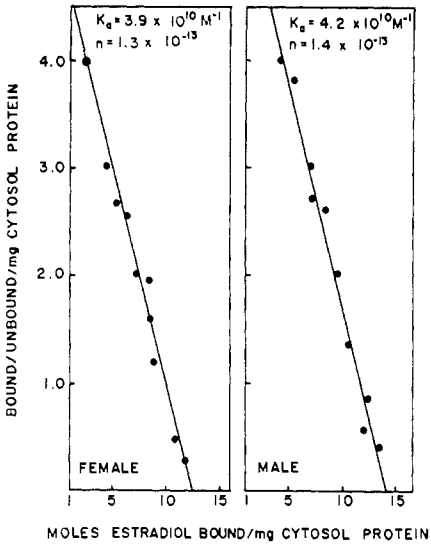

FIGURE 2: Scatchard analysis of specific estradiol binding in anterior pituitary cytosol. The data described in Figure 1B have been normalized to a protein concentration of 1 mg/ml. The saturation level (n), in moles/mg of cytosol protein, was determined as the intercept on the abscissa; the value of K_a was calculated from the slope. Linear correlation in both analyses shown had a coefficient, $r > 0.99$ ($p < 0.001$).

protamine precipitation procedure. Analysis of the number and types of dissociating systems was performed according to the procedures described by Rodbard (1973).

Results

Evaluation of Receptor Site Concentration and Binding Affinity. A representative saturation curve for [^{3}H]estradiol in male rat cytosol is shown in Figure 1A. Following saturation of the receptor system, a second binding system is encountered which is not saturated at even the highest concentrations of steroid added. There is close parallelism between this latter portion of the saturation curve and the line obtained from analysis of samples containing excess unlabeled estradiol, representing nonspecific binding. The difference between the two curves in Figure 1A represents specific interactions and has the characteristics of a single-component saturation curve, as depicted in Figure 1B. The corrected receptor saturation curve for a cytosol sample from female rat pituitary is also shown.

When cytosol was prereacted with protamine and the resulting supernatant fraction was analyzed, no binding of [^{3}H]-estradiol was observed over the range of steroid examined (Figure 1A). This finding demonstrates that protamine precipitates all measurable [^{3}H]estradiol binding proteins present in the cytosol, both specific and nonspecific, and also shows that protamine itself does not bind estradiol, an important point in overall analysis of the binding curves.

Scatchard plot analysis of the data in Figure 1B is shown in Figure 2. The linearity clearly indicates a single species of binding sites. No indication of another binding system could be detected when binding was studied using concentrations of [^{3}H]estradiol up to 10 nM (same protein concentration as shown in Figure 2) or double the cytosol protein concentration shown (same [^{3}H]estradiol concentration as in Figure 2). Freezing to $-20°$ and subsequent thawing were shown to be deleterious to both the concentration of specific binding sites and to the combining affinity of the sites remaining. A number of binding experiments were performed on samples frozen for different periods of time. Although no distinct pattern of changes was discernible, n and K_a were consistently lower than determined for fresh cytosol, even at freezing intervals as short as 3 days.

TABLE I: Anterior Pituitary Cytosol Estradiol–Receptor Interactions: Binding Site Concentration and Equilibrium Association Constants.[a]

Group	n (10^{-13} mol of Estradiol bound/mg of Cytosol Protein)	K_a (10^{10} M^{-1})
Immature female	1.1 ± 0.4	3.5 ± 0.8
Adult intact female	1.2 ± 0.1	3.5 ± 0.4
Adult castrate female	1.5 ± 0.3	3.5 ± 0.1
Immature male	1.1 ± 0.2	3.8 ± 0.3
Adult intact male	1.6 ± 0.3	3.9 ± 0.4
Adult castrate male	1.4 ± 0.2	3.2 ± 1.1

[a] Binding parameters were determined from Scatchard plot analysis of equilibrium binding data. All determinations afforded lines similar to those shown in Figure 2, with a correlation coefficient, $r \geq 0.99$ ($p < 0.001$). Values are mean of at least three separate determinations plus or minus standard error.

Binding as Related to Sex and Endogenous Estradiol Level. The binding of [^{3}H]estradiol by anterior pituitary cytosol from immature, intact adult and castrate adult rats of both sexes was assessed by Scatchard analysis, and the results are shown in Table I. From a total of 35 individual determinations of separate cytosol preparations, the calculated K_a ranged from 2.0 to 5.7 × 10^{10} M^{-1}, and the concentration of binding sites, n, ranged from 0.1 to 0.3 pmol/mg of cytosol protein. The good agreement among the different animal groups indicated that the specific concentrations and binding affinity of cytosol receptor in the rat anterior pituitary are independent of age, sex, or the presence of gonads.

Association Rate Studies. In order to achieve an independent determination of the equilibrium association constant, studies were performed to determine the association (k_{+1}) and dissociation (k_{-1}) rate constants for the reaction between estradiol and its receptor. Since Scatchard analysis showed only a single high-affinity binding component, it was initially presumed that single bimolecular association and monomolecular dissociation kinetics should suffice to describe the complete reaction. The association reaction was therefore examined as a second-order rate function, according to the equation (Frost and Pearson, 1971)

$$\frac{2.3}{[E]_0 - [R]_0} \log \frac{[E]_t}{[R]_t} = k_{+1}t + C \qquad (1)$$

where $[E]_0$, $[R]_0$, $[E]_t$, and $[R]_t$ are the concentrations of estradiol and receptor at the start of the reaction and at time, t, respectively, under conditions where $[E]_0 \neq [R]_0$.

When association rate studies are performed with anterior pituitary cytosol and the data are calculated and plotted according to eq 1, an initial linear relationship, of at least 12–15 min duration, is observed (Figure 3). In order to determine the contribution of nonspecific binding to the measured association reaction, a sample of cytosol was preincubated with a 100-fold molar excess of unlabeled estradiol prior to addition of [^{3}H]estradiol and measurement of the

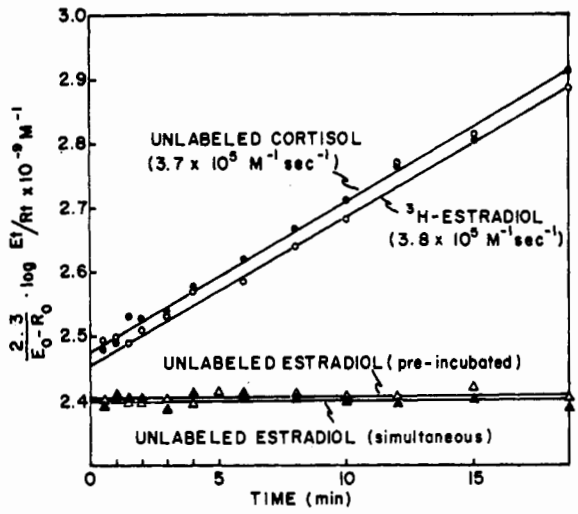

FIGURE 3: Second-order association rate plot of the interaction between estradiol and the pituitary cytosol receptor. The $[R]_0$ value for the cytosol sample used was 0.365 nM, calculated as the product of the protein concentration of the reaction mixture (3.02 mg/ml) and the binding site concentration (n = 0.12 pmol/mg of cytosol protein, determined by Scatchard analysis of saturation data). Initial [^{3}H]estradiol concentration ($[E]_0$) was 0.50 nM. One sample (○) demonstrated the association reaction when [^{3}H]estradiol was added at time zero. Two other samples were preincubated for 2 hr with 50 nM of either unlabeled cortisol (●) or unlabeled estradiol (△), and the reaction was then performed as described in the Experimental Section. In another sample, the labeled and unlabeled estradiol were added together at time zero (▲). Linearity of the plots was established by regression analysis, yielding $r > 0.99$ ($p < 0.001$).

association reaction. As seen in Figure 3, there was no detectable reaction following such pretreatment. The steroidal specificity of the reaction was examined by reacting cytosol samples at time zero with solutions containing [^{3}H]estradiol and 100-fold molar excess of either unlabeled cortisol or unlabeled estradiol. The unlabeled estradiol successfully competed for [^{3}H]estradiol binding sites, decreasing the rate to an unmeasurable level; the sample containing cortisol, on the other hand, was indistinguishable in association rate constant (3.7×10^5 M^{-1} sec^{-1}) from the sample containing only [^{3}H]-estradiol ($k_{+1} = 3.8 \times 10^5$ M^{-1} sec^{-1}).

Validity of the second-order association rate kinetics over the concentration range studied was shown by the independence of the rate constants with respect to changes in the concentrations of the reactants. Figure 4 presents results obtained when $[R]_0$ was varied and $[E]_0$ was kept constant at 0.50 nM. No significant differences in rate constant were observed between male (Figure 4A) and female (Figure 4B) samples, or among the samples examined at different receptor levels. In analogous studies, aliquots of a single cytosol sample (*i.e.*, constant $[R]_0$) were reacted with varying amounts of [^{3}H]-estradiol. The calculated values of k_{+1} were invariant within experimental error. In a representative experiment, at an $[R]_0$ of 0.325 nM, variation of $[E]_0$ from 0.2 to 5.0 nM resulted in values of k_{+1} ranging from 4.3 to 5.2 $\times 10^5$ M^{-1} sec^{-1}.

In the special case wherein $[E]_0 \equiv [R]_0$, the second-order rate equation simplifies to

$$1/[E]_t = k_{+1}t + C \qquad (2)$$

An experiment was designed wherein $[E]_0$ and $[R]_0$ were set equal at 0.5 nM, and the data were analyzed by plotting $1/[E]_t$ as a function of t. The function was linear over the time interval studied, with an association rate constant, calculated from the slope of the line, of 3.0×10^5 M^{-1} sec^{-1}. This value falls within the range of 2.1 to 6.3 $\times 10^5$ M^{-1} sec^{-1} determined for k_{+1} under conditions of varying receptor or steroid concentration. Equation 2 is a valid description of the kinetic reaction, therefore, verifying the second-order nature of the steroid–receptor complex formation.

Dissociation Rate Studies. In the simplest model possible, the dissociation of the complex into free steroid and receptor should obey simple first-order kinetics, as

$$\ln [ER] = -k_{-1}t - C \qquad (3)$$

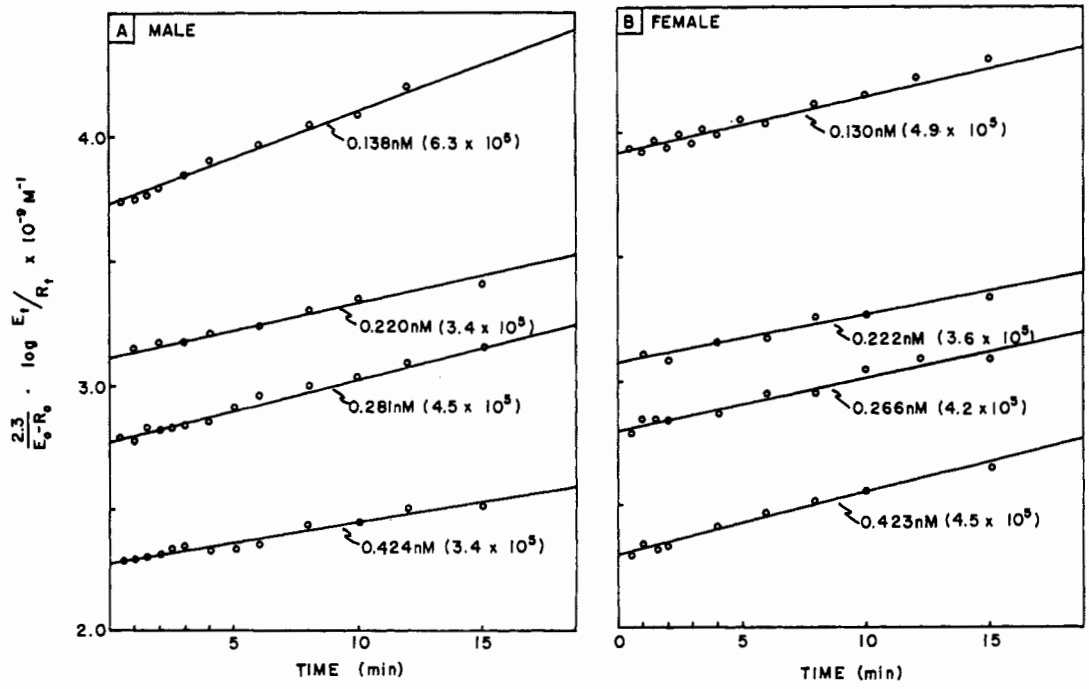

FIGURE 4: Association rate reactions under conditions of constant $[E]_0$ and varying $[R]_0$. Part A of the figure represents samples of male anterior pituitary cytosol; part B, female. Initial [^{3}H]estradiol concentration ($[E]_0$) was constant at 0.50 nM throughout. Receptor site concentration ($[R]_0$) is listed below the line corresponding to its association reaction; the numbers in parentheses are the corresponding association rate constants in M^{-1} sec^{-1}. Linearity was highly significant ($p < 0.001$) in all cases.

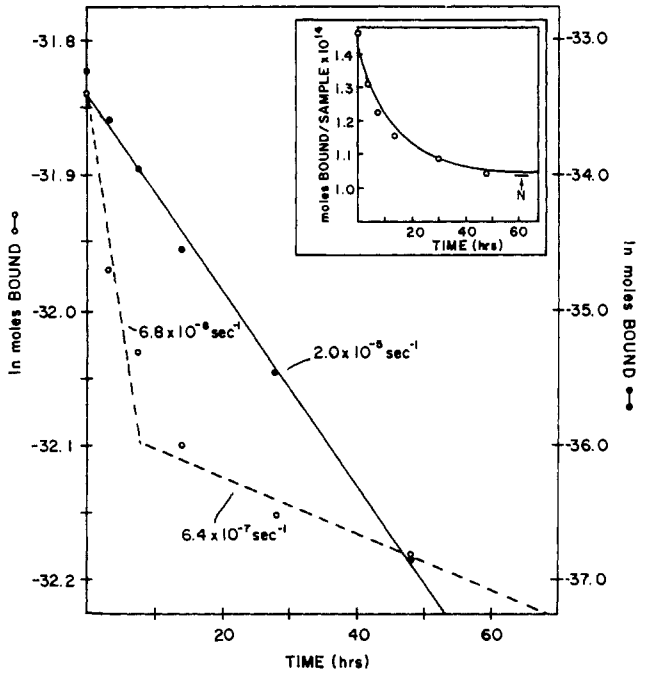

FIGURE 5: Analysis of dissociation rate curve for the pituitary cytosol receptor–estradiol complex. A 1.0-ml sample of female pituitary cytosol (2.51 mg of protein/ml) was incubated with 0.5 pmol of [^{3}H]estradiol for 18 hr at 4°. Free steroid was removed by charcoal adsorption, then 10 pmol of unlabeled estradiol was added, and the exchange reaction was performed (Experimental Section). Moles of steroid bound per aliquot is expressed against the time interval of its sampling in the insert. The level of nonspecific binding for the curve was 1.05×10^{-14} mol/sample and is indicated as "N". Analysis of the raw data (insert) as an exponential function gave a correlation coefficient of 0.853. These raw data were then depicted in a semilogarithmic plot (dotted line). The data were corrected for nonspecific binding and replotted (solid line) with a correlation coefficient of 0.999.

Considering the molar concentration of complex remaining as a function of time of dissociation should then yield a negative exponential curve, the slope of which is the dissociation rate constant.

Representative dissociation data from a sample of female rat cytosol are shown in Figure 5. The dotted line represents the two resolved segments of a direct semilogarithmic plot of the raw data depicted arithmetically in the inset. Clearly, a single-term negative exponential function was not sufficient to describe the overall dissociation reaction measured within the cytosol sample. The nature and contribution of the additional binding system(s) were determined according to the procedures established by Rodbard (1973). Attempts to fit the data to a two-term exponential equation, as would be applicable if two separate rate constants were being measured concomitantly, indicated that the data did not fit such a model.

Alternatively, the deviation from a single exponential function could be attributable to the presence of a set of functionally unsaturable nonspecific sites which bind the labeled steroid as it is released from the specific complex. The contribution of this nonspecific system to the total dissociation measured would be constant throughout the course of the experiment and eq 4 (Rodbard, 1973) would be applicable,

$$[ER]_t = [ER]_0 e^{-k_{-1}t} + N \quad (4)$$

where N is the nonspecific term and may be determined as the horizontal asymptote of the arithmetic plot (Figure 5, inset). When the data were analyzed according to eq 4, a single negative exponential function (solid line, Figure 5) was obtained, indicating that the binding of estradiol within the cytosol could be resolved into a single specific system and a nonspecific system. The dissociation rate constant determined for the corrected data (2.0×10^{-5} sec^{-1}) was appreciably different from that which represented the initial portion of the uncorrected data (6.8×10^{-6} sec^{-1}). Validation of a single exponential function remaining after subtraction of N from the curves was accomplished by examining linearization of the upper and lower halves of the data points separately. The slope and intercept of the two halves were not significantly different.

TABLE II: Association and Dissociation Rate Constants of the Reaction between Estradiol and the Anterior Pituitary Cytosol Receptor.

Group	k_{+1}[a] (10^5 M^{-1} sec^{-1})	k_{-1}[b] (10^{-5} sec^{-1})	k_{+1}/k_{-1} (10^{10} M^{-1})
Immature female	3.3	2.1	1.5
Adult intact female	3.8	1.3	3.0
Adult castrate female	3.7	2.2	1.7
Immature male	3.4	1.5	2.2
Adult intact male	3.6	2.1	1.7
Adult castrate male	3.4	1.5	2.3

[a] Determined from initial linear portions of second-order plots, with $[R]_0$ determined from Scatchard plot. [b] Determined by linearization of exponential function after elimination of nonspecific binding. Values for all rate constants were determined as the slopes of the appropriate lines. In each case, the linear correlation coefficient, $r \geq 0.99$ ($p < 0.001$).

Samples of cytosol which were treated with charcoal to adsorb excess [^{3}H]estradiol just prior to introduction of unlabeled steroid and initiation of the exchange reaction did not differ from samples which had not been exposed to charcoal, with respect to the calculated value of the dissociation rate constant. In addition, experiments were performed to determine the influence of the concentration of perturbing steroid used. Addition of either 5 or 50 nM unlabeled estradiol to identical samples of [^{3}H]estradiol-incubated cytosol was followed by analysis of the dissociation reaction. The rate constants determined for the two samples did not differ.

In Table II are summarized the calculated values of association and dissociation rate constants for cytosol from each of the animal groups for which K_a was determined directly by Scatchard analysis (Table I). The final column of the table represents the value of K_a calculated as the ratio of the rate constants; these values agree well with those obtained from direct analyses.

Receptor Interactions in Other Responsive Tissues. For purposes of comparison, a number of experiments have been performed to examine estrogen binding parameters in the hypothalamus and uterus of the rat. These data are presented in Table III. In these estrogen-responsive tissues, as in the anterior pituitary, only a single set of high-affinity sites was observed. The concentration of binding sites in the hypothalamus is much lower than in either the uterus or the anterior pituitary (Korach and Muldoon, 1974). For this reason, measurement of the association rate constant for the interaction between estradiol and the hypothalamic cytosol receptor gave values which were far less reproducible than those determined for the same parameter in the pituitary and uterus.

TABLE III: Binding Properties of Hypothalamic and Uterine Cytosol Receptors.[a]

Group	K_a (10^{10} M^{-1})	n (mol bd/mg of cytosol protein × 10^{14})	k_{+1} (10^5 M^{-1} sec^{-1})	k_{-1} (10^{-5} sec^{-1})	k_{+1}/k_{-1} (10^{10} M^{-1})
Uterus					
Adult intact	3.2 ± 0.4	10.5 ± 0.3	3.4	1.4	2.5
Adult castrate	3.9 ± 0.1	31.7 ± 8.6	3.1	1.3	2.4
Hypothalamus					
Immature female	5.9 ± 1.2	2.0 ± 0.1			
Intact female	4.4 ± 0.2	1.6 ± 0.2	8.1	2.3	3.5
Castrate female	4.3 ± 0.3	1.8 ± 0.3			
Immature male	5.9 ± 0.2	1.4 ± 0.5			
Intact male	4.4 ± 0.6	1.5 ± 0.4	9.8	1.8	5.2
Castrate male	5.5 ± 0.9	1.9 ± 0.3			

[a] Values for each parameter were determined by identical analyses as used for anterior pituitary cytosol (Tables I and II).

Calculation of K_a from the ratio of the rate constants, however, still afforded a value which was in good agreement with that from Scatchard analysis.

Discussion

In previous studies (Notides, 1970; Kato, 1971), high-affinity cytoplasmic estrogen receptor was detected within the female rat anterior pituitary. Further analysis (Korach and Muldoon, 1973) showed that male rats were very similar to females with respect to the amount of this cytosol receptor. The present study of the chemical nature and extent of these interactions indicates that the uptake mechanism leading to selective accumulation of estradiol and subsequent transfer to the nuclear site of action is the same in males and females. The female does not possess another receptor in addition to that found in the male or a greater concentration of receptor binding sites. Sex-independent receptor binding has also been demonstrated in the rat hypothalamus (Korach and Muldoon, 1974). Thus, cyclicity of gonadotropin secretion in the female must be dictated by some means other than specific tissue uptake of the steroid hormone. Possibilities include differences in nuclear mechanisms, control by higher brain centers, or influences of other endogenous factors, not directly quantifiable in an *in vitro* system, on the extent of interaction of estradiol with its receptors.

The mean value of the equilibrium association constant for complex formation in all three tissues examined, as determined by Scatchard analysis, had a range of 3.2–5.9 × 10^{10} M^{-1}. Calculated values for the same parameter as the ratio of the kinetic constants ranged from 1.5 to 5.2 × 10^{10} M^{-1}. The correlation between the two independent methods strongly supports the validity of the determinations. The agreement among animal groups and among individual tissues within each group compares favorably with analogous data from other studies (Truong and Baulieu, 1971; Sanborn *et al.*, 1971). The diversity of K_a values reported for cytosol samples from species other than the rat, in addition to the complications of multiple specific binding systems and suggestions of binding cooperativity (Sanborn *et al.*, 1971; Best-Belpomme *et al.*, 1970), makes comparison with the present study difficult. In general, however, the strongest binding affinity in all these studies is represented by a K_a of the order of 10^9–10^{11} M^{-1}.

The concentration of specific binding sites in the anterior pituitary, as determined from equilibrium binding experiments in this study, was consistent with values obtained previously using different techniques (Korach and Muldoon, 1973). Values for the hypothalamus were ten times lower than those for the pituitary and also agreed with previous reports (Vertes *et al.*, 1973; Korach and Muldoon, 1974). Although hypothalamic receptors are found primarily in the anterior portion of the gland and in the median eminence region (McGuire and Lisk, 1969; Kato, 1971), the entire hypothalamus was utilized in the present studies in order that the existence of a second binding system might not go undetected. In contrast to the anterior pituitary and hypothalamus, the uterine concentration of binding sites nearly triples following castration. This phenomenon has been observed by others (Feherty *et al.*, 1970) and may be explained, at least in part, by the loss in uterine weight following ovariectomy.

Within the experimental limits of the procedures used herein, specific binding of estradiol within rat anterior pituitary cytosol was confined to a single set of receptor binding sites. This binding appeared to obey second-order association and first-order dissociation rate kinetics. Calculation of dissociation rate constants according to the procedure delineated by Rodbard (1973) permitted analysis of the system as consisting of a high-affinity component in the presence of an unsaturable level of low-affinity binding, rather than possible misinterpretation as a dissociating system composed of two high-affinity components. The dissociation rate constant for the receptor complex did not differ among all groups of animals, having a range of 1.3–2.3 × 10^{-5} sec^{-1}, in good agreement with the values found by Ellis and Ringold (1971) and by Sanborn *et al.* (1971) for high-affinity uterine interactions.

Measurement of the association kinetics of this system has provided consistent results over a variety of experimental conditions. The only measurable association rate within the cytosol was accounted for by the specific receptor interaction, with no observed contribution by low-affinity binding. It would appear that accumulation of the low-affinity complex to the point of protamine precipitativity requires a fair amount of time relative to similar accumulation of the receptor complex. Thus, its formation was not detected within an interval of 15–20 min (Figure 3), but was detected following incubations for longer times (Figure 1A). The specificity with respect to cortisol and estradiol (Figure 3) has been exploited to study inhibition of estradiol binding by androgens (K. S. Korach and T. G. Muldoon, unpublished results), which are known to play some role in hypothalamic–pituitary feedback, but do not appear to participate in specific high-affinity interactions in these tissues (Korach and Muldoon, 1974).

References

Best-Belpomme, M., Fries, J., and Erdos, T. (1970), *Eur. J. Biochem. 17*, 425.
Bogdanove, E. M. (1963), *Endocrinology 73*, 696.
Chamness, G. C., and McGuire, W. L. (1972), *Biochemistry 11*, 2466.
Eisenfeld, A. J., and Axelrod, J. (1966), *Endocrinology 79*, 38.
Ellis, D. J., and Ringold, H. J. (1971), *in* The Sex Steroids, McKerns, K., Ed., New York, N. Y., Appleton-Century-Crofts, pp 73–106.
Feherty, P., Robertson, D. M., Waynforth, H. B., and Kellie, A. E. (1970), *Biochem. J. 120*, 837.
Frost, A. A., and Pearson, R. G. (1971), Kinetics and Mechanism, New York, N. Y., Wiley.
Jensen, E. V., and DeSombre, E. R. (1972), *Annu. Rev. Biochem. 41*, 203.
Kato, J. (1971), *in* Hormonal Steroids, James, V. H. T., and Martini, L., Ed., Amsterdam, Excerpta Medica, pp 764–773.
Kato, J., and Villee, C. A. (1967), *Endocrinology 80*, 567.
King, R. J. B., Gordon, J., and Inman, D. R. (1965), *J. Endocrinol. 32*, 9.
Korach, K. S., and Muldoon, T. G. (1973), *Endocrinology 92*, 322.
Korach, K. S., and Muldoon, T. G. (1974), *Endocrinology* (in press).
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
McGuire, J. L., and Lisk, R. D. (1969), *Neuroendocrinology 4*, 289.
Mowles, T. F., Ashkanazy, B., Mix, E., and Sheppard, H. (1971), *Endocrinology 89*, 484.
Notides, A. C. (1970), *Endocrinology 87*, 987.
Piacsek, B. E., and Meites, J. (1966), *Endocrinology 79*, 432.
Rodbard, D. (1973), *Advan. Exp. Med. Biol. 36*, 289.
Sanborn, B. M., Rao, B. R., and Korenman, S. G. (1971), *Biochemistry 10*, 4955.
Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51*, 660.
Schneider, H. P. G., and McCann, S. M. (1970), *Endocrinology 87*, 330.
Steggles, A. W., and King, R. J. B. (1970), *Biochem. J. 118*, 695.
Thomas, P. J. (1973), *J. Endocrinol. 57*, 333.
Truong, H., and Baulieu, E. E. (1971), *Biochim. Biophys. Acta 237*, 167.
Vertes, M., Barnea, A., Lindner, H. R., and King, R. J. B. (1973), *Advan. Exp. Med. Biol. 36*, 137.
Wakabayashi, K., Ogiso, T., and Tamaoki, B. I. (1968), *Endocrinology 82*, 721.
Williams-Ashman, H. G., and Reddi, A. H. (1971), *Annu. Rev. Physiol. 33*, 31.

Cholesterol and Cholesterol Sulfate as Substrates for the Adrenal Side-Chain Cleavage Enzyme†

R. B. Hochberg,* S. Ladany,‡ M. Welch, and S. Lieberman

ABSTRACT: The rates of the enzymatic cleavage of the side chains of cholesterol and cholesterol sulfate have been studied using subcellular fractions from the adrenal cortex of the rat and cow. Although impermeable to exogenous TPNH, intact mitochondria *in vitro* can convert doubly labeled [^{3}H]-cholesterol [^{35}S]sulfate into [^{3}H]pregnenolone [^{35}S]sulfate. When Ca^{2+} is present, exogenously added TPNH accelerates the rates of side-chain cleavage of both cholesterol sulfate and cholesterol. The addition of isocitrate also increases the oxidation of both substrates. However, succinate accelerates (by about 70%) the rate of side-chain cleavage of only cholesterol sulfate. The rate of oxidation of cholesterol is only slightly affected by succinate. Determination of the kinetic parameters of the two substrates indicated that the apparent K_m for cholesterol sulfate is smaller than that of cholesterol and also that the V_{max} for the conjugate is greater than that of the free sterol. Inhibition studies have shown that each of the substrates can inhibit the cleavage of the other. Cholesterol glucuronide inhibits the oxidation of neither cholesterol nor its sulfate.

One of the metabolic fates of cholesterol sulfate, a ubiquitous compound found in many mammalian tissues and body fluids, is its enzymatic conversion into pregnenolone sulfate[1] (Roberts *et al.*, 1967; Raggatt and Whitehouse, 1966; Young and Hall, 1969). The steroid conjugate serves as a substrate for the side-chain cleavage enzyme systems present in the adrenal and in the testes (Ponticorvo and Lieberman, unpublished results), through mechanisms that have been shown to occur with the retention of the sulfate moiety. In this regard, cholesterol sulfate is unique, since all other known esters of cholesterol are not transformed into C_{21} steroids without prior hydrolysis.

The experiments described in this paper were designed to investigate further the role of cholesterol sulfate as a substrate for the adrenal side-chain cleavage enzyme. Whether cholesterol sulfate can be converted into pregnenolone sulfate by

† From the Departments of Biochemistry, Obstetrics, and Gynecology, and the International Institute for the Study of Human Reproduction, College of Physicians and Surgeons, Columbia University, New York, New York 10032. *Received August 14, 1973.* This work was supported by Grants AM00110, 5T1-HD-0013, and 1P01HD5077 from the National Institutes of Health of the U. S. Public Health Service.

‡ Present address: Department of Biodynamics, The Weizmann Institute of Science, Rehovot, Israel.

[1] Abbreviations or trivial names used are: pregnenolone sulfate, 3β-hydroxypregn-5-en-20-one sulfate; pregnenolone, 3β-hydroxypregn-5-en-20-one; Et_3N, triethylammonium; progesterone, pregn-4-ene-3,20-dione; hbv, hold-back volume.