Journal of Steroid Biochemistry, Vol. 11, pp. 1065 to 1071
Pergamon Press Ltd 1979. Printed in Great Britain

THE THERMOLABILITY OF RAT UTERINE CYTOSOL ESTROGEN RECEPTORS: BASIS FOR A NOVEL CYTOSOL EXCHANGE-DENATURATION ASSAY

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(*Received 5 September* 1978)

SUMMARY

Thermal denaturation of the rat uterine cytosol estrogen receptor obeys simple first-order reaction kinetics in the temperature range from 0–48°C, whether 17β-estradiol is present or not. The rate of inactivation is five times faster in the absence of 17β-estradiol than in its presence, and was shown to be independent of protein concentration over a wide range. Analysis of denaturation data as a function of variable temperature by Arrhenius plots showed that: (1) the stability of the receptor to heat effects is enhanced dramatically by the presence of 17β-estradiol; (2) 5α-dihydrotestosterone exerts a protective effect on the receptor, whereas progesterone does not; and (3) inactivation of binding at temperatures below 30°C is much more rapid than would be predicted from its behavior above 30°C. A cytosol receptor assay has been developed which exploits the fact that denaturation of the estrogen receptor does occur during thermally-accelerated isotope exchange procedures. When receptor site exchange is performed at 40°C, bound radio-labeled steroid achieves a maximal level within 3 min, at which point bound and unbound pools of steroid are at equilibrium, with the same specific activity. For the remainder of a 10-min incubation period at 40°C, binding decreases linearly as the receptor undergoes denaturation. By extrapolation of the receptor denaturation curve to zero time and correction for dilution of specific activity, an accurate determination of total specific estrogen receptor binding site concentration is realized.

INTRODUCTION

The mechanism of action of estrogens is generally conceded to involve interaction between the steroid and cytoplasmic receptor proteins as an obligatory first step. Many features of this association have been described under carefully-regulated conditions in *in vivo* and *in vitro* experiments [1–4]. It is well-known that estrogen receptors are labile and rapidly lose the ability to bind 17β-estradiol unless temperature, pH and buffer composition are maintained within empirically specified limits [5, 6]. The loss of binding activity is thought to be the result of denaturation of the receptor, but the nature and extent of such denaturation have not been investigated. It is a very serious consideration that proper emphasis is not always placed on the possibility that erroneous estimates of estrogen-receptor binding parameters may be obtained as a result of the thermolability of the receptor. The present analysis is therefore focused on a description of thermal inactivation of the rat uterine estrogen receptor, and the effect of ligand binding on this process. We shall also describe a new cytosol receptor exchange assay, the foundation for which is focused primarily on the observed nature of the receptor thermolability.

MATERIALS AND METHODS

Adult female Holtzman rats (Madison, Wisconsin), weighing 240–260 g, were used in this study. Animals were sacrificed by decapitation. Uteri were excised immediately, cleared of adhering fat and placed in cold TE buffer (0.01 M Tris, 0.0015 M Na_2EDTA, pH 8.0). The tissues were homogenized (1 uterus per ml of buffer) in the presence of dextran-coated charcoal for the adsorption of endogenous steroid, using 10-s bursts from a Tekmar tissue grinder. The homogenate was centrifuged (Spinco L2-65B ultracentrifuge) for 60 min at 105,000 *g*, and the cytosol supernatant was collected and stored under liquid nitrogen. Separate analysis has shown that cytosol receptors are fully stable for at least 6 months when preserved in this manner. Cytosol protein was measured using the method of Lowry *et al.*[7], with bovine serum albumin as standard.

[2,4,6,7-^{3}H]-17β-Estradiol (Amersham Searle; specific activity: 100 Ci/mmol) was brought to greater than 98% radiochemical purity by successive descending paper chromatography in two separate solvent systems, according to Mahesh [8]. Unlabeled 17β-estradiol, 5α-dihydrotestosterone and progesterone were obtained from Mann Laboratories and used without further purification.

Cytosol content of unoccupied receptor binding sites was determined by saturation binding experi-

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ments in the presence of [^{3}H]-17β-estradiol (0.05–3.5 nM), with and without the addition of a 100-fold molar excess amount of unlabeled 17β-estradiol. Bound and free species of steroid were separated by dextran-coated charcoal adsorption, essentially according to Korenman[9], except for the concentration of dextran (0.05%) and charcoal (0.5%). Analysis of binding data was performed by graphical representation with the direct linear plot [10, 11]. In the development of the exchange-denaturation assay, the method of Katzenellenbogen *et al.*[12] for measuring total cytosol receptor concentration by exchange was utilized as a basis for comparison of results. Radioactivity was quantified with a Beckman LS 250 liquid scintillation spectrometer having an efficiency of 50% for tritium. Samples were counted in 10 ml of scintillation fluid composed of 5 g of Permablend II (Packard) dissolved in 1 l. toluene. Correction to d.p.m. was accomplished through utilization of the external standards ratio method.

For determination of the reaction order for receptor denaturation, cytosol samples were thawed and diluted to 0.5–1.0 mg protein/ml. Aliquots of 100 μl were incubated in glass tubes at 38 $\pm$ 0.05°C (Gilson Omnibath). Tubes were removed at selected times into an ice bath, and made 1 nM with respect to [^{3}H]-17β-estradiol. Duplicate samples were prepared to which, in addition, was added 1 μM unlabeled 17β-estradiol. Following incubation to equilibrium (18 h, 2°C), bound and unbound steroid were separated and the specific binding level was determined. Experiments designed to permit determination of reaction order in the presence of steroid were performed in a manner identical to the above, except that the steroids were added 1 h prior to thermal denaturation and incubated at 2°C. The effect of temperature on denaturation was assessed by incubation of cytosol for varying time intervals at temperatures ranging from 0° to 48°C, according to the above protocol. To measure the influence of different steroids on the receptor inactivation process, thermal denaturation experiments were conducted using cytosol samples which had been preincubated for 1 h at 2°C with [^{3}H]-17β-estradiol (1 nM) or with progesterone or 5α-dihydrotestosterone at a final concentration of 1 μM.

RESULTS

Since estrogen–receptor interactions are often complex, apparently involving several distinct forms of the receptor molecule, it was necessary initially to determine the kinetic nature of thermally-induced inactivation of receptor binding. This was performed using both 17β-estradiol-free cytosol and cytosol which was preincubated with [^{3}H]-17β-estradiol. The loss of receptor activity for either sample appeared to describe a simple first-order decay pattern when analyzed at 38°C, as shown in Fig. 1. In the presence of hormone, a 4-fold increase in stability was observed, relative to the ligand-free sample. The monophasic nature of the hormone-receptor complex denaturation indicates either that transformation to the nuclear 5S form, which is known to occur at elevated temperature in the presence of steroid [3], does not result in alteration of the rate of denaturation, or that such transformation occurs so rapidly at 38°C that the untransformed complex is not detectable under these experimental conditions, even at the very early time periods of the incubation. Germane to this point is the observation of Notides and Nielsen[13] that transformation at 37°C is essentially complete within 3–5 min.

As an independent method of determining the order of the denaturation process, recourse was made to the general equation:

$$-\frac{dR_0}{dt} = k[R_0]^n,$$

where the rate of loss of initial receptor activity, R_0, is exponentially related to that initial activity by a factor, n, which is equal to the order of the reaction. Thus, a logarithmic plot of $-dR_0/dt$ (or v_0) vs R_0 should give a straight line of slope, n. Receptor concentration was determined as 2–10 nM by direct linear plot analysis [11] of saturation binding data obtained over a range of cytosol dilutions. For each dilution, the initial velocity of denaturation at 38°C was determined as the slope of the linear decay curve. The results, shown in Fig. 2, clearly substantiate the first-order nature of the process, either in the presence or absence of 17β-estradiol, since the slope of each line is equivalent to unity.

Over a 4-fold range in protein concentration, a constant rate of receptor denaturation of ligand-free cytosol was measured, with a half-life of ~3 min (Fig. 3).

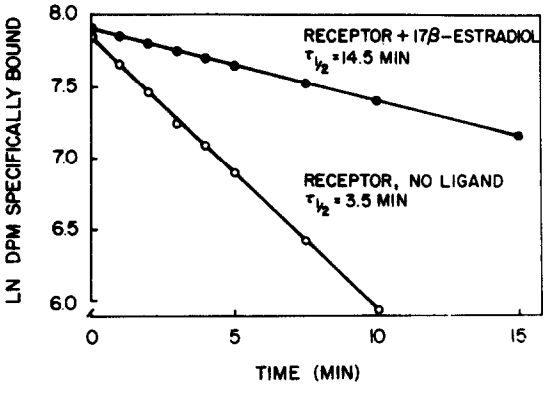

Fig. 1. Kinetics of thermal inactivation of uterine cytosol estrogen receptor in the absence or presence of added 17β-estradiol. Cytosol samples containing 1 mg protein/ml were incubated with 17β-estradiol (1 nM) either 1 h prior to (●) or immediately subsequent to (○) thermal denaturation at 38°C, as described in Materials and Methods. Specific binding was calculated by correcting for nonspecific binding levels assessed from duplicate cytosol samples containing unlabeled 17β-estradiol (1 μM) in addition to the labeled hormone.

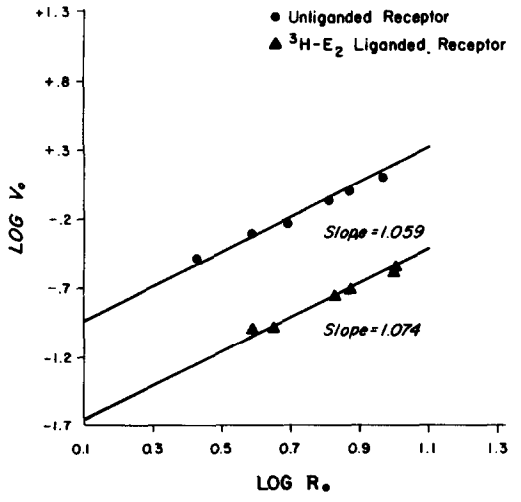

Fig. 2. Receptor decay rate as a function of receptor concentration. The specific binding capacity, R_0, of various dilutions of cytosol was determined by saturation binding analyses as 2–10 nM. For each of these samples, thermal denaturation experiments were performed at 38°C and the initial velocity of decay was measured, and is expressed in units of min^{-1}. In this and three other separate experiments, the slope of every line plotted fell within the range of 1.0 ± 0.04 (mean ± standard error), and the vertical displacement between lines representing ligand-free and ligand-containing samples did not vary.

Within the range of protein concentration used in the present studies (0.5–1.0 mg/ml), the receptor half-life remained constant, both in the presence and absence of 17β-estradiol (Table 1); it is pertinent to note that, within this chosen range, an increase in protein concentration is accompanied by a linear increase in receptor binding activity [14]. Over the course of more than 20 separate determinations, no significant deviations from linearity were observed in the analysis of denaturation rate data.

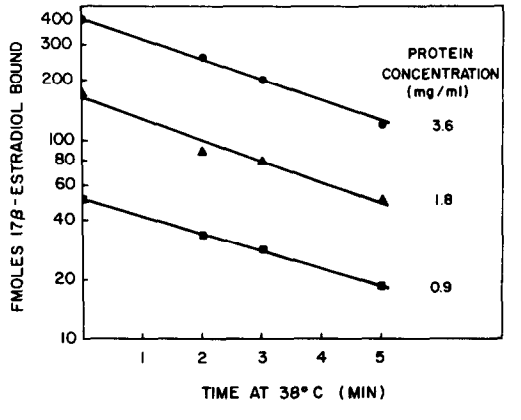

Fig. 3. Constancy of denaturation rate over a broad range of protein concentration. When cytosol samples were prepared at protein concentrations as high as seven times the concentration used in this study, thermal denaturation at 38°C remained a first-order rate function. The lines are linear ($r > 0.98$), and are of very similar slope.

The effect of temperature on ligand-free receptor denaturation was assessed by incubating cytosol for varying time intervals at temperatures ranging from 0° to 48°C. Results for selected temperatures are shown in Fig. 4; additional temperatures used, and not shown in the interest of space limitations, were 0, 16, 20, 30, 44, 46 and 48°C. Denaturation data fit a first order equation throughout the entire range of temperatures employed. An Arrhenius plot [15] was constructed from these data, according to the equation:

$$\log k_{denat} = \log A - \frac{E_A}{2.303RT},$$

where A is an integration constant relating the first order rate constant, k_{denat}, to the energy of activation, E_A, of the denaturation process. R is the gas constant and T is the absolute temperature. Since E_A represents the energy that one mole of reactant must contain before a reaction is possible, the magnitude of E_A reflects the stability of the receptor.

At temperatures greater than 30°C, the Arrhenius plot of the ligand-free receptor data is linear (Fig. 5), indicating that thermal denaturation occurs by a single-step mechanism. The E_A calculated from the slope within this range was 49.90 kcal mol^{-1}. Deviation from linearity was seen at temperatures below 30°C.

From a similar series of temperature-dependency experiments, the activation energy for denaturation of estrogen–receptor complex was determined from an Arrhenius plot (Fig. 6) as 76.40 kcal mol^{-1}. This extensive stabilization of the receptor by its favored ligand was manifested to a slight degree by 5α-dihydrotestosterone (E_A = 54.36 kcal mol^{-1}, Fig. 7A), but was not seen at all with progesterone (E_A = 49.30 kcal mol^{-1}, Fig. 7B).

By application of these observations on receptor denaturation, a simple exchange–denaturation assay has been devised for measurement of total cytoplasmic receptor content. Basically, the procedure takes advantage of the fact that, at elevated temperatures, an equilibrium state will be achieved very rapidly fol-

Table 1. Effect of protein concentration on receptor stability to thermal denaturation

Protein concentration (mg/ml)	Receptor half-life ($t_{\frac{1}{2}}$, min) No ligand	17β-Estradiol
0.5	3.87	14.69
0.6	3.44	14.75
0.7	3.95	13.97
0.8	3.30	14.06
0.9	3.24	13.73
1.0	2.95	14.38

Thermal denaturation analysis was performed as described in the text. Half-life was determined from the first-order decay patterns, all of which displayed linearity with $r > 0.97$.

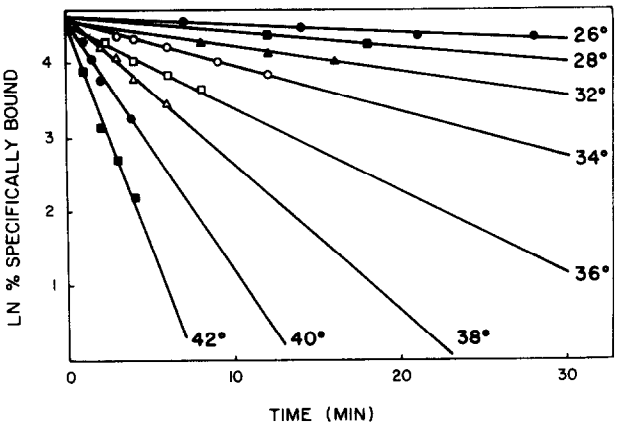

Fig. 4. Thermal inactivation of the estrogen receptor over a range of temperature. Samples of cytosol (1 mg protein/ml) were incubated at the designated temperatures (within 0.05°C) for the time intervals shown. Specific binding was determined at each time point, and the observed loss of binding activity is depicted graphically as a first-order rate function. At all temperatures chosen (including, but not shown, 0, 16, 20, 30, 44, 46 and 48°C), a linear relationship ($r > 0.97$) was apparent.

lowing addition of [^{3}H]-ligand to cytosol containing endogenous ligand-receptor complex in addition to free receptor. This equilibrium is characterized by the fact that both bound and unbound pools of [^{3}H]-ligand have the same specific activity. Beyond this point, no further increase in [^{3}H]-ligand binding to the receptor will occur; rather, the amount of bound [^{3}H]-ligand will decrease as a function of the rate of the receptor's denaturation. Since the denaturation process is linear first-order, extrapolation to the ordinate of the plotted data following achievement of equilibrium should provide an accurate measure of the total initial receptor concentration, corrected for losses due to denaturation.

An example of the manner in which the exchange-denaturation assay applies is illustrated in Fig. 8. Frozen cytosol (9 mg protein/ml) was thawed and made 1 μM with respect to unlabeled 17β-estradiol. The sample was allowed to stand for 1 h at 2°C, treated with dextran-coated charcoal to remove unbound and loosely-associated steroid, and diluted to 1 mg protein/ml. [^{3}H]-17β-Estradiol was added at a final concentration of 1 nM. Aliquots were incubated at 40°C for 0–9 min, then incubated for 5 h at 2°C prior to assay of bound and free steroid. A parallel cytosol sample was prepared with the addition of a 100-fold molar excess of unlabeled 17β-estradiol, and carried through the entire procedure as a correction for nonspecific binding.

The binding data were plotted according to the integrated first order rate equation. During the initial 3 min of incubation at 40°C, the number of binding sites detected increased 6-fold, to a maximal level representing equilibrium. Subsequently, the only measurable reaction was the first-order denaturation of the estrogen-binding sites. This linear portion of

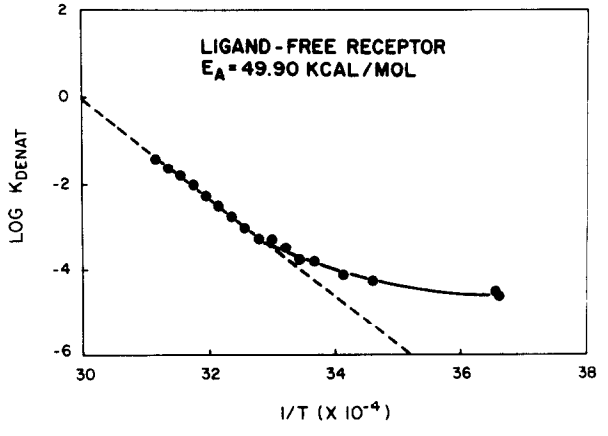

Fig. 5. Arrhenius plot of receptor denaturation in the absence of steroid. The denaturation rate constant (k_{denat}) was determined from the slopes of first-order decay patterns as exemplified in Fig. 4. The logarithm of this parameter was then plotted against the reciprocal of the respective absolute temperature at which denaturation was measured. The activation energy (E_A) was calculated from the slope of the linear portion of the resultant curve.

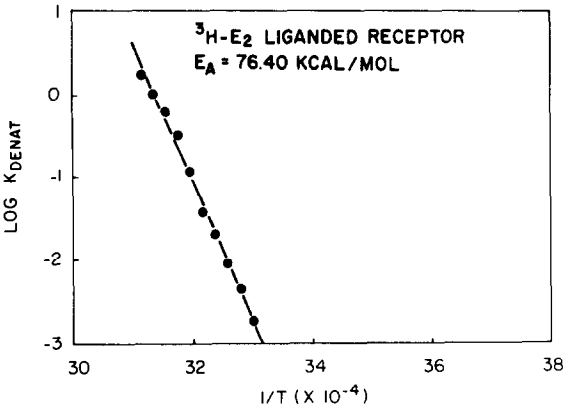

Fig. 6. Arrhenius plot of receptor denaturation in the presence of bound 17β-estradiol. Data are derived and plotted as described in the legend to Fig. 5. Cytosol samples were preincubated for 1 h at 2°C with receptor-saturating concentrations (1 nM) of [^{3}H]-17β-estradiol, with or without concomitant supplementation with unlabeled 17β-estradiol (1 μM).

the curve was extrapolated back to an ordinate value of 300 fmol/mg protein, the presumed total receptor concentration in the original cytosol sample. The values shown have been corrected for the decrease in specific activity of the [^{3}H]-17β-estradiol, according to the equation [12]:

$$P_t = \frac{DPM_f[E_2^* - (DPM_i/SA_i)]}{(SA_i \cdot E_2^*) - DPM_f}.$$

where: P_t is the corrected number of binding sites; DPM_i and DPM_f are, respectively, the disintegrations per min of specifically-bound [^{3}H]-17β-estradiol before and after exchange; E_2* is the amount of [^{3}H]-17β-estradiol added; and SA_i is the initial specific activity of the [^{3}H]-17β-estradiol.

In order to validate this assay, the following experimental protocol was utilized. Duplicate samples of cytosol (9 mg protein/ml) were prepared. One aliquot was made 1 nM in unlabeled 17β-estradiol; the other was made 1 nM in [^{3}H]-17β-estradiol. Following 18 h incubation at 2°C, unbound steroid was removed by adsorption to dextran-coated charcoal, and the samples were diluted to 1 mg/ml. Both samples were then subjected to exchange-denaturation exactly as described above (with the exclusion of the extraneous 1-min incubation point), and the corrected values of receptor binding are shown in Fig. 9. Cytosol containing binding sites partially occupied by unlabeled 17β-estradiol yielded a similar estimate of total binding sites to that obtained from cytosol which contained only [^{3}H]-17β-estradiol-filled sites. These experiments have been repeated numerous times and the results are uniformly reproducible among samples.

In a series of experiments, we have determined that results obtained. At lower temperatures, equilibrium is reached less rapidly than at higher temperatures, and at a higher level of bound steroid. However, subsequent receptor degradation also occurs at a slower linear rate, resulting in a less steep slope for the extrapolated line than observed at higher temperatures. These features should theoretically exactly counterbalance each other, and the data substantiate this; there is no significant difference in receptor levels obtained by extrapolation of data for different temperatures.

DISCUSSION

Thermolability is a known characteristic of the estrogen receptor, but it has generally been accorded the back seat role of a nuisance factor. The extraordinary differences in stability at 0–4°C, where most analyses are performed, as opposed to the physiological temperature of 37°C, dictate the need for a clear analysis of the temperature dependency of binding prerequisite to any correlative interpretations of experimental data. Moreover, this latter knowledge should provide a starting point for determination of the kinetics and loci of intracellular receptor degradation, a process which remains virtually unexplored.

In the present study, we have described the fortuitously simple process of rat uterine estrogen receptor

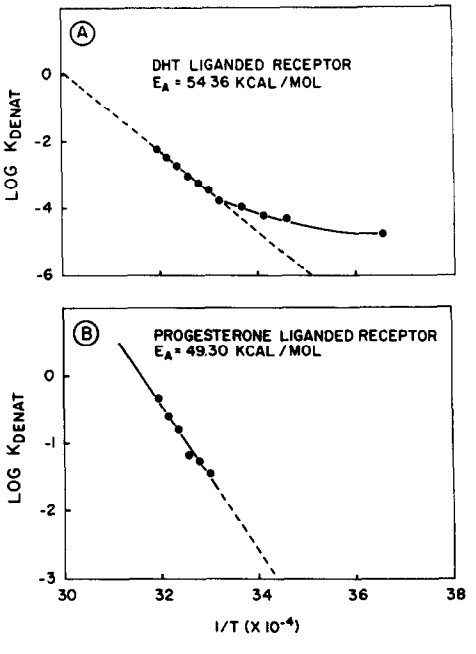

Fig. 7. Analysis of the effects of 5α-dihydrotestosterone or progesterone on the kinetic pattern of uterine cytosol estrogen receptor thermal denaturation. Cytosol samples were incubated for 1 h at 2°C with either 5α-dihydrotestosterone (A) or progesterone (B) at final concentrations of 1 μM. Thermal denaturation was then induced over a range of temperatures. Following subsequent equilibration with 17β-estradiol, the data obtained from first-order

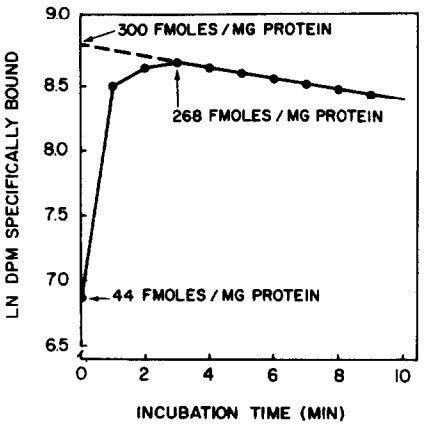

Fig. 8. Application of the exchange-denaturation assay for determination of total cytosol receptor content. Experimental protocol is detailed in the text. The [^{3}H]-17β-estradiol exchange process is plotted as a first-order reaction. At the beginning of the assay, the concentration of unoccupied binding sites was 44 fmol/mg cytosol protein. After 3 min at 40°C, [^{3}H]-17β-estradiol exchange was completed. Correction was made for dilution of specific activity of the [^{3}H]-steroid to obtain a value of 268 fmol of specific binding activity per mg protein. The subsequent linear decrease in binding with time reflected the first order thermal inactivation of the receptor. Extrapolation of this line to zero time, and correction for decreased specific activity, provides an estimate (300 fmol/mg protein) of the amount of receptor activity originally present.

denaturation by heat. The first order nature of the decay process is consistent with a conformational change in the protein [16]. It was not surprising that 17β-estradiol afforded strong protection to the receptor, as this and similar observations in other systems have been variously reported [5, 17]. Somewhat less predictable was the finding that 5α-dihydrotestosterone was capable of conferring a mild, but significant, degree of stability upon the receptor, whereas progesterone could not. It is likely that the previously-described [18, 19] low-affinity interaction between 5α-dihydrotestosterone and the estrogen receptor, not observed with progesterone, accounts for the stabilizing influence of the androgen. The energy of activation levels observed in this study for receptor denaturation are very high, as compared to that for the transformation of cytoplasmic to nuclear receptor form (19–21 kcal mol^{-1} [13]). It is therefore reasonable to expect that *in vitro* analyses of the nuclear transformation phenomenon can be performed readily under conditions where denaturation is a negligible factor.

At temperatures below 30°C, in the case of ligand-free cytosol, denaturation does not occur by the same single mechanism involved at higher temperatures. This might be explained by the presence of a proteolytic enzymatic activity at the lower temperatures, causing a more rapid loss of specific estrogen binding than would be expected from the denaturation behavior at higher temperatures. This putative receptor-degrading action would then necessarily be thermolabile in itself, losing activity at about 30°C and resulting in a homogeneous denaturation mechanism above that temperature.

Determination of total receptor in cytosol preparations is not possible using conventional methodology involving achievement of equilibrium with radiolabeled steroid at low temperatures. Under conditions where the endogenous titer of 17β-estradiol is high and variable, as in mature female animals [20], levels of occupied cytoplasmic sites can be appreciable and significant in determining overall responsiveness to the hormone. Exchange assays have been developed for determination of total nuclear [21] and cytoplasmic [12] receptor binding sites, but both assays are somewhat less than ideal for various reasons.

The nuclear receptor assay [21] entails exchange at 37°C for 1 h in the presence of a very large excess of [^{3}H]-steroid. By maintaining the level of labeled steroid very high relative to that of unlabeled steroid, the necessity for correction of final diluted specific activity becomes minor, and such correction is not made. The degradation of receptor during the exchange reaction at elevated temperature is ignored. Both of these factors tend to produce estimates of total nuclear binding levels which are lower than the true values. Moreover, the assay requires relatively large quantities of expensive radioisotope.

The assay of Katzenellenbogen *et al.*[12] for total cytosol receptor quantification by exchange also requires large amounts of radio-labeled hormone, but

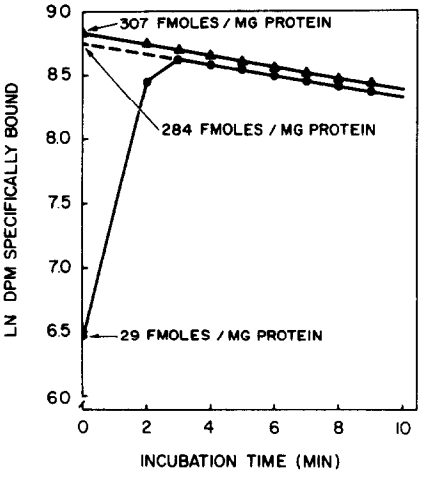

Fig. 9. Validation of the exchange-denaturation assay. Experimental protocol as described in the text; interpretations as discussed in the legend to Fig. 8. In the upper curve (▲), cytosol which had been incubated with [^{3}H]-17β-estradiol was examined. Thus, free [^{3}H]-17β-estradiol was exchanged for bound [^{3}H]-17β-estradiol, and there was consequently no change in the specific activity of the steroid. In the lower curve (●), where cytosol had been incubated with unlabeled 17β-estradiol, it was necessary to correct for decreased specific activity of the subsequently-added [^{3}H]-17β-estradiol occurring during exchange, and this correction has been made for the values shown in the figure.

overcomes the dual problems of denaturation (incubation for 18–24 h at 25–30°C in the presence of large amounts of ligand renders degradation insignificant) and dilution of specific activity (correction made, as performed in the present assay; *cf.* text). The assay does, however, become appreciably more time-consuming.

The exchange-denaturation assay is of the saturation analysis variety. It is not designed to replace classical saturation binding analysis, which must be performed additionally if information on the thermodynamic properties of the receptor system under investigation is desired. The assay will, however, allow determination of an accurate total binding capacity. A multi-point analysis would be possible with this assay if presaturation of the receptor with unlabeled steroid were not used. This would, however, obviate most of the advantages of the assay, and the exchange procedure of Anderson *et al.*[21] would be the method of choice for these purposes. The assay described herein is very rapid, requires very small amounts of radiolabeled steroid and, most significantly, accounts for denaturation occurring during the assay by the very nature of the assay being designed around this parameter. Corrections for change in specific activity are made in the same manner as used by Katzenellenbogen *et al.*[12]. Recent recognition of various classes of cytosol receptors having different nuclear translocation potential [22] emphasizes the need for a good cytosol exchange method; we recommend the exchange-denaturation assay for its speed, accuracy and economy.

Acknowledgement—This study was supported by U.S. Public Health Service grant AM 17650 from the National Institute of Arthritis, Metabolic and Digestive Diseases, NIH.

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