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Estradiol Binding of Exceptionally High Affinity by a Nonhistone Chromatin Protein Fraction*

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ABSTRACT: An estradiol binding system has been found in the nonhistone chromatin fraction of purified nuclei of calf endometrium which had not been exposed to estradiol. It exhibits hormonal and steric specificity since estradiol and diethylstilbestrol are bound to a more significant extent than 17α-estradiol, and neither testosterone nor progesterone compete for the estrogen binding sites. Studies with proteolytic enzymes and nucleases have shown that the binding component is at least in part a protein. Organ specificity is also probable as no specific binding of the hormone could be demonstrated in a similar preparation from calf liver nuclei. With the use of a charcoal adsorbent technique for removing unbound and bound with relatively low-affinity ligand, the binding parameters were studied with the computerized "proportion graph" method. The binding component was found to have an exceptionally high affinity for estradiol ($K \simeq 10^{14}$ M^{-1} at 4°), assuming that it follows the law of mass action, and that it is present only in a small number of sites (<10) per cell. The characteristics (affinity, cellular concentration, and molecular size) which differentiate it from the cytosol and the "neonuclear" estradiol receptors are discussed.

The presence of a receptor[1] for estradiol in the uterine cytosol of the castrated or immature animal is now well established (Talwar *et al.*, 1965; Toft and Gorski, 1966; Jensen *et al.*, 1967; Toft *et al.*, 1967; Baulieu *et al.*, 1967). In nuclei, there is an estradiol receptor, the formation of which appears to be dependent on the presence of both the cytosol receptor and the hormone (Brecher *et al.*, 1967; Jensen *et al.*, 1968; Shyamala and Gorski, 1968) and these dependencies justify its designation as the "neonuclear" receptor (Baulieu *et al.*, 1971). Both cytosol and neonuclear receptors can be detected in *in vitro* experiments using a radioactive estradiol concentration of $1 \times 10^{-9\pm1}$ M, and they have, at equilibrium, an apparent intrinsic association constant of the order of 10^{10} M^{-1} at 4° (Shyamala and Gorski, 1968; Puca and Bresciani, 1969; Truong and Baulieu, 1971). Calculations have shown that the number of binding sites per cell for these receptors is of the order of 10^3.

The possibility of a preexisting nuclear receptor, independent of the arrival of estradiol in the uterine cells and of the cytosol receptor, was considered, and a search was undertaken for its existence. Working with various nuclear extracts at the usual 1-nM level of the hormone, all attempts to find such a specific binding protein were negative. It was felt that this failure may have been due to the concentration of the hormone used in the experiments because if a preexisting receptor was present and had a fairly small number of sites per cell (*i.e.*, <100), the large amount of nonspecific binding found at relatively high levels of estradiol in all nuclear extracts would possibly mask this receptor. In addition, since isolation of the nuclei had to be performed in the absence of hormone and since such a receptor could be sensitive to proteolysis in the absence of its ligand (as is the case for other uterine receptors), its detection was likely to be more difficult after a rather long preparative procedure. Conversely, any exposure of the uterus or homogenate to estradiol in order to provide some degree of protection for the protein could provoke the formation of the neonuclear receptor, which in turn would conceal the presence of any preexisting receptor, especially if per cell, these are very few in numbers.

In order to minimize the effect of these interfering factors in the quest for a nuclear receptor in uteri not previously exposed to estradiol, the binding ability of various nuclear fractions was examined at very low concentrations of estradiol (the order of 1 pM). By so doing, it was possible to demonstrate an estradiol specific binding system in the chromatin nonhistone protein fraction of calf endometrium purified nuclei. The qualitative specificity was derived from studies with various hormones and from its absence in a similar preparation of calf liver nuceli. No such binding system was found in any other uterine nuclear fraction and in addition, there was some evidence that the molecular weight of this receptor was less than that of the cytosol and neonuclear receptors.

The binding parameters were the most striking characteristics of this system. They were determined by the use of a charcoal adsorbent technique and the computerized proportion graph method. The calculated number of estradiol binding sites per cell was found to be less than 10. Assuming that

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[1] An review of the terms cytosol, receptor, neonuclear, specific binding, etc., is found in Baulieu *et al.* (1971).

the law of mass action was obeyed, the affinity constant was found to be greater than that of the other uterine receptors by at least three orders of magnitude. In fact, it has not been demonstrated whether or not binding is reversible.

Materials and Methods

[6,7-^{3}H]Estradiol (50 Ci/mmole) was obtained from the Commissariat à l'Energie Atomique, [2,4,6,7-^{3}H]estradiol (100 Ci/mmole) from Amersham, and [1,2-^{3}H]testosterone (46.5 Ci/mmole) from Tracer Lab. The radioactive hormones were purified by column chromatography and their purity verified by thin-layer chromatography.

Unlabeled steroids were from Roussel-UCLAF.

Enzymes were purchased from Sigma with the exception of trypsin which was obtained from Merck.

All sucrose solutions were prepared in 5 mM Tris·HCl (pH 7.4) and contained 3 mM $CaCl_2$, with the exception of the 0.33 M sucrose solution which had a calcium concentration of 4 mM.

"TEG" buffer contains 10 mM Tris·HCl, 1.5 mM EDTA, and 5 or 20% glycerol (pH 7.4). The percentage of glycerol is indicated in the brackets (for example, TEG (5%)).

Protein was determined by the Folin–phenol method of Lowry *et al.* (1951), and DNA as described by Burton (1956).

Isolation of Nuclei. Nuclei were isolated essentially according to Chauveau's technique (Chauveau *et al.*, 1956) as modified by Wang (1967) and all operations were performed at 4°.

Calf uteri were collected on ice at the slaughterhouse and transported to the laboratory as soon as possible after slaughter. The uteri were slit longitudinally and the endometrium removed by scraping with a scalpel. The yield of endometrium was approximately 1 g/uterus. The tissue was suspended in three volumes of 0.33 M sucrose containing 0.2% octanol to prevent foaming and 1 mM diisopropyl flurophosphate and homogenized by means of an Ultra Turrax for 10–15 sec at a high-speed setting. The homogenate was filtered through four layers of gauze, and centrifuged for 10 min at 600*g*. The crude nuclei were homogenized in three volumes of 0.25 M sucrose with the tight-fitting pestle of a Dounce homogenizer, transferred to a centrifuge tube, underlaid with an equal volume of 0.34 M sucrose and centrifuged for 15 min at 1500*g*. This step was not essential in the isolation procedure but it was employed as a means of reducing the volume of sucrose needed in the next step. The pellet was homogenized in nine volumes of 2.4 M sucrose with the tight pestle of a Dounce and centrifuged in a swinging-bucket rotor for 60 min at 45,000*g*. The pure nuclei obtained at this point were dispersed in 0.25 M sucrose, centrifuged for 10 min at 1500*g* and stored at −20° until needed. The average yield was 1 g of nuclei/10 g of tissue and the recovery based on DNA determinations was between 40 and 50%.

Preparation of Nuclear Protein Fractions (Scheme I). The nuclear fractions were prepared at 4° as described by Wang (1966). Purified nuclei were dispersed in ten volumes of 50 mM Tris·HCl–5 mM $MgCl_2$ (pH 7.4), homogenized with the tight pestle of a Dounce, extracted for 30 min by gentle magnetic stirring, and centrifuged for 10 min at 1500*g*. The supernatant was decanted and saved, and the pellet was subjected to two additional extractions in the same manner. The combined extracts were centrifuged for 60 min at 105,000*g*, and this final supernatant was considered as the soluble protein fraction.

After the removal of the soluble nuclear proteins, the pellet remaining which consists of the deoxyribonucleoproteins and "residual proteins" is usually considered at the chromatin. The deoxyribonucleoproteins were extracted with ten volumes of buffered 1 M NaCl by homogenization followed by stirring for 2 hr. The addition of 1 M NaCl to the chromatin resulted in an immediate and marked change in viscosity and in order to facilitate extraction, the following modification was adopted. The pellet was suspended with the tight pestle of a Dounce in as much 20 mM Tris·HCl–3 mM EDTA (pH 8) as necessary to ensure a fairly homogeneous mixture. An equal volume of 2 M NaCl in 40% glycerol was added to give a final concentration of 1 M NaCl in TEG (20%) and the mixture stirred for 2 hr. The final volume of NaCl was about 20–30 volumes of the original wet weight of nuceli, and a single 2-hr extraction was sufficient for the removal of the majority of the deoxyribonucleoproteins. The extract was centrifuged for 30 min at 47,000*g* and the resulting supernatant which consisted of the dissociated DNA, histones, and nonhistone proteins was decanted and the residue saved for obtaining the residual proteins.

The supernatant was dialyzed overnight against 30–50 volumes of TEG (20%); the reassociated DNA–histone was precipitated and the bulk of the so-called nonhistone chromatin proteins remained in solution. The two fractions were separated by centrifugation for 30 min at 47,000*g*. The nonhistone protein fraction was centrifuged 60 min at 105,000*g* and concentrated either by Diaflo press with a membrane excluding molecular weights lower than 1000, or by placing the extract in washed dialysis tubing and embedding the sac in Sephadex G-200. The final volume was usually two to six times the wet weight of the nuclei and contained 1–2 mg of protein/ml. There was no DNA present in the final extract and there was a constant 40–50% loss in Folin-reacting material during concentration.

In order to obtain residual proteins, the pellet remaining after removal of the soluble protein and deoxyribonucleoprotein fractions was extracted with ten volumes of 50 mM Tris·HCl (pH 8.5) containing 1% deoxycholate by suspension in a Dounce homogenizer followed by 30-min magnetic stirring at 4°. The extract was first centrifuged for 15 min at 1500*g* and the supernatant obtained was recentrifuged for 60 min at 105,000*g*. It was then passed through a small Sephadex G-25 column in order to remove the deoxycholate.

Binding Experiments. DIALYSIS. At the usual concentrations of [^{3}H]estradiol (0.1–1 nM) used for demonstrating estradiol receptors, no indication of specific binding was seen when a nuclear nonhistone protein fraction of uteri not previously exposed to the hormone was dialyzed for 47 hr at 4° against these concentrations. By this is meant that although some binding was detected, the dilution of the radioactive tracer by the addition of 5- to 100-fold of nonradioactive hormone did not show the expected decrease of bound radioactivity. Such a decrease should be observed if, in a concentration range compatible with the solubility of the hormone, the binding obeyed the law of mass action and had sites which could be saturated. This lack of a decrease (provided it was reversible) indicated a nonspecific binding.

However, at the low [^{3}H]hormone levels of 1–10 pM, a decrease was observed, thus implying the presence of a specific binding system. In spite of these promising early results, difficulties encountered in dialysis experiments with low radioactive levels were too numerous and this method had to be abandoned in favor of the dextran-coated charcoal adsorption technique.

ADSORPTION TECHNIQUE. This technique is based on the principle of the "differential dissociation" observed in a mixture of specific and nonspecific binding proteins and ensures

the elimination of almost all, if not all, of the rapidly dissociating (nonspecific) complexes (Milgrom and Baulieu, 1969).

To obtain binding, the extract (1 ml) was incubated at 4° with radioactive hormone or the ^3H tracer plus nonradioactive compounds for various periods of time. In qualitative experiments for hormone and tissue specificity and for the studies of the effect of various enzymes, incubation was for 90 min. It was seen that equilibrium was not reached after this period of time and in quantitative experiments, this incubation was maintained for 18 hr, a time at which equilibrium was obtained.

At the end of the incubation with hormones, an equal volume of 0.06 25% (w/v) charcoal–0.00625% dextran suspension and TEG (20%) was added and the incubation continued for an additional 2–4 hr in order to remove unbound steroid and steroid which had dissociated from nonspecific complexes. For each estradiol concentration, blanks were obtained by incubating 1 ml of buffer with the labeled hormone, and the experiment to blank ratio were at all times greater than 10. In experiments designed to determine the specificity and the effects of various enzymes, the charcoal treatment lasted 2 hr, and for the binding curves 4 hr. At the end of this incubation, the protein–charcoal mixtures were transferred to previously chilled centrifuge tubes and centrifuged for 10 min at 1500*g*. The supernatant containing the protein–radioactive hormone complex was removed by means of a Pasteur pipet and saved for counting. All points for extracts, blanks, and controls for total radioactivity were done in duplicate.

Since in the dialysis experiments, even at low estradiol levels, it was noted that the nonspecific binding detected by the residual bound radioactivity was large in comparison to the specific binding, the charcoal technique was a particular attractive means of reducing this nonspecific binding. Nevertheless, the low levels of binding again presented technical problems.

At concentrations of 0.1–1 nM, the amount of charcoal needed to remove within 2–4 hr the free ligand and/or that released from nonspecific binding was relatively high (0.25% charcoal) and likely to alter or adsorb the specific binding protein (as observed in the transcortin system, Milgrom and Baulieu, 1969). Conversely, with a relatively weak concentration of charcoal, too much free and nonspecifically bound radioactivity would remain. At low estradiol levels, one should expect an almost complete removal of the nonspecific binding. This was found to be true, and with the amount of charcoal used in these experiments, no difficulty was encountered until the level of 50 pM. Above this concentration, it is impossible to guarantee the complete removal of the nonspecific binding by the charcoal concentration used in these studies. However, since the larger part of the nonspecific binding was removed, the specific binding could be detected and its binding parameters determined.

Counting and Calculations. Aliquots of 1.5 ml were counted in 15 ml of Bray's solution (Bray, 1960) for at least two 50-min periods in a Packard liquid scintillation counter. All counts were corrected for quenching by means of an external standard and expressed as disintegrations per minute per milligram of protein.

Binding equilibrium parameters, the concentration of binding sites and the intrinsic association constant, K, were obtained by the computerized "proportion graph" technique (Baulieu and Raynaud, 1970).

This representation was designed to study binding parameters at equilibrium of a given ligand to two (or eventually more) different binding systems simultaneously present in a

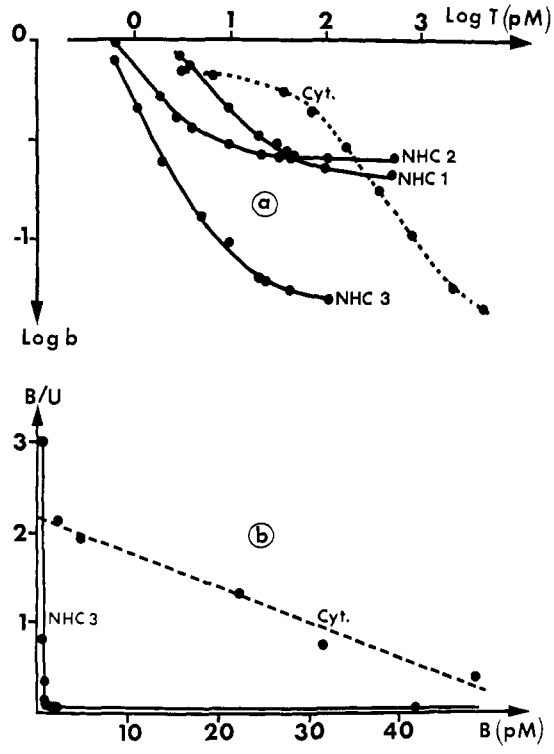

FIGURE 1: Estradiol binding to chromatin nonhistone protein and to cytosol receptor. (a) Proportion graph: log *b* (the proportion of bound ligand) is plotted against log (T) (the total ligand concentration expressed in pM). (b) Scatchard plot: (B)/(U) (bound ligand concentration over unbound ligand concentration) is plotted against (B) (bound ligand concentration expressed in pM). (●—●) Chromatin nonhistone preparation in expt 1, 2, and 3 in part a and 3 in part b. (For individual values, protein concentrations and experimental conditions, see Table VI and text.) (●----●) Calf uterus endometrium cytosol (0.84 mg of protein/ml).

biological medium. It is assumed that they obey the law of mass action and are independent. They are usually "specific" binding system (S) of high affinity, low capacity, and therefore saturable and "nonspecific" system (NS) of low affinity, high capacity, and therefore nonsaturable.

The addition of any binding system to a specific one does not allow linerization of binding data by classical representations such as the Scatchard plot or the reciprocal plot and the coordinates of these methods necessitate the use of only a relatively narrow range of ligand concentration (see Figure 1b).

The new graphical method consists of plotting the log of the proportion of bound ligand *b*, or of unbound ligand *u*, *vs.* log (T), (T) being the total ligand concentration. *b* is equal to (B), the bound ligand concentration divided by (T) and *u* is equal to (U), the unbound ligand concentration divided by (T). The range of ligand concentration should be the largest possible. After graphical estimation of the parameters, there follows a computerized statistical evaluation of the various possible models. In the situation where there is a mixture of one specific and one nonspecific system, K_S, the apparent intrinsic association constant (expressed in M^{-1}) and (N_S), the concentration of binding sites of the specific system, as well as the product $K_{NS}(N_{NS})$ of the nonspecific system, are obtained.

The number of sites per cell was calculated on the basis of the concentration of binding sites in the nonhistone fraction and the corresponding number of cells. The latter was based on the DNA content of the extract, 6 pg of DNA/cell nucleus

TABLE I: Fractionation of Calf Endometrium and Liver Nuclei.[a]

Fraction	Endometrium		Liver	
	mg/g of Nuclei		mg/g of Nuclei	
	Protein	DNA	Protein	DNA
Soluble protein	8	0	8	0.5
Total 1 M NaCl	38	30	28	28
Nonhistone chromatin protein	8	0	6	0
Residual protein	3	1	11	1

[a] Fractions are defined according to Scheme I. The nonhistone chromatin protein values are those observed before concentration (see text). After concentration these values were halved.

TABLE II: Effect of Enzymes on the Binding of [^{3}H]Estradiol by the Nonhistone Chromatin Proteins.[a]

a. Various Enzymes	
[^{3}H]Estradiol	150
[^{3}H]Estradiol + estradiol	77
[^{3}H]Estradiol + Pronase	62
[^{3}H]Estradiol + trypsin	105
[^{3}H]Estradiol + desoxyribonuclease	126
[^{3}H]Estradiol + ribonuclease	126
[^{3}H]Estradiol + lipase	121
b. Pronase	
[^{3}H]Estradiol	110
[^{3}H]Estradiol + estradiol	60
[^{3}H]Estradiol + Pronase	50
[^{3}H]Estradiol + Pronase + estradiol	50

[a] The extracts which contained 0.93 mg of protein/ml in "*a*" and 1.00 mg/ml in "*b*" were incubated at 4° for 2 hr with 6 pM [^{3}H]estradiol or with the same concentration of radioactive hormone plus 2 nM nonradioactive estradiol. Pretreatment with 1 mg/ml of the enzymes was carried out at 4° for 2 hr. The pH of all preparations was 7.4. The trypsin used in these experiments had an optimum pH of 7.5. Binding was measured by the adsorption technique described under Methods and bound radioactivity is expressed in disintegration per milligram of protein.

(Venderly, 1955) and the assumption of no alteration of the binding sites.

Results

Fractionation of Calf Endometrium Nuclei. Table I presents the protein and DNA of the various nuclear fractions of calf endometrium nuclei and, for comparison to a nontarget organ, of calf liver nuclei. It can be seen that although the overall protein content is about the same for the two tissues, there are some differences in their distribution. There is a consistent 25% difference in the total deoxyribonucleoprotein and nonhistone protein fractions and more striking is the higher residual protein contents (fourfold) of the nontarget tissue.

Specific Estradiol Binding by the Acidic Protein Fraction Is Due to a Protein. In Table IIa, it can be seen that in the endometrium nonhistone chromatin fraction, Pronase pretreatment decreased the binding of 6 pM [^{3}H]estradiol by more than 50%. Trypsin also decreased the binding, but to a lesser extent. The small decrease of bound radioactivity observed in the presence of lipase and nucleases might have been due to some protease contamination of these preparations.

In Table IIb, the results of isotopic dilution experiments with intact and Pronase-treated nonhistone chromatin fractions are presented. It can be seen that the decrease due to Pronase pretreatment is of the same order as the decrease provoked by estradiol competition, and this indicates that Pronase may destroy the specific binding while leaving a large part of the nonspecific binding, quantitatively intact. Isotopic dilution experiments on the binding observed after Pronase treatment indicated that it was nonspecific.

Other unpublished experiments with uterine cytosol-containing estradiol receptors have shown an easy destruction of high-affinity specific binding proteins by Pronase (a lesser effect of trypsin) whereas quantitatively, the nonsaturable nonspecific binding was left either intact, or slightly decreased or eventually increased by the proteolytic treatment. Incidently, bovine serum albumin displays the same behavior as a nonspecific binding fraction.

Only the Nonhistone Protein Fraction Displays Estrogen Specific Binding. A comparison of the binding ability of various nuclear fractions is reported in Table III. For this experiment, an additional salt extraction by 0.3 M NaCl was performed before the usual 1 M NaCl step. After removal of the soluble nuclear proteins, the pellet reamining was extracted twice with 0.3 M NaCl in TEG (20%), the extract centrifuged for 60 min at 105,000*g*, and the salt removed by dialysis.

It can be seen that the nonhistone chromatin fraction displayed some saturable binding and that there was some decrease by Pronase but not by nucleases treatment. The extraction by 0.3 M NaCl removed some saturable binding, equivalent to approximately one-fourth of the nonhistone chromatin saturable binding. Although its specific activity was lower than that of the 1 M NaCl extract, the effect of isotopic dilution and enzymes on the binding showed a similar pattern for the two extracts. There was very little estradiol binding

SCHEME I: Flow Diagram for Fractionation of Nuclear Proteins

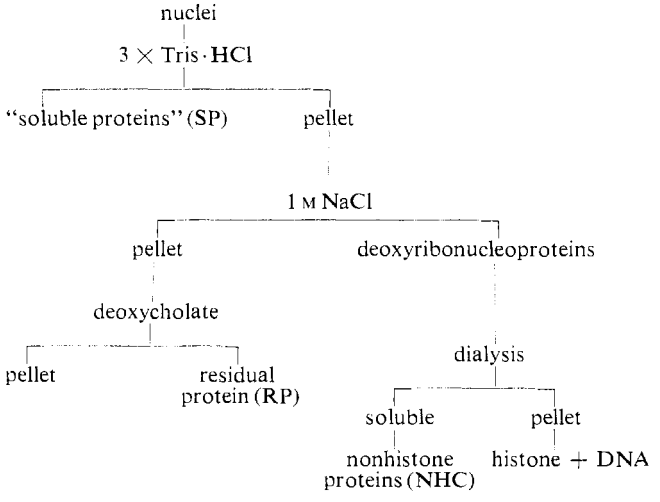

TABLE III: Estradiol Binding by Endometrium Nuclear Fractions.[a]

	SP[b]	0.3 M NaCl	NHC[b]	RP[b]
[^{3}H]Estradiol	62	187	449	382
[^{3}H]Estradiol + estradiol	41	142	302	450
[^{3}H]Estradiol + Pronase	101	99	218	751
[^{3}H]Estradiol + DNase	71	198	443	600
[^{3}H]Estradiol + RNase	111	198	433	600
Protein (mg/ml)	0.79	1.55	0.45	0.29

[a] The extracts (see Methods and Scheme I) were incubated with 6 pM [^{3}H]estradiol or with the same amount of ^{3}H tracer plus 40 mM nonradioactive estradiol. The conditions of incubation and pretreatment with enzymes are the same as described in Table II. Bound radioactivity is expressed in disintegration per minute protein. [b] SP, soluble protein; NHC, nonhistone chromatin protein; RP, residual protein.

TABLE IV: Estradiol Binding in Nuclear Fractions of Different Molecular Weights.[a]

	SP[b] (dpm/mg)		NHC[b] (dpm/mg)	
	>50,000	<50,000	>50,000	<50,000
[^{3}H]Estradiol	65	210	114	151
[^{3}H]Estradiol + estradiol	65	210	170	0
Protein (mg/ml)	0.97	0.30	0.55	1.03

[a] Filtrant and filtrate after Amicon membrane filtration are referred to as >50,000 and <50,000, respectively, since the distinction is based on differences of molecular weights. The soluble and NHC protein fractions were dialyzed 24–36 hr at 4° against 3 pM [^{3}H]estradiol or the same concentration of ^{3}H tracer plus 1 nM estradiol in TEG (20%). Bound radioactivity is expressed in disintegration per minute protein. [b] SP, soluble protein; NHC, nonhistone chromatin protein.

in the soluble protein fraction, and Pronase did not decrease but actually increased the binding. Ribonuclease consistently increased the binding of this fraction. In any case, if there was some specific binding in the soluble protein fraction, it was very small.

In the residual fraction, the binding was relatively high but there was no evidence of specificity as judged by the lack of an effect of isotopic dilution, and all enzymes increased the binding. It should be kept in mind that it is quite difficult to remove all of the deoxycholate from this fraction, and perhaps a trace left in the extract was enough to interfere with the specific binding of the hormone. Other methods of extraction of the residual proteins, excluding the use of detergents, have also been tried and in no case was any saturable specific binding observed in this fraction.

Molecular Size of the Binding Protein. ULTRAFILTRATION EXPERIMENTS. The nonhistone protein fraction and for comparison, the soluble protein fraction, were filtered by Amicon ultrafiltration membrane which excluded molecular weights less than 50,000. The filtrate and the filtrant were tested for their ability to bind estradiol and the results can be seen on Table IV. Binding experiments showed no binding of limited capacity (at least until a concentration of 1 nM) in the soluble protein fraction and in the nonhistone protein fraction mol wt >50,000. Only the proteins found in the histone fraction mol wt <50,000 showed specific estradiol binding.

GLYCEROL GRADIENT ULTRACENTRIFUGATION EXPERIMENTS. In ultracentrifugation studies, the easiest way to detect high-affinity binding is to incubate the protein with radioactive ligand and to use a ligand-free medium. The various complexes dissociate at different speeds and only those of high affinity remain associated. However, due to the very small number of sites and to the possible small size of this binding protein, it was evident that one should work at very low ligand concentration. If not, the peak of bound radioactivity might have been hidden by other binding systems of smaller affinity but larger capacity, which eventually could dissociate during centrifugation. Dissociated ligand and any free hormone already present could then mask the peak of the high-affinity protein near to the top of the gradient.

In these studies, the nonhistone chromatin protein fraction was freed of glycerol by dialysis against 10 mM Tris·HCl–1.5 mM EDTA (pH 7.4) buffer before layering on a Tris·EDTA-buffered 5–35% glycerol gradient. In some experiments, the nonhistone proteins were prepared in TEG (5%) and layered on a buffered 7.5–35% glycerol gradient; both methods gave identical results. Notwithstanding the very low [^{3}H]estradiol concentration which were used, results were often not satisfactory. Under the best circumstances, an extract labeled with 2 pM [^{3}H]estradiol gave a small peak of radioactivity at a sedimentation coefficient of approximately 1.8 S.

Control experiments were performed to determine if the radioactivity found in the 1.8S region of the gradient really represented bound estradiol. [^{3}H]Estradiol-incubated extracts were used in two ways. Either they were treated with charcoal before layering on the gradient in order to remove the free hormone, or they were first ultracentrifuged and each fraction was subjected to the charcoal treatment. In both cases, a peak of bound radioactivity was at all times present in the 1.5–2S region. Since the amount of charcoal used for these studies was more than sufficient for the removal of any free ligand, it was concluded that a small extradiol macromolecule complex had been observed.

Hormone and Organ Specificity of the Estradiol Binding. In parallel with the findings with endometrium nonhistone chromatin protein fraction, in Table V, are reported the results obtained with a corresponding fraction of the liver. In the uterine preparation, testosterone did not bind to the same sites as the estrogen, since no decrease in bound radioactivity was seen when the [^{3}H]estradiol tracer was diluted by the androgen. The competition of transdiethylstilbestrol, a very potent estrogen, for estradiol binding sites was approximately equal to that of estradiol. On the other hand, 17α-estradiol, the epimer of the hormone at C-17 and a weak estrogen, was bound to a lesser extent. The very low [^{3}H]testosterone binding by the uterine nonhistone fraction confirmed the estrogen specificity of the fraction.

If quantitatively the binding of a tracer dose of [^{3}H]estradiol was identical for the two organ preparations, the binding of liver nonhistone chromatin proteins was not decreased by either transdiethylstilbestrol or 17α-estradiol. The slight decrease observed by estradiol isotopic dilution, which could indicate a saturable site of low affinity for estradiol was ob-

TABLE V: Specificity of Estradiol Binding by Endometrium Nonhistone Chromatin Protein Fraction.[a]

	dpm/mg of Protein	
	Endometrium	Liver
[^{3}H]Estradiol	112	148
[^{3}H]Estradiol + estradiol	27	128
[^{3}H]Estradiol + transdiethylstilbestrol	34	152
[^{3}H]Estradiol + 17α-estradiol	60	152
[^{3}H]Estradiol + testosterone	112	155
[^{3}H]Testosterone	25	200

[a] Endometrium nonhistone chromatin proteins (1.62 mg/ml) and liver nonhistone proteins (0.71 mg ml) were incubated with 6 pM [^{3}H]estradiol alone, or along with 2 nM nonradioactive hormones for the uterus, and 40 mM for the liver. The concentration of [^{3}H]testosterone was identical with that of [^{3}H]estradiol. The conditions for incubation are the same as described in Table II, and bound radioactivity is expressed in disintegration per minute per milligram of protein.

TABLE VI: Nonhistone Chromatin Protein–Estradiol Interaction Binding Data.[a]

Expt 1		Expt 2		Expt 3	
(T)	(B)	(T)	(B)	(T)	(B)
1.06	1.08	0.96	0.90	0.63	0.47
2.10	2.10	1.86	0.96	1.11	0.50
3.10	2.48	2.86	q.14	2.27	0.60
4.20	3.04	3.94	1.30	5.20	0.65
10.7	4.64	9.86	2.84	10.0	0.86
21.6	7.24	20.2	5.50	20.5	1.26
31.9	7.96	28.9	7.14	29.0	1.78
42.6	10.05	38.5	9.64	41.8	2.05
102	22.6	95.4	23.2	105	5.38
524	124	490	113		

[a] Nonhistone chromatin (1 ml) preparations was incubated with various concentrations (T) of [^{3}H]estradiol for 18 hr at 4°. Binding was measured by the adsorbent technique. The bound, (B), and the total, (T), ligand concentrations are given in pM.

served in only one out of three experiments. More [^{3}H]testosterone was bound in the liver than in the uterus preparation. Based on these results, one can say that hormone specificity, stereospecificity and quite likely organ specificity were observed.

Under these experimental conditions, there was apparently a background of nonspecific binding of estradiol which was not dissociated during the 2-hr charcoal treatment (Table V, approximately 30 dpm/mg of protein). This could eventually give even more significance to the specificity previously described. The destruction of sites of low capacity by Pronase confirmed this deduction, since after estradiol dilution and protease treatment, the residual binding was similar (see Table IIb).

Quantitative Binding Studies. One of the main limitations in the binding studies was the requirement of the endometrium of more than 100 uteri for each binding curve and consequently, the number of quantitative binding experiments had to be limited. The results of these experiments are found in Table VI.

In no case did the experimental values show an initial plateau for the lowest concentrations (1 pM), implying that even at these very low concentrations, the high-affinity binding sites were saturated and that the number of sites were extremely low.

When the three experiments were calculated, the best fit obtained for each experiment is illustrated on Figure 1a and the binding parameters are listed in Table VII. The model S + NS appeared to be well adapted to the binding data and permitted the evaluation of binding parameters at equilibrium, (N_S), the concentration of specific binding sites, K_S, the intrinsic association constant and K_{NS}(N_{NS}) of the nonspecific system.

The concentration of specific binding sites was evaluated with fairly good precision and in all cases (N_S) was very small. Even when expressed per milligram of protein, this value was not constant from one experiment to another and varied up to a factor of 5. No explanation can be proposed for the observed fluctuation.

The determination of association constant, conversely, resulted in a relatively poor precision and the three binding experiments should be considered separately. In the *first* experiment, the K_S value was obtained after the elimination of the first two points which showed 100% binding. If they had been included, the association constant would have been infinite. From this experiment, it was concluded that the association constant was greater than 1×10^{13} M^{-1}. The *second* experiment gave a negative value for K_S. Such a negative value has no biological significance, but it can be explained mathematically. From the results found in Table VII, it can be seen that all ligand concentrations used in this experiment were greater than the concentration of binding sites. Under these circumstances, the experimental error associated with these points can give a hyperbolic branch, under as well as above the asymptote of the binding function. This concept is detailed in the legend of Figure 2. The association constant can be deduced from the (U) value which shows 50% binding and consequently, K_S is necessarily greater than 1/0.06 pM or 2×10^{13} M^{-1}. In the *third* experiment, also plotted by a more classical representation (Scatchard, 1949) in Figure 1b, if one considers the statistical precision of $1/K_S$ (Table VII), the association constant could not be smaller than 1×10^{14} M^{-1} and could even be much larger.

At this point it is necessary to ask if the model was well adapted to the results. The model used took into account reversible binding and fitted well with the data. However, in no case did the binding curve describe the totality of the phenomenon and since the initial plateau of the highest affinity binding was not observed, the data lined up only along the descending part of the theoretical curve. Consequently, the good fit could not be taken as proof of reversibility. If binding is irreversible, as long as the total ligand concentration (T) is smaller than the concentration of specific binding sites, the unbound ligand (U) will be equal to 0. This was observed in the first experiment for the first two points which were dis-

TABLE VII: Binding Parameters of Estradiol by Nonhistone Chromatin Proteins.[a]

	Expt 1	Expt 2	Expt 3
Protein concentration (mg/ml)	1.30	1.23	0.54
N_S (pM)	3.43 ± 0.17	0.65 ± 0.03	0.48 ± 0.01
$1/K_S$ (pM)	0.29 ± 0.05	−0.016 ± 0.002	0.0041 ± 0.0038
$K_{NS}N_{NS}$	0.24 ± 0.02	0.30 ± 0.01	0.045 ± 0.005

[a] K_S, the association constant and N_S, the concentration of binding sites for the chromatin receptor under study and $K_{NS}N_{NS}$ for the second binding of S + NS system (Baulieu and Raynaud, 1970), calculated from the data found in Table VI.

carded in the calculations; the third point should also have given (T) = (B) if binding was irreversible, but this was not the case. In the other two experiments, the lowest concentrations was already greater than (N_S) and therefore no information could be gained on the question of the nature of the binding.

Although no conclusion can be drawn on whether or not binding is reversible or irreversible, it can be stated that the association constant is $>1 \times 10^{-14}\ M^{-1}$.

It was only *a posteriori* that it was possible to understand why the dialysis technique could not give the results which were obtained by the adsorbent method. It was due to the confrontation of the very low concentration of specific sites with another rather large binding system in the nonhistone protein fraction. In the adsorbent technique, the larger binding system is removed by the charcoal but it remains in dialysis experiments. Most probably this other binding system is also of high affinity since it did not dissociate completely in the 4 hr of charcoal treatment. Its molecular basis is unknown.

Discussion

The estradiol binding system discussed here shares all the features of other previously described uterine-specific estradiol systems, in the cytosol and in nuclei previously exposed to the estradiol–cytosol complex (neonuclear receptor). However, there are special characteristics which differentiate it from the other receptors and four of these will be discussed below.

High Affinity. TECHNIQUE. With the charcoal technique, there is no problem of adsorption or diffusion through membranes as in dialysis experiment. With this technique, some dissociation of the complexes does occur during the duration of the experiment. The low-affinity complexes completely dissociate and only those of high affinity remain. The dissociation of labile complexes, which leads to an artificial increase of unbound ligand, does not affect the specific number of sites but leads to an underestimation of the K value. Dissociation, if any, of the specific binding decreases the number of specific sites and modifies the K value if this dissociation is not identical in all experimental points. However, in the latter case, the function would no longer be a hyperbola and a good fit would not have been obtained with the equation used.

Binding equilibrium was apparently obtained by 18-hr incubation at 4°; if it had not been reached this would eventually have led to an underestimation of the K for the high-affinity binding. One difficulty concerning the K determination is that some extrapolation was needed for calculations (see Quantitative Binding Studies), but this affected only the precision and not the order of magnitude of the K value. Finally, it is also possible to argue that there was some artificial alteration of unknown nature of a normal estradiol specific binding protein, leading to an artificial increase of the K value.

RESULTS. The first and most important argument is the high affinity for estradiol binding found in the nonhistone chroma-

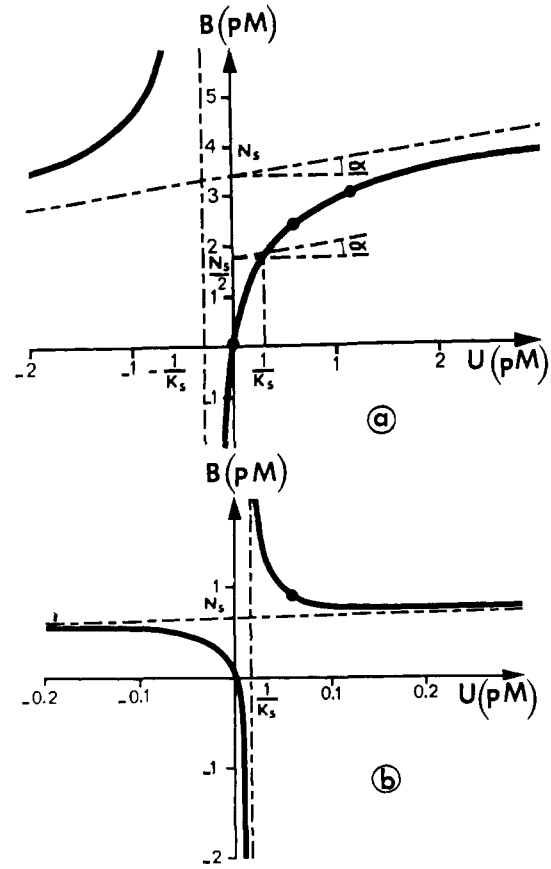

FIGURE 2: Estradiol binding to chromatin nonhistone protein. Plot of (B) *vs.* (U): (B) and (U) are, respectively, the bound and unbound ligand concentrations. (a) Experiment 1: the asymptote of the hyperbola, which for specific binding is horizontal and intersects the ordinate at N_s, has in this case the same intercept, but a positive slope, α, due to the presence of nonspecific binding. The parallel to the asymptote passing through (O, $N_s/2$) intersects the hyperbola at a point with abscissa $1/K_s$. This enables the determination of $1/K_s$. (b) Experiment 2: when the best fit calculated by the computer gives a negative value for $1/K_s$, the branch of the hyperbola necessarily falls in quadrant 1 ((B) > 0, (U) > 0). This implies that the vertical asymptote intersects the (U) axis at (U) > 0. The first experimental point is therefore so close to the oblique asymptote that its imprecision gives rise to this paradoxal situation. However, in any case, its (U) coordinate is greater than the one corresponding to half-saturation and consequently greater than $1/K_s$.

tin protein fraction. This is a unique feature: as visualized in Figure 1a,b (a comparison with cytosol binding is presented) it shows at the same time a smaller concentration of sites and a greater binding for the chromatin receptor than with the cytosol protein which has a K approximately 4×10^{10} M^{-1}. When the cytosol binding was tested at very low estradiol concentration (1 pM) no additional binding was found and the B value was even lower than those observed at greater concentrations. A control experiment was performed consisting in lowering the concentration of cytosol binding sites to a value approximately three times the concentration of specific binding sites of the nonhistone chromatin preparation. No binding was observed in this diluted cytosol following incubation with 1, 10, and 50 pM of [^{3}H]estradiol. One possibility could have been that the dilution of the cytosol receptor sites would have increased its affinity for the ligand (Best-Belpomme *et al.*, 1970) in such a way that it could explain the value found for nonhistone chromatin. In this case, the so-called nonhistone chromatin fraction would have indeed contained only the cytosol receptor or another of similar affinity. The results showed that this was not the case and confirmed the difference of affinity between the two binding systems.

It was also seen that in the nonhistone chromatin fraction treated by charcoal, there is in addition to the high-affinity specific binding, another binding system, not saturated at 0.5 nM and not completely dissociated after 4 hr of charcoal treatment as expected if it was an albumin like nonspecific binding (Milgrom and Baulieu, 1969). Its hormone specificity has not been studied.

Small Number of Sites. The very small number of sites per cell, much smaller than the corresponding value for cytosol and neonuclear receptors, cannot be taken as a definite value. Not only is the number approximate due to the postulates behind the calculations performed to arrive at the number of sites in the original homogenate (see Methods), but obviously the recovery may be low and/or somewhat variable because of inactivation and/or proteolysis during the long procedure in the absence of estradiol needed to obtain the binding preparation. Diisopropyl fluorophosphate did provide some protection of the sites.

In addition, all the high-affinity specific binding may not be present only in the nonhistone chromatin fraction and some may be left in the pellet of the 1 M NaCl extract (see Scheme I), and subsequently not detected. Furthermore, the charcoal treatment in itself may diminish the measured number of sites by adsorption and alteration. The small concentration of charcoal and the relative brief incubation with the adsorbent were chosen in order to minimize these possibilities. However, it seems unlikely that losses were high enough to avoid the conclusion that the concentration of the binding sites discussed here is extremely low, corresponding to <10/cell.

Size. The size of the nonhistone component is difficult to assess. Ultracentrifugation and filtration experiments were in favor of a small protein (<50,000). Moreover, from the work of various laboratories, it has been found that proteins of various sizes (including those obtained by deaggregation and/or proteolysis) are still capable of binding estradiol, and can be found in uterine extracts. Therefore, one can argue that the nonhistone chromatin protein could represent a subunit or a portion of a large molecule. However, if this were the case, the affinity constant should then be identical or smaller than that of the larger cytosol molecule. If not, then a complex mechanism of special entry, proteolysis, or dissociation, and, finally enhancement of affinity would have to be conceived.

Location. The chromatin "location" of the binding system, as indicated by the method of preparation of the nonhistone protein fraction, shows that it could not have been due to a cytoplasmic contamination. The nuclei were purified and no specific estradiol binding was found in the soluble protein fraction. The occurrence of the neonuclear receptor, extractable only by high salt solution as is the chromatin protein, implicates exposure to estradiol which is not secreted in the calf. However, it is difficult to exclude completely estradiol secretion before puberty and there is always a possibility that the animals had received diets containing some estrogen which could provoke the formation of at least a small amount of the neo nuclear receptor. In fact, such a possibility may account for the so called nonspecific binding observed in the nonhistone chromatin protein fraction when the concentration of estradiol was raised to 0.5 nM (see Figure 1). In other words, rather than conclude that the very high-affinity protein is a neonuclear receptor of small abundance (an hypothesis conflicting with the determined K values), it is proposed that in the calf chromatin proteins, there are present at the same time the high-binding entity and some neonuclear receptor. The latter would be responsible for additional binding of relatively high affinity detected even after charcoal treatment and on glycerol gradient at 1 nM estradiol and which could be identical with the binding observed by Jungblut *et al.* (1971) a tan estradiol concentration of 10 nM.

Finally, what could be the significance of such a high-affinity and "rare" estradiol binding protein of the chromatin? From recent findings, it appears that estradiol is transported by the cytosol receptor to nucleus where it appears bound to the neonuclear receptor which may or may not be the modified molecule after translocation. From this position, might not some estradiol be released and selectively and ultimately transferred to a higher affinity protein? Since no precise information is known about the kinetics of estradiol binding proteins at 37°, the speed at which this transfer may occur is unknown. The transfer of estradiol could be eventually facilitated by a mechanism implicating a special recognition of the protein, for example a protein–protein interaction between the neonuclear receptor and the chromatin receptor. In any case there is a disparity in the number of sites of the very high-affinity chromatin protein and the cytosol and neonuclear receptors. The chromatin protein may be part of an enzyme or some other protein playing a role in the RNA fabricating machinery at the chromatin level. The likelihood that it represents a repressor has not been excluded, and in this case, it should have an affinity for DNA, possibly reduced when there is estradiol binding. Its absence in the liver could indicate that a repressor implicated in hormone action is not present in a nontarget organ. Contrary to these exciting hypothesis, the high-affinity protein could simply be another carrier delivering the hormone to a "true" receptive structure, not necessarily a protein.

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Chemical and Physicochemical Studies of the Component Polypeptide Chains of Rabbit Secretory Immunoglobulin A*

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ABSTRACT: The polypeptide chains of rabbit colostral secretory immunoglobulin A—α, light, secretory component, and fast component—are characterized in terms of chemical and physicochemical parameters. Procedures are given to isolate secretory α chain and F component to homogeneity. Molecular weights and amino acid compositions of the component polypeptide chains are discussed. A revised model for the rabbit secretory immunoglobulin A is presented which includes four α chains (54,000 each), four light chains (22,000 each), one secretory component (60,000), and one F component (15,000) to account for 379,000 molecular units.

A salient characteristic of the sIgA[1] molecules found in various mammalian secretions is their sedimentation coefficient of about 11 S (Tomasi *et al.*, 1965; Cebra and Robbins, 1966; Porter and Allen, 1969; Vaerman and Heremanns, 1970; Pahud and Mach, 1970) reflecting a molecular weight of 385,000 (Cebra and Small, 1967; Newcomb *et al.*, 1968). A particular feature that sets the sIgA apart from all other immunoglobulins is its content of "extra-antigenic sites" (Tomasi *et al.*, 1965) which have been taken to define a third polypeptide—the secretory component different from heavy and light chains. Taking advantage of the finding that secretory component dissociates from rabbit sIgA in 5 M guanidine·HCl (Cebra and Small, 1967) this unique polypeptide has been isolated from the parent molecule (O'Daly and Cebra, 1971a) as well as from a fraction of colostrum not containing immunogloublins (O'Daly and Cebra, 1971b). Values for the molecular weight of this polypeptide range between 76,000 (Newcomb *et al.*, 1968) and 58,000 (Tomasi and Bienenstock, 1968) and considerable ambiguity persists regarding molecular characteristics which could be used to clearly distinguish secretory component from other polypeptide chains of the sIgA molecule. Using analytical and preparative zone electrophoresis in polyacrylamide gels a correlation has been found between the antigenic activities used to define component polypeptide chains of sIgA and their electrophoretic mobilities (O'Daly and Cebra, 1971a). Such an approach has led us to confirm the presence of a fast-migrating component of rabbit sIgA (Cebra and Small, 1967). Our analysis also supports the conclusions of Halpern and Koshland (1970) that this component is different from α, light, and secretory component (O'Daly and Cebra, 1971a) and may represent a fourth kind of polypeptide chain of the parent molecule. In the present paper the isolation of secretory α chain, secretory light chain, and fast component[2] (F component) is described. These three polypeptides, as well as the secretory component dissociated from sIgA and the secretory component found free in colostrum, are char-

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[1] WHO meeting on the Nomenclature of Immunoglobulins Šterzl and Riha (1970).