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MACROMOLECULAR BINDING OF ESTRADIOL BY NUCLEI IN A CELL-FREE SYSTEM*

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Abstract—Rat uterine cytosol estradiol-binding protein was labeled with radioactive estradiol and then incubated with highly purified nuclei from an estrogen-binding murine tumor. The nuclei were then extracted with 2 M NaCl–5 M urea, and macromolecular bound estradiol was separated by gel filtration. Nuclear preparations incubated with estradiol-labeled uterine cytosol showed five to ten times more macromolecular binding than corresponding nuclear preparations incubated with buffer, albumin, heart cytosol or uterine cytosol deficient in biologically active binding protein. The process by which nuclei bind estradiol is temperature dependent. Radioactive estradiol attached to protein can be extracted from the nuclei with solutions of 2 M NaCl–5 M urea. Binding of estradiol to isolated nuclei could be distinguished from the binding of the hormone to isolated microsomes or mitochondria in that only the nuclear binding was markedly reduced by prewarming the particulate fraction. Furthermore, nuclear binding was decreased by incubation with several metabolic inhibitors. Most of the nuclear binding appears to occur with the chromatin fraction from which labeled protein-bound estradiol can be extracted. When the cytosol estradiol-binding protein was partially purified by gel filtration there was less nuclear binding of estradiol than resulted from using cytosol. The deficiency of the gel filtration preparation was restored by the addition of unfractionated cytosol. Nuclear binding of estradiol seems to depend on some cofactor present in the cytosol. Nuclei from an estrogen-dependent tumor and the uterus had comparable amounts of nuclear binding that was associated with the 5S fraction as determined by ultracentrifugation on sucrose gradients. Nuclei from liver bound $\frac{1}{3}$ as much estradiol, which was associated with a 3S macromolecular fraction. The nuclear-binding macromolecule in the liver is different from that in the uterus and the dependent tumor. Prior incubation of nuclei with non-radioactive estradiol, diethylstilbestrol or estriol in uterine cytosol reduced the uptake of the nuclei when subsequently incubated in [^{3}H]estradiol containing cytosol. Incubation in cytosol containing MER-25, an anti-estrogen, resulted in no subsequent reduction of [^{3}H]estradiol uptake.

The current concept of estrogen action in the uterus involves the initial interaction of estrogen with a binding protein ("receptor") in the cytoplasm. The entire complex may then enter the nucleus, attach to chromatin and initiate a chain of biochemical events. Recent reviews discuss many of these aspects [1, 2].

Many prior studies of this interaction in cell-free systems have utilized relatively crude preparations of nuclei. In addition, uterine tissue is difficult to homogenize without breakage of nuclei, and the nuclear fraction remains contaminated with myofibrils despite centrifugation through dense sucrose.‡ Furthermore, the uterus consists of several cell types with dissimilar binding characteristics [3]. To circumvent these difficulties, nuclei were purified to a high degree from an estrogen-binding tumor. The tumor line, from which the nuclei were isolated, can concentrate radioactive estradiol 100-fold relative to plasma 1 hr after systemic administration. Autoradiographic and subcellular distribution studies with this tumor showed nuclear localization of the radioactive estradiol [4].

Although the overall sequence of cytoplasmic to nuclear transfer with appearance of a nuclear 5S binding macromolecule is established, the details of the steps involved remain unclarified. Jensen *et al.* [5] have presented evidence that the association of estradiol with the binding protein activates the 4S binding subunit, which then undergoes a pH- and temperature-dependent transformation to a 5S form in the cytoplasm and then binds to nuclei. Puca *et al.* [6], on the other hand, described the cleavage of the receptor by a Ca^{2+} "receptor-transforming factor" to a 4.5S active form. In contrast, Yamamoto and Alberts [7] described the interconversion *in vitro* of the 4S to a 5S estradiol-binding protein and suggested that the 5S form arises through addition of another substance in the cytosol.

Experiments designed to study whether nuclei have an inherent specificity for the binding of the estradiol-binding protein have yielded different results. O'Malley *et al.* [8] and Baulieu *et al.* [9] presented evidence in favor of nuclear specificity. Salt extracts prepared from nuclei which had previously been incubated with steroid-labeled cytosol revealed more macromolecular bound steroid in salt extracts prepared from target tissue. There are, however, contradictory reports. Chamness *et al.* [10] reported no differences in nuclei from target or non-target tissue, neither in their ability to bind the estradiol-binding protein nor in the sedimentation characteristics of the

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nuclear salt extracts. More recently the estrogen receptor binding to isolated nuclei was described as a nonsaturable process, both in regard to target as well as to non-target tissue [11]. These inconsistent findings may, in part, be due to differences in methods, particularly the preparation of nuclei.

A different aspect of this investigation deals with a number of steroids and non-steroidal analogs which will bind the binding protein for estradiol. It is unclear what distinguishes an estrogen from an anti-estrogen. The latter are compounds which will reduce the biological effect and the accumulation of co-administered estrogens. Some drugs may exert their effect by occupying the cytoplasmic-binding protein and thus prevent estradiol binding. Clark *et al.* [12] have shown that some anti-estrogens with partial agonist activity (nafoxidine and clomiphene) can promote the appearance of an estrogen-binding protein in crude nuclear preparation. It is not known if the anti-estrogen promotes attachment of the binding protein to the same potential acceptor in the nucleus as estradiol. These studies have not investigated MER-25, an anti-estrogen with virtually no agonist activity. Conceivably, the binding protein charged with a pure anti-estrogen is unable to penetrate into the nucleus or could be unable to attach to the same nuclear-binding site.

MATERIALS AND METHODS

Preparation of the cytosol fraction (100,000 g supernatant fluid) containing the binding protein. Uteri of normal female noncastrate Sprague–Dawley rats (Camm Research) weighing 175–200 g were minced and homogenized in 6 vol. (w:v) of 10^{-2} M Tris–HCl, pH 7.4, using conical Kontes glass homogenizers. All operations were carried out in ice. The pestle was driven mechanically and the actual homogenization time was 135 sec (3 × 45 sec). The crude homogenate was then centrifuged in a Sorvall rotor SS34 for 10 min at 2400 rev/min (600 *g*). The resulting supernatant fluid was transferred to Spinco centrifugation tubes and centrifuged in a type 40 rotor for 1 hr at 38,000 rev/min (100,000 *g*) at 5°. The supernatant fluid, referred to as a cytosol fraction, was stored in Falcon plastic tubes and kept frozen at −20° until use. The cytosols were used within 1 week of preparation and used only if the cytosol retained high binding activity. The protein content was 0.9 mg/0.2 ml of cytosol.

Preparation of radioactive [^{3}H]estradiol-17β. An aqueous solution of 2,4,6,7-[^{3}H]estradiol-17β (100 Ci/m-mole, Amersham or New England Nuclear Corp.) was prepared by adding 100 μl of the stock solution (benzene-ethanol) to 3.5 ml of a 30% ethanol solution. The organic solvents were flash-evaporated at 40–50°. Distilled water was added to provide an estradiol concentration of 4×10^{-7} M. The purity of tritiated estradiol was established by thin-layer chromatography (80% chloroform, 20% ethyl acetate) on Silica gel (Eastman Co.).

Labeling of the binding protein. Tubes containing the frozen samples of the cytosol fraction were allowed to thaw in ice. Radioactive estradiol was then added to a final concentration of 5×10^{-9} to 10^{-8} M. Unless otherwise specified, the incubation was carried out in ice for 1 hr. The extent of binding of estradiol to the binding protein was determined after each experiment by gel filtration chromatography columns [13].

Characteristics of tumor and purification of its nuclei. Most of the studies on nuclear binding of [^{3}H]estradiol were done with nuclei isolated from an estrogen-induced interstitial cell tumor of the testis. This tumor, line 14LC, has been well characterized in serial passage [4, 14].

The following technique was used for the isolation of nuclei from tumor, uterus and liver. The tissue was rapidly excised, weighed and then minced in 6 vol. of ice-cold sucrose solution (2.2 M sucrose in 10^{-2} M Tris buffer, pH 7.4; 3 mM $CaCl_2$; and 0.2% Triton X-100). The minced tissue (ranging in weight from 3 to 10 g) was then briefly homogenized in two strokes in a round-bottom glass homogenizer with a Teflon pestle (Kontes, clearance 0.009 in.) on a Tri-R Stir-R (model S 630, Tri-R Instruments, Inc.) at intermediate speed. The homogenate was then filtered through four layers of cheesecloth. A drop of this solution was colored with Trypan Blue (1% aqueous solution) and checked under the microscope for the presence of nuclei. In order to obtain pure nuclei, the original Chauveau procedure [15] was modified in the following way: 10 ml of the homogenate was layered over a 3.2-ml cushion of 2.2 M sucrose and the interphase was carefully stirred with the tip of a pasteur pipette to minimize trapping of nuclei. The following step consisted of a 45-min centrifugation in a Spinco ultracentrifuge L2-65B using the SW 40Ti rotor at 40,000 rev/min (284,000 *g*) at 5°. The supernatant was discarded, the sides of the tube were carefully wiped of residual cellular debris, and the whitish nuclear pellets re-suspended in 10^{-2} M TES buffer,* pH 6.6. The nuclei were dispersed with a vortex mixer and centrifuged in a Sorvall rotor for 5 min at 2400 rev/min (600 *g*). The nuclei were washed twice with about 30 ml of the same buffer by dispersing them on a vortex mixer and centrifuging as stated above. The nuclear pellet was then homogenized by hand in a conical glass homogenizer, containing approximately 10 ml of 10^{-2} M TES buffer, pH 6.6. The DNA:RNA ratio was 4.63 and 4.8, as tested on two separate batches of nuclei by the diphenylamine [16] and orcinol reaction [17] respectively.

Under phase microscopy the nuclei appeared to be intact. For electron microscopy the nuclear pellets were fixed in 2.5% glutaraldehyde and post fixed with 1% osmium tetroxide. The electron micrographs were interpreted as containing nuclei free of contamination by other cellular particles. As anticipated, the outer nuclear membrane was removed by the detergent Triton X-100. In the final electron microscopic preparation, some nuclei were intact while others had nuclear material protruding outside of the remaining membrane.

Isolation of mitochondria and microsomes. These cellular constituents were isolated from the same tumors as used for the preparation of nuclei [18]. The tissue was homogenized in 10 vol. of buffer (0.35 M

* *N*-Tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid.

sucrose, 2 mM EDTA, 20 mM K_2HPO_4, and 30 mM nicotinamide, pH 6.8). In order to remove all nuclei, this homogenate was centrifuged twice in a Sorvall rotor SS34 at 2400 rev/min (600 *g*) and the precipitate discarded each time. The next step, a 10-min centrifugation at 8300 rev/min (800 *g*), yielded a crude mitochondrial pellet. The supernatant was saved for preparation of microsomes, while the pellet was rehomogenized in 50 ml of the above buffer. This 10-min recentrifugation was repeated four times, the supernatant discarded and the pellets were rehomogenized in 10^{-2} M TES buffer, pH 6.6. The nuclei and mitochondria-free supernatant were transferred into Spinco tubes for type 40 rotor and centrifuged for 1 hr at 38,000 rev/min (100,000 *g*). The microsomal pellets were homogenized in 10^{-2} M TES buffer, pH 6.6.

Nuclear uptake. After preincubation of the cytosol fraction with [^{3}H]estradiol, 0.2 ml of this cytosol preparation containing the labeled binding protein was added to 0.1 ml of a nuclear suspension. The pH of the incubation mixture was 6.9 to 7.0. The DNA content in the nuclear preparation ranged between 50 and 100 μg. Each experimental group consisted of three or more samples, as indicated. Unless otherwise indicated, the samples were incubated at 25° in a shaking water bath for 30 min. After this period the samples were cooled in ice and the nuclei sedimented for 5 min at 2400 rev/min (600 *g*) in a Sorvall centrifuge RC-2 using the SM rotor. The supernatant was carefully removed using a pasteur pipette and transferred to separate tubes in an ice bucket for determination of the extent of labeling of the binding protein (see below). The nuclear pellet remaining in the original incubation tubes was then washed with 3 ml of 10^{-2} M TES buffer, pH 6.6, and the nuclei were dispersed on the vortex mixer. The nuclei were centrifuged once more under the same conditions. The buffer was carefully aspirated so that the nuclear pellet remained undisturbed. Extraction of macromolecular bound estradiol from the nuclei was achieved by adding 0.3 ml of a high salt solution (5 M urea–2 M NaCl in 10^{-2} M Tris, pH 8.3), which was found to solubilize approximately 90 per cent of the chromosomal proteins [19]. The use of this salt solution overcomes the difficulty of solubilizing only a low percentage of the total amount of macromolecular bound estradiol [20]. Incubation of a nuclear pellet with 0.4 M KCl in 10^{-2} M Tris buffer, pH 8.0, resulted in solubilization of only 34 per cent of the total [^{3}H]estradiol in the nuclear preparation.

After a 10-min incubation on ice, 0.1 ml of a 1% solution of Dextran Blue 2000 (Pharmacia, MW 2×10^6) was added to the samples. They were then chromatographed at room temperature on polyacrylamide columns (Biogel P-10, Biorad 1.1 cm × 10 cm, exclusive MW 20,000) using 10^{-2} M Tris buffer, pH 7.4, as the eluent. One 3-ml fraction was collected from the moment the Dextran Blue front started to elute.

The radioactive estradiol contained in this fraction was extracted by adding 10 ml of isoamyltoluene solution (1:19) to all tubes, which were then shaken at room temperature for 15 min. Five ml of the organic phase was transferred to scintillation vials containing 10 ml of 1,4-bis(2-(5-phenyloxazolyl)benzene and 2,5-diphenyloxazole solution in toluene. Radioactivity was determined in a Packard liquid scintillation counter, model 3320. The results have been corrected for a background of 27 cpm and for the portion of the organic phase which had been counted. The counting efficiency was approximately 43 per cent.

Isolation of chromatin. A test tube containing a purified preparation of nuclei was placed in the chamber of a cell disruption bomb (model 4635, Parr Instrument Co., Moline, Ill.). The sealed chamber was slowly filled with N_2 and the nuclear sample allowed to equilibrate at 1700–1800 psi. After an exposure of the nuclei at this pressure for 20 min the exit valve was carefully opened and the ruptured nuclear suspension collected. This preparation was centrifuged at 2400 rev/min (600 *g*) for 10 min to remove any remaining intact nuclei and aggregates. The resulting supernatant is referred to as crude chromatin preparation. The DNA:RNA:protein ratio was 1:0.21:1.8.

Determination of [^{14}C]amino acid incorporation into trichloroacetic acid (TCA)-insoluble material. A nuclear pellet (0.1 ml containing 220 μg protein) was incubated at 37° for the time periods indicated with 0.2 ml of a [^{14}C]amino acid algal hydrolysate (Amersham, 54 mCi/mAtom of carbon) in 10^{-2} M Tris buffer, pH 7.4 (20°). In different experiments between 0.9 and 0.57 μCi/0.2 ml was added and the amount of TCA-insoluble material was determined as described by Anderson *et al.* [21].

ATP determination. The ATP in nuclear preparations containing approximately 300 μg DNA was extracted as described by McEwen *et al.* [22]. ATP (equine muscle) was purchased from Sigma and ATP determinations were carried out according to McElroy [23]. The luciferin–luciferase enzyme solution (Worthington Biochemical Co.) was prepared by reconstituting 50 mg of lantern extract with 5 ml water. The fluorescence was measured in an Aminco–Bowman spectrophotofluorometer (excitation wave length 370 nm; emission wave length 550 nm).

Preparation of ammonium sulfate-precipitated binding protein. A fraction of the cytosol in the range of 30–50 ml was made 10^{-8} M with [^{3}H]estradiol and incubated on ice for 60 min. Ammonium sulfate (Schwartz/Mann, special enzyme grade) was slowly added to the cytosol preparation to obtain a 30% fraction. After an overnight incubation on ice the preparation was centrifuged at 15,000 rev/min (36,400 *g*) for 20 min at 4°. The pellet was carefully rinsed with cold 10^{-2} M Tris buffer, pH 7.4. Five ml of cold 10^{-2} M Tris buffer, pH 7.4, was then added to suspend the sediment. A 0.2-ml fraction was assayed for macromolecular binding by filtration on columns of Biogel.

RESULTS

Requirement of uterine cytosol for nuclear binding. When purified nuclei incubated with the uterine cytosol containing 10^{-8} M [^{3}H]estradiol, 68,000 ± 200 dis./min/mg of DNA was in the nuclear fraction. The nuclei bound much less radioactivity after incubation with 10^{-8} M [^{3}H]estradiol in bovine serum albumin or in Tris buffer (Table 1). Nuclei incubated in media containing cytosols prepared from the heart or uterus

Table 1. Nuclear binding of [^{3}H]estradiol in the presence of different [^{3}H]estradiol containing media*

	Cytosol binding (dis./min/0.2 ml)	Nuclear binding (dis./min/mg DNA)
(A) Cytosol (0.9 mg protein/0.2 ml)	44,000 (910)	68,000 (2000)
(B) Bovine serum albumin (0.9 mg/0.2 ml)	670 (37)	10,000 (470)
(C) Tris buffer, 10^{-2} M, pH 7.2 (25°)		7,700 (130)

* A suspension of tumor nuclei (89 μg nuclear DNA/0.1 mg) was incubated with 0.2 ml of one of the above solutions. (A) The cytosol was prepared in 6 vol. of 10^{-2} M Tris buffer, pH 7.4. (B) Bovine serum albumin was dissolved in the same buffer. All three groups of solutions were first incubated with 10^{-8} M [^{3}H]estradiol for 1 hr in ice. The amount of macromolecular bound [^{3}H]estradiol was estimated by filtration of the salt extract on columns of Biogel. The results are averaged from three determinations; the figures in parentheses indicate S. E. M.

that had been aged to destroy their estradiol-binding activity supported only about 28 per cent as much nuclear binding as did the active uterine cytosol. With the addition of larger volumes of uterine cytosol to a constant number of nuclei, the amount of nuclear uptake of estradiol was not increased [24].

The radioactive estradiol–cytosol-binding protein complex was partially purified by ammonium sulfate fractionation. Varying amounts of the protein fraction precipitated by ammonium sulfate at 30% of saturation then were added to nuclei at 4° for 20 min. The nuclear binding was proportional to the amount of partially purified binding only up to 80 μg protein. Nuclear binding was the same using 80, 100 or 150 μg of the protein fraction. The apparent saturation level is 58,000 dis./min/mg of DNA. The concentration of binding macromolecules in the nucleus is 0.3 pmole/mg of DNA assuming that each macromolecule binds one estradiol molecule [24].

To examine the nature of the macromolecule binding estradiol in the nucleus, tumor nuclei were incubated with prelabeled cytosol. The nuclei were then washed and a nuclear salt extract was prepared which was filtered on columns of Biogel kept at 4°. Portions of the macromolecular bound fraction from the nucleus were incubated with Pronase (CalBiochem B grade), 10 μg/ml; DNase (Worthington), 5 μg/ml, 5×10^{-3} $MgCl_2$; or RNase (Worthington), 5 μg/ml. All samples including the untreated control were incubated for 15 min at 20° and then reanalyzed for macromolecular binding on columns of Biogel. Nuclear macromolecular binding was destroyed by pronase (11 per cent of control) but not extensively by DNase or RNase (83–85 per cent of control).

Relative binding of estradiol by nuclei, mitochondria and microsomes. Microsomes and mitochondria were isolated from the same tumors as the nuclei and were assayed for binding of [^{3}H]estradiol as described for nuclear binding. The extent of macromolecular binding obtained with these organelles was expressed on the basis of "mg of protein" for comparison. Cytosol fractions were incubated with increasing amounts of labeled [^{3}H]estradiol, a fraction of each prelabeled cytosol was added to the mitochondria or microsomes, and the amount of macromolecular bound [^{3}H]estradiol measured. The mitochondrial binding at a given concentration of estradiol was much less than that found in a nuclear extract (Fig. 1). Microsomes, however, appear to bind approximately to the same extent as nuclei. This extensive binding of estradiol to microsomes, in the presence of cytosol, appears to be a peculiarity of the system *in vitro*, since studies *in vivo* revealed little binding of [^{3}H]estradiol to these organelles [24].

Nuclei, mitochondria or microsomes were heated at 73° for 5 min in sealed tubes. After cooling the preparations in ice, each of the above particulate fractions was incubated with [^{3}H]estradiol-prelabeled cytosol and the amount of macromolecular bound [^{3}H]estradiol was measured. Heated nuclei only contained 25 per cent of control macromolecular binding. In contrast, the binding by heated mitochondria was 82 per cent of control for heated mitochondria and 90 per cent of control for heated microsomes.

The influence of preincubation at 37° on the isolated nuclei was also investigated. The result of this experiment is shown in Fig. 2. The capacity of nuclei to bind [^{3}H]estradiol decreased with increasing duration of preincubation at 37°. Nuclear-binding capacity was not restored by addition of glucose (100 mg/100 ml) to samples incubated at 37° for 30 min. Similarly, the decrease in binding activity of

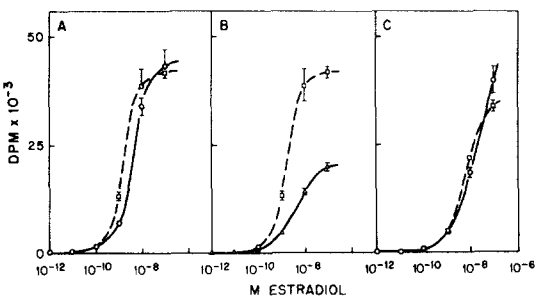

Fig. 1. Binding of [^{3}H]estradiol by nuclei, mitochondria and microsomes in the presence of increasing amounts of [^{3}H]estradiol in cytosol. Increasing amounts of [^{3}H]estradiol were added to 0.2 ml of cytosol preparations which were then incubated at 0–4° for 1 hr. Tumor nuclei (178 μg protein), mitochondria (178 μg protein) or microsomes (178 μg protein) were added in 0.1 ml to the prelabeled cytosol. The samples were incubated at 25° for 30 min. A salt extract was prepared of the washed nuclear and mitochondrial pellets. The amount of macromolecular bound [^{3}H]estradiol was estimated by filtration of the salt extract on columns of Biogel. Dashed lines represent extent of cytosol binding per sample; the solid line in panel A represents nuclear binding; in panel B, mitochondrial binding; and in panel C, microsomal binding of [^{3}H]estradiol. Each point represents the average of three samples; the vertical bars represent S. E. M.

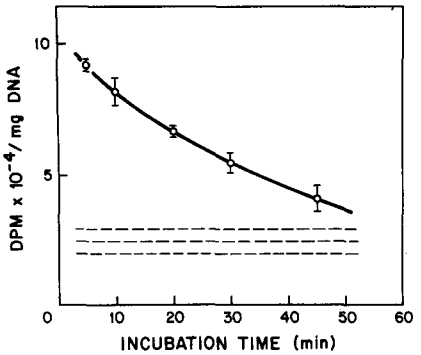

Fig. 2. Effect of temperature on ability of nuclei to bind [^{3}H]estradiol. Incubation tubes containing 0.15 ml of a suspension of tumor nuclei (102 μg DNA) were exposed to 37° for the time periods indicated. Cytosol (0.2 ml) prelabeled with 10^{-8} M [^{3}H]estradiol was then added to each sample, which was subsequently incubated for 30 min at 25°. A salt extract of the washed nuclear pellet was gel filtered to determine the amount of macromolecular bound [^{3}H]estradiol. The center dashed line shows the binding of heat-inactivated nuclei (5 min at 73°). Each point represents the average of three estimates. Vertical bars and top and bottom dashed lines indicate S. E. M.

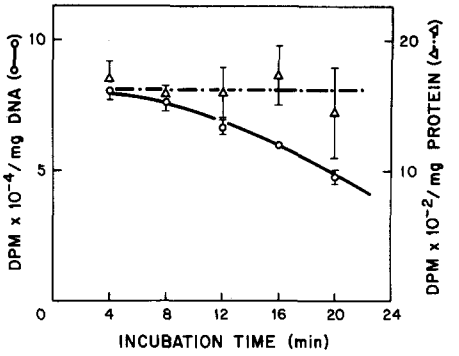

Fig. 4. Effect of short pulses of [^{3}H]estradiol-labeled cytosol on nuclear binding of [^{3}H]estradiol and of [^{14}C]amino acids on nuclear [^{14}C]amino acid incorporation. Nuclear binding of [^{3}H]estradiol (solid line): rat uterine nuclei suspended in a volume of 0.1 ml were incubated at 37°. At 4-min intervals, starting with time zero, cytosol (0.2 ml) prelabeled with 10^{-8} M [^{3}H]estradiol was added and the incubation continued for 4 min, at which time the nuclei were washed, and a salt extract was prepared and analyzed for macromolecular bound [^{3}H]estradiol. Nuclear incorporation of [^{14}C]amino acids into acid-insoluble material (broken line): The same format was followed for the study of nuclear incorporation of [^{14}C]amino acids into acid-insoluble material. [^{14}C]amino acid hydrolysate (0.57 μCi) in 10^{-2} M Tris buffer, pH 7.4 (20°), was added in a volume of 0.2 to 0.1 ml of a nuclear suspension of 4 min as described above. ^{14}C incorporation into protein was then measured. Each point represents the average of three estimates; the vertical bars indicate S. E. M.

the nuclei could not be prevented by addition of glucose prior to exposure to 37°.

The lability of the estradiol-binding protein contained in the cytosol was very pronounced at 37° (Fig. 3). The question arose whether the progressive loss of nuclear-binding activity at 37° was due to concurrent inactivation of the binding protein. To ensure that all samples were exposed to cytosol with indentical levels of biologically active binding protein, a pulse-label experiment was performed (Fig. 4).

Nuclei were incubated at time zero at 37°. At 4 min intervals thereafter, prelabeled cytosol was added to the nuclei for a period of 4 min. As shown by the solid line, nuclear binding of estradiol decreased in a nonlinear fashion, while the ability of nuclei to incorporate [^{14}C]amino acids into the trichloroacetic acid-insoluble material of nuclei remained constant (dashed line). Decreased nuclear binding of estradiol, therefore, does not primarily appear to be due to a general metabolic decline.

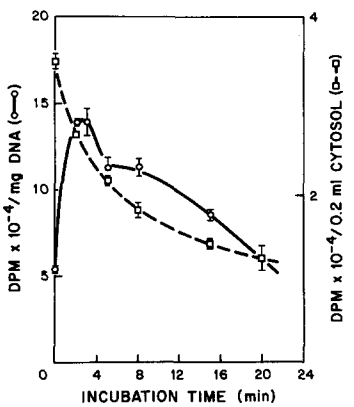

Fig. 3. Effect of incubation of a nuclear preparation with [^{3}H]estradiol-labeled cytosol on nuclear binding of [^{3}H]estradiol and on cytosol binding at 37°. A preparation of tumor nuclei (102 μg DNA/0.1 ml) was incubated at 37° for the time periods indicated with 0.2 ml cytosol which had previously been incubated with 10^{-8} M [^{3}H]estradiol. The 0 min value was obtained by incubating a sample in ice for 20 min. After incubation the supernatant was removed for measurement of cytosol binding. The nuclei were washed, and a salt extract was prepared. Macromolecular bound [^{3}H]estradiol in nuclei and cytosol was measured by gel filtration.

Effect of inhibitors on nuclear binding. Since nuclei have been shown to contain a tricarboxylic acid cycle and an oxidative phosphorylation system [25], it was of interest to determine whether the nuclear binding of estradiol could be influenced by metabolic inhibitors. These experiments were also undertaken in an attempt to distinguish the binding of estradiol to nuclei from the binding of estradiol to mitochondria and microsomes. After a preliminary testing of a number of inhibitors for their effect on nuclear binding of estradiol, only those which did not affect the binding of estradiol to the cytoplasmic-binding protein were studied further. The concentrations used were chosen after performing dose–response experiments and are in the same range as those used by McEwen *et al.* [25].

The reduction in nuclear accumulation of estradiol in the presence of drugs could be due to the interference with the binding process at several points: (1) conversion of the cytoplasmic-binding protein to a different, active form; (2) nuclear entry; and (3) intranuclear binding. To examine the possibility that the observed inhibition was due to a prevention of a conversion from an inactive form to an activated

one in the cytosol, the drugs were added to the [^{3}H]estradiol-prelabeled cytosol for 30 min at 25°. This did not alter the inhibition of nuclear binding observed when inhibitors were added directly to the incubation mix containing nuclei [24]. It appears unlikely, therefore, that the inhibitory effect is due to a temperature-dependent conversion of the binding protein to an active form in the cytosol alone. It is suspected that a nuclear event is influenced by these inhibitors. In the experiments shown, the inhibitors were routinely added to the nuclear preparation 10 min prior to the addition of the cytosol preparation.

In another experiment, the nuclear incorporation of amino acids into trichloroacetic acid-insoluble material in the presence of dinitrophenol (1.2×10^{-3} M) and pyrophosphate (5×10^{-3} M) was studied. Dinitrophenol and pyrophosphate inhibited the incorporation of amino acids into nuclear trichloroacetic acid-insoluble material by 68 and 8 per cent, respectively, of the control. The incorporation of amino acids over a time period of 20 min at 25° was linear with respect to time [24].

The histogram in Fig. 5 summarizes the results of several experiments utilizing inhibitors. The greatest inhibition was obtained with pyrophosphate. However, PO_4^{3-} also inhibited nuclear binding of [^{3}H]estradiol. Anoxia and dinitrophenol decreased nuclear binding of estradiol by approximately 50 per cent, while in the the presence of KCN and ouabain, approximately 70 per cent of the binding of estradiol was obtained. Note that heat-inactivated nuclei (see Fig. 6, column A) exhibited 25 per cent of the binding seen in untreated controls. On the other hand, no decrease in binding of [^{3}H]estradiol was obtained when sodium azide or sodium fluoride was added to nuclear preparations at concentrations ranging from 10^{-5} to 5×10^{-3} M.

Effect of inhibitors on microsomal and mitochondrial binding. When the inhibitors used for the study of nuclear binding of estradiol were tested at the same concentrations with a microsomal pellet, no significant reduction in microsomal binding of labeled estradiol was found. Rotenone (2.5×10^{-5} M) reduced mitochondrial binding by 20 per cent, and acetone extraction by 30 per cent [24].

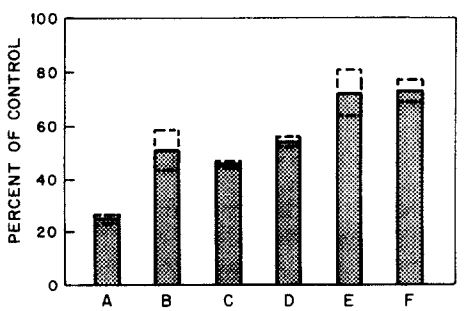

Fig. 5. Comparison of effects of various metabolic inhibitors on nuclear binding of [^{3}H]estradiol. The binding of the untreated control preparations is taken as 100 per cent. (A) Heat-inactivated nuclei (5 min at 73°). (B) Flushed with N_2 for 15 sec. (C) Pyrophosphate, 5×10^{-3} M. (D) DNP, 1.2×10^{-3} M. (E) KCN, 5×10^{-3} M. (F) Ouabain, 5×10^{-5} M. The results are the average of three or four replicate experiments. The dashed lines indicate S. E. M.

Effect of some metabolic inhibitors on nuclear ATP levels. The decreased nuclear binding of labeled estradiol in the presence of potassium cyanide and dinitrophenol may be due to an interruption of nuclear ATP generation. To test this hypothesis, nuclear preparations were incubated in the presence and absence of some metabolic inhibitors [24]. The results indicated that there is no correlation between decreased nuclear binding of labeled estradiol and decreased ATP levels. Furthermore, sodium fluoride, sodium azide and antimycin A did not affect the level of nuclear binding of labeled estradiol.

Association of labeled estradiol with the nuclear macromolecular binding site. The following experiment was performed to provide information concerning the distribution of label between the soluble and particulate components of the nucleus. After incubation with prelabeled cytosol, nuclei were washed in buffer and broken using the Parr pressure bomb. The sample was then centrifuged at 100,000 *g*, and the amount of bound label in an extract of the sedimentable material and in the supernatant was estimated by gel filtration. The nuclear sediment contained 87 per cent of the macromolecular bound radioactivity [24].

To determine whether radioactive estradiol associated with the macromolecular fraction was bound to chromatin, the following experiment was performed. A nuclear preparation was incubated with labeled cytosol and then ruptured as described above. A portion of the pellet was analyzed for estradiol-labeled chromatin using a discontinuous sucrose gradient. Figure 6 shows the profiles of two sucrose gradients. DNase treatment of a sample, prior to centrifugation, destroyed the chromatin and resulted in complete disappearance of the radioactive sedimenting peak.

The effect of some degradative enzymes on macromolecular bound labeled estradiol associated with chromatin (Table 2) was investigated in an experiment similar to the one shown in Fig. 7. The fractions containing chromatin-associated labeled estradiol were pooled and urea and sodium chloride were added (final concentrations 5 and 2 M respectively), thus dissociating the proteins from the DNA [19]. Aliquots were then filtered on Biogel and the fractions containing macromolecular bound estradiol incubated with pronase, DNase or RNase. Only pronase was found to profoundly decrease the amount of macromolecular bound estradiol, indicating that an estradiol–protein complex could be solubilized from the chromatin preparation [24].

Extent of nuclear binding of [^{3}H]estradiol with increasing amounts of nuclei. Figure 7 shows the results of an experiment in which the nuclear binding was measured at 25°, while increasing the concentration of nuclei and holding the volume of cytosol constant. Even at the highest concentration of nuclei used, there is still a considerable amount of label associated with the estradiol-binding protein in the cytosol, yet nuclear binding seems limited. One possible explanation, supported with data below, is the depletion of a cytoplasmic cofactor.

Re-use of cytosol preparation for nuclear binding. Prelabeled cytosol which had previously been incubated with a nuclear pellet was added to a fresh preparation of nuclei. The result, summarized in Table 3, shows that both cytosol binding and nuclear bind-

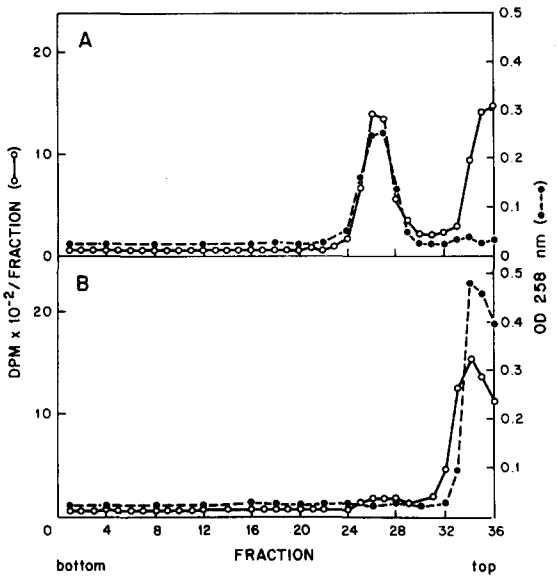

Fig. 6. Sedimentation pattern of crude chromatin preparation obtained after incubation of nuclei with prelabeled cytosol. Tumor nuclei were incubated with prelabeled cytosol for 20 min at 25°, washed and broken by using the Parr pressure bomb. The resulting crude chromatin preparation (200 μg DNA/ml) was divided into two aliquots of 0.4 ml each: panel A, untreated control; panel B, DNase-treated (5 μg/80 μg of nuclear DNA, 5×10^{-3} M $MgCl_2$) for 20 min at 25° prior to centrifugation on the sucrose gradient (4.4 ml of 1.7 M sucrose in 10^{-2} M Tris buffer, pH 7.4), centrifuged for 2 hr at 45,000 rev/min (221,000 *g*) in a Spinco SW 50 rotor at 4°. In panel C, eight drop fractions were collected into 2 ml of 10^{-2} M Tris buffer, pH 7.4, and the absorption was measured at 258 nm in a Beckman spectrophotometer against a buffer-containing blank. The fractions were then extracted with 10 ml of isoamyl-toluene, and the organic phase was counted.

ing of [^{3}H]estradiol are considerably higher with fresh cytosol than with the previously used cytosol. After the first incubation of nuclei, the radioactivity in the cytosol remained unchanged estradiol (thin-layer chromatography), and the cytoplasmic-binding protein sedimented with 5-6 S on salt containing sucrose gradients [24].

Efforts were made to study whether a cytoplasmic cofactor was involved in nuclear binding. Attempts to concentrate Biogel-filtered cytosol preparations with an Amincon concentrating device were unsuccessful, since most of the macromolecular bound [^{3}H]estradiol was lost. Other experiments in which cytosol preparations had been dialysed were hampered by the decrease in binding activity of the cytosol after dialysis. Similarly, experiments with previously used labeled cytosol preparations which were supplemented by addition of fresh, unlabeled cytosol

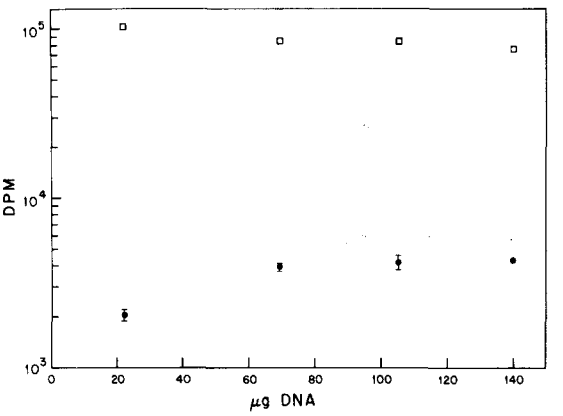

Fig. 7. Effects of increasing amounts of nuclei with constant amount of cytosol on distribution of [^{3}H]estradiol bound in nuclear or cytosol fractions. Increasing amounts of nuclei were added to the tubes in a volume of 0.1 ml. Cytosol (0.2 ml) which had been prelabeled with 10^{-8} M [^{3}H]estradiol was added to the nuclei. After an incubation period of 20 min at 25° the nuclei were washed, a salt extract was prepared and macromolecular binding measured. Cytosol binding was estimated by filtration of the supernatant on columns of Biogel. The squares represent the macromolecular binding remaining in cytosol and the circles the macromolecular binding in the nuclear extract. Each point represents the average of three estimates; vertical bars indicate S.E.M. Where not shown, the values were too small to be graphically depicted.

Table 2. Effects of some degradative enzymes on macromolecular bound [^{3}H]estradiol after extraction from chromatin*

	Macromolecular bound [^{3}H]estradiol (% of control)
(A) Untreated control	100
(B) Pronase (10 μg/ml)	13
(C) DNase (5 μg/ml)	94
(D) RNase (5 μg/ml)	81

* A crude chromatin preparation obtained from tumor nuclei previously incubated with prelabeled cytosol was sedimented on a sucrose gradient under the same conditions as shown in Fig. 7. The fractions containing the DNA were pooled and dialyzed in the cold against 10^{-2} M Tris buffer, pH 7.4. Urea and NaCl were added to a final concentration of 5 and 2 M respectively. Aliquots (0.5 ml) were filtered on columns of Biogel at 4°, and the fractions containing the macromolecular bound [^{3}H]estradiol pooled and divided into four groups. The control was diluted with the same volume of distilled water. All samples were incubated for 15 min at 20° and subsequently assayed for macromolecular binding of [^{3}H]estradiol as described. The binding in the untreated control was 23,000 dis./min/mg of DNA.

Table 3. Effect of repeated use of the same cytosol preparation of nuclear binding of estradiol*

	Cytosol binding (dis./min/0.2 ml)	Nuclear binding dis./min/mg DNA)
(1) First incubation, fresh cytosol		56,000 (2,400)
(2) Second incubation, cytosol of (1)	11,000 (430)	7,700 (390)
(3) Control	21,000 (840)	29,000 (2,000)

* A suspension of tumor nuclei (120 μg DNA/0.1 ml) was incubated with prelabeled cytosol (53,000 dis./min bound in 0.2 ml) for 30 min at 18°. The cytosol was then re-used by adding it to a fresh nuclear preparation. The extent of nuclear binding was determined by filtration of a salt extract of the washed nuclear pellets on columns of Biogel. The control consisted of a cytosol preparation containing no nuclei during the first incubation, but which was kept simultaneously in the waterbath. It was then added to a fresh nuclear pellet and incubated for a second incubation as described above. Cytosol binding was determined by filtration on columns of Biogel. The numbers in parentheses indicate S.E.M. from three separate determinations.

(compared to supplementing with buffer alone) were difficult to interpret. The free [^{3}H]estradiol remaining in the previously used cytosol bound to the freshly added unlabeled cytosol very quickly. This occurred even with short incubation periods of 10 min at 18°, and resulted in higher cytosol binding which in turn gave higher nuclear binding of estradiol.

To overcome these drawbacks, prelabeled cytosol preparations were filtered on columns of Biogel in the cold. The labeled estradiol-binding protein was then precipitated with ammonium sulfate (0–30 per cent fraction) and resuspended in a very small volume of buffer. In order to remove traces of salt from the precipitated proteins, this fraction was then again filtered on another column of Biogel. The fraction containing the macromolecular bound [^{3}H]estradiol is referred to as a preparation of partially purified estradiol-binding protein.

Nuclear binding of [^{3}H]estradiol using cytosol and a preparation of partially purified estradiol-binding protein. When nuclei were incubated with prelabeled cytosol preparations they bound much more [^{3}H]-estradiol than when incubated with the partially purified estradiol-binding protein (Table 4, A and B). The addition of an aliquot of unlabeled cytosol to the partially purified estradiol-binding protein (C) restored the nuclear binding to that attained by the cytosol.

Nuclear binding of partially purified estradiol-binding protein in the presence of uterine and heart cytosol. Since there may be a cofactor or macromolecule which can restore the decreased nuclear binding of [^{3}H]estradiol in the presence of partially purified estradiol-binding protein, the effect of heart cytosol was compared to that of uterine cytosol (Table 5). The result indicated that cytosol prepared from a typical non-target organ, such as heart, was equally effective in restoring the low nuclear binding occurring with partially purified estradiol-binding protein.

Binding of [^{3}H]estradiol by tumor, uterine and liver nuclei. Tumor and uterine nuclei bound approximately the same amount of [^{3}H]estradiol, while the binding by liver nuclei was only 34 per cent of the binding by tumor and uterine nuclei (Table 6). Note that no corrections have been made for the non-specific or background binding of approximately 25 per cent [24]. The liver nuclei preparation showed a similar degree of purity as tumor nuclei, as judged by electron microscopy [24].

Analysis by sucrose gradient centrifugation of nuclear extracts from tumor and liver revealed a labeled

Table 4. Nuclear binding of partially purified [^{3}H]estradiol binding protein*

	[^{3}H]estradiol binding protein suspended in:	Macromolecular bound [^{3}H]E_2 (dis./min) in supernatant	Nuclear binding (dis./min/mg DNA)
(A) Cytosol preparation		53,000 (1,200)	58,000 (2,300)
(B) Partially purified EBP	Tris buffer	52,000 (430)	17,000 (520)
(C) Partially purified EBP	Cytosol	50,000 (2,200)	66,000 (5,400)

* A preparation of cytosol (5 ml) was labeled with 10^{-8} M [^{3}H]estradiol and filtered on columns of Biogel in a cold room. The fraction containing macromolecular bound [^{3}H]estradiol was pooled and the estradiol-binding protein precipitated with ammonium sulfate (0–30 per cent fraction) as described in Materials and Methods. The precipitated proteins were resuspended in 1.0 ml of cold 10^{-2} M Tris buffer, pH 7.1, and again filtered on a column of Biogel in a cold room. An aliquot of the fractions containing macromolecular bound [^{3}H]estradiol (partially purified [^{3}H]estradiol-binding protein) was suspended in unlabeled cytosol or 10^{-2} M Tris buffer as indicated in (B) and (C). The cytosol preparation (A) had been incubated with 2×10^{-9} M [^{3}H]estradiol for 1 hr in ice. The protein concentration of (A) and (C) were equal and amounted to approximately 950 μg/0.25 ml, while (B) contained 56 μg protein. The pH of all three incubation media was adjusted to 7.1 at 25°. Preparations of tumor nuclei (62 μg of nuclear DNA/0.1 ml) were incubated for 20 min at 25° with 0.25 ml of partially purified estradiol-binding protein which had been suspended as described above. The amount of macromolecular bound [^{3}H]estradiol was estimated by filtration of the nuclear salt extract on columns of Biogel. The numbers in parentheses indicate S.E.M.

Table 5. Nuclear binding of partially purified estradiol-binding protein in the presence of uterine and heart cytosol*

Incubation medium	Macromolecular bound [^{3}H]estradiol (dis./min/0.25 ml)	Nuclear binding (dis./min/mg DNA)
10^{-2} M Tris buffer	69,000 (3,500)	17,000 (1,700)
Uterine cytosol	70,000 (2,600)	55,000 (4,300)
Heart cytosol	66,000 (840)	57,000 (560)

* Partially purified estradiol-binding protein was prepared as described in the legend to Table 2. Preparations of tumor nuclei (75 μg nuclear DNA/0.1 ml) were incubated for 20 min at 25° with 0.25-ml aliquots of partially purified estradiol-binding protein (84 μg protein) suspended in 10^{-2} M Tris buffer, pH 7.1 (25°), or in uterine and heart cytosol respectively. The cytosol preparations contained the same amount of protein (0.9 mg). The pH of all incubation media was adjusted to 7.1 at 25°. After incubation, the nuclei were washed, a salt extract was prepared and the amount of macromolecular bound [^{3}H]estradiol assayed on a column of Biogel. The macromolecular bound [^{3}H]estradiol refers to the partially purified estradiol-binding protein. The numbers in parentheses indicate S.E.M.

band having an S value of approximately 5–6 in the case of the tumor extract, while the salt extract from liver nuclei showed a strikingly (approximately 3S) different gradient profile. It has previously been observed after incubation of uterine cytosol and estradiol with nuclei from a non-target organ (diaphragm) that the binding macromolecule extracted from the nucleus sedimented with approximately 3S [26].

Effect of pretreatment of nuclei with cytosol containing estradiol, estriol, diethylstilbestrol or MER-25. Nuclei were incubated for 20 min at 25° with cytosol containing either non-radioactive estradiol, estriol, DES (a non-steroid estrogen) or MER-25 (ethamoxytriphetol), an anti-estrogen.

After incubation the cytosol was removed, the nuclei were washed, and cytosol prelabeled with [^{3}H]estradiol was added for an additional 5 min. Control nuclei were incubated with an aged preparation of cytosol in which binding activity was non-existent. All the samples were 10^{-7} M of each drug to be tested, except MER-25, which was added to a final concentration of 10^{-4} M. MER-25 is a competitive inhibitor of the [^{3}H]estradiol binding to the supernatant EBP. High concentrations of MER-25 markedly reduce the [^{3}H]estradiol binding to the supernatant EBP [24]. The results of this experiment are shown in Fig. 8.

Nuclei do not appear to take up or to efficiently bind MER-25 to the same nuclear site, since nuclear samples preincubated with this drug bound the same amount of [^{3}H]estradiol as control nuclei. In contrast to the results with MER-25, the non-steroidal estrogen, DES, was as effective as non-radioactive estradiol, while estriol was slightly less effective in nuclear binding. These results suggest that prior incubation of nuclei with uterine cytosol and estrogens saturates a nuclear site, so that less [^{3}H]estradiol-labeled binding protein can subsequently bind.

DISCUSSION

When nuclei from the estradiol-binding interstitial cell tumor were incubated with prelabeled uterine cytosol, they bound five to ten times more estradiol than when incubated with heart cytosol, bovine serum albumin or free estradiol. This demonstrates the importance of the cytoplasmic estradiol-binding protein for the intranuclear accumulation of estradiol. The degree of purity of the tumor nuclei represents one of the distinct advantages of the present study, eliminating the potential binding of labeled estradiol to microsomes or mitochondria as it has been shown to occur under assay conditions *in vitro*. Additional support for specific nuclear binding is based on the relative insusceptibility of microsomes and mitochondria to heat treatment prior to the assay. Furthermore, the binding or association of estradiol with microsomes and mitochondria was not affected by the same metabolic inhibitors which were shown to decrease nuclear binding of estradiol. These results not only provide support for a specific binding process in the nucleus but also demonstrate the need for working with nuclear preparations which show the least possible degree of contamination by other cellular organelles. Since these organelles can also bind [^{3}H]estradiol under conditions *in vitro*, simple extraction of relatively crude nuclear pellets with salt solutions cannot be accepted as a measure of nuclear binding of [^{3}H]estradiol. The use of sucrose at a density of 2.2 M (density = 1.28) has been shown to yield very pure nuclei, while a decrease to a density of 1.97 M (density = 1.25) usually results in severe contamination [27]. Thus, the density of the homogenate is one of the most crucial steps in the isolation procedure of nuclei.

The exact biochemical basis for the observed inhibition of nuclear binding of estradiol by metabolic

Table 6. Binding of [^{3}H]estradiol to nuclei from different tissues*

	Nuclear binding (%)
Tumor nuclei	100
Uterine nuclei	99.0 (6.9)
Liver nuclei	33.9 (3.6)

* Nuclei were isolated simultaneously from tumor (LC 14 NB III) and mouse liver on six different occasions and assayed for nuclear binding as described. Uterine nuclei were isolated from rats (two experiments). The nuclear binding is expressed as per cent of binding obtained with tumor nuclei. The numbers in parentheses indicate S.E.M.

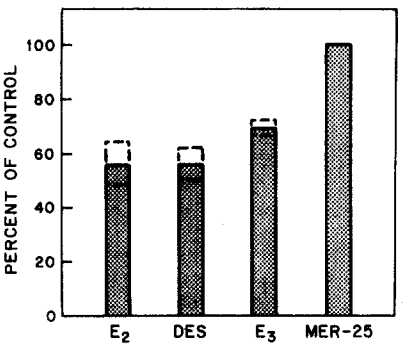

Fig. 8. Effect of prior incubation of nuclei with cytosol containing various drugs. A suspension of rat uterine nuclei was incubated with 0.2 ml cytosol which had been preincubated with non-radioactive estradiol, estriol, DES (10^{-7} M) and MER-25 (10^{-4} M). The amount of DNA/sample in the three different experiments summarized in this figure was 51, 68 and 89 μg/0.1 ml respectively. After an incubation period of 20 min at 25° the nuclei were washed and incubated for a second time at the same temperature for 5 min with 0.2 ml cytosol prelabeled with 10^{-8} M [^{3}H]estradiol. The nuclei were again washed, a salt extract was prepared, and the amount of macromolecular bound [^{3}H]estradiol determined by filtration of the salt extract on columns of Biogel. In order to measure non-specific binding, a pellet of heat-inactivated nuclei (5 min at 90°) was incubated with cytosol preincubated with estradiol (10^{-7} M) and processed as described above. The control consisted of nuclei which were incubated with an aged preparation of cytosol which had also been prelabeled with estradiol. Nuclei were preincubated with estradiol (E_2), DES, estriol (E_3) or MER-25. The data were expressed as per cent binding of the control preparation (87,000 dis./min/mg of DNA) from which the amount of binding of the heat-inactivated nuclei had been deducted. The dashed lines indicate S. E. M.

inhibitors remains unexplained. In view of the observation that there is no correlation between the extent of inhibition of binding and the decrease in nuclear ATP levels [24], and the finding that nuclear ATP levels were also lowered by NaF (which did not affect nuclear binding of estradiol), it is difficult to ascribe the observed inhibition to a depletion of ATP.

The binding of [^{3}H]estradiol to a nuclear protein associated with the chromatin fraction in the present cell-free system mimics the localization *in vivo* of radioactive estradiol.

As noted in Fig. 7 there is the apparent saturated nuclear binding of estradiol while there is still extensive cytosol binding. Possibly a cytoplasmic component is required, for nuclear binding has been depleted and thus limits further nuclear binding of [^{3}H]estradiol. Additional support for the requirement of an unidentified component of the cytosol for nuclear binding was obtained from experiments in which one cytosol preparation was used in two successive incubations with fresh nuclei. This experiment indicated that re-used cytosol, although still containing considerable amounts of estradiol-binding protein, was no longer effective to any significant degree in nuclear binding of estradiol. Experiments in which the nuclear binding of [^{3}H]estradiol was compared using partially purified preparations (by gel filtration) of estradiol-binding protein and labeled cytosol clearly showed that the gel-filtered estradiol-binding protein was considerably less effective than the corresponding crude cytosol preparation. Since the decreased binding with partially purified estradiol-binding protein preparations could be increased to control levels by reconstituting the partially purified estradiol-binding protein with cytosol, it is assumed that Biogel polyacrylamide gel filtration removed some component required for nuclear binding from the cytosol. Alternatively, the Biogel filtration may have converted the estradiol-binding protein to a less active molecule. On the other hand, ammonium sulfate-precipitated estradiol-binding protein, not filtered on Biogel, can be bound by nuclei. Since cytosol prepared from a typical non-target organ could be used with the same result as uterine cytosol for reconstitution of the partially purified estradiol-binding protein, this cofactor is not target tissue specific.

What controls the extreme specificity of target tissue? In the first place, it could be the presence of the binding protein in the cytoplasm. Other possible sites include cytoplasmic cofactors, the nuclear membrane, nuclear acceptor proteins and specific sites on the chromatin. Experiments carried out with tumor, uterine and liver nuclei revealed a lower binding of [^{3}H]estradiol by liver nuclei. Liver nuclei exhibited 34 per cent of the binding obtained with tumor or uterine nuclei. The observation that liver nuclei bind less estradiol suggests that nuclei themselves exert some degree of specificity. Steggles *et al.* [28] showed that uterine chromatin preparations bound two and one-half to three times more estradiol than chromatin preparations from non-target organs. The level of binding by non-target nuclear or chromatin preparations may be predominately non-specific binding. Heat-inactivated tumor nuclei, for instance, bound between 25 and 27 per cent of the amount of control nuclei. Similarly, an aged cytosol preparation (although reduced in binding activity to approximately 10 per cent) will still provide approximately 27 per cent of the binding obtained with fresh cytosol. The reason for this substantial background binding is not clear. This background binding may, however, not be neglected and it becomes especially important in the above studies, where the binding of estradiol by nuclei from different tissues is compared.

A different aspect of this investigation deals with a number of steroids and non-steroidal analogs which will also bind to the binding protein [29]. MER-25 may exert its anti-estrogenic effect by occupying the cytoplasmic-binding protein and therefore preventing estradiol from binding. This had originally been proposed by Jensen [30] as the mechanism of action of this drug. However, it is unclear what distinguishes an estrogen from an anti-estrogen. Conceivably, the binding protein charged with an anti-estrogen is unable to penetrate into the nucleus, or it can penetrate and is unable to attach to chromatin and be retained by it. As Fig. 8 shows, nuclei incubated with cytosol prelabeled with MER-25 showed the same amount of [^{3}H]estradiol macromolecular binding as control preparations. It was concluded, therefore, that this drug was not as capable as estradiol in promoting macromolecular binding to the same site in nuclei. The potent estrogen DES decreased subsequent bind-

ing of [^{3}H]estradiol-labeled binding protein to nuclei to approximately the same extent as estradiol.

Estriol has been termed an "impeded" estrogen, because even massive doses of estriol do not increase uterine weight to the same extent as estradiol [31]. Estriol was less effective than the two potent estrogens in diminishing [^{3}H]estradiol macromolecular binding. It is possible that drugs which bind the binding protein can be divided into the groups of anti-estrogens and estrogens according to their ability to be taken up and retained by nuclei at the same site as promoted by estradiol.

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