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NUCLEAR ESTROGEN RECEPTOR IN THE AVIAN LIVER: CORRELATION WITH BIOLOGIC RESPONSE*

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SUMMARY

Diethylstilbestrol (DES) administration to cockerels resulted in an elevation (7-fold) in the number of high affinity binding sites measured in isolated liver nuclei by the [^{3}H]-estradiol exchange method. Increases were noted within 60 min, reached a maximum by 4 h and by 48 h had returned to control values. Elevation in the quantity of nuclear binding sites was estrogen-specific and 20% of the [^{3}H]-estradiol bound could be extracted by 0.4 M KCl. When isolated chromatin was used to evaluate estrogen–receptor interaction, the binding data were similar to those obtained with intact nuclei. To correlate estrogen binding with a specific hepatic response to this hormone, very low density lipoproteins (VLDL) synthesis was measured by immunochemical techniques in liver slices incubated *in vitro* in the presence of [^{3}H]-lysine following a single injection of DES. An increase in immunoprecipitable VLDL in the liver slices was first noted 2 h after estrogen administration, suggesting that nuclear receptor occupancy occurs prior to the earliest detectable stimulation of protein synthesis. To determine whether receptor binding and protein synthesis were temporally separated by estrogen-induced changes in transcription, RNA polymerase activities were measured in isolated nuclei and RNA synthesis initiation sites were quantified in chromatin preparations. Stimulation of RNA polymerase I and II activities as well as an 85% increase in chromatin initiation sites were demonstrable within 1 h following DES treatment. Thus, these effects were closely correlated in time with increases in nuclear estrogen receptor levels and again preceded enhanced VLDL synthesis. Finally, a dose-related correlation was noted between accumulation of nuclear estrogen-receptor complexes and elevation of plasma triglycerides and VLDL following DES treatment. Collectively, these data provide evidence that estrogen receptor interaction may underwrite the physiologically important regulation of plasma lipoproteins by a mechanism involving both transcription and translation.

INTRODUCTION

Estrogen has been shown to profoundly influence plasma triglyceride levels [1, 2]. In cockerels the administration of estrogen produces an increased hepatic synthesis of very low density lipoproteins (VLDL) [3, 4]. In previous work from this laboratory, a major VLDL protein, apoVLDL-II, had been purified and sequenced [5]. Specific antibodies were prepared against purified apoVLDL-II, and estrogen was shown to increase the level of translatable mRNA specific for apoVLDL-II [6]. This specific response of the cockerel liver to estrogen treatment offers a valuable experimental system for the study of hormonal regulation of an apolipoprotein gene.

In contrast to the enormous amount of work on the estrogen receptor of the mammalian uterus and liver [7–9], relatively little is known about the nature of the receptor in avian liver. A cytoplasmic high affinity estrogen-binding protein has been detected in embryonic chick liver but its level drops to extremely low values after hatching [10]. The presence of nuclear estrogen receptors had been reported by a number of investigators [11–18]. However, information documenting correlations between nuclear receptor accumulation and biologic responses in the VLDL synthesis process are lacking. In this report we describe a systematic study of specific nuclear binding sites for estrogen, and the correlation of occupancy of such sites with steroid-mediated effects on nuclear transcriptional events and the synthesis and secretion of VLDL.

MATERIALS AND METHODS

Animals. Three-week old white Leghorn cockerels (0.2 kg) were used for all the experiments. They were obtained from Rich-Glo Farms and were kept in a controlled environment at 23°C with a light–dark

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Abbreviations used: TE-buffer, 0.01 M Tris–HCl, 0.0015 M EDTA, pH 7.4 at 25°C; TGM-buffer, 0.01 M Tris–HCl, 0.001 M $MgCl_2$, 0.02% sodium azide (w/v), 25% glycerol (v/v), pH 7.4 at 25°C; DES, diethylstilbestrol; R_n, nuclear estradiol receptor; R_n^1E, KCl-extractable nuclear estradiol–receptor complex; R_n^2E, KCl-resistant nuclear estradiol–receptor complex; VLDL, very low density lipoproteins; HAP hydroxylapatite; SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0 at 20°C; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

cycle of 12 h of each. Animals were fed a standard laboratory chicken diet which was provided *ad libitum*. Diethylstilbestrol (DES) was dissolved in sesame oil and was administered subcutaneously in the wing at the doses and times prior to sacrifice as indicated in the appropriate figures and tables. Animals were killed by decapitation and exsanguination. Samples of blood were collected in 0.1% (w/v) EDTA. After removal of the red cells by low-speed centrifugation, the plasma was used for determination of triglyceride by the autoanalyzer technique [6]. Livers were rapidly removed, trimmed, rinsed in ice-cold 0.9% NaCl, and held in ice-cold 0.9% NaCl prior to isolation of nuclei.

Isolation of nuclei. Nuclei were isolated by a modification of the method of Schibler and Weber[19]. Initial steps of the procedure were performed at 4°C. Livers were drained, weighed and finely minced with scissors. Ten volumes of homogenization buffer [10 mM Tris, (pH 7.5): 10 mM NaCl, 1.5 mM $MgCl_2$, and 50% (v/v) glycerol] were added and the tissue was dispersed with a Polytron Pt-10 homogenizer (2–3 bursts of 10 s each at a setting of 5 with intermittent cooling). This homogenate was cooled to −20°C by the use of a NaCl ice bath (1:3, w/w). Temperature was maintained at −20°C throughout the remainder of the isolation procedure. The cooled mixture was further homogenized by 7 strokes in a Dounce homogenizer equipped with a loose fitting pestle. The resulting homogenate was filtered through 4 layers of cheese cloth and one layer of organza. The filtrate was centrifuged at 3900 *g* for 15 min in a Beckman JA-20 rotor. The crude nuclear pellet was resuspended with a Dounce homogenizer in 10 volumes of homogenization buffer which had been adjusted to 0.1% Triton X-100 (v/v). This suspension was filtered through 1 layer of organza and 1 layer of Miracloth (Calbiochem) and centrifuged at 3900 *g* for 8 min. The Triton wash step was repeated once. The purified nuclear pellet was resuspended in 0.01 M Tris–HCl, 0.001 M $MgCl_2$, 25% glycerol (v/v), 0.02% sodium azide (w/v), pH 7.4 at 25°C (TGM Buffer) and centrifuged at 3900 *g* for 8 min at 4°C. Nuclei were suspended in the appropriate buffer for subsequent studies. Purity of the nuclei was monitored by phase contrast microscopy at all stages of isolation. Aliquots of the initial homogenates and the final nuclear preparations were assayed for protein by the procedure described by Lowry *et al.*[20], and for DNA by the diphenylamine method described by Burton[21].

Estrogen receptor assays. The quantity of estrogen–receptor complexes present in liver nuclei was determined by a [^{3}H]-steroid exchange assay [22]. The washed nuclear pellet was suspended in TGM buffer. Portions (250 μl) of this suspension were dispensed into two series of tubes, A and B, containing 100 μl of TE buffer. Series A contained 0.4 to 12 nM [^{3}H]-estradiol and was used to determine the total amount of [^{3}H]-estradiol exchanged. Series B contained [^{3}H]-estradiol as in series A plus a 100-fold excess of nonradioactive DES. Nuclear fractions were incubated for the times and at the temperatures indicated in legends of appropriate figures and tables. Following incubation, 1.0 ml of TE buffer was added and the samples were centrifuged at 800 *g* for 10 min. Pellets were washed three times with cold TE buffer and extracted with 1.0 ml absolute ethanol at 30°C for 30 min. After centrifugation at 800 *g* for 10 min, the radioactivity in the ethanol extract was determined by liquid scintillation spectrometry. Specific nuclear binding was calculated by subtraction of set B from set A.

KCl extraction of nuclear estradiol–receptor complexes. An equal volume of 0.8 M KCl-TE buffer was added to the purified nuclear pellet yielding a suspension with a final KCl concentration of 0.4 M. The suspension was mixed and kept at 0°C on ice for 30 min with vortexing at 5 min intervals. At the end of the incubation period, the suspension was centrifuged at 800 *g* for 10 min in a Beckman JA-20 rotor. The supernatant fraction was recentrifuged at 50,000 *g* for 20 min in a Beckman SW56 rotor. This supernatant fluid was used to quantitate levels of KCl-extractable estrogen–receptor complexes.

Hydroxylapatite assay. Specific [^{3}H]-estradiol binding in 0.4 M KCl extracts of liver nuclei was determined by a modification of the hydroxylapatite (HAP) procedure of Erdos *et al.*[23]. Bio gel HT, hydroxylapatite (Bio-Rad) was washed extensively with TE-buffer at 4°C until the pH of the washes was the same as that of the buffer. It was resuspended in TE-buffer at a ratio of 60% HAP to 40% buffer. Aliquots of KCl-extracts (250 μl) were dispensed in two sets of tubes, A and B. Series A contained varied concentrations of [^{3}H]-estradiol (0.1–12 nM) in 50 μl plus 50 μl of TE buffer while series B contained an equal amount of [^{3}H]-estradiol (50 μl) plus a 100-fold excess of nonlabeled DES (50 μl). The tubes were incubated at 30°C for 30 min. After incubation, tubes were placed in ice, 500 μl of the HAP suspension was added and the samples incubated at 0°C for 15 min with vortexing every 5 min. The tubes were then centrifuged at 800 *g* for 10 min. The resuling pellets were washed three times with TE buffer, and the final pellet extracted with 1.0 ml of absolute ethanol at room temperature for 30 min. The ethanol extract was added to 4.0 ml of ACS scintillation liquid (Amersham-Searle) and total radioactivity measured by scintillation spectrometry. Specific binding was calculated by subtraction of set B from set A.

Sucrose gradient analysis of KCl-extractable estrogen receptor complexes. KCl extracts were divided into two portions. One portion was incubated in the presence of 1.2×10^{-8} M [^{3}H]-estradiol at 30°C for 30 min. The other portion was incubated under identical conditions in the presence of a 100-fold excess of unlabeled DES. Free hormone was removed by treatment with dextran-coated charcoal for 15 min at 4°C [24]. After exposure to charcoal, the samples were centrifuged at 2500 *g* for 10 min.

The supernatant fluid was further centrifuged at 200,000 *g* for 60 min in a Beckman SW56 rotor. The resultant supernatant fractions were concentrated to 600 μl in an Amicon B15 Minicon apparatus. Linear sucrose gradients (4.9 ml) containing 5–20% sucrose in TE buffer plus 0.4 M KCl were prepared. Aliquots of samples (100 μl) were layered, and gradients were centrifuged at 45,000 rev./min for 16 h in a Beckman SW50.1 rotor at 4°C. In parallel gradients the following proteins were included as standards, [^{14}C]-ovalbumin (3.5 *S*), [^{125}I]-Apo-A-I lipoprotein (2.3 *S*), and [^{14}C]-alkaline phosphatase (6.3 *S*). Gradients were fractionated into 0.2 ml fractions and radioactivity was counted in 4.0 ml of ACS (Amersham-Searle).

Assay of endogenous RNA polymerase activities. Endogenous nuclear RNA polymerase activities were measured as described by Hardin *et al.*[25]. An aliquot of the purified nuclei (5–15 μg of DNA) was added to an assay mixture containing 2.5 μmol of Tris–HCl (pH 8.0), 0.05 μmol of $MnCl_2$, 0.1 μmol of $MgCl_2$, 3 μmol of $(NH_4)_2$ SO_4, 0.1 μmol of dithiothreitol, 0.03 μmol each of ATP, CTP and GTP, 0.003 μmol of unlabeled UTP and 2.5 μCi of [^{3}H]-UTP (12–16 Ci/mmol) in a final volume of 50 μl; 0.005 μg α-amanitin was also added to the appropriate tubes. Reactions were incubated at 30°C for 20 min and terminated by removing a 25 μl aliquot from a reaction and spotting it on a 2.5 cm DE-81 filter paper disc. The disc was immediately transferred to a wash solution of 0.5 M Na_2HPO_4. Filters were washed and counted as described by Roeder[26]. Under these assay conditions one pmol of [^{3}H]-UMP incorporated represents 260 c.p.m.

Isolation of liver chromatin. Cockerel liver chromatin was isolated from purified nuclei by a modification of the method of Spelsberg *et al.*[27]. Briefly, purified nuclei were washed three times by resuspension in 80 mM NaCl, 20 mM Na EDTA, pH 6.3, and subjected to centrifugation at 2000 *g* for 10 min. The washed nuclei were then suspended in 50 ml 0.01 × SSC and kept at 0°C until at least 90% of the nuclei had lysed as judged by phase contrast microscopy. The chromatin released from the nuclei was collected by centrifugation at 10,000 *g* for 10 min. It was then resuspended in 0.01 × SSC at a final DNA concentration of 0.5 mg/ml. Chromatin preparations were stored at 4°C and used within 5–7 days of isolation.

Determination of rifampicin-resistant E. coli *RNA polymerase initiation sites.* RNA synthesis on liver chromatin following addition of *E. coli* RNA polymerase was performed in the presence of rifampicin and heparin as described by Tsai *et al.*[28]. *E. coli* RNA polymerase was prepared from 500 g of frozen *E. coli* K12 cells (Grain Processing) by the method of Burgess and Jendrisak[29]. Varying concentrations (0–30 μg) of the enzyme were first incubated at 37°C for 15 min with 3 μg chromatin suspended in 95 μl of preincubation buffer containing 12.5 μg BSA, 10 μmol Tris–HCl, pH 7.9, 1.25 μmol $MnCl_2$, 2.5 μmol β-mercaptoethanol, and 6.25 μmol $(NH_4)_2SO_4$. At the end of the preincubation, RNA synthesis was initiated by the addition of 30 μl of a mixture containing 5 μCi [^{3}H]-UTP [12–16 Ci/mmol], 18.75 nmol each of ATP, CTP, GTP, and UTP, 100 μg heparin, 5 μg rifampicin, and 0.1 μmol potassium phosphate, pH 8.0. The reaction mixtures were incubated for an additional 15 min at 37°C. At the end of this period, 50 μl aliquots were taken from each reaction mixture and spotted on DE-81 filters. The filters were washed and counted by scintillation spectrometry as described in a previous section.

Isolation and sizing of in vitro *synthesized RNA.* RNA from the above reaction mixture was isolated and sized as described by Tsai *et al.*[28] with the following modifications. The synthesized RNA was adjusted to 0.5% SDS, 50 μg of proteinase K was added and the sample was incubated for 15 min at 37°C. The solution was then subjected to phenol extraction, ethanol precipitation and sucrose gradient centrifugation as described previously [28]. The size of the newly synthesized RNA was determined by the c.p.m. incorporated into RNA molecules on the different fractions of the sucrose gradient.

Quantitation of in vitro *synthesized VLDL.* Quantitation of VLDL synthesis by liver slices *in vitro* was performed as previously described [6]. Groups of four cockerels were treated with a single subcutaneous injection of either DES (2.5 mg/0.2 kg) in sesame oil or sesame oil alone. At the indicated times after injection, the right lobe of the liver was removed and was cut into 50–100 mg pieces. The tissue was transferred to 25 ml Erlenmeyer flasks (200–350 mg/flask) and incubated in 2 ml of medium 199 without amino acids (Grand Island Biological Co.); one-eighth the usual amount of amino acids in medium 199 was added. The medium also contained 1.2 mg/ml of $NaHCO_3$, 1 U/ml of penicillin and 1 mg/ml of streptomycin. Twenty-five μCi of L-4,5-[^{3}H]-lysine monohydrochloride (S.A. 19 Ci/mmol, Amersham-Searle) was then added. The tissue was incubated at 37°C with continuous shaking in the presence of 95% O_2–5% CO_2 for 2 h. With these incubation conditions, incorporation of L-[^{3}H]-lysine into immunoprecipitable material was linear for at least 2 h. After incubation, excess (0.5 mmol) unlabeled L-lysine was added, the tissues were weighed, and homogenized in the incubation medium by six strokes in a glass–Teflon homogenizer. The homogenate was then centrifuged at 105,000 *g* for 60 min at 4°C. The supernatant fluid was used for determination of radioactive VLDL.

Aliquots from the 105,000 *g* supernatant were incubated with a rabbit anti-VLDL globulin fraction in the presence of carrier VLDL at a concentration to precipitate 50% of the anti-serum. A typical incubation mixture consisted of the following: sample 200 μl, antisera 75 μl; carrier VLDL (1 mg protein/ml) 5 μl, 20% Triton X-100 400 μl, 10% sodium deoxycholate 100 μl, 100 mM sodium phosphate, pH 7.5, 50 μl, and 100 mM L-lysine 5 μl. Incubation was at 23°C for

3 h or at 23°C for 30 min, and then overnight at 4°C. Both methods were shown to result in maximal precipitation. Immunoprecipitates were collected by centrifugation at 2,000 *g* for 5 min. The supernatant fluid was decanted and the immunoprecipitate was washed three times, once with a standard buffer (10 mM sodium phosphate, pH 7.5, 15 mM sodium chloride, 4 mM L-lysine, 1% sodium deoxycholate and 1% Triton X-100) and twice with 0.9% NaCl. The washed pellet was dissolved in 1 ml of NCS (Amersham-Searle) and counted in 10 ml of spectrofluor toluene. The nonspecific radioactivity was determined by precipitating the supernatant fluid a second time and subtracting this amount of radioactivity from the amount precipitated in the first immunoprecipitate. Immunoprecipitable counts determined by this method were found to give excellent correlation with radioactivity in VLDL quantitated by ultracentrifugal flotation [6].

Preparation of antisera against VLDL and determination of equivalence point were performed as described previously [6].

RESULTS

Binding characteristics of the nuclear estradiol receptor of the chick liver

Saturation curves for specific [^{3}H]-estradiol binding in nuclei from untreated cockerels and from cockerels treated with 2.5 mg of DES per 0.2 kg were obtained by incubating aliquots of purified nuclei at 20°C for 3 h with varying concentrations of [^{3}H]-estradiol (0.2–16 nM) as described in "Methods". Nuclei from unexposed liver contained low concentrations of specific binding sites. Treatment with a single dose of DES (2.5 mg) at 4 h before sacrifice increased the concentration of these sites by 7-fold (Fig. 1). In this analysis, the nonspecific binding was low (15%) in relation to the total binding of hormone and was linear over the range of estradiol concentrations tested. The specific nuclear binding sites were saturated at a hormone concentration of 8 nM and little additional binding was observed at higher concentrations. When the data were analyzed according to the method of Scatchard[30], nuclei from both groups of animals contained a single class of high affinity binding sites with an apparent K_D of 2×10^{-9} M. Liver nuclei from untreated animals contained 0.08 fmol of high affinity binding sites per μg DNA. After DES treatment, the concentration of binding sites increased to 0.60 fmol per μg DNA.

Optimization of [^{3}H]-estradiol exchange assay for liver cell nuclei

Purified nuclei isolated from livers of laying hens were used to determine optimal conditions with respect to time and temperature of incubation for the [^{3}H]-estradiol exchange assay. Nuclei were incubated at 4, 20, 30 and 37°C with a saturating amount of [^{3}H]-estradiol (12 nM), and the specific binding was determined following incubation for varied periods of time.

As shown in Fig. 2, the exchange reached a plateau after 20–30 min at 30–37°C. Decreased binding, due possibly to degradation or inactivation of the receptors, was observed when incubation was carried out beyond 20 min for 37°C and 120 min for 30°C. At 20°C, labeled ligand exchange approached completion within 6 h, while at 4°C extended incubation was required for complete exchange. The conditions for optimum exchange with minimal degradation were 30 min at 30°C, and these conditions were used for all subsequent exchange incubations. No significant change in the K_D of the binding reaction occurred

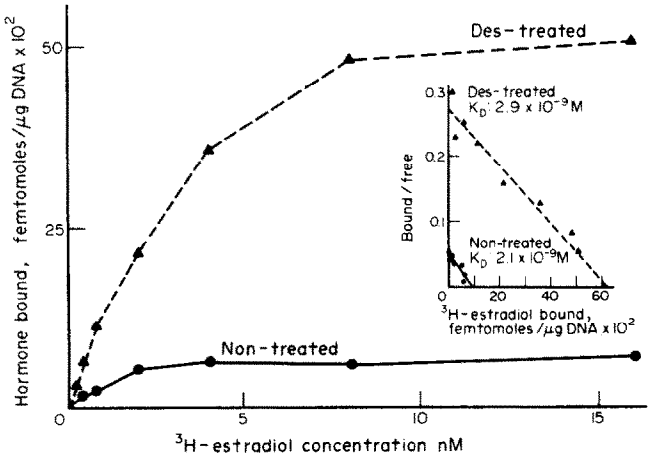

Fig. 1. The binding capacity and affinity of estrogen receptor in liver nuclei from chicks treated with 2.5 mg of DES (▲---▲) and controls (●——●). Groups of three animals were killed 4 h after injection and nuclei were prepared as described in Methods. Aliquots of nuclei were incubated with varied concentrations of [^{3}H]-estradiol at 30°C for 30 min. Specific [^{3}H]-estradiol binding was determined by the [^{3}H]-estradiol exchange assay described under Methods to give bound [^{3}H]-estradiol (fmol/μg DNA × 10^2) versus the concentration of [^{3}H]-estradiol (nM). Inset shows Scatchard plot of the same data.

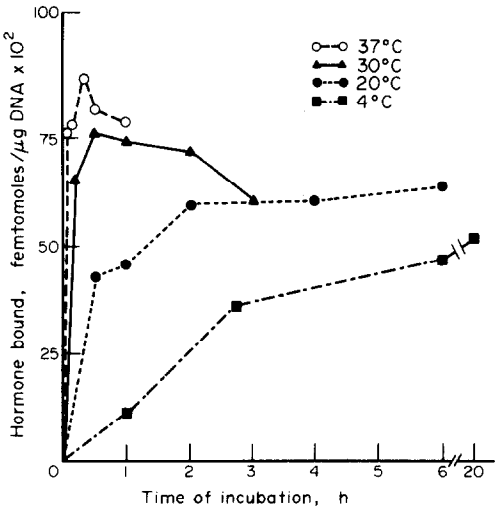

Fig. 2. Optimization of [^{3}H]-estradiol nuclear exchange assay conditions. Liver nuclei from three laying hens were incubated with 12 nM [^{3}H]-estradiol or [^{3}H]-estradiol plus 100-fold excess DES at varied times and temperatures. At the designated times, the exchange of [^{3}H]-estradiol was determined by ethanol extraction of the nuclear pellets as described for the [^{3}H]-estradiol exchange assay in Methods.

when the temperature of the incubation mixture was altered (data not shown). Therefore, the observed increase in bound [^{3}H]-estradiol is not due to an increase in the affinity of the receptor for the hormone.

Characteristics of the nuclear estrogen receptor

The steroid binding specificity of the nuclear estrogen binding sites was determined as follows. Isolated nuclei were incubated with 12 nM [^{3}H]-estradiol both with and without a 100-fold excess of different steroids or steroid analogs at 30°C for 30 min, and the amount of labeled hormone which remained bound was determined (Table 1). Substantial competition was demonstrated only by the estrogenic compounds. The presence of a 100-fold excess of either DES or estradiol obliterated [^{3}H]-estradiol binding. The addition of either estrone or estriol resulted in a 66% and 64% reduction in [^{3}H]-estradiol binding. Non-estrogenic steroid hormones, such as progesterone, corticosterone, or testosterone had no apparent effect on such binding.

Liver nuclei from DES-treated cockerels were prepared, and receptor complexes were extracted with 0.4 M KCl at 4°C for 30 min. The intact nuclear complexes and KCl-resistant complexes were analyzed by the [^{3}H]-estradiol exchange assay, while the KCl-extractable estrogen binding activity was measured by the HAP assay as described in Methods. About 20% of the receptor complexes could be extracted with 0.4 M KCl. As shown in Fig. 3, the K_D for the extractable complex (1.9×10^{-9} M) and the KCl-resistant complex (2.9×10^{-9} M) are similar. Despite some loss of binding capacity during the procedure, the recovery of extractable (0.125 fmol/μg DNA) and nonextractable (0.52 fmol/μg DNA) receptor complex represents about 85% of the initial nuclear binding.

Table 1. Specificity of [^{3}H]-estradiol exchange by isolated liver nuclei from chicks treated with DES

Competitor	Estradiol binding % bound radioactivity remaining
Buffer	100
Estradiol	0
DES	0
Estrone	34
Estriol	36
Progesterone	100
Corticosterone	100
Testosterone	100

Sixteen hours after treatment with 2.5 mg DES/0.2 kg three animals were sacrificed and liver nuclei were isolated. Nuclei (0.31 mg DNA) were incubated with 12 nM [^{3}H]-estradiol plus an 100-fold excess of different competitor at 30°C for 30 min in the nuclear exchange assay described in Methods. Bound radioactivity of 100% represents 32,000 c.p.m.

The KCl-extractable complex was further characterized by sucrose gradient centrifugation. As shown in Fig. 4, a peak of radioactivity migrated in the 5 *S* region on a 5–20% sucrose gradient. The presence of a 100-fold excess of DES abolished the peak.

Chromatin binding of estrogen–receptor complexes

Since the bulk of nuclear estrogen–receptor complexes were resistant to extraction with KCl, we next examined the effect of DES on specific [^{3}H]-estradiol binding to chromatin *in vitro*. Liver chromatin was isolated from control and 16 h DES-treated animals as described in Methods. Specific saturable estradiol binding sites were demonstrated in both groups of animals by the [^{3}H]-estradiol exchange assay. Bind-

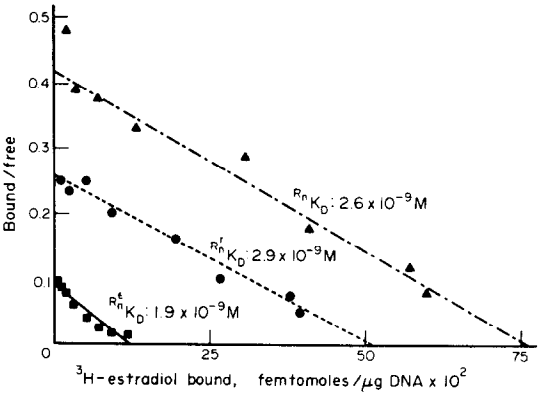

Fig. 3. Determination of K_D and number of specific nuclear KCl-extractable estrogen binding complexes in liver nuclei isolated from DES treated chicks. Three animals were killed 6 h after treatment with 2.5 mg DES/0.2 kg. Nuclei were prepared and extracted with 0.4 M KCl-TE buffer as described in Methods. The receptor content of intact nuclei and nuclear KCl resistant binding sites was determined by [^{3}H]-estradiol exchange assay. The KCl-extractable receptor content was measured by the hydroxyl-apatite assay.

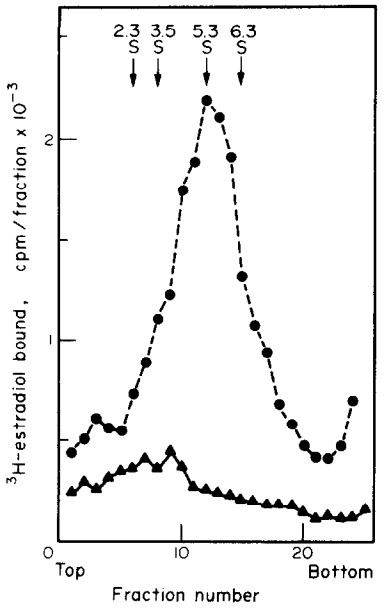

Fig. 4. Sucrose gradient sedimentation profile of KCl-extracted nuclear estrogen receptor. Receptors of isolated nuclei were extracted with 0.4 M KCl at 4°C for 30 min. The extracted DES–receptor complexes were incubated with 12 nM [^{3}H]-estradiol under exchange conditions at 30°C for 30 min prior to centrifugation. The extract was treated with charcoal to remove unbound radioactivity. Sucrose gradients (5–20%) were fractionated in 0.2 ml aliquots and the [^{3}H]-estradiol bound/fraction was determined. [^{14}C]-ovalbumin (3.5 S), [^{125}I]-Apo-A-I lipoprotein (2.3 S), and [^{14}C]-alkaline phosphatase (6.3 S) were used as markers in the gradients.

ing was examined both at 0°C for 60 min and at 30°C for 30 min. The Scatchard analysis of the binding data is shown in Fig. 5. Estradiol binding sites (measured at 0°C and under exchange conditions at 30°C) are substantially increased by DES treatment when measured at both 0 and 30°C. The [^{3}H]-estradiol binding at 0°C by chromatin from DES-treated animals (0.22 fmol/μg DNA) represents 60% of the bound [^{3}H]-estradiol measured under exchange conditions at 30°C for the same chromatin preparation (0.35 fmol/μg DNA). For chromatin from untreated animals, the binding of [^{3}H]-estradiol under exchange conditions at 0°C is extremely low (0.06 fmol/μg DNA) and represents about 75% of the binding observed at 30°C (0.08 fmol/μg DNA). The amount of [^{3}H]-estradiol bound at 30°C is about 4-fold higher for hormone treated compared to untreated animals. At the two temperatures studied, the dissociation constants for the [^{3}H]-estradiol binding assay are similar, 2.5×10^{-9} M at 0°C and 2.6×10^{-9} M at 30°C.

Effect of estrogen on endogenous nuclear RNA polymerase activities

Single injections of DES result in increased activities of both RNA polymerase I and II. Table 2 shows that hormone treatment causes a 100% increase in RNA polymerase II activity and a 40–50% increase in polymerase I activity within 1 h. Although maximal stimulation of both enzymes occurs between 1 and 3 h, enhanced activities are still demonstrable at 24 h.

Effects of estrogen on RNA polymerase initiation sites on liver cell chromatin

Estrogen effects on RNA polymerase initiation sites on liver chromatin were determined. The number of initiation sites were measured by the amount of rifampicin resistant RNA synthesis following preincubation of varying amounts of *E. coli* RNA polymerase with liver chromatin. Figure 6 is a typical titration curve for determination of the number of initiation sites. The transition point of the curves was used to calculate the number of sites on chromatin [28]. The data reveal that DES results, within 3 h after a single injection, in a stimulation of the number of initiation sites from 26,000 to 42,000 per μg of DNA.

The time course of DES stimulation can be seen in Table 3. An 85% stimulation is noted by 60 min, and the number of initiation sites continued to increase thereafter. By 24 h after injection, the hormone-induced enhancement of RNA polymerase initiation sites continues at 200% of the number measured in the unstimulated controls.

Temporal relationship between the increase of nuclear estrogen receptor, rate of VLDL synthesis, and levels of plasma triglycerides

The concentration of estrogen receptor in liver nuclei isolated from DES-treated cockerels was deter-

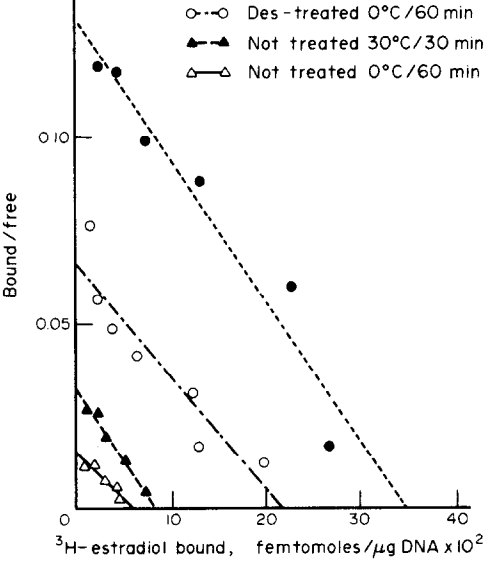

Fig. 5. Livers from DES treated (2.5 mg/0.2 kg) and control chicks were used for chromatin preparation at 16 h after treatment. Portions of the chromatin from each treatment group of three chicks were incubated at 4°C for 1 h in a [^{3}H]-estradiol binding assay or at 30°C for 30 min under exchange conditions in a [^{3}H]-estradiol exchange assay. [^{3}H]-estradiol binding was determined, and the data plotted according to Scatchard [30].

Table 2. Effect of DES on endogenous RNA polymerase activities in isolated nuclei

Time (h)	RNA Polymerase I (pmol [^{3}H]-UMP Incorporated/100 μg DNA)	RNA Polymerase II (pmol [^{3}H]-UMP Incorporated/100 μg DNA)
0	200 ± 2.83	223 ± 4.24
1	278 ± 5.1	424 ± 3.36
3	288 ± 0.89	437 ± 1.41
12	288 ± 9.77	386 ± 1.58
24	266 ± 1.41	326 ± 2.41

Three week old cockerels in groups of three were sacrificed at the times indicated after treatment with 2.5 mg DES/0.2 kg. Liver cell nuclei were prepared and endogenous RNA polymerase activities were measured as described in Materials and Methods. Values represent the mean of six individual determinations ± standard deviation.

mined by the [^{3}H]-estradiol exchange assay at varying times after treatment. As can be seen from Fig. 7, the number of nuclear estrogen binding sites increases rapidly within 1 h, reaching a maximum at 4 h and then declines gradually between 8 and 24 h after treatment approaching control levels by 48 h.

In parallel experiments, liver slices were incubated *in vitro* from control and DES-treated animals as described in Methods. As shown by the interrupted line in Fig. 7, there was a 2-fold increase in the rate of VLDL synthesis within 2 h of hormone injection. The maximum increase in this rate occurred between 12 and 24 h after DES injection and represented a 4-fold increase over the untreated controls. By 48 h, VLDL synthesis had returned to rates similar to those determined in 0 time controls.

Plasma triglyceride levels were measured in blood collected from the same animals used to quantify nuclear estrogen receptors and the rate of VLDL synthesis (Fig. 7). After an initial decline in plasma triglycerides at 1 h following DES treatment the levels began to increase. A maximal 6-fold increase occurs by 24 h. Subsequently, triglyceride concentration declines towards control values.

Relationship between nuclear estradiol receptor concentration and plasma triglyceride levels

A dose response relationship was shown to exist for the concentration of specific estradiol binding sites and plasma triglyceride levels at 16 h after DES treatment (Fig. 8). Chicks were treated with varying amounts of DES from 50 to 2500 μg/0.2 kg. At 16 h

Table 3. Effect of DES on number of RNA synthesis initiation sites on isolated chromatin

Hours after treatment	Number of initiation sites/pg DNA × 10^{-3}
0	26 ± 1.26
1	49 ± 3.15
3	42 ± 1.75
12	50 ± 3.20
24	53 ± 0.60

Three week old cockerels in groups of three were sacrificed at times indicated after treatment with 2.5 mg DES/0.2 kg. Liver chromatin was isolated and *E. coli* RNA polymerase initiation sites were measured as described in Materials and Methods. Values represent the mean of six individual determinations ± standard deviation.

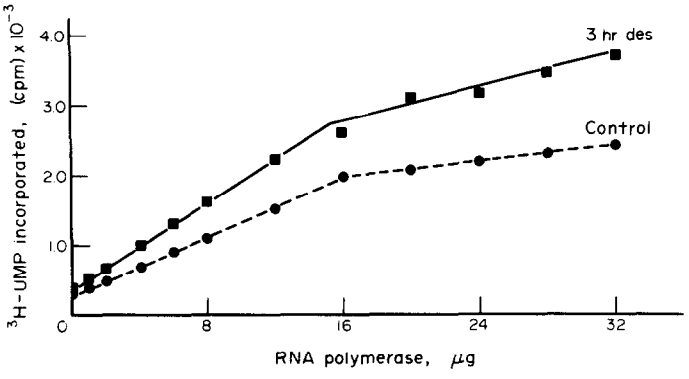

Fig. 6. Rifampicin-resistant RNA synthesis on chromatin. Increasing amounts of *E. coli* RNA polymerase were preincubated for 15 min with 3 μg of liver chromatin isolated from chicks injected with either oil or DES 3 h prior to sacrifice. After the preincubation, RNA synthesis was initiated by the addition of nucleoside triphosphates, rifampicin, and heparin as described in Materials and Methods. The amount of RNA synthesized was determined by the DE-81 filter assay.

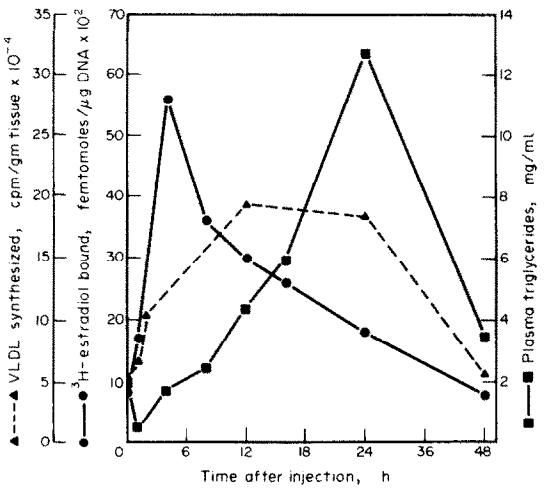

Fig. 7. Effect of DES treatment on nuclear estrogen receptor levels, plasma triglycerides, and liver VLDL synthesis. At intervals after injection with DES, groups of three animals were sacrificed and nuclei were prepared from liver for measurement of nuclear [^{3}H]-estradiol binding by the [^{3}H]-estradiol exchange assay. Plasma samples were taken for determination of triglycerides. In some experiments, liver slices from treated chicks were prepared for organ culture where changes in VLDL synthesis were monitored as described in Methods.

after treatment, plasma triglyceride levels and nuclear estradiol receptor complexes were determined as described in Methods. Total amount of specific estradiol binding for each concentration of DES was determined from Scatchard analysis of the binding data. As shown in Figure 8, elevated plasma triglyceride levels closely correlated with the elevation in nuclear receptor levels. No stimulation of triglyceride levels or [^{3}H]-estradiol binding was detected at a dose of 100 μg/0.2 kg of DES or less. However, levels of plasma triglycerides and nuclear [^{3}H]-estradiol binding were substantially stimulated by injection of 500 μg DES/0.2 kg. The binding of [^{3}H]-estradiol at the highest dose tested, 2500 μg/0.2 kg was only 15% higher than binding observed from a dose of 500 μg. Similarly, the elevation of plasma triglyceride levels plateaued at a treatment dose of about 1000 μg/0.2 kg.

DISCUSSION

In a previous report from this laboratory [6], we presented evidence that estrogen stimulates VLDL synthesis in the cockerel liver. The major site of action of estrogen appears to be at the transcriptional level since administration of the hormone results in the accumulation of the mRNA for a major VLDL protein (apoVLDL-II). In the present communication, we have examined in some detail the nuclear events which precede the increase in VLDL synthesis following estrogen administration.

In many estrogen target organs the hormone initially binds to specific cytoplasmic receptors which then translocate to the nucleus [7, 9]. The avian liver appears to differ from the usual in that cytoplasmic receptors are not readily detectable [11, 12, 14, 17, 31]. In agreement with these reports we have been unable to demonstrate specific cytoplasmic receptors for estrogen in the adult avian liver. However, by employing a modification of the procedure of Schibler and Weber[19] to obtain highly purified nuclei, the nuclear estrogen receptor is easily demonstrated by the [^{3}H]-estradiol exchange assay. This assay permits the quantitation of total nuclear receptor sites [22] and has allowed a quantitative survey of nuclear estradiol receptor response at different times following estrogen administration. In addition, the use of this technique circumvents some of the inherent difficulties in most of the previous studies employing KCl extraction [11, 13, 16, 17] or trypsin digestion [15] of nuclei preparations.

The estradiol binding site characterized in the present study conforms to the characteristics noted for estrogen hormone receptor in other estrogen responsive tissues with respect to hormone specificity, K_D, and sedimentation coefficient [8, 9]. The number of [^{3}H]-estradiol binding sites per cell is approximately 700 with a K_D of 2×10^{-9} M. These observations correlate well with the report of Lebeau *et al.*[12] in which 1100 sites/cell with K_D of 1 nM were observed in detergent-washed liver nuclei. Our studies also show that receptor levels are increased within 60 min following a single injection of hormone (DES). The maximum increase was observed at 4 h when it represented a 7-fold elevation above the control values. Since cytoplasmic receptors were not easily demonstrable in the untreated cockerel liver [11, 12, Snow L. D., unpublished observation], the source of

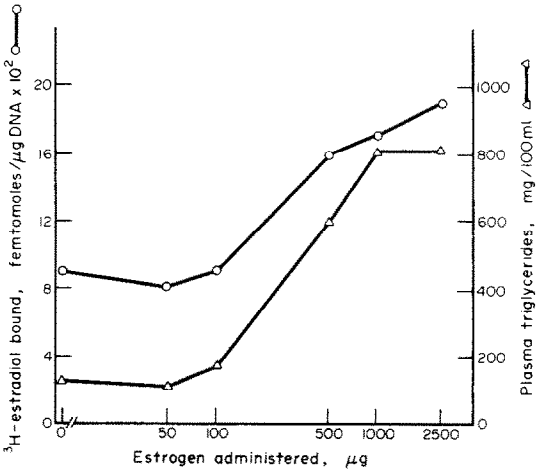

Fig. 8. DES dose response curve for plasma triglyceride levels and nuclear [^{3}H]-estradiol binding. Three chicks per treatment group were injected with DES at 6 h prior to sacrifice. Nuclei were prepared from liver, and nuclear [^{3}H]-estradiol binding was measured by the [^{3}H]-estradiol exchange assay. The bound estradiol (fmol/μg DNA $\times 10^2$) was calculated from Scatchard analysis of the binding data at each dose. Plasma triglycerides were determined as described in the Methods.

the nuclear estradiol binding sites following hormone treatment is uncertain. Even at 24 h nuclear receptor concentrations were considerably greater than the untreated controls. This retention of significant amounts of nuclear estrogen–receptor complex for as long as 24 h is similar to the long term nuclear retention of the receptor complex observed in the rat uterus [32]. In the latter situation, long term nuclear retention is thought to be prerequisite for true uterine growth.

Since the bulk of the [^{3}H]-estradiol bound within the liver cell nucleus was resistant to KCl extraction, we studied the binding of the hormone to isolated liver chromatin by the [^{3}H]-estradiol exchange assay at 30°C. Specific estradiol binding sites were again demonstrated with a K_D of 2×10^{-9} M. The number of binding sites was also increased markedly following DES treatment. Significant binding of [^{3}H]-estradiol to isolated chromatin was also observed at 0°C. These results suggest either that a substantial proportion of estrogen binding sites were exchangeable at 0°C, or that there were uncharged estrogen binding sites which readily bound hormone at this temperature. In contrast, in the only other report concerning the dependency of *in vitro* binding of [^{3}H]-estradiol to liver chromatin of estrogen treated roosters, Gschwendt [14] observed almost no binding at 0°C. This discrepancy may be related to differences in the methods of chromatin isolation or to the different ages of the animals used in the two studies.

If a major action of estrogen in the liver is an increase in specific gene transcription, one might expect changes in the number of RNA synthesis initiation sites on chromatin and/or in endogenous RNA polymerase I and II activities to coincide with the increase in the number of nuclear estrogen receptors. Berg *et al.*[33] reported that the number of chain initiations was elevated 4-fold at 24 h after estradiol administration. However, the assay employed in this study measured nicked DNA as well as nonspecific initiation sites. To circumvent this difficulty, we have adopted the assay of Tsai and coworkers [28] using rifampicin-resistant *E. coli* RNA polymerase-mediated RNA synthesis as a measure of specific initiation sites. This method distinguishes between nicked or single-stranded DNA as well as nonspecific and specific initiation sites [28, 34]. The number of initiation sites in the liver was found to increase from 26,000/pg chromatin DNA to 48,000/pg within 1 h following estrogen injection and remain elevated for at least 24 h. These observations are quite similar to those reported by Tsai and coworkers concerning the effects of estrogen on the immature chick oviduct [28, 34, 35]. They also support the findings of Searcy[36] that sheared liver chromatin from estradiol-treated chicks showed elevated template activity by 2 h of hormone treatment.

Endogenous RNA polymerase I and II activities were measured in chick liver nuclei and found to be stimulated by estrogen over a broad time course. Polymerase II activity increased about 100%, whereas Polymerase I activity increased some 50% within 1 h of DES treatment. Such increase in activities was still evident 24 h later. Previous studies [33, 37–39] employing extracted or partially purified enzyme preparations have stressed the late effect of estradiol treatment (12 h or more) on RNA polymerase activities. Since results from these studies cannot account for the early stimulation of VLDL synthesis, we have examined the early effect of estradiol treatment on these activities. To measure the endogeneous polymerase activities we have avoided extraction or partial purification procedures which might result in differences in the relative recovery of enzyme activity. Indeed, Beri-Bonniot and Dierks-Ventliing[39] have demonstrated this to be the case when the enzymes were studied following extraction from chicken liver.

Taken together, our data suggest that in the avian liver, estrogen acts through mechanisms quite similar to those observed in other classical target organs such as the uterus and oviduct [7–9]. Within a short time of administration (60 min), the hormone appears to be present in the liver cell nucleus bound to specific receptors. The precise nature of the hormone receptors remains to be elucidated. Whether they exist in the cytoplasm as is the case for many other steroid hormone systems, or are integral components of the chromosomal proteins as proposed for thyroid hormone is an open question [40–42]. Nevertheless, interaction of the hormone–receptor complexes with liver chromatin is followed by stimulation of specific gene transcription. This increase in transcriptional activity appears to be a consequence of an elevation in the number of RNA synthesis initiation sites on the chromatin as well as stimulation of RNA Polymerase I and II activities. Subsequently, accumulation of VLDL mRNA occurs which is closely correlated with an increased rate of VLDL synthesis [6]. The newly synthesized VLDL are then secreted and appear in the circulation resulting in elevated plasma VLDL and triglycerides.

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