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INTRACELLULAR LOCALIZATION OF ESTROGENS IN CHICK LIVER: INCREASE OF THE BINDING SITES FOR THE HORMONE ON REPEATED TREATMENT OF THE BIRDS WITH THE HORMONE

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SUMMARY

Binding of [2,4,6,7-^{3}H]-estradiol or its metabolites in the liver tissue of unprimed roosters and those given repeated doses of estradiol has been studied. Electron autoradiographs show the localization of the radioactivity on endoplasmic reticulum, mitochondria, nuclear membrane and nucleolar and extranucleolar chromatin of the hepatocytes. Repeated doses of the hormone to the bird increase the number of binding sites. However, the dissociation constant (K_d) for binding to heavy pellet fraction extractable with 0·4 M KCl remains essentially unchanged.

INTRODUCTION

Estrogens induce the synthesis of phosvitin and other egg-yolk proteins in the avian liver [1–6]. The plasma level of phosvitin as well as the total capacity for synthesis of the protein increases progressively on treatment of an immature male chick with repeated doses of the hormone until plateau levels are attained [4, 7]. Results reported in this communication show that the binding of the hormone to the tissue increases with sequential injections of the hormone. The intracellular sites for binding of the hormone in the chick liver have also been delineated by electron autoradiography.

EXPERIMENTAL

Experiments were conducted on White Leghorn male pullets (10- to 20-day-old). The birds were given either the vehicle (0·2 ml propanediol) or the indicated number of injections of estradiol-17β (2 mg/100 g body weight) intraperitoneally on consecutive days. Ten days after the last injection of the hormone or the vehicle, the birds were killed and the liver taken in 0·32 M sucrose Tris–HCl–EDTA (STE) buffer (10 mM Tris–HCl, pH 7·4, 1·5 mM EDTA). Liver was sliced into thin pieces with a razor blade on a glass slide, rinsed with the buffer and incubated for 15 min at 37°C with 10^{-8} M (S.A. 1 μCi/10^{-8} mmol) of [2,4,6,7-^{3}H]-estradiol in STE buffer. The tissues were washed 3 times with STE buffer. A known weight of the tissue was homogenized and radioactivity was extracted three times with methanol. Pooled extracts were evaporated to near dryness. Ten ml of the scintillation mixture (4 g PPO and 0·5 g POPOP per litre of toluene) was added and the radioactivity counted in Beckman or Packard liquid scintillation counter. The counting efficiency for tritium was 43 and 59% respectively.

Dissociation constant (K_d) and estrogen binding sites

Liver tissue from primed and unprimed birds was homogenized in 3 vol. (w/v) of STE buffer. Aliquots corresponding to a known weight of tissue were taken. The crude nuclear pellet was obtained by centrifugation at 800 *g* for 15 min. The supernatant was centrifuged at 105,000 *g* for 75 min to obtain the cytosol fraction. This fraction was incubated along with the 800 *g* pellet in STE buffer containing different concentrations (1×10^{-10} M to 5×10^{-7} M) of [2,4,6,7-^{3}H]-estradiol-17β for 60 min at 4°C. The suspension was centrifuged at 800 *g* for 15 min, the nuclear pellet was washed thrice with STE buffer and extracted at 4°C twice with 0·4 M KCl Tris–EDTA buffer. The high salt extract (0·2 ml) was mixed with 10 μl (10 μg) of Dextran blue and passed through a Sephadex G-25 column (1·1 cm. × 15 cm.). The radioactivity in the non retarded fraction identified with the blue colour contained the protein bound hormone. The method used for separating the bound hormone from free was the same as used by others [8, 9]. Scatchard plots [10] were drawn using the least square method. Dissociation constant (K_d) was calculated from the 1/slope and the number of binding sites by the intercepts of the plots on the x-axis [6, 11, 12].

Autoradiography

Liver slices after incubation with [^{3}H]-estradiol were washed with STE buffer and fixed in 3% glutaraldehyde in 100 mM phosphate buffer pH 7·3, with the addition of 1% H_2O_2 as suggested by Brokelmann[13]. The fixation was carried for 3 h at room

Table 1. Effect of sequential injections of estradiol-17β on the uptake of [2,4,6,7-^{3}H]-estradiol-17β by chicken liver

Prior Treatment in vivo	d.p.m./mg wet tissue	
	Expt. I	Expt. II
Propanediol	893	1581
Estradiol-17B one injection	1736	2381
Estradiol-17B Two injections	2359	2767
Estradiol-17B Three injections	2816	3255

Ten-day-old birds were primed with 1, 2 or 3 injection of estradiol-17β (2 mg/100 g body weight). After 10 days the binding of [2,4,6,7-^{3}H]-estradiol-17β was determined in liver slices *in vitro* as described in Experimental section.

temperature. The tissue slices were washed in phosphate buffer for about 1 h and post fixed in 1% osmium tetroxide in 100 mM phosphate buffer, pH 7·3 for 2 h at room temperature. The tissue was dehydrated in graded ethanol and embedded in Araldite [14]. Ultrathin sections were cut on a Reichert Om U2 ultramicrotome. Sections were picked up on a bare copper grid. The grids were mounted on glass slices as described earlier [15] and coated with Ilford L_4 emulsion. Grids were exposed for 15 days and developed in Phen-x developer. Sections were stained with uranyl acetate and lead citrate and scanned in Philips EM 300 electron microscope at 80 kV.

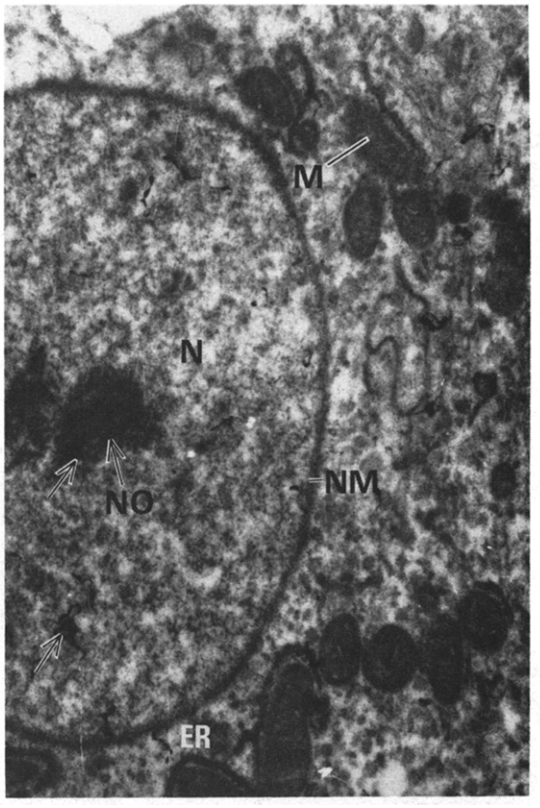

Fig. 1. Liver from unprimed 20-day-old male chick processed as described. 80 kV. × 24,000. Silver grains are indicated by arrows. ER = endoplasmic reticulum, M = mitochondria, N = nucleus, NM = nuclear membrane, NO = Nucleolus.

RESULTS

Uptake of [2,4,6,7-^3H]-*estradiol-17*β

Liver from immature male birds and those given one or more injections of estradiol were incubated in a medium containing 10^{-8} M radioactive estradiol. The radioactivity bound in each case is given in Table 1. The unprimed birds can bind the hormone but this capacity increases 2- to 3-fold on repeated treatment with the hormone.

Table 2. Hormonal specificity of the bound steroid in nuclear fraction

Addition	Protein bound CPM	% binding
Expt. I		
1 x 10^{-8}M [2,4,6,7-^{3}H] Estradiol-17β	468	100
" + 1x10^{-5}M Estradiol-17β	159	33.9
" + 1x10^{-5}M Estrone	127	27.1
" + 1x10^{-5}M Testosterone	452	96.5
Expt. II		
1x10^{-8}M [2,4,6,7-^{3}H] Estradiol-17β	524	100
" + 1x10^{-5}M Diethylstilbestrol	232	44.2
" + 1x10^{-5}M Progesterone	498	95.0
" + 1x10^{-5}M Cortisone	471	89.8

800 *g* pellet obtained from 0·8 g liver was incubated with 105,000 *g* supernatant cytosol fraction and 1 × 10^{-8} M [2,4,6,7-^{3}H]-estradiol-17β (± other non-radioactive steroids) for 60 min at 4°C. The pellets were washed thrice with STE buffer and extracted twice with 1 ml of 0·4 M KCl each time. The protein bound radioactivity in 0·4 M KCl extractable fraction was separated from free steroid by passage of an aliquot (0·2 ml) through Sephadex G-25 column.

Hormonal specificity of binding of the steroid

In order to assess the specificity of the binding of radioactive estradiol to the components of the nuclear pellet, the binding was studied in presence of non-radioactive homologous and heterologous steroid hormones. Data in Table 2 show competition between radioactive estradiol-17β and non-radioactive estradiol-17β, estrone and diethylstilbestrol. Testosterone, progesterone and cortisone did not influence the binding of [^{3}H]-estradiol-17β under the conditions employed.

Localization of the bound hormone

Figures 1–3 show that the radioactive hormone is present in both the nucleus and the cytoplasm. Within the nucleus, the radioactivity grains are prominent in both the nucleolus as well as in the extranucleolar compartments. Radioactivity grains are again discernible on the nuclear membranes. The cytoplasm, the mitochondria and the endoplasmic reticulum were amongst the sites in which radioactivity was present.

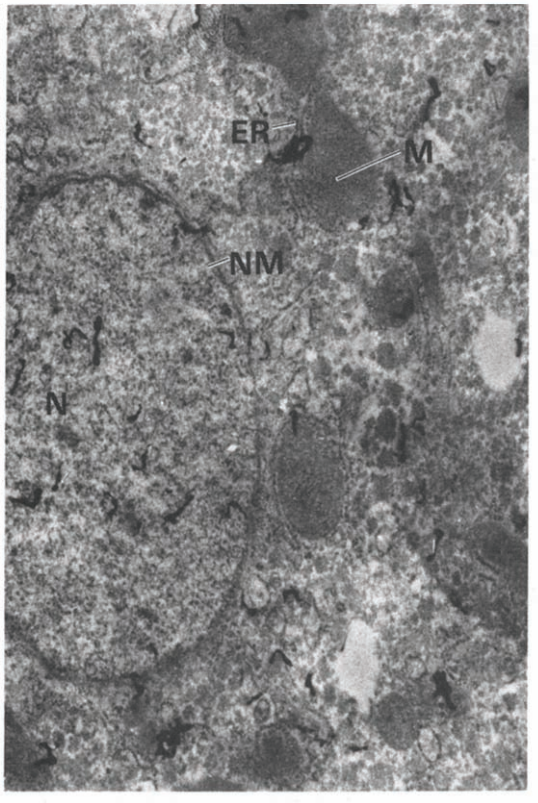

Fig. 2. Ten-day-old chick primed with one injection of estradiol-17β. 80 kV. × 24,000. For abbreviations see Fig. 1.

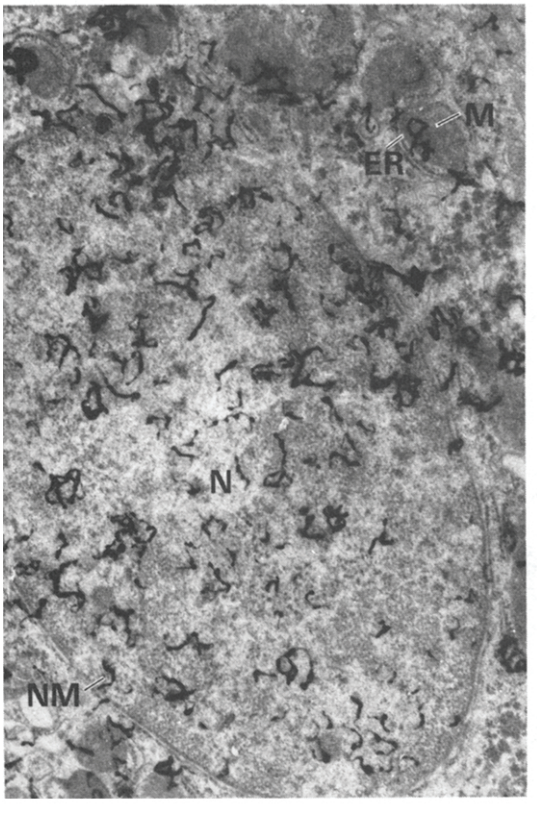

Fig. 3. Ten-day-old male chick primed with three injections of estradiol-17β. 80kV. × 24,000. There is marked increase in silver grains in both the cytoplasmic and nuclear components. For abbreviations see Fig. 1.

In birds that received one or more injections of the hormone, the number of radioactivity grains present in cells was progressively increased (Figs. 2 and 3). There was, however, no clear difference in the distribution pattern of radioactivity in cells from hormone primed and unprimed birds.

The radioactivity grains observed in these figures represent by and large specific binding sites. When the tissue was incubated with a thousand fold excess of non-radioactive estradiol, most though not all of the radioactivity grains of the type seen in Fig. 3 were absent (Fig. 4). The residual radioactivity grains in this case were observable in the nucleus and mitochondria. These organelles may therefore contain not only high affinity but also low affinity high capacity sites for the binding of the hormone.

Increase in the number of binding sites in the heavy particulate fraction

The 800 ***g*** pellet from liver of primed and unprimed birds was incubated with different concentrations of radioactive estradiol in presence of cytosol fraction. The fraction extractable with 0·4 M KCl was passed through Sephadex G-25 columns to evaluate the protein bound hormone. Results in Figure 5 show that the dissociation constant K_d of the hormone bound to the 0·4 M KCl extractable fraction from the heavy pellet was of the order of $1{\cdot}1–1{\cdot}8 \times 10^{-9}$ M. Repeated treatment of the bird with the hormone (until plateau levels are reached) did not change the K_d, although the number of binding sites in this fraction as measured by the method utilized were increased in birds primed with the hormone *in vivo* (Table 3).

DISCUSSION

Earlier studies have shown that in an estrophilic organ such as the uterus, the hormone is essentially bound to components present in the high speed cytosol fraction and in the nuclei [16–20]. These observations were made on subcellular fractions obtained by differential centrifugation of the tissue homogenates. Later low resolution autoradiographic studies of Stumpf[21] have confirmed these findings by showing the presence of radioactivity grains in the nuclear and cytoplasmic compartments.

In the present studies, high resolution electron autoradiography has been employed to delineate the intracellular sites of the binding of the hormone in the avian liver, an organ in which the hormone induces the synthesis of egg-yolk proteins. The radioactivity grains are observed to be associated with organelles such as mitochondria and endoplasmic reticulum in the cytoplasmic compartment. Radioactivity was also discernible on the nuclear membrane and within the nucleus in the nucleolar and extranucleolar regions. Similar distribution of radioactivity grains of estrogens has been noted by Sinha *et al.*[22] in the basal and in the invasive cells of the human prostatic carcinoma.

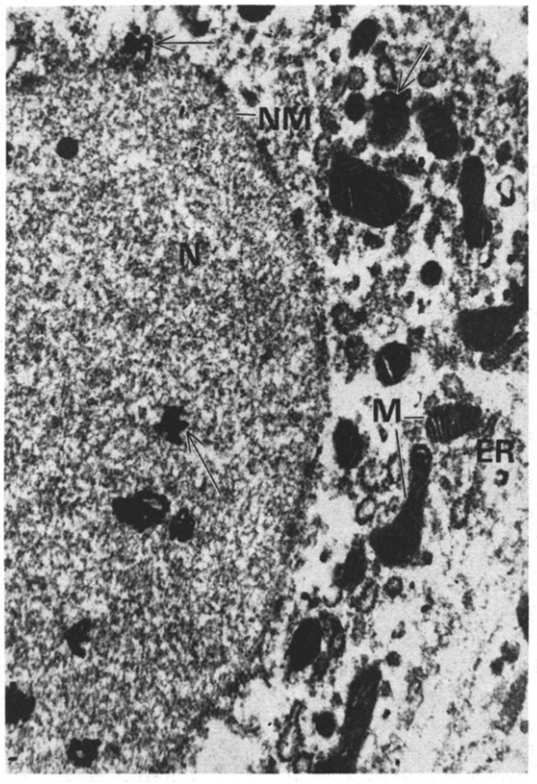

Fig. 4. A portion of the liver from the bird given three injections of estradiol (Fig. 3) was incubated with 10^{-5}M non-radioactive estradiol and 10^{-8}M [2,4,6,7-^{3}H] estradiol. The rest of the procedure was the same as in Fig. 3. 80 kV. ×26,000. The residual radioactivity grains are indicated by arrows.

The disposition of radioactivity solely on organelles and lack of its presence in the cytoplasmic matrix in this tissue raises the question as to whether the radioactivity obtained in the cytosol fraction on homogenization of the tissue be not partly derived by dissociation from the cell organelles. This point needs to be clarified by further investigations. The possibility of solubilization or translocation of the steroid radioactivity in course of processing for electron autoradiography cannot be fully ruled out, although the method utilized for these studies promotes a covalent attachment of the steroid during processing [13, 22]. Consequently it puts in evidence only those sites where this reaction takes place in the tissue. The pattern of distribution of grains was found to be consistent from experiment to experiment. Moreover, preincubation of the tissue with large excess of non-radioactive estradiol (Fig. 4 and Table 2) displaced most of the grains from these sites suggesting that the localization of the radioactive steroid noted under the conditions employed is subject to competition.

Autoradiographic techniques do not permit discrimination between estradiol and its metabolic products. It is, therefore, not unlikely that the radioactivity on some of these sites may be composed of estrone [5, 23] and/or other metabolic products of estradiol [23].

The remarkable increase in the number of binding sites for estradiol on repeated treatment of the bird with the hormone is clearly evident from the data in Table 1 and Figs. 2 and 3. With the increase in

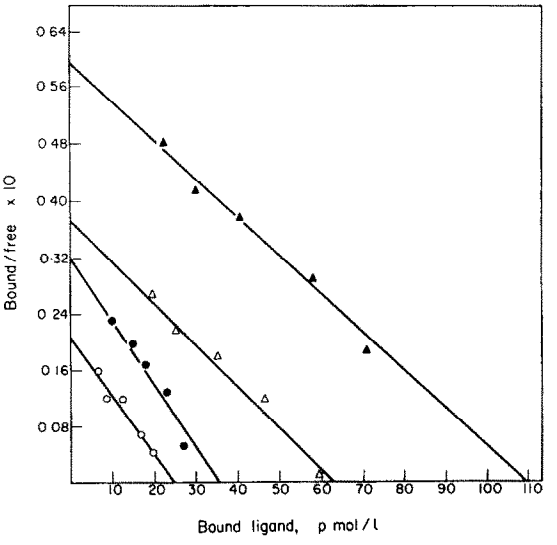

Fig. 5. Scatchard plot of the estradiol bound to 0·4 M KCl 3. 80 kV. ×43,900. The residual radioactivity grains are respectively of liver tissue from unprimed bird (○---○) and birds are given one (●---●), two (△---△) and three (▲---▲) doses of estradiol.

Table 3. Dissociation constant (K_d) and number of binding sites for estradiol in 0·4 m KCl extractable fraction of the 800 *g* pellet obtained from liver of birds before and after treatment with estradiol

Prior treatment* in vivo	K_d	No. of binding sites per ng DNA	No. of binding sites per cell**
Propanediol	1.2 x 10^{-9}M	15368	40
Estradiol-17B one injection	1.1 x 10^{-9}M	21911	57
Estradiol-17B Two injections	1.6 x 10^{-9}M	28077	73
Estradiol-17B Three injections	1.8 x 10^{-9}M	49234	128

* Dose of estradiol was 2 mg per 100 g body wt.

** Computed from data in Fig. 5 on the basis of 2·6 pg/DNA/cell and 2·7 mg DNA/g chick liver [26].

the hormone binding capacity, the pattern of distribution of the hormone remains, however, essentially unchanged. An increase is noticeable in both the cytoplasmic and nuclear compartments.

The dissociation constants for binding of estradiol have been studied with the high salt extractable fraction from 800 *g* pellet. This fraction represents a part but not the whole of estrogens bound in the nucleus. The rest of the steroid bound is extractable with solvents that denature the proteins [24] hence not conducible for such studies. The binding has been studied *in vitro* by incubation of the radioactive steroid with the 800 *g* pellet and with the high speed supernatant. Our own observations in conformity with those of Arias and Warren [5] show the requirement of the cytosol fraction for optimum nuclear binding of the steroid. This procedure was therefore adopted for this study. The exclusion of the mitochondrial and microsomal pellets in these incubations minimized the metabolism of the steroid to other derivatives. The chemical nature of the steroid bound to 0·4 M KCl extractable nuclear fraction was predominantly (above 80%) estradiol-17β [5, 25], although estrone can also bind to this fraction *in vitro*.

The K_d values computed (1·1–1·8 $\times$ 10^{-9} M) were somewhat lower than those reported by Mester and Baulieu[6]. This may be due to the different procedures employed in the two studies. The number of binding sites in this fraction of the nucleus increased with repeated hormone treatment from 40 to 128 per hepatocyte on the basis of the DNA content taken as 2·6 pg per cell [26].

It has been reported elsewhere that the capacity of the bird to synthesize phosvitin increases progressively with injection of the hormone [4, 7]. It is not unlikely that the increase in the number of binding sites for this hormone may constitute one of the mechanisms for the enhanced protein synthesizing capacity.

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