Biochem. J. (1976) **154**, 1–9
Printed in Great Britain

Studies on Sex-Organ Development

OESTROGEN-RECEPTOR TRANSLOCATION IN THE DEVELOPING CHICK MÜLLERIAN DUCT

By CHING SUNG TENG and CHRISTINA T. TENG
Department of Cell Biology, Baylor College of Medicine, Houston, Tex. 77025, U.S.A.

(*Received 9 June* 1975)

After oestradiol administration *in vivo*, 87–95% of the initial concentration of oestradiol receptor in the cytoplasm of the embryonic-chick Müllerian-duct cell was translocated into the nucleus. The process of translocation depends on the amount of oestradiol administered *in vivo*. At 6h after oestradiol administration *in vivo*, about 30% replenishment of the initial content of the cytosol receptor was observed in the cytoplasm. The Müllerian-duct nuclei, after exposure to non-radioactive oestradiol, exhibit saturable exchange with [^{3}H]oestradiol *in vitro*. The exchange of oestradiol is temperature- and time-dependent. The optimal temperature and time for exchange are 37–41°C and 2h respectively. The [^{3}H]oestradiol–receptor complex extracted from the exchanged nuclei is present in 5–6S form, and its isoelectric point is 6.8. The numbers of nuclear oestradiol-binding sites of the developing Müllerian duct are 1.66, 2.22, 2.63 and 2.50pmol/mg of DNA respectively for embryos of 10, 12, 15 and 18 days. The dissociation constants of the nuclear oestradiol receptor of the four observed developmental stages range from 3.0 to 3.1nM.

In the sex organs of several higher organisms, the interaction of the oestrogen molecule with its receptor first occurs in the cytoplasm of the cell; the hormone–receptor complex is subsequently translocated into the nucleus (Shyamala & Gorski, 1967; Jensen *et al.*, 1967). This two-step mechanism for hormone–receptor translocation is temperature-dependent and is involved with the change in the sedimentation coefficient of the hormone-binding macromolecule from 8S to 5–6S (Jensen *et al.*, 1968; Shyamala & Gorski, 1969; Giannopoulos & Gorski, 1971*a*). The characteristic and physiological importances of the retention of oestrogen–receptor in the target-cell nuclei have been reported (King *et al.*, 1965; King & Gordon, 1966; Raynaud-Jammet & Baulieu, 1969; Mohla *et al.*, 1972).

In the first and second papers of this series (Teng & Teng, 1975*a*,*b*), we reported the isolation, characterization and ontogeny of the oestrogen receptor in the cytoplasm of the embryonic-chick Müllerian-duct cell. This embryonic oestrogen-binding protein is tissue- and steroid-specific. Its concentration in the embryonic organ increases as development proceeds, yet its capacity for interaction with oestrogen remains unchanged throughout the development (Teng & Teng, 1975*a*,*b*). Previous studies suggested that the addition of a physiological dose of oestrogen to the culture medium could stimulate the growth of the chick Müllerian duct *in vitro* (Teng, 1964; Hamilton & Teng, 1965). It is therefore pertinent to assume that the oestrogen molecule bound with receptors in the cytoplasm of the Müllerian-duct cell could be translocated into the nucleus and probably exerts its hormonal effect there. Consequently, it is interesting to observe the oestrogen-binding capacity inside the nuclei of the embryonic-chick Müllerian duct.

Materials and Methods

Animals and chemicals

Fertilized white-Leghorn-chick eggs were obtained from Rich-Glo Farms, Houston, Tex., U.S.A., and were incubated at 38°C in a humidified Petersime model 4 incubator, equipped with an egg-turning device which rotated the eggs every 4h. The age of the embryos was determined by the criteria of Hamburger & Hamilton (1951). The normal hatching time under these incubation conditions is 21 days. Immature female Sprague–Dawley rats (22 days old from Timco, Houston, Tex., U.S.A.) were used in one of the studies. [6,7-^{3}H]Oestradiol-17β, with a specific radioactivity of 45Ci/mmol, was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. It was evaporated to dryness under N_2 and redissolved in ethanol before use.

The following chemicals and hormones were obtained from the sources indicated: Tris, bovine γ-globulin, pepsin (hog stomach mucosa), myoglobin (horse heart), calf thymus DNA and ultra-pure sucrose were obtained from Schwarz/Mann (Orangeburg, N.Y., U.S.A.). Aldolase, dextran T-70, Sephadex G-25 and dextran sulphate 2000 were obtained

from Pharmacia (Uppsala, Sweden). EDTA, diphenylamine and propylene glycol were from Mallinckrodt (Los Angeles, Calif., U.S.A.). Oestradiol-17β, collagenase (fraction A from *Clostridium histolyticum*), cytochrome *c* (horse heart) and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Charcoal was purchased from Baker Chemical Co. (Phillipsburg, N.H., U.S.A.), and Ampholine was from LKB Laboratory (Rockville, Md., U.S.A.). All other chemicals were of analytical grade.

Exposure of embryonic Müllerian duct or rat uterus to oestradiol in vivo

Oestradiol was applied to the allantois of the 10-, 12-, 15- and 18-day chick embryos through the shell hole. Before injection, the shell was washed with 70% ethanol. Then a small hole (0.6mm diam.) was made in the shell immediately above the embryo by use of an egg viewer (Breeder's Supply Co., New York, N.Y., U.S.A.). Oestradiol was dissolved in propylene glycol and warmed to 38°C before injection. A single dose of 40μg of oestradiol (or other concentration specified) in 0.1ml was injected with a sterilized syringe and a no. 21G1 needle (Becton-Dickson Co., Rutherford, N.J., U.S.A.); the control group received the same amount of the vehicle minus oestradiol. After the injection was completed, the hole on the shell was sealed with Scotch tape, and the eggs were returned to the incubator. After different lengths of time, the embryos were dissected mid-ventrally, and the left female Müllerian ducts were removed and placed in a beaker on ice for preparation of cytosol or nuclei.

Immature female rats were injected subcutaneously with 2μg of oestradiol per rat in 0.2ml of propylene glycol and were decapitated 1h after the injection. The uteri were removed and placed in a beaker on ice before isolation of nuclei.

Preparation of cytosol and nuclei

The procedures for preparations were essentially as described by Teng & Teng (1975*a*), and all the subsequent processes were done at 0°C. Left Müllerian ducts excised from the female chick embryos exposed to oestradiol *in vivo* were combined, washed twice with buffer (containing 10mM-Tris/HCl and 1.5mM-EDTA, pH7.4), and minced finely with scissors. The minced tissue was suspended in 5vol. of buffer and homogenized for 12 strokes with a Teflon–glass homogenizer (Glenco, Houston, Tex., U.S.A.). Homogenates were centrifuged at 700*g* for 10min at 0°C in a Beckman model 21 J centrifuge; the supernatants were then centrifuged at 105000$g_{av.}$ at 0°C for 30min in a Beckman model L5-65 ultracentrifuge in the SW 50.1 rotor with adaptor model 305527 (Beckman Instruments, Palo Alto, Calif., U.S.A.). The resulting clear supernatants, designated 'cytosol', were adjusted to 4mg of protein/ml with buffer. The sedimented pellet was suspended in 10vol. of 1.8M-sucrose prepared in buffer and was centrifuged at 100000$g_{av.}$ for 30min. The resulting nuclear preparations were washed twice with buffer before they were used for the assay of the nuclear oestrogen-binding sites.

Preparation of the rat uterine nuclei was essentially by the technique of Widnell *et al.* (1967). All succeeding procedures were carried out at 0°C. Uterine horns, after exposure *in vivo* to oestradiol, were stripped of adhering fat and mesenteries, washed twice with buffer, and minced finely with curved scissors. The minced tissues were suspended in 10vol. of buffer and homogenized for 2min at −10°C by a Polytron model P-10 tissue disintegrator (Brinkman, Westbury, N.Y., U.S.A.) run at a rotating speed of 2250rev./min. Homogenates were centrifuged at 700*g* for 10min at 0°C in a Beckman model J-21B centrifuge. The pellet was suspended in 10vol. of 2.0M-sucrose prepared in buffer and again centrifuged at 100000*g* for 60min. The nuclei pellets in the bottom were washed twice with buffer before use. Nuclei isolated from the Müllerian duct or uterus after exposure to oestradiol *in vivo* were designated 'exposed nuclei' thereafter.

Hormone-binding assay

Oestradiol-binding assay was essentially by the procedures previously published (Teng & Teng, 1975*a*). In each experiment, two batches of cytosol (each consisting of 500μg of cytosol protein in 0.125ml final assay volume in buffer) in separate tubes were incubated simultaneously, one with [^{3}H]oestradiol, and the other with [^{3}H]oestradiol together with a 100-fold excess of the non-radioactive oestradiol. After incubation at 0°C for 60min, an equal volume (0.125ml) of dextran-coated charcoal (0.5% charcoal, 0.05% dextran in buffer) was added, and the mixture was stirred with a Vortex mixer three times in 10min. The free steroids absorbed by charcoal were removed by centrifugation at 3000*g* for 15min in a Beckman model J-21B centrifuge. A 200μl volume of the supernatant was removed for radioactivity assay. Specific binding of the cytoplasmic receptor was obtained by subtracting the radioactivity bound in the presence of the competing unlabelled steroid (non-specific binding) from the radioactivity retained in the absence of the competing steroid.

Assay of specific binding of the oestradiol in the nuclei of the Müllerian duct or the rat uterus was by the hormone-exchange assay technique of Anderson *et al.* (1972). The nuclei exposed to oestradiol *in vivo* were obtained by the previous procedure, gently

dispersed and then suspended in buffer to a concentration equivalent to 45–60 μg of DNA/0.125ml per assay tube. Specific binding of oestradiol in the nuclei was measured in a similar manner to that for the cytosol. For each experiment, two assay tubes consisting of the same amount of nuclei were incubated at a concentration of 13nM-[^{3}H]oestradiol (or other concentration specified) to exchange for the non-radioactive oestradiol bound to nuclear receptor, except that in one tube a 100-fold excess of oestradiol was added. After incubation at 37°C for 1h (or other temperature and time-period specified) in an Aquatherm water-bath shaker model G-86 (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.) with constant shaking speed at 150rev./min, the assay tubes were placed immediately in an ice bucket and subsequently were centrifuged at 800g for 10min at 0°C. The nuclei were washed and sedimented at the same speed three times each with 10vol. of buffer at 0°C. Nuclei obtained after this exchange *in vitro* of ^{3}H-labelled hormone, designated 'exchanged nuclei', were smeared with a glass rod and then added to 0.5ml of ethanol overnight to extract the bound [^{3}H]-oestradiol. The ethanol extract was removed for radioactivity assay. Specific binding due to the nuclear receptor was obtained by subtracting the radioactivity bound in the presence of the competing non-radioactive oestradiol from the radioactivity retained in its absence.

Glycerol-gradient centrifugation

The nuclear receptor–hormone complex was extracted from the exchanged nuclei by adding 5vol. of 0.5M-KCl in buffer for 2h. An equal amount of nuclear protein (200 μg of protein in 0.5ml) from the chick Müllerian duct or the rat uterus was layered separately on top of a linear 5–35% (v/v) glycerol gradient (4.5ml) prepared in buffer containing 0.5M-KCl. The gradients were centrifuged at 150000$g_{av.}$ for 18h at 0°C in a Beckman SW 50.1 rotor. In the separate gradients the sedimentation markers (200 μg each of aldolase, γ-globulin, collagenase, bovine serum albumin, pepsin, myoglobin and cytochrome *c*) were run separately. Each gradient was fractionated with a Buchler piercer into 30 fractions (0.16ml per fraction), and each fraction was assayed for radioactivity.

Isoelectric focusing

Isoelectric focusing was done in a small column originally designed by Osterman (1970) and Mainwaring & Irving (1973), and modified by Teng & Teng (1975*a*). Nuclear [^{3}H]oestradiol–receptor protein (the extract of the exchanged nuclei in 0.5M-KCl in buffer) was desalted by gel-exclusion chromatography in a Sephadex G-25 (medium grade) column (2cm×25cm) equilibrated with 5% (w/v) sucrose before use. For analysis, the desalted nuclear [^{3}H]oestradiol–receptor complex (1mg of protein/ml) was treated with dextran sulphate (1.5mg/ml), and a portion of it (0.1ml) was loaded on top of a linear 10–60% (w/v) sucrose gradient (5ml) containing 1% (v/v) ampholytes (pH3–11); it was formed in a 0.8cm×15cm Pyrex glass tube, the bottom of which was previously plugged with 0.25ml of 10% acrylamide plus 0.2% *N,N'*-methylenebisacrylamide, polymerized by adding 0.03ml of *N,N,N',N'*-tetramethylethylenediamine and 0.3ml of 10% (w/v) ammonium persulphate to 40ml of the acrylamide solution. Finally 0.5ml of 2.5% (w/v) sucrose was laid on top of the nuclear protein. Three columns were run simultaneously with a LKB model 3371E D.C. power supply in a Hoefer DE 102 electrophoresis apparatus (Hoefer Scientific Instruments, San Francisco, Calif., U.S.A.). The upper electrode (cathode) contained 1% (w/v) NaOH and the lower reservoir contained 1% (w/v) H_2SO_4. The apparatus was kept in a refrigerator at 0°C; the run was carried out for 3.5h and reached a final field of 150V and 1.5mA. Then 30 fractions (0.2ml/fraction) were collected from the bottom, and the pH of each fraction was measured at 0°C with a Digicord pH-meter (Photovolt, New York, N.Y., U.S.A.) and then assayed for radioactivity.

General procedures

Protein was determined by the procedure of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as a standard. DNA was determined by the diphenylamine reaction (Giles & Myers, 1965) with calf thymus DNA (Schwarz/Mann) as a standard. Radioactivity was determined by adding 50–200 μl samples to 4ml of scintillation fluid {6g of PPO (2,5-diphenyloxazole), 0.15g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]/litre of toluene} in a mini counting vial (Rochester Scientific Co., Rochester, N.Y., U.S.A.). The radioactivity was determined at 60% efficiency in a Beckman model LS-250 liquid-scintillation spectrometer.

Results and Discussion

Effect of oestradiol administration on the translocation of cytoplasmic receptor into nuclei in Müllerian-duct cells

Our previous observation indicated that oestradiol receptors are present in the cytoplasm of the embryonic chick Müllerian-duct cell (Teng & Teng, 1975*a*). To understand more about the function of these receptors, it was necessary to determine whether the receptors were capable of translocation into nuclei *in vivo*. Different concentrations of non-radioactive oestradiol were tested

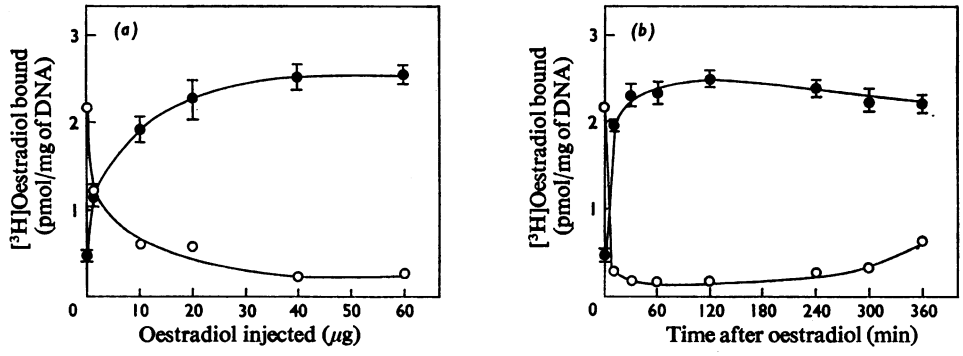

Fig. 1. *Concentration of cytoplasmic and nuclear oestradiol receptor in the 15-day embryonic female chick Müllerian duct after exposure to oestradiol in vivo*

(*a*) Effect of oestradiol concentration on translocation of receptors into nuclei. The experimental group of chick embryos received chorioallantoic injections of various amounts of oestradiol for 2h; the control group of chick embryos received 0.1ml of propylene glycol without oestradiol for 2h. The specific binding of oestradiol to the cytoplasmic or nuclear receptor was measured by incubation with 13nM-[^{3}H]oestradiol. (*b*) Effect of time on receptor translocation into nuclei after oestradiol injection. A single dose of oestradiol (40μg/embryo) was administered, and after various time-periods the Müllerian ducts were removed for assay. The concentrations of the receptor in the cytoplasm and the nuclei were assayed as described in the Materials and Methods section. Each value presented is the specific binding and represents the mean ± S.D. of three determinations. ●, Nuclear receptor concentration; ○, cytoplasmic receptor concentration.

individually *in vivo* to observe the effect on translocation of cytoplasmic receptor into nuclei in the Müllerian duct of 15-day female chick embryos.

At 2h after injection of oestradiol (1μg/egg) *in vivo* the receptor concentration in the cytosol fraction was depleted by about 45%, and the nuclear receptor concentration was correspondingly increased. When the injected dose of oestradiol was increased to the range of 10–40μg/egg for the same period of time, the receptor concentration in the cytosol was depleted by about 73–90%, and the nuclear receptor increased 4–5 times above its initial concentration (Fig. 1*a*). At 10–30min after a single dose of oestradiol (40μg/egg) was administered to the 15-day female chick embryo, the cytosol receptor concentration showed a 85–90% decrease from its original value and remained at that value for 2–3h. The nuclear receptor concentration was highest (5-fold increase) 2h after hormone administration (Fig. 1*b*). Some 87–95% of the depleted receptor in the cytoplasm was recovered in the nuclei within 2h, which contrasts with the low recovery rate (50%) of oestradiol receptor observed in the nuclear fraction of the rat uterus (Mešter & Baulieu, 1975). We attributed this difference to the following possibilities. (*a*) Proteolytic enzyme activity probably is lower in the Müllerian duct than in the rat uterus, and during the process of exchange at 37°C the receptors are probably intact. (*b*) The dose of oestradiol used for this study is sufficiently high to complete the translocation.

To determine the effect of some non-oestrogenic hormones on the quantity of specific oestrogen-binding sites in the Müllerian-duct nuclei, chick embryos were injected with 40μg of testosterone, progesterone, cortisol, or 0.1ml of propylene glycol. At 2h after injection, the nuclear fraction of Müllerian duct was prepared and used in the [^{3}H]oestradiol nuclear exchange assay as described in the Materials and Methods section (Fig. 2). The administration of oestradiol resulted in a 5-fold increase of specific nuclear binding over control values. Testosterone, progesterone and cortisol failed to show any significant effect on the number of nuclear oestradiol-binding sites in the Müllerian duct. This observation indicated that the oestradiol-receptor translocation is steroid-specific. So far our observations indicate that oestradiol–receptor translocation into nuclei depends on the dose of oestradiol, and this is comparable with the observation in the rat hypothalamus and pituitary that the amount of oestradiol–receptor translocation into the nuclei depends on the dose of oestradiol injected *in vivo* (Anderson *et al.*, 1973). In chick Müllerian-duct cell, the dose of oestradiol (10–40μg/egg) used to translocate the cytosol receptor into nuclei is higher than the dose normally required for the translocation of receptor in the mammalian uterine cell. Since in the chick embryonic system the oestradiol injected *in vivo* is not in direct contact with the embryo and the injected hormone can be partially absorbed by the chorioallantoic membrane, the

egg albumin and the egg yolk, we expected that the actual amount of oestradiol received by the embryo would be substantially less.

We observed (Fig. 1*b*) a slight replenishment of cytosol receptor 5h after oestradiol administration and a significant amount of replenishment [30±7% (S.D.) of three preparations, of the initial content of cytosol oestradiol-binding sites] 6h after the hormone injection. Our observation is comparable with the finding in rat uterus by Mešter & Baulieu (1975). They observed that after 6h of oestradiol injection into the immature rat about 50% of the initial content of uterine cytosol oestrogen-binding sites were replenished.

Effect of temperature and time of incubation on [^{3}H]oestradiol exchange with exposed nuclei

Nuclei isolated from the Müllerian duct after exposure to oestradiol *in vivo* were incubated for 60min and at different temperatures with a constant amount of [^{3}H]oestradiol with or without the presence of competing non-radioactive oestradiol. The specific binding of [^{3}H]oestradiol to nuclear binding sites was observed to be highest (2.08–2.05pmol/mg of DNA) at 37° and 41°C; the binding was relatively low (0.43pmol/mg of DNA) at 0°C, which indicated that hormone exchange is temperature-dependent. At a given temperature (37°C), the time required for maximal exchange is 30–60min, and the hormone remains at the same concentration after 120min of incubation (Table 1). This observation indicated that the embryonic hormone receptor is stable at 37°C, which may reflect a low interior proteolytic-enzyme activity in the embryonic sex organ.

Characteristics of the nuclear [^{3}H]oestradiol–receptor complex

On glycerol-gradient centrifugation the [^{3}H]-oestradiol–receptor complex, isolated from the Müllerian-duct nuclei after radioactive hormone exchange, sedimented at fractions 15–16 (Fig. 3*a*). To determine the exact sedimentation coefficient of the hormone–receptor complex, seven purified protein standards were centrifuged through a linear glycerol gradient; experimental conditions were similar to those used for the hormone–receptor complex. Positions of standard proteins in the gradient were determined by the method of Martin & Ames (1961). Fig. 3(*b*) shows the linear

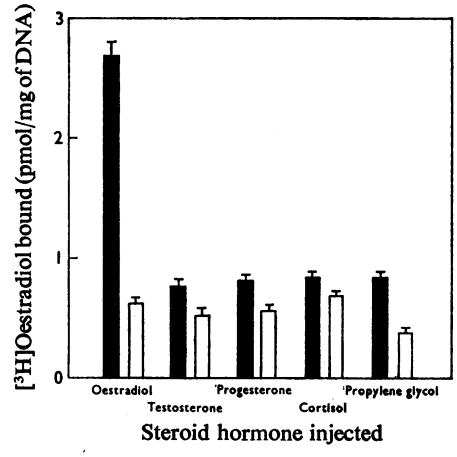

Fig. 2. *Effects of steroid hormones on the quantity of oestradiol-binding sites in the nuclear fraction of female chick Müllerian duct*

Chick embryos were injected with 40μg of oestradiol, testosterone, progesterone, cortisol or 0.1ml of propylene glycol for 2h and the Müllerian ducts were removed for nuclei insolation. ■, The nuclear fraction was incubated with 13nM-[^{3}H]oestradiol; □, the nuclear fraction was incubated with 13nM-[^{3}H]oestradiol plus 100-fold excess of non-labelled oestradiol. The results presented were the means±S.D. of three preparations.

Table 1. *Effect of temperature and time of incubation on the binding of [^{3}H]oestradiol to the nucleus of Müllerian-duct cell*

Oestradiol was administered by chlorioallantoic injection for 2h; then the left Müllerian duct from 15-day female chicks was removed and combined for preparation of nuclei. Nuclei were incubated with 13nM-[^{3}H]oestradiol with or without 100-fold excess of non-radioactive oestradiol at 37°C for 1h. Each value represents the mean±S.D. of three determinations.

	Temperature of incubation (°C)	Time of incubation (min)	Total [^{3}H]oestradiol bound (pmol/mg of DNA)	Non-specific [^{3}H]oestradiol bound (pmol/mg of DNA)	Specific [^{3}H]oestradiol bound (pmol/mg of DNA)
(*a*)	0	60	0.75±0.06	0.32±0.02	0.43±0.03
	21	60	2.55±0.22	0.72±0.04	1.83±0.12
	37	60	2.72±0.21	0.64±0.03	2.08±0.14
	41	60	2.70±0.24	0.65±0.03	2.05±0.13
	60	60	2.15±0.23	0.85±0.04	1.30±0.09
(*b*)	37	15	2.64±0.20	0.79±0.03	1.85±0.13
	37	30	2.70±0.21	0.68±0.04	2.02±0.14
	37	60	2.72±0.21	0.64±0.03	2.08±0.14
	37	120	2.75±0.20	0.68±0.03	2.07±0.15

relationship between the sedimentation coefficient and the extent of migration into the gradient. On the basis of this plot, the nuclear oestrogen receptor has a sedimentation coefficient of about 5–6S.

To compare this system with another well-established system, e.g. the rat uterus, we prepared the nuclear [^{3}H]oestradiol–receptor complex from immature rat uterus and rat it on a glycerol gradient as a marker. The sedimentation pattern of nuclear receptors from rat uterus or chick Müllerian duct are similar. The sedimentation coefficient (5–6S) of Müllerian-duct nuclear receptor is comparable with the previously observed value (5–6S) for the uterine nuclear receptor (Puca & Bresciani, 1969; Giannopoulos & Gorski, 1971*b*). The exchange of [^{3}H]oestradiol with the oestradiol–receptor complex in the exposed nuclei was effectively altered by the addition of 100-fold non-radioactive oestradiol to the incubation medium. In contrast, the addition of testosterone or progesterone failed to compete with the amount of [^{3}H]oestradiol bound (Fig. 3*a*).

Isoelectric focusing indicated that this nuclear oestradiol–receptor complex has pI 6.8 (Fig. 3*c*), which is close to the value obtained for the nuclear oestrogen receptor (pI 6.6–6.8) in the calf uterus (Puca *et al.*, 1972) and for the nuclear 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one) receptor (pI 6.5) in the rat prostate gland (Mainwaring

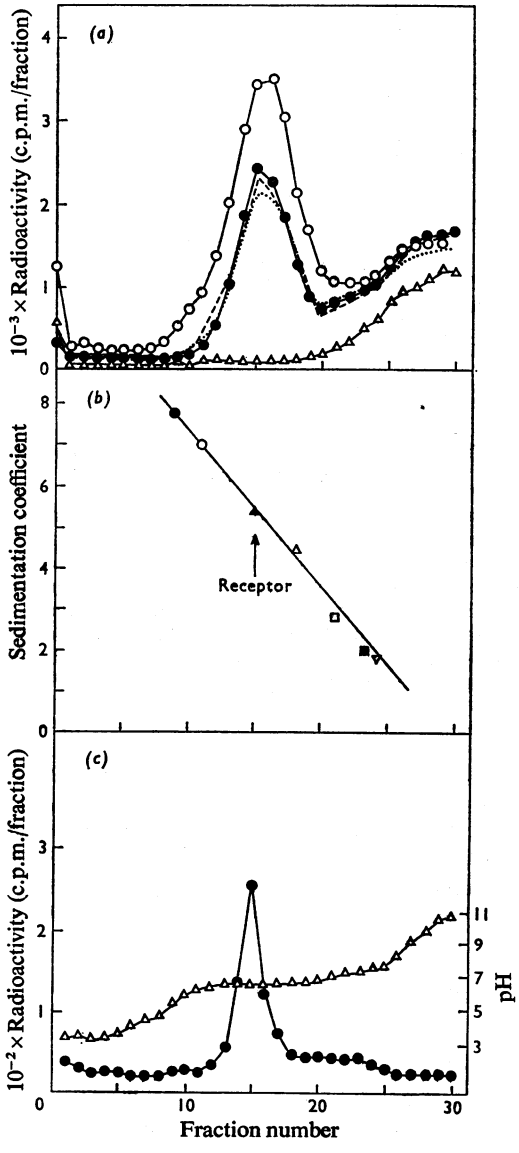

Fig. 3. *Characteristics of the nuclear [^{3}H]oestradiol receptor isolated from the exchanged nuclei of the Müllerian-duct cell*

(*a*) Glycerol-gradient centrifugation of the hormone receptor. Exposed nuclei were incubated at 37°C with 13nM-[^{3}H]oestradiol for 60min to allow the exchange of non-radioactive oestradiol, which complexed with the receptor in the nuclei. Nuclei were then pelleted and washed 3 times with buffer before salt extraction. Nuclear [^{3}H]oestradiol–receptor protein (400μg of each) was extracted with 0.5M-KCl in buffer and then separated on a 4.5ml glycerol gradient for 18h at 150000*g* as detailed in the Materials and Methods section. The preparations were run simultaneously in separate tubes. ○, [^{3}H]Oestradiol–receptor complex extracted from the exchanged nuclei of immature female rat uteri; ●, [^{3}H]oestradiol–receptor complex extracted from the exchanged nuclei of the Müllerian ducts of 15-day embryonic female chick; △, 100-fold excess of non-radioactive oestradiol (0.13μM/assay) present during the exchange of the exposed Müllerian-duct nuclei with radioactive oestradiol; ----, 100-fold excess of testosterone present during the exchange of the exposed Müllerian-duct nuclei with [^{3}H]oestradiol; ····, 100-fold excess of progesterone present during the exchange of exposed Müllerian-duct nuclei with [^{3}H]oestradiol. (*b*) Estimation of the sedimentation coefficient of the [^{3}H]oestradiol–receptor complex in nuclei of the Müllerian-duct cell. Purified protein standards (200μg/gradient) were subjected to glycerol-gradient centrifugation as described in the Materials and Methods section. The location of the protein markers in the gradient were measured by detecting the E_{280}; the nuclear receptors were identified by their radioisotopic profiles. The oestradiol-binding protein from the Müllerian duct or the rat uterus sedimented to approximately tube 15–16 in the gradient. ●, Aldolase (7.8S); ○, γ-globulin (7S); ▲, collagenase (5.5S); △, albumin (4.5S); □, pepsin (2.8S); ■, myoglobin (2S); ▽, cytochrome *c* (1.8S). (*c*) Isoelectric-focusing pattern of the nuclear receptor protein. Receptor protein (100μg) was used for focusing as detailed in the Materials and Methods section. ●, Nuclear receptor; △, pH.

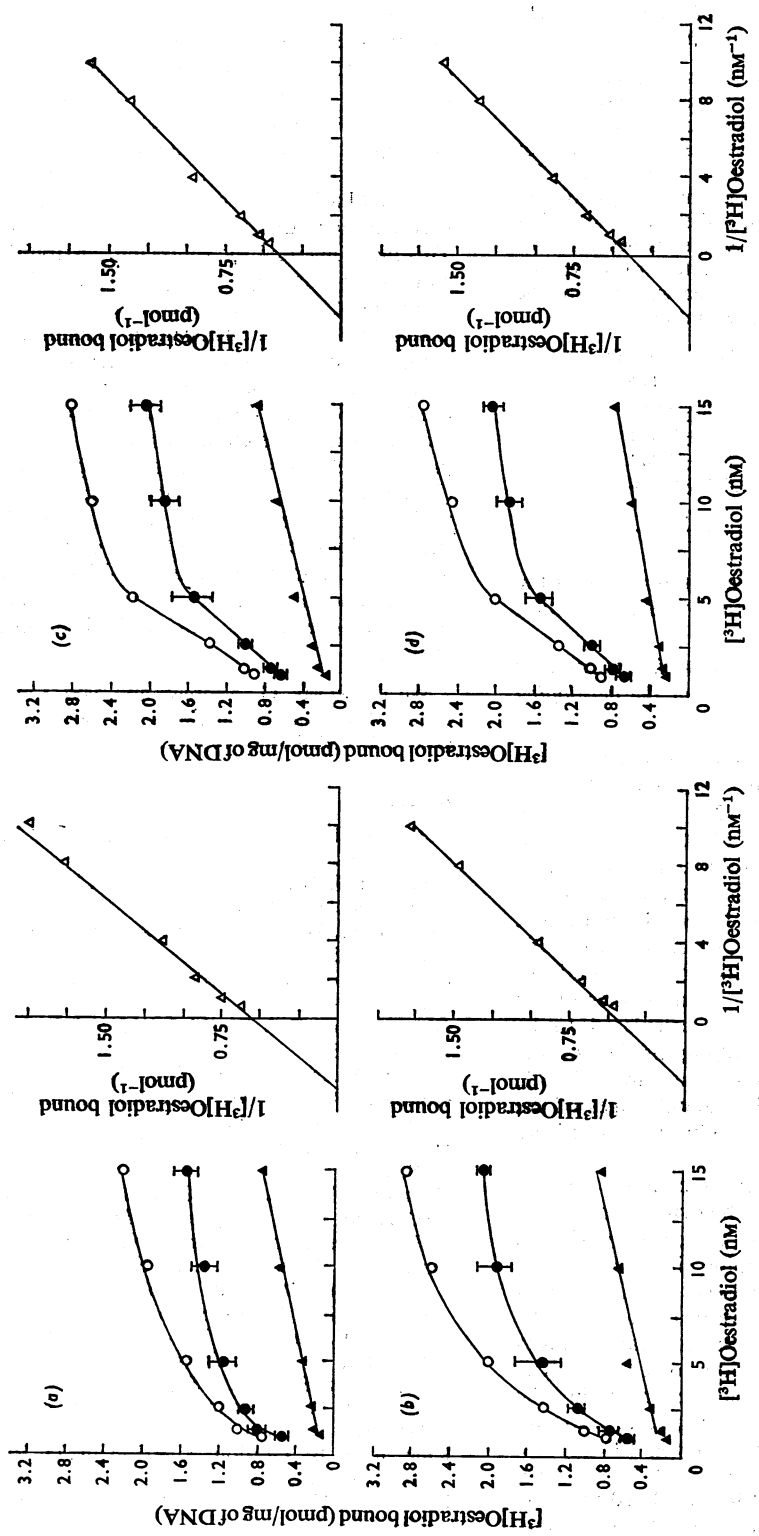

Fig. 4. *Determination of specific oestradiol-binding sites and dissociation constant of nuclear receptor in Müllerian-duct cell by exchange assay*

Nuclear oestradiol binding is shown as a function of added [^{3}H]oestradiol for exchange, and the corresponding Lineweaver–Burk plot of the specific binding is represented on the right-hand side. Nuclei were prepared from left Müllerian ducts of (*a*) 10-day, (*b*) 12-day, (*c*) 15-day and (*d*) 18-day female chick embryos. All embryos were treated with unlabelled oestradiol *in vivo*. The conditions of hormone administration, nuclei isolation and [^{3}H]oestradiol exchange were detailed in the Materials and Methods section. ○, Nuclei incubated with [^{3}H]oestradiol alone (total binding); ▲, nuclei incubated with [^{3}H]oestradiol plus 100-fold non-labelled oestradiol (non-specific binding); ●, specific binding, after the subtraction of non-specific binding from the total binding; △, Lineweaver–Burk plot of the data presented for specific binding.

Table 2. *Endogenous nuclear oestradiol-binding sites in the developing chick Müllerian duct*

Nuclei were prepared from the left Müllerian ducts of female chick embryos at various stages of development and were incubated with 13nM-[^{3}H]oestradiol with or without a 100-fold excess of non-labelled oestradiol for 1h at 37°C. The preparation of nuclei, the procedures for exchange assay, and the assay of the nuclear [^{3}H]oestradiol–receptor complex were as described in the Materials and Methods section, except that the tissues used for nuclei preparation were not subjected to oestradiol exposure *in vivo*. The results presented were the specific binding of mean±S.D. of three preparations.

Period of incubation (days)	Specific [^{3}H]oestradiol bound (pmol/mg of DNA)
10	0.093±0.012
12	0.104±0.014
15	0.536±0.043
18	0.590±0.044

& Irving, 1973). All these findings indicated that the nuclear receptor protein is relatively acidic in nature.

Oestradiol-binding sites and the dissociation constant of oestradiol receptor in the nuclei of the developing chick Müllerian duct

Oestrogen-exposed nuclei were isolated from the Müllerian ducts of female chick embryo at different stages of development (10-, 12-, 15- and 18-day embryos). After incubation of the nuclei with increasing concentrations of radioactive oestradiol, a saturation concentration for maximal exchange was obtained in the range 10–15nM-[^{3}H]oestradiol. A Lineweaver–Burk (1934) plot, based on the specific binding curve of each nuclear receptor, was obtained. The nuclear oestrogen-receptor binding sites of the developing Müllerian duct were calculated to be 1.66, 2.22, 2.63 and 2.50pmol/mg of DNA; the corresponding dissociation constants are 3.0, 3.1, 3.1 and 3.0nM, for the developmental stages of the 10-, 12-, 15- and 18-day embryo respectively (Figs. 4*a*–4*d*).

The nuclear oestrogen–receptor complex has the same dissociation constant as the cytoplasmic form, which ranges from 3.0 to 3.2nM (Teng & Teng, 1975*b*). The number of oestradiol-binding sites obtained by the radioactive-hormone-exchange method represents the sum of (*a*) the receptors translocated from the cytoplasm and (*b*) the endogenous nuclear receptors that existed before translocation took place. The concentrations of cytoplasmic receptor in the developing Müllerian duct are 1.64, 2.25, 2.35 and 2.20pmol/mg of DNA (Teng & Teng, 1975*b*), and the concentrations of endogenous nuclear oestrogen receptor are 0.093, 0.104, 0.536 and 0.590pmol/mg of DNA respectively for the 10-, 12-, 15- and 18-day chick embryo (Table 2). From these values one could assume that the amount of receptor in the nuclei is proportional to the amount of receptor in the cytoplasm. Approx. 87–95% of the cytoplasmic receptor was translocated into the nuclei of the developing chick Müllerian duct.

It is noteworthy that the values of endogenous nuclear binding sites in the Müllerian duct increase gradually according to the age of the embryo (Table 2). This fact suggested the following possibilities. (*a*) As embryonic development progresses the ovarian secretion of oestrogen increases and the endogenous oestrogen could translocate a certain amount of cytoplasmic receptor into the nuclei. This assumption is supported by a previous observation by Guichard *et al.* (1973). They found that the amount of oestradiol formed in the embryonic chick ovary increases progressively corresponding to the age of the embryo. (*b*) The amount of oestradiol retained in the nuclei varies during development, which probably reflects the physiological states of the embryonic organ. The varied amount of oestradiol-binding sites found in the nuclear fraction of rat uteri during the oestrous cycle (Clark *et al.*, 1972) is another similar example.

We thank Mr. G. Andrews and Mrs. Celine Yu for technical assistance. This work was supported by NIH grant HD-08218-02 to C. S. T.

References

Anderson, J., Clark, J. H. & Peck, E. J., Jr. (1972) *Biochem. J.* **126**, 561–567

Anderson, J. N., Peck, E. J., Jr. & Clark, J. H. (1973) *Endocrinology* **93**, 711–717

Clark, J. H., Anderson, J. & Peck, E. J., Jr. (1972) *Science* **176**, 528–530

Giannopoulos, G. & Gorski, J. (1971*a*) *J. Biol. Chem.* **246**, 2524–2529

Giannopoulos, G. & Gorski, J. (1971*b*) *J. Biol. Chem.* **246**, 2530–2536

Giles, K. W. & Myers, A. (1965) *Nature (London)* **206**, 93

Guichard, A., Cedard, L. & Haffen, K. (1973) *Gen. Comp. Endocrinol.* **20**, 16–28

Hamburger, V. & Hamilton, H. L. (1951) *J. Morphol.* **88**, 49–92

Hamilton, T. H. & Teng, C. S. (1965) in *Organogenesis* (DeHaan, R. L. & Upsprung, H., eds.), pp. 681–700, Holt, Rinehart and Winston, New York

Jensen, E. V., DeSombre, E. R., Hurst, D. J., Kawashima, T. & Jungblut, P. W. (1967) *Arch. Anat. Microsc. Morphol. Exp.* **56** (Suppl.), 547–569

Jensen, E. V., Suzuki, T., Kawashima, T., Stumpf, W. E., Jungblut, P. W. & DeSombre, E. R. (1968) *Proc. Natl. Acad. Sci. U.S.A.* **59**, 632–638

King, R. J. B. & Gordon, J. (1966) *J. Endocrinol.* **34**, 431–437

King, R. J. B., Gordon, J. & Inman, D. R. (1965) *J. Endocrinol.* **32**, 9–15

Lineweaver, H. & Burk, D. (1934) *J. Am. Chem. Soc.* **56**, 658–666

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Mainwaring, W. I. P. & Irving, R. (1973) *Biochem. J.* **134**, 113–127
Martin, R. G. & Ames, B. R. (1961) *J. Biol. Chem.* **236**, 1372–1379
Mešter, J. & Baulieu, E. E. (1975) *Biochem. J.* **146**, 617–623
Mohla, S. E., DeSombre, E. R. & Jensen, E. V. (1972) *Biochem. Biophys. Res. Commun.* **46**, 661–667
Osterman, L. (1970) *Sci. Tools* **17**, 31–33
Puca, G. A. & Bresciani, F. (1969) *Nature (London)* **223**, 745–747
Puca, G. A., Nola, E., Sica, V. & Bresciani, F. (1972) *Biochemistry* **11**, 4157–4165
Raynaud-Jammet, C. & Baulieu, E. E. (1969) *C. R. Hebd. Séances Acad. Sci. Ser. D* **271**, 603–605
Shyamala, G. & Gorski, J. (1967) *J. Cell Biol.* **35**, 125A–126A
Shyamala, G. & Gorski, J. (1969) *J. Biol. Chem.* **244**, 1097–1103
Teng, C. S. (1964) M.Sc. Thesis, University of Texas
Teng, C. S. & Teng, C. T. (1975*a*) *Biochem. J.* **150**, 183–190
Teng, C. S. & Teng, C. T. (1975*b*) *Biochem. J.* **150**, 191–194
Widnell, C. C., Hamilton, T. H. & Tata, J. R. (1967) *J. Cell Biol.* **32**, 766–770