Nuclear Binding of Progesterone in Hen Oviduct

BINDING TO MULTIPLE SITES *IN VITRO*

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Steroid hormones, including progesterone, are known to bind with high affinity ($K_d \approx 1 \times 10^{-10}$ M) to receptor proteins once they enter target cells. This complex (the progesterone-receptor) then undergoes a temperature- and/or salt-dependent activation which allows it to migrate to the cell nucleus and to bind to the deoxyribonucleoproteins. The present studies demonstrate that binding the hormone-receptor complex *in vitro* to isolated nuclei from the oviducts of laying hens requires the same conditions as do other studies of binding *in vitro* reported previously, e.g. the hormone must be complexed to intact and activated receptor. The assay of the nuclear binding by using multiple concentrations of progesterone receptor reveals the presence of more than one class of binding site in the oviduct nuclei. The affinity of each of these classes of binding sites range from $K_d \approx 1 \times 10^{-9}$ – 1×10^{-8} M. Assays using free steroid (not complexed with receptor) show no binding to these sites. The binding to each of the classes of sites, displays a differential stability to increasing ionic concentrations, suggesting primarily an ionic-type interaction for all classes. Only the highest-affinity class of binding site is capable of binding progesterone receptor under physiological-saline conditions. This class represents 6000–10000 sites per cell nucleus and resembles the sites detected *in vivo* (Spelsberg, 1976, *Biochem. J.* 156, 391–398) which cause maximal transcriptional response when saturated with the progesterone receptor. The multiple binding sites for the progesterone receptor either are not present or are found in limited numbers in the nuclei of non-target organs. Differences in extent of binding to the nuclear material between a target tissue (oviduct) and other tissues (spleen or erythrocyte) are markedly dependent on the ionic conditions, and are probably due to binding to different classes of sites in the nuclei.

It is now widely accepted that the sequence of events in the mechanism of action of steroid hormones includes: (1) the uptake of the hormone by the target cell, followed by the high-affinity formation of a stereospecific complex with an extranuclear receptor protein, (2) a temperature- and/or ionic-strength-dependent translocation of this hormone-receptor complex to the nucleus, (3) binding of the complex to specific acceptor sites on the chromatin, (4) a qualitative and quantitative stimulation of transcription, resulting in the synthesis of new RNA species, (5) transport of these RNA species to the cytoplasm and subsequent synthesis of new protein on cytoplasmic polyribosomes, and (6) tissue-specific metabolic response (Raspé, 1971). In the chick oviduct system, the cytoplasmic receptor for progesterone has been extensively purified (O'Malley & Schrader, 1972; Schrader & O'Malley, 1972; Schrader *et al.*, 1974; Kuhn *et al.*, 1975; W. T. Schrader, personal communication) and shown to consist of two 4S binding components, termed A and B, which are present in near-equal amounts and have mol.wts. of 110000 and 117000 respectively. Receptor A has been reported to bind to DNA, and receptor B to purified oviduct chromatin

(Schrader & O'Malley, 1972). In the past 8 years, various laboratories have demonstrated binding of steroid-receptor complexes to isolated nuclei or chromatin *in vitro*. This nuclear binding appears to have many of the same properties as that which occurs *in vivo*. Under conditions *in vitro* and *in vivo*, the nuclear binding is of high affinity, and requires a temperature-dependent translocation of the steroid-receptor complex to the nuclei. The steroid-receptor complex can then be dissociated from nuclei under high-ionic-strength conditions.

Previous work in this and in other laboratories using [3 H]progesterone complexed with the oviduct cytosol receptor (progesterone receptor) has suggested that 90% of the total nuclear binding in the system *in vitro* is associated with the nuclear chromatin (Spelsberg *et al.*, 1971b; O'Malley *et al.*, 1972; Spelsberg *et al.*, 1972; Spelsberg, 1974). Under physiological ionic conditions, the extent of binding of the progesterone-receptor complex to the chromatin from the chick oviduct appeared to be greater than that to spleen, heart or other tissue chromatins. The studies presented here demonstrate that the nuclear binding of the progesterone receptor *in vitro* to isolated nuclei from the oviducts of laying

hens has the same properties and requirements as reported for the immature chick system (Spelsberg *et al.*, 1971b). A detailed assessment of this nuclear binding of the progesterone-receptor complex *in vitro* to hen organ nuclei, including many properties of this interaction as well as the tissue specificity, has been made. Further, evidence is presented for the presence of multiple binding sites for the progesterone receptor which probably represent those described for oviduct nuclei *in vivo* in the preceding paper (Spelsberg, 1976).

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Materials and Methods

Isolation and analyses of the nuclear materials

Various organs (oviduct, liver, spleen, lung, heart) of adult laying hens were obtained from a local product company (Jones Produce, Rochester, MN, U.S.A.). Within minutes after death, the organs were excised, stripped of fat and connective tissue, sectioned into smaller pieces, and frozen immediately on solid CO₂. Mature erythrocytes were isolated as reported previously (Neelin, 1968). These tissues were then stored at -80°C in a Revco freezer until used. All subsequent steps were performed at 4°C. The isolation and purification of nuclei were performed essentially as described elsewhere (Spelsberg *et al.*, 1971a,b). The nuclear pellets were resuspended in a 25% (v/v) glycerol solution containing 0.01 M-Tris/HCl (pH 7.5) and 0.001 M-MgCl₂ and stored at -20°C until needed.

Steroids

[1,2-³H]Progesterone (47.8 Ci/mmol) was obtained from New England Nuclear Corp. (Boston, MA, U.S.A.). The degree of chemical purity of this material was checked by chromatography on t.l.c. plates with benzene/ethyl acetate (7:3, v/v) as solvent. The stock steroid solution in benzene was frozen, freeze-dried and redissolved in the original volume with ethanol. As needed, this stock was diluted 1:5 with water and added directly to the cytosol preparations to obtain the progesterone-receptor complexes as described in the next section.

Preparation of progesterone-receptor complex

Immature chicks injected for 20-25 days with stilboesterol were used instead of hens so as to avoid the problem of having endogenous progesterone bound to the partially purified receptor. The oviducts were excised, stripped of fat and immediately homogenized in a Waring blender in 3 vol. (v/w) of cold TESH buffer (0.01 M-Tris/HCl, 0.001 M-EDTA,

0.012 M-thioglycerol, pH 7.4). Homogenization was carried out for 30 s at 2000 rev./min. The homogenate was re-homogenized briefly in a glass homogenizer with a Teflon pestle and then passed through two layers of cheesecloth. The solution was centrifuged at 20000 g_{av.} for 20 min in a J-21 Beckman centrifuge and the supernatant re-centrifuged at 100000 g_{av.} for 1 h in a Beckman L3-50 ultracentrifuge. The 100000 g_{av.} supernatant (cytosol) was then assessed for protein concentration by absorption at 280 nm and by the method of Lowry *et al.* (1951). The solution was then diluted to 20 mg of protein/ml. To every 1 ml of this cytosol was added 5 µl of the progesterone solution [80% water and 20% (v/v) ethanol] described in the preceding section. This gives a concentration of about 20.9 nM-progesterone with 1 µCi of radioactivity/ml. This crude cytosol receptor was then subjected to the (NH₄)₂SO₄-purification procedure described by Schrader & O'Malley (1972). The pellets from the (NH₄)₂SO₄ precipitation were frozen and stored at -80°C. When needed, the receptor pellets were resuspended in TESH buffer at half the volume of the original cytosol solution, dialysed in washed dialysis tubing against 20 vol. of TESH buffer at 4°C for 2 h, and then recentrifuged at 10000 g_{av.} for 5 min to sediment any insoluble material. This hormone-receptor preparation was used directly in the nuclear binding assays. The protein concentration (µg/ml) and radioactivity (d.p.m./ml) were monitored and found to be constant from assay to assay. The integrity of the hormone-receptor complex was monitored as a routine by sucrose-gradient centrifugation and by the charcoal/dextran methods as described previously (Toft & O'Malley, 1972). Before and after incubation, more than 90% of the radioactivity was bound to a protein sedimenting at 4S under the high-salt conditions as determined by the above techniques.

Preparation of cytosol from non-target tissue

The preparation of spleen cytosol was carried out in the same way as that of the oviduct. After the (NH₄)₂SO₄ pellets were resuspended in TESH buffer, dialysed and centrifuged, 100 µl portions were counted for radioactivity. Because the radioactivity was very low, portions of stock [³H]progesterone solution were again added to the solution to bring the radioactivity up to a value approximating that measured in the cytosol preparation from oviduct.

Assays for nuclear binding

The method for binding of the partially purified progesterone-receptor complex to nuclei simply involves the addition of the labelled hormone-receptor complex to the nuclear material, incubation at 4°C for 90 min, several washes of the sedimented

pellet with Tris/EDTA buffer and the assay of bound radioactivity on Millipore filters. The binding reaction contained 25 μ g of DNA as nuclei, 10% (v/v) glycerol, 'half-TESH' buffer (i.e. 0.005 M-Tris/HCl, 0.0005 M-EDTA, 0.006 M-thioglycerol, pH 7.4), the desired KCl concentration, and water to a final volume of 1.0 ml. Small amounts of 50% (v/v) glycerol were used to bring the final concentration of glycerol to 10% in the reactions. The TESH buffer was added in various amounts, depending on the amount of receptor added, to keep the final concentration equal to 'half-TESH' buffer. The reactions in small conical centrifuge tubes were initiated by the addition of the receptor (25–700 μ l per reaction) and the contents of the centrifuge tubes were immediately mixed, and incubated on ice for 90 min with intermittent mixing. The reaction vessels were then centrifuged at 1500 g_{av} . for 5 min. The nuclear pellets were then washed with 2.0 ml of TKM buffer (0.05 M-Tris/HCl, 0.025 M-KCl, 0.002 M-MgCl₂, pH 7.5) containing 0.2% Triton X-100 and 0.50 M-sucrose, resuspended in 2 ml of a dilute Tris/EDTA buffer (0.002 M-Tris/HCl, 0.0001 M-EDTA, pH 7.5), resedimented at 1500 g_{av} , and washed again in 2 ml of the Tris/EDTA solution. The three washes after the hormone binding assay decreased the background radioactivity as well as the radioactivity of the complete reactions to values which only slightly changed with further washing (see the Results section). After the fourth resuspension (in the 2 ml of Tris/EDTA solution), the nuclear material was collected on Millipore filters (0.45 μ m pore size, 24 mm diam.; Millipore Corp., Bedford, MA, U.S.A.). The reaction vessels and filters were washed with 5 ml of the Tris/EDTA solution, and the filters dried and counted for radioactivity in a scintillation spectrometer in 5 ml of the toluene-based PPO (2,5-diphenyloxazole, 6 g/l)/POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene, 0.075 g/l] solution from Amersham/Searle, Arlington Heights, IL, U.S.A. After radioactivity counting (30% efficiency), the filters were removed, dried thoroughly, and the DNA was hydrolysed by incubating the filters in 0.3 M-HClO₄ for 30 min at 90°C. The cooled extracts were then assayed for DNA by the diphenylamine reaction (Burton, 1956). The radioactivity (d.p.m./mg of DNA) was calculated. In some instances the specific radioactivity of the labelled hormone was used to calculate either the pmol of labelled [³H]progesterone (bound or free) per mg of DNA or the molarities of the labelled progesterone (bound or free).

Results

Properties of the nuclear binding *in vitro*

Studies were first performed to examine the properties of the binding of the progesterone–

receptor complex *in vitro* to hen oviduct nuclei. Fig. 1 shows the kinetics of the nuclear uptake and binding of the intermediate concentrations of receptor. When assayed under very-low-ionic-strength conditions, the nuclear uptake of [³H]progesterone–receptor complex came to equilibrium by 90 min of incubation. However, under the higher-ionic-strength conditions, the equilibrium of nuclear uptake is reached within 30 min of incubation. Since all studies were performed between 0.05 and 0.15 M-KCl, subsequent incubations were performed at 4°C for 90 min. It was found that the [³H]progesterone–receptor complex remains essentially intact as measured by sucrose-gradient-sedimentation analysis and charcoal-binding assay (Toft & O'Malley, 1972).

It is known for several steroid hormone systems that the receptor needs to be 'activated', either by mild heat treatment or by exposure to higher ionic strength for optimal binding to isolated nuclei (Jensen & DeSombre, 1972; Buller *et al.*, 1975a,b). It has also been reported that (NH₄)₂SO₄ treatment of the [³H]progesterone–receptor complex from the chick oviduct cytosol appears to simulate this activation process (Buller *et al.*, 1975a,b). As shown in Fig. 2, the crude receptor preparations, not incubated at the higher temperatures (unactivated), show a minimal uptake by

Fig. 1. Kinetics of binding of [³H]progesterone–receptor complex to hen oviduct nuclei

The assays were carried out as described in the Materials and Methods section. The specific radioactivity of the [³H]progesterone (47.8 Ci/mmol) and the value of 2.5 pg of DNA/cell were used to calculate the number of molecules of bound progesterone/cell nucleus. Assays were run with 25 μ g of DNA (as nuclei) with 200 μ l of the partially purified receptor per reaction in (●) 0.01 M-NaCl or (○) 0.15 M-NaCl.

Fig. 2. Binding of crude and purified [³H]progesterone-receptor complex to hen oviduct

The assays were performed as described in the Materials and Methods section with 0.05 M-NaCl and 25 μg of DNA (as nuclei) per reaction by using (a) crude cytosol (100000g_{av} supernatant of oviduct homogenates) or (b) the partially purified receptor [35% (w/v) (NH₄)₂SO₄ precipitate]. The temperature of each incubation was (●) 23°C or (○) 4°C. The range and average of three replicates of analysis for each receptor concentration are shown.

the hen oviduct nuclei. However, exposure of this progesterone-receptor preparation to higher temperature (activation) results in a significant amount of binding. The partially purified [(NH₄)₂SO₄-precipitated] [³H]progesterone-receptor complex from the chick oviduct binds to nuclei at 4°C in a manner similar to that of the crude preparation (whole cytosol) at 23°C. The increased binding of partially purified receptor to the oviduct nuclei at 23°C as opposed to 4°C has been reported by Buller *et al.* (1975b) and has been shown experimentally in our laboratory to be caused in part by the opening of new binding sites in the nucleus, probably by proteolysis. Oviduct nuclei incubated at 23°C for 90 min with the crude or partially purified cytosol receptor show proteolytic activity with respect to degradation of histone species, with an accompanying decrease in thermal denaturation, as well as de-repression of the chromatin DNA with respect to its capacity to serve as a template for DNA-dependent RNA synthesis *in vitro*. Proteolytic activity in nuclei and chromatin has been reported previously (Panyim & Chalkley, 1969). The use of the partially purified [³H]progesterone-receptor complex rather than the crude cytosol preparation in the binding assays was found to be more satisfactory for several reasons. First, the stability during storage of the hormone-receptor complex is much better in the former than in the latter. Also, the integrity of the nuclear chromatin, as measured by chemical analysis, polyacrylamide-gel electrophoresis of the proteins and template activity, is better preserved after incubation with the partially purified receptor preparation at 4°C and, especially, at 23°C. Lastly, in assays using relatively low-ionic-strength conditions, the amount of non-receptor cytosol protein which complexes to the nuclei is much less with the partially purified receptor preparation than with the crude cytosol preparation. Consequently, all subsequent nuclear binding assays were performed with the partially purified receptor at 4°C.

To test the requirement of the receptor protein for nuclear binding, hen oviduct nuclei were incubated with [³H]progesterone either in buffer or complexed to its receptor protein. The amount of free [³H]progesterone in the incubations was determined by assessing the concentration of radioactivity per ml in the [³H]progesterone-receptor preparations. Addition of the equivalent amount of radioactivity as [³H]progesterone to buffer solution was then made. As shown in Table 1 (Expt. A), the binding of the radioactive hormone to oviduct nuclei is negligible when the receptor is omitted from the reaction. Similar negligible uptake and binding of [³H]progesterone to oviduct nuclei is observed when the hormone is incubated with the cytosol of a non-target organ (spleen), which contains no

Table 1. Requirements for nuclei binding in vitro

In Expts. A and B, binding assays were carried out as described in the Materials and Methods section, except that the nuclei were incubated either with free [³H]progesterone or with [³H]progesterone-labelled receptor, partially purified [by (NH₄)₂SO₄ precipitation] from oviduct or spleen cytosol. In Expt. C, oviduct nuclei were incubated as described in the Materials and Methods section with partially purified oviduct cytosol receptor either with or without any complexed [³H]progesterone. After incubation, the nuclei were washed twice in TESH buffer by centrifugation. Any bound receptor was then extracted with 0.4M-KCl in TESH buffer for 30 min at 0°C. The nuclei were again sedimented by centrifugation, and the supernatant was retained. The DNA of the nuclear pellet was determined by the diphenylamine reaction (Burton, 1956). To the 0.4M-KCl nuclear extract was added a saturating amount of [³H]progesterone in TESH buffer. After incubation for 2 h at 0°C, the amount of receptor-bound [³H]progesterone was determined by the charcoal/dextran method (Toft & O'Malley, 1972). The quantity of bound receptor, determined by the concentration of salt-extracted, labelled receptor, is shown as the radioactivity (d.p.m.) of [³H]progesterone/mg of DNA from the extracted nuclei. The results for the oviduct receptor without [³H]progesterone are corrected for the increased stability of the steroid-free receptor during incubation.

Expt.	Oviduct nuclei (μg of DNA)	[³ H]Pro- gesterone alone (μl)	Oviduct receptor		Spleen cytosol With [³ H]pro- gesterone (μl)	KCl (M)	Radioactivity	
			With [³ H]pro- gesterone (μl)	Without [³ H]- progesterone (μl)			(10 ⁻³ × d.p.m./ mg of DNA)	(% of control)
A	25	—	250	—	—	0.05	1693	100
A	25	250	—	—	—	0.05	127	7.5
B	40	—	300	—	—	0.15	520	100
B	40	—	—	—	300	0.15	143	28.0
C	80	—	800	—	—	0.05	236	100
C	80	—	—	800	—	0.05	12	5.2
C	80	—	800	—	—	0.15	13	100
C	80	—	—	800	—	0.15	0.7	5.0

Fig. 3. Binding of the [³H]progesterone-receptor complex to hen oviduct nuclei: effects of ionic strength

The assays were performed as described in the Materials and Methods section in the presence of (●) no KCl, (○) 0.05M-KCl, (Δ) 0.10M-KCl and (▲) 0.15M-KCl. About 25 μg of DNA per assay was used. The range and average of three replicates of analysis for each receptor concentration are shown.

detectable receptor (see Table 1, Expt. B). Consequently, it appears that the nuclear uptake and binding of the labelled progesterone requires the receptor found only in the target tissues. Experiments were also performed to determine whether or not the steroid itself is required for nuclear binding of the receptor. Table 1 (Expt. C) shows minimal nuclear binding by the hormone-free receptor. The lower extent of binding observed in Expt. C is probably due to the difference in the assay method, as described in the legend. It is probably due to the slow dissociation of much of the [³H]progesterone from the receptor. In any case, these studies suggest that both the steroid and the receptor (as an activated complex) are essential for nuclear binding.

Under these conditions, studies were performed to assess the effect of ionic strength on the nuclear binding. Fig. 3 shows results of such binding studies. In the absence of added KCl, no saturation occurs. However, as the ionic strength is increased, saturation occurs, accompanied by a lower degree of binding. Since the increased ionic strength decreases the concentration of progesterone-receptor binding to oviduct nuclei, the question arises as to whether there are different classes of binding sites as found *in vivo* (Spelsberg, 1976) or whether there is decreased binding to the same class of sites.

Identification and characterization of the multiple classes of binding sites in oviduct nuclei

The application of many different amounts of the [³H]progesterone-receptor complex to the binding-

Fig. 4. Effects of ionic strength on the binding of $[^3\text{H}]$ progesterone-receptor complex to hen oviduct nuclei

These experiments were performed as described in the Materials and Methods section, except that the assay mixtures contained either (●) 0.05M-KCl or (▲) 0.15M-KCl (final concn.). The assay was performed by using 25 μg of DNA per assay. The equilibrium dissociation constants (K_d) and the number of binding sites per cell for the first two plateaux were calculated from the Scatchard plot (see Fig. 5). The values for the third plateau were obtained from the estimated saturation (sites/cell) and half-saturation (K_d) concentrations in a plot of bound versus free $[^3\text{H}]$ progesterone-receptor complex. The DNA content per hen organ cell was taken as 2.5 pg/cell. The range and average of three replicates of analysis for each receptor concentration are shown.

assay experiments was then studied to assess the possible presence of more than one class of nuclear binding sites. As shown in Fig. 4, these studies indicate the presence of several classes of binding sites with evident differences in binding affinities. Evidence of even more classes of sites has been obtained by using even greater amounts of the hormone-receptor complex. The presence of these multiple binding sites may be responsible for the non-saturability of the nuclear binding sites under the low-ionic-strength conditions (Fig. 3). Since increasing ionic concentrations cause a decrease in total binding of the $[^3\text{H}]$ progesterone-receptor complex to nuclei, it is possible that different classes of binding sites are involved. Fig. 4 supports this hypothesis in that an increase in the ionic strength from 0.05M- to 0.15M-KCl causes the elimination of the weaker classes of binding sites, leaving only a highest-affinity class. Gradual increase in ionic strength from 0.15M- to 0.25M-KCl caused a decrease in the radioactivity

almost to the background value. The TESH buffer in the reaction mixture additionally adds an equivalent of 0.02M-KCl as determined by conductivity. Not all of the $[^3\text{H}]$ progesterone-receptor complex bound *in vivo* or *in vitro* to oviduct nuclei can be dissociated with 0.25M-KCl. In any case, these results suggest that there is more than one class of binding sites detectable *in vitro*, as *in vivo* (Spelsberg, 1976). All of these interactions between the $[^3\text{H}]$ progesterone-receptor complex and oviduct nuclei are primarily electrostatic in nature, with each selectively dissociated at specific ionic strengths. Under physiological ionic conditions, only the highest-affinity class of binding sites exists.

In Fig. 4, the equilibrium dissociation constants (K_d) for each class of sites are given. These constants were calculated by using Scatchard-plot analysis (Scatchard, 1949) (Fig. 5). An equilibrium dissociation constant, $K_d = 1.2 \times 10^{-9}\text{M}$, representing approx. 5000 sites/cell nucleus, was calculated for the

Fig. 5. Scatchard-plot analysis of $[^3\text{H}]$ progesterone-receptor-complex binding to oviduct nuclei

Binding was carried out as described in the Materials and Methods section with 25 μg of DNA per 1.0 ml assay mixture under conditions of (a) 0.05 M-KCl or (b) 0.15 M-KCl. The results of Fig. 4 were then plotted by the method of Scatchard (1949). The number of binding sites (n) was calculated by assuming 2.5 μg of DNA per oviduct nucleus (Sober, 1970). The K_d was calculated by extrapolating the amount of bound receptor to a 1 litre reaction volume, thus making the K_d expressed in mol/litre per 25 μg of DNA. The average of three replicates of analysis was used in these plots of the results in Fig. 4.

first (highest-affinity) class of binding sites (see Fig. 5a). The second (next-highest-affinity) class of binding sites has a tenfold less affinity with an equilibrium dissociation constant, $K_d = 1.3 \times 10^{-8} \text{ M}$, representing approx. 20 000 sites/cell nucleus (Fig. 5a). Scatchard-plot analysis of the third class of binding sites was too variable, so an estimate of the K_d ($\approx 4.0 \times 10^{-8} \text{ M}$) and the number of sites per cell (approx. 35 000) were roughly calculated from a plot of bound versus free receptor, by using estimated half-saturation and saturation values respectively

of the results in Fig. 4. Fig. 5(b) shows a Scatchard-plot analysis of the binding to the oviduct nuclei under the higher-ionic-strength condition. The equilibrium dissociation constant, K_d ($\approx 6 \times 10^{-9} \text{ M}$), and the number of sites of 10 000/cell nucleus are somewhat higher than those observed for the highest-affinity class of binding sites analysed under low-ionic-strength conditions. Possibly, the inclusion of a few of the sites of the second-highest-affinity group may still occur under these higher salt conditions.

To confirm that these different classes of binding

Fig. 6. Effects of washes on the extent of binding to oviduct nuclei of the [^3H]progesterone-receptor complex

The assays were performed as described in the Materials and Methods section, except that the number of washes of the nuclei after incubation with the [^3H]progesterone-receptor was varied. The assays were divided into groups containing (a) 100 μl , (b) 200 μl , (c) 400 μl or (d) 600 μl of the labelled receptor solution. ●, Whole reactions; ○, blanks containing no nuclei. The assays within each group either were not washed or were washed from one to four times with the Tris/EDTA buffer (0.002M-Tris/0.0001M-EDTA, pH7.5). The radioactivity/filter was then measured. Each value represents the average and the range of values obtained for three separate assays. The amounts of receptor were chosen from experimental results of Fig. 4, and represent the amounts required to saturate each of the classes of binding sites. ○, Blank; ●, nuclei.

sites do not exist as a result of experimental manipulation and that the sites are relatively stable to such manipulation, studies were performed to assess the effects of the number of washes of nuclei on the amount of radioactivity bound to nuclei. The binding was performed under low-ionic-strength conditions and by using amounts of the [^3H]progesterone-receptor which allow binding to each of the classes of sites, based on results in Fig. 4. Fig. 6 shows that the degree of binding to all classes of sites (from the highest-affinity class, with 100 μl of the [^3H]progesterone-receptor, to the lowest-affinity class, with 600 μl of the [^3H]progesterone-receptor), remains unchanged after the second wash. Consequently, the number of washes after the binding assays does not alter the degree of progesterone binding to each of the classes. The different classes of binding sites are also observed when the binding assays are carried out with a constant concentration of the [^3H]progesterone-receptor and with increasing concentrations of DNA as nuclei (results not shown). Consequently, the varying concentrations of [^3H]progesterone-receptor complex in the standard binding assays do not appear to be the cause of the plateau.

Presence of the highest-affinity class of binding sites in nuclei from non-target tissue

Purified nuclei from non-target tissues (spleen, liver, lung and erythrocytes) were incubated with [^3H]progesterone-receptor complex under low- and high-ionic-strength conditions (Figs. 7a and 7b). In Fig. 7(a), the possible presence of a high-affinity class of binding sites is detected in the oviduct (target tissue) nuclei but not in the spleen or erythrocyte (non-target tissue) nuclei. Lower-affinity classes of sites are found in the nuclei of non-target as well as target tissues. By applying the high-ionic-strength conditions to analyse binding selectively to the high-affinity class of sites, it was found that the higher-affinity class of sites in whole nuclei was not absolutely tissue-specific (Fig. 7b). Although spleen and liver nuclei display none of these sites, the presence of a high-affinity class of sites is detected in erythrocyte and lung nuclei, although the extent of binding is lower than that of oviduct nuclei. Under the low-ionic-strength conditions (Fig. 7a), the maximum extent of hormone binding to nuclei is similar for target as well as for non-target tissue, whereas under high-ionic-strength conditions (Fig. 7b) the target-tissue (oviduct) nuclei display greater

Fig. 7. Binding of $[^3\text{H}]$ progesterone-receptor complex to the nuclei from different organs of the hen

The assays were performed as described in the Materials and Methods section with (a) 0.05 M-KCl or (b) 0.18 M-KCl with about 50 μg of DNA (as nuclei)/reaction. The range and average of three replicates of analysis for each receptor concentration are shown. ●, Oviduct; Δ, erythrocytes; ▲, lung; ■, spleen; ○, liver nuclei of mature hens.

binding. These studies suggest that the cell nuclei of non-target tissue contain either no or a decreased number of high-affinity binding sites compared with cell nuclei from target tissues.

Discussion

The present results clearly show the presence *in vitro* of different classes of binding sites for the progesterone-receptor complex in hen oviduct nuclei. These sites probably correspond to those described in the preceding paper (Spelsberg, 1976) for the chick oviduct *in vivo*. The highest-affinity class of binding sites does not appear to be absolutely tissue-specific for whole nuclei, although the extent of binding to these sites is greater for the oviduct (target-cell) nuclei. These results are similar to those reported by Spelsberg *et al.* (1971b) for progesterone-receptor binding to chick oviduct chromatin. Buller *et al.* (1975a) also found measurable binding of progesterone receptor to the nuclei of certain non-target tissues.

It is proposed that only the highest-affinity class of binding sites identified in these experiments represents the physiological nuclear binding for progesterone in the hen oviduct nucleus. The reasons for this are threefold. First, it is only logical to assume that the true binding site for steroid-receptor complexes in the nucleus would be one of highest affinity. Secondly, our results show that under the ionic conditions of the cell cytoplasm and the even higher-ionic-strength conditions proposed to exist in the cell nucleus, only the highest-affinity class of binding sites survives. Finally, experiments in which labelled and/or unlabelled progesterone was injected into chicks (Spelsberg, 1976) have shown that the number of molecules of progesterone bound per cell nucleus can be correlated with the endogenous RNA polymerase activities. Not only were multiple binding sites observed under these conditions *in vivo*, but also, when the number of molecules of progesterone per cell nucleus reached approx. 10000, a maximal alteration in the RNA polymerase activities was observed (Spelsberg, 1976). Thus the high-affinity sites detected *in vitro* and representing 6000–10000 progesterone-receptor binding sites per cell nucleus probably represent the biologically active ones.

The lower-affinity classes of binding sites observed in these assays *in vitro* may or may not have biological significance. Lower-affinity classes of binding sites were observed *in vivo* in the preceding paper (Spelsberg, 1976). In any case, these various classes of binding sites in hen oviduct nuclei are readily distinguishable *in vitro* when the concentration of the hormone-receptor complex is varied. These classes of binding sites display a relatively high affinity ($K_d \approx 10^{-8}$ – 10^{-9} M) for the $[^3\text{H}]$ progesterone-receptor complex, which, however, is much lower

than that of progesterone for its receptor protein ($K_d \approx 10^{-10}$ – 10^{-11} M).

These studies strongly suggest that binding assays involving steroid hormones and isolated nuclei which differ from each other in ionic concentrations are not measuring the same class of nuclear-binding sites. Studies performed under low-ionic-strength conditions are measuring a non-saturable (adsorption-like) interaction, whereas those performed under higher-ionic-strength conditions are measuring a high-affinity, saturable-type binding. The tissue specificity of the latter interaction is much more significant than the former interactions.

These conclusions support those made in studies involving immature chicks (Spelsberg *et al.*, 1971b).

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