Nuclear Binding of Progesterone in Chick Oviduct

MULTIPLE BINDING SITES *IN VIVO* AND TRANSCRIPTIONAL RESPONSE

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(Received 21 August 1975)

1. Varied doses of labelled or unlabelled progesterone were injected into immature chicks which had previously been stimulated with oestrogen. The concentrations of nuclear bound [^3H]progesterone were correlated with the effects of the hormone on endogenous RNA polymerase I and II activities in isolated oviduct nuclei. 2. The extent of nuclear localization of [^3H]progesterone in oviduct (a progesterone target tissue) was shown to be much greater than in lung (non-target tissue). The concentration of bivalent cations in solvents used in the nuclei isolations has a marked effect on the amount of bound hormone in the nuclei. 3. Evidence for the existence of several classes of binding sites for progesterone in the oviduct nuclei is given. These classes represent about 1000, 10000 and 100000 molecules of the hormone per cell nucleus and are saturated by injecting approx. 10, 100 and 1000 μg of progesterone respectively. 4. When saturation of the first (highest affinity) class of nuclear sites occurs, a marked inhibition in RNA polymerase II (but not RNA polymerase I) activity was observed. When the second class of sites was saturated, alterations in both RNA polymerase I and II activities were observed. Binding to the third class of nuclear binding sites was not accompanied by further changes in polymerase activity. It is suggested that the first two classes of nuclear binding sites may represent functional sites for progesterone action in the chick oviduct.

It seems to be universal that the cytoplasm of target cells for all steroid hormones contains specific binding proteins called 'receptors'. Although the complete sequence of events in the response of a tissue to hormone stimulation is still unknown, a postulated primary step is the interaction of the hormone with its receptor in the target tissue. There is evidence that the hormone-receptor complex migrates into the nucleus and binds to chromatin (Raspé, 1971; Spelsberg, 1974). This nuclear binding is followed by an alteration of RNA synthesis which occurs within 15–30 min after the hormone enters the vascular system (Billing *et al.*, 1968; Hamilton *et al.*, 1968; Teng & Hamilton, 1968; Trachewsky & Segal, 1968; Knowler & Smellie, 1971; Glasser *et al.*, 1972; Wilson, 1973; Borthwick & Smellie, 1975).

Although oestrogens produce major growth and developmental responses in the reproductive tract, the effects of progesterone are much more subtle in nature. Progesterone appears to alter the DNA-dependent RNA synthesis in chick oviduct in an altogether different pattern compared with oestrogen (O'Malley *et al.*, 1969). Progesterone decreases the overall DNA-dependent RNA synthesis at 1 h after injection into oestrogen-pretreated immature chicks, whereas oestradiol enhances this synthesis (T. C. Spelsberg & R. F. Cox, unpublished work). Part of the progesterone effect can be assigned to the increased restriction of the DNA in chromatin (as measured by the ability of the chromatin to serve as a template for DNA-dependent RNA synthesis and using isolated

bacterial RNA polymerase as the enzyme). By contrast, progesterone induces the appearance in the polyribosome fraction of specific mRNA coding for egg-white proteins when injected into chicks from which oestrogen has been withdrawn, and acts synergistically with oestrogen to raise these specific mRNA concentrations even further (Palmiter & Smith, 1973). Further, progesterone induces the synthesis of avidin in the chick oviduct (O'Malley *et al.*, 1969). This induction is believed to occur at the transcriptional level, since the amount of avidin mRNA is also enhanced by the hormone (Rosenfeld *et al.*, 1972; O'Malley *et al.*, 1972). It can therefore be assumed that one of the major actions of progesterone occurs at the level of gene expression.

Correlation between the nuclear binding of progesterone and subsequent biological responses has not been studied in detail in the chick oviduct. It was decided to administer increasing doses of progesterone to oestrogen-pretreated chicks and to correlate these doses both with the extent of nuclear binding and with the hormone-induced changes in RNA polymerase activities. This paper presents the results of such studies.

Materials and Methods

Materials

Unlabelled progesterone and stilboestrol were purchased from Schwarz/Mann, Orangeburg, NY,

U.S.A. [^3H]progesterone (44 Ci/mmol) was purchased from New England Nuclear Corp., Boston, MA., U.S.A. Purity of the steroids was determined by t.l.c. with benzene/ethyl acetate (70:30, v/v) as solvent. Unlabelled nucleotide triphosphates as sodium salts were purchased from P-L Biochemicals, Milwaukee, WI, U.S.A. [^3H]UTP (13 Ci/mmol) was purchased from Schwarz/Mann, or from Amersham/Searle, Arlington Heights, IL, U.S.A. α -Amanitin was purchased from Henley and Co., New York, NY, U.S.A.

Source of tissue and treatments

White Leghorn chicks (7 days old) were given daily injections (subcutaneously) of 5 mg of stilboestrol in 0.2 ml of sesame oil. The chicks were kept on a light/dark cycle with a 12 h light-period (8:00–20:00 hours). After 14 days of treatment, the magnum portion of oviducts (0.1 g wet wt. in untreated chicks) attained a wet weight of 1–2 g and morphologically resembled that of fully developed oviducts from laying hens. Full characterization of this oestrogen-induced development has been reported elsewhere (Mason, 1952; Kohler *et al.*, 1969; Oka & Schimke, 1969a,b; O'Malley *et al.*, 1969; Cox & Sauerwein, 1970; Palmiter & Wrenn, 1971; Palmiter, 1972; Socher & O'Malley, 1973). The chicks were then injected subcutaneously with the desired labelled or unlabelled progesterone dissolved in sesame oil, and the magnum portion of the oviducts, and occasionally the lungs, were excised.

Isolation of nuclei by procedures A and B for measurement of [^3H]progesterone binding and RNA polymerase activity respectively

Nuclei which were used for measuring bound [^3H]progesterone were purified by procedure A as described previously (Knowler *et al.*, 1973; Spelsberg *et al.*, 1974), except that diluted buffer solutions and one extra sedimentation through 1.7 M-sucrose were used. The diluted buffer solutions used in these experiments consisted of the specified sucrose concentrations in 1/5 $\times$ TKM buffer (0.01 M-Tris/HCl, pH 7.5, 0.005 M-KCl/0.0004 M-MgCl₂), which is a dilution of 1 $\times$ TKM buffer (0.05 M-Tris/HCl, pH 7.5/0.025 M-KCl/0.002 M-MgCl₂) used in previous work (Spelsberg *et al.*, 1971). The pellets of the purified nuclei were sedimented once more in a solution containing 1.7 M-sucrose in 1/5 $\times$ TKM buffer. The purified nuclei were resuspended in the storage buffer [25% (v/v) glycerol in 10 mM-Tris/HCl (pH 7.5)/1 mM-MgCl₂] and 100 μ l portions assayed for radioactivity by using 10 ml of a solution containing 90% toluene-based PPO (2,5-diphenyloxazole 60 g/l)/POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene (7.5 mg/l)] scintillation fluor and 10% Beckman

Biosolve 3 (v/v). The nuclear suspensions were also assayed for DNA content (Burton, 1956) and the radioactivity per mg of DNA was calculated. The number of molecules of progesterone bound per cell nucleus was calculated by using 2.5 pg of DNA/chick organ cell (Sober, 1970) and a 30% counting efficiency.

Nuclei for the assay of endogenous RNA polymerase I (nucleolar) and II (nucleoplasmic) activities were isolated by procedure B (Glasser *et al.*, 1972; Knowler *et al.*, 1973; Spelsberg *et al.*, 1974). The pellets of partially purified nuclei were then resuspended in 0.2 vol. (1 ml/5 g of tissue) of storage buffer, containing 25% (v/v) glycerol in 10 mM-Tris/HCl (pH 7.5)/1 mM-MgCl₂.

Assay for endogenous RNA polymerase activities in isolated nuclei

To remove glycerol before assay of nuclei for DNA content, nuclear suspensions in storage buffer were diluted fourfold with water, made 0.3 M with respect to HClO₄, and sedimented at 2000g for 10 min. Pellets were then analysed for DNA content (Spelsberg *et al.*, 1971). The assay for endogenous RNA polymerase activity was a modification of that of Roeder & Rutter (1970) and is described elsewhere (Glasser *et al.*, 1972; Glasser & Spelsberg, 1972; Spelsberg *et al.*, 1974). Measurement of radioactivity was as described by Glasser *et al.* (1972), except that the radioactive product was collected on glass-fibre filters and washed additionally with cold 90% (v/v) ethanol before radioactivity counting. The amount of nuclear DNA added to the reaction was used to calculate the amount of radioactivity incorporated per mg of DNA.

Results

Properties of endogenous RNA polymerase activities in isolated nuclei

We have found that isolation conditions affect polymerase activity (Glasser *et al.*, 1972; Knowler *et al.*, 1973; Spelsberg *et al.*, 1974). Triton X-100 treatment results in lowered activities. Concentrations of sucrose of 1.0 M or less cause decreased nucleoplasmic polymerase II activity and have variable effects on nucleolar polymerase I. For optimal polymerase activity, it was necessary to forego complicated purification steps to minimize the time required for isolation. Nuclei can be stored in glycerol solution (storage buffer) without loss of enzyme activity (Glasser *et al.*, 1972; Knowler *et al.*, 1973; Spelsberg *et al.*, 1974). In contrast with other buffers and temperatures studied, this medium preserved both polymerase activities as well as the nuclear morphology for several months of storage at -20°C . Thus glycerol helps to maintain the native structure and biological activity of nuclei.

That the assays are measures of the endogenous polymerase I and II activities is supported by several findings. First, the assays of both RNA polymerase I and II in these experiments are dependent on a DNA template, all four nucleotides and bivalent cations. Secondly, the product is verified to be RNA by its degradation by alkali and ribonuclease. Thirdly, the base analysis of the newly synthesized RNA as well as the extent of inhibition by α -amanitin demonstrate that the two assay conditions represent either RNA polymerase I (synthesizing rRNA) or RNA polymerase II (synthesizing DNA-like RNA). The assays are linear as a function of added DNA: up to 60 μ g of DNA for polymerase II and up to 100 μ g of DNA for polymerase I. Internal absorption of the ^3H radiation is suggested as part of the cause of these restrictions in concentration of nuclei that can be added. In any case, 50 and 100 μ g of nuclear DNA were used for these respective assays. The kinetics of RNA synthesis demonstrate that the nucleotide incorporation is linear for 15 min for polymerase II assays and 30 min for polymerase I assays. These incubation periods were used in all subsequent experiments.

Properties of the nuclear binding of [^3H]progesterone

The effects of different methods of isolation and purification of nuclei on the concentration of nuclear-

bound progesterone after injection of 100 μCi (0.718 μg) of the [^3H]progesterone into chicks were assessed. These concentrations were found to be readily affected by relatively low concentrations of bivalent cations. Table 1 shows some results of these studies. Oviduct nuclei preparations isolated by 'method A' (Spelsberg *et al.*, 1971, 1974), which involves multiple steps using 0.5 M-sucrose in 1 $\times$ TKM buffer, repeatedly display the same concentration of bound hormone as do nuclei preparations isolated by 'method B' (Glasser *et al.*, 1972; Knowler *et al.*, 1973), which involves only a few steps using 2.0 M-sucrose in 1 $\times$ TKM buffer. In short, neither the concentration of sucrose nor the number of steps used in the isolation of the nuclei appears to affect the extent of nuclear binding. However, treatment of the nuclei with detergent lowers the extent of nuclear binding. This decrease in amount of the nuclear-bound hormone probably is a result of removal of cytoplasmic debris from the isolated nuclei, as indicated both by the electron micrographs of the nuclear preparations (see Knowler *et al.*, 1973, for published micrographs) and by the decreases in the protein/DNA ratio [2.47 $\pm$ 0.17 (without Triton treatment) to 2.05 $\pm$ 0.27 (with Triton treatment)] and RNA/DNA ratio [0.346 $\pm$ 0.02 (without Triton treatment) to 0.265 $\pm$ 0.01 (with Triton treatment)]. Interestingly, the use of buffers which contain lower concentrations of bivalent ions results in much higher amounts of nuclear-bound progesterone (Table 1). Indeed, when nuclei isolated and resuspended in 1/5 $\times$ TKM buffer containing 0.0004 M-MgCl₂ were then exposed to 0.002 M-MgCl₂ as contained in the 1 $\times$ TKM buffer, a rapid (about 20 min) loss of about 50% of the nuclear-bound hormone was detected. Studies using additions of MgCl₂ alone also caused similar loss of nuclear-bound hormone. The purity of the preparations in 1/5 $\times$ TKM buffer was equivalent to those preparations isolated in 1 $\times$ TKM buffer as determined by electron microscopy and chemical analysis; however, an extra sedimentation of the nuclei in 1.7 M-sucrose in 1/5 $\times$ TKM buffer was necessary to achieve purity equivalent to that of those isolated in 1 $\times$ TKM buffer. Consequently, the enhanced binding in the 1/5 $\times$ TKM buffer does not appear to be due to an increased cytoplasmic contamination but rather to a lower degree of dissociation of nuclear-bound hormone.

Nuclear localization of [^3H]progesterone

Fig. 1 shows the time-course of the nuclear binding of [^3H]progesterone in chick oviduct and lung nuclei. Immediately after the injection of 100 μCi of the stock of ^3H -labelled hormone (containing 0.718 μg of progesterone), there was an uptake into chick oviduct and lung nuclei. This radioactivity was detected as early as 5 min after injection. Although lung nuclei

Table 1. Amount of nuclear-bound [^3H]progesterone in relation to methods of isolation of nuclei

Immature chicks, previously treated with stilboestrol for 2 weeks, were injected subcutaneously in the neck with 100 μCi of [^3H]progesterone in sesame oil. At 2 h after injection, the oviducts were excised, combined, weighed, rinsed in cold 0.15 M-NaCl and divided into ten groups (two per method of nuclei isolation). Nuclei were then isolated and purified as indicated below and as described in the Materials and Methods section (Spelsberg *et al.*, 1971, 1974; Glasser *et al.*, 1972; Knowler *et al.*, 1973). Radioactivity was measured as described in the Materials and Methods section. The protein/DNA and RNA/DNA ratios of each of the nuclei preparations were determined as described elsewhere (Spelsberg *et al.*, 1974). The d.p.m./mg of DNA was calculated from the c.p.m./mg of DNA and 30% counting efficiency. The average and range of the values obtained for each of the duplicate nuclei preparations is presented.

Group	Method of nuclei isolation	Radioactivity (d.p.m./mg of DNA)
	Method A 1 $\times$ TKM buffer:	
1	-Triton X-100	3995 $\pm$ 120
2	+Triton X-100	2400 $\pm$ 300
	Method B 1 $\times$ TKM buffer:	
3	-Triton X-100	4020 $\pm$ 620
	Method A 1/5 $\times$ TKM buffer:	
4	-Triton X-100	9210 $\pm$ 150
5	+Triton X-100	5820 $\pm$ 980

Fig. 1. Extent of the binding of $[^3\text{H}]$ progesterone to oviduct nuclei *in vivo*

Immature chicks, previously treated with stilboestrol for 2 weeks, were injected (subcutaneously) with $100\mu\text{Ci}$ of $[^3\text{H}]$ progesterone in sesame oil. At various intervals after injection, the oviducts and lungs were excised from two separate groups of four chicks, combined, and the nuclei isolated by procedure A described in the Materials and Methods section. The radioactivity per mg of DNA was measured as described in the Materials and Methods section. The average and range of the values obtained from the two groups at each interval are presented. ●, Oviduct; ○, lung.

showed a rapid depletion of the labelled progesterone, decreasing to undetectable values by 3h, oviduct nuclei showed a fall and a second rise in nuclear binding, with an overall greater retention of the hormone than in the lung. Nuclear-bound hormone is still detectable in oviduct nuclei by 22h after the injection. The dissociation of the hormone from the isolated nuclei with 0.3M-KCl and subsequent sedimentation of the radioactivity in sucrose gradients demonstrated that the first peak of nuclear binding (about 20min after injection) in the chick oviduct largely represents unbound hormone, whereas the label associated with the second peak (between 1 and 3h after injection) is almost entirely associated with receptor-like protein. At all time-periods, the radioactivity associated with the isolated nuclei was shown to be primarily but not entirely progesterone, as determined by t.l.c. as described in the Materials and Methods section. For all subsequent binding experiments, a 2h hormone-treatment period was selected.

Nuclear binding of progesterone in response to dose injected

By selecting a 2h incubation period after injection of $100\mu\text{Ci}$ ($0.718\mu\text{g}$) of labelled progesterone either alone or together with increasing concentrations of

Fig. 2. Binding of $[^3\text{H}]$ progesterone to oviduct nuclei in response to the dose of progesterone

Groups of four chicks were given injections subcutaneously of $100\mu\text{Ci}$ (or $0.718\mu\text{g}$) of $[^3\text{H}]$ progesterone alone or in combination with specified concentrations of unlabelled hormone. Stock $[^3\text{H}]$ progesterone in benzene was mixed with a specified amount of unlabelled progesterone in benzene. The specific radioactivity of the injected hormone is given at the top of the Figure. The solutions were frozen, freeze-dried and resuspended in sesame oil for injection. Each chick received a $100\mu\text{l}$ injection for doses under $100\mu\text{g}$ and a $200\mu\text{l}$ injection for doses over $100\mu\text{g}$. At 2h after injection, the oviducts of each group were excised, combined, and the nuclei isolated by procedure A and their radioactivity was measured as described in the Materials and Methods section. In A, the results are plotted as radioactivity (d.p.m.)/mg DNA; in B, the results are plotted as molecules of $[^3\text{H}]$ progesterone/cell nucleus calculated from the results in (a) as described in the Materials and Methods section. The average and range of three replicates of analysis of values obtained for each group are presented.

unlabelled progesterone, the concentration of radioactivity and the number of molecules of the hormone bound to oviduct nuclei were assessed. As shown in Fig. 2(a), increases in the dose of unlabelled progesterone together with the fixed amount of [^3H]progesterone resulted in a marked increase in the amount of radioactivity bound to oviduct nuclei, with a maximum obtained with an injection of 100 μg of the hormone. As expected, further increases in the dose of

unlabelled progesterone cause a loss of the total radioactivity bound to the nuclei. By using the varying specific radioactivities of the labelled hormone injected (given in Fig. 2a), the number of molecules of labelled progesterone per cell nucleus was calculated. As shown in Fig. 2(b), evidence of more than one type or class of binding sites in the oviduct nucleus for progesterone is suggested. The saturation of the first class of binding sites appears to occur when 50–75 μg of the hormone is injected and involves approx. 1×10^3 – 1×10^4 molecules of progesterone per cell nucleus. The saturation of the second class of binding sites occurs somewhere between the injection of 500 and 1000 μg of the hormone and involves over 10^5 molecules of progesterone per cell nucleus. The cause of the marked increase in the concentration of the hormone in the oviduct caused by increasing the dose of the hormone was investigated briefly. In a separate but similar experiment to that described above for Fig. 2, the effect of increasing the dose of the hormone on the concentrations of progesterone in whole oviduct and serum was assessed. As shown in Fig. 3, the concentrations of radioactivity in the serum and whole oviduct also increase with the increase in dose. However, in these tissues, the maximum concentration of radioactivity is observed at a 50 μg dose of the hormone, with a subsequent decrease in the concentration of radioactivity when greater doses of unlabelled progesterone are given. It appears that at least part of the increase of radioactivity in oviduct nuclei in response to increasing dosage is due to

Fig. 3. Concentration of [^3H]progesterone in oviduct nuclei (a), serum (b) and whole oviduct (c) in response to the dose of progesterone

Chicks (four per group) were each given injections subcutaneously of 100 μCi (0.718 μg) of [^3H]progesterone alone or in combination with specified amounts of unlabelled hormone as described in the legend of Fig. 2. At 2 h after the injection of [^3H]progesterone, blood was taken from the wing vein into heparinized capillary tubes, the tubes were centrifuged for 2 min at 200g, and 10–50 μl portions of the serum removed for measurement of radioactivity and protein analysis (Lowry *et al.*, 1951). The radioactivity (d.p.m.)/mg of protein was then calculated. Oviducts from each group were then excised, combined, and the nuclei isolated by procedure. As described in the Materials and Methods section. For whole-organ measurements, portions of the first homogenate obtained in procedure A for isolation of nuclei were counted for radioactivity. The mg of DNA/ml of the homogenate was measured as described in the Materials and Methods section for the DNA analysis of the nuclei preparation and the radioactivity (d.p.m.)/mg of DNA calculated. In (a), — represents d.p.m./mg of DNA and ---- represents the number of molecules of [^3H]progesterone bound per cell. The average and range of three replicates of analysis for each group are presented.

Table 2. Concentration of [^3H]progesterone in serum compared with the dose of progesterone

Each chick (four per group) was given an injection (subcutaneously) of 100 μCi (0.718 μg) of [^3H]progesterone alone or in combination with specified concentrations of unlabelled hormone. At 2h after the injection of [^3H]progesterone, blood was taken from the wing vein into heparinized capillary tubes, the tubes were centrifuged for 2min at 200g and 10–50 μl portions of the serum removed for measurement of radioactivity and protein analysis (Lowry *et al.*, 1951). The radioactivity (d.p.m./mg of protein) and the amount (ng) of progesterone/ml of serum were calculated. The average and range of two replicate analyses for each group are presented.

Dose of progesterone (μg)	Specific radioactivity ($\mu\text{Ci}/\mu\text{g}$)	Serum concentration of [^3H]progesterone (ng/ml)
0.718	139.66	0.651 $\pm$ 0.016
10.718	9.39	21.97 $\pm$ 0.03
25.718	1.97	89.0 $\pm$ 2.5
100.718	0.99	92.7 $\pm$ 3.2
500.718	0.20	658.0 $\pm$ 21.0
2500.718	0.04	3701.0 $\pm$ 35.0
10000.718	0.01	14214.0 $\pm$ 174.0

increases in radioactivity in serum and whole organ. The maximum radioactivity attained in the nuclei occurs at doses that are greater than those required for maximum radioactivity in the serum and whole organ.

The physiological concentrations of progesterone in the serum of laying hens range between 0.88 and 17.21 ng/ml (O'Malley *et al.*, 1968; Gilbert, 1971). In Table 2, the concentrations (ng/ml) of progesterone in the serum of immature chicks 2h after injections are shown. The injection of 10 μg of progesterone into immature chicks results in concentrations of the hormone in the serum which approximate that measured in the serum of laying hens.

Comparison of nuclear binding and RNA polymerase activities in response to multiple doses of progesterone

Comparisons of the amount of nuclear binding with the RNA polymerase activities by using numerous increasing doses of progesterone was then performed. As shown in Fig. 4(a), an initial enhancement of RNA polymerase I activity over that of controls is observed, beginning at a 10 μg dose of progesterone. At doses greater than 100 μg , a decrease in RNA polymerase I activity compared with that of controls is observed. Doses greater than 200 μg of progesterone do not further alter the RNA polymerase I activity. In Fig. 4(b), the RNA polymerase II activity decreases, beginning at a 6 μg dose of progesterone. The extent of decrease increases up to the 100 μg dose. Doses of the hormone greater than 100 μg fail to alter the extent of this decrease.

Fig. 4. Effects of dose of progesterone on endogenous RNA polymerase activities and on the binding of the hormone in the chick oviduct nuclei

The preparation of the progesterone for injections into chicks is described in legends to Table 2 and Fig. 2. The doses of 200 μg and 1000 μg were administered in 200 μl of sesame oil, and the lower doses in 100 μl . These experiments were carried out as described in the legend to Fig. 2. For the polymerase experiments, groups of six to ten chicks were each injected (subcutaneously) with a specified amount of the hormone. Two groups were injected with vehicle only and used for determining the non-injected controls. At 2h after injection, the oviducts from each group were excised, combined, and the nuclei isolated by procedure B as described in the Materials and Methods section and elsewhere (Glasser *et al.*, 1972; Knowler *et al.*, 1973; Spelsberg *et al.*, 1974). The RNA polymerase I (a) and RNA polymerase II (b) activities were then assayed as described in the Materials and Methods section and elsewhere (Glasser *et al.*, 1972; Spelsberg *et al.*, 1974). The activities are expressed as pmol of [^3H]UMP incorporated/mg of DNA. (c) demonstrates the nuclear concentration of [^3H]progesterone in terms of molecules of progesterone bound per cell nucleus. See the legend to Fig. 2 for details. The average and range of three replicates of analysis are presented for all values.

As an adjunct to these experiments, simultaneous analysis of the amount of nuclear binding of [^3H]progesterone by using similar doses of the hormone

were performed. As shown in Fig. 4(c), the application of these multiple doses of progesterone shows more clearly the presence of several classes of binding sites in the chick oviduct nuclei. The injection of 10–20 μg of the hormone saturates the highest-affinity class of binding site, representing about 1000 molecules per cell. Sites of this kind were not observed in the earlier experiments using a smaller number of doses of the hormone. Further increases in the dose of progesterone revealed a second class of sites which became saturated between 50 and 100 μg of the hormone. This class of sites represents about 10000 molecules per cell. Further increases in the dose of progesterone revealed yet another class of sites representing greater than 100000 molecules per cell. Comparing the amounts of nuclear-bound progesterone with the RNA polymerase activities, it is seen that only the binding of progesterone to the first two higher-affinity classes of sites results in some response in these enzyme activities. The responses of the two RNA polymerase activities are different for each of these classes of sites when they become saturated with the progesterone–receptor complex.

Discussion

In comparison with oestrogen, little work has been published about dosage effects of progesterone on various biological parameters in the chick oviduct. Mason (1952) published preliminary data indicating that as few as ten separate injections of 50 μg of progesterone would act synergistically with large doses (approx. 200 μg) of the stilboestrol in increasing oviduct weight in immature chicks. However, studies by Oka & Schimke (1969a) demonstrated that five daily injections of larger (250 μg) doses of progesterone into immature chicks resulted in a marked inhibition of the oestrogen-induced increases in oviduct weight. Oka & Schimke (1969b) also reported that a single 50 μg dose of the hormone into oestrogen-pretreated chicks resulted in a threefold enhancement of ovalbumin synthesis. Palmiter & Wrenn (1971) reported that a 1 mg dose of progesterone given to oestrogen-pretreated chicks enhanced the oviduct weight and the concentrations of membrane-bound ribosomes in the oviduct. However, if this dose was increased to 3–5 mg of progesterone, net decreases in wet weight and membrane-bound ribosome concentrations were observed. In none of these studies were the doses of progesterone carefully correlated with a biological response.

Several conclusions can be drawn from these experiments. First, maximal radioactivity in the nuclei of target tissues requires the injection of a specified quantity of the labelled hormone (in this case, 100 μg of [^3H]progesterone into 60 g chicks). Whole-body disposal or storage of the hormone may

have something to do with this phenomenon, since increased radioactivity is also observed in serum and whole tissue. Secondly, the buffers used in the isolation of nuclei affect the concentrations of progesterone binding to nuclei. Apparently, low concentrations of the bivalent cations can dissociate much of the labelled hormone from the nucleus. Consequently, buffers with very low concentrations of bivalent ions should be used during isolation of nuclei to preserve binding.

Thirdly, the binding of [^3H]progesterone to oviduct nuclei involves more than one class of binding sites. The first class involves 1000 molecules per cell nucleus, the second class about 10000 molecules per cell nucleus, and the third class, around 100000 molecules per cell nucleus. The first class appears to have the highest affinity for the hormone, followed by the second class and then the third class. Injections of 10 μg of progesterone per chick results in serum concentrations of the hormone (20 ng/ml) which resemble that measured in laying hens (O'Malley *et al.*, 1968; Gilbert, 1971). At this dose, only the highest-affinity class of sites bind the hormone. The isolation of nuclei in the higher-ionic-strength buffers ($1 \times \text{TKM}$) as opposed to the lower-ionic-strength buffers ($1/5 \times \text{TKM}$) appears to decrease the binding to the third class of sites, but has little effect on the extent of binding to the first or second class of sites.

Fourthly, after 2 h of hormone treatment, at least 10 μg of progesterone or more per chick is required to obtain a detectable response in the polymerase activities, whereas doses greater than 200 μg result in no further change in these activities. The 10 μg dose of the hormone which gives serum concentrations of progesterone similar to that of laying hens results in a decrease of polymerase II activity, with little or no effect on polymerase I activity. However, the comparison of effects of equivalent serum concentrations of progesterone between laying hens and immature chicks is not necessarily valid. In laying hens, progesterone is constantly present in the serum, whereas in the immature (stilboestrol-treated) chicks, it is present only for a short period after injection. In addition, the hen oviduct has been exposed for long periods to progesterone and testosterone as well as oestrogen, whereas the chick oviduct has only been exposed to oestrogen for any significant period.

These studies relate the concentration of nuclear-bound [^3H]progesterone to the effects on transcription. The saturation of the highest-affinity class (representing 1000 molecules of progesterone/cell nucleus) results in an effect on the polymerase II activity, with little effect on polymerase I activity, whereas the saturation of the second-highest-affinity class (representing 10000 molecules of progesterone/cell nucleus) results in a response in the RNA polymerase I activity with a further influence on the nucleoplasmic RNA polymerase II activity. Although even

greater amounts of the hormone can be bound to oviduct nuclei with still greater (approx. 200 μ g) doses of progesterone, this increased binding results in no further transcriptional response in the oviduct nuclei. These results suggest that the first two binding sites may represent the true nuclear binding sites required for hormonal response by this tissue. The exact function of each of these classes of binding sites remains to be determined.

The excellent technical assistance of Mrs. Patti Midthun, Mr. Bradley Syverson, Mrs. Barbara Gosse and Mr. Paul Matthai is greatly appreciated. This work was supported by grants HD 8441 and HD 9140-B (from N.I.H.) and CA 14920 (from N.C.I.) and by the Mayo Foundation.

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