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Early Effects of Oestradiol-17β on the Chromatin and Activity of the Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerases (I and II) of the Rat Uterus

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Oestradiol-17β (1.0μg) was injected intravenously into ovariectomized rats. The earliest detectable hormonal response in isolated uterine nuclei was an increase (10–15min) in RNA polymerase II activity (DNA-like RNA synthesis), which reached a peak at 30min and then decreased to control values (by 1–2h) before displaying a second increase over control activity from 2 to 12h. The next response to oestradiol-17β was an increase (30–60min) in polymerase I activity (rRNA synthesis) and template capacity of the chromatin. The concentrations of acidic chromatin proteins did not begin to increase until 1h after injection of oestradiol-17β and histone concentrations showed no significant changes during the 8h period after administration. The early (15min) increase in RNA synthesis in 'high-salt conditions' can be completely eliminated by α-amanitin, an inhibitor of the RNA polymerase II. The exact nature of this early increase in endogenous polymerase II activity remains to be determined, e.g. whether it is caused by the increased availability of transcribable DNA of the chromatin or via direct hormonal activation of the enzyme *per se*.

The analysis of the mechanism of action of steroids on their target cells continues to be hampered by the biological complexity of cells as well as by a lack of knowledge of the primary biochemical events governing cell metabolism. Barring major advances in this area of metabolic control, investigators of the mechanism of steroid-hormone action must be satisfied with the selection and comparison of the earliest measurable chronological events that occur after hormone administration.

Among the various models that have been utilized in the attempt to define steroid-hormone action, the study of the effect of oestrogen on the uterus of either immature or ovariectomized adult rats has been the most prolific. Nevertheless, this area of study is characterized by controversy, contradiction and ambiguity (Tata, 1966; Hamilton, 1968, 1971). The lack of clarity largely stems from the extensive variety of techniques applied by each laboratory, e.g. the amount and route of oestradiol administration, the route of administration of macromolecular precursors, effects of pool size, dynamics of precursor transport etc.

Studies of endogenous RNA polymerase activity in nuclei have revealed the presence of two classes of the polymerase enzyme: I (A) and II (B) (Chambon *et al.*, 1970; Roeder & Rutter, 1970). Recent evidence now suggests each of these classes of the enzyme may in turn be composed of multiple components. In nuclei in 'low-salt' environment the polymerase I (A) class of RNA polymerase, localized in the nucleolus and synthesizing ribosomal RNA, represents the predominant enzyme activity. In nuclei in 'high-salt conditions', the polymerase II (B), localized in the nucleoplasm and synthesizing DNA-like RNA, represents the predominant enzyme activity. An octapeptide, α-amanitin, isolated from a mushroom has been shown to inhibit selectively the majority of the polymerase II enzyme activity. The selective application of varying ionic strengths and α-amanitin allows a fairly specific assay for either polymerase I or polymerase II.

Although there are numerous reports of oestradiol effects on membrane transport and the metabolism of RNA precursors (Means & Hamilton, 1966*a*,*b*,*c*; Hamilton *et al.*, 1968; Munns & Katzman, 1971; Knowler & Smellie, 1971), protein synthesis (Noteboom & Gorski, 1963*a*; Barnea & Gorski, 1970; Barker, 1971) and nuclear RNA transport (Hamilton, 1968; Church & McCarthy, 1970), it is clear that one of the earliest actions of oestradiol on its target cells is to enhance DNA-dependent synthesis of RNA (Hamilton *et al.*, 1965, 1968; Means & Hamilton, 1966*a*,*b*,*c*; Tata, 1966; Trachewsky & Segal, 1967; Hamilton, 1968; Billing *et al.*, 1968; Knowler & Smellie, 1971; Luck & Hamilton, 1972). This initial enhancement of RNA synthesis occurs within minutes, shortly after the entrance of

oestrogen into the nucleus (Hamilton *et al.*, 1968; Teng & Hamilton, 1968) and preceding the earliest rise in specific protein synthesis (Hamilton, 1964; Hamilton *et al.*, 1965; Means & Hamilton, 1966*a*,*b*,*c*; Hamilton *et al.*, 1968; DeAngelo & Gorski, 1970; Knowler & Smellie, 1971). The RNA species reported to exhibit the earliest enhancement of synthesis (30min) after administration of oestradiol) is ribosomal RNA, followed by tRNA (60min) and then DNA-like RNA (at 120min) (Hamilton, 1964; Hamilton *et al.*, 1965, 1968; Trachewsky & Segal, 1967; Billing *et al.*, 1968, 1969). However, recent results of Knowler & Smellie (1971) suggest that a high-molecular-weight RNA (non-ribosomal) is synthesized within 30min after administration of oestradiol before the synthesis of ribosomal RNA is stimulated. In studies with isolated nuclei, the administration of oestradiol-17β to ovariectomized rats for 30–60min caused an increase in the endogenous RNA polymerase I activity (Noteboom & Gorski, 1963*a*; Hamilton *et al.*, 1965, 1968; Nicolette *et al.*, 1968; Barry & Gorski, 1971; Raynaud-Jammet *et al.*, 1971). No response was observed in the other class of endogenous RNA polymerase activity (II) until 12–24h after the injection of the hormone. It is evident that the early hormone-induced synthesis of DNA-like RNA observed *in vivo*, as discussed initially, is not demonstrable in isolated nuclei.

In the present paper we have critically re-examined the overall effect of oestradiol *in vivo* on the chromatin and the activity of the endogenous RNA polymerase in nuclei isolated from the uteri of ovariectomized rats. Rigorous attempts were made to control and account for the possible artifacts and to increase the sensitivity of the polymerase assays.

Materials and Methods

Treatment of animals and isolation of tissues

Female rats (CD strain; Charles River Breeding Laboratories, Wilmington, Mass., U.S.A.) were purchased when 150–175g in weight. After a period of acclimatization to the standardized conditions of the central animal facility, the rats were bilaterally ovariectomized.

The animals were returned to the animal facility and housed six to a colony cage for the 3 weeks preceding experimentation. Temperature (22°C) and humidity were constant, and lights were on a 12h dark–light cycle (on 7 a.m.; off 7 p.m.). Food (Purina Laboratory Chow) and water were offered *ad libitum*.

Solutions of oestradiol-17β (Schwarz–Mann, Orangeburg, N.Y., U.S.A.) containing 10μg of hormone/ml of vehicle were freshly prepared for each experiment. Chromatagraphically pure crystals were dissolved in aq. 95% ethanol and diluted to a final concentration of 20% ethanol with 0.95% NaCl. Each animal was injected intravenously (saphenous vein) under light ether anaesthesia with oestradiol-17β in 0.1ml of vehicle or with vehicle alone. The dose of 1.0μg of oestradiol-17β/0.1ml was experimentally determined from a range of 0.10–10.0μg of oestradiol-17β/0.1ml of vehicle. With the experimental parameters cited in this paper there were no statistically significant differences between 0.1μg or 1.0μg of oestradiol-17β. For this reason the larger dose was used in these studies because it negated any error due to possible loss of hormone during injection. The animals were injected at similar daily time-periods in the various experiments to eliminate differences occurring in the diurnal variations of the endogenous RNA polymerase activities (Glasser & Spelsberg, 1972).

At the appropriate time-period after oestradiol-17β administration the animal was killed, the uterus was rapidly removed and stripped clean of fat and connective tissue. The cervix was then excised. Purified nuclei were prepared immediately for enzyme analysis or the tissue was stored at −20°C for 1–3 days for the isolation of chromatin. Chromatin isolated from frozen tissues showed no alteration from chromatin isolated from unfrozen tissue, provided that the freshly isolated tissues were frozen immediately after the animal was killed. The requirements of each particular experiment dictated the number of uteri that were pooled in a given sample. The minimum number in a pooled sample was 10.

Isolation of nuclei and chromatin

All procedures were carried out at 4°C unless otherwise specified. The nuclei were isolated and purified by modifications of the method of Blobel & Potter (1966). Fresh or frozen uteri were homogenized, at one-third of full speed, in a Polytron apparatus (Brinkman Pt-10, Westbury, N.Y., U.S.A.) in 25vol. of 2.2M-sucrose in TKM buffer (0.01M-tris–HCl–2mM-$MgCl_2$–25mM-KCl, pH7.5). The solution containing the finely minced tissue was then homogenized with several strokes in a Teflon pestle–glass homogenizer (0.005mm clearance). The homogenate was centrifuged for 60min at 60000$g_{av.}$ in a Beckman–Spinco 60 Ti angle rotor to pellet the nuclei. Nuclei to be used for the assay of RNA polymerase activity were resuspended in a small volume of a solution containing 25% (v/v) glycerol in 0.05M-tris–HCl–1mM-$MgCl_2$ (pH7.9) and used in the assay reactions. Nuclei used for the isolation of chromatin were further purified by resuspension (with a Teflon homogenizer) in 0.5M-sucrose in TKM buffer containing 0.1% (v/v) Triton X-100. The homogenate was filtered through organza cloth (100 mesh) and centrifuged for 10min at 10000$g_{av.}$

in a Sorvall RC-2B centrifuge. These nuclear pellets were then subjected to a series of extractions with hypo-osmotic buffer to obtain chromatin as described elsewhere (Spelsberg & Hnilica, 1971*a*; Spelsberg *et al.*, 1971).

Assay of endogenous DNA-dependent RNA polymerase activity

The assay conditions have been described elsewhere (Glasser & Spelsberg, 1972). They were derived from various protocols for the isolation of nuclei (Blobel & Potter, 1966) and the assay of RNA polymerase activity (Roeder & Rutter, 1970; Chambon *et al.*, 1970). These studies are described in the Results section. The DNA concentration of nuclei in the glycerol–tris–$MgCl_2$ buffer (pH7.9) was rapidly determined by measuring the turbidity (at 400nm) of a solution containing 5 or 10μl of nuclei in 1ml of 5% (v/v) trichloroacetic acid. These values were read against a standard curve, which plots turbidity of nuclei in trichloroacetic acid versus the DNA concentration of nuclei as determined by chemical analysis performed with chromatin (Spelsberg & Hnilica, 1971*a*). On the basis of this procedure, samples of nuclei, estimated by turbidity to contain 50μg of DNA, were placed in 5ml conical glass centrifuge tubes together with the reaction mixture used to assay for endogenous RNA polymerase activity. The assay for this endogenous RNA polymerase activity in isolated nuclei is a modification of the method of Roeder & Rutter (1970) and is described elsewhere (Glasser & Spelsberg, 1972). Briefly, 25μl of nuclei (approx. 50μg of DNA), solubilized in the glycerol–tris–$MgCl_2$ buffer (pH7.9), was incubated in the reaction mixture (250μl final volume), which contained: 10μmol of tris–HCl (pH7.9), 0.4μmol of 2-mercaptoethanol, 0.16μmol each of GTP, CTP and ATP, 0.05μmol of ^{3}H-labelled UTP (49Ci/mol), 0.1μmol of sodium phosphate buffer (pH7.5), 2μmol of KCl, 0.4μmol of $MnCl_2$ and 0.5μmol of $MgCl_2$. The reactions, started by the addition of nuclei, were incubated for 10min at 15°C. Assays in low-salt (0.01M-KCl) conditions contained 0.2μg of α-amanitin, whereas those in high-salt [0.25M-$(NH_4)_2SO_4$] conditions contained no α-amanitin.

The reactions were terminated by the addition of 1ml of cold 10% trichloroacetic acid, centrifuging, washing the pellet with 2ml of cold 5% trichloroacetic acid containing 1% (w/w) $Na_4P_2O_7$ and centrifuging again. The pellets, resuspended in the trichloroacetic acid–$Na_4P_2O_7$ solution, were collected on filters (Millipore, 0.45μm pore size). The filters were washed with the trichloroacetic acid–$Na_4P_2O_7$ solution, dried, washed again with toluene to remove trichloroacetic acid, and counted for radioactivity in 5ml of toluene-based 2,5-diphenyloxazole–1,4-bis-(5-phenyloxazol-2-yl)benzene fluor in a Beckman liquid-scintillation spectrometer. The counting efficiency was 60%. The filters were removed from the vials, dried, and hydrolysed in 0.5ml of 0.3M-$HClO_4$ at 90°C for 30min to solubilize DNA (Spelsberg & Hnilica, 1970, 1971*b*). The DNA was determined by the diphenylamine reaction (Burton, 1956) and the specific radioactivity (c.p.m./mg of DNA) was calculated from the c.p.m./filter and the μg of DNA on that same filter. This technique aids in eliminating variations caused by losses of precipitated nuclei (containing labelled RNA) during washing and transfer of the nuclei to the filters.

Analysis of the composition and template capacity of the rat uterine chromatins

The analysis of histone, acidic (non-histone) protein and DNA composition of isolated chromatin has been described in detail elsewhere (Spelsberg & Hnilica, 1971*a*; Spelsberg *et al.*, 1971). The capacity of each of the chromatin preparations to serve as a template for DNA-dependent RNA synthesis *in vitro* with the use of isolated RNA polymerase enzyme (*E. coli*) and DNA (chromatin) were previously determined to establish assay conditions in which the template is rate-limiting (Spelsberg *et al.*, 1971, 1972).

Base composition of the RNA product

An estimation of base composition and changes in the composition of the RNA synthesized by the nuclei under low-salt and high-salt conditions was done by using the approach of Pogo *et al.* (1967). In these experiments the standard reaction assay mixture described above was used except that either ^{14}C-labelled UTP (15μCi/μmol) or ^{14}C-labelled GTP (22μCi/μmol) was present; under the low-salt (with α-amanitin) or the high-salt conditions, $MnCl_2$ or $MgCl_2$ or both were added as described previously (Roeder & Rutter, 1970). The acid-insoluble products on the filters were counted for radioactivity as described above and the GMP and UMP (pmol) incorporated into RNA under the various conditions were calculated. The ratios of UMP to GMP were then calculated to characterize the RNA as described previously (Pogo *et al.*, 1967).

Results

Conditions for the isolation of nuclei and analysis of the nuclear RNA polymerase

Several conditions, critical to the assay of endogenous RNA polymerase activity, were studied. These studies were performed on rat liver and uterus and chick liver and oviduct with essentially the same results.

Isolation of nuclei. Initial studies were concerned with the isolation of nuclei with maximal endogenous RNA polymerase activity. It was found that if isolated nuclei were exposed to buffered solutions containing less than 1.0M-sucrose for short periods (10–20min), losses of 20% or more of the activity occurred. Generally, nuclei that were isolated only in solutions of sucrose of 1.7M and higher concentrations contained over twice as much of the high-salt polymerase activity as nuclei which were exposed to several 0.5M-sucrose solutions during the isolation. The presence of non-ionic detergents enhanced this loss. It appeared that the polymerase enzyme itself diffused out of the nuclei during the exposure to less concentrated (<1.0M) sucrose solutions. Although nuclei prepared by the procedure described in the Materials and Methods section had no microscopically visible cytoplasmic 'material' attached to the nuclear envelopes, some cell debris was observed in the nuclear suspensions. Further purification of the nuclei by repeating the sedimentation through heavy sucrose did not significantly alter the polymerase activity in either high-salt or low-salt conditions.

Elimination of ribonuclease activity. RNA synthesis by our isolated nuclei followed linear kinetics for only 2min when incubated at 37°C. Studies with ribonuclease inhibitors suggested that this was caused by the presence of ribonuclease in the isolated nuclei. To evaluate the extent of ribonuclease activity present in the nuclei, ^{3}H-labelled bacterial RNA from *E. coli* (Miles Laboratories, Elkhart, Ind., U.S.A.) was then incubated at 15° or 37°C with nuclei in a reaction mixture deficient in one of the nucleotides. Selection of the 15°C incubation was based on the previous studies by Chambon *et al.* (1968). As shown in Fig. 1, significant amounts of the labelled RNA became acid-soluble during incubation at 37°C. At 15°C no detectable label was found in the acid-soluble fraction, demonstrating that at 15°C the ribonuclease activity present in isolated nuclei was negligible (Fig. 1). As shown in Fig. 2, the assays of RNA synthesis in isolated nuclei at 15°C support the above results, showing a linear incorporation of labelled UMP into acid-insoluble material for 10min (linear kinetics were observed up to 20min). In addition, the presence of ribonuclease inhibitors, i.e. NaF, bentonite, polyvinyl sulphate, had no effect on the rate of incorporation, as opposed to when the reaction mixture was incubated at 37°C. Consequently, all subsequent assays were performed at 15°C for 10min.

Storage of isolated nuclei. If isolated nuclei were stored at 4°C or frozen (–20°C) in buffered aqueous solutions, the polymerase activity was markedly decreased. However, if the nuclei were resuspended in buffered solutions containing 25% (or more) glycerol, their endogenous polymerase activity was preserved for hours after nuclei isolation and even after storage for weeks at –20°C (Fig. 2). This stability

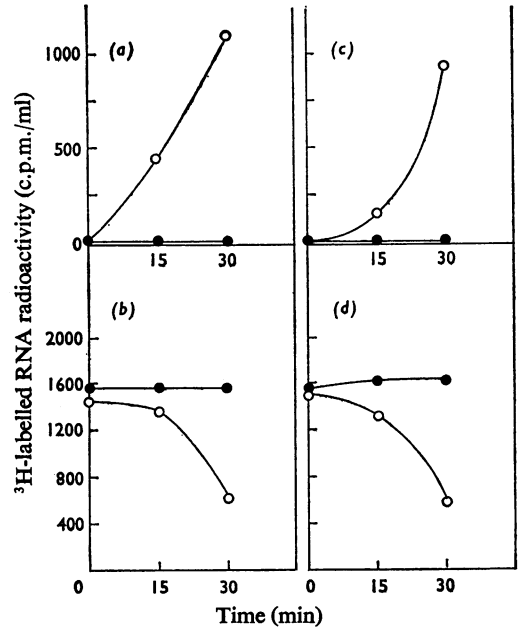

Fig. 1. *Analysis of ribonuclease activity in uterine nuclei of ovariectomized rats under low- and high-salt conditions*

Equal amounts of ^{3}H-labelled RNA (12000c.p.m./μg) were incubated with nuclei (100μg of DNA) at 37°C (○) or 15°C (●) in the standard polymerase reaction mixture minus one of the nucleotides (UTP). Commercial ribonuclease (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was also used in some reactions to test the methodology. After 15 or 30min 200μg of unlabelled yeast RNA was added, followed by 1ml of cold solution containing 10% trichloroacetic acid+1% $Na_4P_2O_7$. The solution was left at 4°C for 20min, and then centrifuged for 10min at 10000$g_{av.}$. The pellets were rinsed in cold 5% (w/v) trichloroacetic acid and collected on Millipore filters (0.45μm pore size) with the same solution. The filters were dried and then counted for radioactivity in a Beckman scintillation spectrometer in 5ml of scintillation fluid prepared by dissolving 4g of 2,5-diphenyloxazole and 50mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene (Packard, Downers Grove, Ill., U.S.A.) per 1 litre of scintillation-grade toluene (Baker, Phillipsburg, N.J., U.S.A.). Samples (200μl) of the supernatant from the original trichloroacetic acid precipitations were applied to Millipore filters, the filters dried and counted for radioactivity. The results are plotted as the radioactivity (c.p.m.)/ml of the original incubation mixture. (*a*) Acid-soluble and (*b*) acid-insoluble radioactivity when nuclei were incubated in low-salt conditions; (*c*) acid-soluble and (*d*) acid-insoluble radioactivity when nuclei were incubated in high-salt conditions.

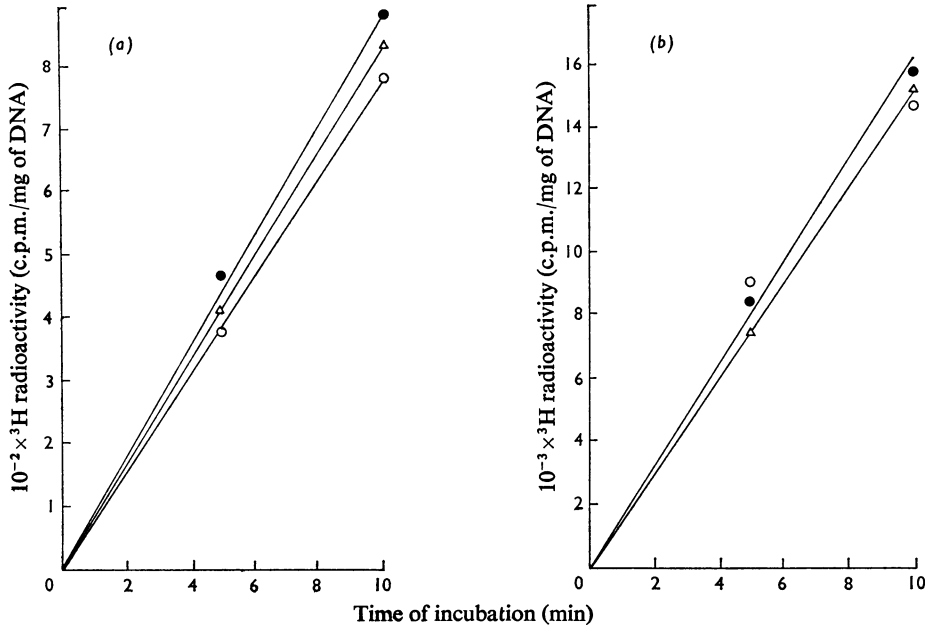

Fig. 2. *Kinetics of endogenous RNA polymerase activity in isolated nuclei at 15°C*

Nuclei, fresh (●) or fresh with ribonuclease inhibitor (NaF at 63 μg per reaction mixture) (○), or after storage in the glycerol medium at −20°C for 7 days (△), were assayed for endogenous RNA polymerase as described in the Materials and Methods section. The reactions were performed in either (*a*) low-salt or (*b*) high-salt conditions.

of polymerase activity of nuclei in glycerol supports the results of Read & Mauritzen (1970). Consequently, nuclei that had been sedimented through 2.2M-sucrose as described above were then resuspended in a buffered 25% glycerol solution (see the Materials and Methods section). Although the activity appeared to be stable at −20°C for weeks, the nuclear preparations were assayed within 2h after their isolation in the experiments reported here.

Components of the reaction mixture. At the 15°C reaction temperature, the presence of an ATP-regenerating system had no effect on the rate of incorporation of nucleotides into acid-insoluble material. Consequently it was not used in subsequent assays. All four nucleotides and a bivalent cation were required for the incorporation. The DNA template was also essential, since treatment of the nuclei with deoxyribonuclease or actinomycin D greatly decreased their ability to incorporate nucleotides.

Acid-insoluble product of the assay. The acid-insoluble radioactivity was labile to ribonuclease but not to protease or deoxyribonuclease. Most of the radioactivity was also made completely acid-soluble by hydrolysis in 0.3M-KOH for 1h at 37°C. These results indicate that the radioactive product of the assay is indeed RNA. Under high-salt conditions, the polymerase activity was decreased to 15% of control by α-amanitin (Fig. 3). This shows that 85% of the polymerase activity of nuclei under high-salt conditions is the polymerase II (or B), which transcribes DNA-like RNA. In contrast, under low-salt conditions the enzyme activity was decreased to only 65% of control, indicating that 35% of the total enzyme activity in this reaction represents the polymerase II (or B), the remaining polymerase being probably the nucleolar enzyme that transcribes ribosomal RNA. Since the non-nucleolar enzyme (polymerase II) contributes as much as 35% of the total enzyme activity of the nuclei under low-salt conditions, α-amanitin (0.2μg per reaction mixture) was included when assays were performed under low-salt conditions. This ensures measurement of only the nucleolar polymerase I (or A) activity.

A second method was used to determine which of the enzymes was active under the various conditions. The nature of the RNA species synthesized *in vitro* was determined by analysing the uridine and guanosine nucleotide composition as described by Pogo *et al.* (1967). When labelled GTP and UTP

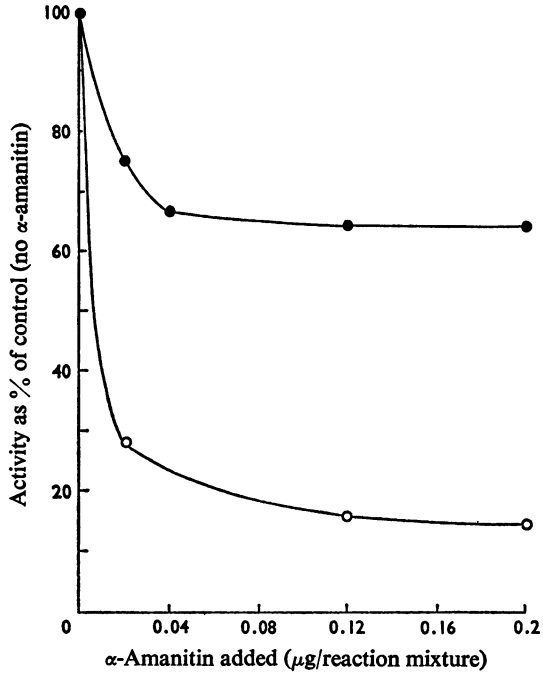

Fig. 3. *Effect of α-amanitin on RNA synthesis in isolated rat uterine nuclei under low-salt and high-salt conditions*

To each reaction mixture used in assaying for endogenous RNA polymerase (described in the Materials and Methods section) was added increasing amounts of α-amanitin. The reactions were begun with the addition of the nuclei. The polymerase activity [acid-insoluble radioactivity in nuclei assayed either in low-salt (●) or high-salt (○) conditions] was normalized for comparative purposes by calculating each value as a percentage of the respective control (the reactions in either low-salt or high-salt conditions with no α-amanitin).

were used, the assays under low-salt conditions with α-amanitin produced RNA with a UMP/GMP ratio of 0.70–0.74, whereas the high-salt conditions produced RNA with UMP/GMP > 1.0 (Table 1). These results support those described by Pogo *et al.* (1967) showing that the nuclei in low-salt conditions with α-amanitin transcribed primarily ribosomal RNA, representing nucleolar polymerase activity I (or A), whereas nuclei in high-salt conditions transcribe DNA-like RNA, representing polymerase II activity.

The use of Mg^{2+} and Mn^{2+} in the same reaction at the concentrations suggested by Roeder & Rutter (1970) resulted in the synthesis of RNA with a base composition similar to that found in the presence of a single ion (Table 1). Consequently the combined ions were used in subsequent assays to enable the use of one stock reaction mixture. It was also found that higher salt concentrations in the assays, e.g. 0.35M-$(NH_4)_2SO_4$, produced RNA with a base composition similar to that of the RNA synthesized with 0.25M-$(NH_4)_2SO_4$, but the former conditions caused more nuclei to rupture; therefore 0.25M-$(NH_4)_2SO_4$ was used for the high-salt conditions.

Measurement of the DNA on the filters. The variation among replicates was improved when the radioactivity per filter was expressed on the basis of recovered DNA on the filter. This was due to (1) the variable amounts of DNA per reaction between the different nuclear preparations (turbidity analyses of DNA were not accurate), and (2) the variable amounts of DNA on the filters due to losses during washes and filtering.

Effects of oestradiol-17β on uterine nuclear RNA polymerase activity

The acute response of uterine nuclear RNA polymerase is depicted in Fig. 4 (*a*, *b*). High-salt (II) polymerase activity (DNA-like RNA synthesis) shows a significant increase within 15min. There is no alteration of low-salt (I) polymerase activity (rRNA synthesis) during this short interval. Polymerase II activity reaches a peak at 30min, at which time the activity of polymerase I begins to rise. The initial increase in polymerase II activity is transient, and this activity falls to the value of unstimulated controls by 1–2h, whereas polymerase I activity continues to rise.

A second and more sustained rise (by the third hour after oestradiol administration) is observed in the activity of polymerase II (Figs. 5*a*, 5*b*). The high point of the restimulated activity occurs at 6h and is followed by a gradual fall until 12h. Low-salt polymerase (I) activity continues to rise, with a small secondary rise to a peak at 6h.

Nature of the early enhancement of RNA polymerase II (DNA-like RNA synthesis)

Further studies were made to elucidate the nature of this oestrogen-induced increase in RNA synthesis in isolated nuclei under high-salt conditions.

Role of chromatin template capacity. Changes in the capacity of uterine chromatin to serve as a primer for the DNA-dependent RNA synthesis (template capacity) could explain the altered RNA-synthetic capacity of the isolated nuclei. Consequently chromatin was isolated and analysed for its composition and template capacity.

The intravenous injection of 1.0μg of oestrogen-17β into ovariectomized rats caused no significant change in the concentration of total histone of uterine chromatin (Figs. 4*c*, 4*d*, 5*c*, 5*d*). However,

Table 1. *Effects of bivalent cations and ammonium sulphate on the uridine/guanosine composition of RNA synthesized in isolated nuclei*

Assays were performed with either ^{3}H-labelled UTP or ^{3}H-labelled GTP under various conditions specified in the Table. In each reaction either 0.5 μmol of $MgCl_2$ or 0.4 μmol of $MnCl_2$ or both were added. The amounts (pmol) of each nucleotide incorporated per mg of DNA were calculated and the ratios of UMP/GMP were calculated.

Concn. of $(NH_4)_2SO_4$ (M)	α-Amanitin (g)	Mg^{2+}	Mn^{2+}	$Mg^{2+}+Mn^{2+}$	Labelled nucleotide	Nucleotide incorporated (pmol/mg of DNA)	Ratio UMP/GMP
–	0.2	+	–	–	UTP	2026	0.79
–	0.2	–	+	–	UTP	1322	0.69
–	0.2	–	–	+	UTP	1482	0.74
–	0.2	+	–	–	GTP	2563	
–	0.2	–	+	–	GTP	1914	
–	0.2	–	–	+	GTP	2011	
0.23	–	+	–	–	UTP	4328	0.51
0.23	–	–	+	–	UTP	11169	1.05
0.23	–	–	–	+	UTP	9490	0.90
0.23	–	–	–	–	GTP	8415	
0.23	–	+	+	–	GTP	10622	
0.23	–	–	–	+	GTP	10670	

concentrations of acidic protein did increase significantly within 1–2h after injection (Fig. 4), reaching a maximum at 4h before decreasing gradually through 7–8h (Fig. 5*c*).

Template capacity of uterine chromatin showed no significant change at 15min but an increase was noted in oestrogen-treated animals within 30min of intravenous injection (Fig. 4*a*). The template capacity was maximal at 1h and it reached a plateau at this value until 4h before it fell to values similar to those noted in untreated animals by 8h (Fig. 5*d*).

Fig. 6 illustrates the activities of both RNA polymerases with chromatin template capacity at various intervals after the injection of oestradiol, expressed as a percentage of the control (non-injected rats). It is clear that the increased availability of the DNA in chromatin for transcription occurs at a time (30–60min) and to a degree (50% above control) that does not account for either the early increase (15–30min) or magnitude (325% above control) of the increase in activity of the high-salt polymerase. The changing template capacity also does not account for the decline and subsequent increase (450% of control at 6h) of enzyme activity at later periods. These results suggest a direct effect of the hormone (oestradiol-17β) on the RNA polymerase enzyme.

Role of ribonuclease activities in isolated nuclei from hormone-stimulated animals. Differences in the ribonuclease activities between the uterine nuclei of treated and untreated rats could also explain the differences in RNA-synthesizing capabilities of the isolated nuclei. Nuclei incubated at 37°C show marked ribonuclease activity (Fig. 1); however, we are concerned only with our assay conditions, i.e. incubation at 15°C. The ribonuclease activity in uterine nuclei at 15°C obtained from oestrogen-treated (30min) rats was found to be negligible, as was shown for uterine nuclei from untreated rats (Fig. 1). Hence the increase in RNA polymerase II activity (high-salt conditions), which we note 15–30min after intravenous injection of oestradiol, could not be due to alterations in ribonuclease activity by the hormone.

Role of RNA polymerase enzyme II. Ovariectomized rats were injected intravenously with α-amanitin (0.7 μg/g body wt.) and oestradiol (1.0 μg) simultaneously. After 30min the uteri were removed and the nuclei isolated. The RNA polymerase II activity of isolated nuclei of rats given both α-amanitin and oestradiol was decreased to that of the respective controls (those given α-amanitin alone).

Similarly, *in vitro*, the oestrogen-enhanced high-salt polymerase activity of uterine nuclei can be decreased to that of control nuclei (from uninjected rats) by the addition of α-amanitin (Fig. 7). These results support the previous results of Knowler & Smellie (1971) in that the early rise in RNA synthesis induced by oestradiol is mediated through a non-nucleolar RNA polymerase enzyme that transcribes DNA-like RNA.

Discussion

The results of these experiments suggest that the early stimulation of RNA synthesis in rat uteri by oestrogen is mediated by activating the RNA polymerase enzyme that synthesizes DNA-like RNA.

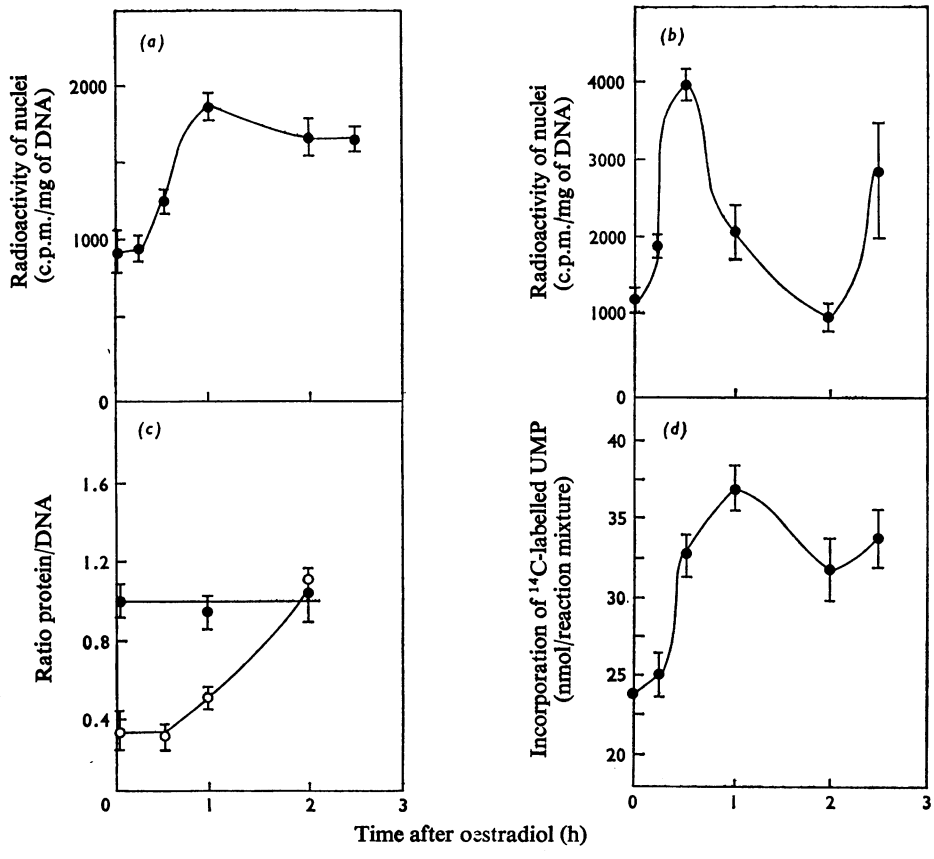

Fig. 4. *Early effects of oestradiol-17β on uterine RNA polymerase and chromatin*

Nuclei and chromatin were isolated from uteri, which were removed from ovariectomized rats at 0–3h after injection of oestradiol-17β. The endogenous RNA polymerase activity of the nuclei was assayed in (*a*) low-salt conditions+α-amanitin, and (*b*) high-salt conditions. (*c*) The content of histones (●) and acidic protein (○) of the uterine chromatin; (*d*) the template capacity of the uterine chromatin was determined with purified RNA polymerase from *E. coli* isolated by the method of Burgess (1969). The reactions were run at 37°C for 10min with 1 μg of chromatin DNA and 5 units of the enzyme. Under these conditions the template (chromatin DNA) is rate-limiting and the incorporation of radioactivity into an acid-insoluble product is linear. The detailed procedures for (*c*) and (*d*) have been described elsewhere (Spelsberg & Hnilica, 1971*a*; Spelsberg *et al.*, 1971). In all experiments mean values with the range for three replicate analyses are given.

The template capacity of the chromatin (Figs. 4*d* and 5*d*) and the polymerase enzyme activity that synthesizes ribosomal RNA (Figs. 4*a* and 5*a*) appear to be stimulated at subsequent periods. This early rise in polymerase II activity (DNA-like RNA synthesis), observed within 15min after injection of oestradiol-17β (Fig. 4*b*), may explain the oestrogen-induced early increase (15–30min) in the synthesis of nuclear RNA *in vivo* in the uteri of immature rats (Means & Hamilton, 1966*a*,*b*; Hamilton, 1968; Hamilton *et al.*, 1968), the very early RNA synthesis reported by DeAngelo & Gorski (1970) and Wira & Baulieu (1972), and the early synthesis of high-molecular-weight RNA reported by Knowler & Smellie (1971). This induced nucleoplasmic polymerase activity may also be responsible for the ultimate synthesis of the induced protein in ovariectomized or immature rats observed at 30–45min

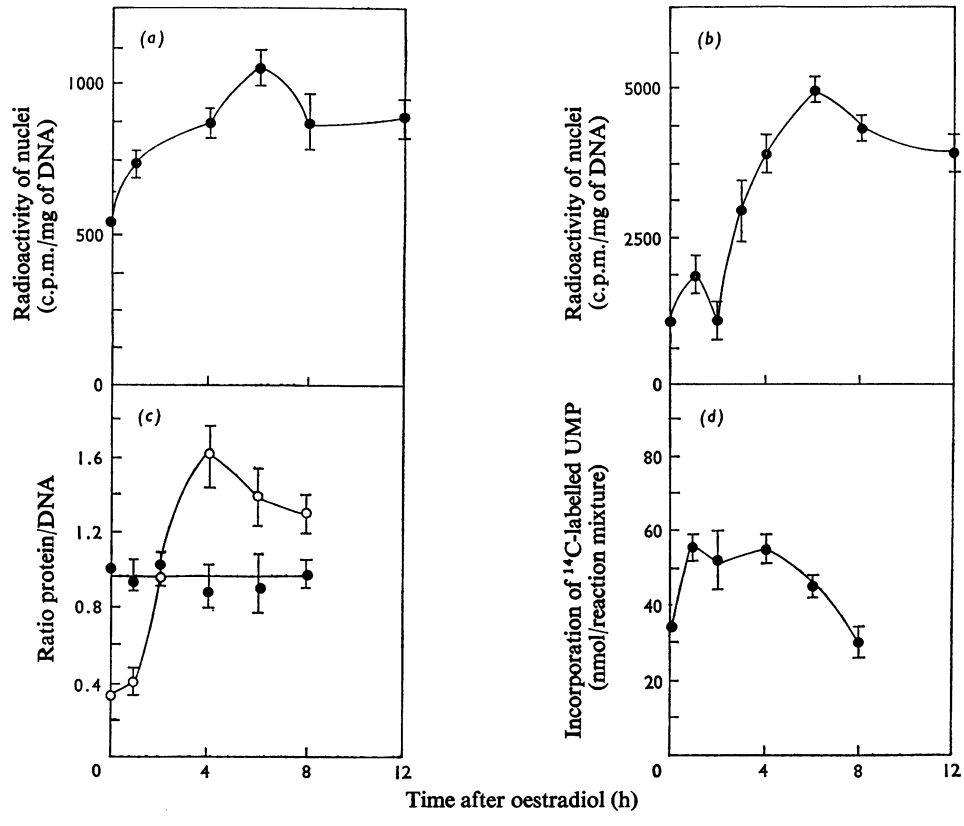

Fig. 5. *Prolonged effects of oestradiol-17β on uterine RNA polymerase and chromatin*

The methods used in these experiments are described in detail in the legend of Fig. 4. This figure depicts the effect of the hormone on the endogenous RNA polymerase activity of uterine nuclei assayed in (*a*) low-salt conditions+α-amanitin and (*b*) high-salt conditions. In (*c*) the histone (●) and acidic protein (○) contents of the chromatin recovered from uteri removed from rats 0–8h after intravenous injection of oestradiol-17β are shown. The template capacity of these chromatins is recorded in (*d*). In all experiments mean values with the range for three replicate analyses are given.

after injection of oestradiol-17β (Notides & Gorski, 1966; DeAngelo & Gorski, 1970; Mayol & Thayer, 1970; Wira & Baulieu, 1971).

The later rise in polymerase I activity (rRNA synthesis) is consistent with the results of other laboratories (Noteboom & Gorski, 1963*a*,*b*; Gorski, 1964; Hamilton *et al.*, 1965, 1968; Widnell & Tata, 1966; Trachewsky & Segal, 1967; Gorski & Moran, 1967; Nicolette *et al.*, 1968; Barry & Gorski, 1971; Raynaud-Jammet *et al.*, 1971). The failure of previous investigations to detect enhanced high-salt polymerase activity may be due to (1) loss of the enzyme during isolation of nuclei, (2) the action of ribonuclease during the assay and/or (3) the increased sensitivity of our assay by careful intravenous injection and decrease in the variability of replicates. The use of the 15°C incubation temperature has been shown to eliminate ribonuclease activity (Chambon *et al.*, 1968).

The intravenous injection of the hormone as performed in our experiments has been reported to result in the greatest early stimulation of nuclear RNA synthesis (Knowler & Smellie, 1971).

The reason for the biphasic response of the high-salt polymerase (II) activity to oestrogen treatment *in vivo* is at present obscure. One can speculate that the first peak of activity (Fig. 6) produces RNA, which in turn induces the later responses of cells to the hormone, e.g. the second increase in polymerase II activity, the initial rise in polymerase I activity, the

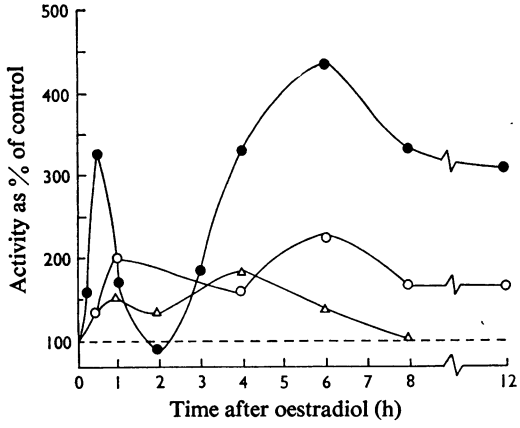

Fig. 6. *Composite of results of effects of oestradiol-17β on uterine RNA polymerase activity and chromatin template capacity*

Details of these experiments are described in the legend of Fig. 4, except that the results in this figure are plotted as percentages of control values (those from nuclei/chromatin obtained from castrated rats who received no oestradiol). The data have been taken from experiments shown in Figs. 4 and 5 and replotted. Endogenous RNA polymerase activity in uterine nuclei was assayed in high-salt conditions (●) or low-salt conditions+α-amanitin (○); △, template capacity (for RNA synthesis) of uterine chromatin.

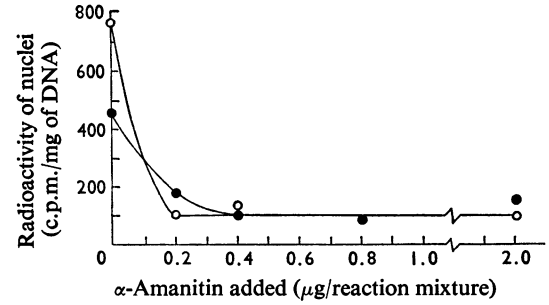

Fig. 7. *Effects of α-amanitin on the oestradiol-17β-enhanced RNA polymerase activity in uterine nuclei in high-salt conditions*

The assay conditions are described in the Materials and Methods section. To a series of reaction tubes were added increasing amounts of α-amanitin as described in the legend of Fig. 3. The reactions were started by the addition of uterine nuclei from ovariectomized rats, which were either injected with the vehicle (●) or injected with oestradiol-17β (○) for 30min.

opening of the DNA for transcription (chromatin template capacity), changes in chromatin composition, the increases in protein synthesis etc. It is noteworthy that the early rise in polymerase activity is very labile. Nine out of ten of our experiments showed the early rise in polymerase II activity, but the extent of enhancement varied from 80% to 600% of control (no oestradiol) between individual experiments. The enhancement of polymerase II activity was usually decreased or abolished when nuclei were subjected to solvents of low osmoticity or assayed at 37°C.

Specifically our present report of the changing chemical composition (increasing acidic protein content) and the increasing template capacity (for RNA synthesis) of the uterine chromatin in response to oestradiol supports previous investigations from several laboratories (Dahmus & Bonner, 1965; Barker & Warren, 1966; Teng & Hamilton, 1968; Church & McCarthy, 1970). However, the changes we observed in rat uterine chromatin did not occur within 10min as noted in the uterus of ovariectomized rabbits by Church & McCarthy (1970). The correlation between template capacity of chromatin and content of acidic proteins has been reported for many tissues (Dingman & Sporn, 1964; Salser & Balis, 1966; Marushige & Ozaki, 1967; Marushige & Dixon, 1969; Spelsberg *et al.*, 1971, 1972). Recent studies of the response of the acidic chromatin proteins to various hormones (Barker & Warren, 1966; Hamilton, 1968, 1969, 1970; King *et al.*, 1969; Shelton & Allfrey, 1970; Spelsberg *et al.*, 1971, 1972) as well as their physical interactions suggest that these proteins may act as mediators between the hormones themselves and the cellular response. The studies in the present paper demonstrate that both the changes in chromatin composition (increase in acidic protein) and the increased availability of chromatin DNA for transcription occur after the initial rise in polymerase II activity (15min after administration of oestradiol) (Figs. 4*a*, 4*b*). The extent of derepression of the DNA, however, does not explain the extent of increase in enzyme activity (Fig. 6). Reservations must be placed on these latter observations, owing to the use of presently employed methods of determining chromatin template capacity, i.e. bacterial polymerase used in an assay *in vitro*. This method may not accurately measure the exact amount of transcribable DNA in cells. In any case within 15min after the injection of oestradiol there is evidence of enhanced RNA synthesis in uterine nuclei in high-salt conditions. The hormone appears to mediate its action on cells through the polymerase II (or B) enzyme, since the RNA product under these conditions is DNA-like, and α-amanitin can completely eliminate the response (Fig. 7).

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