Uterine Ribonucleic Acid Polymerase. Effect of Estrogen on Nucleotide Incorporation into 3′ Chain Termini*

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ABSTRACT: Within 1 hr after an intraperitoneal injection of 5 μg of 17β-estradiol into immature female rats, there is an increase in the rate of incorporation of ribonucleoside triphosphates into RNA by the DNA-dependent RNA polymerase of isolated uterine nuclei. To evaluate the nature of this response, the amount of radiolabeled ribonucleoside triphosphate incorporated into the 3′ chain ends was used as an assay of the number of RNA chains and was shown to be identical in uterine nuclei from both control and estrogen-treated rats. In contrast, the incorporation into the internucleotide positions of the chains was significantly enhanced due to estradiol. This is not due to an estradiol effect on the intranuclear concentration of ribonucleoside triphosphates, nor is it the result of differential RNase activity. Since the number of RNA chains reflects the number of RNA polymerase molecules in the process of synthesis, it appears that estradiol has increased the activity rather than the absolute amount of this enzyme. These data also do not support the notion that an increase in number of templates being transcribed accounts for the estrogen effect on RNA polymerase.

It is well established that within 1 hr after intraperitoneal injection of 17β-estradiol into immature or ovariectomized mature rats there is an increase of 50–100% in the rate of the DNA-dependent RNA polymerase in isolated uterine nuclei when assayed under conditions of low ionic strength (Noteboom and Gorski, 1963; Gorski, 1964; Hamilton *et al.*, 1965). This estrogen-induced portion of the RNA polymerase activity is dependent on a temperature-sensitive process, and it can be prevented or even reversed by cycloheximide and puromycin (Noteboom and Gorski, 1963; Gorski and Morgan, 1967; Nicolette *et al.*, 1968). Thus, protein synthesis appears to be a prerequisite for an estrogen effect on uterine RNA polymerase.

This increased rate of RNA synthesis has been attributed to an increase in the amount of genetic material available for transcription. Measurements of the template capacity of uterine chromatin isolated 1 hr after estrogen are about 25% higher than the control value (Barker and Warren, 1966; Teng and Hamilton, 1968). This increase in template activity is measured with exogenous polymerase and may or may not be related to the one seen when the endogenous DNA-dependent RNA polymerase of whole nuclei is assayed.

Consequently, experiments were designed to distinguish whether the increased rate of endogenous RNA polymerase was the reflection of an increase in the number of growing RNA chains in contrast to the rate of elongation of the chains. The data presented indicate that estrogen does not cause a change in the number of growing chains but does cause an increase in rate of chain elongation. This implies that the activity but not the number of polymerase molecules has changed and that little of the estrogen effect is due to transcription of additional template.

Materials and Methods

Animals and Preparation of Uterine Nuclei. Immature female Holtzman or Sprague–Dawley rats (21–23 days of age) were used. Five micrograms of 17β-estradiol in 0.5 ml of saline was injected intraperitoneally 1 hr before sacrifice; controls received saline alone. Relatively clean nuclei were prepared by a modification of the procedure of Kostyo (1968). Ten to thirty uteri were excised, diced, and homogenized in 7.5 ml of homogenization medium for 35 sec at 23,000 rpm in a Virtis "23" homogenizer equipped with dull blades. The homogenization medium consisted of 0.25 M sucrose, 5 mM $MgCl_2$, 10 mM cysteine, and 0.3% Triton X-100 freshly prepared in 0.01 M Tris-HCl (pH 7.4). The homogenate was diluted with an additional 3.0–4.0 ml of this solution for each ten uteri used beyond the initial ten. The homogenate was stirred for 5 min and then filtered through one layer of washed flannel with gentle suction. The filtrate was centrifuted at 700*g* for 7 min. The supernatant was decanted and the inside walls of the tubes wiped clean. The nuclear pellet was the source of RNA polymerase. All steps in the isolation of the nuceli were performed at 0–5°.

Disruption of Nuclei. Nuclei were disrupted by suspension in 0.2 M Tris (pH 8.0) at 37°, 0.42 M KCl, and 0.07 M NaF for 3 min. The suspending medium was then diluted to 0.01 M Tris, 0.075 M KCl, and 0.035 M NaF for assay of RNA polymerase under standard conditions. Phase microscopy revealed no intact nuceli after this treatment.

Measurement of RNA Polymerase Activity. Nuclei were assayed for RNA polymerase activity in the standard reaction mixture which contained, unless otherwise stated in the figure legends, 100 μmoles of Tris-HCl (pH 8.0 at 37°), 75 μmoles of KCl, 30 μmoles of NaF, 10 μmoles of cysteine, 5 μmoles of $MgCl_2$, 250 μg of methylcellulose, 0.02 μmole each of unlabeled ribonucleoside triphosphate, and either 0.02 or 0.005 μmole of ^{3}H-labeled ribonucleoside triphosphate in a final volume of 1 ml. The reaction temperature was 37°, and the time of incubation is stated in the legends for the figures. Samples (100 μl) were removed from the standard reaction mixture and placed in 4 ml of 15% trichloroacetic acid–saturated sodium pyrophosphate–saturated Na_2HPO_4 (1:1:1, v/v) (acid-washing mixture). The samples were then precipitated on nitrocellulose membrane filters (Schleicher & Schuell, B-6, 27 mm) and washed ten times with the acid-washing mix-

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TABLE I: Breakdown of [^{3}H]CMP into Cytidine during the 16 hr in 0.3 N KOH at 37° and the Subsequent Isolation of These Compounds.

Without Incubation				After Incubation for 16 hr at 37° in 0.3 N KOH and Isolation				
n	CMP (cpm)	Cytidine (cpm)	Cytidine/CMP × 100 (%)	*n*	CMP (cpm)	Cytidine (cpm)	Cytidine/CMP × 100 (%)	Cytidine[c]/CMP × 100 (%)
2	28,460 ± 76[a]	226 ± 6[a]	0.79	5	21,527 ± 312[b]	193 ± 17[b]	0.89	0.10

[a] Range. [b] Standard error. [c] Cytidine due to CMP breakdown.

ture. The filters were dried and counted in 10 ml of toluene containing 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(4-methyl-5-phenoxazolyl)]benzene per l. All samples were corrected for incorporation into zero-time controls. The DNA content of all reaction mixtures was determined by the method of Webb and Levy (1955).

Measurement of RNA Chains. Aliquots of standard reaction mixture containing nuceli equivalent to 100 μg of DNA in a total volume of 0.6 ml were incubated at 37° with [^{3}H]CTP (13.5 or 23.7 Ci per mmole) at a concentration of 0.005 mM or [^{3}H]UTP (21 Ci/mmole) at a concentration of 0.002 mM. The reactions were stopped by chilling and the subsequent addition of 4 ml of the acid-washing mixture mentioned above. The labeled nucleic acid was separated from unincorporated substrate by centrifugation at 1000*g* for 10 min. This cycle of washing and centrifugation was repeated four more times with the acid-washing mixture, once with 95% EtOH, and once with ethanol–ether (2:1, v/v). The dried pellet was hydrolyzed in 1 ml of 0.3 N KOH at 37° for 16 hr. Carrier cytidine or uridine was added either 0.5 or 12 hr after the start of the reaction. The alkaline hydrolysate was chilled, neutralized with 0.2 ml of 10% perchloric acid, and brought to a final concentration of about 5% perchloric acid with 1.2 ml of 10% perchloric acid. The precipitated $KClO_4$ and DNA were collected by centrifugation at 2000 rpm and washed once with an additional 1.4 ml of 5% perchloric acid. The DNA was subsequently determined by the method of Webb and Levy (1955). The nucleotides and nucleosides in the combined perchloric acid supernatants were recovered by the method of Tsuboi and Price (1959) with some modifications. Charcoal (10 mg) was added to each sample; adsorption was for 1 hr on ice; each sample was washed with 2.5 ml of ice-cold H_2O; elution was accomplished with 1.5 ml of 5% pyridine in 50% EtOH for 2 hr; the charcoal in each sample was rinsed with an additional 0.5 ml of elution mixture; complete removal of charcoal was accomplished by filtration through Millipore filters into 10-ml pear-shaped flasks. Samples were then dried *in vacuo* and taken up in 100 μl of 30% EtOH.

Chromatography on PEI-cellulose.[1] Nucleosides were separated from nucleoside monophosphates by ascending chromatography on PEI-cellulose plates prewashed with H_2O. Sample (60 μl) was spotted in four spots 3 cm from one end of the 5 × 20 cm plates, and run to a distance of 3 cm from the top with H_2O as the solvent. Unless some salt carried over in the nucleotide isolation, nucleoside monophosphate was all within 3 cm of the origin. The nucleoside was always found 11–12 cm from the origin and was located by its ultraviolet absorption. The dried plates were fractionated into 2.5–3-cm strips and the PEI-cellulose scraped off and ground into a powder in the scintillation vials. This material was suspended in 12 ml of scintillation fluid consisting of 8 g of 2,5-diphenyloxazole, 0.6 g of 1,4-bis[2-(4-methyl-5-phenoxazolyl)]benzene, 150 g of naphthalene, and 90 g of Cab-O-Sil (Beckman) per l. of 1,4-dioxane–ethylene glycol–2-ethoxy ethanol (880:20:100, v/v). Counting efficiency was 12.7%. Nucleotide/nucleoside recovery for all above procedures was 75%. Counts per minute in the cytidine fraction were corrected for breakdown of CMP into cytidine (Table I), which occurred during the alkaline digestion of RNA and subsequent isolation of the digestion products. Similar corrections were made for UMP breakdown into uridine.

[1] PEI-cellulose = poly(ethylenimine)cellulose (from J. T. Baker Chemical).

Results

Nuclei, isolated from pooled immature rat uteri 1 hr after intraperitoneal injection of 5 μg of 17β-estradiol, exhibit RNA polymerase activity which is sensitive to actinomycin D

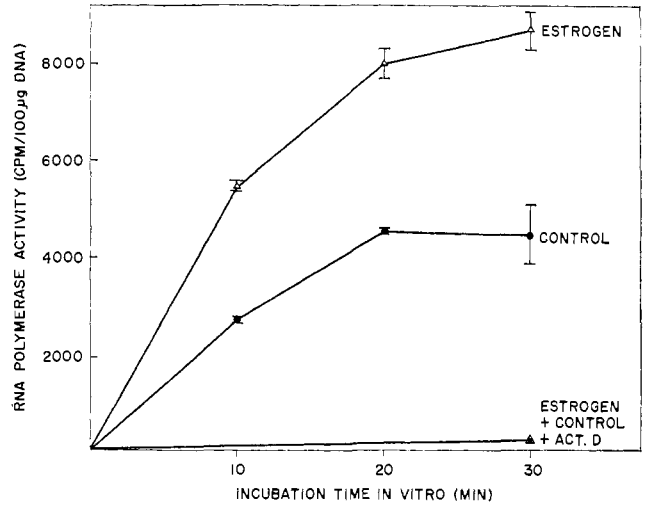

FIGURE 1: Effect of *in vivo* estrogen and *in vitro* actinomycin D on RNA polymerase of uterine nuclei isolated from ten pooled rat uteri 1 hr after estrogen or saline control injections. Aliquots of nuclear suspension were removed for DNA determination and the bulk of the suspension was assayed for RNA polymerase activity in the standard reaction mixture with [^{3}H]CTP (0.02 mM, 13.5 or 2.37 Ci per mmole). Actinomycin D was used at a final concentration of 1 μg/ml. At the indicated times, 100-μl aliquots were removed in triplicate from the reaction mixture for measurement of RNA polymerase activity as described in Materials and Methods. Each point represents the mean of three determinations plus and/or minus standard error. (△) Estradiol and (●) control.

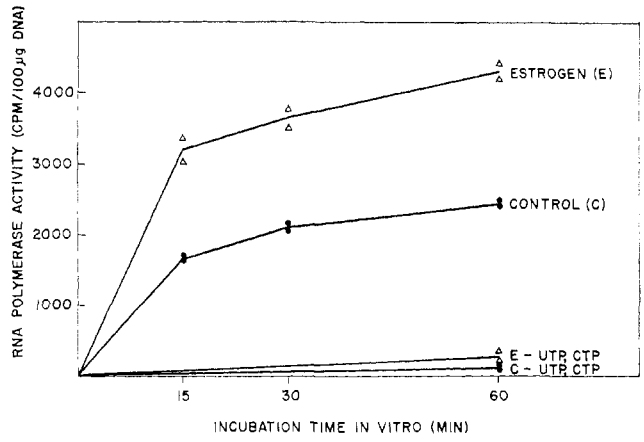

FIGURE 2: Omission of two of the four ribonucleoside triphosphates from the standard reaction mixture. [^{3}H]GTP was the source of the label. Aliquots (100 μl) of reaction mixture were removed for measurement of RNA polymerase activity. Determinations were made in duplicate. (△) Estrogen and (●) control.

(Figure 1) and which is dependent on the presence of the four ribonucleoside triphosphates (Figure 2). Furthermore, the rate of RNA synthesis is significantly enhanced by estradiol. These data indicate that nuceli isolated by this method contain the same RNA polymerase activity characterized earlier in the nuclear myofibrillar fraction from rat uterus (Gorski, 1964). The results in Figure 3 show that the increased rate of RNA polymerase activity due to estradiol is the same whether GTP, CTP, or UTP is used as the source of the label.

Increasing the ribonucleoside triphosphate concentration in an *in vitro* RNA-synthesizing system increased the rate of

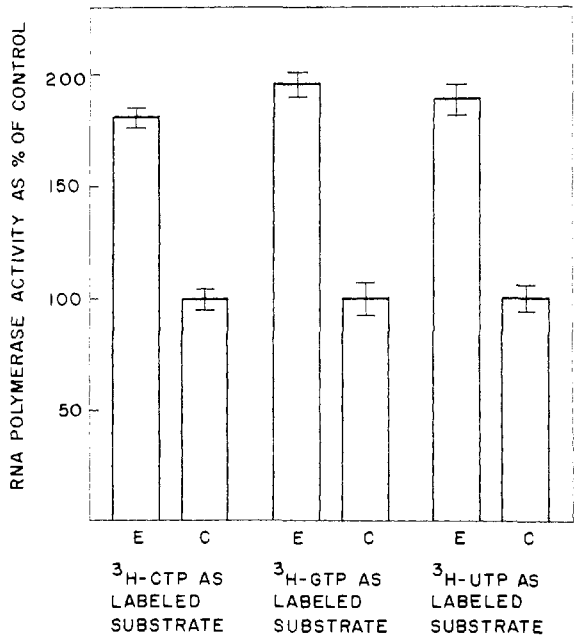

FIGURE 3: Comparison of ^{3}H-labeled UTP, CTP, and GTP as a measure of the estrogen effect on RNA polymerase activity of isolated uterine nuclei. The reaction time was 15 min. Each of the labeled ribonucleoside triphosphates was used at a final concentration of 0.02 mM. Aliquots (100 μl) of the standard reaction mixture were removed for measurement of RNA polymerase activity. Bar heights are means of three determinations plus and/or minus standard error.

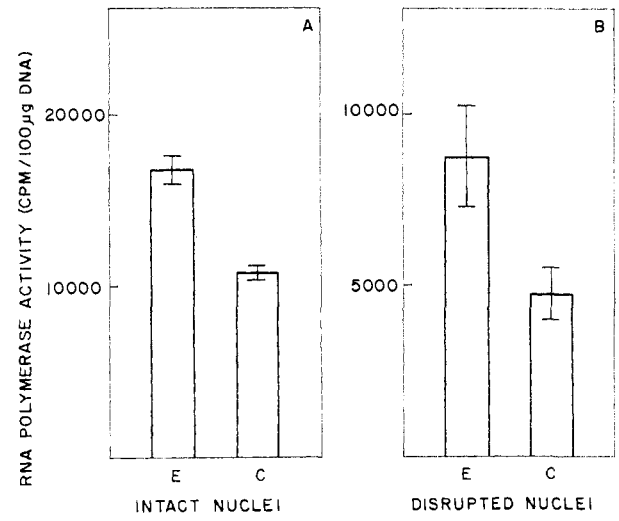

FIGURE 4: RNA polymerase activity in intact (A) and disrupted (B) nuclei. The standard reaction mixture was used with [^{3}H]CTP as the labeled substrate. Reactions were stopped by the addition of 4 ml of 15% trichloroacetic acid–saturated sodium pyrophosphate–saturated sodium monophosphate. The acid-insoluble precipitate from the disrupted nuclei was homogenized in a ground-glass homogenizer. The acid-insoluble material from all reactions was washed six times more in the acid–salt mixture, once with 95% EtOH, and once with EtOH–ether (2:1, v/v). The dried material was dissolved in NCS (Nuclear-Chicago) and counted in 10 ml of toluene containing 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(4-methyl-5-phenoxazolyl)]benzene per ml. Bar heights are means of three determinations plus and/or minus standard error.

RNA synthesis (Bremer, 1967; Maitra *et al.*, 1967; Bremer and Mueller, 1969). With this in mind, the possibility that uterine nuclei from estrogen-treated animals might be more permeable to ribonucleoside triphosphates than nuclei from controls was evaluated. RNA polymerase activity was measured in intact and disrupted nuceli, and the results are shown in Figure 4. Although there was a decrease in the activity of the preparations upon disruption, the effect of estrogen upon the system was still evident.

A differential breakdown of ribonucleoside triphosphate caused by phosphatase activity could change the substrate concentration during the reaction, and thus, the rate of RNA polymerization. However, Table II shows that there is virtually no difference in the breakdown of either GTP, UTP, or CTP between control and estrogen-treated preparations at times when there is a marked difference in RNA polymerase activity due to estrogen.

The effect of endogenous RNase activity on the rate of *in vitro* RNA synthesis was evaluated in the following way. Aliquots of nuclear suspensions were incubated in the standard reaction mixture. After 5- and 20-min reaction, the specific radioactivity of [^{3}H]CTP in some aliquots was diluted 75-fold. This effectively stopped incorporation of [^{3}H]CTP into RNA in those aliquots, and the turnover of RNA while synthesis was still proceeding could be evaluated. Figure 5 shows that a portion of the RNA is unstable and this portion increases with reaction time. Nevertheless, the control RNA (Figure 5B) is clearly not less stable than the estrogen RNA (Figure 5A).

It became of interest to determine whether the estrogen-induced increase in the rate of uterine RNA polymerase was due to an increase in the number of enzymes or an increase in their activity. To answer this question experiments were designed to measure the number of RNA chains as well as the

TABLE II: Breakdown of Nucleoside Triphosphate in Isolated Uterine Nuclei from Control and Estrogen-Treated Rats Compared to RNA Polymerase Activity in These Nuclei.

Triphosphate Used as ^{3}H-Labeled Substrate	Incubation Time *in Vitro* (min)	Estrogen-Induced RNA Polymerase Act. as % of Control	^{3}H-Labeled Triphosphate as % of Total Acid-Soluble Nucleotides[a]	
			Estrogen	Control
GTP	5	164 ± 3[c]	86 ± 1	85 ± 2
GTP	10	180 ± 5	80 ± 1	80 ± 1
UTP[b]	3	184 ± 8	58 ± 1	60 ± 1
CTP[b]	3	230 ± 5	77 ± 1	77 ± 3

[a] Carrier nucleotides (0.2 mM each of the mono-, di-, and triphosphate) were added to the standard reaction mixture which had been acidified with trichloroacetic acid, and the acid-insoluble material was removed for the estimation of RNA polymerase activity (see Methods). To estimate GTP breakdown the trichloroacetic acid was extracted three times with two volumes of ether. The aqueous solution was lyophilized to dryness, and the nucleotides were suspended in 100 ml of 30% EtOH and chromatographed in the descending direction on DE-81 (Whatman). The solvents were 0.1 M NH_4HCOOH (pH 6.0) for 3.5 hr followed by 4 M HCOOH in 0.1 M NH_4HCOOH for 2.5 hr. UTP and CTP breakdown was estimated by recovering the nucleotides by the method of Tsuboi and Price (1959). The nucleotides were suspended in 100 ml of 30% EtOH and separated as described above for GTP, or by chromatography on PEI-cellulose using isobutyric acid–0.5 M NH_4OH, pH 3.6 (10:6, v/v). All chromatograms were dried and the spots corresponding to nucleoside mono-, di-, and triphosphates were located by their extinction of ultraviolet light, cut out, and counted directly in toluene containing 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(4-methyl-5-phenoxazolyl)]benzene per l. [b] Data are from same experiment shown in Figure 8. [c] Range.

total incorporation of labeled ribonucleoside triphosphate into RNA. The number of RNA chains is a reflection of the number of RNA polymerase molecules engaged in transcription (Richardson, 1966; Bremer *et al.*, 1965).

Such analyses seemed well suited to our reaction system. In both control and estrogen nuclear preparations, transcription has virtually ceased by 30 min after the start of the reaction. This is characteristic of RNA-synthesizing systems when the ionic strength of the reaction mixture is below some limiting level; there is little or no release of terminated chains and the polymerase remains bound to the DNA in a complex with the nascent RNA (Bremer and Konrad, 1964; Richardson, 1969). Under these conditions, each transcribing enzyme would be responsible for the synthesis of only one chain.

Aliquots of uterine nuclei equivalent to about 100 μg of DNA were incubated in 0.6 ml of the standard reaction mixture containing each of the unlabeled ribonucleoside triphosphates at a final concentration of 0.02 mM and [^{3}H]CTP at a final concentration of 0.005 mM (13.5 or 23.7 Ci per mmole) or [^{3}H]UTP (21 Ci/mmole) at a final concentration of 0.002 mM. The labeled RNA from this reaction was subjected to alkaline hydrolysis, the products of which are ribonucleoside monophosphate from the chains' interiors, ribonucleoside

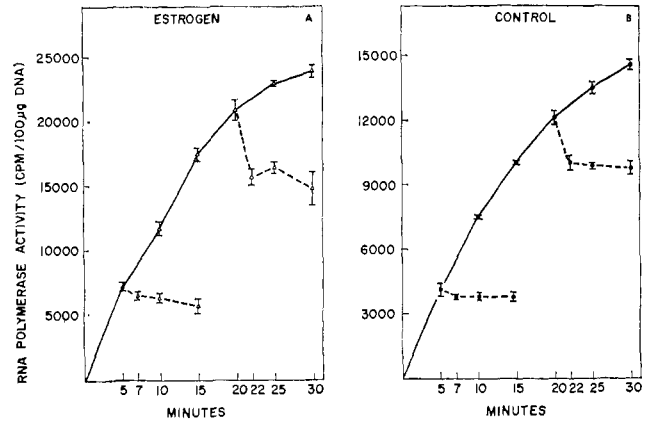

FIGURE 5: Turnover of reaction product of RNA polymerase in nuclei isolated from rat uteri 1 hr after estradiol. At both 5 and 20 min after initiation of the reaction [^{3}H]CTP was diluted 75-fold with unlabeled CTP. Each point is the mean of three determinations plus and/or minus standard error. (△) Estrogen and (●) control.

tetraphosphates from the 5′ ends, and ribonucleosides from the 3′ ends of the RNA chains. These can then be separated chromatographically to provide measurements of the total incorporation into RNA, the number of chains, and the average chain length. In these experiments only the 3′ chain ends were measured, since 5′ chain ends would not measure the number of chains already in progress in the isolated nuclei. Furthermore, difficulty with the measurement was anticipated due to the phosphatase activity in the nuclear preparations. It should be noted that, when carrier nucleoside was added at 0.5 or 12 hr after the start of the alkaline hydrolysis, there was no difference in the amount of labeled nucleoside (3′ chain ends), unlike the results reported by Mueller and Bremer (1969).

Figure 6 shows the results of such an experiment. Although estradiol brings about an increase in the incorporation of [^{3}H]-

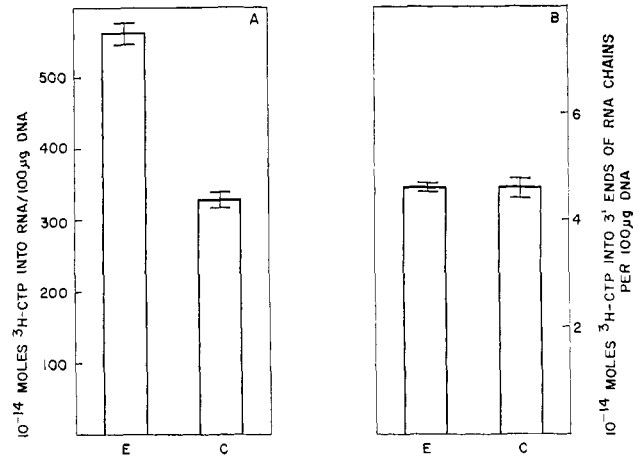

FIGURE 6: The incorporation of [^{3}H]CTP into RNA (A), and 3′ chain ends (B), by uterine nuclear RNA polymerase 1 hr after estrogen. RNA synthesis proceeded for 10 min. The acid-insoluble material from the reactions was subjected to alkaline hydrolysis and the hydrolysis products were isolated. [^{3}H]Cytidine from the 3′ ends of RNA chains was separated from [^{3}H]CMP of the internucleotides on PEI-cellulose. Results are expressed as moles of [^{3}H]CTP incorporated into RNA (CMP) and 3′ chain ends (cytidine) per 100 μg of DNA (nuclei) incubated in the standard reaction mixture. Bar heights are means of three determinations plus and/or minus standard error.

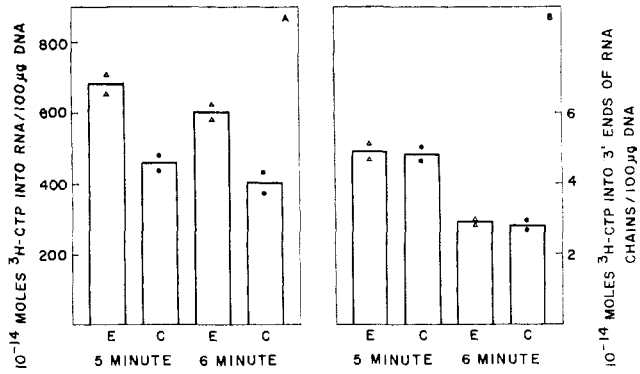

FIGURE 7: Incorporation of [³H]CTP into RNA (CMP) and 3′ chain ends (cytidine) in 5 min. After 5 min [³H]CTP was diluted 100 times with unlabeled CTP and the reaction was allowed to proceed for 1 more min. Determinations were done in duplicate. (△) Estrogen and (●) control.

CTP into RNA (CMP) during a 10-min incorporation (Figure 6A), there is no difference in the incorporation of [³H]CTP into cytidine of the 3′ chain ends (Figure 6B).

In the experiment shown in Figure 7, the specific radioactivity of the [³H]CTP was diluted 100-fold after 5-min reaction, and the reaction was allowed to proceed for 1 more min. Again, estradiol brings about an increase in the incorporation of [³H]CTP into RNA (Figure 7A), this time within 5 min, but does not affect the amount of [³H]CTP incorporated into cytidine of the 3′ chain ends (Figure 7B). During the additional minute of reaction time, there is no more incorporation into RNA in nuclei from either estrogen-treated or control uteri; the slight decrease which is noted is probably due to

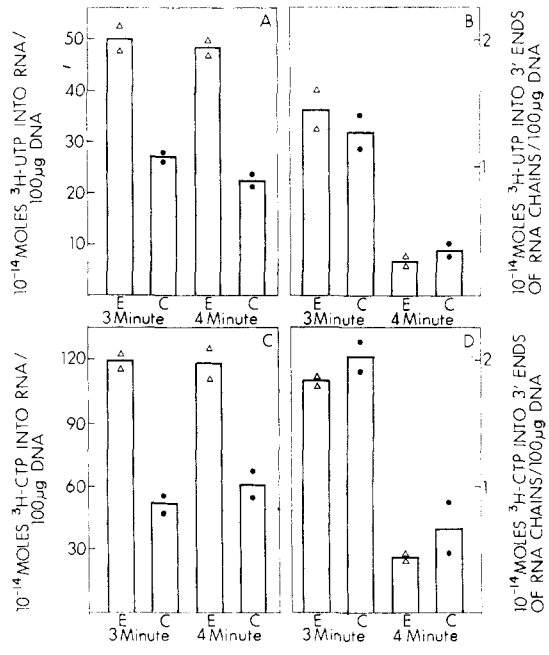

FIGURE 8: Incorporation of [³H]UTP into RNA (UMP) and 3′ chain ends (uridine), (A and B) and of [³H]CTP into RNA (CMP) and 3′ chain ends (cytidine), (C and D) in 3 min *in vitro*. After 3 min the specific activity of each isotope is diluted 100-fold and the reaction is allowed to proceed for 1 more min. Determinations were made in duplicate. The lowest value obtained in the 3-min nucleoside spot was counted at an error of less than 4%. (△) Estrogen and (●) control.

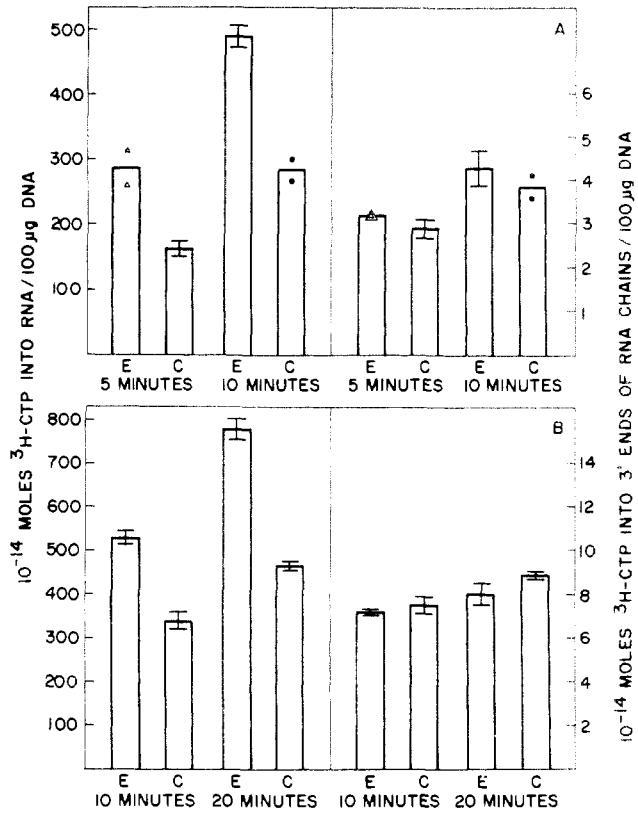

FIGURE 9: Incorporation of [³H]CTP into RNA (CMP) and 3′ chain ends (cytidine). In one experiment (A) the determinations were made after 5 and 10 min of reaction, and in another experiment (B) after 10 and 20 min.

the activity of RNase (Figure 7A). However, there is a 42% decrease in the specific incorporation of [³H]CTP into 3′ chain ends (Figure 7B) in both estrogen and control. This probably indicates that in both control and estrogen reactions, 42% of the chains which are measurable at 5 min are still undergoing elongation.

Figure 8 shows the results of another cold chase experiment. The labeling period was reduced to 3 min, at which time the specific activity of either [³H]UTP (Figure 8A, B) or [³H]CTP (Figure 8C, D) is diluted 100-fold and the reaction is allowed to proceed for 1 additional min. Whether UTP or CTP is used as the labeled substrate, there is an increase in the incorporation of the triphosphate into RNA due to estradiol (Figure 8A, C, 3 min), but the hormone does not affect the amount of incorporation of either triphosphate into the corresponding nucleoside of the 3′ chain end (Figure 8B, D, 3 min). When the specific activity of the nucleoside triphosphate was diluted 100-fold, measurable incorporation was effectively stopped, and the amount of UTP and CTP incorporated into total RNA of both control and estrogen-treated preparations was the same at 4 min as it was at 3 min. However, there was a 70–75% decrease in incorporation from the 3-min values of both CTP and UTP into the corresponding nucleosides at the 3′ chain ends. It appears that with a shorter labeling time it is possible to chase the label out of a greater percentage of the 3′ chain ends (compare Figure 8B, D to 7B).

Figure 9 shows the results of two experiments in which the number of chains was determined at two different times during the reaction. In one experiment a 34% increase in [³H]CTP into cytidine of the 3′ chain ends (Figure 9A) was observed from 5 to 10 min for both estrogen and control preparations.

In another experiment (Figure 9B), the increase in the number of 3′ chain ends from 10 to 20 min was 13% for the estrogen and 16% for the control preparations. The increase in the number of RNA chains between 5 and 10 min and between 10 and 20 min could reflect initiations of new chains. The known presence of RNase in our system which could also increase the number of chain termini makes this interpretation equivocal. However, it can be seen from Figure 5 that 70% of the total incorporation into RNA in both control and estrogen reactions occurs after the first 5 min. Thus, some polymerase molecules not yet active might initiate RNA chains after the first 5 min of synthesis. Nevertheless, there is no significant difference in the number of RNA chains synthesized in the estrogen and control preparations at any one of these times, while the corresponding measurements of RNA synthesis at all these times are significantly different due to estrogen.

Discussion

At a time when incorporation into RNA is significantly enhanced due to estrogen, these data indicate no difference in the number of growing chains. Since the number of RNA chains reflects the number of RNA polymerase molecules, it appears that estrogen has not changed the size of the polymerase population but has increased the rate at which this population of enzymes can polymerize nucleotides into RNA. This increase in activity is not an indirect result of an estrogen effect on substrate concentration, nor is it an artifact due to differential RNase activity. These data also do not support the notion that an increase in the number of templates being transcribed accounts for the estrogen effect on RNA polymerase at 1 hr. A change in a very small population of templates could and likely does occur (DeAngelo and Gorski, 1970), but this would be insignificant in terms of the total polymerase activity. If the entire effect on RNA polymerase were due to an increase in template capacity, one would have to postulate a reduced usage of RNA polymerase at old template sites to account for the invariance of the transcribing polymerase population. It would still be necessary to conclude that estrogen had increased the activity of this population of enzymes.

Figure 5 indicates that there is RNase activity present in both estrogen and control nuclear preparations. This and other experiments have indicated that this activity is present in the same degree in both preparations. If this RNase should be an endonuclease, there is the possibility that the number of chains measured in this system has been amplified due to this activity. However, it should be noted from the data in Figures 7 and 8 that at least half of the RNA chains being measured at 5 min and 75% of the RNA chains bein measured at 3 min truly represent the number of RNA chains being synthesized, since we were able to dilute the specific radioactivity in their 3′ chain ends. And it is significant that this number of chains is equal for both control and estrogen-treated preparations.

The product of RNA synthesis in nuclei incubated in a low ionic strength medium has been shown to be ribosomal in terms of base composition and nearest-neighbor frequency (Widnell and Tata, 1966). In addition, the RNA polymerase activity of uterine nuceli, when assayed in low ionic strength incubation medium, has been localized in the nucleolus (Maul and Hamilton, 1967). Since it is well established that rRNA is synthesized in the nucleolus (Darnell, 1968), we were interested in seeing whether the number of chains synthesized in our reaction was reasonable when compared with the maximum number of chains one could expect to find being synthesized on rRNA genes in rat uterine nuclei. The assumption has been made that the number of genes for 45S rRNA is the same percent of the DNA in rat uterine nuclei as it is in rat liver nuclei. This value is about 0.02% and is based on the fact that 0.01% of rat liver DNA is complementary to 32S rRNA (Quincey and Wilson, 1969) and 45S rRNA has a molecular weight approximately twice that of 32S rRNA. Thus, 2×10^{-2} μg or 4.4×10^{-15} mole of 45S rRNA would just anneal to all gene sites for 45S rRNA in 100 μg of DNA.

Synthesis of 4.4×10^{-15} mole of 45S rRNA on 100 μg of DNA at one time could be accomplished by one RNA polymerase molecule per gene. According to our estimates (multiplying the number of 3′ chain ends due to cytidine by 4), we are synthesizing about 184×10^{-15} mole of RNA molecules/100 μg of DNA (data from Figure 6). If this represents rRNA synthesis, then transcription would involve an average of 42 polymerase molecules per gene. This value is not unreasonable when one considers that the maximum spacing of bacterial RNA polymerase making stable RNA is about one every 54 nucleotides (Manor *et al.*, 1969). If this same spacing were accomplished on genes for 45S rRNA, which is at least 13,000 nucleotides long, then a maximum of 240 polymerase molecules could be in the process of transcription at one time.

In summary, within the limits of detection in these experiments, there is no difference in the number of chains being synthesized in uterine nuclei isolated 1 hr after estrogen, although there is a significant difference in the rate of their elongation. Previous work has shown that the continued synthesis of proteins is necessary for the maintenance of the estrogen effect on RNA synthesis and RNA polymerase activity (Noteboom and Gorski, 1963; Gorski and Morgan, 1967; Nicolette *et al.*, 1968). It is possible that some protein factor is responsible for the faster rate of transcription by RNA polymerase allowing the synthesis of a longer chain before the characteristic inactivation of DNA-dependent RNA polymerase which occurs during low ionic strength reaction conditions.

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Chemical Synthesis and Biological Activity of 8-Substituted Adenosine 3′,5′-Cyclic Monophosphate Derivatives*

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ABSTRACT: 8-Bromoadenosine 3′,5′-cyclic monophosphate served as an intermediate for the chemical synthesis of various 8-substituted derivatives of adenosine 3′,5′-cyclic monophosphate (cAMP). Nucleophilic substitution resulted in displacement of bromine at the 8 position of the purine ring without rupture of the 3′,5′-cyclic nucleotide structure. These 8-substituted derivatives of cAMP have been examined as alternate activators of the cAMP-dependent protein kinase isolated and purified from bovine brain. Structure–activity relationships were established with regard to the functional groups present at C-8 and the accompanying biological activity.

All of the analogs studied possessed some activity toward this enzyme, and several were more effective than cAMP itself. The 8-substituted derivatives of cAMP were also examined as alternate substrates for a cAMP phosphodiesterase isolated from rabbit kidney. With a single exception, 8-amino-cAMP, all of the compounds studied were resistant to degradation by this enzyme. In addition, they were found to be inhibitors of the cAMP phosphodiesterase.

Adenosine 3′,5′-cyclic monophosphate (cAMP) has been shown to stimulate the ATP-dependent phosphorylation of a wide variety of proteins; *e.g.*, casein, histone, protamine, phosphorylase *b*-kinase kinase (Walsh *et al.*, 1968), neurotubular protein (Goodman *et al.*, 1970), fat cell lipase (Huttanen *et al.*, 1970), ribosomal protein (Loeb and Blat, 1970), and σ factor of bacterial RNA polymerase (Martelo *et al.*, 1970). This enzyme has been isolated from cell-free extracts prepared from nearly every single tissue and organism that has been studied (Miyamoto *et al.*, 1969; Kuo *et al.*, 1970), and has been purified from a wide variety of sources, including bovine brain (Miyamoto *et al.*, 1969). It has been postulated that all of the various biological properties attributed to cAMP may be mediated through the stimulation of these protein kinases (Miyamoto *et al.*, 1969). Furthermore, the regulation of cAMP levels both *in vitro* and *in vivo* is in part accomplished by the activity of a class of cyclic nucleotide phosphodiesterases, isolated from most all tissues, that is capable of hydrolyzing several of the cyclic nucleotides to the 5′-monophosphate (Robison *et al.*, 1968).

Examination of variations in biological activity with analogs of cAMP possessing regularly varied structural modifications has had to await the synthesis of sufficient supplies of the pure cyclic nucleotide derivatives. Some reports have appeared in which cyclic nucleotide derivatives of the naturally occurring nucleosides have been studied as alternate activators of the protein kinase. Kuo *et al.* (1970) have shown that cIMP and cGMP had 32 and 7% the activity of cAMP at concentrations of 5×10^{-7} M, using a partially purified preparation of bovine brain protein kinase. The same authors showed that cUMP and cCMP were approximately 7% as active as cAMP, and cdTMP was virtually inactive. Kuo and Greengard (1970a) have also reported on the activity of tubercidin 3′,5′-cyclic monophosphate (cTuMP) which was shown to be about 50% as active as the parent nucleotide, using the same enzyme preparation. Two phosphonate analogs of cAMP (3′-CH_2-cAMP and 5′-CH_2-homo-cAMP) were also examined and found to be relatively inactive toward the bovine brain enzyme. Drummond and Powell (1970) have similarly reported that these two isomeric cyclic phosphonate compounds and adenosine 3′,5′-phosphorothioate were either extremely weak activators or completely inactive with phosphorylase *b*-kinase kinase.

The stability of several cyclic nucleotides to enzymatic hydrolysis has also been studied. Nair (1966) has shown that cdAMP was hydrolyzed about 30% faster than cAMP, while cIMP and cGMP were cleaved at 60 and 33% the rate of cAMP. The pyrimidine cyclic nucleotides cUMP and cCMP were relatively stable to hydrolysis, using a dog heart phosphodiesterase preparation. Eckstein and Bär (1970) have demonstrated that a thio analog of cAMP (adenosine 3′,5′-phosphorothioate) was resistant to diesterase action.

Studies of the effect of structural analogs and derivatives of cAMP on the above enzyme systems are obviously desirable and should lead to useful information regarding the acti-

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