*THE ROLE OF CHROMATIN IN ESTROGEN ACTION IN THE UTERUS, I. THE CONTROL OF TEMPLATE CAPACITY AND CHEMICAL COMPOSITION AND THE BINDING OF H^3-ESTRADIOL-17β**

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Communicated by F. L. Hisaw, May 6, 1968

It is now clearly established that genetic transcription in the mammalian uterus is regulated by the ovarian hormone estradiol-17β (for reviews see ref. 1). Stimulation of nuclear RNA synthesis *in vivo* has been found to be one of the earliest biochemical responses of the uterus of the ovariectomized or immature rat to a single injection of the hormone. This stimulation has been demonstrated for nuclear RNA rapidly labeled *in vivo*,[2] for RNA synthesis assayed *in vitro* in isolated nuclei,[2b, 3] and by the assay *in vitro* of RNA synthesis directed by isolated chromatin and catalyzed by added RNA polymerase.[4a]

The question thus arises: Through what molecular mechanisms of activity or synthesis does estradiol-17β result in an increase in the genetic material available for transcription in the uterus? In an attempt to obtain information of possible value in answering this question, we have undertaken an investigation of the role of uterine chromatin in the early action of the hormone in the ovariectomized rat. We find an early stimulatory effect on the nuclear synthesis and accumulation of chromatin-associated RNA. This effect is manifest 15 minutes after hormone treatment and occurs either prior to *or* simultaneously with a stimulation of the template activity of the isolated and purified chromatin. We further find that at two minutes after treatment *in vivo* H^3-estradiol-17β is bound to the isolated chromatin. The time course for the amount of binding of the hormone *in vivo* parallels nicely the time course for the hormonal stimulation *in vivo* of the template activity of the chromatin, both parameters being maximal at eight hours after treatment. We conclude that estrogen regulates the chemical composition as well as the template capacity of uterine chromatin. The causal relations between these changes in template capacity, chemical composition, and binding of hormone remain to be unraveled.

Materials and Methods.—The intact and ovariectomized adult rats used and their management for hormone treatment were as previously described.[2] The remaining details of experimentation were as follows or appear in the text and relevant figures and tables.

Preparation of purified chromatin: Chromatin was isolated and purified according to two methods. Method 1 was a modification of the procedure of Marushige and Bonner.[5] About 250 mg of minced uterine tissue or 5 gm of minced liver was homogenized at −10°C in 10 ml of saline-ethylenediaminetetraacetate (EDTA) (0.05 *M* NaCl, 0.016 *M* Na_2 EDTA (pH 8.0)). The Ultra Turrax Tp 20/2 tissue disintegrator (Brinkman Instruments) was operated for 30 sec at 110 v and then for 2 min at 60 v. The following preparative steps were at 2°C. The homogenate was filtered through four layers of nylon bolting cloth (110-mesh) and centrifuged at 1500 × *g* for 15 min. The filtrate was washed twice by sedimentation in 12 ml of the saline-EDTA and then twice in 12 ml of tris buffer (0.05 *M*, pH 8.0). The sediment was then suspended in 12 ml of the tris buffer by homogenization in a Dounce homogenizer (Kontes Glass Co.) and centrifuged at 10,000 × *g* for 15 min. The pellet was suspended in 5 ml of the tris buffer, stirred for 30 min by a

Hollow-Spindle electric stirrer (Eberbach) run at 75 v, and then layered over 25 ml of 1.7 *M* sucrose in tris buffer (0.01 *M*, pH 8.0). The upper two thirds of the gradient was gently mixed, and the preparation was centrifuged at 30,000 × *g* for 2 hr. The pellet of chromatin was suspended and washed twice in 12 ml of the tris buffer. It was suspended again in the tris buffer and sheared in the Dounce homogenizer. Fragments of debris were removed by centrifugation at low speed. The supernatant fraction remaining contained the chromatin. Method 2 was the procedure of Dingman and Sporn.[6] Nuclei were isolated from about 450 mg of uterine tissue according to the technique of Widnell, Hamilton, and Tata.[7] Chromatin was then isolated from the nuclei by the exact use of the procedure reported.[6]

Preparation of deproteinized DNA: Purified uterine chromatin was deproteinized by centrifugation in 4 *M* CsCl in the Spinco SW-39 rotor run at 35,000 rpm for 22 hr, as described by Huang and Bonner.[8] The pellet of DNA was then dissolved in tris buffer (0.01 *M*, pH 8.0).

Preparation of RNA polymerase: RNA polymerase was prepared from the early log phase of *Escherichia coli* strain B (Grain Processing Corp., Muscatine, Iowa) by means of the methods of Babinet[9] to the AS_2 stage.

Assay of template activity: The reaction mixture for RNA synthesis directed by uterine or liver chromatin contained in a final volume of 0.25 ml: 10 μmoles of tris buffer (pH 8.0), 1 μmole of $MgCl_2$, 0.25 μmole of $MnCl_2$, 3 μmoles of β-mercaptoethanol, 0.1 μmole each of the triphosphates of cytidine, guanine, and uridine (Schwarz BioResearch), 0.1 μmole of 8-C^{14}-adenosine triphosphate (specific activity, 1.2 μc/μmole; Schwarz BioResearch), and DNA-dependent RNA polymerase. Incubation was carried out at 37°C for 10 min. The reaction was stopped by the addition of 5 ml of 5% trichloroacetic acid (TCA). The TCA-precipitable material was collected on a membrane filter (Millipore HAWP 025) and washed three times with 10 ml of 5% TCA. The filters were dried, treated with 0.5 ml of 80% formic acid, and placed in 15 ml of Bray's[10a] solution for assay of 88% of the total radioactivity in an Anstron scintillation spectrometer.

Chemical determinations and other assays: DNA was determined by the diphenylamine reaction of Burton,[10b] with calf thymus DNA (Sigma) as a standard. RNA was determined by the orcinol reaction described by Dische and Schwarz,[10] with yeast RNA (Sigma) as a standard. Total protein in the chromatin isolated was determined by the method of Lowry *et al.*,[10d] with bovine serum albumin (Sigma) as a standard. Histone was extracted from chromatin with 0.2 *N* HCl at 2–3° for 1 hr, precipitated with 20% TCA, and similarly determined for protein, with the use of calf thymus histone (Sigma) as well as serum albumin for standards. Tritiated uridine and H^3-estradiol-17β (New England Nuclear Corp.) were assayed for 20% of the total radioactivity as described above.

Results.—In Figure 1 are shown the absorption spectra of the chromatin isolated from the uterus of the ovariectomized rat and purified by methods 1 and

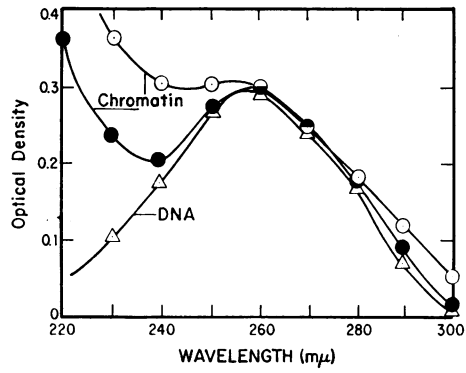

FIG. 1.—Absorption spectra of chromatin and DNA isolated from the uterus of the ovariectomized rat. The procedures for the isolation and purification of chromatin and the preparation of deproteinized DNA were as described under *Materials and Methods*. ●—●, Chromatin prepared by method 1; ○—○, chromatin prepared by method 2.

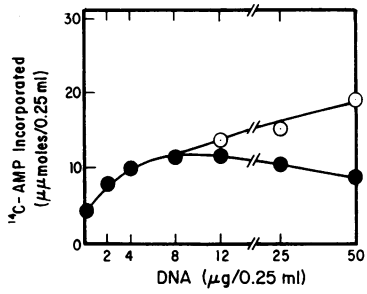

FIG. 2.—Rate of RNA synthesis directed by chromatin isolated from the uterus of the ovariectomized rat by method 1, as a function of template concentration. The reaction mixture contained in 0.25 ml the amount indicated of DNA in the form of purified chromatin. The RNA polymerase added to the reaction mixture was capable of incorporating 60 μμmoles of C^{14}-AMP per 0.25 ml when 50 μg of purified salmonsperm DNA (Sigma) was used as a primer. The incorporation without primer (2.6 μμmoles of C^{14}-AMP per 0.25 ml) was subtracted to obtain the values reported here, in Table 1, and in Figs. 3 and 4. The other conditions were as described under *Materials and Methods*. ●—●, Reaction mixture minus bentonite; ○—○, reaction mixture plus 500 μg of bentonite.

2. The spectra are similar, with that of the preparation obtained by method 1 exhibiting greater fidelity to the spectrum of uterine DNA. The kinetics of the RNA synthesis directed by uterine chromatin obtained by method 1 and catalyzed by added *E. coli* RNA polymerase are shown in Figure 2. There was no further increase in incorporation of C^{14}-AMP into RNA if more than 12 μg of DNA in the form of chromatin was added to the complete reaction mixture. Figure 2 further indicates, however, that RNA synthesis in the system containing more than 12 μg of this DNA was increased proportionately if bentonite was added to the reaction mixture. Whether this enhancement of RNA synthesis was due to inhibition of ribonuclease activity or to deproteinization of the chromatin is unknown. The other kinetics and the principal characteristics of this system for RNA synthesis were as described by Marushige and Bonner[5] for liver chromatin, and by Barker and Warren[4a] for uterine chromatin.

Figure 3 shows that a single injection of 10 μg of estradiol-17β to the ovariectomized rat resulted eight hours later in an increase of 100–125 per cent in the template activity of uterine chromatin obtained by method 1. Uterine chromatin obtained by method 2, however, exhibited only a 50–60 per cent increase in template activity at eight hours after hormone treatment.

The data of Table 1 demonstrate that the chemical composition of the uterine chromatin obtained by method 1 was regulated by hormone action *in vivo*. The amounts of RNA and total protein in the chromatin were less in the preparation from the ovariectomized rat than from the intact animal. In addition, the two components increased in amount after treatment of the ovariectomized animal with the hormone. Furthermore, in the intact animal the components also increased, passing from the diestrous to the estrous phase of the estrous cycle. The increase in the total protein of the chromatin occurred concomitantly with a decrease in the histone component. This was observed for the chromatin isolated from animals stimulated either by endogenous or exogenous hormone.

The earliest stimulatory effect of the hormone *in vivo* on the template activity of uterine chromatin isolated from the ovariectomized rat by method 1 occurred at 30 minutes after treatment (Fig. 4). The activity was elevated 15 per cent over the control. The stimulated activity increased linearly thereafter to a maximal value at eight hours; this represented an increase of 150 per cent over

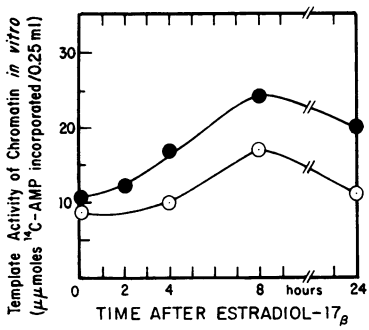

FIG. 3.—Rate of RNA synthesis directed by chromatin isolated from the uterus of the ovariectomized rat, as a function of time after the administration intraperitoneally of 10 μg of estradiol-17β. At the time indicated, the animals were killed, the uteri were pooled (six per group), and the chromatin was isolated and purified as indicated. The constituents of the reaction mixture and the other conditions were as described under *Materials and Methods*. ●—●, Chromatin prepared by method 1; ○—○, chromatin prepared by method 2.

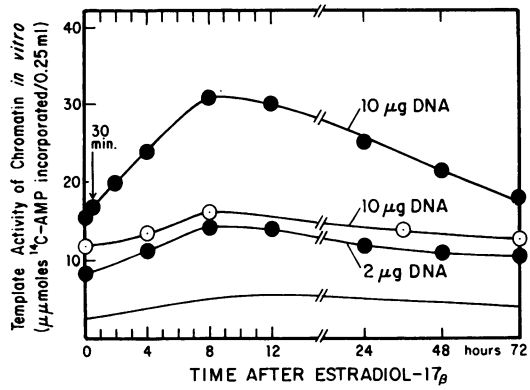

FIG. 4.—Rate of RNA synthesis directed by chromatin isolated from the uterus or the liver of the ovariectomized rat, as a function of time after the administration intraperitoneally of 10 μg of estradiol-17β. At the time indicated, the animals (six per group) were killed, the uteri and liver fragments were pooled respectively, and the chromatin was isolated and purified by method 1. The reaction mixture contained the amount indicated of DNA in the form of chromatin. The other conditions were as described under *Materials and Methods*. ●—●, Incorporation by uterine chromatin; ○—○, incorporation by liver chromatin; ——, incorporation by uterine chromatin (10 μg of DNA) in the absence of RNA polymerase.

the control. At 12–72 hours after treatment, the previously elevated activity of the chromatin decreased. A small but repeatable effect of the hormone increased the template activity of liver chromatin 10–15 per cent at eight hours after treatment.

The changes that occur in the RNA/DNA and total protein/DNA ratios of uterine chromatin isolated by method 1 after hormone treatment of the ovariectomized rat are charted in Figure 5. The RNA component increased 17 per cent in amount during the initial 15 minutes of hormone action. The amount of this component then remained constant for about eight hours. At 12–72 hours after treatment, the RNA/DNA ratio increased again, in close correlation with the relatively delayed increase in the total protein/DNA ratio. There was no detectable change in the RNA/DNA and total protein/DNA ratios of liver chromatin as a result of the hormone treatment.

An analysis of the incorporation of H^3-uridine into chromatin-associated RNA demonstrated that the early increase in the RNA/DNA ratio described in Figure 5 was truly a result of nuclear RNA synthesis *in vivo*, and not a result of redeployment of RNA within the nucleus (Table 2). Increases in the specific radioactivity of the RNA of the chromatin isolated were observed at 15 minutes to eight hours after hormone treatment.

Because considerable interest has recently been expressed in the binding of

TABLE 1. *Chemical composition and template activity of uterine chromatin.*

Component	Source of Uterine Chromatin: Ovariectomized Animals* Control	Hormone-treated 8 hr	Hormone-treated 48 hr	Intact Animals† Diestrus	Intact Animals† Estrus
			Mass Ratio		
DNA	1.0	1.0	1.0	1.0	1.0
RNA	0.12	0.14	0.17	0.17	0.21
Total protein	1.12	1.14	1.32	2.45	2.65
Histone	0.70	0.55	0.65	0.92	0.75
			Template Activity‡		
Chromatin	14.5	30.5	22.5	12.5	16.0

The experimental animals were given 10 μg of estradiol-17β intraperitoneally and were killed at the time indicated. The uteri of each group were pooled, and the chromatin was isolated and purified by method 1. The values cited are representative of replicate experiments.

* Six animals per group.

† Three animals per group.

‡ Expressed as $\mu\mu$moles of C^{14}-adenosine monophosphate incorporated per 0.25 ml of the complete reaction mixture containing 10 μg of DNA in the form of chromatin (see *Materials and Methods*).

radioactive estradiol-17β to receptor molecules in uterine cells,[11] we measured the time course for the binding *in vivo* of H^3-estradiol-17β to the chromatin of the uterus of the ovariectomized rat. Figure 6 shows that the labeled hormone was bound to the chromatin two minutes after treatment. The binding was maximal at eight hours, a time when the template activity of the chromatin was also maximal (Figs. 3 and 4). The labeled hormone was considered bound to the chromatin, in accordance with the procedure of Maurer and Chalkley,[11] if it was not released by dialysis for 18 hours at 0°C against tris buffer (0.03 *M*, pH 7.3). Figure 6 also demonstrates a significant but lesser binding, also maximal at eight hours, of the hormone to liver chromatin.

Discussion.—The experiments reported here indicate that estrogen in its early action in the uterus of the ovariectomized rat controls not only the amount of genetic material available for transcription but also the chemical composition of the genetic material isolated in the form of chromatin. Our experiments also show that the template activity and chemical composition of uterine chromatin vary with the phase of the estrous cycle in the intact rat, presumably in correlation with cyclical fluctuations in the endogenous titers of ovarian hormones.

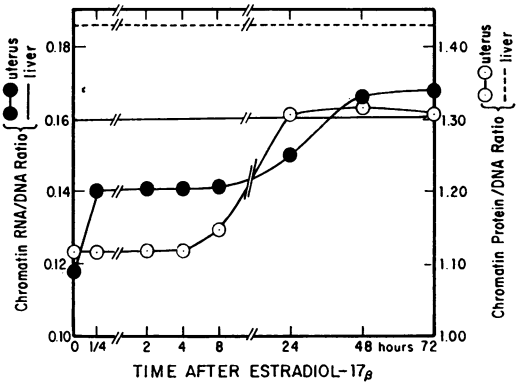

FIG. 5.—RNA/DNA and total protein/DNA ratios of chromatin isolated from the uterus and the liver of the ovariectomized rat, as a function of time after the administration intraperitoneally of 10 μg of estradiol-17β. At the time indicated, the animals were killed (six per group), the uteri and liver fragments were pooled respectively, and the chromatin was isolated and purified by method 1. The amounts of the components of the chromatin were determined chemically as already described.

TABLE 2. *Incorporation of H^3-uridine into chromatin-associated RNA in vivo in the uterus of the ovariectomized rat.*

Specific radioactivity	Hours after Hormone Treatment				
	0 (control)	1/4	2	4	8
Cpm/mg RNA	660	965	989	2170	9210
Cpm/mg DNA	98	117	138	248	1658

All animals received 100 μc of H^3-uridine (2.0 c/mmole) 8 hr before being killed. At the time indicated after hormone treatment as already described, the animals (four per group) were killed, the uteri were pooled, and the chromatin was isolated and purified by method 1. The TCA-precipitable radioactivity of the chromatin was expressed as the cpm/mg of the RNA or DNA determined chemically.

The results reported concerning hormonal control of uterine template activity support the work from Warren's laboratory dealing with the control in the ovariectomized rat[4a] and in the intact hamster,[4b] and are also consistent with the demonstration by Dahmus and Bonner[12] of the control by hydrocortisone of the template activity of liver chromatin.

Our discovery of a very early effect of the hormone (at 15 minutes after treatment) on the synthesis and accumulation of chromatin-associated RNA in the uterus of the ovariectomized animal was unexpected but is compatible with recent work in this laboratory demonstrating that nuclear RNA synthesis *in vivo* is maximal at 20 minutes after treatment.[2] The characterization of this RNA synthesized early in the action of the hormone and the determination as to

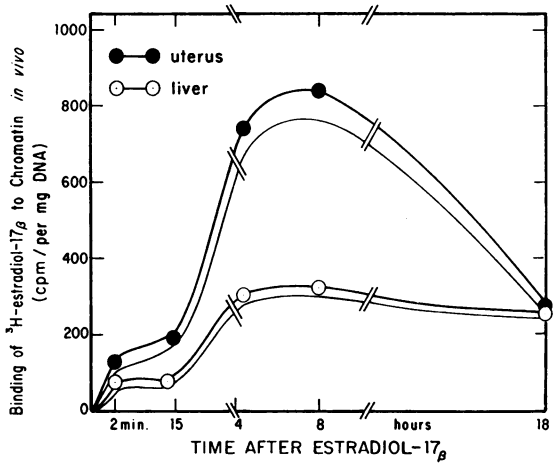

FIG. 6.—Binding of H^3-estradiol-17β to chromatin isolated from the uterus or liver of theovariectomized rat, as a function of time after the administration intraperitoneally of the hormone. All animals received 1 μg (150 μc) of the hormone. At the time indicated, the animals were killed (four per group), the uteri and liver fragments were pooled respectively, and the chromatin was isolated and purified by method 1. The DNA of the preparation was determined chemically, and equal portions were taken. One portion was suspended directly in Bray's solution for measurement of radioactivity. The other portion was dialyzed for 18 hours at 0°C against tris buffer (0.03 *M*, pH 7.3) and similarly measured for radioactivity. The binding was expressed as cpm/mg of DNA in the form of chromatin. The lines without points indicate the binding occurring after dialysis.

whether it is truly the chromosomal RNA described by Bonner and Widholm[13] remain to be completed. We have, however, examined by centrifugation in 2.09 *M* CsCl (see ref. 8) the P^{32}-labeled RNA of uterine chromatin, isolated by method 1 at eight hours after administration *in vivo* of the hormone and the radioisotope. The resulting profiles of radioactivity indicated a heavy DNA-associated RNA peak and a lighter histone-associated RNA peak. Both peaks were greater than those of the control observed after centrifugation of the chromatin obtained from animals receiving the radioisotope only.[14]

Our finding that both the template activity and the chemical composition of uterine chromatin are regulated by estrogen has a clear parallel in the findings of Marushige and Ozaki[15] for developmental changes in the sea urchin embryo. Comparing the chromatin isolated from the more advanced pluteus to that from the blastula, they also observed increases in the template activity and the RNA and total protein components, correlated with a decrease in the amount of histone. Since the total protein increases but the histone decreases in the stimulated or induced chromatin isolated from both systems, it can only be surmised that acidic protein components in particular are increased. We are currently investigating this problem for the uterine system.

In conclusion, we acknowledge a dilemma raised by our findings. Is the stimulation by estrogen of the template capacity of uterine chromatin a cause or consequence of the initial changes in the chemical composition of the chromatin? Given the limited data presently available for the system, we draw no conclusions concerning this problem. Also, we are cautious concerning the physiological significance of the observed binding of the hormone to chromatin.[16] The relation of this observation to the binding of the hormone to the 9.5*S* and 5*S* receptor proteins recently described by Jensen, Gorski, and their respective coworkers[11] remains to be investigated. We note only that the binding of the hormone to uterine chromatin *in vivo* is at a maximum eight hours after treatment, a time when the template activity of the chromatin is maximal and its RNA and histone components are respectively elevated and reduced. At the minimum, our observations still indicate that the induction of RNA synthesis is a major feature in the mediation of hormone action.

Summary.—Estrogen regulates the amount of genetic material available for transcription in the mammalian uterus. The chemical composition of the chromatin is also regulated, with the concentrations of the RNA and total protein components increasing sequentially and the histone content decreasing as a result of hormonal stimulation. The amount of binding of the hormone to chromatin also increases in the process. The causal basis for these genomic variations in template capacity, chemical composition, and binding of hormone remains to be discovered.

* This research was supported by research grants from the Lalor Foundation of Wilmington, Delaware, and the U.S. Public Health Service (HD-00726-07 and CDA-1-F1-GM-9997-01).

[1] (*a*) Mueller, G. C., in *Mechanisms of Hormone Action*, ed. P. Karlson (Stuttgart: Georg Thieme Verlag, 1965), p. 228; (*b*) Gorski, J., W. D. Noteboom, and J. A. Nicolette, *J. Cell. Comp. Physiol.*, **66**, 91 (1965); (*c*) Segal, S. J., and W. Scher, in *Cellular Biology of the Uterus*, ed. R. M. Wynn (New York: Meredith Publishing Co., 1967), p. 114; (*d*) Hamilton, T. H., *Science*, in press.

[2] (*a*) Means, A. R., and T. H. Hamilton, these PROCEEDINGS, **56,** 1594 (1966); (*b*) Hamilton, T. H., C. C. Widnell, and J. R. Tata, *J. Biol. Chem.*, **243,** 408 (1968); (*c*) Hamilton, T. H., C. S. Teng, and A. R. Means, these PROCEEDINGS, **59,** 1265 (1968).

[3] Gorski, J., *J. Biol. Chem.*, **239,** 889 (1964).

[4] (*a*) Barker, K. L., and J. C. Warren, these PROCEEDINGS, **56,** 1298 (1966); (*b*) Warren, J. C., and K. L. Barker, *Biochim. Biophys. Acta*, **138,** 421 (1967).

[5] Marushige, K., and J. Bonner, *J. Mol. Biol.*, **15,** 160 (1966).

[6] Dingman, C. W., and M. B. Sporn, *J. Biol. Chem.*, **239,** 3483 (1964).

[7] Widnell, C. C., T. H. Hamilton, and J. R. Tata, *J. Cell Biol.*, **32,** 766 (1967).

[8] Huang, R. C., and J. Bonner, these PROCEEDINGS, **54,** 960 (1965).

[9] Babinet, C., *Biochem. Biophys. Res. Commun.*, **26,** 639 (1967).

[10] (*a*) Bray, G. A., *Anal. Biochem.*, **1,** 279 (1960); (*b*) Burton, K., *Biochem. J.*, **62,** 315 (1956); (*c*) Dische, Z., and K. Schwarz, *Mikrochim. Acta*, **2,** 13 (1937); (*d*) Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193,** 265 (1951).

[11] (*a*) Toft, D., G. Shyamala, and J. Gorski, these PROCEEDINGS, **57,** 1740 (1967); (*b*) Maurer, H. R., and G. R. Chalkley, *J. Mol. Biol.*, **27,** 431 (1967). (*c*) Jensen, E. V., T. Suzuki, T. Kawashima, W. E. Stumpf, P. W. Jungblut, and E. R. DeSombre, these PROCEEDINGS, **59,** 632 (1968).

[12] Dahmus, M. E., and J. Bonner, these PROCEEDINGS, **54,** 1370 (1965).

[13] Bonner, J., and J. Widholm, these PROCEEDINGS, **57,** 1379 (1967).

[14] Teng, C. S., and T. H. Hamilton, unpublished.

[15] Marushige, K., and H. Ozaki, *Develop. Biol.*, **16,** 474 (1967).

[16] Maurer and Chalkley[11] reported evidence that the binding *in vitro* of the hormone to chromatin in incubated calf endometrial cells represents a noncovalent binding to an undescribed protein component of the chromatin.