*ROLE OF CHROMATIN IN ESTROGEN ACTION IN THE UTERUS, II. HORMONE-INDUCED SYNTHESIS OF NONHISTONE ACIDIC PROTEINS WHICH RESTORE HISTONE-INHIBITED DNA-DEPENDENT RNA SYNTHESIS**

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Abstract.—Nonhistone acidic proteins were isolated, by equilibrium density centrifugation in 4 *M* cesium chloride, from the chromatin isolated and purified from the uterus of the ovariectomized rat or from calf endometrium. Evidence is presented to show (1) that arginine-rich histones are more effective inhibitors of chromatin-directed RNA synthesis *in vitro* than lysine-rich histones, (2) that the nonhistone acidic proteins of chromatin do not inhibit the synthesis of RNA directed by chromatin *in vitro*, (3) that added nonhistone acidic chromatin proteins effect a restoration of histone-inhibited RNA synthesis directed by chromatin *in vitro*, and (4) that the synthesis of nonhistone acidic chromatin proteins is under estrogen control in the uterus, but not in the liver. It is concluded that a major feature of the early action of estrogen in the uterus of the ovariectomized rat is the stimulation of synthesis and the accumulation in the interphase chromosomes of nonhistone acidic proteins which counter the inhibitory effect of histone on transcription by RNA polymerase. Presumably this would permit more and perhaps a new synthesis of RNA programmed for transport to the cytoplasm.

We previously demonstrated that the amount of genetic material available for transcription in the mammalian uterus is regulated by estrogen.[1] The chemical composition of the chromatin in the organ is also regulated by the hormone. The levels of chromatin-associated RNA and of total chromatin protein increased sequentially as a consequence of estrogen acting *in vivo* in the ovariectomized rat. Since the level of histone in the chromatin decreased in the course of this action of the hormone, we inferred that acidic chromatin proteins in particular were increased. In this report, we present evidence for the early action of estrogen in the uterus that the rate of synthesis of nonhistone acidic chromatin proteins is increased and that these proteins accumulate in the chromatin of the organ. The increase in the rate of synthesis of the nonhistone chromatin proteins correlates nicely with the previously reported rise in the template activity of the chromatin, both parameters being maximum at eight hours after administration of hormone to the ovariectomized rat. We now demonstrate that the addition of nonhistone acidic chromatin proteins together with histone to the complete system for chromatin-directed RNA synthesis *in vitro* counters the inhibitory effect of the histone, resulting in a restoration of rate of transcription. We conclude that a major feature of the early action of estrogen in the uterus is a stimulation of synthesis of nonhistone acidic chromatin proteins which accumulate in the interphase chromosomes and negate the

inhibitory effect of histone on transcription, thereby resulting in more and perhaps a new synthesis of RNA.

Materials and Methods.—Nearly all the materials and methods used in the isolation and purification of chromatin from the uterus, liver, or spleen of the ovariectomized rat or from the endometrium of the calf were as previously reported.[1] Other details of procedure and experimentation follow or are described in the relevant figures and tables.

Preparation of histone from chromatin: The total histone of isolated and purified chromatin was extracted in 0.25 *N* HCl at 2–4°C for 30 min. The histone extracted was dialyzed against water at 6°C before use. Fractionation of the total histone in 0.25 *N* HCl was by the acetone procedure of Tidwell, Allfrey, and Mirsky.[2] In one experiment, the extracted histone was acetylated by the procedure of Reid.[3]

Preparation of acidic proteins from nuclei or chromatin: Acidic proteins were isolated by a modification of the procedure of Benjamin and Gellhorn.[4] Isolated nuclei or chromatin were extracted for 2 hr with three 5-ml volumes of 0.2 *N* HCl at 2–4°C. The acid-insoluble precipitate was extracted with 4 *M* CsCl, 0.02 *M* lysine (pH 11.5), 0.005 *M* Na_2 EDTA, and 0.01 *M* mercaptoethanol. The mixture was separated into the protein and DNA fractions by equilibrium density centrifugation in 4 *M* CsCl for 60 hr at 6°C in a SW65 rotor at 50,000 rpm in the Spinco model L2 ultracentrifuge. The upper 1.0 ml of solution of each centrifuge tube was collected and dialyzed against 0.01 *M* lysine buffer, pH 11.5, containing 0.002 *M* Na_2 EDTA, and 0.005 *M* mercaptoethanol.

Preparation of nonhistone proteins from chromatin: This was by a modification of the procedure of Paul and Gilmour.[5] Isolated chromatin was suspended in 4 *M* CsCl and centrifuged for 24 hr at 6°C in a SW39 rotor at 39,000 rpm in the Spinco model L2 ultracentrifuge. The top pellicle, containing proteins, was removed from each centrifuge tube and extracted with 0.25 *N* HCl for 2 hr at 2–4°C. The remaining proteins were washed twice with 1 *N* HCl at 2–4°C (see ref. 6). The proteins precipitated by the acid wash were dissolved in 1 *M* NaCl and 0.05 *M* Tris buffer at pH 8.0.

Terminology: The proteins obtained by the modification of Benjamin and Gellhorn's procedure[4] are termed acidic nuclear or chromatin proteins. Those extracted from chromatin by the modification of Paul and Gilmour's procedure[5] are termed nonhistone proteins. The use of the expression acidic or nonhistone is a categorical one for the purpose of this paper only. The proteins extracted by either procedure are rich in acidic amino acids and contain tryptophan. The preparation of acidic chromatin proteins appears to contain less residual protein than that of the extracted nonhistone chromatin proteins. The results of an analysis of the amino acid composition and of the zonal distribution on polyacrylamide gel of the nonhistone acidic chromatin proteins of the rat uterus or the calf endometrium will be reported elsewhere.[7]

Results.—Inhibition of RNA synthesis in vitro by added histone: It is well known that DNA-dependent RNA synthesis *in vitro* is inhibited by the addition of histone to the cell-free system containing RNA polymerase and either DNA or DNA in the form of chromatin.[8] The data presented in Figure 1 show that histone prepared from chromatin isolated from calf endometrium inhibits RNA synthesis when added to the complete system containing uterine chromatin and RNA polymerase of *Escherichia coli*. In the system containing 10 μg of DNA in the form of chromatin, there was a progressive decrease in the rate of transcription upon addition of increasing amounts of total histone (5–100 μg). Addition of 100 μg of total histone resulted in about 75 per cent inhibition of RNA synthesis. When the several histone fractions (*f1–f5*) were each similarly tested, *f5* and *f4* histone were the most effective in inhibiting transcription. Addition of 100 μg of either fraction resulted in about 90 per cent inhibition of RNA synthesis. The other fractions of histone (*f3–f1*) were less effective in inhibiting RNA synthesis, their inhibitory effect decreasing in the order listed.

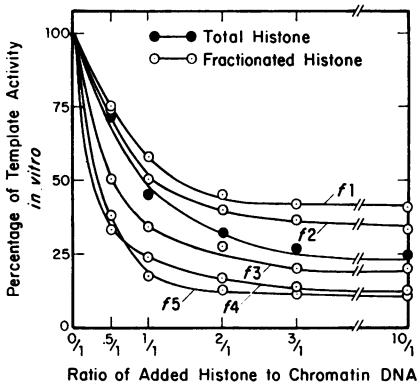


FIG. 1.—Inhibition by added histone of chromatin-directed RNA synthesis *in vitro*. Histone was prepared and fractionated from chromatin isolated and purified from calf endometrium, as described under *Materials and Methods*. The chromatin used for RNA synthesis *in vitro* was isolated and purified from the uterus of the ovariectomized rat as previously reported.[1] The complete system contained, prior to addition of total or fractionated histone in the amount indicated, 10 μg of DNA in the form of chromatin, a constant amount of *E. coli* RNA polymerase, and the other requisite constituents for RNA synthesis. Template activity was measured as $\mu\mu$moles of ^{14}C-AMP incorporated per 0.25 ml of reaction mixture incubated for 10 min at 37°C. The measurements are expressed as percentage of the activity (15 $\mu\mu$moles of ^{14}C-AMP incorporated) of the complete system in the absence of added histone. The remaining details of experimentation and of measurement of template activity were as described in the text or previously.[1]

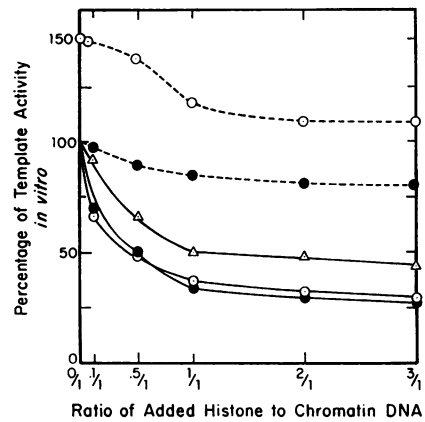


FIG. 2.—Inhibition by added uterine, hepatic, or splenic histone of RNA synthesis *in vitro*. Histone was prepared from chromatin isolated and purified from the uterus, liver, or spleen of the ovariectomized rat. The chromatin used for RNA synthesis *in vitro* was isolated and purified from the uterus of the ovariectomized rat or of the ovariectomized rat administered 10 μg of estradiol-17β at 8 hr before killing. The design of the experiments was similar to that described in Fig. 1, and the template activity is expressed as percentage of that (15 $\mu\mu$moles of ^{14}C-AMP incorporated) of the complete system containing no added histone and 10 μg of DNA in the form of chromatin isolated from the uterus of the untreated ovariectomized rat.

●——●, Uterine histone at the concentration indicated; ○——○, hepatic histone △——△, splenic histone; ●- - - -●, acetylated uterine histone; ○- - - -○, uterine histone added to chromatin stimulated by estrogen *in vivo*.

The amino acid composition of each of the five histone fractions was also determined. Table 1 summarizes the ratios of lysine to arginine for these fractions, and shows a decrease in the ratio for the fractions listed in the order *f1* to *f5*. It is clear that the inhibitory effect of a given histone fraction on chromatin-directed RNA synthesis *in vitro* is inversely correlated with its lysine to arginine ratio (cf. Fig. 1 and Table 1).

TABLE 1. *Ratio of lysine to arginine for fractionated endometrial histone.*

Fraction	Lysine (moles per cent)	Arginine (moles per cent)	Lysine/arginine ratio
f1	24.2	4.4	5.5
f2	14.7	5.1	2.9
f3	12.8	8.3	1.5
f4	10.5	10.9	1.0
f5	10.1	11.7	0.9

Histone was prepared and fractionated from calf endometrium, as described under *Materials and Methods*. The amino acid composition of each fraction was then determined.

Figure 2 shows the inhibitory effect on RNA synthesis directed by uterine chromatin *in vitro* of added histone prepared from the chromatin of the uterus, liver, or spleen of the ovariectomized rat. Uterine and hepatic histone were almost identical in their inhibitory effects on transcription, whereas splenic histone was somewhat less effective in inhibiting transcription. Figure 2 also shows that acetylation of uterine histone, prior to its addition to the complete system, markedly diminished its inhibitory effect on RNA synthesis. Figure 2 further shows that stimulation by estrogen *in vivo* of the template activity of chromatin also results in a protection from the inhibitory effect *in vitro* of added histone. The significance of this observation remains to be elucidated, but conceivably it could reflect the rise in the level of the nonhistone chromatin proteins effected by the hormone acting *in vivo*, as described below. We have, however, determined that addition of estradiol-17β to a final concentration of 10^{-5} or 10^{-6} M in the complete system for chromatin-directed RNA synthesis does not alter the inhibitory effect of added histone.[9]

Restoration by nonhistone acidic chromatin proteins of histone-inhibited RNA synthesis in vitro: We examined the effect on chromatin-directed RNA synthesis *in vitro* of added acidic or nonhistone chromatin proteins (see *Materials and Methods*), in the presence or absence of added histone. The data of Figure 3 show that either the acidic or nonhistone proteins prepared from chromatin isolated from calf endometrium can effect a restoration of the template activity of rat-uterus chromatin in the presence of added uterine histone. A near-complete restoration of the rate of transcription of the chromatin was dependent upon the addition to the complete system of the acidic or nonhistone chromatin proteins *immediately* prior to the addition of the histone. If, however, the histone was added to the complete system immediately prior to the addition of the acidic or nonhistone chromatin proteins, a small inhibition of RNA synthesis occurred. This inhibition, however, was less than that occurring if only histone was added to the complete system.

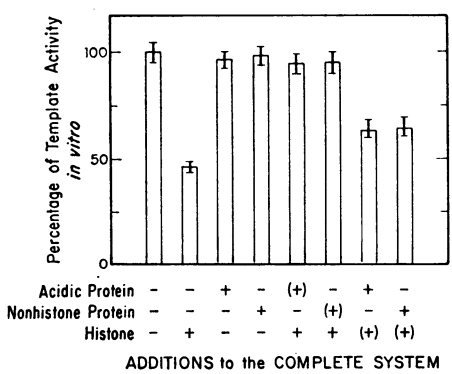
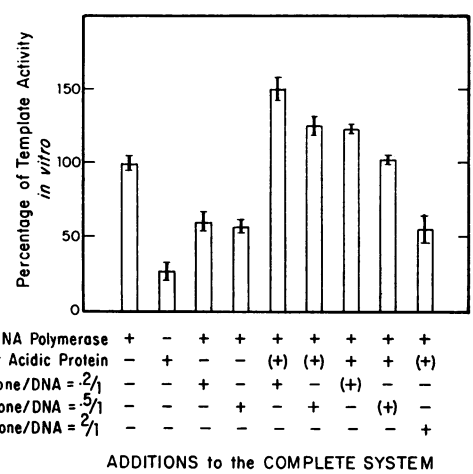
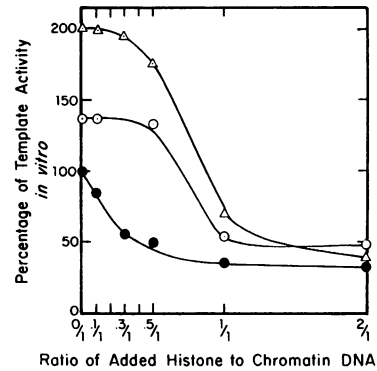


FIG. 3.—Restoration by added acidic or nonhistone chromatin proteins of histone-inhibited RNA synthesis *in vitro*. Histone, acidic proteins, and nonhistone proteins were prepared from chromatin isolated and purified from calf endometrium, as described under *Materials and Methods*. The experimental design was similar to that described in Fig. 1, and the template activity is expressed as percentage of that (15 $\mu\mu$moles of ^{14}C-AMP incorporated) of the complete system in the absence of additions of histone and/or chromatin proteins. The indicated additions to the complete system containing 10 μg of DNA in the form of chromatin were: histone, 10 μg; acidic proteins, 20 μg; nonhistone proteins, 20 μg. Parentheses denote the addition to the complete system that immediately preceded the second addition. The brackets denote the ranges of template activity for triplicate experiments.





(*Left*) FIG. 4.—Restoration by added nuclear acidic chromatin proteins of histone-inhibited RNA synthesis *in vitro*. The experimental design was similar to that described in Figs. 1 and 3, except that acidic proteins were prepared from whole nuclei isolated[1] from calf endometrium. The measurements of template activity are expressed as percentage of that (15 $\mu\mu$moles of ^{14}C-AMP incorporated) of the complete system containing 10 μg of DNA in the form of chromatin isolated and purified from the uterus of the ovariectomized rat. The amount of nuclear acidic proteins added to the complete system where indicated was 20 μg. The parentheses indicate whether nuclear acidic proteins or histone was first added to the complete system. The brackets denote the ranges of template activities for triplicate experiments.

(*Right*) FIG. 5.—Effect of concentration of added histone on chromatin-directed RNA synthesis *in vitro* in the presence of added nuclear acidic proteins. Nuclear acidic proteins were prepared from whole nuclei isolated[1] from the uterus or liver of the ovariectomized rat, and added to the complete system at a constant concentration. Histone was prepared from chromatin isolated and purified from the uterus of the ovariectomized rat. The chromatin used for RNA synthesis *in vitro* was also isolated from the uterus of the ovariectomized rat. The measurements of template activity are expressed as percentage of that (15 $\mu\mu$moles of ^{14}C-AMP incorporated) of the complete system containing no additions of nuclear acidic proteins and 10 μg of DNA in the form of chromatin.

●——●, Uterine histone at the concentration indicated; ○——○, uterine histone and 20 μg of added uterine nuclear acidic proteins; △——△, uterine histone and 20 μg of added hepatic nuclear acidic proteins.

Restoration of histone-inhibited RNA synthesis by nuclear acidic proteins: Figure 4 shows that the acidic proteins extracted from whole nuclei isolated from calf endometrium restore histone-inhibited chromatin-directed RNA synthesis. The results of the experiments described in Figure 4 are similar to those obtained by use of proteins prepared from isolated chromatin and described in Figure 3. It is especially interesting to note that the nuclear acidic proteins, in addition to countering the effect of histone, appeared to stimulate the template activity of the uterine chromatin. However, deletion of RNA polymerase from the complete system containing added nuclear acidic protein resulted in a synthesis of RNA at a rate about 25 per cent of that occurring in the complete system only. It is unknown whether the increase in template activity of the complete system resulting from the addition of nuclear acidic protein is due to RNA polymerase or to some other stimulatory nuclear protein.

Figure 5 also shows an increase in the template activity of uterine chromatin,

upon addition to the complete system of acidic proteins prepared from whole nuclei isolated from the rat uterus. An even greater increase in the rate of RNA synthesis occurred if acidic proteins prepared from rat-liver nuclei were added to the complete system. Finally, Figure 5 shows that increasing the amount of histone added, while holding constant the amount of added uterine or hepatic nuclear acidic proteins, resulted in increasing inhibition of RNA synthesis.

Hormonal control of synthesis of nonhistone chromatin proteins in vivo in the uterus: The rate of incorporation *in vivo* of C^{14}-amino acids into the nonhistone proteins of the uterine chromatin was increased 30 per cent at eight hours after administration to the ovariectomized rat of estrogen (Fig. 6). All animals received 20 μc of C^{14}-labeled acid protein hydrolysate at 24 hours prior to killing. The stimulation of nonhistone protein was true whether the incorporation was expressed as radioactivity per milligram of the nonhistone protein prepared from the isolated chromatin or as the radioactivity of the nonhistone protein expressed per milligram of DNA in the isolated chromatin. The rate of incorporation of

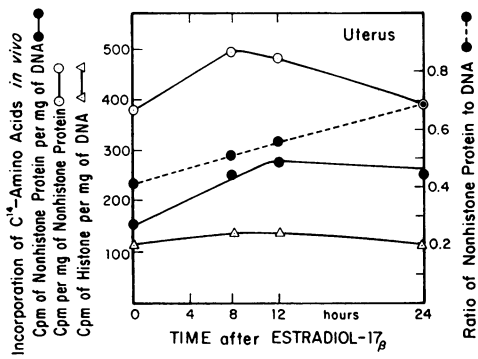


FIG. 6.—Rate of incorporation of ^{14}C-amino acids *in vivo* into the nonhistone proteins and histone of the chromatin of the uterus of the ovariectomized rat, as a function of time after hormone treatment. All animals (six per group) received intraperitoneally 10 μg of estradiol-17β at time zero. All animals received intraperitoneally 20 μc of ^{14}C-labeled acid protein hydrolysate (New England Nuclear) at 24 hr before killing. At the time indicated after hormone treatment, the animals were killed and the uterine chromatin isolated and purified. The nonhistone proteins of the chromatin were extracted, determined chemically, and measured for incorporated radioactivity. The remaining details of experimentation and procedure were as described in the text or previously.[1]

the C^{14}-amino acids into the histone of the uterine chromatin was increased by the hormone no more than 5 per cent at 8 and 12 hours after treatment. That the increased specific activity of the nonhistone chromatin proteins truly indicated a net synthesis was demonstrated by the observed increase in the level of nonhistone proteins in the chromatin. Figure 6 shows that the ratio of the nonhistone protein to DNA increased linearly about 70 per cent during the 24 hours following hormone treatment. The liver of the animals used in these experiments demonstrated neither an increased incorporation of C^{14}-amino acids into the nonhistone chromatin proteins nor an increase in the ratio of the nonhistone proteins to DNA in the chromatin as a result of hormone treatment (Fig. 7).

The experiment described in Figure 6 was repeated with H^3-tryptophan rather than with the mixture of C^{14}-amino acids as radioactive precursor. The data of Table 2 show that most of the radioactivity was incorporated into the nonhistone proteins rather than the histone of the uterine chromatin, and that the stim-

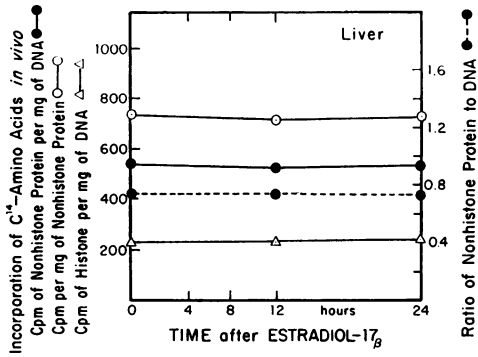


FIG. 7.—Rate of incorporation of C^{14}-amino acids *in vivo* into the nonhistone proteins and histone of the liver of the ovariectomized rat, as a function of time after hormone treatment. The design of the experiment was identical to that described in Fig. 6, except that the livers rather than the uteri of the animals used were examined for incorporation of ^{14}C-amino acids into the nonhistone and histone proteins of chromatin and for the ratio of nonhistone proteins to DNA.

ulation by estrogen of the specific activity of the nonhistone chromatin proteins was maximum at 12 hours after administration of the hormone.

Discussion.—The demonstration that the nonhistone acidic chromatin proteins of the uterus do not inhibit RNA synthesis directed by uterine chromatin *in vitro* is consistent with comparable observations recently made for rat-liver DNA and nonhistone chromatin proteins by Marushige, Brutlag, and Bonner.[10] Our finding that nonhistone acidic chromatin proteins of the uterus restore histone-inhibited chromatin-directed RNA synthesis is also consistent with the recent work of Wang.[11] That synthesis of nonhistone chromatin proteins in the uterus of the ovariectomized rat was stimulated early in the organ's response to estrogen (Fig. 6) suggests that nonhistone acidic chromatin proteins play a role, perhaps an antagonistic one with regard to histone, in controlling the amount of genetic material available for transcription by RNA polymerase. Our observations and the conclusion derived from them seem compatible with the idea of hormonal stimulation resulting in a conversion of compact chromatin to a more diffuse or active state, as suggested by Frenster,[12] King,[13] Berendes,[14] Allfrey and his coworkers,[15] and others. Our conclusion is also compatible with the suggestion of Nicolette, Lemahieu, and Mueller[16] that estrogen may affect the availability of a protein involved in the intranuclear processing of RNA.

Although a variety of conclusions could be drawn from the data now reported, we choose to emphasize only the following topic. The earliest effect of estrogen *in vivo* on the chemical composition of uterine chromatin we have detected is an increase in the rate of synthesis and in the level of chromatin-associated RNA.[1] This stimulation of RNA synthesis either precedes or coincides with the initial

TABLE 2. *Rate of incorporation of H^3-tryptophan in vivo into the nonhistone proteins and histone of the chromatin of the uterus of the ovariectomized rat.*

Specific radioactivity	Hours after Hormone Treatment 0 (control)	12	24
Cpm of nonhistone proteins/mg DNA	253	370	530
Cpm/mg nonhistone proteins	484	642	544
Cpm of histone/mg DNA	76	89	113
Cpm/mg histone	93	113	97

All animals received intraperitoneally 100 μc (6.6 c/mmole) of H^3-tryptophan (Schwarz) 24 hr prior to killing, at the time indicated following administration of 10 μg of estradiol-17β at time zero. Otherwise the experiment was as described in Fig. 6.

increase in the template activity of the chromatin. At later times after administration of estrogen to the ovariectomized rat, the levels of RNA and of nonhistone protein in the chromatin increase in correlation with the continued increase in the template activity of the chromatin. At eight hours after hormone treatment, both the template activity and the rate of synthesis of nonhistone chromatin protein are increased to maximal values. There is only a minor increase in the rate of synthesis of histone at this period of hormone action. At 12–24 hours after hormone treatment, both the rate of chromatin-directed RNA synthesis *in vitro* and the rate of synthesis of nonhistone chromatin proteins *in vivo* decrease. However, the levels of RNA and of nonhistone protein in the chromatin continue to increase during this later period of hormone action (cf. Fig. 6 and ref. 1). The data cited are interpreted to mean that hormone-induced alterations in the metabolism of the RNA and proteins in the interphase chromosomes of the uterus may in turn control the amount of genetic material available for transcription. Parallels of the data cited are to be found in recent studies of ecdysone-induced puffs in salivary gland chromosomes of *Drosophila hydei*,[14] of the effect of senescence on chromatin of barley leaves,[17] and of the effect of hydrocortisone on rat-liver chromatin.[18]

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