HISTONE ACETYLATION BY CELL-FREE PREPARATIONS FROM RAT UTERUS: IN VITRO STIMULATION BY ESTRADIOL-17β[1]

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Histone acetylation has been shown to be a normal component of the metabolism of these nuclear proteins. Pogo, Allfrey, and Mirsky (1966) showed that human lymphocytes acetylated their nuclear histones during culture, and that the acetylation was increased during exposure to phytohemagglutinin (PHA). Allfrey et al. (1966) have also described the stimulation of histone acetylation in liver by cortisol. Nohara et al. (1966) have demonstrated that partially purified preparations from liver have the ability to acetylate histones. It was of interest to examine the capacity of the uterus of the immature rat to acetylate histones. The results described in this paper demonstrate the presence in the uterus of the immature rat of an enzyme system that acetylates histones. In addition, in vitro stimulation of the cell-free system by the female sex hormone, estradiol-17β, is described.

Materials and methods. ATP and Coenzyme A were purchased from P-L Laboratories, Cleland's reagent (dithiothreitol) from Calbiochem, sodium acetate-1-C^{14} (specific activities of 53.7 and 59 millicuries/millimole) from New England Nuclear Corporation, Sepraphore III from the Gelman Instrument Co., and calf thymus histone from Worthington Biochemical Co. Weanling rats, 21 to 30 days old, supplied by the Holtzman Co., Madison, Wisconsin, were used in these studies.

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Rats were killed by cervical dislocation and the uteri were excised, weighed and homogenized in 9 volumes of a medium which was made from 26 ml of Robinson's medium (Robinson, 1949), 9 ml of glycerol, and 35 mg of Cleland's reagent. Substitution of 0.1 M mercaptoethanol for the Cleland's reagent gave a preparation with slightly lowered activity, whereas omission of glycerol, or use of 0.25 M sucrose rather than Robinson's medium gave a preparation with greatly decreased activity. Routinely, the enzyme source was the supernatant from a centrifugation at 10,000 X g for 10 minutes at 1°C.

After incubation at 37°C for 30 minutes, the reaction was stopped by addition of 4 volumes of ethanol. The precipitated proteins (including histones) were washed 2X with 80% ethanol. The histones were then extracted twice with 0.2 N HCl for 10 minutes at 0°. Histones were precipitated from the acid solution by the addition of 10 volumes of acetone. The histones were dissolved at a concentration of 20 mg/ml in 0.05 M barbital buffer, pH 9.0, containing 7.5 M urea. Ten microliters of the solution were placed on a strip of Sepraphore III which had been soaked in the same buffer. Electrophoresis was carried out for 45 minutes at 300 V. The histones were fixed with picric acid and stained with Fast Green (Pogo et al. 1966).

Two main bands were found, a narrow fast band and a broader slow one. They were cut apart and dissolved in a mixture of dioxane and 2 N HCl (10:1). The histone concentration was determined from the absorbance at 625 millimicrons. Aliquots were then transferred to scintillation vials and counted in 10 ml of a scintillation solution, consisting of 4 g 2,5-diphenyloxazole plus 50 mg of 1,4-phenylenebis[2-(5-phenyoxazole)] and 80 g of naphthalene in 600 ml of toluene plus 400 ml of 2-methoxylethanol, after adding sufficient 1 N hyamine hydroxide to neutralize the HCl in the solution. The vials were counted in a Nuclear Chicago Scintillation Counter equipped with an automatic external standard. Efficiencies of approximately 50% were found. Results were calculated as micromicromoles of acetate incorporated per mg of histone.

Results and Discussion. The incubation of radioactive acetate with histones in the presence of the uterine preparations and the appropriate cofactors (Table I) led to the incorporation of appreciable amounts of acetate into the histones. The association of the acetate with the histones was shown by the travel of the radioactivity with the histones during the purification procedure (during which the label showed a net positive charge at pH 9) and the precipitation of the label, along with the histones, by 20% trichloroacetic acid.

The cofactor requirements of the system are shown in Table I. For the incorporation of acetate into the histones Coenzyme A, ATP, and magnesium ions are needed. The data in Table I show the absolute requirement of the reaction for both Coenzyme A and ATP. In addition, omission of magnesium ions led to a 90% loss of activity. For the preparation of the uterine enzyme for this experiment, $MgSO_4$ was omitted from the Robinson's medium used to make up the homogenization solution, so that the dependence on magnesium ions could be assessed.

Table I

Requirement for Cofactors

Experimental Conditions	Incorporation into	
	Slow histones (μμmoles	Fast histones acetate/mg)
Complete System	1076	749
-ATP	17	9
$-Mg^{++}$	97	60
-ATP, $-Mg^{++}$	3	0
-CoA	5	3

The preparation was made in the Mg^{++}-less medium given in the text, and 0.05 ml of the 10,000 X g supernatant (0.9 mg protein) was incubated for 30 minutes at 37°C with 100 μmoles phosphate buffer, pH 7.7, 5 μmoles ATP, 3 μmoles $MgSO_4$, 0.05 μmoles CoA, 5 μcuries (0.1 μmole) Na acetate-1-C^{14}, and 1 mg. calf thymus histone in a final volume of 1.0 ml.

Table II

Subcellular Localization of Enzyme Activity

Source	Incorporation into	
	Slow histones	Fast histones
	(μμmoles acetate/mg)	
Homogenate	114	75.4
Nuclei	27	20
Mitochondria	11	9
Microsomes	2	3
Supernatant	178	111

A known aliquot of a uterine homogenate was centrifuged successively at 780 X g for 10 minutes, 10,000 X g for 15 minutes and at 105,000 X g for 60 minutes to sediment the nuclear, mitochondrial, and microsomal fractions, respectively. These pellets and the final supernatant were made to volume with the homogenization medium and 0.05 ml incubated under the conditions of Table I.

As shown in Tables I and II and the figure, the incorporation of acetate into the slow histones and the fast histones is not equal, greater incorporation being shown by the slow-running histones. If the slow histones can be identified with the arginine-rich histones and the fast histones with the lysine-rich (Pogo, Allfrey, and Mirsky, 1966), the greater incorporation of acetate into the "arginine-rich" histones becomes reasonable, since Phillips (1963) has found that histones do contain acetate, and that the end groups of the lysine-rich histones are about 73-80% acetylated, whereas the end groups of the arginine-rich histones are only about 36-48% acetylated, leaving substantially more end groups available for acetylation in the arginine-rich histones.

Table II shows the subcellular localization of the enzymes. Although there is some activity in every fraction but the microsomes, the bulk of the activity is found in the supernatant and most of the remainder is found in

the nuclear fraction. The results are parallel for the two bands, the slow-running histone band and the fast-running band. The reason for the apparent doubling of activity when the sum of the activities of the separated fractions is compared to the activity of the whole homogenate is not known. One possible explanation is the possibility of mitochondrial oxidation or other metabolism of the added acetate.

Although one would expect a histone metabolizing system to be found in the nucleus, the distribution of the system is not unexpected. DNA polymerase is another enzyme which was expected to be confined to the nucleus, but which was found to be mostly in the supernatant fraction. A second possibility may be that the histone transacetylase is mostly in the nucleus, but the nucleus is very low in aceto-CoA synthetase, the acetyl-CoA being supplied by the cytoplasm *in vivo*.

Figure I shows the effect of adding low concentrations of estradiol-17β to the incubation medium. As may be seen from Figure IA, the presence of low

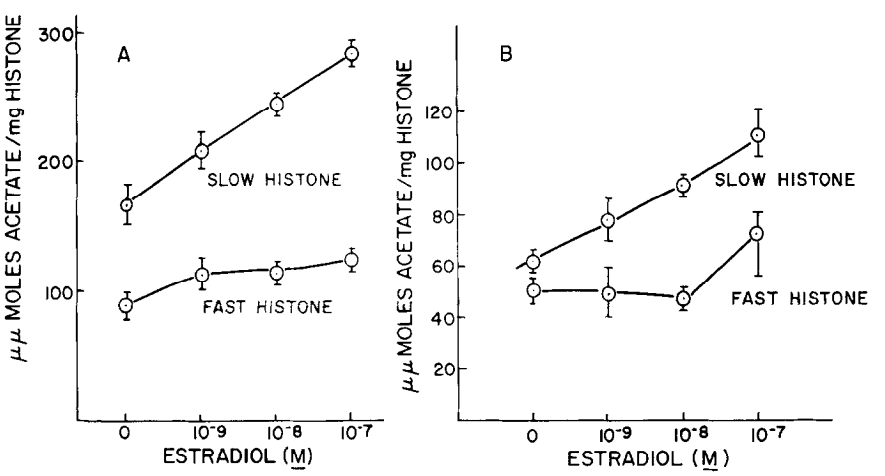

Fig. 1. Effect of estradiol-17β on the histone acetylation. A freshly prepared 0.01 M solution of estradiol was diluted to 10^{-6} M, 10^{-7} M, and 10^{-8} M with water. Addition of 0.1 ml of each of these solutions to the reaction mixture gave the concentrations shown in the figure. Control tubes were incubated with 0.001% ethanol, which had no effect on the rate of reaction. (A) Enzyme used was 0.3 mg protein from a 10,000 X g supernatant of the homogenate. (B) Enzyme used was 0.16 mg protein from the nuclear pellet. The bars show the range of triplicate determinations.

concentrations of estradiol (10^{-9} M) during the course of the incubation with the supernatant fraction causes an increase in the acetylation of the slow histones. Increasing the concentration of estradiol to 10^{-7} M causes a stimulation of acetylation of the slow fraction to 66% over the control value in this experiment. At the same time, the effect of estradiol on the acetylation of the fast-running histones is negligible at these concentrations.

In Figure IB it is seen that the effect of estradiol is the same on the acetylation of the histones using the nuclear fraction as a source of enzyme. In this case also, estradiol stimulates the acetylation at 10^{-9} M and the stimulation is greatly enhanced by increasing the concentration to 10^{-7} M. As is the case with the supernatant enzyme, the nuclear enzyme is stimulated by estradiol to increase the acetylation of the slow histones, but not the fast histones.

This effect of the estradiol, to increase the incorporation of the acetate into the slow histones but not the fast histones is similar to the results found by Pogo, Allfrey, and Mirsky (1966). These authors found that PHA caused a stimulation of histone acetylation in human lymphocytes, and that this stimulation was found in the slow histones, which these authors identified as the arginine-rich histones.

The action of estradiol to increase the acetylation of the slow histones may possibly be equated to its overall action on the uterus, to increase growth, because one of the earliest actions of estradiol on the uterus is to increase RNA synthesis. Means and Hamilton (1966) have found that estradiol increases uterine nuclear RNA synthesis, Gorski (1964) found that estradiol increases the apparent rate of uterine RNA polymerase, and Barker and Warren (1967) found that chromatin from the uterus of animals treated with estradiol was a more efficient primer for E. coli RNA polymerase than was chromatin from the uterus of control animals. These results, especially those of Barker and Warren, may be related to histone acetylation because of the earlier findings of Allfrey, Faulkner, and Mirsky (1964). These authors

demonstrated that, after acetylation of arginine-rich histones by chemical means, formation of a nucleohistone complex with DNA led to a material which, while still less efficient as a primer than the original DNA for E. coli RNA polymerase, was more efficient as a primer than was a nucleohistone complex formed from DNA and non-acetylated arginine-rich histone. The two nucleohistone complexes were identical in all their properties except their priming ability.

Thus, since estradiol increases the rate of histone acetylation and this increase is apparent mainly in the slow histones, which are presumably the arginine-rich histones, the action of estradiol to increase RNA synthesis is, first, followed by increases in protein synthesis, and finally growth, may be explained by a stimulation of the histone acetylation, leading to an increase in the priming ability of the uterine chromatin, and thus an increase in RNA synthesis.

The site of action of the estradiol cannot be determined in these crude systems. Four possibilities are available for the site, if only one site is involved. The hormone could stimulate either of the two enzymes involved in the reaction, the aceto-CoA synthetase or the histone transacetylase or the hormone could inhibit either the breakdown of acetyl-CoA or a deacylase. Further study will be required to differentiate between these possibilities.

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