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EFFECTS OF OESTRADIOL ON COVALENT MODIFICATIONS OF CHROMOSOMAL PROTEINS AND TRANSCRIPTION OF CHROMATIN OF THE BRAIN OF RATS OF VARIOUS AGES

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SUMMARY

In vitro phosphorylation, acetylation and methylation of histones and nonhistone chromosomal proteins (NHCP) and their modulation by oestradiol were studied in the cerebral cortex of female rats of different ages. All the three modifications of histones are highest in immature rats. Phosphorylation and acetylation of histones decrease with age, but methylation is higher in the old than in the adult. NHCP show an age-dependent increase in phosphorylation, but a decrease in acetylation and methylation. Phosphorylation of H1 and H4, and acetylation of H3, H4, H2A and H2B decreases with age. H3 and H4 methylation is highest in the immature and decreases with age. Modifications of only a few NHCP change as a function of age. Oestradiol stimulates phosphorylation of total histones and NHCP of immature and adult rats, but has no significant effect in old age. Acetylation of histones and NHCP of immature rats is stimulated by the hormone. Nuclei were purified from tissues after acetylation with and without 17β-oestradiol and incubated with [^{3}H]-UTP. Transcription was significantly higher after oestradiol treatment Oestradiol stimulates methylation of chromosomal proteins of the adult only. Thus, alterations in covalent modifications of chromosomal proteins and their specific modulation by oestradiol may cause differential expression of genes and influence the aging process.

INTRODUCTION

Chromosomal proteins associated with DNA play a key role in regulating the pattern of gene expression in higher organisms [1–3]. Their modifications through phosphorylation, acetylation and methylation cause conformational changes in the chromatin, and thereby influence its functional activity [4–6]. This may modulate the expression of specific genes. Histones are regarded as unspecific repressors of genes [7]. The nonhistone chromosomal proteins (NHCP), on the other hand, are believed to control the expression of specific genes [1, 8]. Regulation of gene expression is thought to play a key role during development and normal functioning of cells of higher organisms.

Steroid hormones exert their effects on the chromatin after binding to acidic proteins along with specific receptor proteins [9]. They regulate the number of transcription sites available on DNA, and thus control gene function [10, 11]. They also stimulate enzymatic modifications of side chains of chromosomal proteins. This may alter gene expression [12]. We have found that the incorporation of different radioactive labels into individual histones and NHCP varies during different stages of the life span of the rat. Covalent modifications of both types of chromosomal proteins are specifically modulated by oestradiol. Also, oestradiol not only stimulates acetylation of these proteins, but this modification stimulates transcription as well.

MATERIALS AND METHODS

Animals. Immature (2-week), adult (15-week) and old (84-week) female albino rats of Wistar strain, maintained under standard conditions in a rat colony, were used.

Incubation of cortical slices and isolation of nuclei. The cerebral cortex was removed and immediately sliced [13]. The methods used for the study of phosphorylation and acetylation have been previously described [14, 15]. *In vitro* phosphorylation of chromosomal proteins was studied by incubating the slices with 25 μCi/ml of [^{32}P]-orthophosphate (Bhabha Atomic Research Centre, Trombay) in phosphate-free Krebs–Ringer buffer [16]. For acetylation and methylation of chromosomal proteins, the slices were first preincubated with 2×10^{-4} M cycloheximide for 30 min [17]. Then 20 μCi/ml of [^{14}C]-sodium acetate (Bhabha Atomic Research Centre, Trombay, 41.8 mCi/mM) or 5 μCi/ml of methyl-[^{14}C]-methionine (Radiochemical Centre, Amersham, England) was added to the medium. The incubation was continued for 1 h at 37°C with constant shaking after addition of the radioactive isotope. 17β-Oestradiol (1 μmol) was added to the incubation medium 30 min prior to the addition of the isotope.

The control and experimental sets were run parallel in duplicate. The slices were then taken out and washed three times with cold Krebs–Ringer phosphate buffer. They were homogenized in saline-EDTA, centrifuged at 1500 *g* in an IEC high-speed

refrigerated centrifuge for 15 min and the nuclear pellet was sedimented.

Purification of chromatin and fractionation of chromosomal proteins. The nuclei were lysed in 0.05 M Tris buffer, pH 8.0, and centrifuged at 10,000 *g* for 15 min. The chromatin was purified [18] through sucrose gradient at 30,000 *g* in a VAC 601 ultracentrifuge for 3 h. The pellets were suspended in 0.01 M Tris buffer, dialysed overnight and centrifuged at 10,000 *g* for 30 min. The supernatant was sheared and histones were removed [19] with 0.4 N H_2SO_4. The dehistonized chromatin was digested with bovine pancreatic DNase I (Sigma, U.S.A.) to separate DNA from NHCP [20].

Polyacrylamide gel electrophoresis and other methods. The histone and NHCP contents were determined [21] and radioactivity was counted in a Beckman LS-100C liquid scintillation system. The histone fraction was pelleted with absolute ethanol and analysed electrophoretically [18, 22] in 6.25 M urea–5.4% (v/v) acetic acid. The NHCP were analysed by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis [23] in SDS–Tris–glycine buffer, pH 8.3. The protein bands were sliced and digested with 30% H_2O_2 overnight at 37°C. The radioactivity in each band was counted using triton–toluene scintillation fluid.

Studies on transcription. The nuclei were isolated by sucrose gradient after acetylation. The template activity of chromatin for RNA synthesis was studied[24] by placing the nuclei in a reaction mixture (0.25 ml) consisting of 20 mM Tris–HCl buffer, pH 8.0; 120 mM KCl; 0.1 mM EDTA; 2.5 mM $MnCl_2$; 0.1 mM dithiothreitol; 0.8 mM each of ATP, GTP and CTP; 0.2 mM [^{3}H]-UTP (S.A. 50 μCi/mmol, Radiochemical Centre, Amersham). The concentration of nuclei in each assay was 50 μg equivalent of DNA. Nuclei were incubated for 30 min at 37°C, and the reaction was stopped by adding 5 ml of 10% TCA. The reaction in the control tube was stopped at zero time. The acid insoluble material was filtered through glass filtered discs and counted. The incorporation of [^{3}H]-UMP into RNA was expressed as specific activity.

RESULTS

Phosphorylation

Table 1 shows that the specific activity of phosphorylation of histones of cerebral cortex decreases with increasing age of the rat. Even though the electrophoretic pattern of histones of rats of three ages was similar [14], the phosphorylation patterns of individual histones show considerable change with age (Fig. 1). Phosphorylation of H1 and H4, in particular, is higher than that of other histones. Also, the phosphorylation of these two histones decreases with age. Oestradiol stimulates phosphorylation of histones in immature and adult rats, but not in the old. It significantly activates phosphorylation of H3 of immature and adult rats. On the other hand, phosphorylation of H1 and H4 is decreased by the hormone in immature rats. Oestradiol has no appreciable effect on histone phosphorylation in old age.

Phosphorylation of total NHCP is considerably lower in adult rats than in immature and old rats. Different NHCP show different degree of incorporation of [^{32}P]. Also, phosphorylation of individual NHCP is age-dependent. An NHCP that is highly phosphorylated at one age is repressed at another and vice versa. In old rats, phosphorylation of individual NHCP is generally higher (Fig. 2). Oestradiol significantly stimulates, phosphorylation of total NHCP only in immature and adult rats. Also, oestradiol stimulates specific NHCP. In immature rats, phosphorylation of 18th and 21st NHCP is considerably higher. However, phosphorylation of 13th NHCP is inhibited. In the adult, the 21st NHCP is stimulated as in the immature. Also, the 13th and 27th NHCP are stimulated, and the 15th is considerably inhibited. In old age, no significant change in their phosphorylation was observed (Fig. 2).

Acetylation

The specific activity of acetylation of histones declines gradually with age. The degree of acetylation is highest in the immature. Although the acetylation of H1 and H2A is not affected with age, that of H3, H4 and H2B decreases gradually (Fig. 3). Oestradiol significantly stimulates acetylation of histones in immature rats and has no apparent effect in the adult and the old. Individual histones have different responses to oestradiol. Whereas acetylation of H1 and H3 is stimulated, that of H4 is inhibited in immature rats. In the adult also, acetylation of H1 and H3 is stimulated, but the degree of stimulation is lower than

Table 1. Phosphorylation, acetylation and methylation of total histones and NHCP (c.p.m./μg protein) of the cerebral cortex of female rats of three ages and their modulation by oestradiol

Age (weeks)	Experimental condition	Phosphorylation		Acetylation		Methylation	
		Histone	NHCP	Histone	NHCP	Histone	NHCP
2	Normal	25.41	2.00	0.94	2.20	3.30	0.38
	+ oestradiol	30.69	3.39	1.35	3.18	3.22	0.35
15	Normal	32.11	1.67	0.70	0.28	2.26	0.15
	+ oestradiol	51.40	2.06	0.73	0.31	2.76	0.19
84	Normal	9.64	3.03	0.55	0.12	3.97	0.11
	+ oestradiol	9.82	3.32	0.53	0.13	3.94	0.10

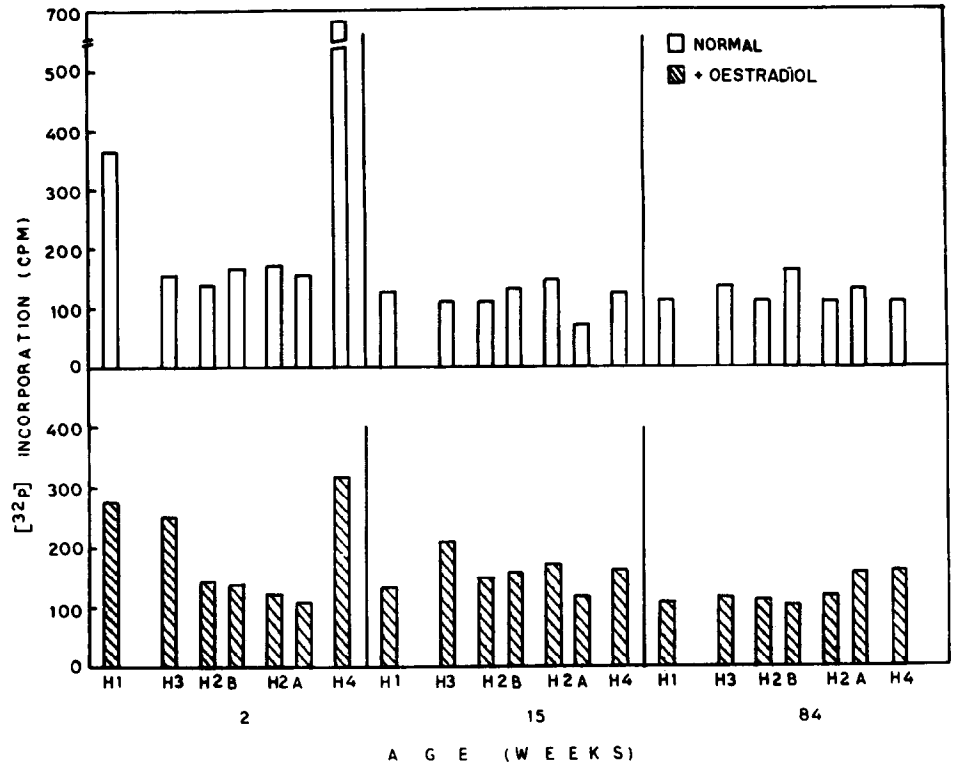

Fig. 1. Effect of oestradiol on the phosphorylation of individual histones of the cerebral cortex of female rats of different ages.

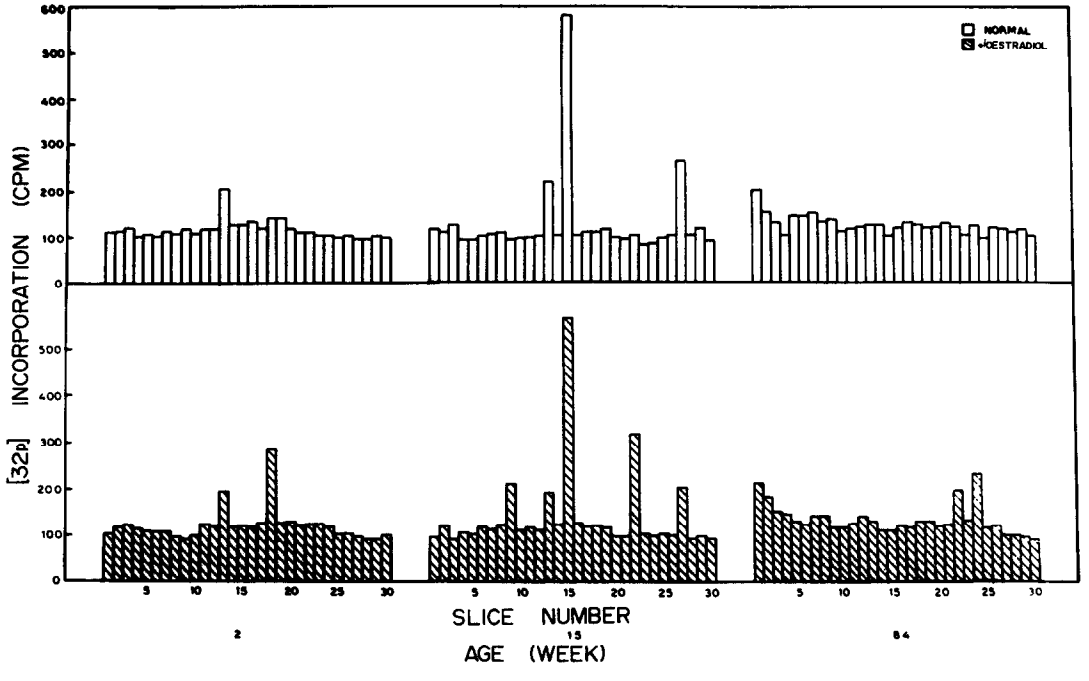

Fig. 2. Effect of oestradiol on the phosphorylation of individual NHCP of the cerebral cortex of female rats of different ages.

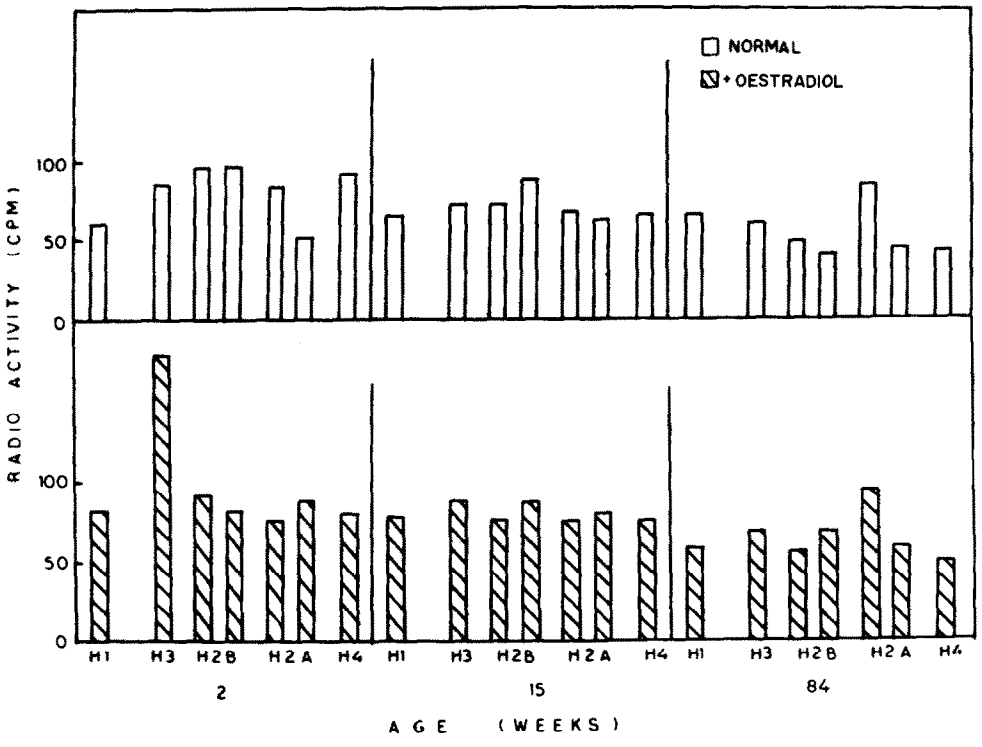

Fig. 3. Effect of oestradiol on the acetylation of individual histones of the cerebral cortex of female rats of different ages.

that of immature rats. In old rats, acetylation of H2B and H3 is slightly stimulated by oestradiol.

Acetylation of NHCP is highest in immature rats and decreases thereafter precipitously with increasing age. Acetylation of individual NHCP is considerably lower in adult and old rats than in the immature. No marked difference is found in the acetylation of individual NHCP of adult and old rats, except a few which are stimulated in the adult and inhibited in the old or vice versa (Fig. 4). Oestradiol enhances acetylation of NHCP. The degree of stimulation is highest in the immature and decreases thereafter. It stimulates the acetylation of the 2nd, 14th and 19th polypeptides in immature rats. In the adult, it stimu-

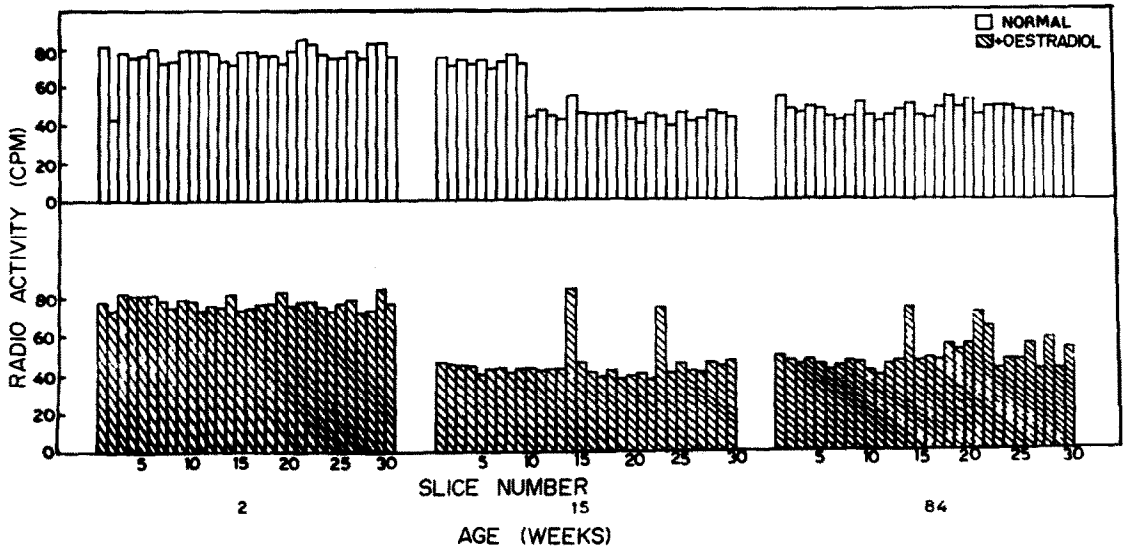

Fig. 4. Effect of oestradiol on the acetylation of individual NHCP of the cerebral cortex of female rats of different ages.

Table 2. Incorporation of [^{3}H]-UMP (nmol) into RNA of acetylated nuclei of cerebral cortex of female rats of different ages, and its modulation by oestradiol

Age (weeks)	Slice	Slice + Ac^-	Slice + oestradiol + Ac^-
4	8.86	17.29	20.68
32	7.41	9.30	14.78
110	6.14	6.51	6.16

lates acetylation of the 14th and 23rd NHCP, but inhibits that of high molecular weight NHCP. In the old, the 14th and 21st NHCP are stimulated.

Table 2 shows that there is a gradual decrease in RNA synthesis with increasing age. Transcription is greatly increased after acetylation, which is further potentiated by oestradiol. Not only does transcription after acetylation decreases with age, the stimulatory effect of oestradiol also declines.

Methylation

Methylation of histones was higher in immature and old rats than in the adult. The degree of methylation of H3 and H4 declines gradually with increasing age (Fig. 5). Oestradiol enhances methylation of histones of adult rats. It has no effect on methylation of histones of immature and old rats. Methylation of H2B is stimulated by the hormone in immature and adult rats, but not in the old.

Methylation of NHCP is highest in immature rats and falls thereafter with increasing age. The incorporation of methyl groups into individual NHCP varies during different stages of the life span. Almost all NHCP bands showed lower methylation in adult and old rats as compared to that of the immature (Fig. 6). Oestradiol stimulates methylation of total NHCP of adult rats, but not of the immature and the old. It inhibits incorporation of methyl groups into the 1st, 9th, 18th, 20th and 30th NHCP in immature rats. Methylation of several NHCP of adult and old rats is stimulated by oestradiol.

DISCUSSION

Phosphorylation of chromosomal proteins and its modulation by oestradiol

Phosphorylation of histones weakens their interaction with DNA and dissociates them from it. The region of DNA devoid of histone is capable of transcription. Thus, greater phosphorylation reflects a higher degree of transcription. Phosphorylation of histones is highest in immature rats and declines with increasing age. A higher level of phosphorylation during the early phase of the life span indicates a significantly higher synthesis of RNA and proteins. This may be because the developing brain may need not only more proteins but also several different types of proteins. An age-dependent decline in phosphoryla-

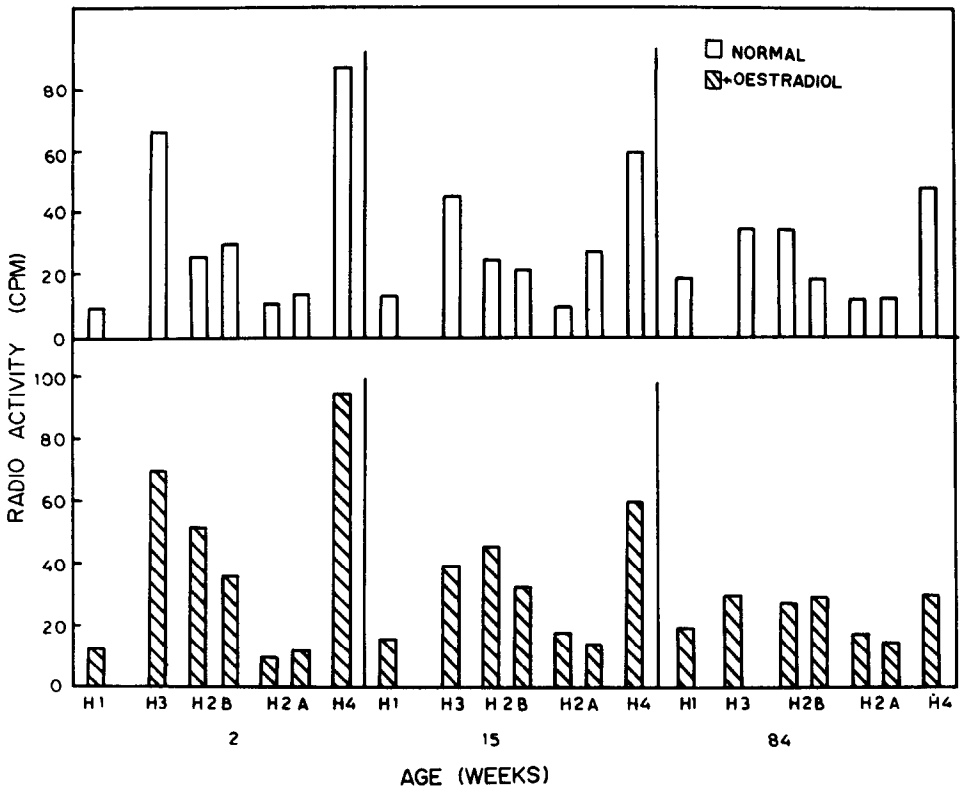

Fig. 5. Effect of oestradiol on the methylation of individual histones of the cerebral cortex of female rats of different ages.

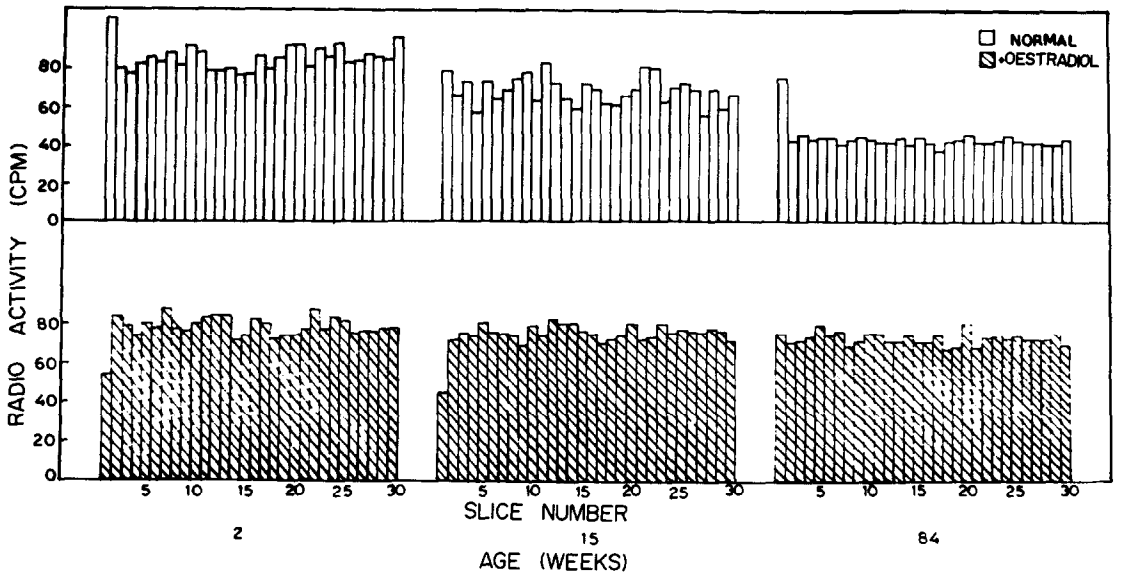

Fig. 6. Effect of oestradiol on the methylation of individual NHCP of the cerebral cortex of female rats of different ages.

tion of histones indicates a decrease in the template activity of the chromatin. This is consistent with the finding [25] that the template activity of chromatin declines with age. Also, the differences in the degree of phosphorylation of different histones seen at each age may have much significance on the template activity of the chromatin. The general decrease in the phosphorylation of histones with increasing age may be due to conformational changes in the chromatin that may mask serine and threonine residues which serve as sites for phosphorylation.

Incorporation of phosphate into individual NHCP may result in the activation or inhibition of expression of specific segments of DNA. Phosphorylation of NHCP is correlated with gene activity. Analysis of phosphorylated NHCP by SDS-polyacrylamide gel electrophoresis showed significant variations in the labelling pattern during ageing. Such age-dependent differences in phosphorylation of NHCP may be due to differences in protein kinases and phosphatases which control phosphorylation of individual NHCP. Our observations, therefore, indicate differential transcription of genes at different phases of the life span. The finding that a specific NHCP which was highly phosphorylated at one age was not phosphorylated at another and vice versa is of much significance. It indicates that different genes are activated and repressed at different phases of the life span. Thus, our observation is in agreement with the hypothesis of Kanungo [26, 27] according to which differential activation and repression of genes is responsible for differentiation, development and senescence of an organism.

Oestradiol stimulates *in vitro* phosphorylation of both histones and NHCP of the cerebral cortex of immature and adult rats. The hormone has no effect in the old. This is well correlated with the finding of Kanungo *et al.* [28] that the specificity of cytoplasmic receptor protein for 17β-oestradiol of the brain of rats decreases with increasing age. These studies are consistent with the observations that the response of the rat to hormones decreases with age. Doughty *et al.* [29] have reported that the brain of the newborn rat is highly sensitive to oestrogen which may be related to the number and specificity of receptor molecules in the cytoplasm. The level of 8S oestradiol receptor of rat uterus reaches the peak on the 10th day after birth [30]. The young animal may also have a higher number of chromatin acceptor sites than the old.

Electrophoretic analysis of phosphorylated chromosomal proteins reveals that the stimulatory effect of oestradiol is very specific. It significantly activates phosphorylation of H3 histone. In case of NHCP, only the 21st polypeptide is strikingly phosphorylated. These studies show that oestradiol stimulates phosphorylation of specific histones and NHCP which are different from those influenced by calcium [14]. Thus differential modulation of phosphorylation of chromosomal proteins by different effectors may influence transcription of specific genes.

Acetylation of chromosomal proteins and its modulation by oestradiol

Acetylation of lysyl residues of —NH_2 terminal regions of histones leads to a decrease in their positive charge density. Since the interaction of histones with DNA is largely ionic in nature, this modification may well weaken histone-DNA interaction and alter the conformation of DNA-histone complexes. Also,

acetylation of histones may alter histone–histone interaction. This could result in the conversion of nucleosome to a conformation more suitable for transcription of the associated DNA. In this way acetylation of histones may play a direct role in the activation of genes [31, 32].

Our observation of a decrease in acetylation of histones with age is consistent with the observation of Ryan and Cristofalo [33] who reported an age-dependent decrease in acetylation of histones in human diploid cells in culture. It is interesting to note that histones H2A, H2B, H3 and H4, which form the core of the nucleosome, show a higher degree of acetylation in the immature than H1 which has an internucleosomal location. Our data show that H1 of the brain is acetylated in all the ages, but the degree of acetylation does not change with age unlike the nucleosomal histones. Also, acetylation of H3, H4 and H2B decreases with age. This would increase the interaction of these histones with DNA and render it transcriptionally less active.

The highest level of acetylation of NHCP is seen in immature rats. This may account for higher transcription of chromatin in the young [25, 34]. Acetylation of specific NHCP may be of particular significance since each NHCP has its own role to play in the expression of genes. Age-dependent decrease in acetylation of individual histones and NHCP may be due to changes in the conformation of chromatin that may render lysyl groups less accessible for acetylation. Alternatively a decrease in the level of acetyltransferase may be responsible for this change.

Our studies show that oestradiol stimulates acetylation of histones of immature rats, but not of the adult and the old. This may be responsible for the higher template activity of the chromatin in immature rats. Stimulation of acetylation of specific histones which may have definite role in chromatin structure may lead to conformational changes. This may stimulate the activity of the genome by exposing specific sites for transcription. It is seen that the hormone specifically stimulates H3 and H1 acetylation which decreases with age. Whereas acetylation of H3 would expose the DNA located in the nucleosome for transcription, that of H1 may make the chromatin more diffuse which may be required for its template function.

Oestradiol stimulates acetylation of NHCP in immature rats, but has no effect in the adult and the old. Furthermore, acetylation of only a few NHCP is stimulated.

Greater stimulation of acetylation of chromosomal proteins of immature rats by oestradiol and a decline thereafter may be due to a decrease in acceptor sites on chromatin and/or a decrease in the concentration of oestradiol receptors. Higher concentration of oestradiol receptors in immature rats as shown by Kanungo *et al.* [28] may account for higher degree of acetylation. Variation in the degree of acetylation of individual histones and NHCP may be of particular significance for the expression of specific genes at different phases of the life span [27].

The above observation is corroborated by our finding that oestradiol not only stimulates acetylation but also potentiates transcription of the acetylated chromatin. Transcription is higher in the immature and the adult after acetylation, but it is significantly stimulated after oestradiol treatment. This effect is lost in old rats. Hence oestradiol may stimulate transcription by increasing acetylation of chromosomal proteins.

Methylation of chromosomal proteins and its modulation

Methylation increases hydrophobicity and cationic charge of the amino groups situated in the polar regions of histones. This strengthens the binding of these polar regions of histones to DNA. Incorporation of methyl groups into lysine residues also raises the pK of the ϵ-NH_2 group. This increases the basicity of histones and thereby strengthens their binding to DNA. These modifications may, therefore, alter histone–histone interaction. Such changes, therefore, would alter the overall conformation of the chromatin and affect its template activity, and hence the expression of genes.

Methylation of histones of the adult brain is lower than that of the immature. Surprisingly, methylation in the old is greater than that of the adult. Repeated experiments gave the same pattern of data. Higher activity of methylase III in the young than in the adult may account for greater methylation of histones in the former [35], but how methylation increases in the old cannot be accounted for from these experiments. The availability of specific sites on histones to accept methyl groups may also differ with age due to conformational changes in the chromatin. Even the degree of methylation of the same active site on a histone may depend upon the functional status of the chromatin. Methylation of H3 and H4 which are known to be the most dominant forms of methylated histones is highest in the immature and decreases significantly with age. Methylation of other histones is lower than that of H3 and H4, and also does not change markedly with age. This is of much significance since $(H3)_2(H4)_2$ tetramer defines the length and basic fold of the core DNA [36–38]. Stronger binding of H3 and H4 to DNA in old age may be responsible for the unavailability of methylation sites. Methylation of certain histones has been implicated in the process of development [39]. Likewise, differential incorporation of methyl groups into different histones may play a significant role in the process of senescence.

Unlike methylation of histones, that of NHCP declines progressively with age. This appears to be due to a general decrease of methylation of all NHCP fractions. Such a general pattern of change may be due to a decrease in methylase responsible for methylation of NHCP.

Oestradiol stimulates methylation of histones of the adult, but has no significant effect in immature and old rats. Also, H2B methylation is specifically stimulated. It is of interest that calcium stimulates methylation of H3 and oestradiol that of H2B (unpublished data). Such differential stimulatory effects by two distinct effectors may have different effects on gene expression.

Stimulation of methylation of NHCP by oestradiol is seen only in the adult. No specific effect on any particular NHCP is observed. The stimulatory effect on total NHCP is only 27% and it is possible that methylation of NHCP may not be significantly influenced by oestradiol. However, specific effect of oestradiol on methylation of histones may influence gene expression.

Alterations in various post-synthetic modifications of histones and NHCP may account for changes not only in the structure but also in the function of the genome. The specific modulatory effects of oestradiol on these covalent modifications indicate that this endogenous steroid hormone may be involved in differential expression of genes. Variations in the level of oestradiol with age may produce variations in the modifications of individual histones and NHCP. This may alter the expression of specific genes and account for the alterations in the levels of different enzymes that occur during the life span. Such differential effects of hormones and other factors may be responsible for the aging process [27].

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