Aldosterone-Induced Alterations in the Synthesis of Renal Cortical Chromatin Components and Acetylation of Histones (36707)

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Administration of aldosterone to either the isolated toad bladder system or the adrenalectomized rat is known to regulate sodium transport through an early stimulation of the incorporation of radioactive RNA precursors into bladder or kidney RNA, respectively, followed by an increase in incorporation of labeled amino acids into protein (1). Some later experiments showed no effect on protein biosynthesis after aldosterone administration (2) while more recent results have been consistent with a mineralocorticoid effect on biosynthesis (3–5). We have demonstrated (6) that there was no increase in the capacity of the rat renal cortical chromatin to act as template for DNA-dependent RNA polymerase after aldosterone treatment. This same study (6) showed, however, that the state of the chromatin had changed after aldosterone administration since labeled actinomycin D bound considerably less to the chromatin from the hormone-treated rats compared to the chromatin from the untreated adrenalectomized controls.

Histones (7), chromosomal RNA (8), and nonhistone chromosomal proteins (9) have all been implicated as possible regulators of transcription in eukaryotes. Several studies (10–15) have already demonstrated that a major feature of the early action of some steroids was an alteration in the synthesis of various chromatin components in the target organs.

Interactions between DNA and its associated proteins would be expected to change at times of gene activation and repression. Studies on histone metabolism in the cells of higher organisms indicated that the basic proteins of the nucleus are modified by attachment of acetyl groups (16). Acetylation of the histones has been found to be one of the earliest chemical events in the process of gene activation for RNA synthesis in different systems (16). A strong correlation has been noted between the patterns of RNA synthesis and histone acetylation in liver responding to stimulation by cortisol (16). Estradiol-17β, which stimulates RNA synthesis in the uterus (17, 18), has also been reported to stimulate the acetylation of histones by cell-free extracts of rat uterus (19). In the present investigation changes in the synthesis of chromatin components and the acetylation of histones were measured in order to shed further light on the mechanism by which aldosterone brings about known increases (1) in the rates of RNA synthesis in the nuclei of the rat kidney cortex.

Materials and Methods. Experiments on the synthesis of chromatin components. Male bilaterally adrenalectomized hooded rats (Royal Victoria Hospital strain) weighing 150–175 g (4/group) each received an ip injection of 4 μg of *d*-aldosterone in saline at zero time. The control animals received an injection of saline at zero time, too. Each rat was also injected ip at 4 hr before sacrifice with 15 μCi of a (^{14}C)-protein hydrolyzate from chlorella (45 mCi/m atom of each carbon labeled); 1 μCi each of L-(^{14}C)-lysine monohydrochloride (312 mCi/mmole), L-(^{14}C)-arginine monohydrochloride (312 mCi/mmole), L-(^{14}C)-aspartate (208 mCi/mmole), and L-(^{14}C)-glutamate (260 mCi/mmole), and 100 μCi of 5-(^{3}H)-uridine (17.25 Ci/mmole). The labeled compounds were purchased from Amersham/Searle. The mixture of 15 different (^{14}C)-amino acids [(^{14}C)-protein hydrolyzate] was employed to minimize possible hormone-induced gross

changes in pool sizes that might occur with any one amino acid to increase the extent of overall protein labeling and to eliminate the escape of any one protein from being labeled by being deficient in a single amino acid. At various times after aldosterone or control treatments (0 to 240 min), the rats were decapitated and the renal cortical chromatins were isolated and purified by the procedure of Marushige and Bonner (20). In all experiments the total recovery of chromatin DNA for each group of four rats was relatively constant within a variation of 15%. The criterion used for purity of the chromatin from the control animals were spectral qualities (260/240 $m\mu = 1.45 \pm 0.05$ and 260/280 $m\mu = 1.75 \pm 0.05$) (21).

The total RNA was removed from the isolated and purified chromatins after alkaline hydrolysis (1 *N* KOH for 20 hr at 37°) and precipitation of the DNA and proteins in the cold with 1 *N* HCl and 5% trichloroacetic acid (TCA) (22). The RNA contents of the supernatants were determined after centrifugation by the orcinol method (22). The DNA was hydrolyzed in 0.5 *N* perchloric acid at 70° for 15 min; its content was measured according to Burton (23). The total histone (acid-soluble proteins) of the isolated chromatin was twice extracted in 0.2 *N* HCl at 2° for 30 min (12). The histone extracted was then precipitated with 20% TCA, the precipitate heated at 90° for 15 min with 16% TCA to remove polysaccharides, washed with ethanol, ethanol: ether (3:1) and ether, air dried, and resuspended in 0.01 *M* Tris–HCl, pH 8.0. The protein content was measured by the method of Lowry *et al.* (24). The sediment remaining after HCl extraction of the chromatin was washed twice with 1 *N* HCl at 2° (25), extracted with 0.5 *N* perchloric acid at 70° for 15 min to remove nucleic acids, and the remaining insoluble fraction was washed with ethanol, ethanol:ether (3:1) and ether, air dried, resuspended in a solution containing 1 *N* NaCl and 0.05 *M* Tris–HCl (pH 8.0), and its protein content was determined (24). This was termed the nonhistone protein fraction (acid-insoluble proteins). Radioactivity was measured in an automatic refrigerated liquid scintillation spectrometer after an aliquot of each of the RNA, histone, and nonhistone protein fractions was mixed with 10 ml of Instagel (Packard). Under our experimental conditions (^3H) was counted at approximately 25% efficiency and (^{14}C) at 70% efficiency.

Experiments on the acetylation of histones. Male bilaterally adrenalectomized hooded rats weighing 150–175 g (10/group) each received an iv injection of 4 μg of *d*-aldosterone in saline and 1.5 mCi of sodium (methyl-^3H) acetate at zero time. The control animals received an injection of saline and 1.5 mCi of sodium (methyl-^3H) acetate at zero time, too. The labeled acetate (sp act 100 mCi or 1.1 Ci/mmole) was purchased from New England Nuclear Corp. or ICN. Fifteen minutes after the injections the rats were decapitated and the renal cortical chromatins were isolated and purified as already described above. The total histone (acid-soluble proteins) of the isolated chromatin was also prepared, purified and measured as mentioned above.

The presence of radioactive acetyl groups in the histones was verified by acid hydrolysis and steam distillation of volatile (^3H)-acetic acid as described by Allfrey (16). That incorporation of (^3H)-acetate into histones truly represents acetylation of the histones has been verified in a couple of ways. Apart from the presence of (^3H)-acetate in the acid-soluble protein fraction of the chromatin, the acetyl groups have been recovered as (^3H)-acetic acid by steam distillation of 6 *N* H_3PO_4 hydrolyzates of the acid-soluble (histone) fraction. Treatment of the histone fraction with 16% TCA at 90° for 15 min extracts polysaccharides and nucleic acids but does not remove radioactivity from the histone fraction. This is an important point because polysaccharides associated with some proteins can be acetylated after synthesis (26).

In order to determine the extent of *O*-acetylation the amount of radioactivity in the histone fraction which was unstable to treatment with 2 *M* neutral hydroxylamine for 1 hr was established. It has already been shown that 2 *M* neutral hydroxylamine hydrolyzes

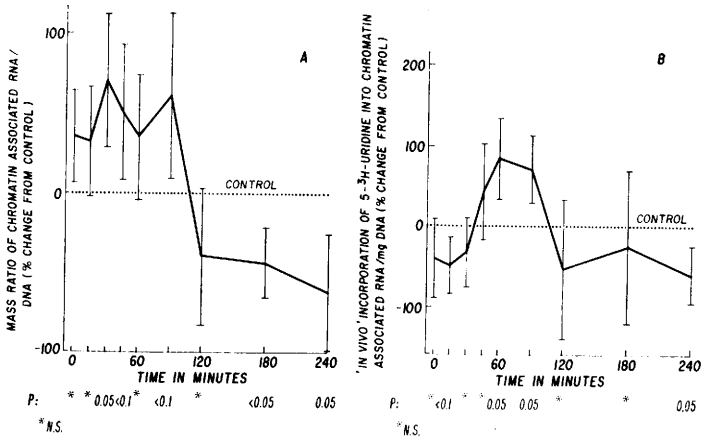

FIG. 1. Chromatin associated RNA. Each point on the curve for the aldosterone treated rats represents the mean ± S.D. of three separate experiments. The values of the points for sham-injected rats (controls) have been averaged out to give straight base lines. p values represent levels of significance of difference calculated from t test. *NS, $p > 0.10$. Time is minutes after aldosterone administration. (A) Mass ratio (mg RNA/mg DNA)—% change from control values. The average value from 0 to 240 min for the control rats was 0.112 ± 0.021 (SD) mg RNA/mg DNA. (B) *In vivo* incorporation of 5-(^{3}H)-uridine dpm into chromatin associated RNA/mg DNA—% change from control values. The average value from 0 to 240 min for the control rats was 5104 ± 664 (SD) dpm of RNA/mg DNA.

O-acetyl but not *N*-acetyl linkages (16).

Results. In this study three separate experiments were carried out using aldosterone-treated rats and sham-injected rats, respectively. Thus each point in Figs. 1, 2, and 3 represents the average ± SD of three separate determinations. The points for the sham-injected rats (controls) have been averaged out to give straight base lines. The chromatin preparations were reasonably reproducible as shown by the standard deviations (SD) of the mass ratios of the control chromatins in Figs. 1A, 2A, 3A. They were also pure as shown by their spectral qualities.

Synthesis of chromatin components. If one takes a look at the shapes of the graphs in Fig. 1A and B, a generalized increase in the synthesis of chromatin associated RNA is clearly indicated from 30 to 90 min following aldosterone administration. The decrease in (^{3}H)-uridine incorporation around 240 min may possibly be due to an increased "turnover" of the RNA with the hormone treatment.

Again, the shapes of the graphs in Fig. 2A and B indicate that from 45 to 120 or 180 min after the mineralocorticoid injection there is also a generalized increase in the synthesis of histones (acid-soluble proteins) in the chromatin material. A point of interest is the fact that there may be a decreased synthesis of histones during the first 30 min.

Figure 3A and B suggest a generalized increase in the synthesis of nonhistone chromatin proteins (acid-insoluble proteins) from 15 to 60 or 90 min following aldosterone treatment. The decrease in (^{14}C)-protein hydrolyzate incorporation from 90 to 240 min may possibly indicate an increased "turnover" of the nonhistone proteins with the mineralocorticoid treatment.

However, it should be borne in mind that the time course and magnitude of increase in renal cortical chromatin "RNA, histone, and nonhistone protein synthesis" measured "*in vivo*" remain open to question since the effect of aldosterone on the specific activities of the immediate precursors of the RNA and chromatin associated proteins have not been measured.

Acetylation of histones. Table I shows that 15 min after aldosterone administration there was a significant 150% increase in the presence of radioactive acetyl groups in the chromatin associated histones. For both the

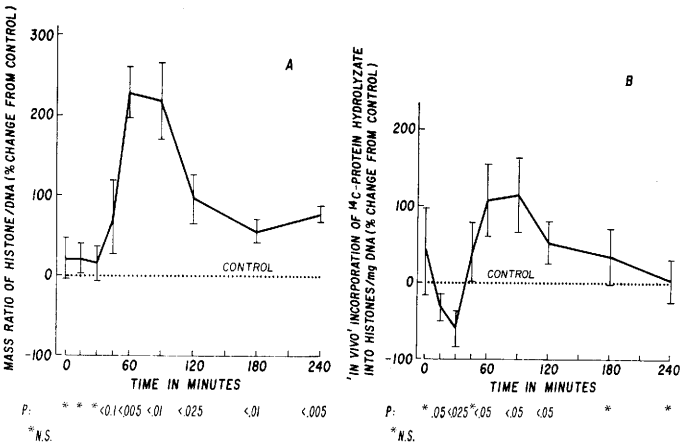

FIG. 2. Histones (acid-soluble proteins). Each point on the curve for the aldosterone-treated rats represents the mean ± SD of three separate experiments. The values of the points for the sham-injected rats (controls) have been averaged out to give straight base lines. p values represent levels of significance of difference calculated from t test. *NS, $p > 0.10$. Time is minutes after aldosterone administration. (A) Mass ratio (mg histone/mg DNA)—% change from control values. The average value from 0 to 240 min for the control rats was 0.525 ± 0.057 (SD) mg histone/mg DNA. (B) *In vivo* incorporation of (^{14}C)-protein hydrolyzate dpm into histones/mg DNA—% change from control values. The average value from 0 to 240 min for the control rats was 488 ± 78 (SD) dpm of histone/mg DNA.

control and hormone-treated animals approximately 33% of the radioactivity in the histones were present as amino acids (nonvolatile ^{3}H-acetate) derived from acetate by metabolic conversion. Unlike acetyl groups in histones, this radioactivity cannot be recovered as acetic acid by steam distillation of acid hydrolyzates (27). Again, it should be noted that we have not measured the acetate "pool" sizes in the renal cortices of the two groups of animals.

In order to establish the site of acetylation it was found that approximately 25% of the volatile (^{3}H)-acetic acid in both groups of rats were unstable to treatment with 2 *M* neutral hydroxylamine for 1 hr, a procedure which would hydrolyze *O*-acetyl but not *N*-acetyl linkages (16). Thus approximately 75% of the volatile (^{3}H)-acetate were in *N*-aceyl linkages. It has already been shown that 50% of the acetyl groups in the arginine-rich F3 histone fraction are *O*-acetylated (16). Furthermore about 60% of the acetyl groups of histones are to be found in the arginine-rich F2al histone fraction (16). Virtually all of the acetyl groups are recovered in the amino-terminal residue or as ε-*N*-acetyllysine, which occurs internally at position-16 of fraction F2al (16).

Discussion. We have made some attempts to shed further light on the mechanism by which aldosterone brings about known increases (1) in the rates of RNA synthesis in the nuclei of the kidney cortex by attempting to study the changes in the synthesis of chromatin components and the acetylation of histones. Edelman (1) demonstrated that renal cortical RNA synthesis *in vivo* was significantly increased 90 min after aldosterone treatment. Utilizing the technique of DNA–RNA hybridization we have also reported (28) that 45 min after aldosterone administration there was a qualitative change in the renal cortical nuclear RNA.

The present data suggests that 15, 30, and 45 min following aldosterone treatment there appears to be a generalized increase in the synthesis of the nonhistone proteins, RNA, and histones, respectively, in the chromatin material. These findings may explain some of the results we obtained earlier (6). We found that 90 min after aldosterone administration 1.2% of the guanine–cytosine nucleotide pairs of the renal cortical chroma-

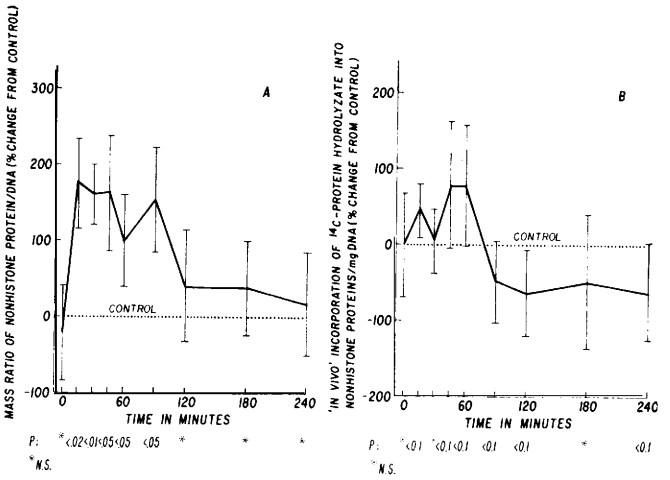

FIG. 3. Chromatin associated nonhistone proteins (acid-insoluble proteins). Each point on the curve for the aldosterone treated rats represents the mean ± SD of three separate experiments. The values of the points for the sham-injected rats (controls) have been averaged out to give straight base lines. p values represent levels of significance of difference calculated from t test. *NS, $p > 0.10$. Time is minutes after aldosterone administration. (A) Mass ratio (mg nonhistone protein/mg DNA)—% change from control values. The average value from 0 to 240 min for the control rats was 0.271 ± 0.038 (SD) mg nonhistone protein/mg DNA. (B) *In vivo* incorporation of (^{14}C)-protein hydrolyzate dpm into nonhistone proteins/mg DNA—% change from control values. The average value from 0 to 240 min for the control rats was 668 ± 56 (SD) dpm of nonhistone protein/mg DNA.

tin DNA from the adrenalectomized and sham-injected rats bound (^{14}C)-actinomycin D while only 0.6% of the same nucleotide pairs of the chromatin DNA from the hormone-treated animals did so. This could possibly be explained by the large accumulation of histones and nonhistone chromatin proteins 90 min after the mineralcorticoid injection. Both proteins, but especially histones, are known to restrict the binding of actinomycin D to DNA (29, 30).

Histones (7), chromosomal RNA (8), and nonhistone chromatin proteins (9) have all been implicated as possible regulators of transcription in eukaryotes. Acidic nuclear proteins have been suggested as regulatory intermediates in the restriction of DNA-template activity by histones. Specific acidic nuclear proteins which are covalently linked to unique species of chromosomal RNA have been implicated in contributing tissue specificity to chromatin constructed from histones and DNA through *in vitro* reannealing processes (8, 9).

Our present data seem to concur with those of several previous studies. These former investigations have already demonstrated that the early action of some steroid hormones was an alteration in the synthesis of the various chromatin components in the target organs (10–15). At least one study suggested that synthesis of the arginine-rich histones in calf endometrium nuclei is associated with periods of enhanced RNA synthesis following estrogen treatment (15). Thus aldosterone appears to behave similarly to several other steroids in that it has a very early influence on the synthesis of the nonhistone proteins, RNA, and histones in the chromatin material. All three of these components are believed to be involved in the specificity of gene activation and repression (7–9).

The experiments described in this investigation also show that 15 min following aldosterone treatment there was a significant 150% increase in the presence of radioactive acetyl groups in the chromatin associated histones. In both the control and hormone-treated animals approximately 75% of the

TABLE I. Effect of Aldosterone on the *"in Vivo"* Acetylation of Histones from the Chromatin of Rat Kidney Cortex 15 min after the Hormone Administration.

Conditions of expt	Sodium (methyl-^{3}H) acetate incorporated into histones[a] (dpm/mg histone)	Significance[b] p	Percentage of sodium (methyl-^{3}H) acetate incorporated into *O*-acetyl linkage[c] (%)	Significance[b] p
Aldosterone-treated	11,206 ± 3242[d](10)[e]	<.005	21.5 ± 8.1 (4)	<.45(NS)
Control	4424 ± 675 (10)		25.3 ± 11.8 (4)	

[a] The presence of radioactive acetyl groups in the histones was verified by acid hydrolysis and steam distillation of volatile (^{3}H)-acetic acid.

[b] According to the unpaired t test; NS = not significant.

[c] Percent = [Amount of sodium (methyl-^{3}H) acetate in histones unstable to treatment with 2 *M* neutral hydroxylamine/total volatile (^{3}H)-acetic acid from histones] × 100.

[d] Mean values ± 1 standard deviation (SD).

[e] The number of experiments is given in parentheses.

acetyl groups were in *N*-acetyl linkages.

Recent studies of histone metabolism in the cells of higher organisms have indicated that the basic proteins of the nucleus are modified by the attachment of acetyl groups (16). The biological significance of these structural modifications of proteins associated with DNA in the chromatin remains an unsolved problem, but there is growing evidence (16) that acetylation of the histones represents one of the earliest chemical events in the process of gene activation for RNA synthesis.

Allfrey (16) has shown a strong correlation between the patterns of RNA synthesis and histone acetylation in liver responding to stimulation by cortisol. The acetylation of the F2al fraction was increased within 15 min after administration of the hormone. Cortisol stimulation of the liver is only one instance of hormone-induced gene activation. Another example is provided by estradiol-17β, which stimulates RNA synthesis in the uterus (17, 18). Here too, there is a correlation with histone acetylation, because estradiol-17β has been reported to stimulate the acetylation of histones by cell-free extracts of rat uterus (19).

Thus it is quite significant that we find this increase in the rate of histone acetylation 15 min after aldosterone administration, since Edelman (1) found a significant increase in RNA synthesis only 90 min after the mineralocorticoid treatment while we (28) have shown a change in the quality of renal cortical nuclear RNA 45 min later; in the present study as well, we have found an increase in chromatin associated RNA by only 30 min. In other words, histone acetylation precedes RNA synthesis.

Summary. This investigation was undertaken in order to obtain some knowledge of the mechanism by which aldosterone brings about known increases in the rates of RNA synthesis in the nuclei of the kidney cortex by attempting to study the changes in the synthesis of the chromatin components as well as changes in the acetylation of histones. Specificity of gene activation and repression in eukaryotes is believed to involve these chromatin components and the acetylation of histones. The present data suggests that following aldosterone administration there appears to be a generalized sequential increase in the synthesis of the nonhistone proteins, RNA, and histones in the chromatin material. Furthermore the mineralocorticoid treatment leads to a very early increase in the rate of histone acetylation.

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