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Modification of Histone Binding in Calf Thymus Chromatin and in the Chromatin–Protamine Complex by Acetic Anhydride†

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ABSTRACT: A relationship between side-chain modification of histones and their displaceability from DNA has been investigated using calf thymus chromatin which was chemically acetylated with acetic anhydride. When the chromatin is treated with increasingly higher concentrations of the reagent, histones become acetylated to an increasingly greater extent, attaining the modification at 23–24 sites for histone I, 5–6 for IIb_1, 9–10 for IIb_2, 5–6 for III, and 3–4 for IV. As the chromatin becomes more acetylated, NaCl concentrations required for histone removal are lowered. Saturation binding of protamine does not bring about either an increase in the number of acetylation sites of histones in chromatin or a decrease of the NaCl requirement for dissociation of the acetylated chromatins. A comparison of the present results with the extents of histone acetylation known to occur enzymatically in vivo indicates that the complete removal of somatic histones during transformation of chromatin in spermiogenesis cannot be explained on the basis of decreased binding of the histone to DNA by acetylation or by a combination of acetylation and protamine binding, suggesting that the displacement process may require some additional processes.

The process of histone displacement during the transformation of nucleohistone into nucleoprotamine in spermiogenesis may involve a combination of different mechanisms which would weaken interactions between histones and DNA. When nucleohistone is exposed in vitro to protamine, the protein binds stoichiometrically to DNA, weakening the electrostatic interactions between histones and DNA (Wong and Marushige, 1975). The saturated binding of protamine does not, however, cause major displacement of any of the histone fractions (Wong and Marushige, 1975), and only histone I is completely dissociated from the DNA by addition of excess protamine (Evans et al., 1970; Marushige and Dixon, 1971; Wong and Marushige, 1975). Phosphorylation (Marushige et al., 1969; Sung and Dixon, 1970; Louie and Dixon, 1972) and acetylation (Candido and Dixon, 1971, 1972a–c) of histones have been observed in developing trout testes. These modifications which are found in the spermatids (Marushige et al., 1969; Candido and Dixon, 1972b) are possibly associated with the replacement process. In an attempt to elucidate the mechanisms involved in the displacement of the histones during the replacement process, a further investigation of the chemical acetylation of chromatin, first described by Simpson (1971), has been done. The relationship between the extent of side-chain modification of histones and their displaceability from DNA is examined in conjunction with protamine binding to chromatin.

Materials and Methods

Preparation of Chromatin. Calf thymus chromatin was prepared by the method of Marushige and Bonner (1966) with a modification previously described (Wong and Marushige, 1975), and stored in 50% glycerol at −20 °C. Just before use, the chromatin was diluted five times with 5 mM sodium borate (pH 8.2) and centrifuged at 17 000*g* for 20 min. The sediment was suspended in the borate buffer at a concentration equivalent to approximately 1 mg of DNA/ml and then sheared in a Waring blender at 100 V for 3 min. After centrifugation at 17 000*g* for 20 min, the supernatant was removed and used as the chromatin.

Calf thymus chromatin–protamine complex was prepared by dropwise addition of concentrated protamine solution to the calf thymus chromatin solution which was vigorously stirred in an ice bath. The final concentration of protamine was ad-

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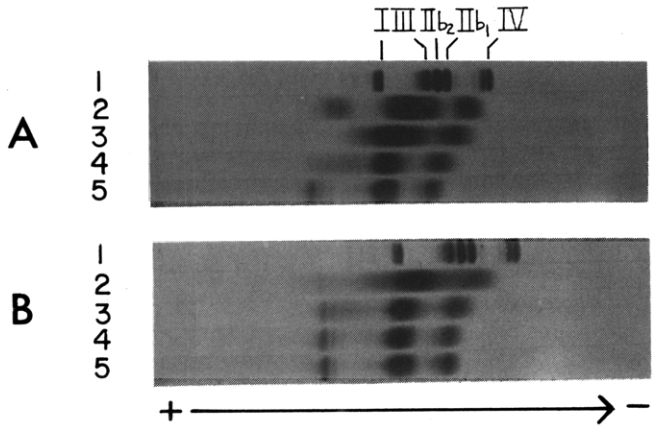

FIGURE 1: Acetylation of chromatin. In A, calf thymus chromatin was treated with 0 (1), 0.7 mM (2), 1.4 mM (3), 3.5 mM (4), and 7 mM (5) acetic anhydride in the presence of 0.15 M NaCl. In B, the chromatin was treated with 7 mM acetic anhydride in the presence of 0 (2), 0.05 M (3), 0.10 M (4) and 0.15 M (5) NaCl. B-1 represents histones of control (unacetylated) chromatin. Acid-soluble proteins from control chromatin equivalent to 18 μg of DNA and from acetylated chromatins equivalent to 54 μg of DNA were applied to the gels, which were then electrophoresed at 150 V for 240 min.

justed to 150 μg/ml at the chromatin concentration equivalent to 300 μg of DNA/ml. Under these conditions all of the added protamine was bound to the chromatin, and the protamine binding to chromatin was saturated (Wong and Marushige, 1975).

Acetylation of Chromatin with Acetic Anhydride. Calf thymus chromatin in 5 mM sodium borate (pH 8.2) was treated with 0.7, 1.4, 3.5, and 7 mM acetic anhydride at the chromatin concentration equivalent to 300 μg of DNA/ml in a total volume of 30 ml. In some experiments, [^{3}H]acetic anhydride (2 mCi/mmol) was used. While the chromatin solution was vigorously stirred, 5 M NaCl was added dropwise to a final concentration of 0.15 M unless otherwise indicated. The resulting solution was stirred for 10 min further before the addition of 0.02 ml of acetic anhydride which had been appropriately diluted with benzene. The reaction was allowed to proceed for 20 min, while maintaining the pH between 8.0 and 8.2 by addition of 0.1 M NaOH. Chromatin thus acetylated was either immediately treated with 0.2 M HCl for characterization of histones or dialyzed overnight against 10 mM Tris buffer (pH 7.0) for usage in dissociation experiment.

Characterization of Acetylated Histones. Acetylated chromatins were treated (0 °C, 30 min) with 0.2 M HCl and centrifuged at 17 000*g* for 20 min. Aliquots of acid extracts thus obtained were dialyzed against 0.87 M acetic acid containing 20% sucrose and analyzed by polyacrylamide gel electrophoresis according to Panyim and Chalkley (1969). The gels (5 × 100 mm) contained 2.5 M urea.

In order to obtain individual histone fractions, acid extracts containing 8–18 mg of histones were treated with 20% trichloroacetic acid, followed by centrifugation at 17 000*g* for 20 min. The precipitate was washed successively with acidified acetone (0.1 ml of concentrated HCl in 200 ml of acetone) and acetone and dried under vacuum. The precipitate was then dissolved in 0.01 M HCl (1 ml) and fractionated by chromatography on a Bio-Gel P-60 column (1.2 × 90 cm) as previously described (Marushige and Dixon, 1971). The chromatographic fractions comprising each histone were combined, lyophilized, and further characterized by polyacrylamide gel electrophoresis. When the extent of acetylation was examined using [^{3}H]acetic anhydride, each histone fraction was dissolved in H_2O and assayed for protein by the method of Lowry et al. (1951) and for radioactivity in a Beckman LS-250 liquid scintillation counter using a dioxane-based scintillation fluid.

Dissociation of Chromatin. Acetylated chromatins, which had been dialyzed against 0.01 M Tris buffer (pH 7), were appropriately diluted with the same buffer, and 5 M NaCl was then added dropwise to the stirred chromatin solutions to final concentrations ranging from 0.15 M to 1.2 M. The final chromatin concentration was equivalent to 120 μg of DNA/ml. In order to examine the effect of protamine binding on histone dissociation, acetylated chromatins were treated with saturating amount of protamine (the input ratio of protamine to DNA by weight, 0.5). The resultant chromatin–protamine complexes were subjected to the same NaCl treatment as that for the corresponding control acetylated chromatins. The acetylated chromatins and the complexes were then centrifuged at 60 000 rpm for 5 h in a Spinco Type 65 rotor. After centrifugation, the upper 10 ml of the supernatant was pipetted off, dialyzed exhaustively against 0.1 M acetic acid at 4 °C, and lyophilized. Histones thus obtained were then treated (16–24 h at 4 °C) with 5 M urea–0.2 M 2-mercaptoethanol, adjusted to 0.87 M acetic acid, and analyzed by polyacrylamide gel electrophoresis.

Results

Acetylation of Chromatin. Calf thymus chromatin has been treated with various concentrations (0.7–7 mM) of acetic anhydride in the presence of 0.15 M NaCl (pH 8.0–8.2) and histones isolated thereupon have been analyzed by electrophoresis. As seen in Figure 1A, the histones show increasingly slower electrophoretic mobilities as the acetic anhydride concentration increases. As also seen in this figure, histones isolated from the chromatins which have been treated at lower concentrations of acetic anhydride (0.7 and 1.4 mM; Figure 1A, 2 and 3, respectively) are electrophoretically highly heterogeneous, while at higher acetic anhydride concentrations (3.5 and 7 mM; Figure 1A, 4 and 5, respectively) the histone bands are less diffused and more sharply focused. As shown in Figure 1B, acetylation of histones is markedly affected by the presence of relatively low concentrations of NaCl during the treatment of chromatin with acetic anhydride. At 7 mM acetic anhydride calf thymus chromatin acetylated in the absence of the salt gives very heterogeneous histone bands (Figure 1B, 2). The heterogeneity diminishes when the chromatins are acetylated in the presence of 0.05–0.15 M of NaCl (Figure 1B, 3–5). Although dissociation of histones from the chromatin does not occur at these salt concentrations, the binding of histones to the DNA appears to be altered at these ionic strengths in such a manner that the basic proteins in the chromatin become more readily available for chemical acetylation. In order to characterize individual histone fractions with respect to their side-chain modifications, histones isolated from calf thymus chromatin which had been treated with various concentrations of acetic anhydride have next been fractionated by chromatography on Bio-Gel P-60 columns. As seen in Figure 2A, histones from control (unacetylated) chromatin are eluted in the order of I, IIb_1, IIb_2, III, and IV. Histones IIb_1, IIb_2, and III, which are eluted closely together in this system, can be purified further by rechromatography on the same column (dotted lines in Figure 2A). When histones obtained from chromatins which have been treated with various concentrations of acetic anhydride are fractionated on the Bio-Gel P-60 column (Figure 2, B–C), the three major peaks are present, but the positions and appearance of the peaks have changed. As the histones become more acetylated, they are

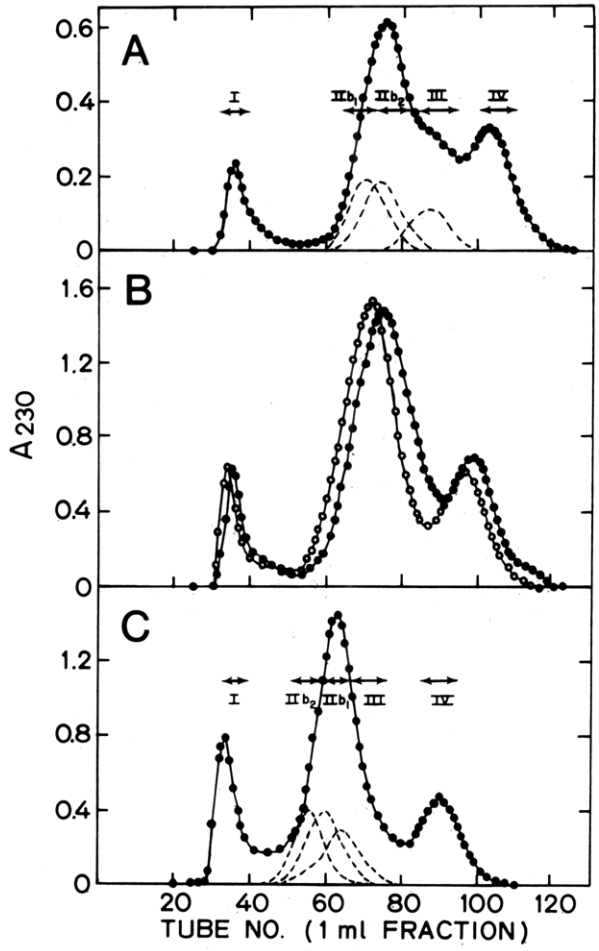

FIGURE 2: Bio-Gel P-60 column chromatography of acetylated histones. Histones obtained from control chromatin (A) and from chromatins acetylated at 0.7 mM (B, solid circles), 1.4 mM (B, open circles), and 7 mM (C) acetic anhydride were applied to Bio-Gel P-60 columns (1.2 × 90 cm) and eluted with 0.01 M HCl at a flow rate of 10–12 ml/h. The dotted lines beneath the middle peak represent the rechromatography of histones IIb_1, IIb_2, and III (cf. text).

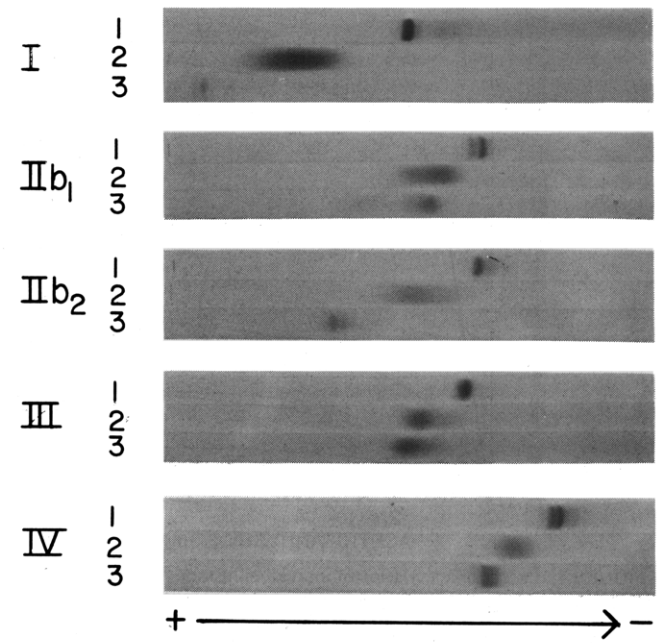

FIGURE 3: Polyacrylamide gel electrophoresis of acetylated histone fractions. Histones obtained from control (unacetylated) chromatin (1) and from chromatins treated with 1.4 mM (2) and 7 mM (3) acetic anhydride were fractionated into five major fractions (I, IIb_1, IIb_2, III and IV) by Bio-Gel P-60 column chromatography and analyzed by electrophoresis. For control histones, a 4-μg sample of each of the five major histones was applied to the gels. For acetylated histones, 20 μg of histone I, 11–13 μg each of histones IIb_1 and IIb_2, and 6–7 μg of histones III and IV were used. Electrophoreses were carried out at 150 V for 240 min.

eluted increasingly earlier, and the shoulder region between the middle peak and the histone IV peak is absent, making the middle peak much more symmetrical. The earlier elution of acetylated histones is presumably due to weak cation-exchange properties of the Bio-Gel P series resins (cf. Ling et al., 1969; Candido and Dixon, 1972a). In the case of histones from chromatins acetylated at 1.4 mM (open circles in Figure 2B) and 7mM (Figure 2C) acetic anhydride, rechromatography of the front, middle, and tail portions of the middle peak containing histones IIb_1, IIb_2, and III (dotted lines in Figure 2C) gives three electrophoretically distinguishable components. Oxidation of these three components followed by electrophoretic analysis have identified cysteine-containing histone III to be eluted last. Further examination of the first two fractions by cyanogen bromide cleavage has revealed that methionine-containing histone IIb_2 is eluted first, followed by histone IIb_1. For histones from 0.7 mM acetic anhydride treated chromatin (solid circles in Figure 2B), rechromatography of the middle peak can separate histone III from histone II, but the method has been found to be incapable of fractionating histones IIb_1 from IIb_2. Electrophoretic profiles of individual histone fractions obtained from calf thymus chromatins which have been acetylated with 1.4 and 7 mM acetic anhydride, as well as those from control chromatin, are presented in Figure 3. It is apparent that all five major histone fractions are acetylated.

When chromatin is acetylated with 1.4 mM acetic anhydride, all of the histones appear to be quite heterogeneous. In contrast, chromatin acetylated at the higher concentration gives sharper electrophoretic bands. Under conditions employed in the present experiments, acetic anhydride acetylates primarily amino groups, and phenolic hydroxyl groups of tyrosyl residues (cf. Riordan and Vallee, 1972 a,b). The observed changes of electrophoretic profiles of histones are therefore due mainly to acetylation of ϵ-amino group of lysyl residues to different extents. Sharpening of electrophoretic bands of histones at higher concentrations of the reagent suggests that certain restricted numbers of lysyl residues of each histone fraction in the chromatin are accessible to chemical acetylation.

Extent of chemical acetylation of individual histone fractions is next examined. After fractionation of acetylated histones extracted from chromatins which had been treated with various concentrations of [^{3}H]acetic anhydride, moles of acetate incorporated per mole of histone have been calculated on the basis of specific radioactivity and the molecular weight of each histone fraction. In this experiment, total histones have been treated (60 min at room temperature) with 0.5 M hydroxylamine (pH 7.5) in order to deblock *O*-acetyltyrosine (cf. Riordan and Vallee, 1972b) prior to chromatography on Bio-Gel P-60 columns. Approximately 10% of the radioactivity incorporated into the histone fractions is removed by this treatment. As summarized in Figure 4, the extent of acetylation as a function of acetic anhydride concentrations gives hyperbolic curves. The maximum acetylation of all of the histone fractions occurs essentially at 7 mM acetic anhydride, leading to the modification of, on the average, 23.2 sites in histone I, 5.7 sites in histone IIb_1, 9.5 sites in histone IIb_2, 5.5 sites in histone III, and 2.7 sites in histone IV. A correlation between the electrophoretic band of the histones (Figure 3) and

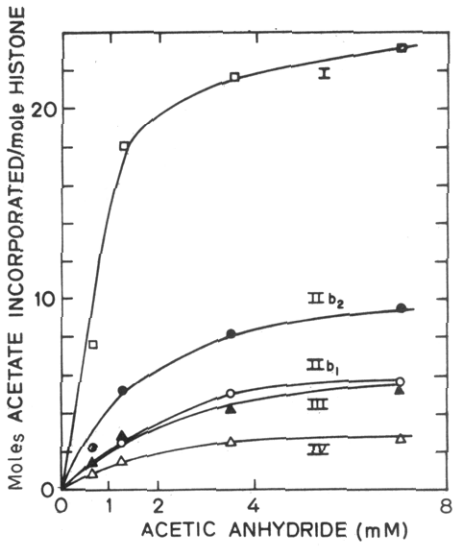

FIGURE 4: Extent of acetylation of individual histone fractions. Histones obtained from chromatins which had been treated with 0.7, 1.4, 3.5, and 7 mM [^{3}H]acetic anhydride were first treated with 0.5 M hydroxylamine (pH 7.5) and fractionated by chromatography on Bio-Gel P-60 columns. Fractions comprising histones I (□), IIb$_1$ (○), IIb$_2$ (●), III (▲), and IV (△) were pooled, lyophilized, and assayed for protein and radioactivity. No separation of histones IIb$_1$ and IIb$_2$ of 0.7 mM acetic anhydride treated chromatin was obtained and, therefore, the value obtained for the mixture of these histone fractions was presented (◑).

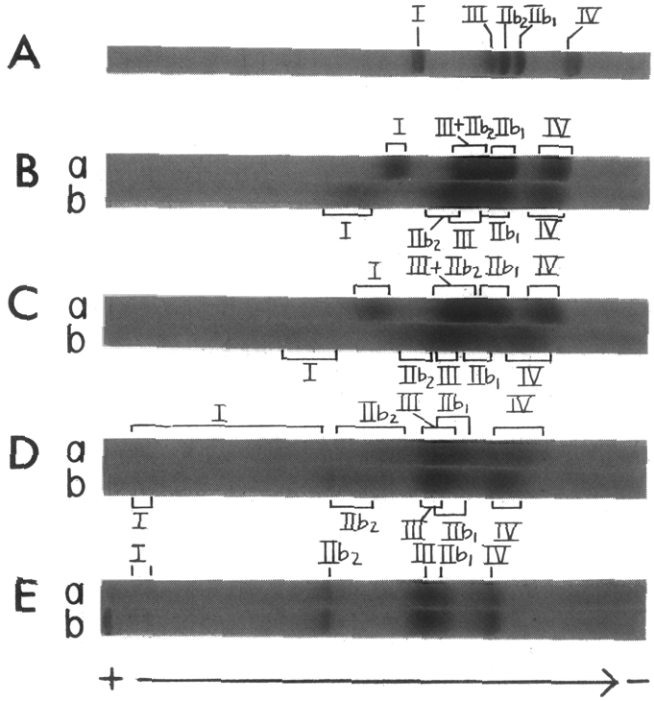

FIGURE 5: Effect of protamine binding on acetylation of chromatin. Calf thymus chromatin (a), and the chromatin–protamine complex (b), were acetylated either with 0.7 mM (B), 1.4 mM (C), and 7 mM (D) acetic anhydride in the absence of 0.15 M NaCl or with 7 mM acetic anhydride in the presence of 0.15 M NaCl (E). Acid-soluble proteins from acetylated chromatins equivalent to 70 μg of DNA as well as 25 μg of control histones (A) were applied to the gels and electrophoresed at 150 V for 255 min. Protamine ran off from the gels under the present electrophoretic conditions. The histone classes were identified from fractionation on Bio-Gel P-60 columns.

their acetyl content (Figure 4) cannot, however, be clearly understood at present. As particularly evident in the case of histone IV, this histone fraction obtained from the chromatin which has been acetylated with 7 mM acetic anhydride (Figure 3, IV-3) appears to migrate much slower than expected from the difference between monoacetyl histone IV (the slower migrating band) and the unmodified species (the faster migrating band) in the control chromatin (Figure 3, IV-1). Moreover, histone IV (similarly, histones IIb$_1$, IIb$_2$ and III) obtained from the chromatin which has been acetylated with 1.4 mM acetic anhydride gives a highly diffused electrophoretic band (Figure 3, IV-2) rather than a multiple of discrete bands.

Effect of Protamine on Acetylation of Chromatin. When calf thymus chromatin is exposed to protamine, the protein binds to the chromatin, weakening the interactions between histones and DNA in the chromatin (Wong and Marushige, 1975). Thus, it seems possible that the binding of protamine to chromatin may affect accessibility of histones to chemical acetylation. In order to test this possibility, calf thymus chromatin and the chromatin–protamine complex have been treated with acetic anhydride and their histones examined by polyacrylamide gel electrophoresis. The electrophoretic profile of histones from unacetylated calf thymus chromatin is shown in Figure 5A. In Figures 5B–D, acetylations of the chromatin and of the chromatin–protamine complex which have been treated with 0.7, 1.4, and 7 mM acetic anhydride in 5 mM borate buffer (pH 8.2) but without NaCl are compared. At 0.7 and 1.4 mM acetic anhydride, effects of protamine binding are seen in the histones of the acetylated complexes which are more heterogeneous and more retarded than those of the corresponding acetylated chromatin (compare Figure 5B-b with 5B-a; Figure 5C-b with 5C-a). The chromatin–protamine complex acetylated at 7 mM acetic anhydride gives histone bands which are more retarded, but more sharply focused (Figure 5D-b) when compared with the corresponding acetylated chromatin (Figure 5D-a). However, when calf thymus chromatin and the chromatin–protamine complex are acetylated with 7 mM acetic anhydride in the presence of 0.15 M NaCl, there is no difference between the electrophoretic profiles of histones obtained from these two chromatins (Figure 5E). It appears therefore that there is no increase of acetylation sites of histones as a consequence of protamine binding to the chromatin. The greater acetylation of the chromatin–protamine complexes in the absence, but not in the presence, of 0.15 M NaCl suggests that both the salt and protamine binding destabilize similar regions of the histone molecules in the chromatin.

Dissociation of Acetylated Chromatin. When calf thymus chromatin is treated with increasing concentrations of NaCl, the histones dissociate in the order of I (mainly at 0.4–0.6 M), IIb$_1$ and IIb$_2$ (0.6–1.2 M), and III and IV (1.2-2 M) (cf. Ohlenbusch et al., 1967; Fambrough and Bonner, 1968; Wilhelm and Champagne, 1969; Marushige and Dixon, 1971). To ascertain the effect of acetylation of histones on their dissociations from DNA, calf thymus chromatins acetylated with 1.4 and 7 mM acetic anhydride have been treated with various concentrations of NaCl, and the histones dissociated have been analyzed by polyacrylamide gel electrophoresis. As seen in Figure 6A-a and 6B-a, histone I is dissociated completely at 0.15 M NaCl when the chromatin is acetylated at either of the two acetic anhydride concentrations. For chromatin acetylated with 1.4 mM acetic anhydride (Figure 6A-a), histones IIb$_1$ and IIb$_2$ are removed by 0.6 M NaCl, while histones III and IV dissociate between 0.6 and 1.2 M NaCl. For chromatin acetylated with 7 mM acetic anhydride (Figure 6B-a), histones IIb$_1$ and IIb$_2$ are dissociated at 0.15–0.3 M NaCl, and histones III and IV dissociate at 0.6–0.9 M NaCl. Calf thymus chro-

matins acetylated with 1.4 and 7 mM acetic anhydride have been further treated with a saturated amount of protamine, and histone dissociation of these acetylated chromatin–protamine complexes has also been examined (Figure 6A-b and 6B-b). As clearly seen by comparing Figure 6A-b and -a as well as Figure 6B-b with -a, protamine binding to both acetylated chromatins has essentially no effect on the dissociation of histones. The dissociation of protamine from both complexes occurs at 0.9 M NaCl.

Discussion

When calf thymus chromatin is treated with increasing concentrations of acetic anhydride, all of the histone fractions become acetylated to an increasingly greater extent, attaining essentially their maximum modifications at 7 mM under the conditions used in the present study (Figures 1–4). Thus, each histone fraction maximally acetylated electrophoreses relatively homogeneously (Figure 3). Moles of acetate incorporated per mole of histone, which are resistant to hydroxylamine treatment, have been found to be 23.2, 5.7, 9.5, 5.4, and 2.7 for histones I, IIb_1, IIb_2, III, and IV, respectively (Figure 4). These values presumably represent the numbers of lysyl residues whose ϵ-amino group has been chemically modified. The exceptions are histones IIb_2 and III, where the α-amino group of the N-terminal amino acids can also be acetylated. Using competitive labeling techniques, Malchy and Kaplan (1974) have reported that the N-terminal amino acid of histone IIb_2, Pro, is more reactive toward acetic anhydride than the ϵ-amino group of lysyl resides of histones in calf thymus chromatin. In calf thymus histone IV, one-half of the population of molecules is acetylated at a single site (DeLange et al., 1969), and the total number of the acetylation sites after the chemical acetylation should be between 3 and 4. Histones can be enzymatically acetylated in vivo (Allfrey et al., 1964), and the acetylation occurs at ϵ-amino group of lysyl residues (Gershey et al., 1968; Vidali et al., 1968; DeLange et al., 1968). Further studies (Candido and Dixon, 1971, 1972a,c; DeLange et al., 1972) have shown that one site (lysyl residue 5) can be acetylated in histone IIb_1, four sites (lysyl residues 5, 10, 13, 18) in histone IIb_2, four sites (lysyl residues 9, 14, 18, 23) in histone III, and four sites (lysyl residues 5, 8, 12, 16) in histone IV. It is immediately apparent that the number of the sites accessible to chemical acetylation in vitro and those to enzymatic acetylation in vivo are quite similar in both histones III and IV, whereas histones IIb_1 and IIb_2 can be acetylated chemically to a much greater extent. In histone I, the internal lysyl residues are available for chemical acetylation in vitro but not for enzymatic acetylation in the cell. Although the sites of the chemical acetylation of histones remain to be investigated, the ϵ-amino groups of these lysyl residues that are available for enzymatic acetylation in vivo may also be accessible to chemical acetylation. In both instances, the groups being modified are likely to be accessible within the chromatin structure.

Examination of salt dissociation of chemically acetylated chromatin indicates that, as the histones become more acetylated, they require lower concentrations of NaCl for their removal from DNA (Figure 6). When chromatin is acetylated maximally at 7 mM acetic anhydride, the majority of histones IIb_1 and IIb_2 becomes displaceable at 0.15 M NaCl. However, the numbers of their modified sites (5.7 and 9.5 for IIb_1 and IIb_2, respectively) are far greater than that which can be achieved in vivo by enzymatic acetylation (1 and 4 for IIb_1 and IIb_2, respectively). Acetylation of chromatin with 1.4 mM acetic anhydride brings about the modification of 2.7 sites for

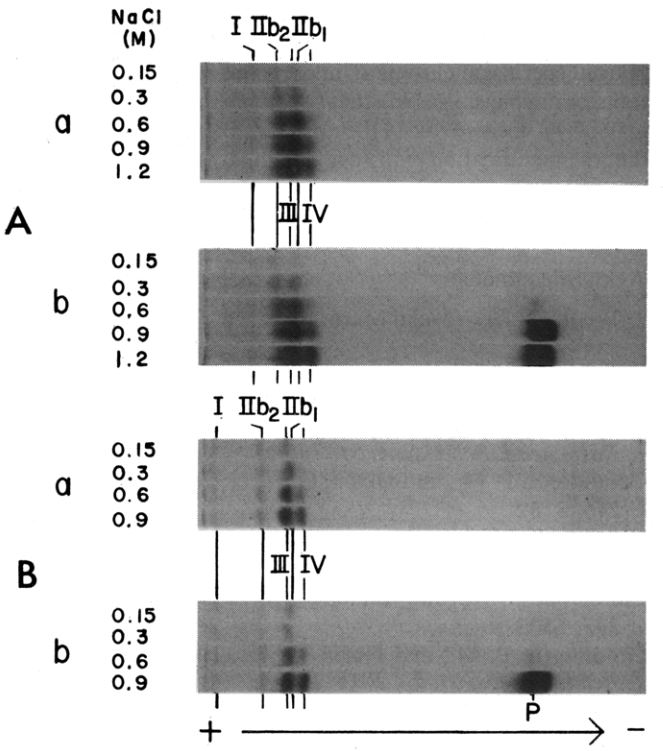

FIGURE 6: Dissociation of acetylated chromatin. Calf thymus chromatin was acetylated with 1.4 mM (A) and 7 mM (B) acetic anhydride. The acetylated chromatins (a) and those subsequently treated with protamine at the weight ratio of protamine to DNA equivalent to 0.5 (b) were treated with various concentrations of NaCl as indicated in the figure. Acid-soluble proteins dissociated from the acetylated chromatins equivalent to 50 μg of DNA were applied to the gels and electrophoresed at 1.5 mA/tube for 120 min. P, protamine.

histones IIb_1 and 5.2 sites for histone IIb_2. These modified histones, however, still require 0.6 M NaCl for their dissociation from DNA. Histones III and IV chemically acetylated at 5.4 and 2.7 sites dissociate from DNA only when the NaCl concentration is raised to 0.6–0.9 M. As shown in Figure 6, acetylated chromatins which are treated with saturating amount of protamine exhibit identical dissociation profiles as that of the corresponding untreated acetylated chromatins. These results seem reasonable considering the observation (Figure 5) that the regions of histone molecules, whose association to DNA is affected by protamine binding, appear to be similar to those regions which can be modified by acetylation. Histones IIb_1, IIb_2, III, and IV, even when acetylated enzymatically at all the known sites, would therefore, not be displaceable from DNA under physiological ionic conditions. The present results suggest that, while acetylation of histones IIb_1, IIb_2, III, and IV in spermatids (Candido and Dixon, 1972b) is likely to play a key role in the displacement of these histones during the transformation of nucleohistone into nucleoprotamine in spermiogenesis, the histone displacement cannot be explained by either acetylation or a combination of acetylation and protamine binding to transforming chromatins and requires some additional mechanisms. All of the major histone fractions are also known to be phosphorylated in developing testes (Marushige et al., 1969; Sung and Dixon, 1970). The sites of phosphorylation are highly restricted (Sung and Dixon, 1970), and the dissociation profile of phosphorylated histones is essentially identical with that of the unmodified species (Marushige et al., 1969). The question as to whether a combination of acetylation and phosphorylation of histones can explain their displacement remains to be investigated.

Marushige and Dixon (1971) have reported that highly heterogeneous and small basic protein components are detectable in trout spermatid chromatin undergoing active chromosomal transformation, suggesting that selective proteolysis of histones may play a decisive role in their displacement. It seems possible that the modification of histone binding by acetylation and/or phosphorylation may be a prerequisite for proteolytic removal of histones from the DNA.

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