Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mariman, E. C. M., van Eekelen, C. A. G., Reinders, R. J., Berns, A. J. M., & van Venrooij, W. J. (1982) *J. Mol. Biol. 154*, 103–119.
Morris, T., Marashi, F., Weber, L., Hickey, E., Greenspan, D., Bonner, J., Stein, J., & Stein, G. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 981–985.
Parker, M. I., & Fitschen, W. (1980) *Cell Differ. 9*, 23–30.
Plumb, M. A., Stein, G., & Stein, J. L. (1983a) *Nucleic Acids Res. 11*, 2391–2410.
Plumb, M. A., Stein, J. L., & Stein, G. (1983b) *Nucleic Acids Res. 11*, 7927–7945.
Prescott, D. M. (1966) *J. Cell Biol. 31*, 1–9.
Rickles, R., Marashi, F., Sierra, F., Clark, S., Wells, J., Stein, J., & Stein, G. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 749–753.
Robbins, E., & Borun, T. W. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57*, 409–416.
Sierra, F., Lichtler, A., Marashi, F., Rickles, R., van Dyke, T., Clark, S., Wells, J., Stein, G., & Stein, J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1795–1797.
Sittman, D. B., Graves, R. A., & Marzluff, W. F. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1849–1853.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.
Spaulding, J., Kajiwara, K., & Mueller, G. C. (1966) *Proc. Natl. Acad. Sci. U.S.A. 56*, 1535–1542.
Stein, G. S., & Borun, T. W. (1972) *J. Cell Biol. 52*, 292–307.5.
Stein, G. S., & Stein, J. L. (1984) *BioEssays 1*, 202–205.
Stein, G. S., Park, W. D., Thrall, C. L., Mans, R. J., & Stein, J. L. (1975) *Nature (London) 257*, 764–767.
van Eekelen, C. A. G., & van Venrooij, W. (1981) *J. Cell Biol. 88*, 554–563.
Weaver, R. F., & Weissmann, C. (1979) *Nucleic Acids Res. 7*, 1175–1193.
Wu, R. S., & Bonner, W. M. (1981) *Cell (Cambridge, Mass.) 27*, 321–330.

Structural Studies of Acetylated and Control Inner Core Histones†

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ABSTRACT: The role of acetylation on the conformation and association state of the inner core histone octamer isolated from HeLa cells was examined. Preparation of suitable quantities of pure acetylated and control inner core histones from HeLa cells required the development of a new preparative procedure. The results from size-exclusion high-performance liquid chromatography and sedimentation equilibrium studies indicated that acetylated inner core histones associate to species larger than the octamer and form a more stable complex. Circular dichroism studies demonstrated that the amount of α-helix increases with increasing association of the histones. Furthermore, acetylation results in an increase in the amount of α-helix, perhaps coupled through its effect on the association state. At high protein concentration and elevated temperature, the acetylated sample displays a greater increase in β-sheet content, relative to the control sample. This increase in β-sheet content may be induced during the association of the acetylated sample to species larger than the octamer. There is a marked effect on the conformation of both acetylated and control inner core histones as a function of protein concentration, ionic strength, and temperature. The difference in conformational flexibility and association state of the acetylated vs. the control inner histone core may play a significant role in the control of transcription in the nucleus.

Despite considerable effort, the effect of acetylation on the properties of chromatin is not clear. The original observation that histones H3 and H4 were acetylated (Allfrey et al., 1964) suggested that acetylation might be a mechanism for modulation of transcription. Hyperacetylation of histones H3 and H4 has been correlated with increased periods of transcription during development (Ruiz-Carrillo et al., 1974, 1976; Sung & Dixon, 1970; Christensen et al., 1984; Christensen & Dixon, 1982; Oliva & Mezquita, 1982; Grimes & Henderson, 1983; Burdick & Taylor, 1976). A number of schemes are available to fractionate active from inactive chromatin, and the active fractions seem to be enriched in acetylated material (Levy-Wilson et al., 1979; Davie & Candido, 1980; Davie & Saunders, 1981).

Conversely, the transcriptional activity of chromatin has been reported to have decreased (Mathis et al., 1978) or not to be affected (Lilley & Berendt, 1979) by acetylation of the histones.

The action of DNase I on acetylated chromatin is reported to be preferential and much more rapid than control chromatin (Mathis et al., 1978; Nelson et al., 1978; Simpson, 1978; Sealy & Chaulkley, 1978; Vidali et al., 1978; Davie & Candido, 1980), suggesting that acetylation alters chromatin conformation. The DNase digestion experiments, and the observed

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correlations of acetylation with increased transcriptional activity, have prompted much research to investigate the effect of acetylation on the conformation of chromatin.

The discovery that *n*-butyrate-treated HeLa and Friend erythroleukemia cells contained highly increased amounts of acetylated histones opened the way for extensive studies on the effects of acetylation (Riggs et al., 1977). The results of physical studies on the effect of acetylation on chromatin and core particles are not conclusive, and the observed structural changes are small [see Simpson (1978), Bode et al. (1980), and Reczek et al. (1982)]. It has been reported that control and acetylated chromatin, stripped of H1 and reconstituted with chicken erythrocyte H1, showed thermal denaturation profiles that indicated that the acetylated reconstitute was destabilized, compared to the control reconstitute. Hüvos et al. (1984) and Yau et al. (1982, 1983) reported that the premelt transition of mono-, di-, and trinucleosomes was not affected by acetylation; however, the intermediate and major thermal denaturation transitions were broader and at a lower T_m for acetylated material. This would suggest a lowering of stability for the acetylated samples. An unfolded nucleosome, termed a "lexisome", was recently isolated from transcribing rDNA chromatin from *Physarum Polycephalum* (Prior et al., 1983). A study by McGhee et al. (1983) indicated that histone hyperacetylation does not, in itself, prevent formation of higher order folding of chromatin. A recent paper by Ausio and van Holde (1985) concluded that in a low physiological ionic strength, there were small differences in hydrodynamic properties and circular dichroism spectra between acetylated chromatin (7–17 acetyls/nucleosome) and control chromatin but there was a significant difference in thermal denaturation and nuclease sensitivity. It was concluded that at physiological ionic strength, in the absence of factors other than acetylation, highly hyperacetylated nucleosomes remain essentially folded. Recently, neutron scattering studies on core particles acetylated to different levels showed no evidence of unfolding (Imae et al., 1986).

The dissociation/association pathway for histones has been well-defined using inner core histones obtained from calf thymus and chicken erythrocytes (Eickbush & Moudrianakis, 1978; Godfrey et al., 1980; Stein & Page, 1980). Under conditions of high ionic strength and high concentration, the histones exist as an octamer with a molecular weight of 108 000. Upon lowering the ionic strength, or the protein concentration, the octamer dissociates into a dimer containing H2A and H2B and a hexamer containing a tetramer made up of two each of H3 and H4 and a dimer made up of H2A and H2B (Eickbush & Moudrianakis, 1978; Godfrey et al., 1980). Further decrease in either the concentration or the ionic strength leads to the dissociation of the hexamer into an H3/H4 tetramer and an H2A/H2B dimer (Eickbush & Moudrianakis, 1978; Godfrey et al., 1980). These are the limiting species under most conditions. In solution, these species exist in a rapid and reversible equilibrium. Temperature and urea perturbation studies have suggested that the dimer/tetramer interaction is due to hydrogen bonding (Eickbush & Moudrianakis, 1978). Additional support for this comes from precise thermodynamic measurements of the enthalpy of association which is consistent with a predominance of hydrogen bonding (Benedict et al., 1984).

In an attempt to precisely determine the effect of acetylation on both histone conformation and histone/histone interactions, an investigation was undertaken to examine the association state of the isolated histone octamer in both the control and hyperacetylated state in solution. In the study herein, the techniques of gel chromatography and sedimentation equilibrium were used to determine the effect of acetylation on the dynamic equilibrium between the octamer and its component species. Circular dichroism was also employed to determine the effect of acetylation on the conformation of the histone proteins.

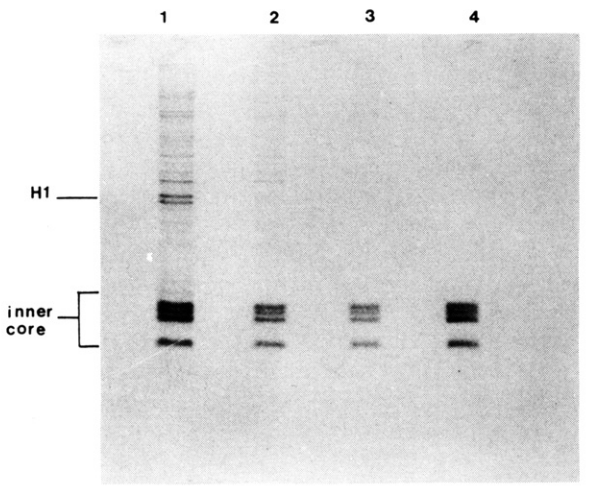

FIGURE 1: SDS–polyacrylamide gel electrophoresis of samples from combined Dowex–hydroxylapatite preparation. Lane 1, pooled extracts (CSK/AS/EDTA) of digested nuclei, "starting material"; lane 2, after treatment with Dowex AG 50W-X2 using 0.6 M NaCl/0.5 M urea, pH 8.0, elution; lane 3, after chromatography on Bio-Gel A-0.5m, after removal of 0.5 M urea by dialysis; chromatography was in 0.6 M NaCl buffer; lane 4, after removal of DNA by dialysis into 2 M KCl buffer and chromatography on Bio-Gel HT hydroxylapatite in 2 M KCl buffer.

Materials and Methods

Cell Culture. HeLa S3 cells were grown by the MIT Cell Culture Center (Cambridge, MA) as previously described (Reczek et al., 1982).

Preparation of Nuclei. The nuclei were isolated as previously described (Reczek et al., 1982).

Micrococcal Nuclease Digestion of Nuclei. The digestion was carried out as previously described (Noll et al., 1975) using a nuclease concentration of 10 units/mL for 3 min.

Extraction of Chromatin. The micrococcal nuclease digested nuclei were centrifuged at 3000*g* for 5 min at 4 °C, and the supernatant fraction was decanted. Modified CSK[1] was made 0.25 M in ammonium sulfate (AS) and 10 mM in EDTA through the addition of the crystalline forms of both, followed by adjusting the pH to 7.8; 20 mL of this modified CSK/AS/EDTA was used to extract the pellet for 30 min at 4 °C. Following extraction, the sample was centrifuged at 3000*g* for 5 min at 4 °C. The supernatant was decanted and saved and the process repeated. The first extract contained 65–100% of the total A_{260}, while both the digest supernatant and the second extract typically contained less than 10%. Analytical gel electrophoresis was routinely performed on this "starting material" (Figure 1, lane 1).

Extraction of Histone H1 and Non-histones. The first and, where appropriate, the second CSK/AS/EDTA extracts were pooled and dialyzed against 2 × 500 mL of 600 mM NaCl,

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; CD, circular dichroism; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; HPLC, high-pressure liquid chromatography; PBS, phosphate-buffered saline (150 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7); modified CSK, 2 × 20 mL of 300 mM sucrose, 3 mM $MgCl_2$, 100 mM NaCl, 2 mM $CaCl_2$, and 10 mM Tris, pH 7.8; AS, ammonium sulfate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.

10 mM Tris, 10 mM EDTA, and 0.1 mM DTT (0.6 M NaCl buffer) supplemented with 0.5 M urea, pH 8.0, in Spec 6 tubing (25 000 molecular weight cutoff) (Spectrum Medical Industries, Los Angeles, CA) at 4 °C. The Na^+ form of Dowex AG50W-X2 (Bio-Rad Industries, Richmond, CA) was equilibrated with 0.6 M NaCl buffer plus 0.5 M urea and dried by suction filtering. A 1-mL volume of Dowex was added to the sample for every 15 A_{260} units of DNA present. The sample was allowed to sit with the Dowex for 1 h at 4 °C with occasional stirring. After this time period, the sample was collected by suction filtration and the Dowex washed up to 3 times with approximately 1 volume of 0.6 M NaCl buffer plus urea. UV spectra were recorded, and samples with significant DNA content were pooled. Analytical SDS gel electrophoresis was performed on the "ion-exchanged material" (Figure 1, lane 2). Analytical gel electrophoresis of this material showed no loss of inner core histones but significant removal of histone H1. At higher urea concentrations (1 M), SDS gel electrophoresis showed some depletion of H2A and H2B, and at 2 M urea, H2A and H2B were almost completely removed.

Bio-Gel A-0.5m Chromatography. While the ion-exchange resin removed many non-histone proteins, there was still considerable non-histone contamination. In order to remove the non-histones the sample was chromatographed on a Bio-Gel A-0.5m column. Surprisingly, it was found that the sample first had to be dialyzed out of the 0.5 M urea or loss of H2A and H2B resulted. This was accomplished by dialysis against 2 L of 0.60 M NaCl buffer in Spec 6 tubing (Spectrapor Medical Industries).

The dialyzed stripped chromatin (≈30 mL) was layered on a Bio-Gel A-0.5m column (2.5 × 100 cm) and eluted with the dialysate at ≈30 mL/h, collecting 3-mL fractions. Fractions containing A_{260} were pooled, and analytical gel electrophoresis was performed. The gel results indicated that the chromatin was substantially free of contaminating proteins. The presence of some non-histone bands was noted (Figure 1, lane 3).

Removal of DNA from Stripped Chromatin. The pooled fractions from the Bio-Gel column were dialyzed (Spectrapor 3 tubing) against 2 M KCl, 10 mM potassium phosphate, 1 mM EDTA, and 0.1 mM DTT, pH 6.8. This sample was applied to a Bio-Gel HT hydroxylapatite column (2.5 × 25 cm) equilibrated in 2 M KCl, 10 mM phosphate, 1 mM EDTA, and 0.1 mM DTT, pH 6.8, and eluted at ≈20 mL/h. Six-milliliter fractions were collected, and the fractions containing proteins, as assayed by A_{230}, were pooled. Analytical gel electrophoresis revealed pure core histones (Figure 1, lane 4). The contaminating non-histones that passed through the Bio-Gel column appear to remain bound to the DNA even in high salt as they could also be removed when the DNA was pelleted by centrifugation.

The core histones thus obtained were concentrated by using an Amicon stirred cell equipped with a PM-10 membrane under 20–30 psi N_2 at 4 °C. The concentrated core histones were then dialyzed in Spec 3 tubing against the appropriate buffer for experimentation, typically 2 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25 at 4 °C. The final yield was typically 5 mg from 2 × 10^9 cells.

Preparation of Acetylated Core Histones. The procedure to prepare acetylated core histones is identical with that described above except that the butyrate-treated cells are employed and all buffers were supplemented with 5 mM sodium butyrate, pH 7.0.

Analytical Procedures. (A) HPLC Studies. (1) Preparation of Histones for HPLC Studies. Purified HeLa core histones were extensively dialyzed (Spectrapor 3 tubing) against the required final solvent at 4 °C. Dialyzed HeLa core histones were concentrated to ≈3 mg/mL by using an Amicon 8 MC micro-ultrafiltration system equipped with a microvolume accessory (Amicon Industries Inc., Danvers, MA). The apparatus was fitted with an Amicon PM-10 membrane, and the core histones were concentrated under 20 psi of N_2 at 4 °C with gentle stirring. Alternatively, the sample was concentrated by using a Centricon 10 concentrator (Amicon Industries) at 5000*g* at 4 °C. The filters were not changed during the course of a single days run in order to maximize sample recovery. Samples were removed from the concentrator and filtered through a Nucleopore 0.2-μm filter, or a Millex HV_4 (Millipore Corp, Bedford, MA) affixed to the end of a 1-mL disposable syringe. Following filtration, UV spectra were recorded, and the concentration of the core histones was adjusted to the desired concentration through the addition of dialysate.

(2) High-Performance Liquid Chromatography of HeLa Core Histones. High-performance liquid chromatography (HPLC) of HeLa core histones employed an Altex Spherogel TSK 3000 SW column (7.5 × 300 mm) jacketed for temperature control. Typically, 150 μL of sample was loaded into the sample loop and injected onto the column. The elution profile was monitored by using a Gilford 2600 spectrophotometer equipped with a 0.5-cm path-length flow cell. Absorbance was monitored at 230 nm, and the flow rate was typically 0.3 mL/min. Molecular weight calibration curves were generated by using the following protein standards: bovine serum albumin, M_r 65 000 (Pentex); ovalbumin, M_r 44 000 (Mann); cytochrome *c*, M_r 13 500 (Sigma). The retention time was independent of the ionic strength of the buffer. All buffers were filtered through Millipore type GS 0.22-μm filters prior to use. When not in use, the column was stored in 10% HPLC-grade methanol in glass-distilled water.

(B) Polyacrylamide–SDS Slab Gel Electrophoresis. Histone purity and integrity were assessed by SDS gel electrophoresis on 15% polyacrylamide gels (Laemmli, 1970; Maizel, 1971). The gels were scanned, and area determinations employed an E-C densitometer (E-C Apparatus, St. Petersburg, FL) fitted with a Hewlett-Packard 3390A integrator.

(C) Acid–Urea Gel Electrophoresis. The extent of acetylation of the inner core histones was assessed by a modification of the gel electrophoresis method of Panyim and Chalkley (1969) as described by Reczek et al. (1982). Following electrophoresis, the gels were stained overnight in 0.1% amido black in methanol/acetic acid/water (5:1:5) and diffusion-destained in a destainer (Bio-Rad) in methanol/acetic acid/water (5:1:5) until the interband region was clear (2–3 days).

(D) Assay for the Presence of Contaminating DNA. To assay for the presence of contaminating DNA, a sensitive (0.01 μL/mL) assay employing ethidium bromide was used (LePecq & Paoletti, 1966). The DNA contamination was demonstrated to be less than 1 part in 1000.

(E) Circular Dichroism Spectroscopy. Circular dichroism (CD) measurements were made with a Jobin-Yvon Mark V Autodichrograph. The instrument was calibrated with *d*-10-camphorsulfonic acid, using an ellipticity of $[\theta]_{292.5}$ = 7600 deg·cm^2·dmol^{-1} (Chen & Yang, 1977; Gillen & Williams, 1975). Measurements were made in jacketed cylindrical quartz cells (Optical Cell Corp., Woodbine, MD) employing path lengths and sensitivities as given in the text. A minimum of three scans were taken and averaged for each sample with a typical sampling interval of 0.2 nm. The time constant and

integration time were held equal and were either 1 or 2 s. The band-pass was held constant at 20 Å.

(*F*) *Absorption Spectroscopy.* Absorption spectra were recorded on either a Cary 14 spectrophotometer or a Gilford 2600 spectrophotometer.

(*G*) *Equilibrium Sedimentation.* Meniscus depletion sedimentation equilibrium (Yphantis, 1964) experiments were performed in a Beckman Model E analytical ultracentrifuge equipped with a UV scanner and electronic speed control. Double-sector cells fitted with 12-mm aluminum-filled epon centerpieces and quartz windows were used in conjunction with an An-D rotor and absorbance counterbalance. The sample sector contained 130 μL of sample layered over 10 μL of FC-43 oil (Beckman). The reference sector was filled with 150 μL of dialysate. Centrifugation was at 26 000 rpm, and equilibrium was obtained in less than 20 h as determined by repeated scanning at 4-h intervals. The temperature was constant during this interval, and the temperature for all experiments was 14 ± 2 °C.

UV scans at 280 nm were captured and digitized into 2049 8-bit data points at a rate of 50 ms/point by a Biomation Model 805 waveform recorder (Biomation Inc., Cupertino, CA), triggered on the rising slope. The data were then punched on paper tape, read by a PDP 11/34 computer equipped with a PDP-11 paper tape reader/punch (Digital Equipment Corp., Maynard, MA), edited by using the TECO text editor, and printed. Typically, there were 300 points across the region of sample distribution. A Fortran program was written which plotted the sample absorbance as a function of radius from the center of rotation on a Complot DR-1 incremental plotter (Houston Instruments, Austin, TX).

The sample was exhaustively dialyzed (Spectrapor 3 tubing) against the appropriate solvent at 4 °C and filtered through Millex HV_4 filter units, and then the absorbance spectra at 280 nm were recorded. An extinction coefficient ($A_{1mg/mL}$ = 0.45) was determined on the basis of the Lowry protein assay. The sample was diluted to a concentration of 0.35 mg/mL with dialysate and used directly.

For each run, four scans were taken, two each after ≈20 and 24 h. The four scans were hand-smoothed, and average absorbances were determined at 0.002-cm intervals from the center of rotation. This process netted about 100 data points. These data were then analyzed to give apparent number-, weight-, and z-average molecular weights by using the computer program written by Roark and Yphantis (1969). The partial specific volume for both acetylated and control inner core histones was taken as 0.753 (Godfrey et al., 1980).

(*H*) *Miscellaneous.* Solutions were prepared at room temperature with glass-distilled water. The chemicals used throughout were reagent grade unless otherwise stated. Unless otherwise stated, the dialysis tubing used throughout was Spectrapor 1 or Spectrapor 3 (Spectrum Medical Industries) that had been boiled in 10 mM $NaHCO_3$/1 mM EDTA for 60 min followed by extensive washing in distilled water and boiling in glass-distilled water for 60 min, followed by rinsing in glass-distilled water. Protein concentrations were determined by the method of Lowry et al. (1951), and an extinction coefficient ($A^{280nm}_{1mg/mL}$ = 0.45) was determined and used for routine concentration determination.

Preparative Results. The preparation of inner core histones, $(H2A/H2B/H3/H4)_2$, from HeLa cells required the development of a new preparative procedure as previous methods gave low yields with many protein contaminants. In Figure 1 is seen an SDS–PAGE gel of the inner core histones at various stages during the preparation. Lane 1 of Figure 1 contains the starting material, generated by extracting micrococcal nuclease digested nuclei with buffer containing 0.25 M ammonium sulfate. The inclusion of the ammonium sulfate resulted in the release of approximately 90% of the total nucleic acid present in the nuclei. In the absence of ammonium sulfate, the yield was approximately 10%. It is thought that ammonium sulfate acts to break the bonds which anchor the chromatin to the nuclear matrix.

Table I: Relative Ratios of Inner Core Histones after Ion-Exchange Treatment in the Presence of Two Urea Concentrations[a]

[urea] (M)	H3	H2B	H2A	H4
0.5	1.15	0.91	0.95	1.00
1.0	1.26	0.76	0.77	1.00

[a] Based on SDS–polyacrylamide gels stained with 0.05% Coomassie Blue in 5:1:5 (water/acetic acid/ethanol). Gels were destained in the same solvent and scanned on a E-C densitometer fitted with a Hewlett-Packard 3390A integrator. Values represent the ratio of peak areas for the respective histone bands normalized to histone H4.

Table II: Relative Ratios of Inner Core Histones in Starting Material[a] and Purified Core Histones[b]

	H3	H2B	H2A	H4
control				
starting material	1.15	0.94	1.13	1.00
purified core	1.27	1.01	0.95	1.00
acetylated				
starting material	1.26	1.16	1.48	1.00
purified core	1.25	1.20	1.38	1.00

[a] Starting material is the pooled CSK/AS/EDTA extracts. [b] Based on SDS–polyacrylamide gels stained with 0.05% Coomassie Blue in 5:1:5 (water/acetic acid/ethanol). Gels were destained in the same solvent and scanned on a E-C densitometer fitted with a Hewlett-Packard 3390A integrator. Values represent the ratio of peak areas for the respective histone bands normalized to histone H4.

The starting material contained the core histones and significant quantities of both non-histone chromosomal proteins and histone H1. To remove H1 and some non-histone chromosomal proteins, the chromatin was dialyzed against buffer containing 0.6 M NaCl and 0.5 M urea and stirred over the Na^+ form of the Dowex AG50W ion-exchange resin. In Figure 1, lane 2, it can be seen that this process resulted in a drastic reduction in the amount of non-histone chromosomal proteins and effectively removed all the histone H1. In the absence of urea, the histone H1 was not adequately removed. Urea concentrations above 0.5 M resulted in a stripping of histones H2A and H2B (Table I).

In order to remove the remainder of the non-histone proteins, urea was removed from the chromatin by dialysis vs. 0.6 M NaCl buffer, and the chromatin was passed down a Bio-Gel A-0.5m column. This removed significant amounts of non-histone proteins (Figure 1, lane 3). Some tightly bound non-histones remained.

The chromatin, now free of histone H1 and most non-histone proteins, was dialyzed against 2 M KCl buffer in order to dissociate the DNA and passed down a hydroxylapatite column which adsorbed the DNA (Beaudette et al., 1981). The protein solution that eluted was free of both DNA and the tightly bound non-histone proteins (Figure 1, lane 4). Subsequent experiments revealed that the extremely tightly bound non-histone proteins remained bound to the DNA even in 2 M KCl.

In order to assess the relative ratios of the four inner core histones, the SDS–PAGE slab gels were scanned and the relative ratios determined (Table II). There was no systematic loss of any individual histone during the preparative procedure. An apparent slight increase in the relative amounts of H2A

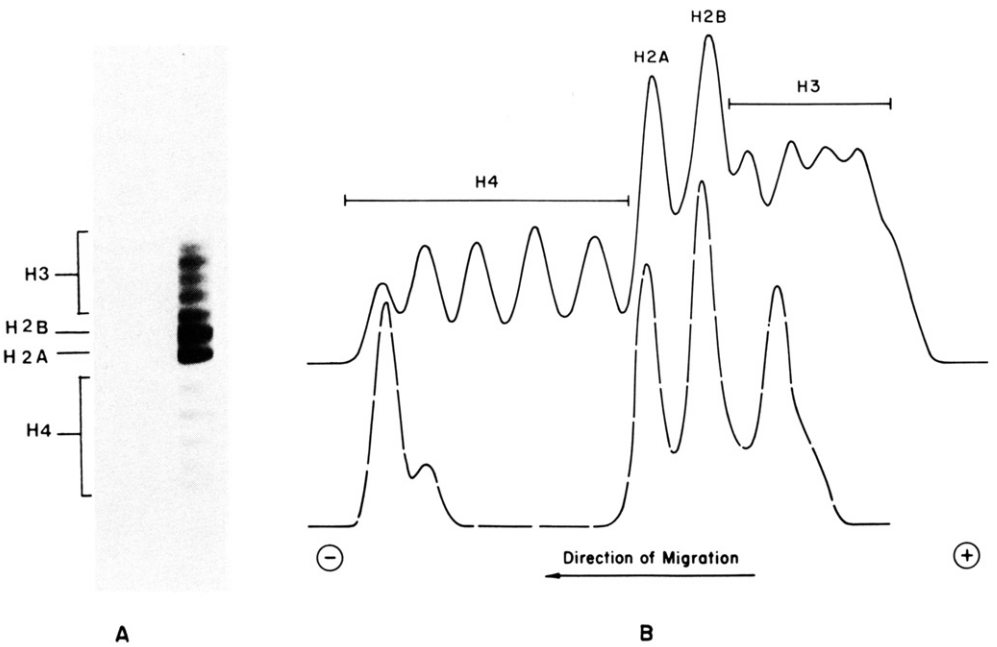

FIGURE 2: (A) Acetic acid–urea–20% polyacrylamide gel of acetylated inner core histones from HeLa S3 cells. Gels were stained with 0.1% amido black in 5:1:5 methanol/acetic acid/water and diffusion-destained in the same solvent. (B) Electrophoretograms of control and acetylated inner core histones prepared from HeLa S3 cells. Scans of acetic acid–urea–20% polyacrylamide gels: (– –) control inner core; (—) acetylated inner core.

and H2B in the acetylated chromatin was observed.

In order to assess the level and stability of acetylation in the purified histones in the absence of butyrate, the histones were stored for 3 days at 4 °C in 2 M NaCl, 10 mM sodium phosphate, 0.25 mM EDTA, 0.1 mM DTT, and 0.1 mM PMSF and then analyzed on acid–urea gels. The gels, shown in Figure 2, demonstrate that the purified histones obtained following butyrate treatment were hyperacetylated and that this acetylation was stable with time in the absence of butyrate in the above buffer system. Therefore, the histone preparation was functionally free of deacetylase activity.

Results

Size-Exclusion HPLC Studies. Size-exclusion HPLC was performed on a TSK 3000 SW column with acetylated and control inner core histones as a function of protein concentration and ionic strength.

The elution profiles obtained for acetylated inner core histones in 2 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25 at 4 °C (Figure 3) displayed a hypersharp leading boundary. The profile was bimodal, and the two peaks were not fully resolved. As the initial loading concentration was decreased, the relative ratios of the two peaks shifted. Material from the high molecular weight peak shifted into the lower molecular weight peak. This is indicative of a rapidly and reversibly associating system. Similar results were obtained for the control inner core histones under identical conditions (data not shown).

The apparent molecular weights of the various peaks were determined by running a calibration curve under identical conditions (Table III). The apparent molecular weight of the high molecular weight peak decreased in concert with the decrease in initial loading concentration. The lower molecular weight peak did not seem to vary in any systematic way with the initial loading concentration.

The apparent molecular weight of the acetylated inner core histones varied from approximately 124 000 at 2 mg/mL to approximately 73 000 at an initial loading concentration of 0.06 mg/mL. The apparent molecular weight of the control inner

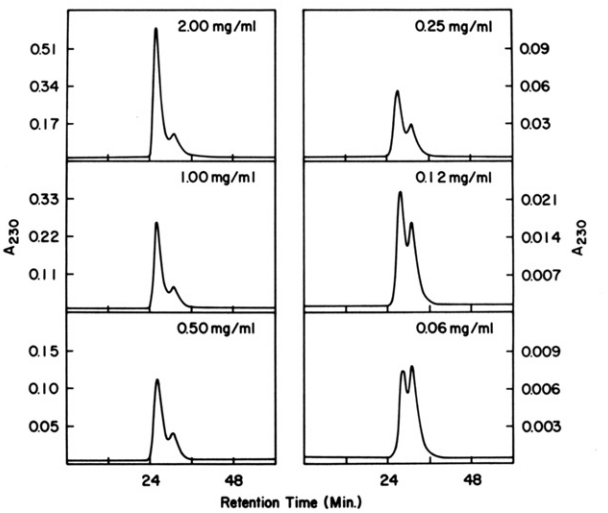

FIGURE 3: Size-exclusion HPLC of acetylated inner core histones as a function of concentration. 150 μL of histone at the indicated concentration was injected on a TSK 3000 SW column and eluted at 0.3 mL/min with 2 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25, 4 °C.

Table III: Apparent Molecular Weights of Acetylated and Control Inner Core Histones as a Function of Initial Loading Concentration As Determined by Size-Exclusion HPLC[a]

	acetylated		control	
concn (mg/mL)	peak 1 mol wt	peak 2 mol wt	peak 1 mol wt	peak 2 mol wt
2.00	123 800	47 200	109 750	47 000
1.00	115 900	47 200	110 200	50 000
0.50	110 200	47 200	95 900	48 000
0.25	96 800	47 200	82 300	45 000
0.12	85 300	47 200	78 000	45 000
0.06	72 700	47 200	65 600	44 000

[a] Solvent was 2 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25, 4 °C.

core histones (Table III) varied from a limiting value of approximately 110 000 at 2 mg/mL to approximately 66 000 at 0.06 mg/mL initial loading concentration. The dilution factor

Table IV: Apparent Molecular Weights of Acetylated and Control Inner Core Histones as a Function of Ionic Strength As Determined by Size-Exclusion HPLC[a]

[NaCl] (M)	acetylated peak 1 mol wt	acetylated peak 2 mol wt	control peak 1 mol wt	control peak 2 mol wt
2.0	115 900	47 200	109 600	49 000
1.2	96 300	47 200	93 300	55 000
0.8	84 600	47 200	75 800	49 000
0.6	72 400	49 000	75 800	49 000

[a] Solvents were indicated concentrations of NaCl plus 10 mM sodium phosphate/0.1 mM DTT, pH 7.25, 4 °C. All initial protein concentrations are 1 mg/mL.

during the course of the run was approximately 7-fold.

Quantitative interpretation of the apparent molecular weight of a small-zone experiment involving an associating system is complicated. The species present in the equilibrium mixture at any given point on the column are a function of the protein concentration at that point, and the protein concentration at any point will depend upon the initial loading concentration, the flow rate, the diffusion rate, and the solute elution rate. The use of size-exclusion HPLC afforded the advantage of extreme reproducibility. Great care was taken to control the flow rate, initial loading volume, and concentration. Therefore, comparisons between runs are reliable. At all initial loading concentrations, the acetylated material eluted with a higher apparent molecular weight than did the control. The observation that the apparent molecular weight for the acetylated histones was 124 000, greater than that of a histone octamer, suggested that the acetylated core histones form high molecular weight aggregates and it was the presence of these aggregates that resulted in the high apparent molecular weight of the acetylated relative to the control at all concentrations.

The lower molecular weight peak had a molecular weight that seemed to be invariant with concentration. The identity of this peak was determined by SDS–PAGE to be H2A/H2B (data not shown). Since purified H2A/H2B eluted with the expected molecular weight, the apparent increase observed in the histone complex must be due to interaction between the various species to form higher molecular weight species, presumably the octamer and hexamer forms. Why this peak did not shift with concentration as expected is not clear, though identical results were obtained on Sephadex G-100.

The effect of ionic strength on the molecular weight of both acetylated and control inner core histones was investigated. The profiles were qualitatively very similar to those obtained as a function of concentration. As the ionic strength was increased, the hypersharp leading peak increased in area, and the trailing low molecular weight peak decreased in area. The average molecular weight of the leading peak increased with increasing ionic strength, while the apparent molecular weight of the minor peak remained relatively constant (Table IV).

The increase in apparent molecular weight with increasing ionic strength is most likely due to the fact that high ionic strength should act to reduce charge–charge repulsion between the individual histones in the octamer. This will serve to stabilize species with a higher average degree of association.

Sedimentation Equilibrium Studies. Sedimentation equilibrium studies using a Beckman Model E ultracentrifuge were performed on inner core histones in the acetylated and control state at both 2 M NaCl and 0.6 M NaCl at 14 °C. Both NaCl solutions were buffered with 10 mM sodium phosphate, pH 7.25, and supplemented with 0.1 mM DTT. For each sample, two runs, each consisting of four scans, were performed, and the data were averaged.

For the acetylated and control inner core histones at both 2 M NaCl and 0.6 M NaCl, the ln c vs. r^2 plots displayed upward curvature. Since upward curvature is generally considered indicative of polydispersity in the sample, the data were analyzed by using the computer program of Roarke and Yphantis (1969). The computed number-, weight-, and z-average molecular weights are plotted in Figure 4A–D as a function of concentration. In no case were the number-, weight-, and z-average molecular weight averages equal. All three types of molecular weight averages increase with increasing concentration. These results indicate polydispersity in the sample.

In 2 M NaCl, the weight-average molecular weight at 0.25 mg/mL was approximately 57 000 for both the acetylated and control samples (Table V). At higher concentration, the weight- and z-average molecular weights of the acetylated sample were consistently greater than that of the control sample.

The acetylated weight-average molecular weight was greater than 100 000 at 1.5 mg/mL, and the z-average molecular weight was greater than 105 000 at 0.75 mg/mL (Table V). The control core histones appear to reach a lower limiting molecular weight with a weight-average molecular weight of approximately 82 000 at 1.5 mg/mL. Neither the number-, weight-, nor z-average molecular weight can take on a value greater than the molecular weight of the largest species present. Therefore, the observation that the acetylated material displays a z-average molecular weight of 105 000 at 0.75 mg/mL, which was still increasing, suggested the presence of species larger than the octamer. The presence of these species would result in an increase in the apparent molecular weight at all concentrations and would be particularly pronounced in the z-average molecular weights.

At low concentration, the octamer should have largely dissociated, and the observed molecular weights should approach those of the component species, specifically the H2A/H2B dimer and the H3/H4 tetramer. Since the weight-average molecular weight expected for a mixture of H3/H4 tetramer and a 1:2 ratio of H2A/H2B dimer is 36 000, it appeared that there was still considerable associated material. The similarity of the control and acetylated samples at low concentration suggested that both have similar populations of species under these conditions. If the high molecular weight aggregate present in the acetylated sample arose from the octamer, this would be the expected result since there

Table V: Weight-Average Molecular Weights as a Function of Total Protein Concentration for Acetylated and Control HeLa Core Histones As Determined by Sedimentation Equilibrium Studies

concn (mg/mL)	2 M NaCl control	2 M NaCl acyl	0.6 M NaCl control	0.6 M NaCl acyl
0.25	56 500 ± 7 000	57 500 ± 4 500	<35 500 ± 3 000	38 000 ± 4 000
0.50	62 400 ± 4 000	68 100 ± 3 000	37 700 ± 2 600	43 500 ± 3 000
0.75	68 000 ± 2 000	73 400 ± 3 000	41 200 ± 2 500	51 400 ± 2 000
1.00	74 500 ± 2 000	81 400 ± 6 000	47 200 ± 1 600	58 100 ± 2 000
1.25	78 500 ± 3 000	93 500 ± 7 000	53 500 ± 1 900	65 700 ± 3 000
1.50	81 500 ± 6 000	101 000 ± 8 000	58 700 ± 2 800	71 150 ± 8 000

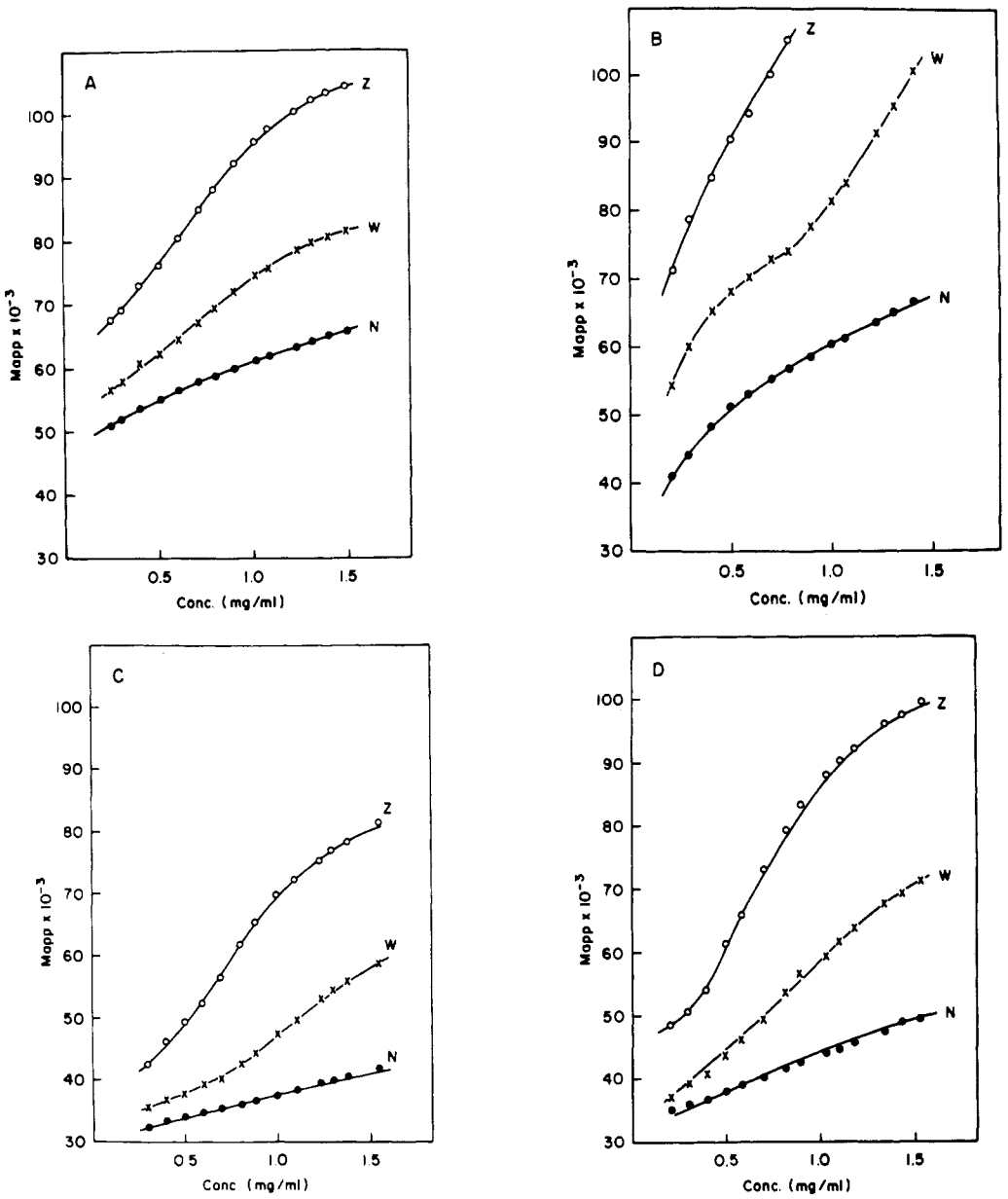

FIGURE 4: (A) Meniscus depletion sedimentation equilibrium sedimentation of control HeLa core histones in 2 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25. Apparent number- (●), weight- (×), and z-average (○) molecular weights as a function of protein concentration. Samples were at 0.35 mg/mL, 26 000 rpm. (B) Meniscus depletion sedimentation equilibrium sedimentation of acetylated HeLa core histones in 2 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25. Apparent number- (●), weight- (×), and z-average (○) molecular weights as a function of protein concentration. Samples were at 0.35 mg/mL, 26 000 rpm. (C) Meniscus depletion sedimentation equilibrium sedimentation of control HeLa core histones in 0.6 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25. Apparent number- (●), weight- (×), and z-average (○) molecular weights as a function of protein concentration. Samples were at 0.35 mg/mL, 26 000 rpm. (D) Meniscus depletion sedimentation equilibrium sedimentation of acetylated HeLa core histones in 0.6 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25. Apparent number- (●), weight- (×), and z-average (○) molecular weights as a function of protein concentration. Samples were at 0.35 mg/mL, 26 000 rpm.

would be relatively little octamer present.

In 0.6 M NaCl, the weight-average molecular weight at low concentration was approximately 37 000 (Table V). This is close to the value of 36 000 expected for a system in which the only species present are the H2A/H2B dimer and the H3/H4 tetramer in a 1:2 molar ratio. For such a system, the number-average and z-average molecular weights would be expected to be 32 400 and 40 500, respectively. The experimental values are in reasonable agreement with this model. As was the case in the 2 M NaCl buffer, the acetylated sample had a higher average molecular weight at high concentrations. These results clearly demonstrate that the association state of the inner core histones is ionic strength dependent and that at 0.6 M NaCl and low total protein concentration the octamer was present in very low concentration. Under these conditions, both the acetylated and control core histones displayed similar molecular weights, providing further evidence that the observed increase was not due to the presence of a contaminating high molecular weight species in the acetylated sample.

Circular Dichroism Studies. Far-UV CD spectra were recorded for acetylated and control inner core histones as a function of ionic strength, concentration, and temperature.

The CD spectra obtained for control inner core histones in 2 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25, as a function of concentration and temperature are seen in Figure 5. There was a decrease in ellipticity upon dilution of the protein or elevation of the temperature (Figure 5A). The $[\theta]_{222}$ values varied from $-17\,700$ deg cm^2 dmol^{-1} at 1

Table VI: Ellipticity Values for Control and Acetylated Inner Core Histones Purified from HeLa Cells[a]

concn (mg/mL)	temp (°C)	solvent[b]	control		acetylated	
			$[\theta]_{208}$	$[\theta]_{222}$	$[\theta]_{208}$	$[\theta]_{222}$
1.0	5	2 M NaCl	−19 400	−17 700	−20 000	−18 400
1.0	30	2 M NaCl	−18 200	−16 500	−18 000	−16 900
0.1	5	2 M NaCl	−18 000	−16 600	−18 300	−17 500
0.1	30	2 M NaCl	−16 700	−15 500	−17 400	−16 800
1.0	5	0.6 M NaCl	−17 900	−17 000	−17 500	−16 500
1.0	30	0.6 M NaCl	−17 000	−15 800	−16 000	−15 100
0.1	5	0.6 M NaCl	−15 200	−14 600	−14 600	−13 700
0.1	30	0.6 M NaCl	−14 000	−13 700	−13 200	−12 300

[a] For concentrations of 1.0 mg/mL, the cell path length was 0.1 mm. For concentrations of 0.1 mg/mL, the cell path length was 1.0 mm. Units are deg cm^2 dmol^{-1}. Error is ±300 deg cm^2 dmol^{-1}. [b] Solvent is stated concentration of NaCl plus 10 mM sodium phosphate and 0.1 mM DTT, pH 7.25.

Table VII: Estimates of Secondary Structure in Acetylated and Control Inner Core Histones Purified from HeLa Cells by Analysis of CD Spectra[a]

concn (mg/mL)	temp (°C)	solvent[b]	control (%)			acetylated (%)		
			α	β	random	α	β	random
1.0	5	2 M NaCl	43	22	35	51	7	42
1.0	30	2 M NaCl	36	26	38	39	31	30
0.1	5	2 M NaCl	41	14	45	44	11	45
0.1	30	2 M NaCl	38	13	49	43	9	48
1.0	5	0.6 M NaCl	40	23	37	40	21	39
1.0	30	0.6 M NaCl	37	17	46	34	26	40
0.1	5	0.6 M NaCl	32	27	41	28	25	47
0.1	30	0.6 M NaCl	26	19	55	22	29	49

[a] Percents of α-helix and β-sheet were determined from the CD spectra from 201 to 250 nm. Ellipticities were compiled in ≈2-nm increments and processed by computer. Reference spectra were poly(L-lysine) (Greenfield & Fasman, 1969) and random-coil reference spectra taken as the average of spectra of individual core histones in 10^{-3} N HCl (Beaudette et al., 1981). [b] Solvent was indicated concentration of NaCl plus 10 mM sodium phosphate and 0.1 mM DTT, pH 7.25.

mg/mL, at 5 °C, to −15 500 deg cm^2 dmol^{-1} at 0.1 mg/mL, at 30 °C (Table VI). The effect of temperature between 5 and 30 °C was reversible in all cases. Since dilution of the protein resulted in its dissociation, it is likely that the observed spectral changes are correlated with the association state of the proteins. The spectra were analyzed for secondary structure composition by using the procedure of Greenfield and Fasman (1969). The results (Table VII) indicate that there was a progressive decrease in the α-helical content during subunit dissociation. The sample at higher concentration appears to have more β-sheet than it did at low concentration. Both the increase in β-sheet and the increase in α-helix were due to loss of random regions.

A study was performed to evaluate the change of ellipticity as a function of both histone core concentration and the ionic strength of the medium. In Figure 6A are seen the $[\theta]_{222}$ and $[\theta]_{208}$ values as a function of core histone concentration (0.2–2.5 mg/mL) in 2 M NaCl. As is evident, there is an increase in ellipticity with increasing histone core concentration, indicating an increase in helical content. In Figure 6B are seen the $[\theta]_{222}$ and $[\theta]_{208}$ values as a function of the ionic strength of the media, at a histone core protein concentration of 0.37 mg/mL. Again, it is evident there is an increase in ellipticity with increasing ionic strength, implying an increase in helical content. Note there is a continuous increase from 2 M NaCl to 4 M NaCl.

Acetylated inner core histones also undergo a decrease in ellipticity upon either dilution of the protein concentration or elevation of the temperature (Figure 5B and Table VI). Secondary structural analysis indicated a progressive decrease in helical content accompanying dissociation. In contrast to the control core, there does not appear to be an elevated β-sheet content at increased concentration except at the highest concentration and temperature. At this point, a dramatic increase in β-sheet was seen. This may represent a reversible aggregation phenomenon.

When the data obtained in 2 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25, for acetylated and control inner core histones were compared, several trends were evident. First, under all conditions, the acetylated material displayed more negative dichroism (deeper minima) at both 208 and 222 nm than did the control. Upon analysis, this appeared to be due to an increased α-helical content. The acetylated material displayed a pronounced increase in β-sheet at 1 mg/mL at 30 °C whereas the control material did not.

CD spectra were also recorded for acetylated and control inner core histones in 0.6 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25 (Figure 5C,D, Table VI). There was a decrease in ellipticity with decreasing concentration and increasing temperature; structural analysis indicated that this was due to a loss of α-helical content. The β-sheet content also decreased at elevated temperature.

The results obtained for the acetylated inner core histones were similar. However, the acetylated inner core histones displayed an increase in β-sheet content with increasing temperature (Table VII).

It should be noted that at identical concentration and temperature, both the acetylated and control samples in 0.6 M NaCl had lower ellipticity values than do the equivalent samples in 2 M NaCl. This presumably reflects the lower average association state of the histones at lower ionic strength.

Discussion

Size-Exclusion HPLC. Size-exclusion HPLC was performed on acetylated and control inner core histones as a function of concentration and ionic strength. The use of HPLC afforded the advantages of economy of sample, speed of separation, increased resolution, and extreme reproducibility.

Despite the difficulties attendant in the interpretation of small-zone experiments with associating systems, they can provide valuable qualitative information regarding the experimental system. Rapidly associating systems display

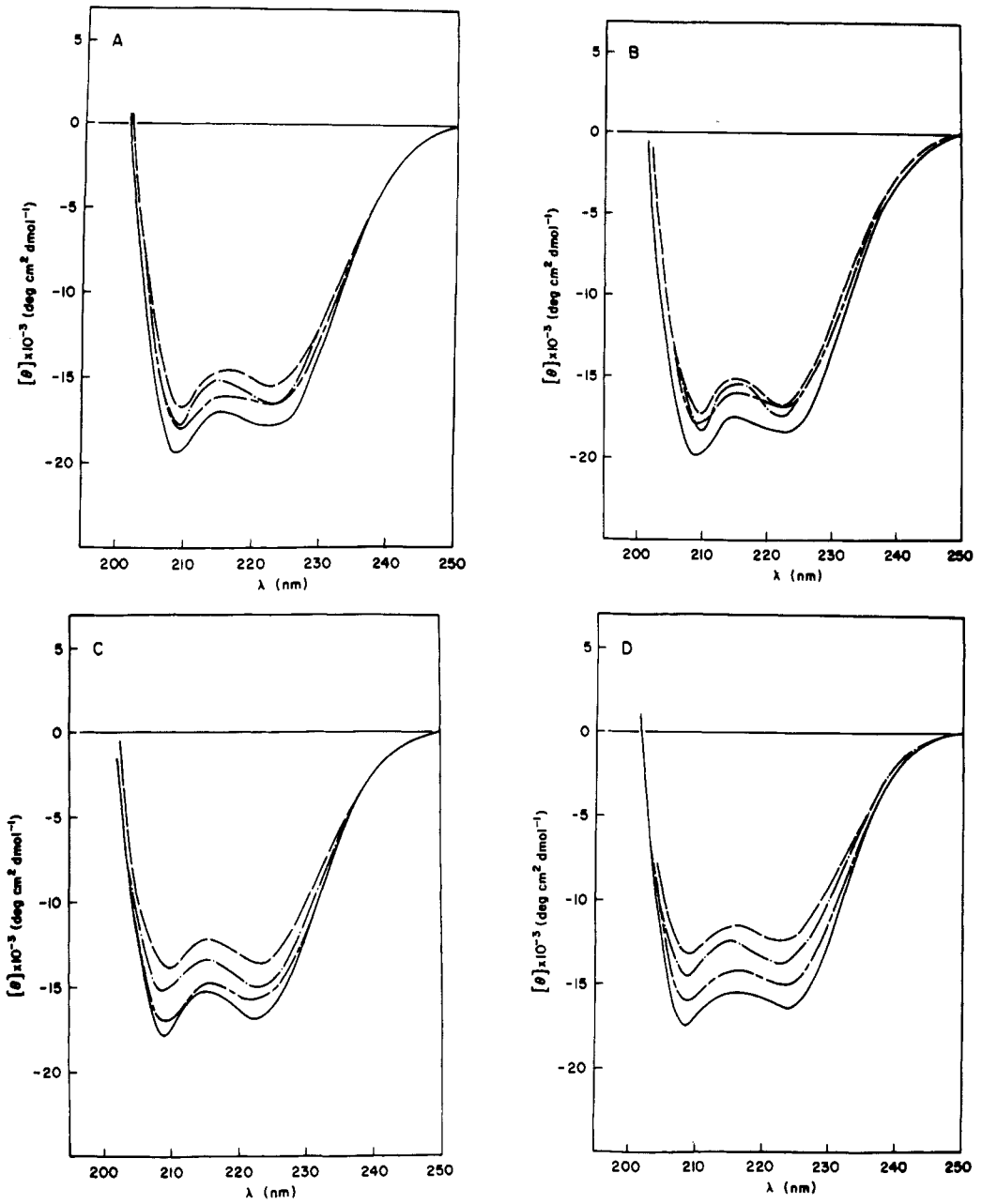

FIGURE 5: (A) Circular dichroism spectra of control inner core histones purified from HeLa cells as a function of concentration and temperature in 2 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25. (—) 1.0 mg/mL, 5 °C, 0.1-mm cell path length; (- - -) 1.0 mg/mL, 30 °C, 0.1-mm cell path length; (-·-) 0.1 mg/mL, 5 °C, 1.0-mm cell path length; (· · ·) 0.1 mg/mL, 30 °C, 1.0-mm cell path length. (B) Circular dichroism spectra of acetylated inner core histones purified from HeLa cells as a function of concentration and temperature in 2 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25. (—) 1.0 mg/mL, 5 °C, 0.1-mm cell path length; (- - -) 1.0 mg/mL, 30 °C, 0.1-mm cell path length; (-·-) 0.1 mg/mL, 5 °C, 1.0-mm cell path length; (· · ·) 0.1 mg/mL, 30 °C, 1.0-mm cell path length. (C) Circular dichroism spectra of control inner core histones purified from HeLa cells as a function of concentration and temperature in 0.6 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25. (—) 1.0 mg/mL, 5 °C, 0.1-mm cell path length; (- - -) 1.0 mg/mL, 30 °C, 0.1-mm cell path length; (-·-) 0.1 mg/mL, 5 °C, 1.0-mm cell path length; (· · ·) 0.1 mg/mL, 30 °C, 1.0-mm cell path length. (D) Circular dichroism spectra of acetylated inner core histones purified from HeLa cells as a function of concentration and temperature in 0.6 M NaCl, 10 mM sodium phosphate, and 0.1 mM DTT, pH 7.25. (—) 1.0 mg/mL, 5 °C, 0.1-mm cell path length; (- - -) 1.0 mg/mL, 30 °C, 0.1-mm cell path length; (-·-) 0.1 mg/mL, 5 °C, 1.0-mm cell path length; (· · ·) 0.1 mg/mL, 30 °C, 1.0-mm cell path length.

characteristic boundary shapes including a hypersharp leading edge and a diffuse trailing edge (Ackers, 1970; Winzor & Scheraga, 1963). The apparent molecular weight observed represents a weight average of all the species present (Ackers, 1970). Conditions which favor association, such as increasing concentration, will result in a larger apparent molecular weight, while conditions favoring dissociation will result in a smaller apparent molecular weight. The data in Tables III and IV show that both the acetylated and control core histones dissociate with decreasing concentration or ionic strength. At NaCl concentrations below 2 M, the octamer dissociates as witnessed by an increase in the amount of material in the trailing peak and a decrease in the apparent molecular weight of the leading peak.

When the results obtained for the acetylated and control inner core histones in 2 M NaCl as a function of concentration are compared, the acetylated appears to be more stable than the control. Furthermore, the apparent molecular weight of the acetylated species at 2 mg/mL was greater than that expected for an octamer and suggests that larger species are present.

The acetylated histones seem more stable at most ionic

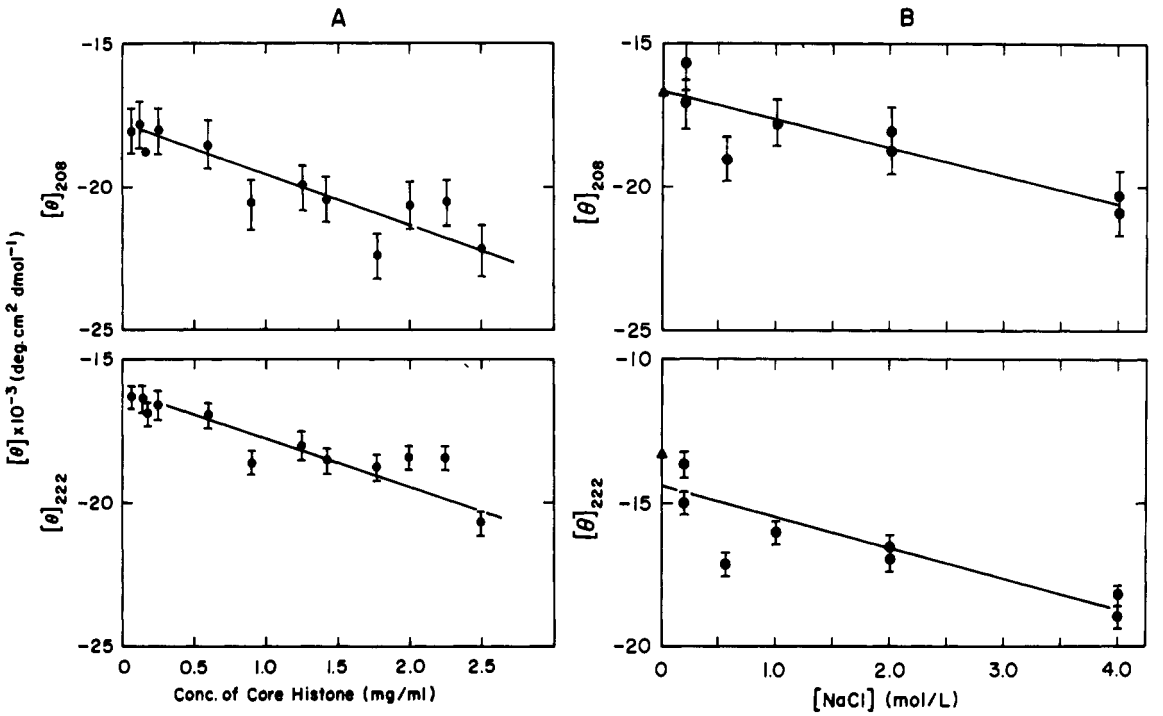

FIGURE 6: (A) $[\theta]_{208}$ and $[\theta]_{222}$ of histone core complex vs. protein concentration. Salt-extracted core histone complex in 2.0 M NaCl, 10 mM sodium phosphate, 0.25 mM EDTA, and 0.1 mM DTT, pH 7.2 at 4 °C. Solutions were prepared by dilution of a concentrated stock solution of core histones. Path length of cell varied from 0.01 to 0.1 cm. Chicken erythrocyte core histones were used. (B) $[\theta]_{208}$ and $[\theta]_{222}$ of histone core complex vs. NaCl concentration. Salt-extracted core histones. Protein concentration in the range of 0.18–0.25 mg/mL. Closed triangles (▲) give the values of the ellipticities of the core complex (concentration = 0.37 mg/mL in 5 mM sodium phosphate, 0.25 mM EDTA, and 0.05 mM DTT, pH 7.2). Path length of cell, 0.05 cm.

strengths. The exception to this was in 0.6 M NaCl. It is possible that a conformational change is occurring at this ionic strength in the acetylated material which results in a change in the apparent molecular weight. Some support for this idea is found in the CD data.

Sedimentation Equilibrium Studies. These experiments were undertaken because size-exclusion HPLC studies suggested that acetylated core histones have a higher apparent molecular weight under a variety of conditions than do control core histones. The sedimentation studies bear this out.

At both 2 and 0.6 M NaCl, the acetylated core histones have a higher average molecular weight than do the control histones. This result strongly suggests that the acetylated material contains species with a molecular weight greater than that of the control. Furthermore, the z-average molecular weight obtained at 2 mg/mL in 2 M NaCl for the acetylated material was greater than that of the octamer. This requires that there be species present which were larger than an octamer.

The control core histones appear to reach a limiting molecular weight of approximately 82 000 at 1.5 mg/mL. The values obtained for the control samples are consistent with the model of the octamer as the end point of association and are similar to data obtained for calf thymus inner core histones (Godfrey et al., 1980).

Under dilute conditions, the acetylated and control samples have roughly the same molecular weight. This suggests that the fundamental stable species were the same for both the acetylated and control samples. Since the apparent molecular weights for the control and the acetylated species were very similar at low concentration, this argues against the increase in molecular weight in the acetylated material being due to the presence of contaminants.

Even under the dilute concentration conditions in 2 M NaCl, there was significant associated material. Evidence for this comes from the fact that the weight-average molecular weight obtained for both the acetylated and the control samples was approximately 57 000. The fully dissociated species (H3/H4 tetramer and H2A/H2B dimer) should display a weight-average molecular weight of 37 000. This is what is seen at low concentration in 0.6 M NaCl. Furthermore, since sedimentation equilibrium experiments are shape independent, the decrease at low concentration with decreasing ionic strength must be due to dissociation.

It is worth noting that in 0.6 M NaCl the acetylated histones have higher weight-average molecular weights than do the control. In the HPLC experiments, the acetylated material displayed a lower molecular weight under these conditions. It is possible that a change in tertiary structure rather than a change in quaternary structure is responsible for this effect on the HPLC column. Sedimentation equilibrium would not be sensitive to such changes.

Circular Dichroism. In 2 M NaCl, both acetylated and control inner core histones exhibit a temperature- and concentration-dependent change in conformation (Table VI, Figures 5 and 6). At lower temperature or increased protein concentration, the magnitude of the CD spectrum increases. Secondary structural analysis indicates that this was due to an increase in the α-helical content (Table VII). Increasing the concentration or decreasing the temperature has been shown to shift the equilibrium between histone species toward the octamer (Ruiz-Carrillo & Jorcano, 1979; Eickbush & Moudrianakis, 1978; Stein & Page, 1980; Godfrey et al., 1980; Thomas & Kornberg, 1975). It has also been demonstrated that complex formation between certain histone subunits is accompanied by an increase in the α-helical content (D'Anna & Isenberg, 1974a,b). In the case of the observed increase in α-helix with increasing concentration, the most likely interpretation is that there is helix formed during the association of the subunits into the octamer. It is difficult to see how concentration would affect the conformation of the subunits

themselves. The effect of changes in temperature is less simply interpreted. It is possible that temperature plays a role in the observed spectral changes both by changing the assembly state of the proteins and by changing the conformation of the subunits directly.

It has been shown that the individual histones display an increase in the amount of α-helix with increasing ionic strength (Adler et al., 1974, 1975a,b; Guzzo, 1965). Therefore, it is difficult to separate the changes due to ionic strength effects on the subunits and those due to ionic strength effects on the association state of the subunits.

It has previously been shown that the H3/H4 tetramer complex, as isolated from calf thymus chromatin in 2.0 M KCl/0.1 M potassium phosphate, pH 6.7, undergoes a time-dependent conformational change upon dilution to low salt (0.14 M NaCl, pH 7). This conformational change was evident from CD studies and the exposure of antigenic sites on the complex. A decrease of $\approx$30% α-helix was observed. It was suggested that the existence of multiple forms of the H3/H4 complex may be related to the dynamic equilibrium of nucleosome structure in vivo (Feldman et al., 1980). Studies on the H3/H4 complex, the H2/H2B complex, and the inner core complex, obtained from chicken erythrocyte chromatin, had previously shown the pH, ionic strength, and concentration dependence of the conformation of these various complexes. The reversibility of the totally denatured histone complexes (in 1.0 mM HCl) to the native structure was also demonstrated, showing the conformational mobility of the core histone complex and products of dissociation, the H2A/H2B dimer and H3/H4 tetramer (Beaudette et al., 1981).

The reversible increase in β-sheet seen for the acetylated material at 30 °C may represent an increase in β-sheet during oligomerization to species of higher molecular weight than the octamer. The fact that this change is seen at high concentration and high ionic strength conditions, which favor oligomerization, would tend to support this. However, it has been shown that an increase in temperature tends to destabilize the octamer.

Conclusions

This study was undertaken to examine the effect of acetylation on the subunit/subunit interactions in the histone octamer. The results from the HPLC and sedimentation equilibrium studies suggest that the acetylation results in the increased formation of a species with a molecular weight greater than that of the octamer.

These results indicate that acetylation has a drastic effect on the association state of HeLa inner core histones. Upon acetylation, the histones tend to oligomerize into structures larger than the octamer. Since acetylation occurs at the ϵ-amino of lysine residues, the resulting charge neutralization may allow for increased interparticle interaction. This study also demonstrates that there is an increase in helical content upon association of the H2A/H2B dimer and the H3/H4 tetramer into the octamer. The results of this study can be summarized as follows:

(1) Acetylation of the inner core histones causes a greater degree of association between subunits than exhibited by control samples.

(2) Acetylation causes an increase in helical content of the core complex at 4 °C. In part, this may be due to the higher degree of association. At 30 °C, the acetylated core displayed a slightly higher β-composition than did the control complex.

(3) HPLC studies indicated that acetylated inner core histone complexes are more stable at most ionic strengths investigated than the control.

(4) Sedimentation equilibrium studies confirm that acetylated inner core histones (at 2.0 and 0.6 M NaCl) have a higher average molecular weight than the control core histone complex.

(5) There is a marked effect on the conformation of both acetylated and control inner core histone complexes as a function of the concentration of protein, the ionic strength of the media, and the temperature of the environment.

The difference in the conformational flexibility and association state of the acetylated vs. the control inner histone core complex may play a significant role in the control of the transcription of chromatin in the nucleus.

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References

Ackers, G. K. (1970) *Adv. Protein Chem. 24*, 343–446.

Adler, A. J., Ross, D. G., Chen, K., Stafford, P. A., Woiszwillo, M. J., & Fasman, G. D. (1974) *Biochemistry 13*, 616–623.

Adler, A. J., Fulmer, A. W., & Fasman, G. D. (1975a) *Biochemistry 14*, 1445–1454.

Adler, A. J., Moran, E. C., & Fasman, G. D. (1975b) *Biochemistry 14*, 4179–4185.

Allfrey, V. G., Faulkner, R. M., & Mirsky, A. E. (1964) *Proc. Natl. Acad. Sci. U.S.A. 51*, 786–794.

Ausio, J., & van Holde, K. (1986) *Biochemistry 25*, 1421–1428.

Beaudette, N. V., Fulmer, A. W., Okabayashi, H., & Fasman, G. D. (1981) *Biochemistry 20*, 6526–6535.

Benedict, R. C., Moudrianakis, E. N., & Ackers, G. H. (1984) *Biochemistry 23*, 1214–1218.

Bode, J., Henco, K., & Wingender, E. (1980) *Eur. J. Biochem. 110*, 143–152.

Burdick, C. J., & Taylor, B. A. (1976) *Exp. Cell Res. 100*, 428–432.

Christensen, M. E., & Dixon, G. H. (1982) *Dev. Biol. 93*, 404–415.

Christensen, M. E., Rattner, J. B., & Dixon, G. H. (1984) *Nucleic Acids Res. 12*, 4575–4591.

D'Anna, J. A., Jr., & Isenberg, I. (1974a) *Biochemistry 13*, 2098–2104.

D'Anna, J. A., Jr., & Isenberg, I. (1974b) *Biochemistry 13*, 4992–4997.

Davie, J. R., & Candido, E. P. M. (1980) *FEBS Lett. 110*, 164–168.

Davie, J. R., & Saunders, C. A. (1981) *J. Biol. Chem. 256*, 12574–12580.

Eickbush, T. H., & Moudrianakis, E. N. (1978) *Biochemistry 17*, 4955–4964.

Feldman, L., Beaudette, N. V., Stollar, B. D., & Fasman, G. D. (1980) *J. Biol. Chem. 255*, 7059–7062.

Godfrey, J. E., Eickbush, T. H., & Moudrianakis, E. N. (1980) *Biochemistry 19*, 1339–1346.

Greenfield, N., & Fasman, G. D. (1969) *Biochemistry 8*, 4108–4116.

Grimes, S. R., & Henderson, N. (1983) *Arch. Biochem. Biophys. 221*, 108–116.

Guzzo, A. V. (1965) *Biophys. J. 5*, 809–822.

Hüvos, P., Sasi, R., & Fasman, G. D. (1984) *Biopolymers 23*, 2195–2210.

Imai, B. S., Yau, P., Baldwin, J. P., Ibel, K., May, R. P., & Bradbury, E. M. (1986) *J. Biol. Chem. 261*, 8784–8792.

Levy-Wilson, B., Watson, D. C., & Dixon, G. H. (1979) *Nucleic Acids Res. 6*, 259–274.
Lilley, D. M., & Berendt, A. R. (1979) *Biochem. Biophys. Res. Commun. 90*, 917–923.
Mathis, D. J., Oudet, P., Wasylyk, B., & Chambon, P. (1978) *Nucleic Acids Res. 5*, 3523–3547.
McGhee, J. D., Nickol, J. M., Felsenfeld, G., & Rau, D. C. (1983) *Nucleic Acids Res. 11*, 4065–4075.
Nelson, D. A., Perry, M., Sealy, L., & Chalkley, R. (1978) *Biochem. Biophys. Res. Commun. 82*, 1346–1353.
Noll, M., Thomas, J. O., & Kornberg, R. D. (1975) *Science (Washington, D.C.) 187*, 1203–1206.
Oliva, R., & Mezquita, C. (1982) *Nucleic Acids Res. 10*, 8049–8059.
Prior, C. P., Cantor, C. R., Johnson, E. M., Littau, C. V., & Allfrey, V. G. (1983) *Cell (Cambridge, Mass.) 34*, 1033–1042.
Reczek, P. R., Weissman, D., Hüvos, P. E., & Fasman, G. D. (1982) *Biochemistry 21*, 993–1002.
Riggs, M. G., Whittaker, R. G., Neumann, J. R., & Ingram, V. M. (1977) *Nature (London) 268*, 462–464.
Roarke, D. E., & Yphantis, D. A. (1969) *Ann. N.Y. Acad. Sci. 164*, 245–278.
Ruiz-Carrillo, A., & Jorcano, J. L. (1979) *Biochemistry 18*, 760–768.
Ruiz-Carrillo, A., Wangh, L. J., Littau, V. C., & Allfrey, V. G. (1974) *J. Biol. Chem. 249*, 7358–7368.
Ruiz-Carrillo, A., Wangh, L. J., & Allfrey, V. G. (1976) *Arch. Biochem. Biophys. 174*, 272–290.
Sealy, S., & Chalkley, R. (1978) *Nucleic Acids Res. 6*, 1863–1876.
Simpson, R. T. (1978) *Cell (Cambridge, Mass.) 13*, 691–699.
Stein, A., & Page, D. (1980) *J. Biol. Chem. 255*, 3629–3637.
Sung, M. T., & Dixon, G. H. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 1616–1623.
Thomas, J. O., & Kornberg, R. D. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2626–2630.
Vidali, G., Boffa, L. C., Bradbury, E. M., & Allfrey, V. G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2239–2243.
Winzor, D. J., & Scheraga, H. A. (1963) *Biochemistry 2*, 1263–1267.
Yau, P., Thorn, A. W., Imai, B. S., Matthews, H. R., & Bradbury, E. M. (1982) *Eur. J. Biochem. 129*, 281–288.
Yau, P., Imai, B. S., Thorne, A. W., Goodwin, G. M., & Bradbury, E. M. (1983) *Nucleic Acids Res. 11*, 2651–2664.

Kinetic Reaction Mechanism for Magnesium Binding to Membrane-Bound and Soluble Catechol *O*-Methyltransferase†

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ABSTRACT: Catechol *O*-methyltransferase (COMT, EC 2.1.1.6) from human brain occurs in both a membrane-bound (MB-COMT) and a soluble form (SOL-COMT). While these enzymes appear to be distinct molecular entities, both catalyze the O-methylation of catecholamines through an ordered reaction mechanism in which *S*-adenosylmethionine (SAM) is the leading substrate [Rivett, A. J., & Roth, J. A. (1982) *Biochemistry 21*, 1740–1742; Jeffery, D. R., & Roth, J. A. (1985) *J. Neurochem. 44*, 881–885]. Both MB-COMT and SOL-COMT require the presence of divalent cations for catalytic activity. This series of experiments provides evidence indicating that magnesium ions bind to both MB-COMT and SOL-COMT in a rapid equilibrium sequence prior to the addition of SAM. An equation is presented that predicts the qualitative results obtained in all kinetic experiments carried out with either MB-COMT or SOL-COMT.

Catechol *O*-methyltransferase (COMT;[1] EC 2.1.1.6) catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to a ring hydroxyl group on catecholamine neurotransmitters or other xenobiotic catechols. The presence of divalent cations is essential for enzymatic activity, and magnesium ions are believed to be the physiological cofactor (Axelrod & Tomchick, 1958). While the molecular and kinetic mechanisms of the methyltransfer reaction have been studied in some detail (Hegazi et al., 1976, 1979; Rivett & Roth, 1982; Jeffrey & Roth, 1985), the role of magnesium ions in the reaction has received little attention.

Senoh et al. (1962) studied the effects of various divalent cations on the base- and COMT-catalyzed O-methylation of catecholamines and found that the total yield of O-methylated products was dependent upon the nature of the cation. Magnesium appeared to stimulate activity to the greatest extent whereas copper was the least effective cation. The order of effectiveness in promoting O-methylation was the same for the enzymatic and nonenzymatic, base-catalyzed reaction. The authors suggested that magnesium ions form a bridge between a catechol hydroxyl group and *S*-adenosylmethionine and act to align the methyl group from SAM with a ring hydroxyl group. In agreement with the conclusions reached by Senoh et al. (1962), Belleau and Burba (1963) provided evidence suggesting that tropolones (dead-end inhibitors of COMT)

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[1] Abbreviations: COMT, catechol *O*-methyltransferase; SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; MB, membrane bound; SOL, soluble; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DA, dopamine.

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