A Description of Two Procedures Which Avoid the Use of Extreme pH Conditions for the Resolution of Components Isolated from Chromatins Prepared from Pig Cerebellar and Pituitary Nuclei*

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ABSTRACT: Utilizing pig cerebellar and pituitary tissues, procedures for preparing nuclei, chromatin, and the major components from the latter are described. Almost complete recovery of the proteins from these chromatins was achieved using two different methods which avoid an acid extraction step. The first of these is centrifugation of the chromatins in 7 M urea–3 M NaCl by which 90–95% of the chromatin proteins are separated from the DNA. The proteins prepared by these procedures were analyzed by urea—and sodium dodecyl sulfate—polyacrylamide gel electrophoresis. Nonhistone proteins were well separated from the histones in the urea–polyacrylamide gel system (pH 2.7) and this technique served as a basis for preparing the nonhistone proteins for analytical purposes. Cerebellar and pituitary nonhistone proteins consisted of 19.9–23.8% and 22.4–24.4%, respectively, of the total chromatin proteins.

The histone electrophoretic patterns of both chromatins are qualitatively similar to that obtained for calf thymus histones except for the presence of a band which has an electrophoretic mobility intermediate to that of histone F1 and histone F3. The low molecular weight RNA fraction prepared from the two chromatins consisted of RNA whose electrophoretic mobility was faster compared to that of transfer RNA in polyacrylamide gels.

The complete separation, with good recovery, of the major known classes of interphase chromatin components (DNA, RNA, histone, and nonhistone proteins) is an essential prerequisite to our understanding of the interactions between these components and their role in the structural organization and function of chromatin. Much progress has been made in the characterization of histones from a number of tissues (summarized by Stellwagen and Cole, 1969, and Fambrough, 1969). Most of the histone extraction work has been based on histone solubility in acid, either dilute HCl or H_2SO_4. By the acid extraction technique the recovery of histone is complete and contamination with DNA, RNA, and nonhistone protein is small. Since the DNA, RNA, and nonhistone proteins are denatured by the acid extraction procedure, we have undertaken a number of studies in our laboratory seeking procedures which would extract all of the components under milder conditions (Shirey and Huang, 1969; L. Kleiman and R. C. Huang, 1970, manuscript in preparation).

In the present paper, utilizing pig cerebellar and pituitary tissues as the source for our chromatin preparations, we will report our procedures for the isolation of nuclei and chromatin and the major components extractable from the latter. Two simple and rapid procedures have been adapted to separate total chromatin proteins and low molecular weight RNA. The further separation of histones from nonhistone proteins was accomplished by polyacrylamide disc gel electrophoresis. We hope that the present study can serve as a basis for future experiments (1) which will be designed to probe the physical basis of the interactions between the major chromatin components in reconstitution studies; (2) to further characterize the individual nonhistone proteins within the total population of this class of chromatin proteins and to determine their role in the structure and function of chromatin.

Experimental Section

Isolation of Pig Cerebellar and Pituitary Nuclei. Fresh cerebellar and pituitary tissues were obtained from Esskay Meat Co., Baltimore, Md. The pituitaries were quick frozen on Dry Ice immediately after being excised from the pig brains, whereas the cerebellar tissue was not. These tissue samples were kept in plastic bags in an ice bucket at 0° during transport to the laboratory. The method used for preparing nuclei is essentially that of Busch (1967). Cerebellar grey matter and whole pituitaries were cut into small pieces with a scissor and homogenized with a Teflon-to-glass homogenizer in 9 volumes of 0.25 M sucrose–0.0033 M calcium acetate. Homogenization of cerebellar grey matter was achieved with 7–8 up and down strokes of the Teflon homogenizer at 1725 rpm; pituitaries (a much tougher tissue) required 10–12 strokes for homogenization and it has subsequently been found (preparation 4) that the homogenization is much easier if the pituitaries are first passed twice through a Dormeyer meat grinder. After passing the homogenates through 4 layers of cheesecloth, crude nuclear pellets were obtained by centrifugation at 3500*g* for 20 min in the SS-34 rotor, Sorvall centrifuge in

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the cold (4°). The crude nuclear pellets from 30 g wet weight of tissue were suspended to a total volume of 80 ml with cold 2.0 M sucrose–0.0033 M calcium acetate. Aliquots (25 ml) were placed in each of 3 cellulose nitrate tubes and 5 ml of 2.0 M sucrose–0.0033 M calcium acetate was layered under each suspension. The tubes were placed in buckets and centrifuged in the SW 25.1 Spinco rotor at 40,000*g* for 60 min. The resulting nuclear pellets were utilized for the preparation of chromatin. The purity of the nuclear preparations was checked by examination with the phase microscope (Zeiss Photomicroscope) and one cerebellar nuclear preparation was looked at under the electron microscope. For electron microscopic analysis a portion of a cerebellar nuclear pellet was placed in 0.25 M sucrose–0.0033 M calcium acetate–5% glutaraldehyde–2.26% sodium phosphate, pH 7.2, and kept in this solution for 40 min at 4° followed by standing in 0.25 M sucrose–0.0033 M calcium acetate–2.26% sodium phosphate, pH 7.2, overnight at 4°. The nuclei were then postfixed with 1% OsO_4 in 2.26% sodium phosphate, pH 7.2, for 1.5 hr followed by dehydration through an ethanol–propylene oxide series. The nuclear sample was embedded in Mollenhauers Epon-Araldite mixture. For observation under the electron microscope (RCA EMU-3) the sections of embedded nuclei were stained for 20 min in 5% uranyl acetate followed by staining with lead citrate (Reynolds (1963)) for 1 min. Nuclear yield from cerebellar tissue was 38–69%, based on the recovery of DNA from the original homogenate, but was somewhat lower from pituitary (20–30%).

Preparation of Cerebellar and Pituitary Chromatin. Chromatin was prepared from the isolated nuclei obtained from the cerebellar and pituitary tissues using essentially the procedure of Paoletti and Huang (1969). Nuclear pellets from 30 g wet weight of tissue were suspended in 40 ml of 0.075 M NaCl–0.024 M EDTA, pH 8.0, and centrifuged at 7700*g* for 15 min. This step was repeated three times more. The resulting pellets were suspended in 40 ml of 0.05 M Tris, pH 8.0, and centrifuged at 7700*g* for 10 min. This wash was repeated once and followed by two washes each in 0.01 M Tris, pH 8.0, 0.002 M Tris, and 0.0004 M Tris. The chromatin was then allowed to swell overnight in a total volume of 100 ml of ice-cold distilled water, pH 8.0, placed in an ice bucket, and stored in a refrigerator. After stirring the chromatin into solution it was not pelleted by centrifugation at 10,000*g* for 30 min. For larger scale preparations (nuclei from 60 or 120 g wet weight of tissue) the volumes of the wash media were increased proportionally. The recovery of DNA, as determined by the Burton (1956) method, in the chromatins prepared in this way was 91–99%.

Chemical Analyses of Chromatin. DNA, RNA, histone, and nonhistone protein were assayed according to the procedures of Paoletti and Huang (1969) with the following modification for assaying RNA. For the measurement of RNA in chromatin, chromatin subfractions, and nuclei the sample to be analyzed was hydrolyzed in 0.3 M NaOH at 37° for 18 hr. DNA, if present, and protein were then precipitated from the solution by adding 72% $HClO_4$ to a final concentration of 4%. This mixture was allowed to stand in an ice bucket for 15 min and the precipitate centrifuged and washed once with cold 4% $HClO_4$. The two supernatants were combined and utilized for the RNA determination. Total chick embryo brain RNA was used as the standard. Pig pituitary DNA prepared by the procedure of Marmur (1961) was employed as the DNA standard and crystalline bovine serum albumin (Sigma, fraction V) as the protein standard.

Preparation of Chromatin Components. A. EXTRACTION OF CHROMATIN WITH 7 M UREA, 3 M NaCl. Into a solution of chromatin is poured a mixture of urea and NaCl in a small volume of distilled water. This mixture is stirred in an ice bucket and the final concentration of urea and NaCl made to 7 and 3 M, respectively, by adding deionized water to the appropriate final volume. The chromatin concentration for all such preparations was approximately 2 optical density units per ml at 260 mμ. This clear solution was then centrifuged in a 40 rotor at 85,487*g* for 48 hr at 4° in a Spinco ultracentrifuge. Under these conditions DNA was completely pelleted and none was detected in the supernatant. In order to further analyze the supernatants they were dialyzed at 4° against three 6-l. changes of 0.05 N acetic acid and then lyophilized to dryness. The DNA pellets were recovered by resuspension in either distilled H_2O or dilute saline citrate (0.015 M NaCl–0.0015 M sodium citrate) and gentle stirring for 24 hr at 4°. Some material does not go into solution and for measurements of DNA, RNA, and protein the suspension was homogenized by hand with a loose-fitting Teflon-to-glass homogenizer and aliquots of the suspension were taken for the analyses.

B. EXTRACTION OF CHROMATIN WITH 3 M NaCl ON A BIO-GEL A-50 COLUMN. Optical density 260-mμ units (270–500) of cerebellar chromatin were made to 3 M in NaCl in a final volume of 40–60 ml. After stirring the NaCl slurry into solution the chromatin solution was sheared in a small blender at 80 V in a 4° cold room for 1 min. The sheared chromatin was then stored in an ice bath for 16 hr. After clarifying this sheared chromatin solution by centrifuging it at 12,100*g* for 30 min, it was applied to a Bio-Gel A-50 (Bio Rad) column, preequilibrated in 3 M NaCl, in a cold room. The size of the column was 4.0 × 74 cm. Optical density 260-mμ units (170–200) of pituitary chromatin was made to 3 M in NaCl in a final volume of 40–50 ml and treated in the same way as the cerebellar chromatin prior to application to a somewhat smaller Bio-Gel A-50 column (4.0 × 60 cm). Column fractions were pooled and dialyzed against three 6-l. changes of deionized water and then lyophilized to dryness.

C. PREPARATION OF HISTONES BY EXTRACTION WITH H_2SO_4. Histones were extracted from cerebellar chromatin by the procedure described by Bonner *et al.* (1968a) except that the initial extraction was done with 0.4 N H_2SO_4 instead of with 0.2 N H_2SO_4.

Analytical Gel Electrophoresis of Proteins. A. IN THE CATHODE DIRECTION. The method of Panyim and Chalkley (1969a) was used. The gels were 15% acrylamide in 2.5 M urea and 0.9 M acetic acid. Acetic acid (0.9 N) is the electrophoresis buffer and is placed in the upper and lower chambers of the electrophoretic apparatus. The pH of 0.9 N acetic acid is 2.2. Gels (0.6 × 8.0 cm) were preelectrophoresed for 2 hr at 2 mA/gel. The pH of preelectrophoresed gels is 2.7. Electrophoresis, unless otherwise stated, was conducted at 100 V for 3 hr. Staining of the gels was achieved by soaking them in 1% Amido Black–7% acetic acid for 1 hr. After staining, the gels were rinsed with H_2O to free them of excess dye and destained by standing in several changes of 7% acetic acid. All samples run in this system were incubated in the presence of β-mercaptoethanol prior to electrophoresis. The samples were dissolved in 10 M urea–0.9 N acetic acid–0.5 M β-mercaptoethanol and incubated at room temperature for 12–14

hr. Sample volume was 10–30 μl and 40–120 μg of protein applied unless noted otherwise.

The procedure of Johns (1967) was used to determine the quantities of protein fractions in some of the Amido Black stained gels. The gels to be analyzed were immersed in an ethanol–Dry Ice bath for 15 sec and then the stained bands cut with a single-edge razor blade. These gel sections were then placed in tubes and broken into small pieces with a glass rod; 1–3 ml of dimethyl sulfoxide was added to each. This mixture was incubated at room temperature for 24 hr. After centrifuging the pieces of gel down, the color intensity was measured at 600 mμ. The quantities of the protein fractions were expressed as a per cent of the total color eluted from the gels or, in the case of the individual histones, as a per cent of the total color bound to just the histone bands. This method involves a determination of the quantity of Amido Black bound to different proteins. As such it is very useful for the comparison of individual histone bands of one chromatin (cerebellar) with the corresponding bands of another chromatin (pituitary) as well as comparing the amount of nonhistone from each chromatin. The quantitation of any given protein fraction can only be approximate since we do not know for each individual protein how much dye is bound per unit weight of protein.

B. In the anode direction. The sodium dodecyl sulfate–polyacrylamide gel electrophoresis procedure (Weber and Osborn, 1969) was used for electrophoresis of proteins in the anode direction. Gels (0.6 × 8.0 cm) were 10% acrylamide, 0.1% sodium dodecyl sulfate. Protein (50–100 μg) was applied to a gel. The protein sample was first dissolved in 0.01 M sodium phosphate buffer, pH 7.0, 1% sodium dodecyl sulfate, and 0.1% β-mercaptoethanol. An aliquot of the protein solution was added to a solution containing 50 μl of buffer (0.1% sodium dodecyl sulfate–0.1% β-mercaptoethanol–0.01 M sodium phosphate, pH 7.0), 5 μl of β-mercaptoethanol, and 1 μl of tracking dye (0.1% brom phenol blue in H_2O). The total volume of sample applied to a gel was 60–80 μl. Staining of the gels was achieved by soaking them for 2 hr in 0.25% coomassie brilliant blue in 50% methanol–7% acetic acid. The gels were then rinsed with H_2O, stored overnight in 5% methanol–7.5% acetic acid, and then electrolytically destained in the 5% methanol–7.5% acetic acid.

Analytical Gel Electrophoresis of RNA. The electrophoresis system of Loening (1967) was used for the electrophoresis of RNA. The gels were 8% in acrylamide. *N,N'*-Methylenebisacrylamide concentration was 2.5% that of acrylamide. The reagents were added to one another in the following proportions: 5.33 ml of 15% acrylamide, 1.0 ml of *N,N'*-methylenebisacrylamide, 1.0 ml of buffer (0.4 M Tris–0.2 M sodium acetate–0.02 M sodium EDTA, pH 7.4), 2.4 ml of distilled H_2O, 0.0264 ml of tetramethylenediamine, 0.264 ml of 10% w/v ammonium persulfate. Size of the gels was 0.4 × 6.5 cm. RNA samples were dissolved in 0.05 ml of buffer (0.04 M Tris–0.02 M sodium acetate–0.002 M sodium EDTA, pH 7.4) and applied to the gels which had been preelectrophoresed for 1 hr at 2.5 mA/gel. Electrophoresis was conducted for 2.5 hr at 2.5 mA/gel. After the gels were removed from the glass tubes their pH was lowered by soaking them in 0.9 M acetic acid for 5 min. Then the gels were placed in staining solution (0.2% methylene blue in 0.2 M sodium acetate–0.2 M acetic acid) for 2 hr. Destaining was achieved by standing the gels in distilled H_2O. This is essentially the same procedure developed by Peacock and Dingman (1967) for fixing and staining RNA in polyacrylamide gels.

Preparation of Nonhistones. A. By the method of Marushige *et al.* (1968) with some modifications. Cerebellar chromatin (25 ml, 14.5 optical density, at 260 mμ, per ml) was adjusted to 0.2 N HCl in the cold with ice-cold 1 N HCl. This mixture was stirred for 0.5 hr and then centrifuged for 20 min. The pellets were suspended in 10 ml of 1% sodium dodecyl sulfate–0.05 M Tris, pH 7.9, and stirred at room temperature overnight until all of the material dissolved. The solution was then adjusted to 20 ml with distilled H_2O and put into 2 tubes in the Spinco 40 rotor. DNA was pelleted by centrifugation at 85,487*g* for 30 hr at room temperature. An aliquot of this supernatant was dialyzed against 250 ml of 0.01 M sodium phosphate buffer, pH 7.0, 0.1% sodium dodecyl sulfate, and 0.1% β-mercaptoethanol for 5 hr and utilized for electrophoresis in the sodium dodecyl sulfate–polyacrylamide gel system. Another aliquot, 1.6 ml, of the supernatant was lyophilized. The lyophilized material was adjusted to 0.5 ml with distilled H_2O and then 9 volumes of cold acetone added to precipitate the proteins according to the procedure of Weber and Osborn (1969). This precipitate was used for amino acid analysis as well as for electrophoresis in urea–polyacrylamide gels.

B. By elution from polyacrylamide gels. Proteins obtained by centrifuging cerebellar and pituitary chromatins in 7 M urea–3 M NaCl as described earlier were electrophoresed in the cathode direction according to the procedure given above. After electrophoresis of a set of gels one gel was stained and destained and used as a template to cut the nonhistone region from each of the other gels which had been quick frozen on a slab of Dry Ice and kept in a freezer. The nonhistone containing pieces of gel were cut into small pieces, placed in 10 M urea, and homogenized with a Teflon-to-glass homogenizer. The resulting slurry was centrifuged in a Spinco 40 rotor at 26,400*g* for 30 min at room temperature and the supernatants were combined, dialyzed against distilled H_2O (four 6-l. changes), and then lyophilized to dryness.

Hydrolyses of nonhistones prepared by methods A and B were done by dissolving the protein samples in 12 N HCl, diluting this to 6 N HCl, and placing them in vials which were then flushed with N_2, sealed, and maintained at 110° for 18 hr. The hydrolysates were diluted to 2 N HCl with distilled H_2O, lyophilized, dissolved in 0.2 N sodium citrate, pH 2.2, and chromatographed on a Beckman Spinco amino acid analyzer, Model 120. Hydrolyses of 0.4 N H_2SO_4 extracted cerebellar histone and of histone F1 were achieved by placing the samples in 6 N HCl and proceeding with the hydrolysis and subsequent amino acid analyses in the same way as was done with the nonhistone samples.

Preparation of Low Molecular Weight RNA. After lyophilizing the Bio-Gel A-50 chromatin protein peaks, which contain a portion of the RNA associated with cerebellar and pituitary chromatins, the technique of Huang and Huang (1969) for further purifying this RNA was used. In this procedure the protein and RNA mixture was dissolved in 40% guanidinium chloride–0.1 M sodium acetate, pH 6.5. Then solid CsCl was added and the solution adjusted to 20% guanidinium chloride–0.05 M sodium acetate–4 M CsCl. This solution was then centrifuged for 72 hr in a 40 rotor at 85,487*g* in a Spinco ultracentrifuge. The contents of the tubes were fractionated by inserting them in the Büchler dripping

TABLE I: Chemical Composition of Cerebellar and Pituitary Chromatin Preparations.[a]

	Cerebellar				Pituitary			
Preparation	DNA	RNA	Histone	Nonhistone	DNA	RNA	Histone	Nonhistone
1	1.00	0.10	1.48	0.73	1.00	0.092	1.71	0.40
2	1.00	0.13	1.83	0.53	1.00	0.10	1.66	0.52
3	1.00	0.14	1.47	0.32	1.00	0.099	1.46	0.39
4	1.00	0.15	1.37	0.48	1.00	0.14	1.41	0.48
5	1.00	0.15	1.85	0.46				
Mean ± SE[b]		0.13 ± 0.01	1.60 ± 0.10	0.50 ± 0.07		0.108 ± 0.01	1.56 ± 0.07	0.45 ± 0.03

[a] The values given are the mass ratios of each component to DNA. Determination of DNA, RNA, and histone and nonhistone protein were done as described in the text on 5 different cerebellar and 4 different pituitary chromatin preparations. [b] Standard error = $[\Sigma(x - \bar{x})^2/n(n-1)]^{1/2}$.

device and collecting 30-drop fractions. Usually 36 fractions were collected per centrifuge tube. For recovery of RNA which has been separated from the histone and nonhistone proteins on the basis of density difference the first 15 fractions (high density fractions) were pooled, dialyzed against distilled H_2O (three 6-l. changes), and then lyophilized to dryness. The partially purified RNA was then further purified by chromatography on DEAE-cellulose. The RNA sample was first dissolved in 2 ml of 0.2 M NaCl–0.05 M sodium acetate, pH 6.5, and applied to a 1.0 × 5.0 cm DEAE-cellulose column preequilibrated with 0.2 M NaCl–0.05 M sodium acetate, pH 6.5. The column was then washed successively with 15-ml aliquots of 0.2 M NaCl, 0.05 M sodium acetate, pH 6.5, 0.3 M NaCl, 0.05 M sodium acetate, pH 6.5. The RNA was then eluted with 0.7 M NaCl and some contaminant protein was eluted in the runoff fraction.

Results

I. CHEMICAL AND PHYSICAL PROPERTIES OF CEREBELLAR AND PITUITARY CHROMATIN. In order to minimize cytoplasmic contamination of the chromatin preparations, nuclei were first isolated from the cerebellar and pituitary tissues and were checked for possible contamination with other cell organelles, whole cells, myelin, and erythrocytes using phase contrast microscopy and, for one preparation of cerebellar nuclei, electron microscopy. The nuclei were found to be quite clean and free of such contamination according to the criteria of purity of Busch (1967) (see Figure 1). Cerebellar nuclei appear to be spherically symmetrical with intact membranes. Pituitary nuclei, on the other hand, appear somewhat distorted, although the membranes are intact. This distortion may be due to the fact that the pituitaries were quick frozen on Dry Ice before use.

Our definition of isolated chromatin is that nucleoprotein complex which results after four saline–EDTA[1] washes and successive Tris, pH 8.0, washes of the isolated nuclei followed by swelling of the chromatin in the cold overnight. After stirring the chromatin into solution by gentle stirring with a glass rod, the chromatin remains in solution after centrifugation at 10,000*g* for 30 min. It is to be noted that our chromatin preparation has not been mechanically sheared at any point in this procedure.

The chemical compositions of 5 cerebellar chromatin preparations and 4 pituitary preparations are presented in Table I. The quantities of DNA, RNA, histone, and nonhistone protein in the two chromatins appear to be similar and reproducible as are the melting curves and spectra of these chromatins (Figure 2). A noteworthy characteristic of the melting profiles is the fact that both cerebellar and pituitary chromatins have a hyperchromicity of 25 and 23%, respectively, which is substantially less than that obtained for the DNA prepared from these chromatins (Figure 2A). This observed hyperchromicity of chromatin compared to DNA is consistent with the data of Tuan and Bonner (1969) for calf thymus chromatin in which they reported both a higher molar extinction coefficient at 260 mμ and lower hyperchromicity on melting of chromatin compared with purified DNA. One noticeable consistent difference between these two chromatins is that cerebellar chromatin is very

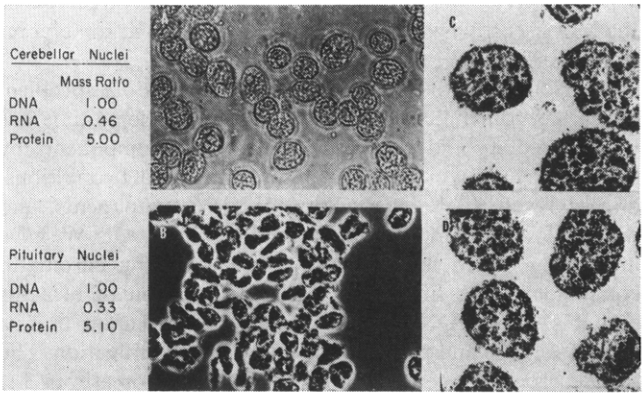

FIGURE 1: Phase and electron microscopy of purified nuclei. Nuclei were prepared and analyzed as described in the text. Photographs of nuclei were taken with the Zeiss photomicroscope at a magnification of 790× for the cerebellar nuclei (A) and 500× for the pituitary nuclei (B). C and D are electron micrographs of cerebellar nuclei taken at a magnification of 8200×.

[1] Abbreviation used is: saline–EDTA, 0.075 M NaCl–0.024 M disodium EDTA, pH 8.0.

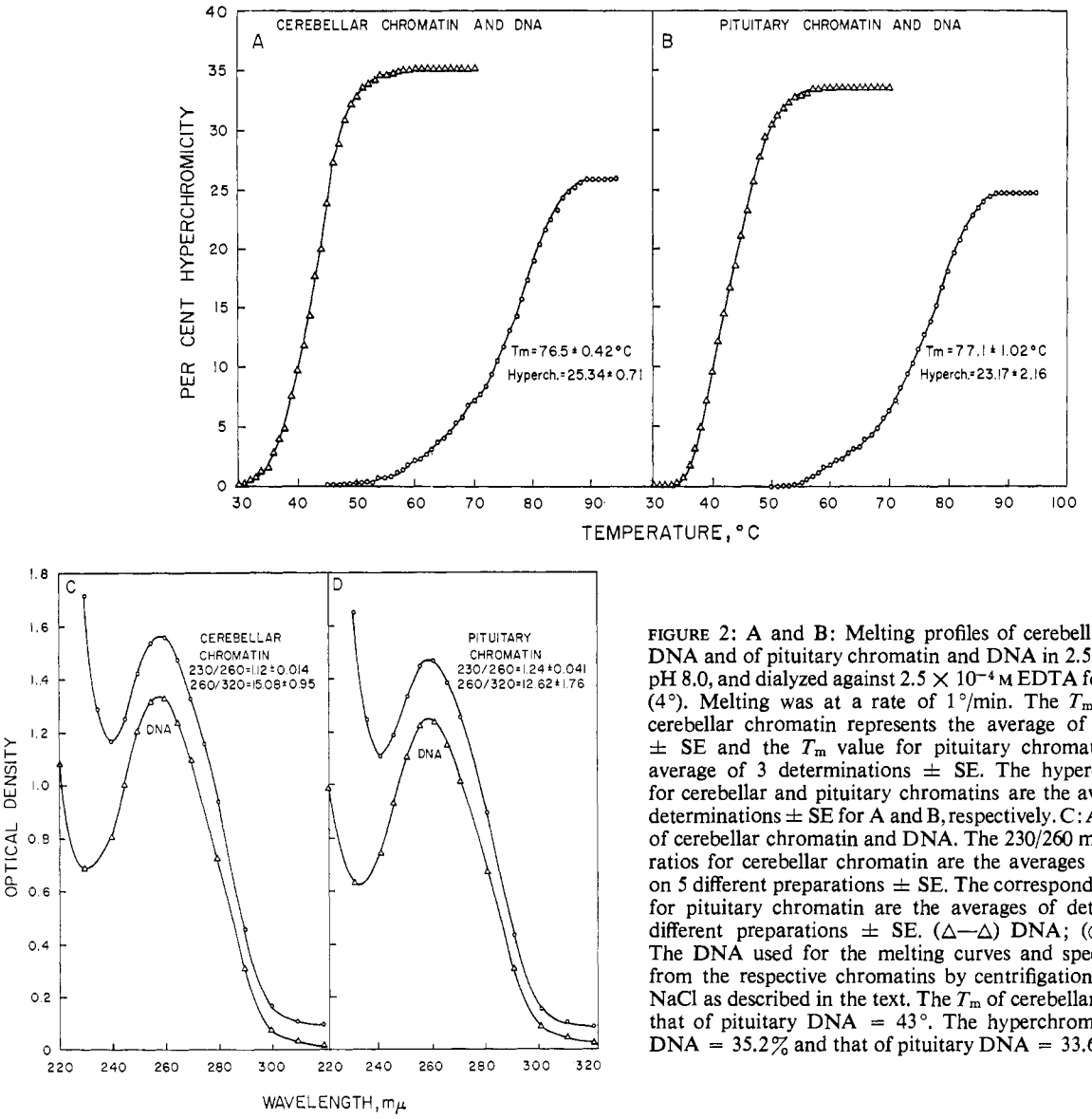

FIGURE 2: A and B: Melting profiles of cerebellar chromatin and DNA and of pituitary chromatin and DNA in 2.5 × 10^{-4} M EDTA, pH 8.0, and dialyzed against 2.5 × 10^{-4} M EDTA for 24 hr in the cold (4°). Melting was at a rate of 1°/min. The T_m value shown for cerebellar chromatin represents the average of 5 determinations ± SE and the T_m value for pituitary chromatin represents the average of 3 determinations ± SE. The hyperchromicity values for cerebellar and pituitary chromatins are the averages of 5 and 3 determinations ± SE for A and B, respectively. C: Absorption spectra of cerebellar chromatin and DNA. The 230/260 mμ and 260/320 mμ ratios for cerebellar chromatin are the averages of determinations on 5 different preparations ± SE. The corresponding spectral ratios for pituitary chromatin are the averages of determinations on 3 different preparations ± SE. (△—△) DNA; (○—○) chromatin. The DNA used for the melting curves and spectra was prepared from the respective chromatins by centrifugation in 7 M urea–3 M NaCl as described in the text. The T_m of cerebellar DNA = 43° and that of pituitary DNA = 43°. The hyperchromicity of cerebellar DNA = 35.2% and that of pituitary DNA = 33.6%.

viscous whereas pituitary chromatin is not. It is possible that the low viscosity is due to the action of DNase at some point in the preparation of the pituitary chromatin.

II. Isolation of chromatin components. A problem which has confronted the chromatin field for some time has been to isolate and characterize chromatin components by mild procedures which do not involve extreme pH conditions, do not result in the aggregation of these components, and which allow the complete recovery of the components. We have utilized two such mild procedures for isolating chromatin components. The first involves the dissociation of proteins and RNA from DNA by dissolving the chromatin in 7 M urea–3 M NaCl and pelleting the DNA by centrifugation. The second procedure consists of dissolving the chromatin in 3 M NaCl, shearing the solution, and separating DNA from protein and RNA by gel filtration chromatography on Bio-Gel A-50.

As can be seen in Table II dissociation of cerebellar and pituitary chromatins with 7 M urea–3 M NaCl followed by centrifugation results in the recovery of 90–95% of the chromosomal proteins and a smaller and more variable percentage of the chromosomal RNA. The pellets obtained by this procedure contain in 100% recovery the chromatin DNA plus 5–10% of the chromatin protein and a substantial amount of RNA. Most of the pellet is soluble in dilute saline citrate (0.15 M NaCl–0.015 M sodium citrate) or distilled in H_2O. However, a small portion of the pellet remains as an insoluble aggregate.

Dissociation of the chromatin preparations with 3 M NaCl and the subsequent separation of the components on a Bio-Gel A-50 column (Figure 3A,B) resulted in the recovery of 90–96% of the chromosomal proteins and a higher percentage of the RNA than obtained by centrifugation in 7 M urea–3 M NaCl. The DNA (Figure 3A fractions 14–35, 3B fractions 11–20) obtained by this method does not contain any aggregated material. Chromosomal proteins and RNA (Figure 3A fractions 36–53, 3B fractions 34–51) were recovered by dialysis against distilled H_2O and lyophilized. The DNA and its associated RNA were recovered in this way also.

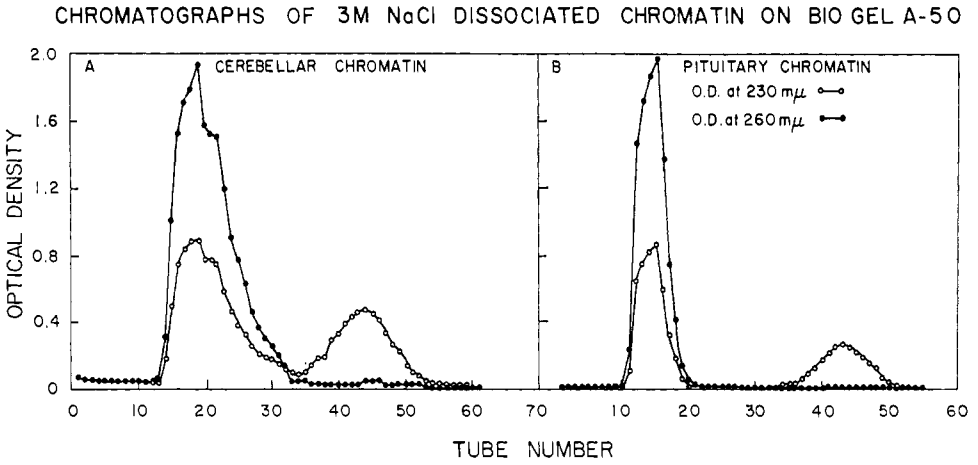

FIGURE 3: Chromatographic fractionation of cerebellar and pituitary chromatins in 3 M NaCl on Bio-Gel A-50 columns. The 3 M NaC dissociated chromatins were fractionated on the columns as described in the text.

TABLE II: Recovery of Protein and RNA from Chromatin by Centrifugation in 7 M urea–3 M NaCl and from Bio-Gel A-50 Columns in 3 M NaCl.

	Cerebellar						Pituitary					
	Supernatant from Centrifugation			Bio-Gel A-50 column			Supernatant from Centrifugation			Bio-Gel A-50 column		
Protein	85[a]	88	94	89	94[a]	90	82	95	95	94	96	95
RNA			17	12	42	32			61	30	57	70

[a] These numbers are the per cent of protein and RNA recovered in the supernatant resulting after centrifugation of the chromatin preparations in 7 M urea–3 M NaCl or in the protein peak obtained by chromatography of the chromatin preparations in 3 M NaCl on Bio-Gel A-50 columns. For the centrifugation experiments they were calculated by dividing the quantity of protein or RNA in the supernatant by the sum of this quantity plus the quantity of protein or RNA in the pellet × 100. For the Bio-Gel A-50 columns the per cent of protein or RNA values were calculated by dividing the quantity of protein or RNA in the proteinl peak (see Figure 3A,B) by the sum of this quantity plus the quantity of protein or RNA in the DNA peak × 100.

III. ANALYSIS OF CHROMATIN COMPONENTS. A. Preparation and analysis of nonhistone chromatin proteins. Two methods were used for preparing nonhistone chromatin proteins. The method of Marushige *et al.* (1968) was first employed. This procedure involved extraction of histones from cerebellar chromatin with 0.2 N HCl and subsequent recovery of the nonhistones which were not acid extractable by dissolving the DNA–nonhistone pellet in sodium dodecyl sulfate and pelleting the DNA by centrifugation. These proteins were then analyzed for amino acid content and also for their electrophoretic patterns in both the sodium dodecyl sulfate–polyacrylamide and urea–polyacrylamide (pH 2.7) gel electrophoresis systems. According to the amino acid analysis, (Table III), glutamic and aspartic acids consisted of 22.5 moles % of the total recovered amino acids whereas lysine, histidine, and arginine consisted of 15.6 moles %. The electrophoretic patterns of this chromatin protein fraction demonstrate its heterogeneity (Figures 4c and 5c).

The second method employed for the preparation of nonhistones utilized the milder procedure for extracting chromosomal proteins with 7 M urea–3 M NaCl as described above. Total chromatin proteins, extracted by this procedure, were analyzed in the urea–polyacrylamide gel electrophoresis system. The electrophoretic patterns obtained for both cerebellar and pituitary chromatin proteins are shown in Figure 5a,b. For comparative purposes the electrophoresis in this gel system of a well-characterized acidic protein, bovine serum albumin, is shown in Figure 6h. Bovine serum albumin has a pI of 4.7 (Longsworth and Jacobsen, 1949) and an Asp + Glu/Lys + His + Arg ratio of 1.3 (Table III). In order to further separate the nonhistone bands from one another electrophoresis was conducted at a higher voltage (130 V) and longer time (5 hr) compared with the gels in Figure 5a,b. Total cerebellar chromatin proteins extracted with 7 M urea–3 M NaCl as well as cerebellar chromatin nonhistones, prepared by sodium dodecyl sulfate extraction of acid extracted chromatin, were electrophoresed in this way (Figure 5c,d). The nonhistone banding patterns are extremely complex and at least 20 bands are present. Also, total cerebellar chromatin proteins were electrophoresed in the sodium dodecyl sulfate–polyacrylamide gel system (Figure 4a). Since the nonhistones were clearly separated from the histones in

TABLE III: Amino Acid Analyses of Nonhistones Prepared from Cerebellar and Pituitary Chromatins.[a]

	Nonhistones from[b] Residue after Acid Extraction of Cerebellar Chromatin	Nonhistones from[c] 7 M Urea–3 M NaCl Supernatant of Cerebellar Chromatin	Nonhistones from[c] 7 M Urea–3 M NaCl Supernatant of Pituitary Chromatin	Bovine[d] Serum Albumin
Lysine	7.4	8.8	8.4	9.4
Histidine	2.2	1.6	2.1	2.2
Arginine	6.0	4.2	5.0	4.2
Aspartic	9.6	8.9	8.0	8.4
Threonine	4.2	4.8	5.0	5.5
Serine	7.2	9.6	10.1	4.8
Glutamic	12.9	10.4	13.3	11.6
Proline	7.6	6.2	4.8	5.0
Glycine	10.8	13.1	10.8	3.0
Alanine	7.4	7.6	7.5	8.0
Half-cystine	Trace	1.0	1.0	5.1
Valine	5.3	5.5	5.0	5.7
Methionine	1.3	0.4	1.2	0.5
Isoleucine	3.7	3.9	3.7	2.2
Leucine	7.6	6.6	7.6	12.4
Tyrosine	3.1	1.9	2.4	3.9
Phenylalanine	3.8	3.3	3.0	4.5
Phosphoserine		1.8	1.0	
$\frac{\text{Glutamic + aspartic}}{\text{Lysine + histidine + arginine}}$	1.4	1.3	1.4	1.3

[a] Values expressed in moles per hundred moles of recovered amino acids. [b] Nonhistones prepared from the precipitate remaining after extraction of cerebellar chromatin with 0.2 N HCl as described in the text. [c] Nonhistones prepared by elution from urea–polyacrylamide gels after electrophoresis of cerebellar and pituitary chromatin proteins extracted from the chromatins with 7 M urea–3 M NaCl as described in the text. [d] Bovine serum albumin (Sigma, fraction V) was hydrolyzed and analyzed using the same procedure used for the other samples as described in the text.

the urea–polyacrylamide gels, they were prepared by elution from these gels and then subjected to amino acid analysis as well as reelectrophoresis in the sodium dodecyl sulfate–polyacrylamide gel system (Figure 4b).

As is shown in Table III the amino acid profile of the cerebellar chromatin nonhistones is similar although not identical with that of cerebellar nonhistones prepared by the acid extraction and subsequent sodium dodecyl sulfate extraction of the nonhistones remaining associated with the DNA. The amino acid profile of pituitary chromatin nonhistones, prepared by elution from urea–polyacrylamide gels, is quite similar to that of cerebellar nonhistones as is their electrophoretic pattern (Figure 5a,b). In addition to the greater quantity of glutamic and aspartic acid residues compared to the lysine, histidine, and arginine content is the high percentage of serine in these nonhistone fractions. These properties of nonhistone chromosomal proteins were also found in the nuclear phosphoprotein fraction of calf thymus (Kleinsmith and Allfrey, 1969) and rat liver (Langan, 1967), in the nuclear acidic protein fractions prepared from rat liver nuclei (Steele and Busch, 1963), in the euchromatin and nuclear acidic proteins prepared from rat liver (Benjamin and Gellhorn, 1968) and in the nonhistone fraction isolated from rat liver chromatin (Wang, 1967; Marushige *et al.*, 1968; S. C. Elgin and J. Bonner, 1970, submitted for publication). It should also be noted that an amino acid peak with a retention time identical with that for authentic phosphoserine was detected in both the cerebellar and pituitary nonhistone amino acid analyses. Since phosphoserine would not be expected to survive the strong acid hydrolysis conditions in the amino acid analyses, further chemical analyses employing milder hydrolysis conditions will be necessary to rigorously establish the structure and quantity of this amino acid.

Also it should be emphasized that since we do not know the isoelectric points of our nonhistones or the per cent of the glutamic and aspartic acid residues in the amide form we can only tentatively classify them as acidic proteins.

B. Quantitation of nonhistone proteins in cerebellar and pituitary chromatins. The conventional method used for the quantitation of nonhistone protein involves the use of acid for extracting histone from chromatin and assaying the protein residue not soluble in the acid as the total nonhistone protein. Our quantitation of the nonhistone contents of cerebellar and pituitary chromatins by this method are presented in Table I (expressed as a mass ratio to DNA) and are also presented in Table IV (expressed as a per cent of the total

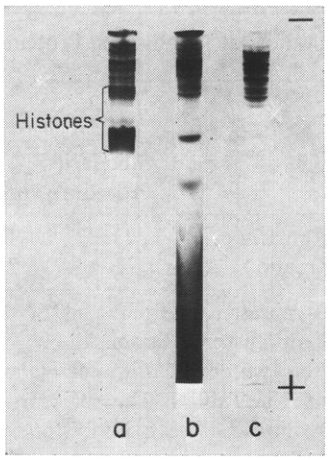

FIGURE 4: Electrophoresis of cerebellar chromatin protein fractions in sodium dodecyl sulfate–10% polyacrylamide gels: (a) 100 μg of cerebellar chromatin proteins extracted with 7 M urea–3 M NaCl; (b) 50 μg of cerebellar chromatin nonhistone proteins obtained by elution from polyacrylamide gels after electrophoresis of 7 M urea–3 M NaCl extracted proteins in the cathode direction as described in the text; (c) 50 μg of cerebellar chromatin nonhistone proteins prepared by sodium dodecyl sulfate extraction of the DNA–nonhistone residue which remains after acid extraction of the chromatin. The histone bands indicated in (a) have mobilities with respect to the marker dye of 0.68 for the fastest moving one and 0.38 for the other band. At lower concentrations both of these histone bands consist of two bands whose mobilities are close to each other. The mobilities of the histone bands of 0.4 N H_2SO_4 extracted histones in this gel system are the same as those in 7 M urea–3 M NaCl extracted proteins (gel not shown).

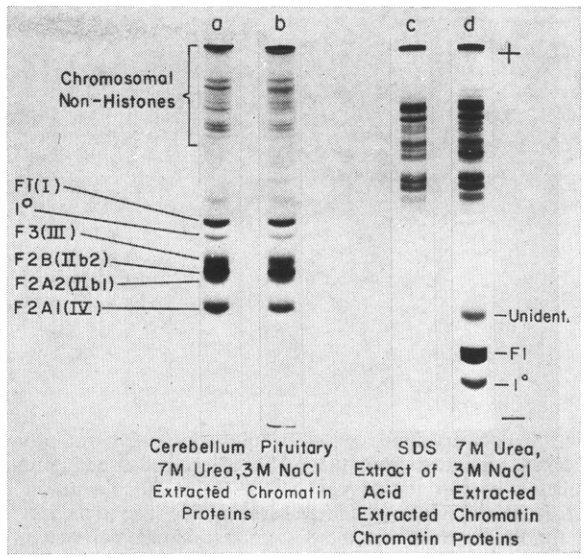

FIGURE 5: Electrophoresis of cerebellar and pituitary chromatin proteins. These proteins were electrophoresed in 15% polyacrylamide gels which were 2.5 M in urea and 0.9 N acetic acid, pH 2.7. The gels were preelectrophoresed and electrophoresis was conducted as described in the text. Direction of migration was toward the cathode. (a) Total cerebellar and (b) pituitary chromatin proteins extracted with 7 M urea–3 M NaCl were applied to the gels. Cerebellar (110 μg) and pituitary (85 μg) proteins were applied. The duration of electrophoresis was 3 hr. Histone bands are designated according to the nomenclature of Johns, Phillips, and Butler (Butler, 1966) and the nomenclature of Luck *et al.* (1958) is given in parentheses for reference purposes (c and d). Electrophoresis of cerebellar chromatin proteins was for a longer time, 5 hr. Extracted cerebellar chromatin proteins (270 μg, 7 M urea–3 M NaCl) (c) and 96 μg of cerebellar chromatin nonhistones prepared by sodium dodecyl sulfate extraction of acid extracted chromatin (d) were applied to the gels. In this experiment the gels were electrophoresed at 130 V for 5 hr in order to further resolve the nonhistone protein bands.

chromatin protein). Since, as can be seen in the electrophoretic pattern of 0.4 N H_2SO_4 extracted cerebellar histones (Figure 6i), some nonhistone protein was also extracted, the measurement of the nonhistone protein by the conventional method will be somewhat lower than it should be. We have determined that approximately 9.8% of the acid extracted total protein is nonhistone by eluting the Amido Black stain of all the bands on the gel and measuring the ratio of color in the nonhistone bands to total color eluted. Also, therefore, the histone to DNA mass ratios reported in Table I are somewhat higher than they should be.

Two additional procedures were used to measure the quantity of nonhistone protein present in the 7 M urea–3 M NaCl extracted proteins. Both measure the quantity of nonhistone protein which enters the urea–polyacrylamide gel system at pH 2.7. We have determined that 96% of the applied 7 M urea–3 M NaCl supernatant proteins enter this gel system by conducting the electrophoresis with a piece of dialysis tubing tightly secured over the gel glass tubing and analyzing the buffer solution above the gel, after electrophoresis, for protein by the Lowry (1951) procedure; 4% of the applied protein sample, by this analysis, was present in the buffer above the gel after electrophoresis. Thus, the urea–polyacrylamide gel system, pH 2.7, is capable of analyzing virtually all of our chromatin proteins. The first of these procedures is the Amido Black stain elution technique of Johns (1967) by which the quantity of Amido Black eluted from the nonhistone region of the gel divided by the total color eluted from the gel $\times$ 100 gives the per cent nonhistone protein. Since the capacity for binding the Amido Black stain by the

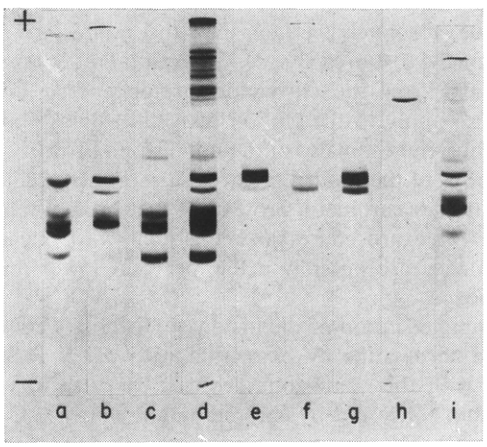

FIGURE 6: Electrophoresis of proteins in the urea–polyacrylamide gel system: (a) 0.4 N H_2SO_4 extracted calf thymus histones; (b) cerebellar chromatin histones extracted with 3.5% $HClO_4$; (c) cerebellar chromatin histones remaining in the nucleoprotein pellet after 3.5% $HClO_4$ extraction. These histones were extracted from the pellet with 0.4 N H_2SO_4; (d) protein extracted from cerebellar chromatin with 7 M urea–3 M NaCl; (e) histone F1 prepared by elution from urea–polyacrylamide gels; (f) histone band 1° was prepared by elution from gels; (g) F1 + 1° prepared as in e and f and electrophoresed together; (h) 5 μg of bovine serum albumin (Sigma fraction V); (i) 0.4 N H_2SO_4 extracted histones from cerebellar chromatin.

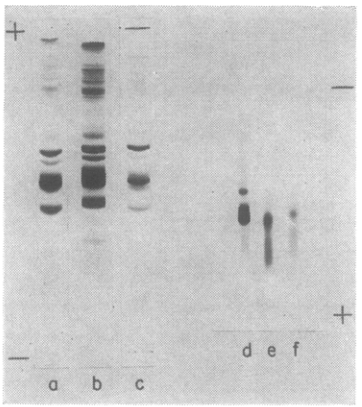

FIGURE 7: Polyacrylamide gel electrophoretic patterns of proteins and RNA separated from the DNA of cerebellar and pituitary chromatins by chromatography on Bio-Gel A-50 columns in 3 M NaCl. The urea–polyacrylamide gel system described in the text was used for the proteins and the 8% polyacrylamide gel system described in the text was used for the RNA: (a and b) 70 and 120 μg of total cerebellar chromatin proteins, respectively; (c) 40 μg of total pituitary chromatin proteins (the histone and nonhistone bands seen in these gels are further described in Figure 5a); (d) 16 μg of commercially purified tRNA (Schwarz Bio Research, Inc., *E. coli* B); (e) 40 μg of low molecular weight RNA fraction prepared from cerebellar chromatin as described in the text; (f) 20 μg of low molecular weight RNA fraction prepared from pituitary chromatin as described in the text. The 8% polyacrylamide gels used for these RNA samples were preelectrophoresed for 1 hr at 2.5 mA/gel, pH 7.4, and electrophoresis conducted for 2.5 hr at 2.5 mA/gel.

TABLE IV: Quantitation of Nonhistone Protein.

	Standard[a] Procedure	% Stain on gels	Urea Elution from Gels
Cerebellar chromatin	23.8%	19.9	23.8
Pituitary chromatin	22.4	22.7	24.4

[a] These values represent the per cent of total chromatin protein which is nonhistone. Standard procedure refers to the analyses of the two chromatins, cerebellar and pituitary, which are presented in Table I. The per cent nonhistone was calculated by dividing the mean nonhistone to DNA mass ratio by the sum of the mean histone plus nonhistone to DNA mass ratios × 100. Per cent stain on the gels is the per cent of dimethyl sulfoxide extracted Amido Black stain in the nonhistone protein region of the urea–polyacrylamide gels as described in Table V. This value is obtained by dividing the color (total optical density at 600 mμ) eluted from the nonhistone protein region of the gels by the total color eluted from all of the bands in the gel. Urea elution from gels refers to the quantity of protein eluted from the nonhistone region of polyacrylamide gels with 10 M urea divided by the total quantity of protein applied on the gels × 100. The protein samples used for nonhistones quantitation on urea–polyacrylamide gels by assaying the per cent stain and by urea elution were the supernatant fractions obtained by centrifugation of the chromatins in 7 M urea–3 M NaCl as described in the text. The electrophoresis of these proteins in the cathode direction and the procedures for elution of Amido Black from the gels and elution of nonhistones from the gels are described in the text.

different proteins is not known this method can only be approximate.

The technique of eluting the nonhistone protein from unstained urea–polyacrylamide gels should give an accurate estimate of the nonhistone protein content of the 7 M urea–3 M NaCl supernatant fraction although, since some 5–10% of the chromatin proteins are sedimented with the DNA; we cannot be absolutely certain that the nonhistone protein content of the 7 M urea–3 M NaCl supernatant is representative of 100% of the chromatin proteins. This limitation would also apply to the elution of Amido Black stain technique. Thus, our estimates of the nonhistone protein contents (19.9–23.8% of the cerebellar chromatin protein, 22.4–24.4% of the pituitary chromatin protein) of the chromatin preparations by three procedures are consistent with one another although they are possibly a few per cent lower than they should be.

C. Characterization of the histones. Histones were isolated from the chromatins by extraction with 0.4 N H_2SO_4 and together with the nonhistone proteins by extraction with 7 M urea–3 M NaCl and by chromatography on Bio-Gel A-50 in 3 M NaCl. The electrophoretic patterns of the 7 M urea–3 M NaCl extracted proteins from both cerebellar and pituitary chromatins are shown in Figure 5a,b. The electrophoretic patterns of the 0.4 N H_2SO_4 extracted cerebellar histones and the 3 M NaCl extracted proteins from cerebellar and pituitary chromatins are shown in Figures 6i 7a,b,c, respectively. We have designated the histones using the nomenclature of Johns, Phillips and Butler (Butler, 1966) (Figure 5a). The histone patterns are similar to those obtained by Panyim and Chalkley (1969a,b) using 0.4 N H_2SO_4 extracted histones from a variety of calf and mouse tissues. A noteworthy point for cerebellar and pituitary histones is the presence of a band, 1°, whose mobility is intermediate between that of histones F1 and F3. This band is not present in calf thymus histones (Figure 6a). Panyim and Chalkley have reported that a histone band with a mobility the same as this one is lysine rich and apparently it is present in chromatins isolated from tissues not undergoing rapid cell division (1969a,b). Quantitative analysis of the Amido Black in the histone bands reveals that 4.1% and 3.4% of the total color bound to histones are present in this band in cerebellar and pituitary histones, respectively (Table V). Both histones F1 and 1° are completely extracted with 3.5% $HClO_4$ according to the selective acid extraction technique of Johns (1964) (Figure 6b,c). As can be seen F1 and 1° are extracted with 3.5% $HClO_4$ along with some of the other histones but no F1 and 1° is present in the extracted nucleoprotein residue while the other histones are and these were extracted from the residue with 0.4 N H_2SO_4.

Amino acid analyses of 0.4 N H_2SO_4 extracted cerebellar histones and of histone F1 prepared by elution from the urea–polyacrylamide gels are presented in Table VI. (See Figure 6e,f,g for electrophoretic analysis of purified F1 and that of 1° whose electrophoretic mobility is close to that of F1.) The amino acid profile of total histone is comparable

TABLE V: Colorimetric Quantitation of Protein Bands on Urea–Polyacrylamide Gels.[a]

	Cerebellar Chromatin Proteins		Pituitary Chromatin Proteins	
	% of Total Histone	% of Total Protein	% of Total Histone	% of Total Protein
Nonhistones		19.90		22.70
F1	11.65		11.00	
1°	4.06		3.37	
		77.14		75.73
F3 + F2B + F2A2	66.40		67.90	
F2A1	17.80		17.60	
Unidentified		2.96		1.57

[a] Chromatin proteins obtained from the supernatant after centrifugation in 7 M urea–3 M NaCl as described in the text were electrophoresed in the cathode direction (Figure 5a,b). After staining and destaining with Amido Black the designated bands were cut from the gels and the stain was eluted by the procedure of Johns (1967). The F3, F2B, and F2A2 bands were combined due to the fact that they were close together. Unidentified refers to the faintly stained band which migrates slower than histone F1 but faster than the nonhistone proteins. It can be seen in Figure 6b,c that this protein is not extracted from chromatin with 3.5% $HClO_4$ but is extracted with 0.4 N H_2SO_4. Also included in unidentified is a very faint band which migrates considerably faster than histone F2A1.

to that of calf thymus, also presented in Table VI, and the amino acid profile of F1 is comparable to that obtained from a number of tissues (Fambrough, 1969) and has the high Lys/Arg ratio (22:1) and high Ala content (21.8 moles %) typical of lysine rich histones.

D. Isolation and characterization of the low molecular weight RNA from cerebellar and pituitary chromatins. The RNA fraction which is extractable from cerebellar and pituitary chromatins along with the chromatin proteins on Bio-Gel A-50 columns (Figure 3A,B) was further purified using the density gradient (4 M CsCl, 20% guanidinium chloride, 0.05 M sodium acetate, pH 6.5; centrifuged 72 hr at 85,487*g*) centrifugation technique (Huang and Huang, 1969; Huang and Smith, 1970). This procedure separates the RNA from chromatin proteins on the basis of the density difference between the RNA and proteins. The RNA was further purified by chromatography on DEAE-cellulose and then electrophoresed on 8% polyacrylamide gels (Figure 7d,e,f). It is apparent from the electrophoretic mobilities of the cerebellar and pituitary chromatin low molecular weight RNAs compared with the mobility of *Escherichia coli* transfer RNA that this fraction consists of RNA of molecular weights ranging between that of tRNA and lower. This result is consistent with the finding of Huang and Smith (1970) that the low molecular weight RNA fraction isolated from chicken embryo chromatin (dihydropyrimidine rich, peptide bound RNA) has a heterogeneous molecular weight distribution with a range of 15,200–23,600 and a weight-average molecular weight of 17,000 as determined by sedimentation equilibrium. The electrophoretic pattern of chicken embryo chromatin RNA and that of chicken embryo brain chromatin RNA (L. M. J. Shaw and R. C. Huang, unpublished observation, 1970) are both similar to the patterns of cerebellar and pituitary low molecular weight RNAs.

Since some chromosomal RNA is eluted from the Bio-Gel A-50 columns with the DNA peak, it is apparent that this RNA fraction is either a higher molecular weight species or is an RNA species which is tightly bound to the DNA and not dissociable with 3 M NaCl. We have not attempted to further isolate and characterize this RNA fraction.

Discussion

Rationale for Using Isolated Nuclei for Chromatin Preparations. We have prepared cerebellar and pituitary chromatins from isolated nuclei prepared by the sucrose-calcium procedure since initial attempts using the "direct" procedure, in which the saline–EDTA washes are initiated on the whole tissue homogenate, gave what appeared to be highly contaminated chromatin. The latter conclusion was drawn from the fact that the spectral ratio 230/260 mμ was as high as 2:1 and the 260/320 mμ ratio was between 2:1 and 3:1. These ratios would indicate that the preparations were contaminated with protein (Bonner *et al.*, 1968a), and perhaps pieces of cells such as myelin fragments. The latter were found to be contaminants of crude nuclear preparations from adult chicken brain and, as judged by inspection with phase-contrast microscopy, were entirely removed by sedimenting the crude nuclei through high-density (*ca.* 1.58 M) sucrose (Dingman and Sporn, 1964). Thus for the pig cerebellar and pituitary tissues the direct procedure for chromatin preparation was not used. The latter procedure, however, has been successfully used in our laboratory for preparing highly purified chromatins from the sea urchin, *Arbacia punctulata* (R. A. Paoletti and R. C. Huang, 1969; T. Shirey and R. C. Huang, 1969), and from calf thymus (L. Kleiman and R. C. Huang, 1970, manuscript in preparation). The nuclei from these two sources occupy a large portion of the total cellular volume. A nuclear to cytoplasm ratio of approximately 3:2 has been found for calf thymus (Fambrough, 1969).

Advantages and Disadvantages of the 7 M Urea–3 M NaCl Procedure for Extracting Chromatin Components. The advantages of this extraction procedure are that it extracts 90–95% of the total chromatin proteins (histones and nonhistone proteins) in soluble form. The 7 M urea–3 M NaCl protein solution can then be dialyzed against 0.05 N acetic acid or

TABLE VI: Amino Acid Analyses of Histones[a]

	Cerebellar[b]	Calf Thymus[c]	Cerebellar[d] F1
Lysine	14.0	14.5	24.9
Histidine	2.2	2.2	0.2
Arginine	8.0	8.9	1.1
Aspartic	5.0	5.1	3.3
Threonine	5.6	5.6	5.4
Serine	8.4	5.2	10.0
Glutamic	8.3	8.6	5.6
Proline	5.8	4.5	6.7
Glycine	9.1	8.7	10.3
Alanine	12.7	12.9	21.8
Half-cystine	Trace	0.4	0
Valine	4.9	6.1	4.6
Methionine	0.7	0.6	0
Isoleucine	3.8	4.4	1.2
Leucine	7.3	8.0	3.5
Tyrosine	2.4	2.5	0.6
Phenylalanine	1.8	1.8	0.7
Lys + Arg + His/Asp + Glu	1.8	1.9	2.9
Lys/Arg	1.8	1.6	22.0

[a] Values expressed in moles per 100 moles of amino acids. [b] Total cerebellar histones were prepared by extracting cerebellar chromatin with 0.4 N H_2SO_4. [c] Data from Johns and Forrester (1969); calf thymus total histones were extracted from calf thymus chromatin with 0.25 N HCl. [d] Cerebellar histone F1 was prepared by elution from urea–polyacrylamide gels.

distilled H_2O and lyophilized to dryness or it can be dialyzed against 7 M urea until all of the salt has been removed and then utilized directly for further fractionation on ion exchange columns. These proteins are completely soluble under the latter conditions. The disadvantages of this procedure lie in the fact that it is a time-consuming one in that 48 hr are required for complete pelleting of the DNA followed by an additional 3–4 days for dialysis and lyophilization. Also, as noted in the Experimental Section, the pellet cannot be completely solubilized.

Advantages and Disadvantages of the Bio-Gel A-50 Procedure for Fractionating Chromatin Components in 3 M NaCl. This procedure is capable of extracting 90–96% of the total chromatin proteins. The dialyzed and lyophilized protein mixture is completely soluble in urea. Another advantage of this procedure is that large quantities of chromatin can be fractionated in short periods of time. For example, 270–500 optical density 260 mμ units of cerebellar chromatin in 3 M NaCl were readily fractionated on a 4.0 × 74 cm column in 13 hr. Larger quantities of chromatin could be fractionated on proportionately larger columns. In addition to the usefulness of this procedure for the preparation of chromatin proteins, the low molecular weight chromatin RNA fraction is extractable with the proteins and can then be further purified and analyzed. A possible disadvantage of this procedure lies in the fact that the chromatin is sheared prior to chromatography. This results in the formation of a small quantity of aggregated chromatin (approximately 5–10%) which needed to be centrifuged out of the chromatin solution before chromatography on the Bio-Gel A-50 columns.

Description of the Chromatin Associated Nonhistone Proteins. There are numerous reports in the literature in which the quantity of nonhistone proteins in different chromatins were determined (data summarized in Bonner *et al.*, 1968b) and in some cases amino acid analyses were reported on the further purification and analysis of these proteins compared to the histones. Several recent reports have used polyacrylamide gel electrophoresis for analyzing nonhistone protein (Benjamin and Gellhorn, 1968; Loeb and Creuzet, 1969; S. C. Elgin and J. Bonner, 1970, manuscript submitted for publication). We have reported the quantitation, amino acid analysis, polyacrylamide gel electrophoresis in urea—and sodium dodecyl sulfate—polyacrylamide gels as well as two extraction procedures which do not involve the extremes of pH as would be encountered using a procedure utilizing acid extraction. We are now actively pursuing investigations into procedures by which we can further purify the nonhistone proteins utilizing these mild extraction procedures as a first step in the procedure. In addition to the possibility of separating these proteins on ion-exchange columns we will also pursue the possibility of separating them utilizing the technique of polyacrylamide gel electrophoresis. Since the histone and nonhistone proteins were well separated on the analytical urea–polyacrylamide gel system of Panyim and Chalkley the use of this gel system on a preparative scale utilizing the preparative disc electrophoresis apparatus (Canalco) may provide a simple and rapid means for the further purification of chromatin proteins.

Several questions need to be answered regarding the role individual nonhistone proteins play in the structure and function of chromatin. (1) Of the nonhistone proteins present in the chromatin isolated from tissues such as cerebellum and pituitary which protein(s) are essential to interphase chromatin structure? (2) Which of the nonhistone proteins are enzymes? (3) Are any of the nonhistone proteins regulatory proteins of the type which have been shown to function in bacteria (*e.g.*, β-galactosidase repressor, Zubay *et al.*, 1967)? An interesting and apparently reproducible observation is that the nonhistone protein fraction is present in greater amount in chromatin isolated from tissues which are very active in protein synthesis (*e.g.*, liver, Seligy and Miyagi, 1969; sea urchin embryo plutei, Marushige and Osaki, 1967; trout testis in stages 1 and 2 of spermatogenesis, Marushige and Dixon, 1969) compared to the chromatin isolated from tissues which are much less active in protein synthesis (*e.g.*, chicken erythrocytes, Seligy and Miyagi, 1969; sea urchin sperm, Paoletti and Huang, 1969; sea urchin blastulae, Marushige and Osaki, 1967; trout testis in stages subsequent to 1 and 2 of spermatogenesis, Marushige and Dixon, 1969). (4) Which of the nonhistone proteins are phosphoproteins? A number of studies have been reported in which a nuclear phosphoprotein fraction was isolated and characterized (Kleinsmith *et al.*, 1966; Kleinsmith and Allfrey, 1969; Langan, 1967; Wang, 1967). Kleinsmith, *et al.* (1966) have suggested that protein phosphorylation may be related to the physical structure and genetic function of chromatin. (5) It has been proposed by Johns and Forrester (1969) that isolated chromatin (prepared by either the direct procedure or by

first isolating nuclei) may in fact have adsorbed proteins from the cytoplasm and/or the nucleoplasm during the preparative procedure. Thus future studies of the function of nonhistone proteins will have to be designed to distinguish between those nonhistone proteins which are essential to the structure and function of chromatin and those which are not.

Description of the Chromatin-Associated Low Molecular Weight RNA. A unique low molecular weight RNA species (*ca.* 3S) has been isolated from a variety of tissues (Huang and Bonner, 1965; Dahmus and McConnell, 1969; Huang and Smith, 1970; De Filippes, 1970; Getz and Saunders, 1970). In addition to its small size this class of RNA is characterized by its association (possibly by covalent linkage) with a protein and by its high content of dihydropyrimidine nucleotide. The nature of the possible mode of covalent linkage of this RNA to protein has been studied in the case of chick embryo chromatin RNA (Huang and Smith, 1970). The function of this class of RNA in conferring specificity to the interactions between chromosomal proteins and DNA has been suggested (Huang and Huang, 1969; Bekhor *et al.*, 1969; Getz and Saunders, 1970).

One of the major efforts of our laboratory has been to develop procedures which would achieve good recovery of the low molecular weight RNA fraction associated with chromatin. Previous studies (indicated in the reference above) utilized the technique of density gradient equilibrium centrifugation to separate this RNA, along with the chromatin proteins, from DNA. The RNA could then be further purified by digesting the proteins with a proteolytic enzyme followed by phenol extraction of the RNA (Dahmus and McConnell, 1969). De Filippes (1970) has recently reported a method for isolating this class of RNA. His procedure consisted of subjecting HeLa cell chromatin to DNase digestion followed by phenol extraction. The RNA thus purified was then analyzed on polyacrylamide gels.

Thus far two procedures have been utilized in our laboratory for preparing this RNA fraction from chromatin. The first procedure utilized sodium dodecyl sulfate or high salt to dissociate the low molecular weight RNA from DNA and subsequent centrifugation to separate the RNA along with the chromatin proteins. Preparative disc electrophoresis (6.5% acrylamide, Ornstein, 1964) was then used as the major means for further purification (Huang and Smith, 1970; Shirey and Huang, 1970). The second method, reported in this paper, utilizes molecular sieve chromatography to separate the low molecular weight RNA and chromatin proteins from DNA. Density gradient centrifugation and DEAE-cellulose column chromatography were then used for the further purification of the RNA. Both methods resulted in the complete separation of this RNA fraction from DNA, histones, and nonhistone proteins. In the case of pig cerebellar and pituitary chromatins the low molecular weight RNA fraction migrates faster than transfer RNA in the polyacrylamide gel electrophoresis system of Loening (1967; 8% polyacrylamide). This result confirms the finding of De Filippes that the low molecular weight RNA prepared from HeLa cell chromatin migrated faster than transfer RNA in the Loening electrophoresis system.

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Inhibition of Peptide-Chain Initiation in *Escherichia coli* by Hydroxylamine and Effects on Ribonucleic Acid Synthesis*

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ABSTRACT: In an *Escherichia coli* extract which was prepared from cells exposed to 10^{-2} M hydroxylamine and was supplemented with 10^{-2} M hydroxylamine, bacteriophage f2 RNA did not promote protein synthesis unless either formyltetrahydrofolate or fMet-tRNA$_f^{Met}$ was added to the reaction mixture. For studies on the nature of the hydroxylamine effect, an extract free of tetrahydrofolate was prepared from cells treated with trimethoprim, an inhibitor of dihydrofolate reductase. In order to make possible protein synthesis directed by f2 RNA in this extract, it had to be supplemented with either fMet-tRNA$_f^{Met}$ or formyltetrahydrofolate. Hydroxylamine (1.5×10^{-2} M) had no effect upon protein synthesis if the extract was supplemented with fMet-tRNA$_f^{Met}$. Hydroxylamine inhibited protein synthesis however if the extract was supplemented with formyltetrahydrofolate. Hydroxylamine blocked peptide-chain initiation but had no effect on peptide-chain elongation. Hydroxylamine was found to form a compound with the formyl residue of formyltetrahydrofolate in a nonenzymic reaction. (The nature of this reaction is described in the accompanying paper by Nixon, P. F., and Bertino, J. R. (1970), *Biochemistry* (in press).) These results indicate that hydroxylamine blocks protein synthesis *in vitro* by causing depletion of the formyltetrahydrofolate pool and thus inhibiting the formation of fMet-tRNA$_f^{Met}$, the initiator of peptide chains. The effect of hydroxylamine on protein and RNA synthesis *in vivo* was tested in an *E. coli* strain (CP 78) in which the RNA synthesis is under "stringent" control of amino acids and in a strain (CP 79) in which the control is "relaxed." Hydroxylamine, 5×10^{-4} M, blocked protein synthesis in both the stringent and the relaxed *E. coli* strain growing in a medium supplemented with twenty amino acids and five purine and pyrimidine bases. The same concentration of hydroxylamine inhibited net RNA synthesis greatly (80%) in the stringent and only slightly (10%) in the relaxed *E. coli* strain. These effects of hydroxylamine upon RNA and protein synthesis are similar to the effects resulting from the removal of a required amino acid from the growth medium. It remains to be seen whether these results reflect a dependence of net RNA synthesis upon fMet-tRNA$_f^{Met}$ in a stringent but not in a relaxed strain or if other phenomena are involved.

Hydroxylamine is a highly reactive nucleophilic reagent. It is a mutagen for bacteria and bacterial viruses, and stops the growth of a number of microorganisms (Borek *et al.*, 1951; Price *et al.*, 1960; Phillips and Brown, 1967). At a concentration of 10^{-3} M it was found to stop the synthesis of DNA, RNA, and protein (Rosenkranz and Bendich, 1964); at lower concentrations it preferentially inhibits protein synthesis (Beguin and Kepes, 1964). Kepes and Beguin (1965) studied the effect of inhibitors on the kinetics of the various phases of β-galactosidase induction. They concluded that hydroxylamine blocked a (at the time unknown) phase of protein synthesis, occurring between the synthesis of mRNA and the growth of the peptide chain, that is, peptide-chain initiation.[1] More recently, insight into the details of initiation was obtained and it thus became feasible to study the mode in which hydroxylamine inhibits. The major, if not the sole, peptide-

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[1] According to a recent communication, catabolite repression contributes to the effect of hydroxylamine upon protein synthesis in certain *E. coli* strains (Basu *et al.*, 1967).