Electron Microscopy and Circular Dichroism Studies on Chromatin†

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ABSTRACT: Sheared native calf thymus chromatin has been examined by electron microscopy (EM) and circular dichroism (CD). Substantial EM morphological and CD spectral changes were found to occur as a function of ionic strength, in the range of 10^{-3} to 0.14 M NH_4OAc. At 0.07 M NH_4OAc, large globular aggregates (0.1–0.3 μ in diameter) with thickened flat regions and occasional large flat aggregates are present; at 10^{-3} M NH_4OAc the large masses have been replaced by uniform rod-like material, 2–3 nm in diameter, and nodular elements or thickened regions. Concurrent sedimentation studies confirmed the relationship between the state of aggregation and the EM and CD changes: the greater the aggregation, the larger the deviation from the conservative CD of free DNA. Ionic strength effects were shown to be reversible. Fractionation of chromatin by sedimentation demonstrated an uneven distribution of protein on the DNA, as revealed by EM and CD data. Results obtained from reconstituted histone–DNA complexes at a given ionic strength showed the f1–DNA complex formed small spherical clusters, while the f2a1–DNA complex formed large globular aggregates. The CD changes associated with these complexes may be related to the average radius of the aggregate. The morphological state of chromatin, as viewed by EM, is highly dependent on its ionic environment; thus, comparisons of chromosomal material in various stages of mitosis must be examined under stringent conditions.

In eucaryotic cells DNA is found complexed to basic proteins, the histones, as well as other components. Possible function for these proteins may be to stabilize the condensed structure of DNA, and possibly to regulate transcription (for a review, see Elgin *et al.*, 1971). In spite of a variety of approaches using many physical techniques, the molecular structure of nucleohistone is not explicitly known (Pardon and Richards, 1972).

The electron microscope evidence concerning the structure of native nucleohistone is extremely variable. Commerford *et al.* (1963) showed electron micrographs (of Hall) indicating a 3 ± 1 nm thread interpreted as being consistent with DNA having an even distribution of histone along its length. Upon inspection of these micrographs, one sees numerous 15-nm thickenings. Dupraw (1965), in investigating honeybee embryonic cells, found nucleohistone, after trypsin treatment, to consist of a thin fibrous core of about 5 nm, attached to which were various sized lobules, presumably consisting of remaining histone. It is doubtful that the 5-nm measurement reflects a correction for metal shadowing material which could be expected to reduce the dimension by about 2.5 nm to about 2–3 nm. Zubay and Doty (1959) showed Hall's micrographs comparing DNA and deoxynucleoprotein (DNP) in selected small fields. The conclusion was that the DNP was substantially thicker than the DNA. Ris and Chandler (1963) compared nucleohistone prepared by two methods and concluded that the spread chromatin of calf thymus contains a fundamental fibrillar unit about 4-nm thick, which was thought to associate with other strands to produce thicker fibers. Utilizing lysates from red blood cells of the newt spread on water, Gall (1963) found 40–60-nm fibers whose appearance is the same whether shadow cast or critical point dried. Solari (1965), in studying sea urchin chromatin, found elementary fibers, negatively stained with uranyl acetate, which were about 4-nm wide; whereas after trypsin digestion or salt extraction, DNA-like strands were obtained. Hay and Revel (1963) showed, in thin sections of epidermal cell nucleus, a filamentous network consisting of approximately 5-nm fibers which are described as "punctate" (*i.e.*, interspersed with thickenings). Davies (1967) examined the fine structure of heterochromatin in cell nuclei in thin sections, and he interpreted the photographs as showing a basic structure of threads and granules about 15-nm wide. Bram and Ris (1971) published electron micrographs of nucleohistone which, according to these authors, do not contain any individually dispersed nucleohistone molecules, but rather a network of fibers about 10 nm in diameter with protuberances of 20–30 nm. Bram (1971a,b) concluded that his results support the suggestion of Pardon *et al.* (1967), to the effect that histone molecules may form links at regular intervals along the DNA, imposing physical restraints analogous to those present in closed, circular, viral DNA, such that subsequent changes in the pitch of the helix induce supercoiling.

In spite of the variability of the results of electron microscopic studies of chromatin and their susceptibility to artifact (such as distortion, contamination in the electron beam in the case of critical point drying, or exaggeration of dimensions of fine structure due to protein film embedment or defocus), there emerges the concept of strands varying in diameter from 2 up to about 50 nm, with or without thickened

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regions which appear along the axis of the strand more or less irregularly.

Circular dichroism[1] (CD) has been utilized in studies of native chromatin (the interphase chromosomal material) and of complexes reconstituted from purified components. The conformation of DNA in chromatin has been found by CD studies to be different from that of isolated DNA in solution (Shih and Fasman, 1970; Permogorov *et al.*, 1970; Wagner and Spelsberg, 1971; Matsuyama *et al.*, 1971; Fric and Sponar, 1971). The large, positive CD band at 277 nm, characteristic of native DNA, is greatly reduced in chromatin. Upon partial dissociation of the histones from chromatin by salt, intermediate CD values are obtained (Simpson and Sober, 1970; Wilhelm *et al.*, 1970; Henson and Walker, 1970).

Other investigations, particularly X-ray diffraction investigations on isolated nucleohistone, seemed to suggest a native, supercoiled conformation (Wilkins *et al.*, 1959; Pardon, 1966, 1967; Wilkins, 1956; Garrett, 1971a). By removal of certain histone fractions, others have been able to show a dependence of the supposed supercoiling upon three specific histone fractions, as well as upon ionic strength and elevated temperature (destruction of supercoil occurs at approximately 0.9–1.1 M NaCl, 64–80°) (Richards and Pardon, 1970; Garrett, 1971b; Varshavsky *et al.*, 1971).

The studies of Clark and Felsenfeld (1971) suggested that the histones were unevenly distributed along the DNA in chromatin so that about 50% of the phosphates of the DNA were exposed. However, Itzhaki (1971) concluded that although most of the free phosphates of DNA are "hidden" beneath covering histone, they are surprisingly accessible to very large molecules. The exchange studies of Clark and Felsenfeld revealed that at moderately high salt concentrations, where some or all of the histone species remained tightly bound, many of these species were in labile equilibirum with unbound histones, implying the possibility of rearrangement. Crick (1971) suggested a model for the distribution of DNA in the chromatid as alternating globular control DNA and fibrous coding DNA, the latter presumed to constitute the roughly 300-nm long interband region in chromosomes stained for light microscopy. Recently, Comings and Riggs (1971) proposed that allosteric proteins, after binding to specific DNA sites, undergo conformational changes, thus facilitating cross-linking in a way which could result in pairing and condensation, and thus an alteration in the genetic function of chromosomes.

Another approach to an understanding of the structure of chromatin is through studies of reconstituted complexes of histone fractions with DNA. Haynes *et al.* (1970) performed such a study using CD, X-ray diffraction, and electron microscopy in which they concluded that DNA has a propensity to form circular structures and that it is this propensity which determines the morphology of chromatin. Olins and Olins (1971) have conducted physical studies, including electron microscopy, on reconstituted complexes of f1 and f2a1 histones with T7 DNA. In the case of f1–DNA prepared from urea–NaCl or from guanidine, the complex exhibited a salt-dependent structural change between 10^{-3} and 10^{-1} M sodium ion. At low ionic strength, complexes sedimented like naked DNA and appeared to be a loose network of DNA fibers when examined by electron microscopy. At high ionic strength, the complexes exhibited rapid and heterogeneous sedimentation and an altered circular dichoric spectrum (see above). These appeared in the electron microscope as 100–300-nm diameter masses with a possible hole in the center. Complexes of f2a1 with T7 DNA did not reveal the salt-dependent structural changes of the magnitude seen with f1–DNA complexes.

Altered CD spectra of DNA in reconstituted complexes with f1 or f2a1 histone in 0.14 M salts have been reported (Fasman *et al.*, 1970; Shih and Fasman, 1971; Adler and Fasman, 1971; Olins and Olins, 1971). However, under certain other experimental conditions, such as complexes formed at low ionic strength, the CD spectrum of the DNA is unchanged (Olins, 1969; Wagner,1970; Li *et al.*, 1971; Fasman *et al.*, 1970; Shih and Fasman, 1971). It is not clear how the structural changes induced by histones contribute to the CD of chromatin.

In the studies reported herein an attempt was made to correlate CD with EM studies at the molecular level in order to deduce whether any distinct morphological changes could be associated with substantial spectral changes. In this manner, a study was made on a number of native sheared chromatin preparations as well as f1 and f2a1 reconstituted complexes. We have also carried out sucrose gradient fractionations of native sheared chromatin to see whether or not different fractions displayed different properties.

Experimental Section

Preparation of Chromatin. Calf thymus was shipped to the laboratory on crushed ice immediately after the animals were killed. Fats and other contaminating tissues were carefully removed, and thymuses were divided into pieces of about 20–50 g, along the lobule membrane. The tissue was then stored at −20° until used.

Chromatin was isolated essentially by the method of Shih and Bonner (1969). About 20 g of calf thymus was homogenized in a Waring blender with 100 ml of grinding medium composed of 0.25 M sucrose–1 mM magnesium acetate–0.01 M Tris (pH 7.8). Homogenization was carried out at 50 V for 2 min, 60 V for 1 min, 70 V for 30 sec, and 80 V for 30 sec with addition of 100 ml of fresh grinding medium each time. The homogenate was filtered first through two layers and then through four layers of Miracloth (Chicapee Mills, New York, N. Y.). The filtrate was then centrifuged at 1935*g* for 20 min with Sorvall SS-34 rotor. The pellet was washed once more with an equal volume of grinding medium. The pellet was then lysed by suspension in 0.01 M Tris (pH 8.0) and centrifuged at 12,100*g* for 20 minutes. This pellet was washed three times with the same volume of 0.01 M Tris (pH 8.0). The sucrose-pelleting step (Shih and Bonner, 1969) was omitted, as it was found, with calf thymus chromatin, that the DNA:protein ratio was essentially unaltered by omitting this step. The resulting chromatin at a concentration of about 20 OD_{260} was then sheared with a Waring blender at full speed for three 1-min blending periods separated by cooling periods. The sheared chromatin was then centrifuged at 12,100*g* for 20 min. The pellet was discarded, and the supernatant (in 0.01 M Tris) was the *sheared native chromatin*. This chromatin was then dialyzed *vs.* two or three changes of ammonium acetate buffer at a given concentration, over a period of 48 hr. Total protein in the chromatin was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. The presence of DNA and ammonium acetate did not interfere with the assay.

Fractionation on Sucrose Gradients. Sheared native chromatin preparations were fractionated at various ionic strengths

[1] Abbreviations used are: CD, circular dichroism; EM, electron microscope.

on 5–10% sucrose gradients prepared in the appropriate buffer solution. Gradients were spun in the Beckman SW25 rotor at 55,000*g* for 6 hr and fractions collected. The three regions examined were: the peak, which sedimented at about 12 ± 3 S (according to empirical calibration against standard particles run concurrently); the pellet, which was resuspended from the bottom of the gradient tube; and material taken from the region between the peak and the pellet.

F2a1 complex with DNA was fractionated in an analogous manner, although 1 hr at 11,000*g* pelleted a substantial amount of the material, leaving a supernatant and a small peak. The shape of the SG profile at 5 and 15 min indicated a substantial heterogeneity.

Cyclical Reversal of Ionic Strength with Chromatin. Sheared native chromatin prepared as described from calf thymus in 0.01 M Tris was dialyzed 24 hr *vs.* 1 l. of 0.001 M NH_4OAc with two changes of solvent. At the same time some of the chromatin material at 0.001 M NH_4OAc was dialyzed in a parallel manner, first *vs.* 0.01 M Tris (pH 7.5) for 5 hr and then *vs.* 0.14 M NH_4OAc overnight, then 6 hr again *vs.* 1000 volumes of 0.14 M NH_4OAc. (The intermediate step into 0.01 M Tris was necessary due to the tendency of the material to precipitate upon direct dialysis into 0.14 M NH_4OAc from 0.001 M NH_4OAc.) Finally, some of the material at 0.14 M NH_4OAc was returned by dialysis directly to 0.001 M NH_4OAc. At the end all three different preparations were prepared for electron microscopy at once.

Preparation of DNA–Histone Complexes. Preparations and procedures are those of Shih and Fasman (1971). DNA and histones were mixed in 2 M NaCl–5 M urea–0.01 M Tris (pH 7.0) at final DNA concentration of 1.5×10^{-3} M. The mixtures were then dialyzed in 5 M urea with a decreasing NaCl gradient, *i.e.*, 0.9 M, 0.6 M, 0.3 M, and 0.15 M. Each step was of a 3-hr duration except that at 0.3 M, which was carried out overnight. Urea was removed by dialysis against 0.15 M NaCl–0.01 M Tris (pH 7.0). This is the dialysis A procedure. For some experiments the salt gradient dialysis in urea was extended to 0.015 M NaCl, and urea was then removed by dialysis against 0.015 M NaCl (dialysis B).

Electron Microscopy. Electron microscopy was carried out with a Siemens 1A electron microscope, at a magnification of 27,000. Calibration was based on photographs of diffraction grating replicas prepared at the same time as a given series of micrographs of the particles. Micrographs were recorded sufficiently close to focus to make metal grain at the 2-nm level clearly resolved on the original plates. Plates were enlarged 4 to 6 times photographically using a contact intermediate to reverse contrast so that the shadowing metal would appear light. A 70-μ objective aperture was used to enhance contrast. Unless otherwise specified, chromatin and complexes were exhaustively dialyzed into ammonium acetate (pH 7.2) at an appropriate ionic strength. Preparations of chromatin or complex at a concentration of 0.05 mg/ml were sprayed through a high-pressure spray gun at freshly cleaved mica. Specimens were evacuated to a pressure in the 10^{-7} Torr range and shadow cast with platinum evaporated from a tungsten filament at a shadow to height ratio of 10:1. Amounts of metal evaporated were minimized to permit optimum resolving power consistent with adequate contrast enhancement as previously described (Slayter and Lowey, 1967; Slayter, 1971).

Since a replica is the actual object photographed, a correction must be applied to account for the distortion of the shape of the original particle by the replicating cap of metal. Due to unavoidable variations in procedure from one experiment to the next, such a correction cannot be applied unambiguously, unless a number of particles of known size and shape have been examined by the same method (H. S. Slayter, unpublished results). Sufficient consistency has been established in our experiments to suggest that a correction of 25 Å be applied to measurements of length and diameter of the various structures seen in these chromatin preparations.

In addition negatively and positively contrasted preparations of chromatin were prepared by applying solutions of chromatin at about 0.3 mg/ml to carbon-coated collodion grids, drawing off excess material, and staining with 1% uranyl acetate in water at pH 4.5. Because of the sensitivity of chromatin to ionic conditions, it was concluded that the mica replica method described above was superior.

Circular Dichroism. CD measurements were performed with a Cary 60 recording spectropolarimeter with 6001 CD accessory, as described in Shih and Fasman (1970) and in Adler and Fasman (1971). All CD spectra were obtained at 23° in 1-cm fused quartz cells. Concentrations of DNA in chromatin or complexes were usually $(1–2) \times 10^{-4}$ M nucleotide residues. Mean residue ellipiticity $[\theta]$ is reported in deg cm^2 $dmole^{-1}$ on the basis of DNA nucleotide residue concentration. The concentration of DNA in chromatin or complex was determined by ultraviolet absorption at 260 nm in the presence of 0.1% (w/v) sodium dodecyl sulfate, using ϵ_p 6800 (Shih and Fasman, 1971).

Results

In 0.07 M NH_4OAc. Electron micrographs of shadow cast and negatively stained native chromatin in 0.07 M NH_4OAc are shown in Plate 1. Certain features of the appearance of chromatin are variable in any given specimen, as well as from preparation to preparation. Features common to all preparations at moderate ionic strength are large globular aggregates 0.1–0.3 μ in diameter (Plate 1d), thickened flat regions of various lengths along the single strands (Plate 2a), and large flat aggregates of material varying up to several tenths of a micrometer across (Plate 1a). Frequently, single strands connect the larger masses (see Plate 8). We shall refer to the variable feature in this type of preparation as a nodular element, which is essentially a small, approximately spherical nodule about 15 nm in diameter occuring axially on single strands, perhaps formed by small loops of the nucleohistone strand (Plate 1a,e, f). Specific features are indexed in the caption to Plate 1. Plates 1e and 1f show negatively and positively contrasted fields of native chromatin prepared at 0.07 M NH_4OAc. Granularities equivalent in size to the nodular elements of the shadowed preparations are obvious in both. The diameter varies in the range of 10–20 nm. The nodular elements and their aggregates found in native chromatin are virtually absent in complexes between native DNA and certain specific histone fractions. For example, negatively contrasted f1 complex showed no such granulation (Plate 6c). Histograms summarizing measurements of the dimensions of nodular elements, widths of strands of DNA or nucleohistone, lengths of these strands between either nodular elements or substantial thickenings, and the linear extent of the limited thickenings seen frequently along single nucleohistone strands are found in Figure 1. This shows that the peak strand length is about 150 nm, that the nodular element diameters peak at about 15.5 nm, that the strand width is about 2.5 nm, and that the thickened strand width is about 10 nm (corrected measurements). Plate 1c shows an example of an object less frequently seen in these preparations, which

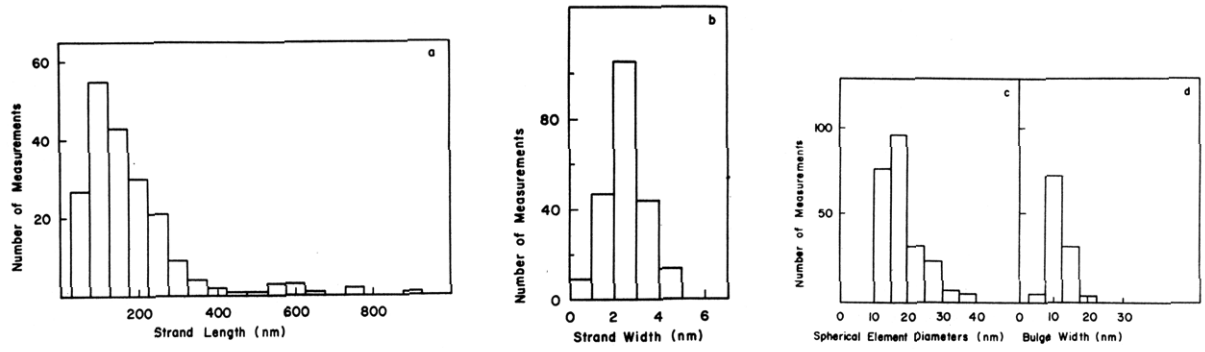

FIGURE 1: Histograms of sheared chromatin showing measurements on various characteristic features: (a) lengths of single nucleohistone strands; (b) widths of single nucleohistone strands; (c) diameters of nodular (spherical) elements; (d) bulge widths along single nucleohistone strands.

seems to consist of equally spaced globular units about ribosome size, connected by what appears to be multiple strands. It is tempting to think of them as a peculiar configuration of polyribosomal complex.

The circular dichroism spectrum of native sheared chromatin (Shih and Fasman, 1970; Permogorov *et al.*, 1970) is characterized by a positive band at about 278 nm (attributable to nucleotide chromophores) and two negative bands at 222 and 209 nm (arising mainly from peptide group asymmetry). The CD curve for such chromatin in 0.07 M NH_4OAc is given in Figure 2; it differs slightly from that of chromatin in 0.01 M Tris ± 0.14 M NaF ($[\theta]_{278}$ = 4000, Shih and Fasman, 1970). In 0.07 M NH_4OAc the peak ellipticity values are $[\theta]_{278}$ = 3400, $[\theta]_{222}$ = −48,800, $[\theta]_{209}$ = −53,500. The CD spectrum of isolated DNA is shown in Figure 2 for comparison. The

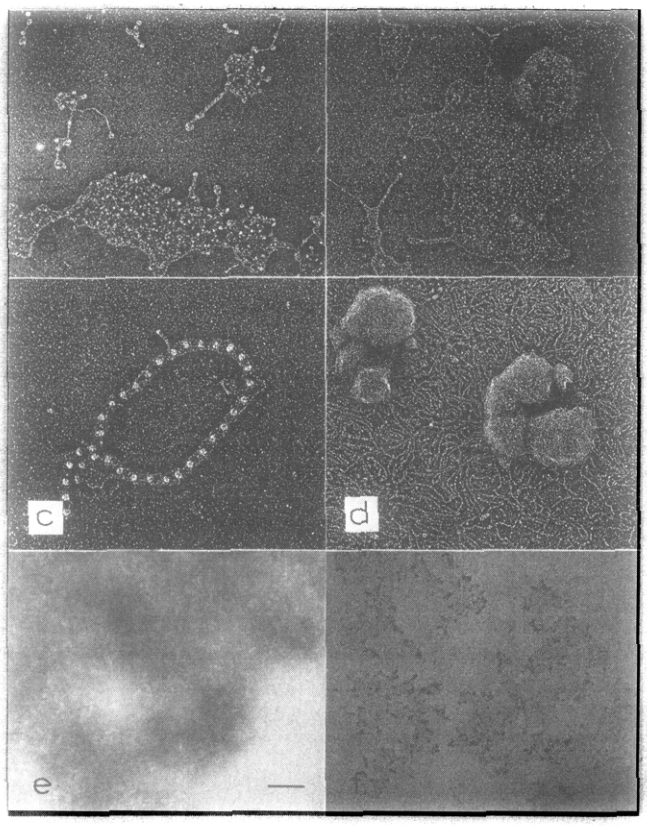

PLATE 1: Various features of native chromatin in 0.07 M NH_4OAc: (a) large flat aggregates showing nodular elements (36,191×); (b) large, condensed granule at the center of a larger, flat aggregate (36,191×); (c) circular array of nodules apparently held together by several strands (36,191×); (d) large, condensed granule surrounded by ~2-nm strands (36,191×); (e) same material as a–d negatively contrasted with uranyl acetate showing nodular elements (47,619×); (f) as e, but positively contrasted with uranyl acetate (47,619×). Bars indicate 100 nm.

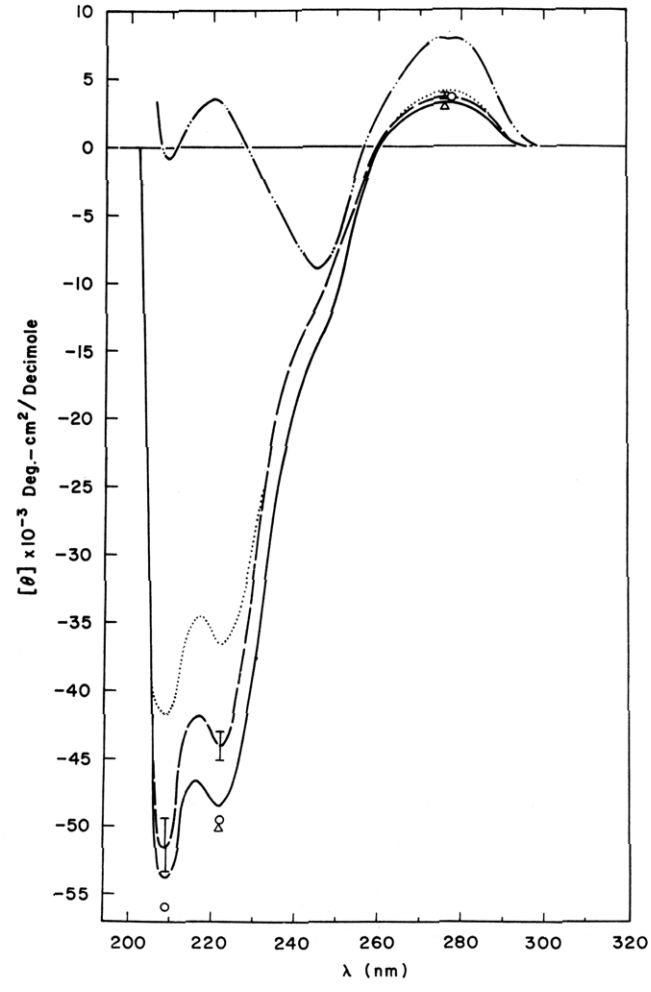

FIGURE 2: Circular dichroism spectra of native sheared chromatin in various salts and ionic strengths: . . . , 0.01 M Tris with or without added 0.14 M NaF; - - -, 0.001 M NH_4OAc; O, 0.005 M NH_4OAc; —, 0.07 M NH_4OAc; Δ, 0.14 M NH_4OAc. The CD of isolated DNA in 0.14 M NaF is given by – · · — · · ·.

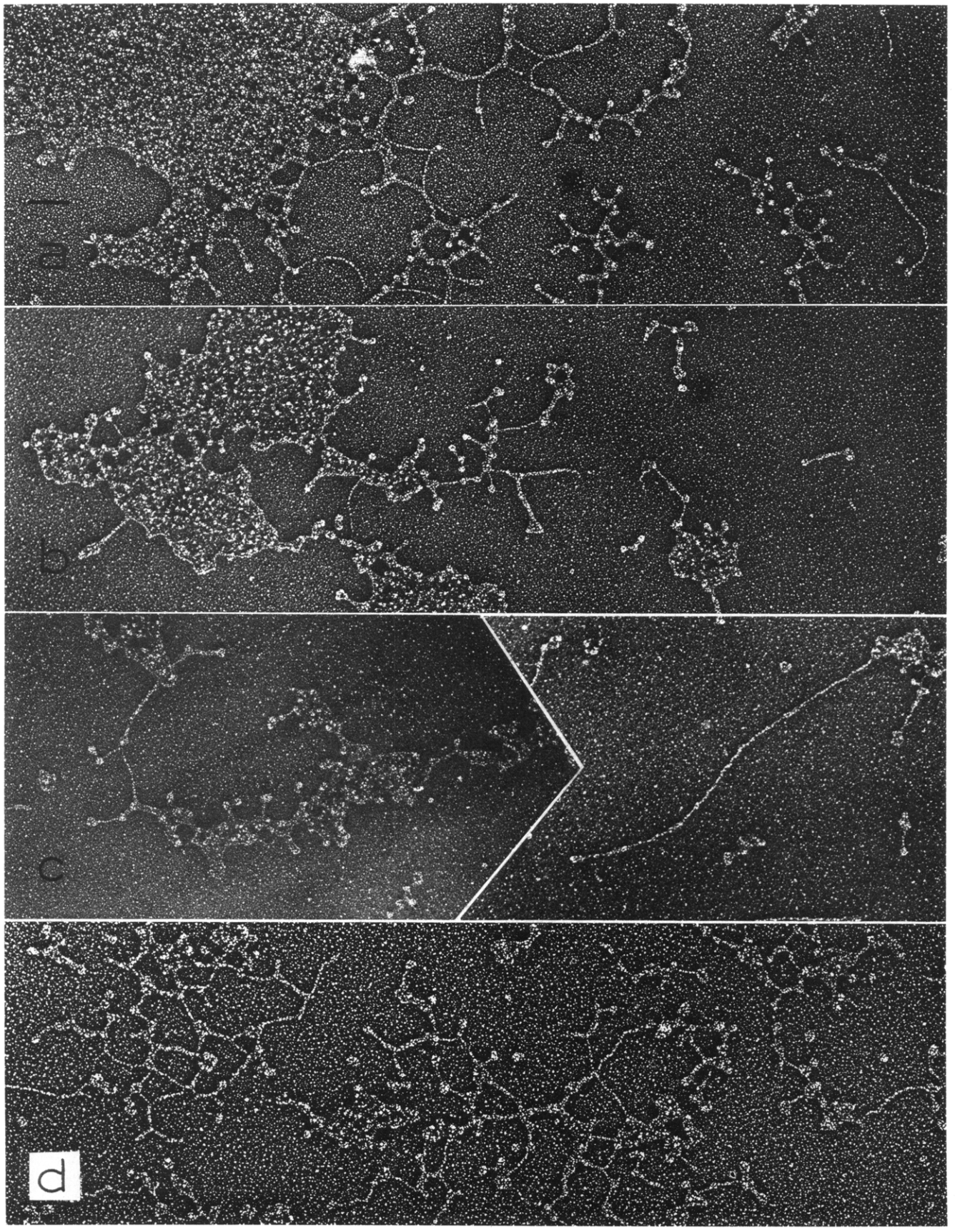

PLATE 2: Micrographs of chromatin reflecting changes of ionic strength upon structure, rotary shadow cast with platinum (75,000×): (a) 0.14 M NH_4OAc; (b) 0.07 M NH_4OAc; (c) 0.005 M NH_4OAc; (d) 0.001 M NH_4OAc. Bar indicates 100 nm.

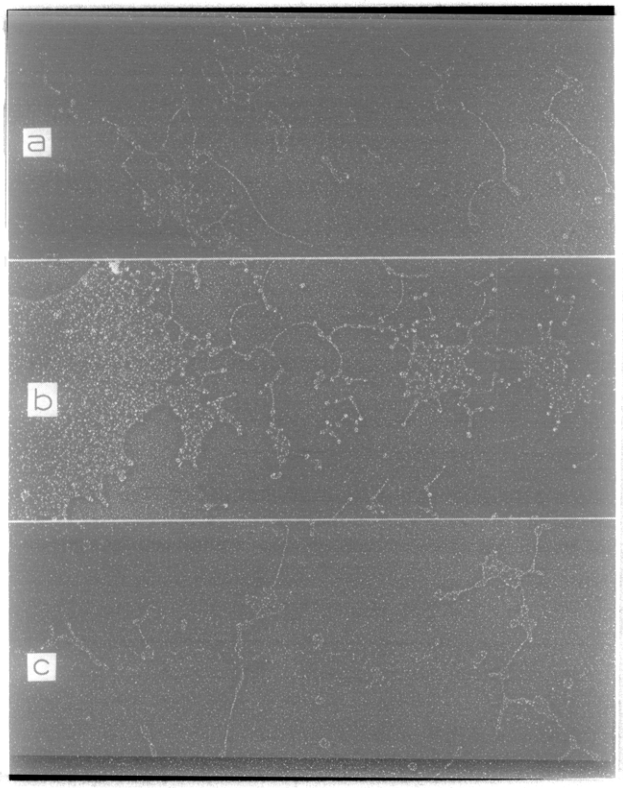

PLATE 3: Reversal of chromatin structure as a function of ionic strength. Micrographs of rotary shadow cast material (36,191×): (a) 0.001 M NH_4OAc; (b) 0.14 M NH_4OAc; (c) reversal to 0.001 M NH_4OAc. Bar indicates 100 nm.

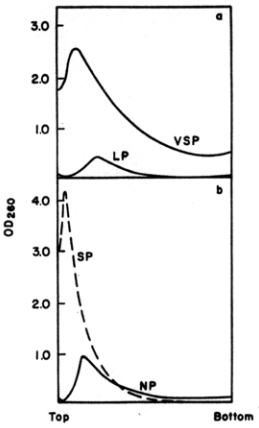

FIGURE 3: Sedimentation profiles of native sheared chromatin (a) on a sucrose gradient at 0.07 M NH_4OAc: lower curve, 360 min at 55,000*g*; upper curve, 60 min at 11,000*g*. LP, SP, VSP, and NP refer to larger pellet, small pellet, very small pellet and no pellet, respectively; (b) on a 5–10% sucrose gradient at 0.005 M NH_4OAc: dashed curve, 60 min at 11,000*g*; solid curve, 360 min at 55,000*g*.

difference between the optical activity of chromatin and DNA (especially in the 280-nm region) probably indicates a conformational change of the DNA when it is incorporated into chromatin.

Ionic Strength Effect. The effect of ionic strength, varying from 0.14 to 10^{-3} M NH_4OAc, upon the conformation of native chromatin is seen in Plate 2. The large masses (Plate 2a) disappear as ionic strength decreases, and what appears to be the fundamental strand becomes more obvious (Plate 2c and d). This is an assembly in which uniform, rod-like material, 2–3 nm in diameter, alternates with masses of nodular elements or with thickened regions. A substantial fraction of the total material at high ionic strength seems to consist of the massed aggregates, including nodular elements in some preparations. The material is maximally dispersed at low ionic strength (Plate 2d). It should be emphasized that while in some experiments, such as the one depicted in Plate 2, nodular elements characterized material at all levels of ionic strength; in other experiments, either no nodular elements were seen, or else they appeared irregularly as a function of ionic conditions. Part of the difficulty with variability may be due to lack of preservation of these structures due to the age of the preparation or its treatment. The question of the possible significance of these structures will be raised in the discussion.

It can be seen from Figure 2 that the circular dichroism of chromatin varies with the concentration of NH_4OAc. The higher the [NH_4OAc], the smaller the magnitude of the positive band [$[\theta]_{278}$ ranges from 4000 ± 300 in 0.01 M Tris (with or without 0.14 M NaF) to 2900 ± 300 in 0.14 M NH_4OAc, based on at least three independent CD spectra at each of these NH_4OAc concentrations], and the larger the magnitude of the negative CD bands. The percentage changes in both the 278-nm region and the 220–210-nm region are comparable (28%), thus indicating a conformational change in the DNP and not of any particular component. A similar effect of this salt upon CD was observed with f1–DNA complexes and with pure DNA itself (Adler and Fasman, 1971). The CD spectra at higher [NH_4OAc] are indicative of increased aggregation and of greater conformational change (using DNA as reference), both functions of ionic strength; this is consistent with EM data. The weight ratio of protein to DNA for these chromatin preparations is 1.47 ± 0.07, independent of [NH_4OAc]. Therefore, the differences seen in CD and EM cannot be attributed to variations in protein content.

Salt Reversal. A reverse cycle of ionic strength variation from 10^{-3} to 0.14 M and back to 10^{-3} M NH_4OAc was executed to determine whether the same conformation would be obtained in both low ionic strength preparations. The results of this type of experiment are shown in Plate 3. No clear morphological differences between low ionic strength preparations, before and after cycling through high ionic strength, were found (see Plate 3a, c). On the other hand, the characteristic differences (as described under ionic strength effects) between low and high ionic strength preparations are clearly in evidence (Plate 3b). One additional difference in this case is that nodular elements are found in abundance here only at high ionic strength (Plate 3b).

The CD spectra for chromatin in 0.001 M NH_4OAc are identical (within experimental error) before and after dialysis through 0.14 M NH_4OAc. On the other hand, the CD spectrum of this chromatin sample in 0.14 M NH_4OAc (after dialysis from 0.001 M) is typical of chromatin at this higher NH_4OAc concentration. Chromatin has previously been shown to exhibit different spectra at these two ionic strengths (see Figure 2). Therefore, there is no indication from CD of irreversible change in secondary structure during cyclical reversal of ionic strength. There is an analogy in CD to the gross changes in aggregation seen in EM upon cycling chromatin into and out of 0.001 M NH_4OAc.

Fractionation of Chromatin. Sheared native chromatin, after exhaustive dialysis into 0.07 M NH_4OAc, was fractionated on sucrose gradients as described. Figure 3a shows the sedimentation profile and distribution of material on sucrose

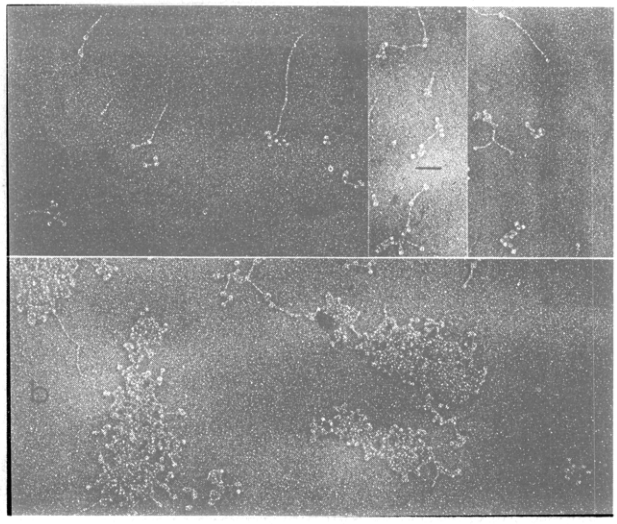

PLATE 4: Fractionation of chromatin in 0.07 M NH_4OAc: (a) material from the peak region, rotary shadow cast with platinum (36,191 ×); (b) material resuspended from the pellet, shadow cast with platinum (36,191 ×). Bar indicates 100 nm.

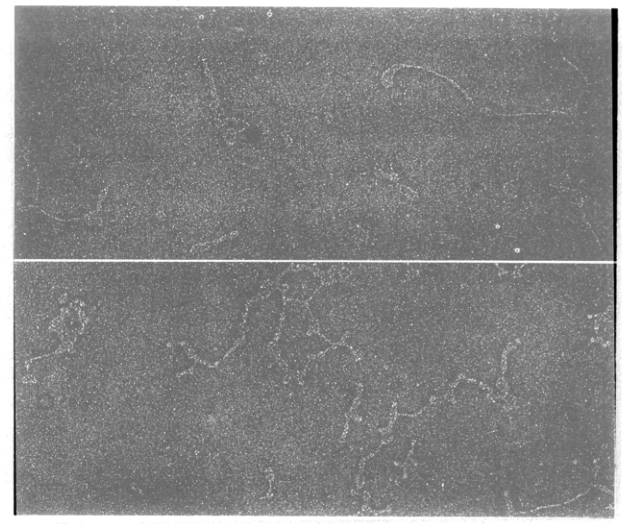

PLATE 5: Fractionation of chromatin in 0.005 M NH_4OAc: (a) material from the peak region, rotary shadow cast with platinum (36,191 ×); (b) material resuspended from the pellet, rotary shadow cast with platinum (36,191 ×). Bar indicates 100 nm.

gradients. Under these conditions, a substantial pellet was formed and a broad peak appeared at about 12 ± 3 S. Figure 3b shows a profile of chromatin at 0.005 M NH_4OAc applied to a gradient under the same conditions. In this case, there was a larger, slower sedimenting peak and much less pellet, indicating less aggregation.

Plate 4a shows electron micrographs of material taken from the 12S peak from the sucrose gradient fractionation of chromatin in 0.07 M NH_4OAc. Short fragments showing characteristic features of the material seen attached to the larger masses found at high ionic strength, and dispersed throughout fields of material at low ionic strength in unfractionated preparations, were characteristic. Generally more extended strands were seen. The pellet fraction, when resuspended, seemed characteristic of what was found in a normal chromatin preparation at 0.07 M NH_4OAc, with somewhat less aggregate (Figure 4b). In material fractionated at 0.005 M NH_4OAc, approximately four times as much material was found in the peak as at 0.07 M NH_4OAc, and there was a concomitant decrease in the size of the pellet. Plate 5a shows an electron micrograph of material taken from the peak of the sucrose gradient fractionation of chromatin in 0.005 M NH_4OAc (shown in Figure 3b). Again the peak fraction was characterized by shorter fragments showing substantial single-stranded regions of nucleohistone. The pellet material consisted of long, irregularly thickened strands of greater average width, which, while somewhat cross-linked compared to the peak material, nevertheless showed substantially greater dispersion than the 0.07 M NH_4OAc pellet (Plate 5b). Hints of nodular elements are found only in the pellet material of the low ionic strength preparation.

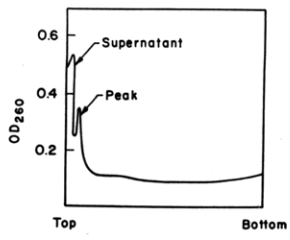

FIGURE 4: Fractionation of f2a1–DNA complex on a 5–10% sucrose gradient.

Protein analysis shows that chromatin fractions from the top of the sucrose gradient have much less protein than unfractionated chromatin, whereas material at the bottom is somewhat enriched in protein. For example, the weight ratios of protein to DNA for a fractionation experiment in 0.005 M NH_4OAc are: peak, 0.81; unfractionated, 1.47; pellet, 1.58.

The CD spectra of fractionated chromatin are consistent with the EM findings. Peak fractions from sucrose gradients, for both fractionation experiments (in 0.005 and 0.07 M NH_4OAc), displayed CD curves intermediate between those typical of native chromatin and of pure DNA in the same solvent. For example, the ellipticity bands for the peak fraction in 0.005 M NH_4OAc are $[\theta]_{278} = 4870$, $[\theta]_{222} = -21{,}100$, $[\theta]_{209} = -27{,}700$, which are very different from native chromatin in the same solvent (Figure 2). (Similar results were obtained for the peak fraction in 0.07 M NH_4OAc; for comparison, the CD parameters for DNA in 0.07 M NH_4OAc are $[\theta]_{277} = 6500$, $[\theta]_{245} = -8300$). Thus, both the CD and EM data for the fractionated chromatin peaks correlate in terms of loss of characteristic aggregated chromatin structure, and demonstrate an uneven distribution of protein along the DNA.

The CD of the pellet fractions is similar to that of native chromatin. For example, the redissolved pellet from the gradient in 0.005 M NH_4OAc had bands: $[\theta]_{278} = 2440$, $[\theta]_{222} = -44{,}300$. Again there is correlation with the EM data (Plate 5b) which shows that fractionated pellets have a structure similar to that of native chromatin. The CD shows that the pellet material, in addition to containing somewhat more protein, may have its DNA in a conformation more unlike free DNA than is true for unfractionated chromatin.

Synthetic DNA–Histone Complexes. The strikingly different appearances of electron microscope preparations of f1–DNA and f2a1–DNA complexes in 0.07 M NH_4OAc are shown in Plate 6a and b, respectively. Whereas the f2a1 material consisted largely of 0.3–0.5-μm globular aggregates, the aggregates of f1 complex were substantially smaller (20–

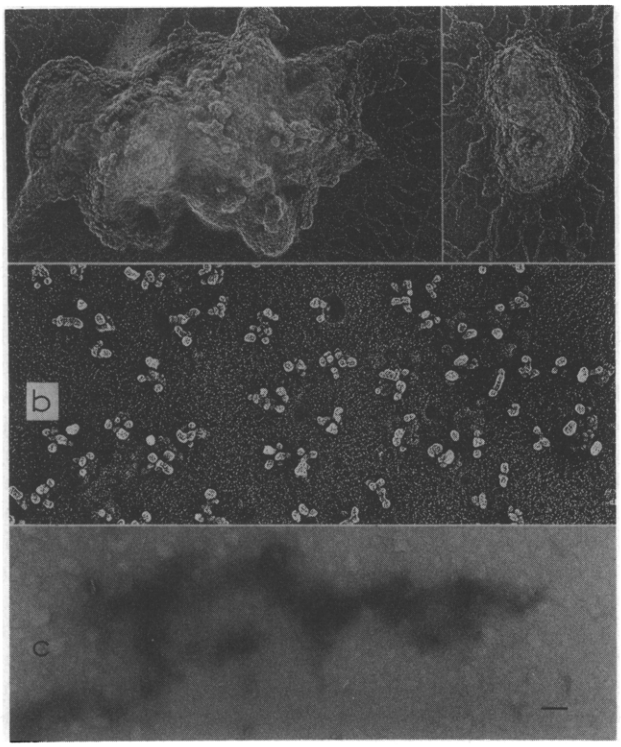

PLATE 6: Comparison of f1–DNA and f2a1–DNA complexes at high ionic strength in 0.07 M NH_4OAc: (a) f2a1–DNA complex, rotary shadow cast (36,191×); (b) f1–DNA complex, rotary shadow cast (36,191×); (c) f1–DNA complex, negatively contrasted with uranyl acetate (36,191×). Bar indicates 100 nm.

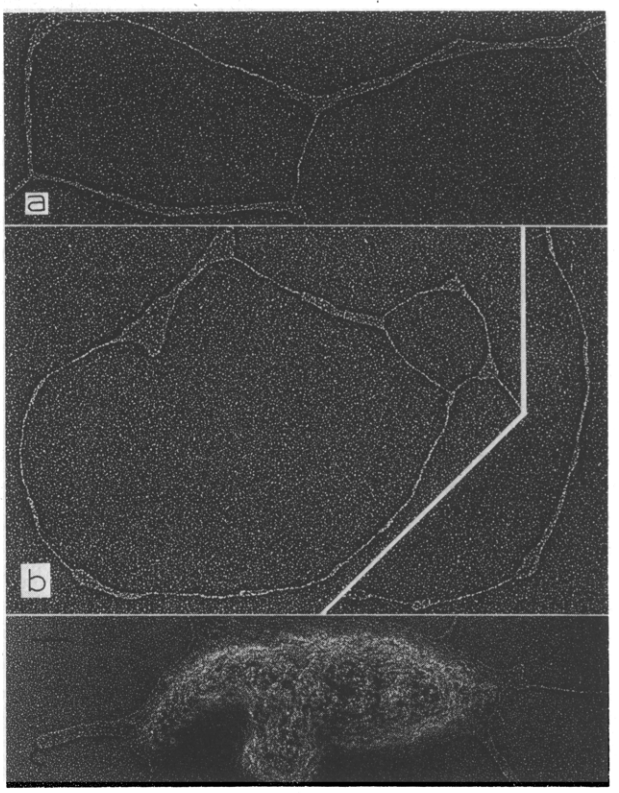

PLATE 7: f2a1–DNA complex fractionated in 0.07 M NH_4OAc, rotary shadow cast with platinum (47,619×): (a) supernatant; (b) peak; (c) pellet. Bar indicates 100 nm.

40 nm), occurring as small clusters. At high ionic strength, there is a substantial background composed of strands in both complexes. As is discussed below under fractionation, a substantial amount of uncomplexed DNA is known to be present in this type of preparation. A field of stained f1 complex at 0.07 M NH_4OAc is shown in Plate 6c. Stain-excluding strands about the size of DNA are visible in well-stained regions. No 15-nm particles are seen within these regions as was the case with stained native chromatin. At low ionic strength (10^{-3} M NH_4OAc), complex material was spread out primarily as strands, in electron microscope preparations, similar in appearance to the peak material from the fractionation at high ionic strength shown in Plate 7b.

The CD spectra of the synthetic complexes examined in this study agree well with previous CD data for f1–DNA complexes in NH_4OAc (Adler and Fasman, 1971) and for f2a1–DNA complexes (Shih and Fasman, 1971). For example, the peak ellipticity values for the f1–DNA complex, $r = 0.5$ (amino acid residue/base residue), in 0.07 M NH_4OAc, whose EM is shown in Plate 6b, are $[\theta]_{297} = 2800$, $[\theta]_{252} = -25{,}500$. Values for the f2a1–DNA complex, $r = 1.5$, in 0.07 M NH_4OAc, of Plate 6a, are $[\theta]_{303} = -2700$, $[\theta]_{268} = 16{,}800$, $[\theta]_{240} = -4100$. Both of these CD spectra indicate complexes in which the conformation of DNA may be very different from that of the uncomplexed DNA, and from each other. This is confirmed by the substantial morphological differences.

In contrast, the CD spectrum of f2a1–DNA complex, $r = 1.5$, in 0.001 M NH_4OAc, prepared by dialysis B (see Methods) looks very similar to that of DNA: $[\theta]_{303} = 0$, $[\theta]_{275} = 7250$, $[\theta]_{245} = -10{,}300$. This similarity in conformation to that of DNA is shown also by EM data.

Fractionation of the f2a1 complex, $r = 1.5$, in 0.07 M NH_4OAc, was carried out on sucrose gradients as described (Figure 4). In a typical fractionation, about 53% of the material (as OD_{260}) was found in the pellet (after 60 min at 10,000g on a 5–10% sucrose gradient in a SW 25 head), about 18% of the OD_{260} was found in the supernatant, about 6% was found in the top peak, and about 25% was found distributed between the top peak and the bottom. Material in the lower gradient and pellet consisted largely of 0.3–0.5-μm globules with associated strands sometimes seen to coil parallel to the base as shown in Plate 7c. Well-dispersed material, showing thickenings randomly distributed along the strand axis, characterized the sedimenting peak, as shown in Plate 7b. There is no measurable significant difference in diameter between the single strands in the sedimenting peak fraction and the supernatant material, depicted in Plate 7a, which is indistingishable morphologically from control preparations of DNA. This is borne out by the CD spectra of the supernatant which were also indistinguishable from DNA ($[\theta]_{277} = 7500$, $[\theta]_{245} = -10{,}000$, $[\theta]_{303} = 0$). The top peak displayed a CD curve intermediate between those for the intact complex and free DNA: $[\theta]_{303} = -1300$, $[\theta]_{270} = 9600$, $[\theta]_{242} = -9000$; while the resuspended pellet CD looks like intact complex: $[\theta]_{303} = -3100$, $[\theta]_{267} = 16{,}000$, $[\theta]_{242} = -6700$. These results indicate that f2a1–DNA complexes can be fractionated by centrifugation to yield complex in which DNA is associated with a full complement of f2a1 and a smaller portion of less covered complex, as well as free DNA (which remains at the top of the gradient).

Discussion

Our results with sheared native chromatin indicate that substantial CD spectral and morphological changes occur

as a function of ionic strength in the range of 10^{-3} to 0.14 M NH_4OAc. It should be emphasized that a high degree of variability of the effect of specific ions is observed for CD spectra of synthetic complexes of DNA–histone (Adler and Fasman, 1971), and studies on chromatin should be designed with this in mind. NH_4^+ ions were found to be very effective in producing aggregation. Sedimentation studies confirm that under the conditions used a much larger proportion of the material is incorporated into the pellet at moderate ionic strength (0.07–0.14 M NH_4OAc) than at low ionic strength (0.001 M NH_4OAc). Furthermore, material sedimenting as a peak on sucrose gradients is faster sedimenting at high ionic strength. The CD spectra at high ionic strength are consistent with increased aggregation and greater conformational change of the associated DNA. The gross electron microscope appearance of preparations at high ionic strength indicates a mixture of a larger proportion of tightly aggregated material in the size range 0.1–0.3 μ, with an increasing proportion of loosely aggregated material and single-stranded nucleohistone at lower ionic strength. These results are consistent with the recent work of Itzhaki and Rowe (1969) and Varshavsky *et al.* (1971). At intermediate values of ionic strength most of the large, formed elements disappear, leaving the latter two species. Often there is evidence that the single nucleohistone strand has a tendency to form zig-zag arrays as seen in electron microscope preparations, which is suggestive of a superasymmetric arrangement. At low ionic strength virtually all of the material is found in the form of single nucleohistone strands. We are led to the conclusion that since there is a definite correlation between the changes seen in the CD spectra and the conformational changes reflected in the large aggregates produced at high ionic strength, as seen by EM, this gross aggregation is probably closely related to the conformational change, which then results in the change in the CD spectra. Comparable changes in the morphology of nuclei have been reported (Olins and Olins, 1972; Brasch *et al.*, 1971) as a function of ionic strength.

The question of reversibility of the ionic strength dependent conformational change was investigated and complete reversibility was found within the limits of accuracy of both the electron microscopic and CD methodology employed.

Fractionation of native chromatin was carried out in order to determine whether elements separable on sucrose gradients showed different properties. The morphological appearance indicated that the difference between the pellet and peak fractions from the gradient was that the material left behind in the sedimenting peak appeared to contain more apparently bare "DNA-like regions" and was depleted in protein. Duerksen and McCarthy (1971) have differentially centrifuged sheared mouse chromatin in a similar manner, and have found other differences between the fractions. The CD information indicated that the material in the peak was intermediate in character between native DNA and nucleohistone. On the other hand, the pellet showed even stronger nucleohistone characteristics than unfractionated chromatin. These findings along with protein analysis are interpreted as demonstrating an uneven distribution of protein on the DNA in chromatin. While large changes in CD are noted for the pellet fraction of chromatin compared to unfractionated chromatin, no significant differences were noted between equivalent fractions at high and low ionic strength. Perhaps small differences in CD are undetectable for samples having large CD alterations. The original change in CD of native chromatin as a function of ionic strength was very small (see Figure 2). Therefore, the above result is not unexpected, in spite of the apparently significant morphological differences. It may be more significant that there was much less pellet material in the low ionic strength fractionation.

The salient features of chromatin as examined here are as follows: chromatin, prepared by procedures which in our experience tend to best preserve fine structural details at the molecular level, and which provide adequate contrast enhancement, emerges morphologically as a structural complex which undergoes gross changes in its state of aggregation as a function of ionic conditions. At high ionic strength, fine structural features in the aggregated chromatin complexes are virtually obscured by the sheer size of the complex, which is far too thick to allow observation of detail at the molecular level by electron microscopy. On the other hand, at low ionic strength, chromatin, while still aggregated somewhat, is far more amenable to high-resolution electron microscopy of its molecular features. What is clearly indicated from the combined EM and spectroscopic information is that the relatively aggregated state contributes to the characteristic chromatin-like CD spectrum. With respect to the distribution of histones on DNA, it is not possible to determine whether minimal amounts of histone, closely associated with DNA, may be present on apparently bare regions. On the basis of combined EM and CD data, it is concluded that histone is probably attached discontinuously as characteristic aggregates and bulges along the DNA axis. This view is substantiated by observed differences between fractionated and unfractionated material. Caution should be used in extrapolating these results to apply to unsheared chromatin in the intact mammalian cell nucleus. Mirsky (1971) has shown that susceptibility of DNP to DNase digestion (which reflects protein attachment and distribution) varies with the preparative method.

With regard to the nodular elements which are found on native chromatin in a somewhat inconsistent fashion, it is believed that these are related to the form in which histones are complexed to the DNA, presumably in a discontinuous fashion. The way in which this occurs may be complicated by intrastrand interactions, possibly in some superasymmetric arrangement. The fact that nodular elements of this size are found interspersed as a salient feature of sectioned chromosomes (Moens, 1969; Guenin, 1965) leads us to believe that they are real when seen; but due to their labile nature, they may disappear as a function of age and/or treatment after extraction from the cellular environment. It was noted in the salt reversal experiment that the nodular elements were found at high ionic strength, whereas they were not seen at low ionic strength, either before or after the high salt dialysis, followed by return to low salt. Whether such an exact relationship exists for these features is moot, but it may be related to the first stage in molecular rearrangement, which leads to ultimate condensation of chromatin. At low ionic strength, the pellet fractions of chromatin examined by electron microscopy often indicated nodular elements at the ends of characteristic twisted folds of nucleohistone strand. The corrected size of these elements is about 15 nm, which is suggestive of their being a large enzyme such as RNA polymerase. However, particles of this size are not found in significant numbers in the background of such photographs, as is the case with RNA polymerase–DNA complexes (Slayter and Hall, 1966). This suggests that the nodular elements are an integral part of the nucleohistone complex, perhaps high molecular weight aggregates of histones, asymmetrically distributed. In the sucrose gradient fractionations, attached nodular elements were found both in the peak and pellet fraction at low ionic

strength. This is another indication that at low ionic strength they may be unstable.

In the case of the synthetic complexes, at high ionic strength, the f2a1 complex forms large particles of roughly 100–300 nm diameter, with a relatively small conformational change as deduced by CD. On the other hand, the f1 complex at the same ionic strength produces groups of smaller (20–40 nm) nodular particles which show a substantially larger conformational change by CD. In the fractionation of sheared native chromatin on sucrose gradients no supernatant material appears, as indicated by the spectrophotometric trace at 260 nm. On the other hand, the fractionation of reconstituted f2a1–DNA complex produces a supernatant with characteristics very much like DNA.

The work with synthetic complexes of DNA with histone indicates several possible conclusions. First, it appears that the f2a1 complex at high ionic strength produces aggregates with a much larger radius than the f1 complex. A correspondingly small difference in CD was observed. It is therefore possible that the conformational change induced by bending through a certain average radius into a spherical particle of any given size leads to a characteristic change in CD. A second feature noted with f2a1–DNA complex is that upon fractionation at high ionic strength a supernatant appears which is indistinguishable from DNA, a small sedimenting peak appears, and a large pellet is produced which is characteristic of complex. This indicates that a small fraction of the DNA binds to less than a full complement of histone and implies that the interaction between f2a1 and DNA to form complex is largely, but not entirely, cooperative, a suggestion made previously by Shih and Fasman (1972). It is difficult to compare and contrast the various electron microscope studies previously carried out with chromatin due to the variability of source material, ionic conditions, methods of preparation, poor statistical sampling in the limited areas shown in the published photographs, and lack of studies carried out concurrently, varying only one parameter such as ionic strength (see introduction). However, Olins and Olins (1971), in their electron micrographs of f1 complex with T7 DNA, found large aggregates whose gross morphological features did change as a function of ionic strength, tending toward greater dispersion at low ionic strength; and similar results at extremely low concentrations were reported for chromatin by Varshavsky *et al.* (1971).

Recently Crick (1971) proposed a model for the structure of chromatin with respect to its incorporation into the functional chromosome. This model suggested that most of the DNA in higher organisms (called globular control DNA) does not code for protein, but is used for control purposes. The coding sequences of DNA were postulated to be primarily in the interband regions—the bands constituting the regions where the bulk of the control DNA is found. Both types of DNA were presumed to be incorporated into nucleohistone. In the type of preparation used in the work presented here, single nucleohistone strands, 200–300 nm long, were frequently found connecting large masses, as shown in Plate 8, in a manner consistent with Crick's (1971) model, as well as Mirsky's (1971) data for a minimal accessibility of DNA to DNase. These results may be useful in the interpretation of the X-ray diffraction studies on isolated nucleohistone. Regions of DNA which have small amounts of associated protein could be responsible for the reflections attributable to the B form of DNA. The other reflections may be due to regions which have more associated proteins and thus have other possible conformations, *e.g.*, super coil, etc.

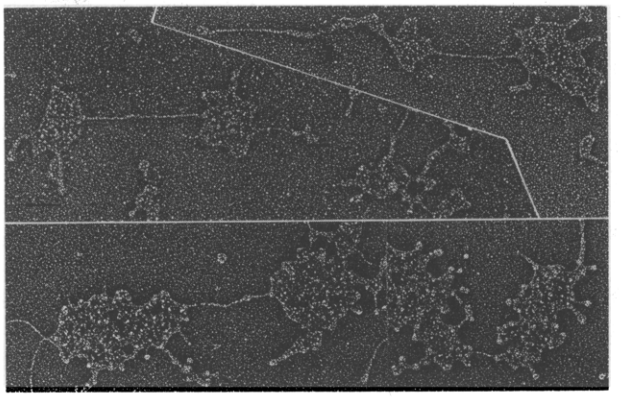

PLATE 8: Selected areas showing aggregates connected by ~2-nm strands, rotary shadow cast with platinum (47,619×). Bar indicates 100 nm.

The results presented here support the proposition that a reversible conformational change in the DNA of chromatin, induced by moderate ionic strength (0.07 M NH_4OAc), is inversely related to the radius of curvature of the DNA when it is associated into a globular complex aggregate. Results of fractionation studies indicate a grossly discontinuous distribution of histone along the DNA axis. The state of aggregation in chromatin was shown to be dependent on the ionic strength, with longer aggregates present at the higher ionic strengths. The gross conformational changes induced in chromatin by altering the ionic strength displays the ease of structural change within the chromosome and the ease with which significant morphological changes may be encountered. Although alteration in the ionic media in which the nucleus is bathed is not impossible, other mechanisms of equal simplicity can be simulated in the manner studied herein to account for the morphological changes during mitosis.

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Stabilization and Breakdown of *Escherichia coli* Messenger Ribonucleic Acid in the Presence of Chloramphenicol†

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ABSTRACT: In *Escherichia coli* chloramphenicol stabilizes the fraction of preexisting mRNA which is associated with polyribosomes. mRNA not bound to ribosomes is not protected against breakdown. mRNA synthesized in the presence of chloramphenicol enters into polyribosomes and is turning over as rapidly as in exponentially growing cells. Consequently there is no significant accumulation of mRNA in chloramphenicol-treated cells.

Chloramphenicol at appropriate concentrations stops protein synthesis abruptly but allows RNA synthesis to continue for a considerable period. RNA synthesized in the presence of chloramphenicol contains all the RNA species encountered in normal, exponentially growing cells. Ribosomal RNA accumulates and is mainly localized in the so-called chloramphenicol particles, the nature of which is still unclear (Osawa, 1968; Schleif, 1968). The preexisting total mRNA (Levinthal *et al.*, 1963) as well as the specific lactose mRNA (Varmus *et al.*, 1971) and tryptophan mRNA (Morse *et al.*, 1969) seem to be stabilized by chloramphenicol. The data concerning the fate of mRNA synthesized in the presence of chloramphenicol are controversial. Several reports indicate that under these conditions lactose mRNA is either not synthesized at all (Artman and Ennis, 1972) or is rapidly degraded (Varmus *et al.*, 1971). On the other hand, Gurgo *et al.* (1969) reported that there was an accumulation of mRNA in polyribosomes of cells exposed to chloramphenicol. This conclusion was inferred

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