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Structure Studies on Chromatin and Nucleohistones. Thermal Denaturation Profiles Recorded in the Presence of Urea*

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ABSTRACT: Structural information about chromatin and artificial nucleohistones was revealed by plots of the temperature derivative of hyperchromicity *vs.* the temperature (derivative denaturation profiles) from thermal denaturation experiments. Soluble preparations of rat thymus or liver chromatin displayed derivative denaturation profiles with a characteristic pattern of ascending steps when studied in a medium containing 3.6 M urea. Such profiles provide a convenient test for the intactness of chromatin since degraded preparations showed distinctly different patterns, characterized by more sharply defined maxima and minima. Comparisons of denaturation profiles of treated and untreated nucleoproteins in various denaturation media revealed that: (1) profiles of natural chromatin were more complex than those of artificial nucleohistones; (2) less than 10% of the nucleic acid within fresh chromatin denatured as extended stretches of free DNA; (3) both autolysis of chromatin and trypsin digestion exposed DNA and destabilized distinctive regions in the denaturation profile; (4) fixation of chromatin by formaldehyde elevated denaturation temperatures; (5) derivative denaturation profiles appear to reflect physical as well as chemical features of nucleoprotein complexes; (6) a cacodylate buffer containing 3.6 M urea resolves the structure of chromatin better than a buffer containing only 5 mM sodium cacodylate, and reveals the structure of lysine-rich nucleohistones better than 0.25 mM Versenate buffer; (7) spontaneous formation of ammonium cyanate in a urea-containing medium does not produce major artifacts that prevent the interpretation of denaturation profiles; (8) the nucleohistones reconstituted at low ionic strength in this laboratory appeared to have greater structural variety than related complexes reconstituted elsewhere by the gradient dialysis method.

The formation of complexes between basic proteins and DNA is known to increase the thermal stability of DNA (Huang and Bonner, 1962; Allfrey *et al.*, 1964; Ohba, 1966; Huang *et al.*, 1964; Sponar *et al.*, 1967; Olins *et al.*, 1967, 1968). Since the denaturation of DNA, either free or within a complex, is accompanied by an increase in uv optical absorbance, complexes may conveniently be detected by means

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at an increase in the temperature at which the absorbance rises as samples are slowly heated. Ordinarily, a single transition is found for pure DNA, but nucleoproteins show thermal transitions that may be broad, biphasic, or monophasic, depending on the composition of the preparation. Recent techniques for recording and analyzing data from such thermal denaturation experiments (Shih and Bonner, 1970a; Ansevin and Brown, 1971) make it possible to obtain more detailed information about the transitions, especially in the case of those that appear relatively broad. In the improved technique, many observations of uv absorbance are recorded as a sample is gradually heated from room temperature to about 100° and a "derivative" thermal denaturation profile is plotted that shows the temperature derivative of hyperchromicity $[A_{(25\to\Delta)}°/A_{25}°]$ *vs.* the temperature, where A is the optical absorbance (usually measured at 260 nm).

We have observed that derivative denaturation profiles obtained in a medium containing urea can be used to characterize broad thermal transitions in reconstituted nucleohistones (Ansevin and Brown, 1971) and also in natural chromatin. Related, but nonidentical findings were reported for nucleohistones by Shih and Bonner (1970a,b) and by Li and Bonner (1971) for plant chromatin and reconstituted mammalian nucleohistones. It is the purpose of this paper, to describe the appearance of thermal denaturation profiles of chromatin preparations from a mammalian source and to compare these to profiles of simple nucleohistones. In addition we consider the degree of complexity that is resolvable in different denaturation media, and examine especially the possible existence of artifacts in the denaturation method employed here. We show that the denaturation profile of mammalian chromatin is consistent with that expected for a complicated nucleohistone and present evidence that distinctive changes in the denaturation profiles of chromatin accompany several types of modification in normal structure. We have attempted to determine whether or not differences between the denaturation medium employed by Ansevin and Brown (1971) and that of Shih and Bonner (1970a) could have been responsible for a more sensitive resolution of components in reconstituted nucleohistones prepared in this laboratory. In the experiments to be reported we conclude that the medium of Ansevin and Brown (1971) does not produce artifacts that would lead to a major misinterpretation of denaturation profiles and that a 5 mM cacodylate buffer containing 3.6 M urea can give a distinctly better resolution in thermal denaturation profiles for certain nucleoproteins.

Materials and Methods

Nucleoproteins. Mammalian chromatin was prepared as described earlier (Spelsberg and Hnilica, 1971). Briefly, cells were broken in a medium containing 0.25 M sucrose, 0.025 M KCl, 5 mM magnesium chloride, and 0.05 M Tris-chloride buffer (pH 7.5). Nuclei were isolated by sedimenting filtered particulates in a similar medium in which the sucrose concentration was increased to 1.7 M; in some cases 0.1% Triton X-100 was included in this medium. Chromatin was prepared by disrupting the nuclei in 0.075 M sodium chloride–0.024 M sodium Versenate at pH 6.3, by means of a glass and Teflon homogenizer. Pelleted chromatin was washed twice in this buffer, once in 0.3 M sodium chloride, once in SSC/100,[1] once in SSC/10, and finally was resuspended in SSC/100. It was then stored at −20°. Thawed chromatin was resuspended by gentle mixing in a homogenizer. The chemical composition of such preparations was slightly different for liver and thymus of rats. For each gram of DNA, liver chromatin had approximately 1.2 g of basic proteins and 1.0 g of nonhistone proteins, while thymus chromatin had 1.0 g of basic proteins and 0.5 g of nonhistone proteins (Spelsberg and Hnilica, 1971).

Nucleohistone complexes were formed as described in a previous publication (Ansevin and Brown, 1971). Individual calf thymus histone fractions of high purity were slowly added to DNA in a pH 7 buffer containing 5 mM sodium cacodylate, 0.3 mM Versene, and 3.6 M urea. Dehistonized DNA used for most reconstitution experiments was gently prepared by sedimenting chromatin in the presence of high salt and urea. Residual acidic proteins, amounting to about 20% of the weight of the final preparation, did not influence thermal profiles of the DNA.

Chemicals. Urea for the thermal denaturation medium was the Ultra Pure grade prepared by Mann, cacodylic acid was a Purified grade obtained from Fisher. Formaldehyde was the Certified grade obtained from Fisher, containing about 37% formaldehyde stabilized with methanol. Formamide was freshly redistilled and dimethylformamide was Spectroquality reagent from Matheson Coleman & Bell. Ammonium cyanate, nominally free of urea, was prepared by distilling cyanuric acid into an ammonium hydroxide solution. Ammonium cyanate with urea was formed by heating a 3.6 M urea solution at 100° for 1 hr. Unless otherwise indicated, other chemicals were reagent grade or best available purity.

Thermal Denaturation. The denaturation of DNA, both free and complexed, was detected by the change with temperature of absorbance at 260 nm, using an automated, digitally recording spectrophotometer (Ansevin and Brown, 1971). Samples were heated by circulating fluid from a bath with a programmed temperature rise. The heating rate usually was 0.5°/min, but in some cases was 1°/min; for special experiments, the heating rate was reduced to 0.25°/min. Temperature was recorded by a carefully calibrated thermistor couple and bridge circuit that gave a linear voltage response from 20 to 100°; the thermistor elements were located just above the optical path in the "blank" cuvet. Optical density at 320 nm was attributed to turbidity and was monitored periodically either by operator intervention or, in more recent experiments, by automatic operation of the spectrophotometer to give one observation at 320 nm for every four observations at 260 nm. Estimates of the influence of turbidity on initial hyperchromicity were obtained by extrapolations of the type made by Englander and Epstein (1957), and changes in the contribution to absorbance at 260 nm were deduced by monitoring optical density at 320 nm (where neither DNA nor histones absorb), assuming that the slope of the scattering curve remained constant with time.

Net absorbance of samples was corrected for volume changes assuming that the heated samples expanded as pure water. Initial absorbance of samples usually was about 0.6 A at 260 nm. Hyperchromicity was found by dividing corrected net absorbance at any temperature by the mean of the three lowest values, usually observed at room temperature. Hyperchromicity and the temperature derivative of hyperchromicity were calculated and plotted by computer as described earlier (Ansevin and Brown, 1971). Curves intentionally were left unnormalized so that any imperfections of the experiments would not be overlooked. The ordinates of all derivative plots have been multiplied by a scale factor of

[1] SSC is "standard saline citrate" solution, composed of 0.15 M sodium chloride and 0.015 M trisodium citrate; SSC/100 is a 1–100 dilution of SSC and SSC/10, a 1–10 dilution of SSC.

1000. Both integral and derivative plots in all figures show symbols only at each fifth point, however, all points are used in drawing the curves. With very few exceptions, the four intermediate points are located at evenly spaced temperature intervals between the symbols on the curves.

Experimental Section

Denaturaton Profiles of Chromatin in Solvents Containing Urea. A thermal denaturation medium containing 3.6 M urea–0.005 M sodium cacodylate buffer (pH 7) and 1.5×10^{-4} Versene (urea–cacodylate buffer) was used by Ansevin and Brown (1971) to study nucleohistones formed with each of five pure histone fractions from calf thymus, and also by Spelsberg *et al.* (1971) to show that reconstitution had taken place in experiments in which dissociated chromosomal proteins were added to DNA. Urea was included in this buffered medium because it favored the solubilization of nucleoproteins and because it helped to lower the temperature at which DNA and nucleoprotein complexes denatured without requiring that ion concentrations be kept at extremely low levels. Although spontaneous rearrangement of urea molecules caused properties of the solvent to change with temperature (Ansevin and Brown, 1971), melting profiles of DNA and nucleohistones were reproduced within a few degrees from one experiment to another for buffer preparations that had been stored frozen or kept at 4° for as long as 2 weeks.

We have employed the same buffer system to study the denaturation of mammalian chromatin and observe a regularity of form in derivative denaturation profiles that appears to be useful in identifying undegraded chromatin preparations from thymus and liver of rats. The characteristic shape of the derivative denaturation profile of rat chromatin in a relatively undenatured state is compared in Figure 1 (filled circles) to less completely stabilized complexes with single histones (open squares and circles). The prominent peak near 50° in the profiles for F1 and F3 is located at the melting temperature of uncomplexed DNA, while peaks falling near 65 and 75° are produced by the unstacking of DNA that was thermally stabilized by attachment to histones. In the case of the chromatin in Figure 1, only a small fraction of the area is superimposed on the thermal region for the melting of uncomplexed DNA and thus not more than about 2% of the area can be attributed to extended stretches of completely free DNA with a representative base composition. Alternatively, not more than 10% of the total profile can be explained by the possible existence of DNA with a melting point 5° higher than normal and not more than 15%, if a sum is made for a 5 and 10° shift. (See Ansevin and Brown, 1971, for a discussion of the method employed for curve resolution.) Most of the profile corresponds to moderately and highly stabilized DNA. The profiles for rat thymus and liver chromatin show an ascending stair-step arrangement of thermal transitions with maxima centered approximately at 60, 70, and 80°. Some quantitative variations exist in the patterns from sample to sample and, in Figure 1, the second peak at 70° is less prominent than in many profiles. The area between 75 and 80° frequently is slightly exaggerated by an increase in light scattering. If denaturation is accomplished at a heating rate of 1.0°/min, instead of 0.5°/min as in Figure 1, a final low maximum usually appears around 90° in the profile for rat chromatin.

When appreciable turbidity was observed (320/260 $\geq$ 0.1) at 30°, the hyperchromicity plot usually declined in a temperature region from about 85 to 100° because of a terminal decrease in turbidity. Finally, it should be remembered in viewing Figure 1 and all others to follow that only each fifth point is emphasized with a drawn symbol. However, all points are plotted and individually connected with *straight* lines; no useful information is lost and the resolution is unaffected by this practice.

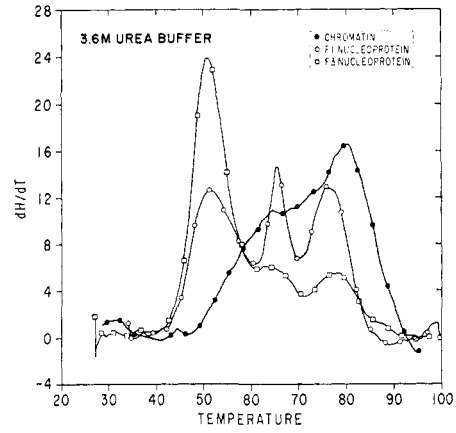

FIGURE 1: Derivative thermal denaturation profiles of mammalian chromatin and reconstituted nucleohistones recorded at a heating rate of 0.5°/min in a medium containing 3.6 M urea–0.005 M sodium cacodylate buffer at pH 7, and 1.5×10^{-4} M Versene (urea–cacodylate buffer). Rat chromatin was prepared as described in the Methods section; nucleoproteins were reconstituted from rat DNA and (0.5:1.0, w/w) F1 (lysine rich)–F3 (arginine rich) histone from calf thymus. The ordinate in this and subsequent derivative profiles is the temperature derivative of hyperchromicity at 260 nm times a scale factor of 1000; symbols are drawn only at every fifth point, although all observations are used in constructing the plots. The peak located near 50° in the F1 and F3 nucleoprotein preparations results from uncomplexed DNA and indicates that reconstitution was not complete at this ratio of protein to DNA. The fraction of the area under the chromatin curve that could be contributed by a DNA component with the same shape as that for free DNA and located at 50° is about 2%. If the peak were to be displaced to 55° the fraction of the total area included in this region and thence attributed to DNA would be about 10%.

The influence of urea on the denaturation of rat thymus chromatin is illustrated in Figure 2 with both integral and derivative profiles, for urea concentrations from 0 to 5 M in a 5 mM cacodylate buffer. The sample without urea had a high initial turbidity which elevated the first portion of the hyperchromicity plot (filled squares in Figure 2) and lowered the apparent final hyperchromicity. Changes in 320-nm readings for this sample (no urea) showed that the drop in hyperchromicity between 50 and 55° is explained by a decrease of turbidity. It is apparent from both integral and derivative profiles that denaturation temperatures are lowered by increasing urea concentrations. The derivative profiles indicate that the early melting regions are influenced more than the late melting portions. The denaturation temperature of free DNA also is lowered by urea. If the melting temperature in 3.6 M urea is taken as 49.5° (see dashed lines in Figure 2), the melting temperatures expected for DNA at 5, 2, 1, and 0 M urea are about 46, 54.5, 57.5, and 60°, respectively, according to calculations based on the data of Bekhor *et al.* (1969). Close examination of the curves indicates that, in each case, the early melting portions of the chromatin curves were displaced to approximately the same extent as would be expected for DNA. However, not more than a few per cent of the area of any derivative profile could be attributed to completely free DNA. Striking rises were found in the derivative

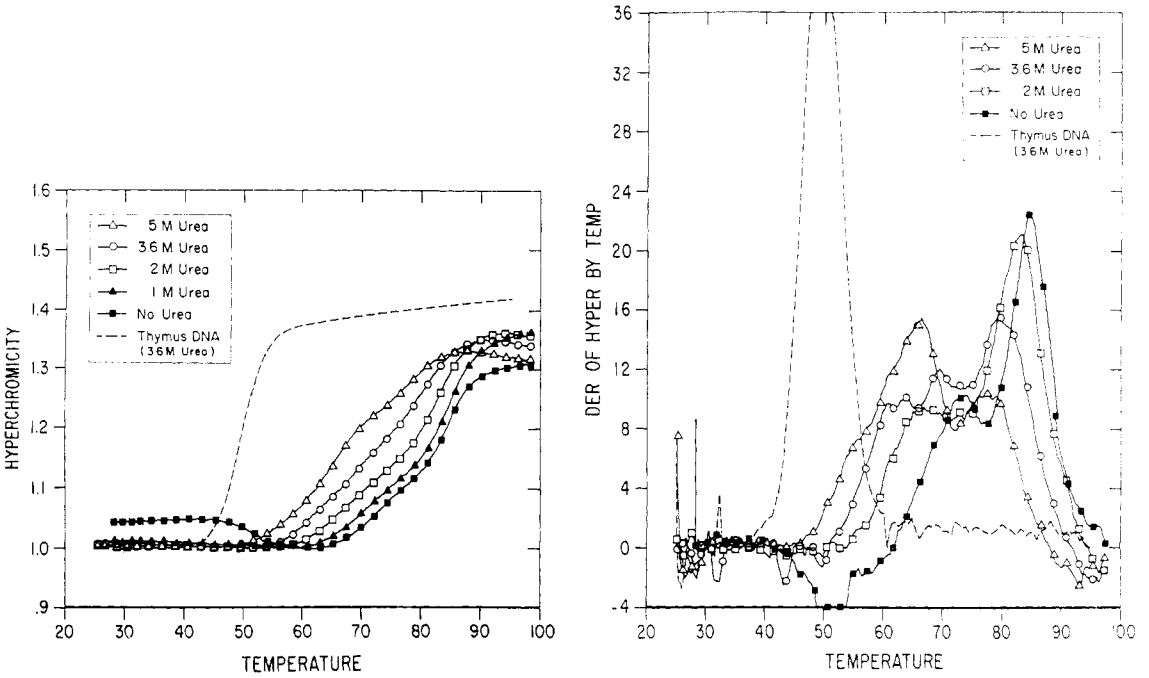

FIGURE 2: Corresponding integral and derivative plots of hyperchromicity at 260 nm for thermal denaturation of rat thymus chromatin (solid lines) or rat thymus DNA (dashed line) in a medium containing 0.005 M sodium cacodylate buffer at pH 7 and 1.5×10^{-4} M Versene with the addition of 0, 2, 3.6, or 5 M urea, as indicated. Heating rate was 0.5°/min. For the medium with 3.6 M urea, the area under the derivative curve rises in three steps, which has been found to be characteristic for fresh preparations of rat chromatin. The derivative profiles of chromatin are seen to change their shape as a function of urea concentrations. The initial level of the integral curve for no urea (filled squares) was abnormally elevated by a high starting turbidity and a decline in apparent absorbance accompanied a loss of turbidity in a temperature range starting at 45° and extending to 65°. Initial turbidity decreases were much smaller in urea-containing samples.

curves around 85°. An extremely high peak seen in the absence of urea become progressively lower as the urea concentration was increased to 5 M, and corresponding areas were shifted to lower temperature regions. The 65° peak then became the chief melting region in 5 M urea. This suggests that concentrated urea converts tightly complexed histones into moderately and weakly bound histones. Both 3.6 and 5 M urea appeared to give very good resolution although the character of the profiles was quite different. It can be estimated from 320-nm readings that increases of turbidity produced about a 5% enlargement of the area under the derivative curves in the temperature region 70–90°. The high 85° peaks in these samples thus cannot be attributed to a scattering artifact. The better resolved curves of chromatin in 3.6 and 5 M urea are obviously more complicated than those for the simple nucleoproteins shown in Figure 1.

Correlation of Denaturation Profiles with Structure in Chromatin. Figures 1 and 2 demonstrate that derivative thermal denaturation profiles of nucleoproteins show a variety of distinctive shapes very unlike that of the simple curve for uncomplexed DNA. Several types of experiments indicate that such derivative profiles provide a convenient means for obtaining information about the internal organization of nucleoproteins. For instance, it has been shown that the area under the first peak for partially reconstituted nucleohistones can be related to the quantity of uncomplexed DNA in the preparation (Ansevin and Brown, 1971). Thus, the amount of DNA remaining uncomplexed along an extended stretch of base pairs is a structural feature that should be recognized also in derivative denaturation profiles of chromatin.

A second type of observation concerns the distinctive modifications that accompany enzymatic attack on the proteins in chromatin. One illustration of this is given in Figure 3, which shows a progressive shift in denaturation profiles related to the length of time a preparation of liver chromatin was held at 4°. Components initially denaturing at 70°, as well as smaller amounts of those with a transition at 75°, appeared to be destabilized so that they denatured at 55–65° after standing; at the same time, DNA became exposed in a thermally unprotected form. We ascribe these changes to autolysis of the chromatin by bound endogenous enzymes which in part may involve a protease described by Furlan and Jericijo (1967) and Bartley and Chalkley (1970). Similar changes have been observed with some preparations of liver chromatin stored for longer times in a frozen condition (−20°). Degradation has not regularly been seen for rat thymus chromatin frozen in SSC/100 buffer but has been recognized after 1 week at 4°. Related changes in profiles were observed (see Figure 4) when successively larger concentrations of trypsin were used to attack proteins in a purified preparation of rat thymus chromatin for approximately 1.5 hr at room temperature. Although a number of attempts to stop the reaction with trypsin inhibitor were unsuccessful, one relatively low concentration of enzyme was partially inhibited (2 μg of trypsin/mg of chromosomal DNA). Trypsin digestion also exposed DNA in a thermally unprotected state and its extended action eliminated complexes stable at temperatures of 75° and above.

Another type of structural change that modifies thermal denaturation profiles is fixation by formaldehyde. Chromatin samples were reacted with 1% formaldehyde at 35° for 1 hr in SSC/100 medium or in urea–cacodylate buffer. One portion of each of the preparations was then melted in the presence of 1% formaldehyde using the medium in which it had been treated. Another portion of each preparation was dialyzed against

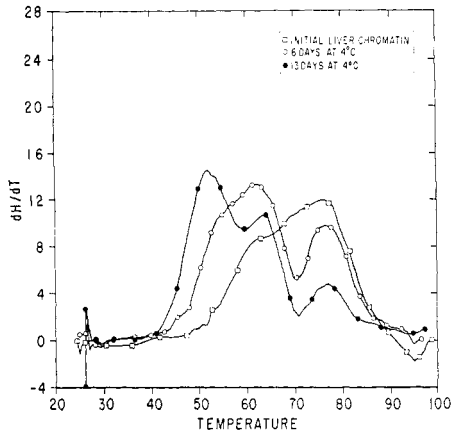

FIGURE 3: Derivative thermal denaturation profiles of rat liver chromatin held in a thawed condition for 0, 6, and 13 days. Denaturation was observed in urea–cacodylate buffer at a heating rate of 0.5°/min. Drastic changes in profiles are attributed to the autolysis of proteins and accompanying exposure of free DNA.

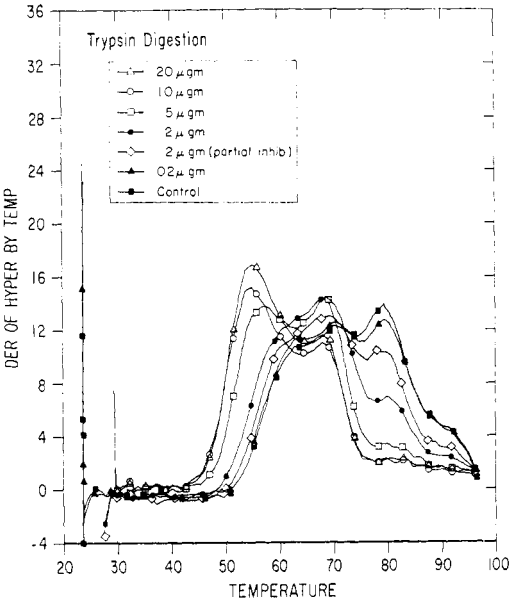

FIGURE 4: Derivative thermal denaturation profiles of trypsin-treated rat thymus chromatin. Samples were held at room temperature for about 1.5 hr following the addition of various amounts of trypsin (or no trypsin, in the control), as indicated on the figure inset in terms of micrograms per milligram of chromatin DNA. One of the 2-μg samples was partially inhibited by the addition of soybean trypsin inhibitor immediately following the trypsin. Denaturation profiles were recorded in urea–cacodylate buffer at a heating rate of 1.0°/min. Modification of a characteristic stair-step profile for fresh chromatin proceeded with greater enzyme action, showing a progressive depletion of highly stabilized areas and an increasing exposure of unprotected DNA

SSC/100 and then melted in its corresponding treatment medium, but with formaldehyde absent. One might expect the structural integrity of fixed chromatin to be enhanced through the formation of protein–protein methylene bridges (French and Edsall, 1945), but it also seems possible that the capacity of the protein to interact with DNA could be reduced by the alteration of amino groups on histones and that instead the melting temperature of DNA could be lowered when formaldehyde was present in the medium (Haselkorn and Doty, 1961; Grossman *et al.*, 1961). Figure 5a,b shows that in both solvents, formaldehyde treatment stabilized the chromatin if the formaldehyde was removed before the melting experiment. Both in SSC/100 and in urea cacodylate buffer, low melting regions were shifted to the right; in SSC/100 it appeared that some of the more thermally stable regions also were shifted to higher temperatures. When formaldehyde remained in the melting medium, the effect depended on the denaturation solvent. In SSC/100 all regions of chromatin apparently were heat stabilized. When urea was present along with formaldehyde in the denaturing solvent, thermally labile portions of the chromatin seemed little influenced and the more thermally resistant parts were shifted to lower temperatures; however, no denaturation occurred at temperatures where naked DNA normally melts. Because many relatively large aggregates were formed during reaction with formaldehyde, these experiments had to be conducted on a soluble 700*g* supernatant portion that in some cases represented only 50% of the total sample. The inaccessible precipitates may well have been more fully stabilized.

These results are consistent with the observations of other investigators who found that treatment of chromatin with 1% or higher concentrations of formaldehyde caused the proteins to remain bound to DNA even in concentrated salt solutions (Brutlag *et al.*, 1969; Ilyin and Georgiev, 1969; Hancock, 1970). The denaturation profiles of Figure 5 indicate the existence of a more complicated interaction between formaldehyde and chromatin than could be detected by melting studies in the earlier experiments of Brutlag *et al.* (1969). That the effect of formaldehyde does not appear entirely simple may be a consequence of competition between cross-linking reactions and the destabilizing effects of formaldehyde on DNA. It is noteworthy, however, that the inclusion of formaldehyde in the melting buffer did not shift any portion of the profile to a region melting as free or partially denatured DNA. Presmuably this indicates either that the chromosomal proteins hold the bases in a stacked conformation even when hydrogen bonds between paired bases are broken, or that the hydrogen bonds in DNA have reduced reactivity toward formaldehyde in the presence of associated protein.

Another quite different change in the structure of chromatin has been investigated; this is the breakage of strands to form nucleoproteins of shorter length. The size of the chromatin strands isolated by the methods outlined has not been extensively studied but better preparations contained nucleic acid having a modal sedimentation value corresponding to DNA molecular weights between 8 and 17 million. The further breakage of chromatin strands was achieved by sonication or by spray fragmentation. The sonication was carried out for a total of 120 sec using 10-sec irradiation periods in the cold at the maximum power setting for the microprobe of a Bronwill Biosonik III sonifier. Spray fragmentation involved an adaptation of the method of Cavalieri and Rosenberg (1959) in which an 8-ml volume of chromatin solution (0.35 mg/ml) in dilute buffer was sprayed and recycled for 15 min in a DeVilbis atomizer. Modal molecular weights for the DNA were reduced to about 2 million by spraying and to 0.60 million by sonication.

Denaturation profiles for spray fragmented and control chromatin were found to be nearly identical. Sonication, however, produced small but detectable changes in the denaturation profile; the changes were interpreted as indicating a small exposure of DNA and an increased tendency for aggregation (230-nm readings were higher after sonication

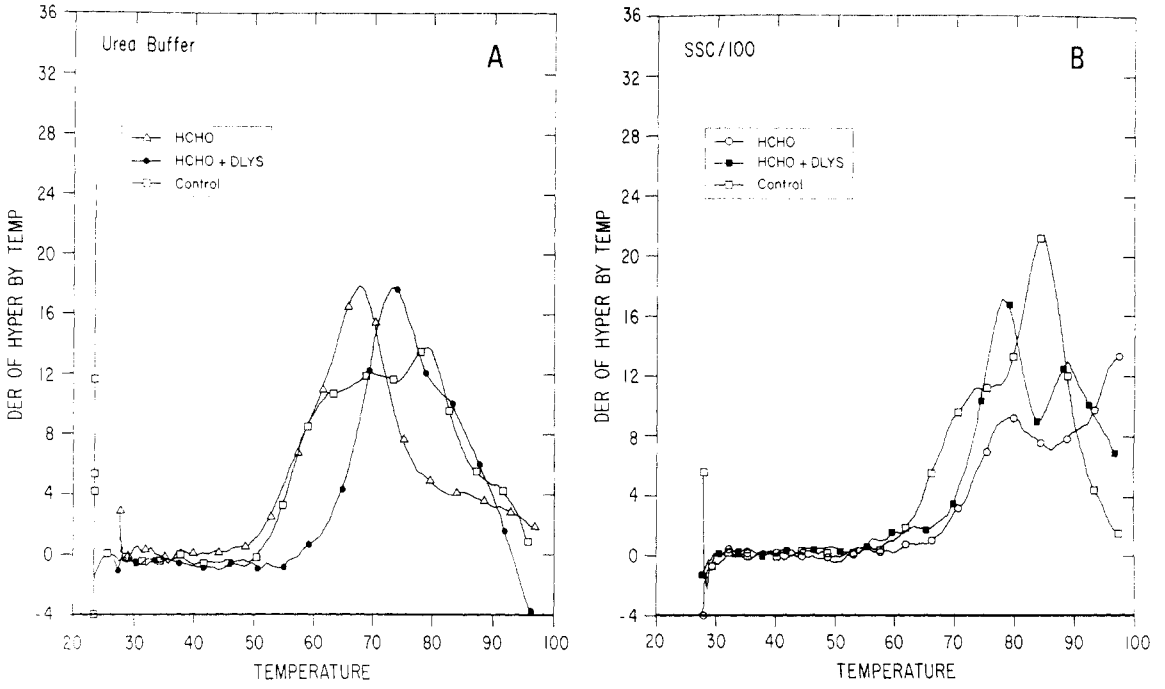

FIGURE 5: Derivative thermal denaturation profiles of formaldehyde-treated rat thymus chromatin, recorded at a heating rate of 1°/min in the presence or absence of urea. All preparations in graph A were denatured in urea–cacodylate buffer: the HCHO sample had 1% formaldehyde added to the denaturation medium; the HCHO + D-Lys sample was reacted with 1% formaldehyde for 1 hr at 35° in urea–cacodylate buffer, then was dialyzed against SSC/100 to remove the formaldehyde, and finally was denatured in urea–cacodylate buffer; the control sample was untreated rat thymus chromatin. All preparations in graph B were denatured in a medium containing 1.5 mM sodium chloride–0.15 mM trisodium citrate (SSC/100); the HCHO sample was denatured in the presence of added 1% formaldehyde; the HCHO + D-Lys sample was reacted with 1% formaldehyde for 1 hr at 35° in SSC/100 medium, and then was dialyzed against SSC/100 to remove the formaldehyde before being denatured; the control sample was untreated rat thymus chromatin.

suggesting larger particle sizes). Such modifications following sonication might be caused by a limited rearrangement of protein components within the chromatin. It is not surprising that freezing and thawing of chromatin, which might be expected to introduce occasional linear breaks, has not been observed to change the shape of derivative profiles, although aggregation (presumably, side by side) is increased by this treatment and samples routinely require resuspension by mild shearing in a hand-operated glass and Teflon homogenizer. This absence of prominent effects from strand scission is readily understood since it is reasonable to assume that thermal stabilization should reflect the organization of nucleoproteins in a direction radial to the DNA strand, rather than along its length.

Comparison of Urea–Cacodylate Medium to Other Denaturation Solvents. A denaturation medium with 3.6 M urea, 0.005 M cacodylate buffer, and 1.5×10^{-4} M Versene (urea–cacodylate buffer) has been found to give a favorable resolution of details in melting profiles of nucleoproteins when compared to a number of alternative solvents. The addition of 3.6 M urea to the dilute cacodylate buffer employed in Figure 2 revealed more complexity in the derivative profile of chromatin than was found in cacodylate buffer alone, and also prevented the initial turbidity artifact of the hyperchromicity plot in this figure.

Denaturation in SSC/100, a medium often used to melt DNA, gave a profile which was laterally compressed when compared to that in urea–cacodylate buffer.

We have compared also the nature of nucleohistone patterns in the present urea buffer to those produced in a very dilute solution of sodium Versenate. This comparison has particular interest because denaturation profiles reported by Ansevin and Brown (1971) for nucleohistones were more complex than those shown by Shih and Bonner (1970a), who employed an aqueous solvent containing 0.00025 M sodium Versenate. Figure 6 shows a series of analyses on the same three preparations of nucleohistones that had been reconstituted in low ionic strength medium. Portions of the preparations were retained for initial denaturation in urea–cacodylate buffer; the preparations were then dialyzed into 0.00025 M Versenate solution and aliquots were denatured in this buffer. Finally, the remaining portions of the preparations were dialyzed back to urea–cacodylate buffer, where the samples were again denatured, to indicate whether the manipulations had resulted in any permanent change in the structure of the nucleoproteins. An examination of Figure 6 reveals that: (1) the nucleohistone complexes apparently were little influenced by transfer to the Versene solutions and subsequent retransfer to urea–cacodylate medium, and (2) derivative patterns in urea medium may show more complexity than those obtained in dilute Versenate buffer.

Investigations of Possible Artifacts in a Denaturation Medium Containing Urea. A firm conclusion about the desirability of using the urea-containing medium requires the presentation of evidence that the greater complexity referred to above is not an artifact. As was shown in an earlier publication (Figure 2 of Ansevin and Brown, 1971), the rearrangement of urea to ammonium cyanate during heating leads to an increase in both ionic strength and pH. This accumulation of ammonium cyanate was found to have an exponential course such that the concentration remained below 0.005 M (on the basis of the equivalent conductivity of sodium chloride) at 70° but rose to about 0.013 M at 80°, and doubled this value near 90°, when the temperature of the heating bath

was elevated at the rate of 0.5°/min. The following experiments were conducted to determine whether any artifacts existed and whether they were of major importance in evaluating derivative thermal denaturation patterns. Altogether, three classes of evidence were considered: (1) the effect of the heating rate, (2) possible chemical influence of cyanate ions, and (3) variations in the shape of profiles in the absence of chemical differences in composition.

For the first test, it was reasoned that experiments taking greatly different times for completion should show discordant results if increases in pH or ionic strength were responsible for artifacts in denaturation profiles. Such an argument is valid under the condition that the ammonium cyanate production does not attain equilibrium values at the fastest heating rate. Denaturation profiles for DNA and chromatin preparations were compared at two heating rates, either twice or one-half that usually employed (0.5°/min). The two sets of denaturation patterns were found to be similar for the extremes of heating rate tested. The denaturation of DNA (40–55° range) was almost uninfluenced by the different rates, in agreement with earlier conductivity measurements indicating that the ionic strength due to cyanate remains low for temperatures up to 60°. Greater scattering was observed at 75–85° in some slowly heated samples where the accumulation of higher ion content could be expected; below 0.1 M, higher ion concentration generally is found to increase the turbidity of nucleoproteins. The small quantitative differences observed were consistent with the assumption that the urea–cyanate equilibrium lagged behind thermal changes of 1°/min.

The greatest cause for concern in the use of a medium containing urea came from the fact that the accumulating cyanate could react with sulfhydryl and amino groups on protein (Stark *et al.*, 1960) and possibly with phosphate groups in DNA (Allen and Jones, 1964). Such chemically modified nucleoproteins might well exhibit denaturation complexity that was derived from a mixture of reacted and unreacted groups. A series of three different tests was conducted to determine the influence of cyanate ions, when present either during the denaturation experiment or in a pretreatment period. In one of these, a denaturation medium containing 50% ethylene glycol plus dilute Versene and 0.005 M cacodylate buffer, but not containing urea, was made 0.035 M in KOCN; a control contained added 0.035 M KCl, instead of cyanate. This concentration of KOCN is approximately the same as that found at 88° in urea–cacodylate buffer heated at 0.5°/min. Rat thymus chromatin and two reconstituted nucleohistone preparations were denatured in both media. It is easily seen from the comparison of graphs in Figure 7 that the profiles were significantly changed by 0.035 M KOCN present throughout the heating period. But the complexity of the profiles was decreased rather than increased by the cyanate. It seems likely that some of this difference was due to an elevated pH (apparent pH in cyanate medium around 9.5) since potassium cyanate is an alkaline salt. Similar experiments involving treatment with ammonium cyanate or ammonium cyanate plus urea showed only minor differences between reacted and control samples, suggesting that internally generated cyanates do not cause spurious peaks.

It is interesting that the denaturation temperature of free DNA in the solutions of F1 and F3 nucleoproteins examined in Figure 7 was only one or two degrees lower in 0.035 M potassium cyanate than in the control; this suggests that if there is any interaction of cyanate with DNA, it is inconsequential to melting. In view of the absence of anticipated

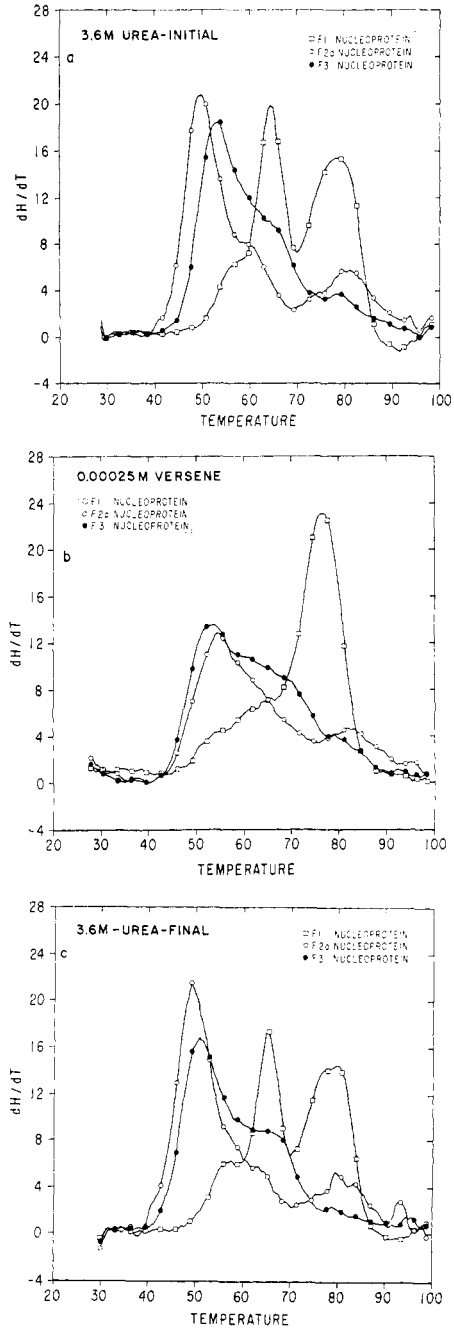

FIGURE 6: Comparative resolving powers of dilute Versene solution and of a medium containing 3.6 M urea for revealing thermal transitions in nucleohistones. Corresponding integral and derivative thermal denaturation curves are presented for the denaturation of nucleohistones in 0.00025 M sodium Versenate at pH 8, the denaturation medium of Shih and Bonner (1970a), or in a solvent containing 3.6 M urea + 0.005 M sodium cacodylate buffer at pH 7 + 0.00015 M sodium Versenate (urea–cacodylate buffer), the denaturation medium of Ansevin and Brown (1971). "Initial" profiles were obtained by denaturing the nucleohistones in urea–cacodylate buffer, a medium similar to the solution in which the preparations were reconstituted; profiles in dilute Versene were obtained after dialyzing the reconstituted samples into 0.00025 M Versene; "final" profiles were obtained after portions of the nucleohistone preparations in dilute Versene had been dialyzed back into urea–cacodylate buffer, to see whether any changes had occurred during handling of the samples. Nucleoproteins were reconstituted from rat DNA and calf thymus histones at a weight ratio of histone to DNA of 0.75 for F1, F2a, and F3 nucleohistone. Histones F1 and F3 were highly purified fractions; histone F2a contained approximately equal amounts of F2a1 and F2a2. All profiles were recorded at a heating rate of 0.5°/min. Lower than normal hyperchromicity for F2a2 and F3 nucleohistones denatured in dilute Versene was due to high initial turbidity levels in this solvent. The profile shape for F1 nucleohistone is strikingly influenced by the denaturation medium.

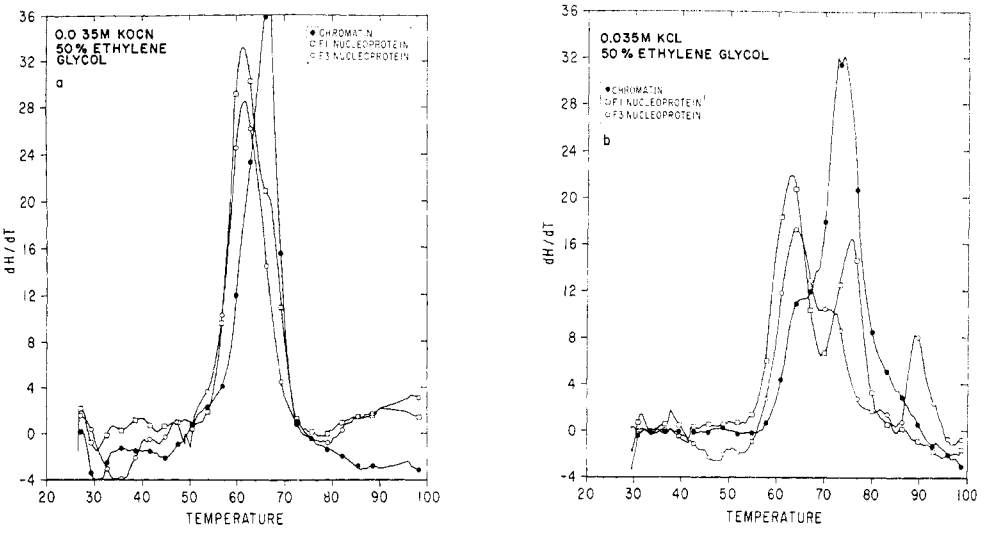

FIGURE 7: Influence of potassium cyanate in the denaturation medium on derivative thermal profiles of chromatin and reconstituted nucleoproteins. Denaturation media contained 50 vol % of ethylene glycol, 5 mM sodium cacodylate buffer (pH 7 in aqueous medium), 0.15 mM Versene, and 0.035 M potassium cyanate or potassium chloride (control), as indicated. Heating rate was 0.5°/min. Denaturation in cyanate medium was influenced by pH, which was more alkaline than in the control.

chemical effects on denaturation profiles when cyanates were added, it is possible that lysine amino groups have reduced chemical reactivity when they are closely associated with DNA or that they revert to a free form when dialyzed.

Lastly, we ask the question whether the complexity observed in our denaturation profiles is derived from intrinsic physical properties of the samples or, instead, could result from reproducible, but uninterpretable rearrangements of protein subunits on the DNA during the denaturation experiment. This is answered by the curves of Figure 8 in which two different preparations of F1 nucleoprotein were simultaneously denatured in the same urea–cacodylate medium. The patterns are distinctly different but were closely duplicated in other denaturation experiments with these samples. Each preparation was reconstituted to the nominal value of protein DNA (0.75:1.0, w/w), but by slightly different techniques. Since, to our knowledge, the preparations were chemically identical and they were simultaneously melted in the same urea–cacodylate medium, we conclude that the complexity in the derivative patterns is inherent to the samples and is a consequence of the physical organization of the nucleohistones in each preparation.

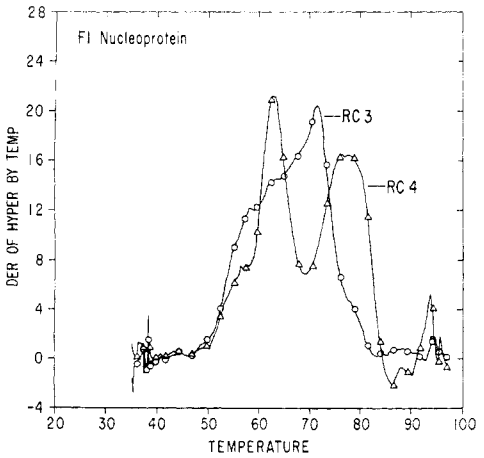

FIGURE 8: Variety in thermal denturation profiles of F1 nucleoproteins reconstituted in different experiments. Nucleoproteins were reconstituted in low ionic strength medium from purified F1 histone and protein-free DNA at a weight ratio of 0.75 (protein:DNA). Nucleoproteins RC3 and RC4 nominally have the same chemical composition. The two profiles were simultaneously recorded in the same denaturation medium (urea–cacodylate buffer) at a heating rate of 0.5°/min.

A rather large variability in the shape of F1 nucleohistone profiles has been observed in the course of many reconstitution experiments and the more extreme examples illustrated in Figure 8 make it apparent that an important aspect of the association is hard to control in the "direct" reconstitution method employed here, even though the F1 fraction combines with about the same amount of DNA each time. In the cases illustrated, the only major difference recognized at the time of reconstitution was that the rate of protein addition for RC-4 (10 μl/min) was about 50% faster than for RC-3. Other histone fractions, however, did not show the great variability in profile shape seen with F1 histone.

The observation important to this discussion is that different preparations of F1 nucleohistone with the same apparent chemical composition may give different profiles. We conclude that physical as well as chemical features of nucleoprotein structure affect the shape of derivative profiles and that thermal denaturation experiments represent one of the more revealing techniques for studying the architecture of nucleoproteins.

Discussion

Profile Analysis. The investigation of nucleoprotein structure obviously is greatly aided by plotting the temperature derivative of hyperchromicity *vs.* temperature as well as the customary hyperchromicity profile of thermal denaturation. Although the area under a peak in a derivative profile can be related to the fraction of DNA base pairs broken in that thermal region, we presently believe that it is difficult to justify making a quantitative resolution of complex profiles into individual components, except for the peak corresponding to exposed DNA. Both theoretical and practical reasons can

be cited for caution in going beyond relatively simple comparisons in the interpretation of derivative patterns. These include the possibility that the relationship between hyperchromicity and extent of denaturation is nonlinear (Applequist, 1961; Applequist and Damle, 1965; Leng and Felsenfeld, 1966); difficulties in accurately accounting for scattering effects, especially with chromatin, which often shows turbidity changes during denaturation; variability of hyperchromicity; lack of truly reversible conditions in the present buffer; ambiguity in the profile if any of the components is bound in a noncooperative manner (Olins *et al.*, 1968; Inoue and Ando, 1970); and lack of knowledge about the precise shape of single-component nucleoprotein profiles. It can be hoped that many of these uncertainties will be worked out in the future.

Despite these limitations, profile shape can be correlated with certain aspects of nucleoprotein structure, as was demonstrated by experiments involving autolysis, trypsin digestion, and fixation. Both autolysis of chromatin and trypsin treatment were seen to produce progressive changes suggesting rather specific modifications of structure. The chemical nature of the changes taking place in these enzyme-digested samples was not investigated in this study. However, Marks and Schumaker (1968) examined the peptides remaining after trypsin digestion and found them to be rich in arginine. Their chromatin apparently was more extensively degraded than that examined here, since only a quarter of the original protein remained and the DNA was no longer thermally stabilized. Autolytic changes in chromatin were studied by Furlan and Jericijo (1967) and by Bartley and Chalkley (1970). Both groups concluded that an endogenous protease tightly bound to nucleohistone from a variety of tissues rapidly attacks lysine-rich histone, while other fractions are degraded more slowly. Similarly, Combard and Vendrely (1970) found initial autolysis of lysine-rich fractions which left a residual arginine-rich nucleoprotein. The pronounced dip near 70° seen in the derivative profiles of chromatin after standing at 4°, but not observed in the trypsin-treated chromatin, may reflect this preferential removal of F1 histone by an endogenous enzyme.

Denaturation of Artificial Nucleoproteins in Different Solvents. In a number of earlier studies complexes between DNA and various proteins were found to display up to two inflection points in plots of optical absorbance *vs.* temperatur (Tsuboi *et al.*, 1966; Olins *et al.*, 1967, 1968; Samis *et al.*, 1968; Inoue and Ando, 1970; Shih and Bonner, 1970b). The occurrence of two transitions often has been attributed to the melting of uncomplexed DNA followed by the denaturation of a homogeneous nucleoprotein complex. Derivative denaturation patterns with only two prominent transitions were reported by Shih and Bonner (1970a) for reconstituted nucleohistones even with complexes containing DNA and more than one histone fraction. Qualitatively different results were obtained with nucleohistones in the present study and also in an earlier one (Ansevin and Brown, 1971) in which the derivative denaturation profiles of F1, F2a1, F2a2, and F3 nucleohistones were shown to display at least three partially separated regions of thermal transition. The three regions are presumed to correspond to the melting of free DNA and two or more types of DNA–histone complexes. F2b nucleohistone profiles were found to be less complicated. A recent paper by Li and Bonner (1971) also emphasizes the observation of a multistage denaturation for nucleohistones reconstituted with a mixed fraction of calf thymus histones as well as for preparations of pea plant chromatin.

A resolution of any question about the degree of compositional complexity that is discerniable in thermal denaturation profiles is important to the analysis of reconstituted nucleohistones and especially to the study of chromatin, which is known to have a most complicated structure. Therefore, we have attempted to identify the reason for greater complexity in the denaturation profiles of simple nucleohistones studied by Ansevin and Brown (1971), in comparison to that reported by Shih and Bonner (1970a). Although several differences existed between the two studies, including the manner of reconstituting the nucleoproteins, in the present experiments we have asked specifically whether the urea–cacodylate denaturation medium of Ansevin and Brown (1971) was able to give a more sensitive resolution of components in reconstituted preparations and whether the urea–cacodylate was responsible for any artifacts that could have imposed spurious complexity.

A number of comparisons were made between denaturation profiles obtained in urea–cacodylate buffer and those in alternative media both with artificial nucleohistones and with chromatin. No solvent appeared more revealing than the one we have used most often: 3.6 M urea, 0.005 M sodium cacodylate buffer at pH 7, plus 1.5×10^{-4} M sodium Versenate. Alternative media considered include 5 mM cacodylate plus 50% glycerol, 50% ethylene glycol, or 50% methanol; 4 M *N*,*N*-dimethylformamide, 4 M formamide, 4 M dimethyl sulfoxide; 3×10^{-4} M sodium Versenate; SSC/100; and 1, 2, and 5 M urea in 5 mM cacodylate buffer. The direct comparison between urea–cacodylate buffer and dilute Versene buffer showed strikingly better detail with the urea–cacodylate buffer only in the case of F1 nucleohistone samples.

As mentioned earlier, a full description of the properties of a solvent containing urea is complicated by the conversion of urea to ammonium cyanate to cause time-dependent changes in pH and ionic strength. One of the conclusions of the present study was that such changes in the medium do not preclude its use for analytical work if conditions during experiments are adequately reproduced. In this respect, the dynamic character of the urea buffer discussed here might be compared to the variations in a buffer gradient used to elute different components bound to a chromatography column. However, an additional possibility which had to be carefully considered was that cyanate formed during the experiment might introduce artifacts by chemical reaction with DNA or proteins. Reaction of cyanate with both α- and ϵ-amino groups of proteins readily occurs at pH 8 and is facilitated in the presence of a denaturant such as concentrated urea (Stark and Smyth, 1963). In view of this anticipated high reactivity of cyanates with amino groups and the fact that most side-chain amino groups of histones appear to be directly associated with DNA phosphates (Walker, 1965), it would not have been surprising to find that results in urea solvents were poorly reproduced.

Instead, we have observed a great regularity in the shape of profiles for the same nucleoprotein preparation if the composition of the denaturation buffer and the rate of heating were kept constant. With rat thymus or liver chromatin, deviations from the usual ascending stair-step pattern in the derivative profile were easily correlated with treatments expected to induce structural change.

Although profile shape is well preserved from experiment to experiment, a moderate degree of variability has been experienced for the temperature at which exposed DNA denatures in nucleohistone preparations. Discrepancies in the location of the DNA peak appear to be caused by small

differences in the salt content of the denaturation medium. Stabilized portions of the profile are less affected.

A number of experiments were performed to show that internally generated cyanates do not appear to cause significant artifacts in denaturation profiles. These tests revealed that: (1) complexity in the stabilization profiles of experimentally formed F1 nucleohistones is intrinsic to the preparations; (2) complexity in stabilization profiles of chromatin and F1 or F3 nucleoproteins was decreased rather than increased for denaturation in the presence of potassium cyanate, and little or no increase of complexity was observed for samples pretreated with ammonium cyanate, in the presence or absence of urea; and (3) changes in the duration of denaturation experiments to twice or to one-half as long as the usual time produced relatively small quantitative alterations in the profiles of DNA and chromatin without changing the general character of the profile. We conclude from the foregoing results that the complexity of patterns illustrated here and in earlier studies (Ansevin and Brown, 1971; Spelsberg *et al.*, 1971) was not produced by cyanate ions in the denaturation medium.

With the above conclusion in mind, we are now able to comment on differences between the derivative denaturation profiles of simple nucleoproteins presented by Ansevin and Brown and those illustrated by Shih and Bonner (1970a,b). We believe that the greater complexity seen by Ansevin and Brown in F1 nucleoproteins was due in part to a superior resolution capability of the urea–cacodylate buffer. N. T. Van and A. T. Ansevin (in preparation) have found that the higher ionic strength of the urea–cacodylate medium is important to this better resolution of denaturation profiles of F1 nucleohistones. Distinctions between other nucleoprotein patterns probably are attributable to differences in the methods of reconstitution used by the two groups. It would appear that more variety in the mode of interaction of histone with DNA is exhibited in nucleohistones formed by the slow addition of protein to DNA in a solvent of relatively low ionic strength.

Mammalian Chromatin. A particularly interesting observation with chromatin was that very little uncomplexed DNA could be detected by thermal denaturation. However, a thermally unstable peak rapidly appeared following enzyme digestion, at the expense of well-stabilized regions of the denaturation profile. This result is consistent with information showing a limited availability of DNA as a template for RNA polymerase, but is in superficial disagreement with the results of Itzhaki (1970, 1971) and inconsistent with the conclusion of Clark and Felsenfeld (1971) that as much as 50% of the DNA in chromatin is in a free state. It seems possible that thermal denaturation experiments might show no free DNA even though a sizable fraction of the DNA phosphate groups actually were exposed, under the condition that free segments were staggered with protected regions so that the length of free segments did not exceed some limit, on the order of 50–100 nucleotide pairs (about 17–35 nm in DNA length). Since it has been proposed that melting of free DNA occurs simultaneously for all base pairs over a chain extent of approximately 50 units (Geol and Maitra, 1969; Crothers, 1968), regular incorporation of a stabilizing molecule at a shorter distance might elevate the denaturation temperature for the whole, as is observed, for instance, with tetralysine (Olins *et al.*, 1968). However, a mechanism that would produce a selective deposition of histones at the required spacing is not known, nor does it appear that any of the five histone fractions is automatically bound in a random pattern that would resemble the attachment of small oligolysines (Ansevin and Brown, 1971). It may be that short stretches of DNA are partially exposed under nonbasic segments of histones, as suggested by Itzhaki (1971).

Derivative thermal denaturation profiles for pea bud chromatin were recently published by Li and Bonner (1971). There appear to be significant differences between these results with plant chromatin and ours with mammalian chromatin. The derivative profiles of Li and Bonner (1971) could be resolved into three thermal regions of stabilized melting, the second two of which show high maxima separated by a deep minimum, giving a profile shape that is quite distinct from the stair-step pattern which we found for rat chromatin. Furthermore, the authors have interpreted the profile of plant chromatin differently, suggesting that the regions of major interest with respect to direct histone–DNA interaction are the two prominent peaks occurring at 66 and 81° in their medium. They have postulated a model for histone–DNA interaction in which each histone molecule is considered to bind to DNA with two different binding strengths, one for a region with high density of positive charge and the other for a region with low charge. This view is supported in part by profiles of salt-extracted chromatin and the observation of a double-peaked profile for their reconstituted calf thymus fraction IIb, which shows a striking resemblance to certain of the chromatin curves. The model would appear to be at variance with the results of Shih and Bonner (1970a) for the denaturation of reconstituted fraction IIb, which along with fractions Ia and Ib (F1) and IV (F2a1), appeared to lack clearly biomodal regions of thermal stabilization. The model is not fully consistent with the observations of Ansevin and Brown (1971) on the melting behavior of the five major histones, especially the pure fraction F2b where initial stabilization seemed to involve a single thermal peak, although variety was seen for each of the others. We do not question an interpretation that most histones may be able to interact with DNA in more than one way (although, with the exception of fraction F2b, we have no evidence indicating that this involves differential attachment of two halves of the same molecule) but we favor a view that different melting regions supply distinctive information about the organizational state of natural chromatin. According to this view, our preparations of mammalian chromatin must be significantly different in composition or structure from the preparations of pea bud chromatin examined by Li and Bonner (1971). This conclusion is based on the arguments already elaborated in this paper and our observation that denaturation of rat thymus or liver chromatin in dilute Versene buffer gives profiles quite similar to those shown above in urea–cacodylate buffer and essentially unlike those of Li and Bonner (1971). It seems likely that these differences arise partly from the manner in which the chromatin is prepared and partly from intrinsic differences between the sources.

We have tested the effect of omitting Ca^{2+} from the sucrose solution used in early stages of nuclear isolation, as recommended by von Hahn *et al.* (1970), and Anderson and Moudrianakis (1971), and also of including Versene at this point. We do not find a significant modification in the shape of the denaturation profile of thymus chromatin melted in urea–cacodylate buffer. Similarly, we do not see well-resolved peaks in 3×10^{-4} M Versene medium with chromatin isolated under conditions where divalent ions were either included or withheld. Although we have found that chromatin from other organisms may show patterns of thermal stabilization that are distinct from those reported here for rat, in our experience, the observation of well-resolved peaks in the derivative de-

naturation profile of chromatin always could be associated with degraded preparations.

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