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A Physical Study of the Stability of the Native Nucleohistone Conformation to Salt Dissociation and Heating*

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ABSTRACT: The subjection of nucleohistone to both salt dissociation and heating results in a loss of supercoiled structure over the salt range 0.9–1.1 M NaCl and the temperature range 64–80°. The loss of structure commences when about 60% of the histones are dissociated by salt, and approximately at the temperature at which the DNA in nucleohistone melts. The conformation of F1-histone-deficient-soluble nucleohistone was lost in approximately the same temperature range. No concurrent conformational change was detected in the B-DNA after either salt dissociating the histones, or heating and cooling the nucleohistone, but in both circumstances conformational changes were detected in the histones.

X-Ray diffraction investigations on isolated nucleohistone now provide very strong evidence for the presence of a native supercoiled conformation of individual nucleohistone molecules. The evidence rests on the interpretation of a simple partially oriented X-ray diffraction pattern with semimeridionally oriented reflections at 110, 55, 37, 27, 22, and 18 Å, where the 110-Å reflection is assumed to derive from the pitch of the superhelix and the other reflections are secondary maxima (Wilkins *et al.*, 1959; Pardon, 1966; Pardon *et al.*, 1967; Pardon, 1967; J. Pardon and M. H. F. Wilkins, manuscript in preparation). While this evidence is strong in itself, it is not conclusive. However, supporting evidence for this structure is available. First, X-ray diffraction patterns from whole nuclei show the same secondary maxima as those from extracted nucleohistone (Wilkins, 1956). Second, electron microscope investigations on chromatin reveal hollow tubular structures which are compatible with supercoiled nucleohistone of the above dimensions (Davies, 1968; Davies and Small, 1968). Third, a sedimentation study of F1-deficient-soluble nucleohistone at increasing stages of salt dissociation indicated a large conformational change compatible with the loss of supercoiled conformation (Garrett, 1970).

The main purpose of this work was to follow in detail the loss of conformation with salt dissociation and heat melting of the complex using the characteristic X-ray pattern as a criterion for the presence of the native conformation (Richards *et al.*, 1970).

The chemistry of the dissociation of histones by salt has been thoroughly investigated (Ohlenbusch *et al.*, 1967), and was further characterized by optical rotatory dispersion measurements. The heat melting of soluble nucleohistone was characterized by measuring hyperchromicity and sedimentation coefficient changes.

Materials and Methods

Preparation of Soluble Nucleohistone. The modified standard procedure was used (Zubay and Doty, 1959; Garrett, 1970). The source of nucleohistone was calf thymus. This was frozen in Dry Ice within 30 min of the calf's death. It was stored in Dry Ice and used within 3 months of collection. Observations were made on several preparations over a period of 1 year. The final concentration of nucleohistone was approximately 0.4 mg/ml in 0.7 mM sodium phosphate buffer at pH 6.8. F1-histone-deficient-soluble nucleohistone was prepared as previously described (Garrett, 1970). Both soluble nucleohistone, and F1-histone-deficient nucleohistone, preparations were invariably characterized by their respective sedimentation coefficients of 26 (±3) S and 55 (±5) S; in the extraction buffer, and by their capacity, in fibers to yield the characteristic nucleohistone low-angle X-ray pattern (Figure 1A). The properties of these nucleohistones have been described elsewhere (Garrett, 1970, 1971).

Salt-Dissociation Experiments. Gradual loss of histones from the nucleohistone complex was achieved by increasing the salt concentration of F1-deficient nucleohistone solutions.

Increments of 2.6 M NaCl–0.1 M sodium phosphate buffer (pH 6.8) were added with rapid stirring to 25-ml aliquots of the 0.6 M NaCl extract. Solutions were adjusted to 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, and 1.8 M NaCl so as to cover completely the histone dissociation salt range (Ohlenbusch *et al.*, 1967). The partially dissociated nucleohistone was pelleted in 12 hr at 40,000 rpm in a Spinco "40" rotor. Solutions were investigated for optical rotatory dispersion differences. Fibers of the gels were examined by X-ray diffraction.

Heating Experiments. Aliquots (25 ml) of nucleohistone

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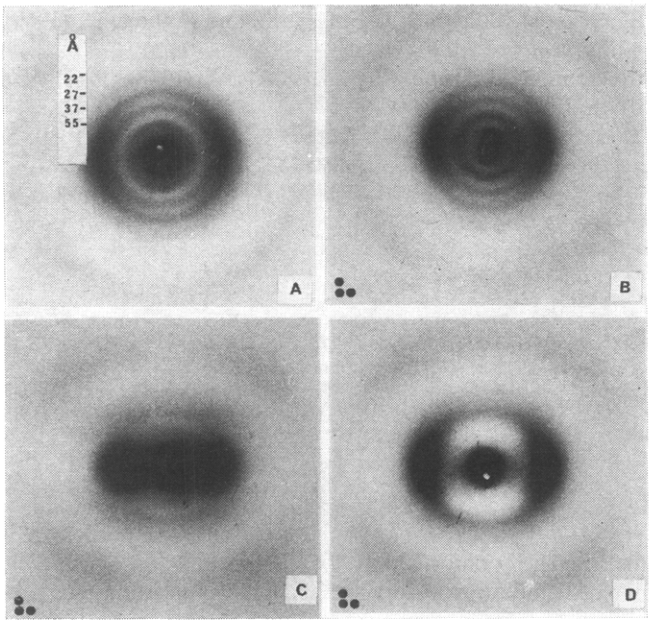

FIGURE 1: Low-angle X-ray diffraction patterns for soluble nucleohistone extracted in 0.6 M NaCl and prepared at different salt concentrations. The characteristic maxima are present on A (0.6 M NaCl) and B (0.9 M NaCl), but are only partially present on C (1.0 M NaCl) and have disappeared on D (1.1 M NaCl). The exposures were obtained on an Elliott toroidal camera in 18 hr at 98% relative humidity and room temperature. The specimen to film distance was 8.6 cm. (●) Number of independent samples examined.

solutions in 0.7 mM sodium phosphate buffer (pH 6.8) were maintained at a constant temperature (±0.5°) for 10 min at 20, 40, 50, 55, 60, 64, 68, 72, 76, 80, and 90° in a water bath. The solutions were cooled in ice. Sedimentation coefficients were measured at 20°. Fibers for X-ray diffraction analysis were obtained from gels precipitated in 0.15 M NaCl. Solutions of F1-deficient nucleohistone in 0.6 M NaCl were heated under similar conditions at 79, 85, 96, and 100°. After cooling sedimentation coefficients were measured and then the soluble nucleohistone was precipitated by adjusting the salt concentration to 0.14 M NaCl–0.01 M sodium phosphate buffer (pH 6.8). Fibers were pulled from the precipitate for X-ray diffraction analysis.

X-Ray Diffraction. Nucleohistone and partially dissociated nucleohistone were prepared for fiber diffraction as described above. Fibers were pulled (Wilkins *et al.*, 1959). In order to avoid mechanically breaking down the supercoiled structure (Pardon *et al.*, 1967) these fibers were not stretched excessively. They were exposed on pinhole collimating (Langridge *et al.*, 1960b) or on low-angle Franks' (Franks, 1966) cameras for 2–3 days, or on Elliott toroidal cameras (Elliott, 1965) for 18–22 hr. Exposures were at 92–98% relative humidity in a helium atmosphere. A Hilger and Watts X-ray source with a low-power filament was used.

Freshly prepared material invariably generated the characteristic reflections at 55, 37, 27, and 18 Å. In the relative humidity range 92–98%, the supercoil transform (Pardon, 1967) appears to be modulated such that the 110-Å reflection is missing (J. Pardon, personal communication). The structures of the partially dissociated nucleohistone complexes were investigated after pelleting. As a fiber was pulled it was occasionally rinsed in the appropriate salt solution until almost dry. The heated complexes were examined after salt precipitation from the cooled solutions. Long exposures were required for the 80 and 90° samples (24–26 hr on a toroidal camera) because the material diffracted weakly.

Analytical Ultracentrifugation. A Spinco Model E analytical ultracentrifuge was used. Uv optics were used and runs were performed at 20 (±2)° between 29,500 and 52,640 rpm. The heated and cooled water-extracted and F1-histone-deficient nucleohistone solutions were diluted to $A_{260\ nm}$ = 0.6–0.8 (1-cm light path) with the appropriate buffer (pH 6.8). Sedimentation coefficients were not corrected for the nucleohistone concentration since the effects are very small in this range (Giannoni and Peacocke, 1963). The sedimentation rate of the water-extracted nucleohistone is dependent on both primary and secondary charge effects because much of the DNA is not covered by histones and is therefore highly charged (Itzhaki, 1970). The F1-histone-deficient nucleohistone sedimentation rate was corrected for the density of the NaCl solution (Svedberg and Pedersen, 1959).

Optical Rotatory Dispersion. Measurements were made on a Bendix recording spectropolarimeter (Polarmatic 62) which was calibrated with sucrose. Partially salt-dissociated nucleohistone solutions were adjusted to $A_{260\ nm}$ = 1 with the appropriate high-salt solution. Verdet wavelength corrections were made to all readings. Specific optical rotation values were calculated. The α-helix content of the total histones in solution was estimated by the method of Oriel (1966). This method assumes that the optical effects of histones and DNA are additive in nucleohistone and that by subtracting the DNA contribution one obtains the histone cotton effect and subsequently the total percentage helicity of the histones. The form of the optical rotatory dispersion curves was the same as reported by Oriel (1966) for water-extracted soluble nucleohistone. The optical rotatory dispersion of isolated histones and histone fractions have been well characterized (Bradbury *et al.*, 1965, 1967). In dilute salt each fraction, except F1, has 20–30% α-helix content. F1-histone has only 0–10%.

Melting Profiles. Solutions of water- and 0.6 M NaCl-extracted nucleohistone were diluted to $A_{260\ nm}$ = 0.5 (1-cm light path). 1-cm silica cells with Teflon stoppers were sealed with RTV silicone rubber (Jacobsen van den Berg, Ltd.). After heating, cells were checked to ensure that no precipitation, pH change, or leakage had occurred. A scattering correction was made. Relative melting profiles were plotted at 257 and 282 nm.

Results

Salt Dissociation. Figure 1 illustrates the disappearance of the characteristic X-ray pattern over the salt range 0.9–1.1 M NaCl. At 0.6, 0.7, 0.8, and 0.9 M NaCl the X-ray diffraction patterns were indistinguishable with respect to spacings. Relative intensities varied slightly, within the range which normally occurs with slight variations in relative humidity. It is clear from the disappearance of the low-angle X-ray pattern, that the nucleohistone conformation is lost over the salt range 0.9–1.1 M NaCl. The low-angle reflections were not detected between 1.2 and 1.8 M NaCl; only the 24- to 25-Å equatorial B-DNA reflection (Langridge *et al.*, 1960a) was observed. Although these results were reproducible, the nucleohistone conformation was relatively unstable at 0.8 and 0.9 M NaCl and was easily disrupted by mechanically stretching (Pardon *et al.*, 1967) the fibers.

Fibers of approximately equal birefringence, prepared at 0.1 M NaCl intervals over the histone dissociation range, showed no appreciable change in the intensity of the partially

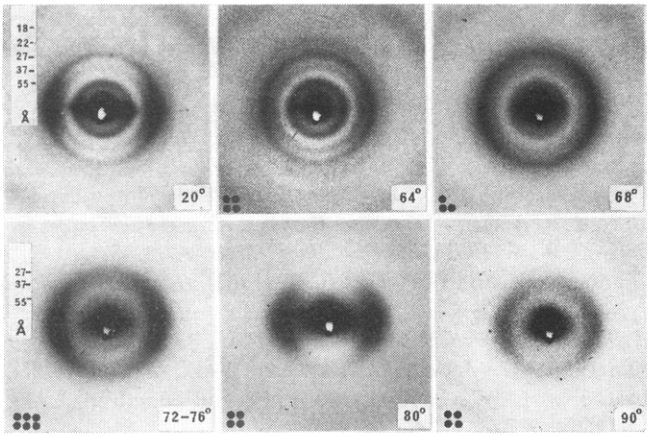

FIGURE 2: Low-angle X-ray diffraction patterns which derived from water-extracted-soluble nucleohistone heated at different temperatures in 0.7 mM sodium phosphate buffer (pH 6.8). The characteristic pattern is well preserved up to 64°, but at 68° it has markedly deteriorated and by 80° none of the nucleohistone low-angle reflections are present. The fibers were exposed on X-ray cameras as described under Figure 1. (●) Number of independent samples examined.

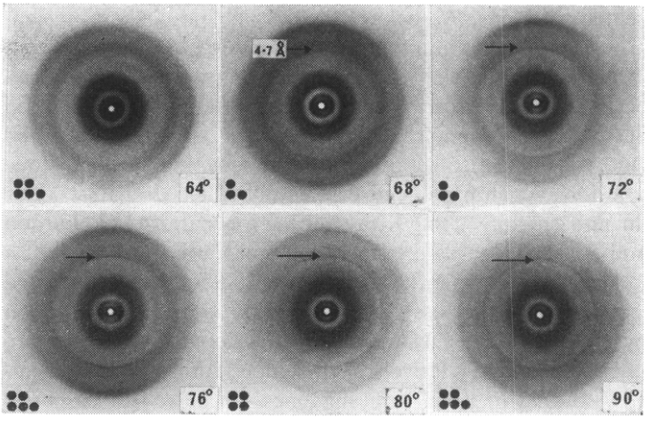

FIGURE 3: High-angle X-ray diffraction patterns obtained on pinhole collimating cameras. The 4.7-Å reflection is first apparent at 68° and is very weak. It gradually increases in intensity to 76°, above which it is more disoriented. (●) Number of independent samples examined.

oriented 3.4-Å reflection. Since this reflection is characteristic only of the B-DNA conformation, it was inferred that the majority of the DNA was in this form in both the supercoiled and the uncoiled nucleohistone.

Estimates of the percentage of α helix in the total histones of the partially dissociated nucleohistone solutions yielded the following results: 0.6 M NaCl, 37% α helix; 0.8 M, 36.5%; 1.0 M, 37%; 1.2 M, 33%; 1.4 M, 32.5%; 1.6 M, 31%; and 1.8, 2.0, and 2.2 M, 30%. The limits of error were estimated at $\pm 3\%$ at each salt concentration. This suggests that there is some change in the α-helix content of the histones as they are increasingly dissociated from the DNA.

Heating. Figure 2 shows the gradual loss of the nucleohistone conformation over the temperature range 64–80°. The DNA in soluble nucleohistone melted over the range 69.5–100°. Clearly, therefore, some loss of supercoiling preceded melting of the DNA, but much of it occurred in the first half of the DNA melting curve.

In an attempt to detect this loss of conformation in solution, sedimentation coefficients were measured for the water-extracted nucleohistone solutions in 0.7 mM sodium phosphate buffer (pH 6.8) which had been heated at 20, 79, 85, and 92°. The respective sedimentation coefficients were 25, 25, 26, and 25 S, with an estimated experimental error of (± 2 S); they do not support the occurrence of a large conformational change.

As judged by the intensity of the 3.4-Å reflection, before and after heating, the DNA was predominantly in the B conformation.

Figure 3 shows the high-angle X-ray patterns from the heated complexes. At 68° and higher temperatures a 4.7-Å meridional reflection is clearly exhibited. This reflection was first detected in isolated whole histones and was attributed to a denatured "cross-β" structure in the histones (Zubay and Wilkins, 1962). It increases in intensity with higher temperature, as the low-angle reflections become weaker.

For the F1-histone-deficient-soluble nucleohistone, the loss of conformation was also complete at 80°, although the melting range of the solution was much higher, over the range 87–100°. Sedimentation coefficients showed a marked decrease with increasing temperature, suggesting that a large conformational change had occurred. The respective sedimentation coefficients measured at 20, 79, 85, 92, 96, and 100°, were 56, 16, 16, 19, 20, and 19 S. The DNA was predominantly in the B conformation and the intensity of the 4.7-Å reflection increased as the low-angle reflection intensity decreased.

Discussion

Evidently, the native nucleohistone conformation is destabilized over a small salt range 0.9–1.1 M NaCl, during which histone dissociation increases from approximately 48 to 63% (Fambrough and Bonner, 1968). However, the conformation was clearly less stable at 0.8 M NaCl, where approximately 38% of the histones were dissociated. These results are in excellent agreement with the sedimentation study of nucleohistone in high salt solutions where the loss of conformation was detected in the range 0.6–1.1 M NaCl. Furthermore, it is in agreement with the results of Richards *et al.* (1970; and unpublished work), who showed that only three histone fractions are responsible for supercoiling, all of which are predominantly attached to the DNA at 0.9 M NaCl (Ohlenbusch *et al.*, 1967).

The conclusion that the DNA is predominantly in the B form is in agreement with the inference of Wilkins *et al.* (1959) who only investigated fibers of water-extracted nucleohistone. Moreover, the change in the conformation of the histones on dissociation from the DNA is in agreement with the conclusions of Bradbury *et al.* (1967) who maintained that isolated histones have a variable secondary structure in solution. Furthermore, our value of 37% α helix in nucleohistone in 1 M NaCl, is substantially higher than the value for isolated histones in 1 M NaCl of 23% α helix (Bradbury *et al.*, 1967), which suggests that the histones on the DNA, under these salt conditions, have a higher α-helix content than when detached. Our results are rather higher than the estimates of Oriel (1966) who found about 20% α helix in water-extracted nucleohistone in 0.7 mM sodium phosphate buffer (pH 6.8) and in 2 M NaCl. However, there is an increasing amount of circumstantial evidence to support the concept that the nucleohistone conformation is lost in 0.7 mM sodium phosphate buffer (pH 6.8) (Bradbury *et al.*, 1967). This is further supported by our failure to detect any change in sedimentation coefficient of the

heated solutions. We believe the reason that fibers of nucleohistone prepared from these solutions contain the native conformation is that the increase in concentration of salt which occurs when fibers are prepared is essential for the formation of the native conformation (R. A. Garrett, unpublished work). This is also supported by the fact that nucleohistone solutions which have been extensively dialyzed against water do not generate good X-ray diffraction patterns (J. Pardon and B. M. Richards, personal communication).

The heating experiments indicate that for water extracted nucleohistone the loss of conformation is probably initiated by melting of the DNA, and short regions of super coil are preserved under relatively high temperatures (80°). Presumably, during melting, some histones are detached, or partly detached, from the nucleohistone and form cross-β structures. For the F1-histone-deficient material the large change in conformation was manifest in the large decrease in sedimentation coefficient, 56–20 S, and by the change in the X-ray pattern. The change occurred over a temperature range similar to that of the water-extracted material.

Acknowledgments

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