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Effects of Shearing on Chromatin Structure†

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ABSTRACT: The effects of mechanical shearing on chromatin structure were investigated by using thermal denaturation and circular dichroism (CD) spectroscopy. Under ordinary conditions of mechanical shearing used for preparation of soluble chromatin, we observed only minor changes (less than 10%) of chromatin properties with respect to (a) absorption melting curves, (b) CD spectra, (c) CD melting curves and (d) histone transfer from chromatin to exogenous DNA. Such small perturbation of structural properties could be due to the generation of free ends when a large chromatin was cut into smaller fragments and by weakening the binding of histones to DNA near these free ends. In addition to mechanical shearing, sonication was used to shear some samples of chromatin. The effect of sonication on chromatin structure was investigated by the same physical methods used for mechanically sheared chromatin. The results indicate that sonication only slightly changes the chromatin properties with respect to CD spectra, similar to the results obtained by mechanical shearing, but sonication at high settings has a greater effect on the thermal denaturation property of chromatin as contrasted to our results from mechanically sheared chromatin.

Mechanical shearing of chromatin has been used as a method for isolating soluble chromatin for structural studies (Bonner et al., 1968) as well as fractionation of chromatin into components for transcriptional studies (Frenster et al., 1963; Chalkley & Jensen, 1968; McCarthy et al., 1973; Gottesfeld et al., 1974; Simpson, 1974). Noll et al. (1975) reported disruption of chromatin structure after mechanical shearing as judged by nuclease digestion patterns. Circular dichroism (CD) spectroscopy (Nicolini et al., 1976) as well as CD and thermal denaturation properties (Miller et al., 1976) of chro-

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matin were reported to be altered greatly after shearing by sonication. Sliding of histones caused by mechanical shearing has also been demonstrated (Doenecke & McCarthy, 1976). Since many structural studies of chromatin were made on mechanically sheared chromatin, and, if it is true that the shearing conditions used in the experimental procedures cause severe structural changes in chromatin, all the previous experiments made on sheared chromatin have to be reexamined. The impact of artifacts caused by shearing was so great that we were directed to examine the effects of shearing by either mechanical or sonication methods on chromatin structure with respect to (a) CD spectra, (b) thermal denaturation properties of DNA in chromatin measured by absorption at 260 nm and CD at 280 nm, and of proteins in chromatin measured by CD at 220 nm, and (c) release of histones from chromatin. Our results showed no significant changes in the above properties for mechanically sheared chromatin but slightly greater changes in the absorbance melting profiles for highly sonicated chromatin.

Materials and Methods

Unsheared chromatin from calf thymus was prepared according to the methods of Marushige & Bonner (1969) as modified by Seligy & Miyagi (1969) except that 0.1 mM $PhCH_2SO_2F$ (phenylmethanesulfonyl fluoride) in 2-propanol was added as a protease inhibitor in the buffers used subsequent to the isolation of nuclei. Isolated chromatin was dialyzed to 0.25 mM EDTA (pH 8.0) and was mechanically sheared in a Virtis homogenizer Model 45 at a speed setting of 20 or 50 at 15-s intervals, for a total shearing time of 75 s or sheared by sonication in a Sonifer cell disrupter Model W140D with a macrotip at a setting of 3 or 5 at 15-s intervals, for a total sonication time of 60 s.

CD spectra of the samples were taken with a Jasco spectropolarimeter Model J-41C. A water-jacketed cell connected to a constant temperature circulator was used for all the CD measurements. The CD spectrum at each given temperature was recorded after the sample was equilibrated at that temperature for about 10 min. The temperature in the cell filled with buffer solution was calibrated in a separate run. The CD results are reported either as $\Delta\epsilon = \epsilon_L - \epsilon_R$, or molar ellipticity $[\theta]$. The units of $\Delta\epsilon$ are M^{-1} cm^{-1}, where M is the number of moles of nucleotide per liter.

Thermal denaturation experiments were performed with a Gilford spectrophotometer Model 2400-2. The hyperchromicity (h) was recorded at 260 nm and the plot of its first derivative, dh/dT, was reported as previously described (Li & Bonner, 1971).

The viscosity of each sample was measured with a Beckman rotating cylinder viscometer of Zimm & Crothers (1962). The temperature was controlled at 23 °C by a constant temperature circulator. Intrinsic viscosity, $[\eta] = \lim_{C\to 0}[(\eta_{rel} - 1)/C]$, is reported, where C is the chromatin concentration in moles of nucleotide per liter (M) and η_{rel} is the relative viscosity.

Results

Effect of Mechanical Shearing and Sonication on Intrinsic Viscosity of Chromatin. The viscosity dependence of a polymer on its concentration can be expressed in terms of the following relation.

$$\frac{\eta_{sp}}{C} = \frac{\eta_{rel} - 1}{C} = [\eta] + K[\eta]^2 C \qquad (1)$$

where η_{rel}, η_{sp}, and $[\eta]$ are respectively relative, specific, and intrinsic viscosity of the polymer, C is the concentration, and K is a constant (Tanford, 1961).

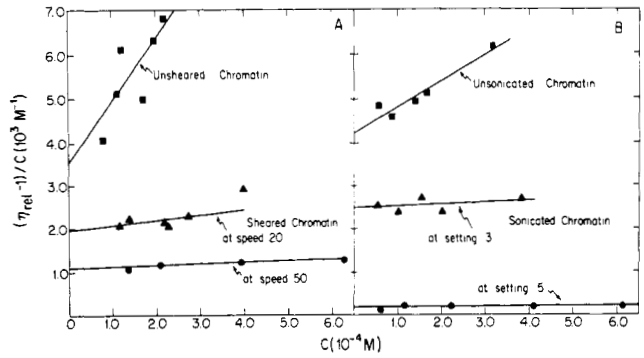

FIGURE 1: The viscosity of native, mechanically sheared, and sonicated chromatin. (A) Native unsheared chromatin (■—■). Chromatin sheared by Virtis homogenizer at speed 20 (▲—▲) or speed 50 (●—●). (B) Native unsonicated chromatin (■—■). Chromatin sonicated in a Sonifer cell disrupter at setting 3 (▲—▲) or setting 5 (●—●). The unit of $[\eta]$ in M^{-1} can be converted to dL/g by dividing the present values by 30.8.

The plots of eq 1 for native, mechanically sheared, and sonicated chromatin in 0.25 mM EDTA, and the intrinsic viscosity, $[\eta]$, determined from the intercept at zero concentration are shown in Figure 1. Figure 1A shows that mechanical shearing at speed 20 reduces the intrinsic viscosity from 3500 to 2000 M^{-1}, while at speed 50 the intrinsic viscosity was reduced further to 1100 M^{-1}. Figure 1B shows that sonication reduced the intrinsic viscosity of chromatin even more drastically than mechanical shearing. For instance, at setting 3 the intrinsic viscosity of native chromatin was reduced from 4200 to 2500 M^{-1}. It was reduced further to 200 by increasing the sonication setting to 5. At the top setting, 10, on the sonifier, the chromatin was sheared to an intrinsic viscosity too small to be measured reliably. The difference in intrinsic viscosity for the native chromatin was due to slight differences in preparation and also to nonhomogeneity in the chromatin solution. Shearing by either mechanical or sonicating methods basically cuts the large chromatin into smaller fragments.

Effect of Shearing on Thermal Denaturation and CD Properties of Chromatin. Previously Li et al. (1973) reported very minor variations in the derivative melting profiles of unsheared chromatin and sheared chromatin obtained either from the supernatant after a low-speed centrifugation or the pellet after a high-speed sucrose-gradient centrifugation. Since centrifugation would probably remove a portion of materials from the original chromatin, the physical properties measured from those sheared chromatins might not represent all the materials in the original chromatin. Therefore, no centrifugation was made on chromatin after mechanical shearing or sonication when the effects of shearing on chromatin were to be examined.

Figure 2A shows derivative melting profiles of unsheared chromatin and chromatin sheared at speed 20 or 50 and Figure 2B of unsonicated chromatin and chromatin sonicated at settings of 3 or 5. In order to avoid any variation in the conditions of melting, the three samples (native, setting A, and setting B) for either mechanically sheared or sonicated chromatin were simultaneously denatured in the instrument. Within experimental error, the detectable variations were very small: for mechanically sheared samples shearing caused a slight shift of hyperchromicity from the high temperature side of the profile, about 90–95 °C, to the lower temperature side, about 50–60 °C. As to be discussed below, this shift could be due to weakening of binding of a few percent of histones bound to the

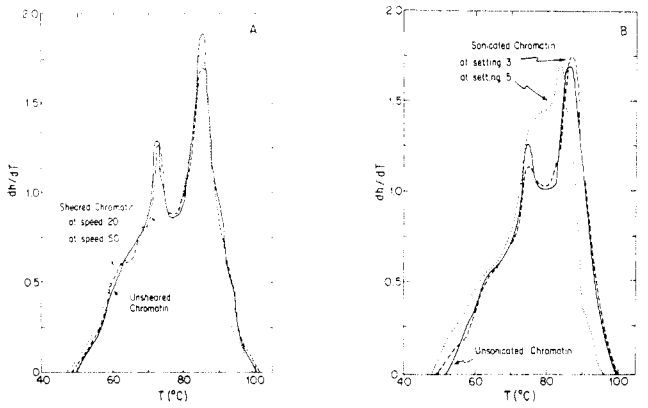

FIGURE 2: Derivative melting profiles of native, mechanically sheared, and sonicated chromatin. (A) Native unsheared chormatin (—). Chromatin sheared by Virtis homogenizer at speed 20 (----) or speed 50 (····). (B) Native unsonicated chromatin (—). Chromatin sonicated in a Sonifer cell disrupter at setting 3 (----) or setting 5 (····).

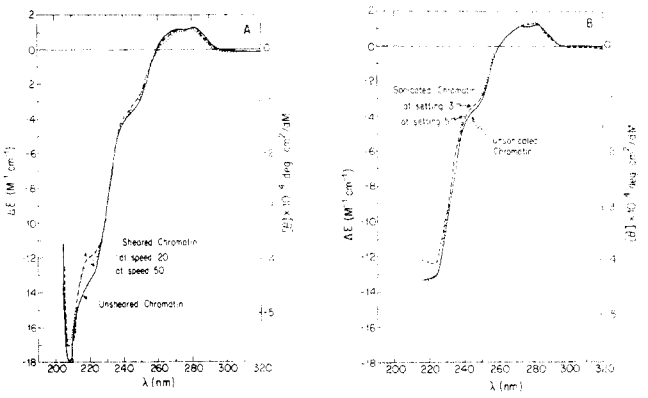

FIGURE 3: Circular dichroism spectra of native, mechanically sheared, and sonicated chromatin. Samples were those of Figure 2.

opening ends of sheared chromatin. Therefore, except for these very minor effects, mechanical shearing used for preparation of soluble chromatin does not alter chromatin structure as measured by melting profiles which are sensitive to the primary binding of the two halves of histone molecules to DNA in chromatin (Li & Bonner, 1971; Li et al., 1973).

Sonication of the chromatin at setting 3 caused very little perturbation on the melting profile of the chromatin and the small changes observed were similar to those of mechanical shearing. But at the higher setting of 5 a more substantial change was observed. The melting band at 86 °C was shifted to a band at 83 °C with a broad shoulder from 70 to 80 °C that is increased in amplitude over the 75 °C band for native chromatin. In addition to the above effect there is an increase in hyperchromicity at the lower temperature side, about 48–60 °C. This increase in perturbation of the structure could be due to the small fragment size of the sonicated chromatin (Figure 1B) as well as a breakage in the binding of some of the more basic region of the histones with the DNA resulting in a decrease in the 86 °C peak and a concomitant increase from 48 to 75 °C.

Although shearing does not alter melting profiles of chromatin, it might alter the conformation of either DNA or chromosomal proteins. To examine these effects, CD spectra of unsheared, mechanically sheared, and sonicated chromatins were measured. They are shown in Figure 3. Mechanical shearing (Figure 3A) and sonication (Figure 3B) caused only minor changes in the CD spectrum of chromatin (about 5–10%) both in the positive band near 275 nm for DNA and in the negative band near 220 nm for proteins. These changes are small considering variations in concentration determination and in baseline as well as in signal-to-noise ratio.

The results shown in Figure 3 differ greatly from those reported by Nicolini et al. (1976) and Miller et al. (1976). For instance, Nicolini et al. (1976) reported that mechanical shearing or sonication caused an increase of about 80% of the ellipticity at 275 nm of chromatin DNA, i.e., θ_{275}(sheared)/θ_{275}(unsheared) = 1.84. A summary of CD values of chromatin from the literature is presented in Table I in order to examine the differences among these reports. The critical parameters summarized include: (a) molar ellipticity $[\theta]_{280}$ and $[\theta]_{245}$, for chromatin DNA, and $[\theta]_{220}$ for chromatin proteins which are subject to errors in the determination of concentration; (b) ellipticity ratio, $[\theta]_{280}/-[\theta]_{245}$, which depends upon the shape and is not subject to possible errors in concentration determination; and (c) ellipticity ratio at 280 nm of chromatin to DNA, $[\theta]_{280}$(Chr)/$[\theta]_{280}$(DNA), which depends upon conformational distortion on DNA rendered by proteins.

As shown in Table I, all the CD parameters of unsheared, mechanically sheared, and sonicated calf thymus chromatin in this paper agree in all respects with those of sheared calf thymus chromatin reported in the literature. Variation in amplitude ($[\theta]_{280}$) was reported when chromatin from different origins was used, e.g., calf liver (Simpson & Sober, 1970) and chick embryo brain (Hjelm & Huang, 1974).

$[\theta]_{280}$ or $[\theta]_{276}$ reported by Nicolini et al. (1976) and by Miller et al. (1976) varied greatly among chromatins. For each chromatin they studied there was a great increase in ellipticity after shearing. Unfortunately the other CD parameters were not reported by these authors. For instance, it is impossible to examine from their papers whether or not the protein CD at 220 nm, $[\theta]_{220}$, and the shape of the CD spectrum of chromatin DNA as measured by $[\theta]_{270}/-[\theta]_{245}$ were changed after shearing. The CD ratio of chromatin to DNA, $[\theta]_{280}$(Chr)/$[\theta]_{280}$(DNA), was reported to be increased from 0.44 for unsheared chromatin to 0.80 for sheared chromatin (Miller et al., 1976). The 0.44 of their unsheared chromatin is close to 0.45–0.50 obtained from unsheared and sheared calf thymus chromatin, while the 0.80 from their sheared chromatin is much higher than all the other reported values (Table I).

More Stringent Test of Effects of Shearing on Chromatin Structure. Although the results in Figure 2 and 3 show no substantial changes in both absorption melting curves and CD spectra of chromatin at room temperature, the former measures only thermal stabilization of DNA against denaturation by the binding of proteins while the latter measures only the overall conformation of both DNA and proteins in the chromatin at room temperature. A more stringent test would be the examination of any conformational changes of DNA both in the native state (such as C → B transition) and in the helix–coil transition. The CD melting curve monitored at 220 nm, on the other hand, measures changes in secondary structure of chromosomal proteins either bound to/or dissociated from DNA during melting.

Figure 4a shows the CD melting curves (280 nm) of unsheared chromatin and chromatin sheared at speed 20 or 50. Within experimental errors they were essentially identical with one another with a major increase in rotational strength near 60 °C and a decrease after 70 °C. These curves are similar to those reported for sheared erythrocyte chromatin (Wilhelm et al., 1974) and oligomers of nucleosomes isolated by nuclease digestion of calf thymus nuclei (Mandel & Fasman, 1976). The agreement with the latter report is even more striking because

TABLE I: Circular Dichroism Parameters of Sheared and Unsheared Chromatin.

Chromatin	Shearing[b]	$[\theta]$ (10^3 deg cm^2 dmol^{-1}) 280 nm	245 nm	220 nm	$[\theta]_{280}/-[\theta]_{245}$[a]	$[\theta]_{280}$(Chr)/$[\theta]_{245}$(DNA)[a]	Reference
Calf thymus	Yes (M)	3.8	−11.5	−37.0	0.33	0.46	Shih & Fasman (1970)
Calf thymus	Yes (M)	4.3	−11.9	−42.0	0.36	0.53	Hensen & Walker (1970)
Calf thymus	Yes (M)	3.8	−12.0	−41.0	0.32	0.47	Johnson et al. (1972)
Calf thymus	Yes (M)	3.8	−9.5	−35.3	0.40	0.45	Chang & Li (1974)
Calf thymus	No	4.2	−11.6	−44.0	0.36	0.48	This paper
Calf thymus	Yes (M, speed 20)	4.2	−11.0	−39.0	0.38	0.48	This paper
Calf thymus	Yes (M, speed 50)	3.8	−11.4	−40.0	0.33	0.43	This paper
Calf thymus	No	4.0	−11.7	−42.9	0.34	0.46	This paper
Calf thymus	Yes (S, setting 3)	4.2	−10.7	−35.1	0.39	0.48	This paper
Calf thymus	Yes (S, setting 5)	4.2	−10.2	−41.6	0.41	0.48	This paper
Bovine thymus	Yes (M)	4.1	−11.5	NA	0.36	0.55	Bartley & Chalkley (1973)
Calf liver[c]	Yes (M)	5.5					Simpson & Sober (1970)
Chick embryo brain[c]	Yes (M)	5.2					Hjelm & Huang (1974)
AF-8 cells[d] (39 °C)	No	2.0	NA	NA	NA	NA	Nicolini et al. (1976)
AF-8 cells[d] (39 °C)	Yes (M)	3.4	NA	NA	NA	NA	Nicolini et al. (1976)
AF-8 cells[d] (34 °C)	No	3.0	NA	NA	NA	NA	Nicolini et al. (1976)
H 615 cells[d]	No	3.6 (276 nm)	NA	NA	NA	0.44	Miller et al. (1976)
H 615 cells[d]	Yes (S)	7.1 (276 nm)	NA	NA	NA	0.80	Miller et al. (1976)

[a] $[\theta]$ in deg cm^2 dmol^{-1} = 3300 $\Delta\epsilon$ in M^{-1} cm^{-1}. Molar ellipticity $[\theta]$ is used in this table because the majority of the original reports used $[\theta]$. [b] M, mechanical shearing (Virtis homogenizer or Waring blender). S, sonication. [c] $[\theta]$ with $\lambda < 250$ nm was reported based upon protein concentration which was not used for comparison in this table because $[\theta]$ given here was calculated against nucleotide concentration. [d] NA, data not available.

these authors used calf thymus as we did in this report and also used a procedure suggested by Noll et al. (1975) to prepare soluble chromatin, i.e., nuclease digestion of nuclei. Figure 4a also shows that shearing does not affect the renaturation properties of chromatin CD monitored at 280 nm.

Denaturation curves of chromatin proteins as monitored by chromatin CD at 220 nm (Figure 4b) were essentially the same for both unsheared and sheared chromatin at either speed 20 or 50, with a major denaturation occurring between 50 and 60 °C and a minor one between 65 and 75 °C. The portion of secondary structures which had been denatured could not be renatured as the temperature was lowered. Therefore, shearing used for preparation of soluble chromatin does not alter the denaturation properties of chromatin proteins.

Doenecke & McCarthy (1976) demonstrated that mechanical shearing caused sliding of histones along DNA. Although no quantitative data were given with regard to percent of histones involved in such movement, their observations suggested that histone binding to DNA could be weakened by shearing which led to histone movement.

To study histone movement caused by shearing, thermal denaturation was used. As reported earlier (Polacow et al., 1976), this method is sensitive in detecting the transport of histones from one DNA to another whenever these two DNAs have different G + C contents. If the binding of histones to DNA is indeed weakened by shearing, thermodynamically, these histones could become available for binding to an exogenous DNA added to the chromatin solution. To examine this transport of histones during shearing, an equimolar mixture of *Cl. perfringens* DNA and native calf thymus chromatin was used as the control. Portions of this mixed solution were then mechanically sheared at speed 20 or 50 or sonicated at settings of 3 or 5. These three samples (either the set of mechanically sheared or sonicated) were thermally denatured in the instrument at the same time to minimize experimental errors. This extra caution is important since the effect we expected to observe could be small.

Figure 5 shows typical results of the above experiments for

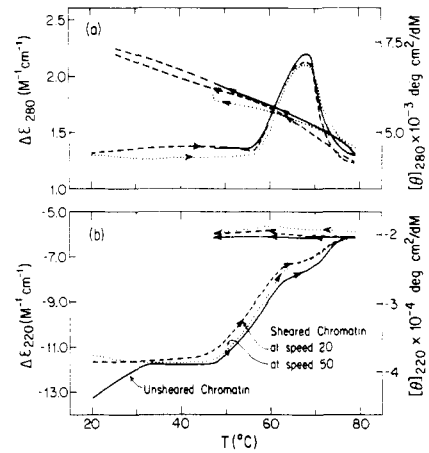

FIGURE 4: Thermal denaturation and renaturation profiles of native and sheared chromatin monitored by circular dichroism. Measured at 280 nm (a) and at 220 nm (b). Samples were those of Figure 2a.

mechanically sheared and sonicated mixtures. The control for both showed a melting band at 40 °C corresponding to *Cl. perfringens* DNA and the melting profile of unsheared calf thymus chromatin. Mechanical shearing of this mixed solution (Figure 5A) caused a slight reduction of the *Cl. perfringens* band at 40 °C as well as of the hyperchromicity at higher temperatures (above 90 °C) with a concomitant increase of the hyperchromicity from 50 to 70 °C. These changes suggest a shift of a small amount of histones from calf thymus chromatin to *Cl. perfringens* DNA, i.e., a slight decrease of free *Cl. perfringens* DNA (at 40 °C) and a slight increase of free calf thymus DNA (about 50 °C). Based upon the hyperchromicity changes at 50–60 °C, obtained from three experiments, it was observed that shearing at speeds 20 or 50, 5 or 8% of histones originally bound to calf thymus DNA became loosened and then bound to *Cl. perfringens* DNA. However, it is stressed that, in an ordinary preparation of sheared chromatin, the amount of histones released from the chromatin should be

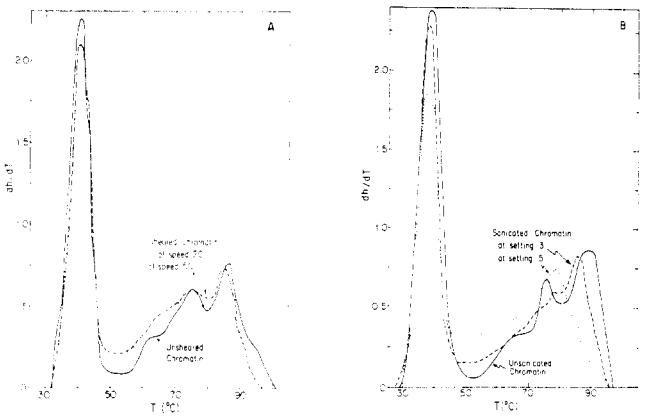

FIGURE 5: Effect of shearing on derivative melting profiles of a mixture of calf thymus chromatin and *Cl. perfringens* DNA. (A) Control mixture with native unsheared chromatin (--) and the mixture sheared at speed 20 (----) or speed 50 (····). (B) Control mixture with native unsonicated chromatin (—) and the mixture sonicated at setting 3 (----) or setting 5 (····).

lower than the value of 5–8% because thermodynamically the presence of *Cl. perfringens* DNA provides an additional driving force to facilitate histone dissociation. In accordance with this prediction, Figure 2 shows a smaller effect on the melting profile of chromatin when *Cl. perfringens* DNA was absent during shearing.

As shown in Figure 5B, sonication of the *Cl. perfringens* DNA and chromatin mixture at setting 3 gave similar results to the mechanical shearing, but at the higher setting of 5 the high melting band of the chromatin at about 90 °C was lost and the hyperchromicity from 50 to 70 °C was increased as before. From hyperchromicity changes at 50–60 °C obtained from four experiments, it was found that sonication at settings 3 and 5 gave respectively histone exchanges of 3 and 11%. The difference in amount of histone exchange can be directly related to the amount of shearing (Figure 1); e.g., more shearing either mechanically or by sonication results in more histone exchange, less shearing results in less histone exchange.

Discussion

Mechanically sheared chromatin has been used by many laboratories in the studies of chromatin structure and function. If shearing primarily cuts a large chromatin into smaller fragments for the convenience of experimental manipulation, the results and conclusions obtained from sheared chromatin legitimately can be applied to native chromatin. However, if severe structural changes on chromatin occurred during shearing, those experiments dealing with structural properties of sheared chromatin should be reevaluated.

As reported in this paper, under mild conditions of mechanical shearing (using a Virtis homogenizer, for example), the chromatin structure is not significantly changed (within 10%) with respect to (a) the binding of histone to DNA (Figure 2A), (b) the conformation of both DNA and histones (Figure 3A), (c) the detailed temperature dependence of the conformation of both DNA and proteins (Figure 4), and (d) the histone redistribution (Figure 5A). Therefore, if 5–10% errors are allowed in experimentation, which is quite reasonable for a complex system like chromatin, the above properties can be considered unchanged by shearing and that previous studies on sheared chromatin are still valid. Sonication of chromatin at low settings (as 3 on the sonifier used in this study) caused only small changes in the chromatin structure as monitored by the methods used above (Figures 2B, 3B, and 5B). But when the setting was increased and the chromatin sheared to smaller fragments, histone binding to DNA was changed significantly (Figure 2B). Therefore at lower sonication settings the change is not significant but at higher settings more perturbation is observed.

The 5–10% perturbation of chromatin structure as observed in this report could be a direct consequence of fragmentation of chromatin. If a chromatin is cut into n fragments, the number of free ends will be increased from 2 to $2n$. Due to Brownian motion, both DNA base pairs and histones close to the free ends could have properties slightly different from the central regions. For instance, a slight DNA unwinding and a weaker binding affinity between histones and DNA could probably happen near those ends such that their structural properties in the fragmented state deviate from those in the original unsheared state. Such deviation would then depend upon the extent of fragmentation, the smaller the fragments the greater the effect. If such effects are natural consequences of fragmentation of chromatin, they are expected to occur when the fragmentation of chromatin is made by mechanical shearing, sonication, or nuclease digestion.

The above conclusions are applicable to structural determination wherein the averaged properties of a large population of chromatin fragments are measured; ordinarily a variation of 5–10% in structural properties is not considered to be large. On the other hand, when a specific sequence of DNA is dealt with, such as transcription of a particular gene, the 5–10% perturbation in the overall population of chromatin fragments could be severe if it occurred selectively on those specific regions. Therefore, our observations and our view on shearing are compatible with those of Doenecke & McCarthy (1976).

It is possible that the cut of chromatin made by shearing is more random while nuclease digestion is more specific. For instance, there might be a great difference in susceptibility of a DNA sequence toward nuclease digestion depending upon whether it is free or bound by an octamer of histones, histone H1, or nonhistone proteins. Thus the enzyme can cut certain regions more frequently than the others and preserves the same arrangements of histones in chromatin fragments. On the other hand, if shearing occurs more randomly, it may cut a DNA bound by a histone octamer, by histone H1 or nonhistone proteins or other regions. According to the thermal denaturation results (Figure 2B), it seems that the more basic regions of histones are affected more, especially by sonication at high settings. Therefore, it is likely that the fragments obtained by shearing are more heterogeneous with respect to histone arrangement than those fragments obtained by nuclease digestion. Thus the above discussion explains the observations of Noll et al. (1975). It is emphasized, however, that some physical properties of chromatin may be sensitive to homogeneity in histone distribution in individual chromatin fragments, e.g., discrete fragments of DNA protected against nuclease digestion, while the others may not be, e.g., binding of the two halves of histones to DNA, length of DNA bound by histones and conformation of histone and conformations of DNA in chromatin fragments (Li et al., 1973; Li, 1975). One has to consider these differences before a general statement about the validity of using sheared chromatin for structural studies of chromatin is made.

Nicolini et al. (1976) have reported severe changes in the CD spectra of sonicated chromatin isolated from AF-8 cells. Miller et al. (1976), while using sonicated chromatin isolated from H 615 cells, saw changes in CD and thermal denaturation of this chromatin upon sonication. These conclusions, however, could not be reproduced in our studies of calf thymus chro-

matin, except that thermal denaturation of chromatin sonicated to a high extent showed substantial changes in the melting profile. These differences could possibly be caused by the use of a different type of sonifier, the extent of sonication, or the type of chromatin used. For example, the unsheared chromatin used was reported to have a very strong light scattering. They, therefore, had to correct for this in their determination of concentration. Such corrections were not applied in our experiments since light scattering from our unsheared chromatin was minimal ($A_{320}/A_{260} = 0.03$). This difference in light scattering could contribute to the differences in CD observed in their report and ours.

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