Volume 5 Number 2 February 1978 Nucleic Acids Research

Superstructure and CD spectrum as probes of chromatin integrity

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Received 22 November 1977

ABSTRACT

Two types of chromatin were extracted from the same stock of rat liver nuclei by a short exposure to micrococcal nuclease and by shearing respectively. These two materials which are identical in their protein/DNA content and by the presence of the five histones, were compared by means of circular dichroïsm and electron microscopy. Under the electron microscope and in absence of any divalent cation a superstructure of the unfixed chromatin fiber can be viewed only with native material but is no more present in sheared one. The increase of CD signal at 280 nm (from 2000 to about 4000 cm^2 $deg.dmole^{-1}$) in the case of sheared chromatin is not related to the loss of superstructure but to the structural changes of DNA inside the nucleosomal core which are always produced by shearing. These two correlated observations offer new sensitive probes of the integrity of any native or reconstituted chromatin.

INTRODUCTION

Since the proposition of the subunit structure of chromatin (1, 2,3) a number of reports have been made pointing out the differences between the unsheared and sheared chromatin. In general, the authors are of the opinion that the shearing of chromatin causes significant changes in its biological and physical properties. These modifications mostly include the loss of repeating unit structure (4), change in the template activity (5,6), alteration of chromatin conformation as measured by circular dichroïsm (7,8,9,10), thermal denaturation (11) and by accessibility of ethidium bromide intercalation sites (12).

Unfortunately, it is difficult to correlate all the data in the literature. Authors have used the term "unsheared chromatin" for preparations in which shearing forces were necessary to solubilize the nuclear material (10,14). On the other hand shearing was performed on partially digested chromatin (13) but as we have shown (results not presented) shearing is ineffective when applied to too short segments of chromatin. More-

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over, no direct and systematic comparison of the physical properties has yet been made between the unsheared and sheared chromatin prepared from the same stock of nuclei. Recent electron microscopic studies (15,16,17) show that a higher order structure is present in chromatin prepared by short exposure of nuclei to micrococcal nuclease. No electron microscopic comparison between sheared and unsheared chromatin has yet been made.

In the present work the nuclease digested chromatin and the sheared chromatin which have been prepared in a standard way are extracted from the same stock of rat liver nuclei. Both preparations characterized by the same protein/DNA ratio and the presence of all five histones, are then compared by using several and biophysical assays : DNA digestion pattern, thermal denaturation, circular dichroism and electron microscopy.

The results demonstrate that the presence of higher order structure or superstructure (15) as chiefly seen by electron microscopy is strongly dependent upon the mode of preparation of the chromatin samples rather than upon the presence of divalent cations. The contribution of the superstructure to the CD signal is negligible. However, the disappearance of the superstructure which is observed in the case of sheared chromatin is accompanied by a structural alteration at the nucleosomal level detected both by Micrococcal nuclease digestion and circular dichroism.
In this connection, circular dichroïsm, as related to the integrity of the nucleosomes, can be used as a probe of the native state of the chromatin.

EXPERIMENTAL PROCEDURES

A. Chromatin preparation

For the present study nuclei were isolated from rat liver as described by Hewish and Burgoyne (1). The nuclei suspension was checked for cytoplasmic contamination and the integrity of the nuclear membrane was examined under an optical microscope. The stock nuclei suspension was then divided into two equal parts :

(i) one part was used for the extraction of unsheared chromatin according to the procedure of Noll (3,4) with the use of micrococcal nuclease (0.3 unit/1 OD_{260} unit of lysed nuclei for 1 mn at 37°C). About 100 OD_{260} units of unsheared material were recovered in the lysis medium (0.2 mM EDTA 0.2 mM PMSF pH 7) corresponding to 40 to 50 percent of the total nuclear DNA content. Acid soluble DNA was determined by the addition of perchloric acid to 0,8 N and NaCl to 0.8 M followed by 15 min in ice

and centrifugation for 15 min at 3000 g. The absorbance at 260 nm of the supernatant was measured and multiplied by 0.60 to correct for the hyperchromic effect. Under these conditions, long fragments having an average length of about 50 nucleosomes with a range roughly from 10 - 200 nucleosomes were obtained from 1% digested nuclei. This chromatin sample will be referred to as native unsheared chromatin in the following text.

(ii) The second part of the stock nuclei was used to prepare sheared chromatin by a stepwise reduction in ionic strength (18). The nuclei pellet was first washed in 0.25 M NaCl, 25 mM EDTA, pH 6 and then it was transferred, step by step, to lower molarity of Tris-HCl buffer, pH 7.4, from 50 to 0.5 mM. Homogenization at each step was realized by a Dounce homogenizer. Finally, the chromatin pellet was dissolved in 0.5 mM Tris HCl, pH 7.4 to form a gel. The recovery was 80% in DNA of the starting nuclei. This sample is referred to as sheared chromatin.

B. Analysis

DNA concentration was determined from the absorbance value, using the molar extinction coefficient per nucleotide residue of 6600 M^{-1} cm^{-1} at 260 nm.

Protein was estimated by the procedure of Lowry (19). The method was calibrated with a preparation of whole histone.

SDS-gel electrophoresis for histones was carried out according to the method of Weintraub (21).

For DNA extraction, the samples (10 to 20 OD_{260} units) were incubated (1 h 37°C) with 100 µg/ml of Proteinase K (E. Merck) in 0.4% SDS, 0.8 M NaCl, 0.5 M EDTA. The solution was then extracted with one volume of buffered phenol and two volumes of chloroform isoamyl alcool (24:1) mixture. DNA in the aqueous phase was precipitated with two volumes of absolute ethanol (overnight, -20°C), collected by centrifugation and redissolved in the appropriate medium. DNA electrophoresis was performed on 2.5% acrylamide, 0.5% agarose gels as described by Loenig (20). Running buffer was 40 mM Tris-HCl (pH 7.8) 20 mM sodium acetate, 2 mM EDTA. Bromophenol blue was used as markers indicating the extent of migration. Gels were stained in ethidium bromide (1 µg/ml) in running buffer overnight and destained for 1 hour in the same buffer. Gels were photographed under ultra violet light at 254 nm through an orange filter (Kodak).

C. Physical measurements

For absorption melting curves, circular dichroïsm (CD) measure-

ments and CD melting curves, the chromatin samples were transferred to 1 mM phosphate buffer, 0.2 mM EDTA (pH 7.0) by overnight dialysis.

Absorbance melting curves were recorded at 260 nm as previously described (22).

Circular dichroïsm spectra were recorded at 20°C with a Roussel-Jouan DC III in 1 cm thermostated quartz cell. For CD melting curves a special jacketed cell (1 cm pathlength) was used. Temperature was recorded with a platinum resistance connected to a digital thermometer the signal of which was applied to the X-way of a Sefram TGM 164 X-Y recorder. The heating rate was 0.2°/mn. Maximum absorbance of the samples was never higher than 1.0 OD_{260} unit. The results are presented in terms of molar ellipticities, $|\theta|$, based on the molar nucleotide concentration.

D. Electron microscopy

Carbon coated grids (400 Mesh) were positively charged according to Dubochet's technique (23). A 50 µl drop of unfixed chromatin solution (made 0.5 µg/ml with Tris 10 mM, pH 7.4) was deposited on a parafilm sheet and immediately covered with the grid. After 5 minutes adsorption, the specimen was positively stained with a 50 µl drop of distilled water (30 sec.), air dried and finally rotary shadowed with platinum at an angle of 7°. Grids were examined in a Siemens 101 Elmiskop. Calibration was checked with the diffraction grating replicas photographed after each series of measurements. Yield of adsorption of the positively charged carbon film was routinely checked by counting the number of SV 40 form I DNA/μm^2, spread under the same conditions as the chromatin samples (17).

RESULTS

In the present investigation, we carried out all the measurements on five sets of independent preparations. For the sake of better clarity, we shall describe the results of a particular set, pointing out the differences, if any, with that of the other sets of measurements. The U.V spectra of the chromatin preparations -both native and sheared- exhibited very little light scattering effect up to OD_{260} value of 20. The A_{230}/A_{260} ratio was always in the range of 0.74 - 0.78 which was in good agreement with the protein/DNA ratio estimated by Lowry's procedure. In all the cases, the protein/DNA ratio was around 1.6 for both chromatin samples and the gel electrophoresis patterns of native and sheared chromatin indicates the presence of all of the five histones in both preparations (data not shown).

In order to find a criterion in solution of the native state of chromatin, CD measurements were carried out to examine the organisation of DNA inside the native and sheared chromatin preparations. Figure 1 shows that there is a significant difference between the CD spectra of the two chromatin samples. The spectrum for native chromatin exhibits two well resolved peaks at 282 and 272 nm in the positive band which are absent in the case of sheared chromatin. The maximum molar ellipticities value at 280 nm increases from 2085 for native chromatin to around 4000 for sheared one, still much lower than the corresponding value for DNA (8000). A small negative peak at 295 nm is observed in the spectrum of native chromatin which is highly reproducible but the corresponding spectrum of sheared chromatin does not exhibit any signal in this region. The absolute magnitude of the large negative peak at 227 nm is lower for sheared chromatin than for the native one.

In addition to the CD spectra, we also recorded the variation of $|\theta|_{280}$ with temperature for the two chromatin preparations and also for the control DNA. These CD melting curves as shown in figure 2 can be divided into three parts :

(i) "Premelting" change - Sheared chromatin shows a slight "premelting" effect in the temperature range of 45-55°C which is completely absent in the case of native nuclease digested chromatin.

(ii) Cooperative transition - Both the chromatin preparations exibit a cooperative increase in molar ellipticity in the temperature range of 65-80°C. This process is less cooperative in the case of sheared chromatin than the native one having the midpoints of transition at the temperature of 68° and 74°C respectively. The temperature at which the molar ellipticity value reaches its maximum are 78°C and 81°C for sheared and native chromatin respectively.

(iii) Helix-coil transition - At the end of the cooperative transition a sharp decrease in molar ellipticity upto about 90°C is observed which involves the helix-coil transition of DNA inside the nucleosome. All the results of CD measurements have been summarized in table I in which are also given the data relative to OD melting.

Figure 3 displays the electron micrographs of the sheared chromatin samples, which show an irregular distribution of nucleosomes of 125 Å diameter, leaving frequently some free DNA like regions. No quantitative estimation of the amount of free DNA was performed because of the complexity of the spread molecules. However, in some cases (shown by arrows in

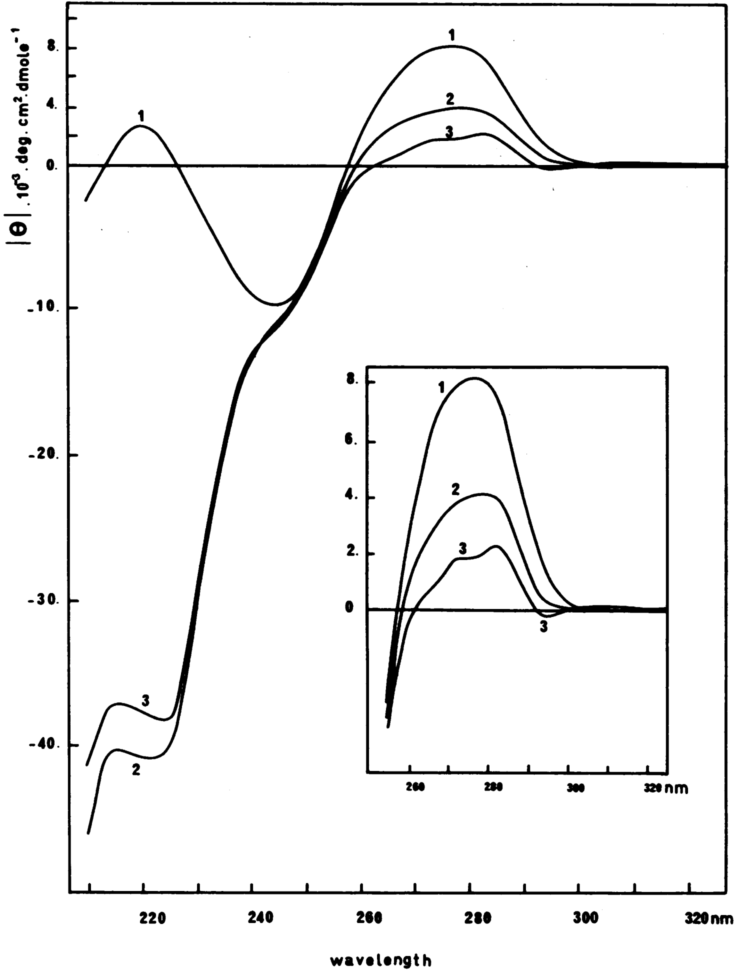

Figure 1 - Circular dichroism spectra of Rat liver DNA (1), sheared chromatin (2) and native chromatin (3). The solvent was 1 mM phosphate buffer, 0.2 mM EDTA pH 7.0. The inset shows the positive part of the spectra on a magnified scale.

figure 3b) loops of nucleosomal DNA are observed outside a dense core. In a larger field of the grid (fig. 3d) the same qualitative nature of the micrographs is retained.

In contrast to the sheared chromatin the electron micrographs of the native preparation (fig. 4 - a - e) show an organized structure of closely packed nucleosomes. The chromatin superstructure, which is completely absent in the case of the sheared samples can be described, in our

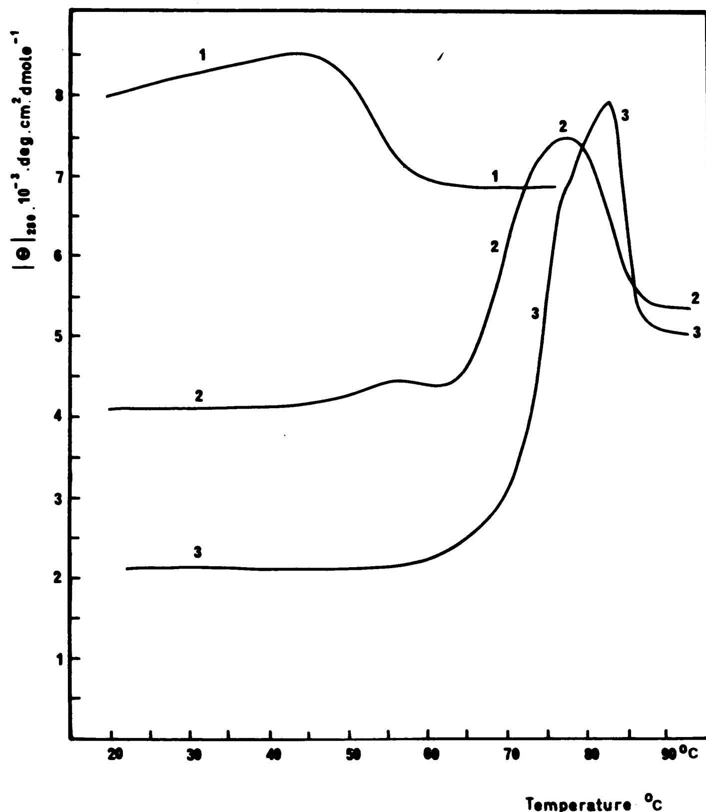

Figure 2 - C.D. melting profiles as monitored by $|\theta|$ 280 nm of Rat liver DNA (1), sheared chromatin (2) and native chromatin (3). The solvent is the same as mentioned in fig. 1.

Table 1

Physical parameters of native and sheared chromatin and rat liver DNA

Sample	A_{230}/A_{260}	Protein/DNA	Tm °C	Hyperchromicity %	[a] $\lvert\theta\rvert^{20°C}_{280nm}$	[b] $\lvert\theta\rvert^{max,t°}_{280nm}$	[c] $\Delta\lvert\theta\rvert^{t-20°}_{280nm}$
Native Chromatin	0.76±0.01	1.65	81.3±0.2	42%±0.5	[d] 2085±200	7825±200 (81°)	5740±200
Sheared Chromatin	0.73	1.67	77.5	45.6%	[e] 4050	7345 (78°)	3295
Rat liver DNA	0.41	< 0.01	51.7	41%	8000	8500 (48°)	500

Ellipticity values are expressed in degree.cm^2.decimole^{-1}. (a) $|\theta|^{20°}_{280nm}$: Molar ellipticity measured at 280 nm at 20°C - (b) $|\theta|^{max,t}_{280nm}$: maximum molar ellipticity attained at temperature t°C and at 280 nm - (c) $\Delta|\theta|^{t-20}_{280nm}$: difference between $|\theta|^{max,t}_{280nm}$ and $|\theta|^{20°}_{280}$ - The standard deviation of $|\theta|^{20°C}_{280nm}$ among five different sets of independent preparations is : (d) : ± 150 and (e) ± 250

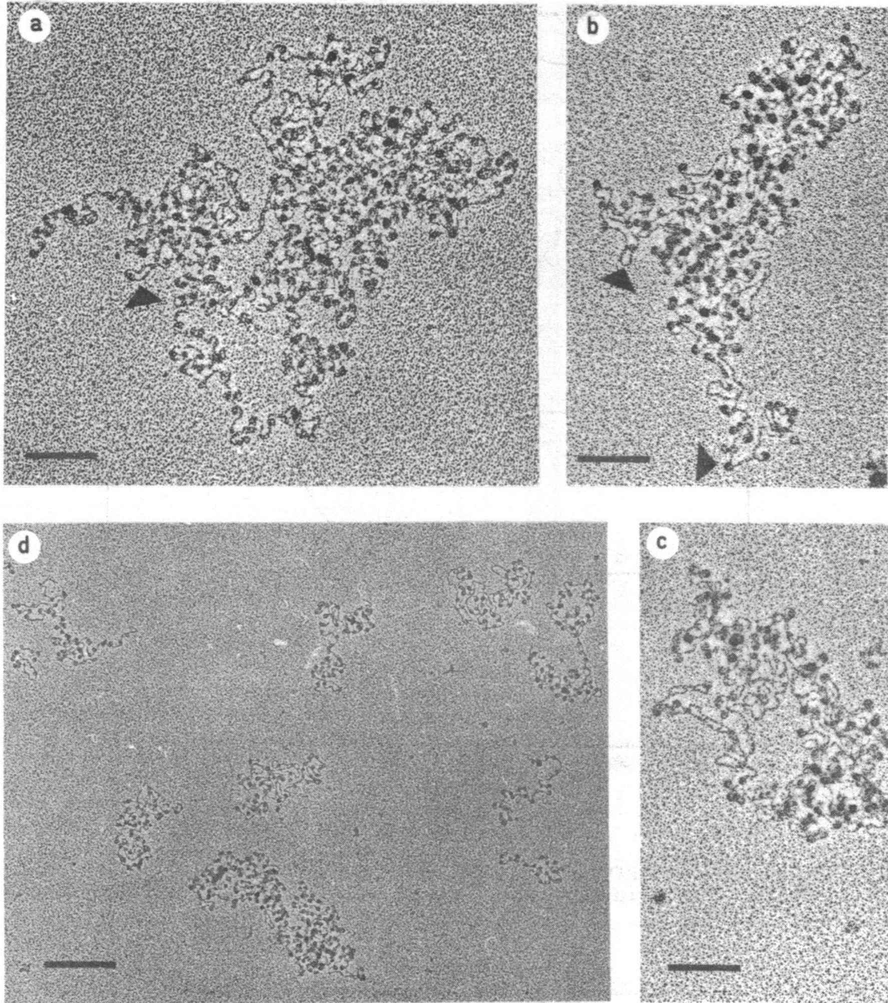

Figure 3 - Electron micrographs of sheared chromatin. The samples, for electron microscopy were prepared as mentioned in Materials and Methods. The bar represents 1000 Å in fig. 3a, b, c, and 2000 Å in fig. 3d.

conditions, as a fiber of regular width of 340 Å, composed of successive rows of nucleosomes in close contact. In spite of the two dimensional representation of the chromatin structure on the carbon surface of the grid, an helicoïdal arrangement of the nucleosomes seems to be the most probable configuration (figs 4a,b) as recently proposed by Finch and Klug but in the presence of 0.2 mM Mg^{++} (15). We have measured by atomic absorption spectroscopy the concentration of Mg^{++} and Ca^{++} ions in our chromatin sam-

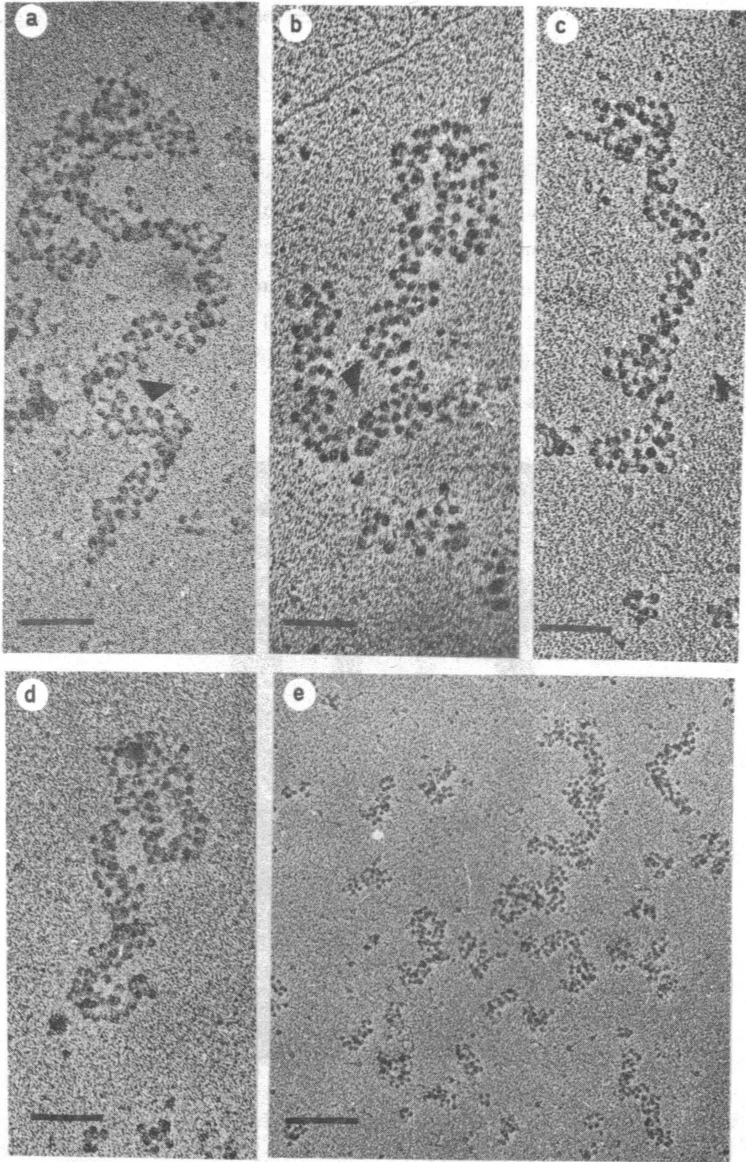

Figure 4 - Electron micrographs of native chromatin. Experimental conditions are the same as mentioned in fig. 3. The bar represents 1000 Å in figure 4a, b, c, d and 2000 Å in fig. 4e.

ples, and it was found to be less than 10^{-6} M. The chromatin superstructure is not therefore correlated with the presence of divalent cations but simply reflects the absence of any shearing forces during the preparation.

A detailed electron microscopic study of the chromatin superstructure will be published elsewhere (17).

Finally, as a control experiment, comparison of the state of chromatin in the nuclei with that in the native and sheared preparations was made by the partial digestion experiment with micrococcal nuclease. DNA electrophoresis pattern as presented in Fig. 5 clearly shows that on further digestion with micrococcal nuclease native chromatin gives the same pattern of bands as obtained with the digestion of the nuclei whereas sheared chromatin does not give any band. This loss of periodicity in sheared chromatin is consistent with the original finding of Noll (4) and also with our electron microscopic results.

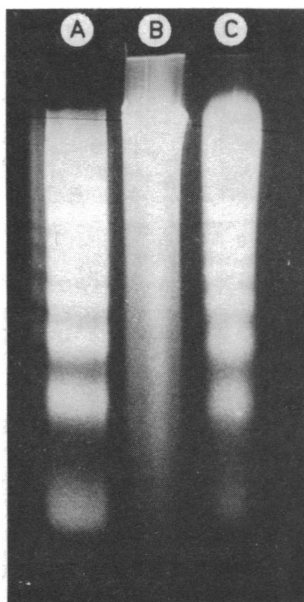

Figure 5 - Gel electrophoresis of DNA extracted from the digest of native chromatin (A), sheared chromatin (B) and nuclei (C). In the three cases the percentage of digestion is 3% (see Materials and Methods).

DISCUSSION

Since the original finding of Noll et al. (4) it is generally accepted that the chromatin prepared by a short exposure of nuclei to micrococcal nuclease closely resembles the chromatin in nuclei as judged by the nuclease digestion pattern.

1. The electron microscopic results presented in this work clearly demonstrate the preservation of a higher order structure in long fragments of native unfixed chromatin in the absence of any divalent cation in the buffer. Similar results have been recently published by Thoma and Koller (24). It is unlikely that the aspect and the diameter of the fibers presented in this study (fig. 4) are dependent upon the spreading method since the same results were obtained under the same conditions of solvant by freeze etching or freeze drying experiments (data not shown). It appears from our study that the presence or not of a superstructure is primarily correlated to the mode of fragmentation used to solubilize the chromatin.

2. The molar ellipticity value of this chromatin when measured in 1 mM phosphate buffer (or in 10 mM Tris buffer, i.e. the same medium as used in electron microscopy), is always around 2000 ± 200 degrees cm^2 $decimole^{-1}$. The same results were obtained with chicken erythrocyte chromatin and chicken liver chromatin prepared by nuclease digestion. When this value is compared with that of the mononucleosome, which is about 1850 ± 200 degrees cm^2 $decimole^{-1}$ (14,26,27) it appears that at room temperature the superstructure of chromatin does not contribute (or only in a negligible way) to the CD signal. An opposite conclusion was drawn by Mandel and Fasman (14). This contradiction can easily be explained since the contribution attributed by these authors to the superstructure was calculated by substracting the value of the mononucleosome to the molar ellipticity value of a sheared sample (i.e. ∿ 4000 degrees cm^2 $decimole^{-1}$ as we have found in our study). In line with this, the cooperative increase in ellipticity as observed in CD melting curves, is correlated with the unfolding of nucleosomes but not with the breakup of superstructure since the same pattern is observed with sheared chromatin (fig. 3). Actually, the higher ellipticity of sheared chromatin can be readily explained by the existence of free DNA-like regions for which ellipticity values are much higher and close to that of the B-form. Many experimental evidence support this interpretation :

a) the appearance of a premelting region in CD melting profile (fig. 2) as well as the decreased thermal stability (table I) is indicative of free DNA like regions in sheared chromatin.

b) The small DNA loops embracing the nucleosome which sometimes are visible in electron micrographs of sheared chromatin and the irregular distribution of nucleosomes separated by long DNA spacers (fig. 3) indicative that the tightly coiled DNA regions around the histone core might

be partially stripped off.

However, free DNA-like regions are too long to be considered as only made up with the so-called "linkers" i.e. the 60 base pair regions of DNA which are not involved in the building of the core particle (30). In line with this observation, CD measurements offer another interesting clue. The two closely spaced but well resolved peaks at 272 and 282 nm and the small negative one at 295 nm which are observed as well with mononucleosomes (26,27) and core particles (29) are no more present in sheared chromatin. Since they can be considered as a characteristic feature of DNA-histone structure inside the core particle, their disappearance is a qualitative but clear indication of a modification in the interactions between DNA and histone octamer.

It is interesting to note that native chromatin exibits discrete bands in DNA gel when digested with micrococcal nuclease (28) whereas sheared chromatin does not give any band. Most probable reason of it is that the changes at the nucleosomal level destroys the unique structure of the cleavage sites which makes the template unsuitable for recognition by this enzyme. One is thus led to the conclusion that during the shearing process, a structural rearrangement of the nucleosome takes place, related likely to the partial dissociation of histones followed by their unspecific reassociation and to the sliding of nucleosomes along the DNA (25).

Beside the classical characterizations of chromatin, i.e. presence of nucleosomes under the electron microscope and digestion pattern of micrococcal nuclease, it appears from the present work that the existence of a superstructure (without adding divalent cations or fixing reagent) and the qualitative and quantitative aspect of CD spectrum can be considered so far as the most sensitive probes of a chromatin preparation. Though CD spectrum of DNA appears to be insensitive to its tertiary structure inside chromatin, we have shown that any shearing process which affects the superstructure will also modify the CD spectrum, because any disruption of superstructure by shearing forces is always accompanied by structural modifications of the nucleosomal core.

These two experiments are therefore well reflecting the integrity of chromatin and would have to be used systematically as a control of native or reconstituted material.

A preliminary account of this study has been presented in the IIIrd CMEA Symposium on Structural and Functional aspects of protein - nucleic acid interactions. September 3-9, 1977, Brno, Tchecoslovaquia.

ACKNOWLEDGEMENTS

The authors are grateful to Drs J. Pouyet and J. Neimark for their help in CD and OD melting curves and to Dr M. Champagne for critical reading of the manuscript. They are also thankful to Dr M.T. Strosser and Mrs D. Buhr for their helpful cooperation. One of the authors (G.C.D.) is indebted to the French Ministry of Foreign Affairs for offering him a postdoctoral fellowship.

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