Molecular Arrangement of DNA in Nucleohistone

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Deoxyribonucleic Acid (DNA), Nucleohistone, Shear Orientation (q), Formaldehyde Reaction, Dichroism, Polarization and Rotational Diffusion

Several optical and hydrodynamic properties of purified nucleohistone have indicated that the secondary structure of DNA in nucleohistone is slightly different both from the structures of native and denatured DNA in solution. It has been suggested that while histones stabilize the overall DNA-structure they incorporate into the latter some denaturation defects.

Rotational diffusion studies of acridine-orange complexes of whole and dehistonized nucleohistones have indicated that nucleohistone should contain a single continuous DNA molecule. The contour length of the DNA molecule would be about 2 to 3 times the end-to-end distance of nucleohistone.

Polarized fluorescence microscopy and linear dichroism studies of whole nucleohistone have given an average estimate (about 30% of the total DNA) of oriented DNA in nucleohistone. The degree and the direction of maximum polarization of fluorescence from the dye complexes of whole and partial (*i. e.* partially dissociated) nucleohistones have been studied. From these studies, it is proposed that no single supercoiled arrangement of the DNA in nucleohistone is tenable.

The combined results of all these studies suggest that the basic nucleohistone molecule contains coiled and extended DNA regions. The proportion of the lengths of the DNA constrained in the coiled and the extended regions is about 7:3. Only two probable models or classes have this quantitative feature of the DNA-arrangement and have been discussed.

1. Introduction

Several direct and indirect studies have indicated distinctive features which imply structural alterations of DNA in purified cell free nucleohistone [1–9]. These conformational features have been characterized differently by various authors [5–9] but they have not been related to the features of the DNA-packing in nucleohistone. Physicochemical, optical and hydrodynamic properties of purified nucleohistone in suspension have suggested that the DNA of nucleohistone is combined with an equal weight of different classes of histones to form a fibrous structure of intermediate length [10–13]. Also, on association with histones the number of DNA base pairs perpendicular to the axis of the molecule decreased by about 60%. More recently, evidence of DNA-supercoils in nucleohistone has been obtained from low angle x-ray scattering studies (Parton *et al.* [4], Bram and Ris [14]). Much less is known concerning the architecture of DNA in chromatin *in situ* in cells. Under electron microscope the chromatin appears as fibrils and the fibrils themselves appear to be much less than the length of DNA in them [14–17]. Various models have been proposed, such as DNA parallel to the chromatin fiber [17, 18], DNA coiled into a superhelix [19] and DNA parallel to the chromatin fiber with frequent side loops [20]. At the present time, it is difficult to decide whether the DNA-arrangement in purified nucleohistone is the same as that of chromatin fiber *in situ* because the DNA arrangement in neither case (*i. e. in vitro* and *in situ*) has been well established.

In taking up the aforesaid problem, the present author has characterized the conformation (*i. e.* secondary structure and the length of the phosphate backbone) of the DNA in nucleohistone by two techniques, namely, spectrophotometry and thermal depolarization of fluorescence. In the first technique formaldehyde has been used to test any denaturation defect in the DNA of nucleohistone. Formaldehyde at any concentration in the range 1 – 10% (w/v) reacts very fast at room temperature with denatured DNA. Spectroscopically, this reaction indicates a red shift (3 – 8 nm) and a hyperchromicity (up to 15%) at the DNA-absorption peak (260 nm) [21–25], along with a wide dispersion [26] (*i. e.* broadening) of the DNA-spectrum on the long wavelengths side. The reagent does not react with native DNA under similar conditions. In the second technique acridine orange (AO) has been used as the fluorescent probe, at a very low dye-to-DNA (*i. e.* the DNA of nucleohistone) proportion [27]. Such proportion promotes

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intercalation of the dye into the DNA of nucleohistone. The estimates of rotational relaxation time of whole and stripped nucleohistones have been obtained also from the second technique, *i. e.* from Perrin's [28] plots derived from the polarization transitions of these two materials. From these estimates, the contour length of the total DNA in a nucleohistone molecule has been approximately determined with respect to the end-to-end distance of nucleohistone.

The main features of the DNA-arrangement in nucleohistone, such as DNA-orientation, type of packing, have been determined by measurements of linear polarization (R) of fluorescence [27–30] of nucleohistone-AO complex and of the linear dichroic ratio (E_1/E_{11}) [31] at 257 and 280 nm of nucleohistone, aligned in various ways.

2. Materials and Methods

a. Materials

The preparation and characterization of whole and dehistonized calf thymus nucleohistones, calf thymus DNA and of histones have been described previously [9]. Acridine orange (AO) (Harleco, Philadelphia) has been freed from contaminating metal ions and from other acridines according to a modification of the method of Freifelder and Uretz [32]. The dye has been added to nucleohistone solution to a final concentration of 0.5 μg/ml so that the dye-to-nucleotide ratio was at about 1/150 [27]. For the studies of linear polarization of fluorescence, each of the nucleohistone and DNA solutions containing the same amount of dye was warmed up at 37 °C for two hours in order to promote intercalation of the dye into the DNA of nucleohistone [27, 29]. For rotational diffusion and fluorometric studies, the dye was added at room temperature immediately before the measurements of polarization (P) and fluorescent intensity.

b. Methods

1. Spectrophotometry
   (See previous report [9].)

2. Fluorescence, rotational diffusion and spectrofluorometry

The methodology, instrumentation and principles underlying the measurements of fluorescence and rotational diffusion (thermal depolarization of florescence) have been described elsewhere [27]. For the studies of the spectroscopic emission from the AO-complexes, an Amino Bowman spectrofluorometer has been used.

The effect of scattering depolarization on the measured P-values of dye-complexes has been tested by changing the refractive index of the medium. Glycerol or bovine serum albumin was added each at a time to dye-complexes in solution but no detectable change in P-values was noticed. Since the scattered excitation light also contributes to the fluorescent signals, concentration (DNA and nucleohistone) dependence of polarization was studied. The relative concentration of dye and DNA (*i. e.* nucleotides) in each complex has been fixed (ratio 1/150) such that P just reached a saturation value with nucleohistone (or DNA) concentration [27]. No further increase in nucleohistone (or DNA) concentration has been made. Unless specified the concentration of DNA and nucleohistone has been kept in the range .05 – .15 mg/ml.

3. Polarization of fluorescence (R) and linear dichroism (D)

The nucleohistone-AO complex has been aligned in various ways, *viz.*, by stroking on quartz cover slips, smearing gently between two cover slips, and by flow in a capillary and by drawing fibers. These were studied for the degree of linear polarization of fluorescence (R) from the oriented dye in the dye complex. (For details of instrumentation, see refs. [27, 29, 30].) R is defined as $R = (I_{max} - I_{min})/I_{max}$, where the maximal ($I_{max}$) and the minimal ($I_{min}$) polarization intensities correspond to the emitted vectors which vibrate parallel (in-plane) and perpendicular (off-plane) to the planar intercalated dye. The directions of maximal and minimal polarizations were measured with respect to the axis of alignment of nucleohistone molecules. R is assumed to be positive if the maximal polarization is oriented perpendicular to the axis of alignment, and negative if polarization is directed along the axis of alignment. The linear dichroic ratio (D), *i. e.* ratio of the perpendicular-to-parallel absorption (E_1/E_{11}), of nucleohistone has been measured mainly at 257 nm in a microspectrophotometer. The latter was equipped with quartz lenses (Spencer reflecting condenser and objective, each 50X, NA, 0.56; Ramsden eyepiece, 10X), a Bausch & Lomb grating monochromator, a polacoat UV polarizer and a Pacific photometer. The light source (Hanovia mercury arc) had a discrete energy spectrum (peaks at 257 and 280 nm). Orientation by flow was achieved by maintaining a to-and-fro motion of the nucleohistone solution through quartz capillaries (diameter 0.01 – 0.023 cm). The capillaries for the sample and for the reference solvent have been matched at 257 nm

before use. The maximal instrumental error in the measurements of D and R was about $\pm 5\%$.

The hydrodynamic conditions of flow [33], *e.g.*, critical shear (*viz.* $11.2 \times 10^5\,\mathrm{sec}^{-1}$ corresponding to Reynold's number 2100), frictional distance from the edges of the capillaries, lower limit to relaxation time (*viz.* 2.9×10^{-4} sec) etc., have been checked so that the steady values of R and D may be valid.

The general mathematical part providing the analysis of R and D, theoretical assumptions in estimating R and D values for various DNA-arrangements are given elsewhere [30, 31].

3. Results and Explanation

Conformation of DNA in nucleohistone

There are various direct and indirect approaches to characterize the complex nature and the nativity of nucleohistone. Tuan and Bonner [5] have established spectrophotometrically the characteristics of native nucleohistone in solution. These characteristics have been fulfilled by the nucleohistone in the present studies. These properties have been tested as they were considered to be the pre-requisites to any undertaking of the DNA-arrangement in this nucleohistone. Briefly, the present nucleohistone had a large histone-to-DNA ratio (*viz.* 1.52) as determined by the ratio of the absorbances of the material (water) at 260 nm and 230 nm. Secondly, the DNA of this nucleohistone was slightly hyperchromic (8 – 10%) in absorption (or molar extinction) relative the DNA obtained either by complete dissociation of histone in high salt or by selective precipitation of DNA from this dissociated mixture by use of isopropanol (see ref. [9]). In addition to this, the ratios of the absorbances (260 nm and 230 nm) of whole nucleohistone and of partially histone removed nucleohistone(s) were studied as a function of histone-to-DNA ratios. These results agreed quite well with the data of Tuan and Bonner [5]. Finally, the position of the DNA minimum near 230 nm was also noted in those materials (*i.e.* various histone-to-DNA ratios). The minimum remained steady at 237 nm for histone-to-DNA ratios in the range 1.0 – 1.52. At smaller ratios (less than 1.0) the minimum shifted linearly with the decrease in histone content, finally, to about 230 nm when the ratio was 0.2 only. The optical rotatory dispersion of the present nucleohistone was also of standard type.

In as much as native nucleohistone has a 'hyperchromic' DNA, the reaction of formaldehyde with nucleohistone (histone-to-DNA ratio equals 1.52) has been studied spectrophotometrically at different reagent concentrations (1, 4 and 10%). The absorption spectrum of nucleohistone (Fig. 1) did not indicate any red shift. Nor any hyperchromicity has been seen at 260 nm in three hours. By subtracting the absorption spectrum of nucleohistone (curve c's) from the spectrum of the nucleohistone mixed with formaldehyde (curve b's, Fig. 1) a spectrum (curve a_2's, Fig. 1) was obtained to show the

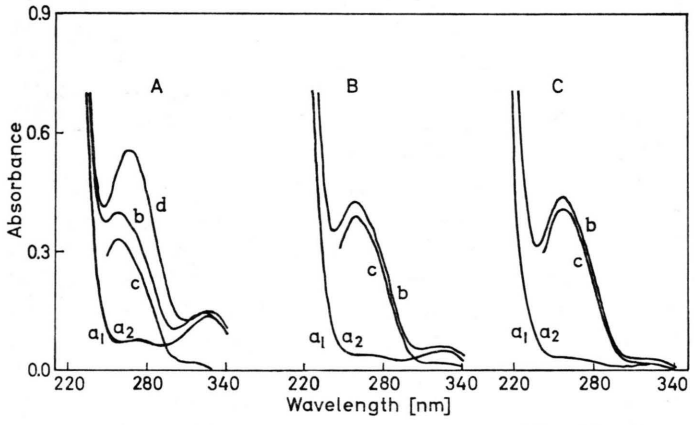

Fig. 1. Reaction of formaldehyde with nucleohistone (*P/D* ratio equals 1.52) at different reagent concentrations: Suite A, 10%, Suite B, 4% and Suite C, 1%.
Suite A: curve (a_1), free formaldehyde (10%); curve (a_2), explained below; curve (b), reaction ($\frac{1}{2}$ h, 25 °C) mixture containing nucleohistone and formaldehyde (10%); curve (c), nucleohistone only, and curve (d) nucleohistone heated at 90 °C in the presence of formaldehyde (10% conc., 10 min) and then chilled in ice-water mixture (0 °C). Curve (a_2) represents the spectral differences between curves (b) and (c) (*i.e.* curve (b) *minus* curve (c)). In other words curve (a_2) measures the spectra absorbances of the formaldehyde component of the reaction mixture after $1\frac{1}{2}$ h at 25 °C.
Suites B and C: legends are the same as in Suite A except that formaldehyde concentrations should be read as 4% (B) and 1% (C), respectively. The (d) curves are not shown. (a_1) and (a_2) curves are coincident, and therefore shown by a single line curve.
The concentration of nucleohistone was about .04 mg/ml starting from suite A to B and C. For (c) curves, nucleohistone has been diluted with solvent (distilled water, pH brought to 6.0 – 6.5 by boiling and then degassing) to the same extent as was caused by formaldehyde of various proportions (pH neutralized before addition). Path length: 1 cm.

spectral absorption of the reagent component in the reaction mixture. This differential spectrum identified the reagent to be mostly in the free state (*cf.* curve a_1's, Fig. 1; see legends section). There was no evidence at 270 – 280 nm of formaldehyde-protein cross linkage.

If the nucleohistone was heated at 90 °C in the presence of 1 – 10% formaldehyde and then rapidly cooled to 0 °C, a red shift of 3 – 8 nm occurred at the absorption maximum (260 nm). Also, a hyperchromicity only about 30% relative to the measurements on the initial nucleohistone was observed (Fig. 1 A, curve d). Under similar conditions of heat denaturation, in the presence of formaldehyde, DNA and dissociated nucleohistone (histone-free) showed a hyperchromicity of 40% or more, with a concomitant red shift of about 3 – 8 nm. These effects on DNA are also supported by other reports [21, 22, 25]. Formaldehyde at any concentration in the range of 1 – 10% reacts fast at room temperature with denatured DNA, whereas the rate of reaction of the reagent with native DNA is negligible [21–25]. The two results *i. e.* the lack of reaction with formaldehyde and the 'hyperchromism' of native nucleohistone suggest that the secondary structure of the DNA in native nucleohistone is different both from the structures of native and denatured DNA.

Rotational diffusion and fluorometric studies of unoriented nucleohistone-AO complexes (Table I, Figs 2 and 3):

The lack of polarization of histone-AO suggests a higher rotational diffusion rate of bound dye in this complex. This may be due to the low molecular weight of these compounds and to the negligible effect of the fluid viscosity [34, 35]. The diminished magnitude of polarization from nucleophistone-AO (Table I) as opposed to that of DNA-AO may be due to a compact configuration of nucleohistone provided the dye-binding to the DNA of nucleohistone has occurred *via* a stronger mode (*e. g.* intercalation). However, the small *P*-value of nucleohistone-AO may have a second reason *i. e.* a large amount of dye-binding in the weaker modes (*viz.* stacking, edge-wise binding, etc.). This is because the *P*-value is determined both by the configuration of the dye-complex [34–36] as well as by the quantum efficiency of the mode of dye-binding [37]. In order to eliminate one of these two possible interpretations further measurements of the nucleohistone-AO and DNA-AO complexes have been undertaken in a fluorometer and in a spectrofluorometer. The integrated intensity of emission (450 – 600 nm) from the two dye-complexes (*i. e.* nucleohistone-AO and DNA-AO) was found to be equal. The maximal emission from the bound dye in the two complexes occurred at 530 – 534 nm, whereas free dye emitted at 528 nm. The total intensity of emission from each dye complex was about twice the intensity of the same amount of free dye in solution as present in any dye complex. The absorption maximum (490 nm) of AO in either complexes shifted toward the longer wavelengths (506 nm). The enhancement of the fluorescent intensity and the red shift in the absorption maximum of AO were evidence of the stronger mode (*viz*, intercalation) of dye-binding to the DNA in nucleohistone (*cf.* Basu [27]). This, therefore, eliminates the second (2) interpretation given above. Stacking and edgewise binding of the dye to the histones are not probable because the quantum efficiencies of these weaker modes of interaction are negligible compared to that of intercalation of the dye into the DNA of nucleohistone [37] (for various other reference, see Basu [9]), and they will contribute very little to the polarization and to the enhanced intensity of fluorescence of the nucleohistone-AO complex. In the high molarity solvent (*viz.* 2.5 M NaCl) both the dissociated nucleohistone and the native DNA represented similar *P*-values (*viz.* 0.14 for the DNA and 0.13 for nucleohistone). So the two compounds (*viz.* DNA and DNP) had quite similar degrees of aggregation in 2.5 M NaCl because in both of them there was excess amount of counterions. The *P*-value

Table I.

	NaCl conc. in molarities *	*P*-Value
Calf thymus	0 (dist. water)	0.10
Nucleohistone	0.15	0.12
(protein-to-DNA	1.0	0.17
ration 1.52)	1.8	0.15
	2.5	0.13
Free histones	0 (dist. water)	0.02
(10 – 25 μg/ml	2.5	0.00
in water)		
Calf thymus DNA	0	0.18
	2.5	0.21 – .25
	1	0.21
	1.8	0.17
	2.5	0.14

* Salt was added directly to the materials.

of nucleohistone increased up to 0.17 in 1 M NaCl. This means that the DNA in nucleohistone was unfolded to a large degree (*cf.* Ohba[68]; see also, Discussion) in 1 M NaCl. Some of the interpretations above have been substantiated from thermal depolarization studies in the following section.

In Fig. 2 thermal polarization transitions of native nucleohistone-AO and of a partial nucleohistone-AO have been represented by curves (d) and (e). The two materials were suspended in distilled water. In either materials P decreased for heating up to 47 °C and, then, it suddenly increased to a maximum near 58 °C, and finally decreased near the melting region of calf thymus DNA. Although an exact correlation has not been obtained, a similar feature in the change in relative fluorescent intensity (F_t/F_0, F_0 means fluorescent intensity at 25 °C; F_t is the fluorescent intensity at any other temperature) is shown in Fig. 3 (curve (d) or (e)). Most probably the DNA in nucleohistone was slightly unfolded at about 58 °C or the histones were partly dissociated as a result of DNA-breathing[29]. The results of heating free DNA-dye complex in water (*cf.* curve (b)'s, Figs 2 and 3) and in 2.5 M NaCl (*cf.* curve (c)'s, Figs 2 and 3) were slightly different. The DNA in water had suffered partial denaturation (about 10% hyperchromicity) due to lack of counterions before thermal transitions were obtained. Since the transitions of nucleohistone in water and of DNA in 2.5 M NaCl were not alike it is clear that the tertiary structures of the DNA in these two cases were primarily different. This contradicts the suggestion of Permogorov *et al.*[6]. Only in the cases of DNA-AO and nucleohistone-AO – both in 2.5 M NaCl –, the thermal transitions were similar. We shall refer to the configuration of DNA in these two materials in the next section. The polarization transitions of heat denatured (31% hyperchromicity) DNA were also different from the transitions of native nucleohistone in water (*cf.* curve (d) *vs* curve (f) in Figs 2 and 3).

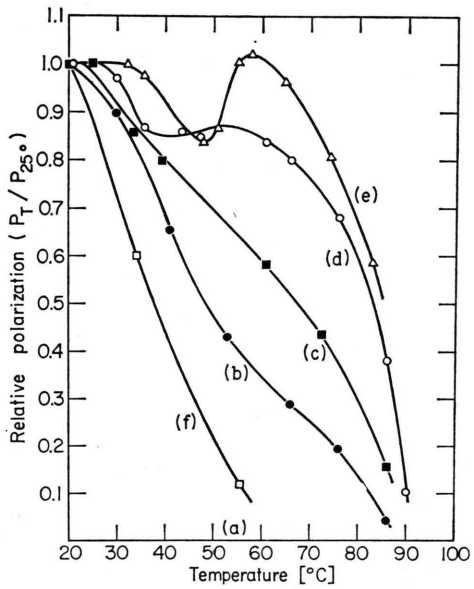

Fig. 2. Thermal depolarization of fluorescence from acridine orange molecules bound either to DNA or to nucleohistone (dye-to-nucleotide ratio 1/150). Curve (a), free dye (0.5 μg/ml); curve (b), calf thymus DNA-dye complex in distilled water; curve (c), calf thymus DNA-dye complex 2.5 M NaCl. The curve for dissociated nucleohistone-dye complex in 2.5 M NaCl was identical to curve (c). They are represented by one curve. Curve (d), nucleohistone (protein-to-DNA ratio equals 1.35)-dye complex in 0.15 M NaCl; curve (e), nucleohistone (protein-to-DNA ratio equals 1.52)-dye complex in distilled and curve (f), calf thymus DNA-dye complex heat denatured in water. The differential between curves (e) and (d) might be due to some configurational change in nucleohistone, as some histones are released from the native nucleohistone (protein-to-DNA ratio 1.52). This effect is continued up to 2.5 M NaCl where nucleohistone is principally represented by curve (c).

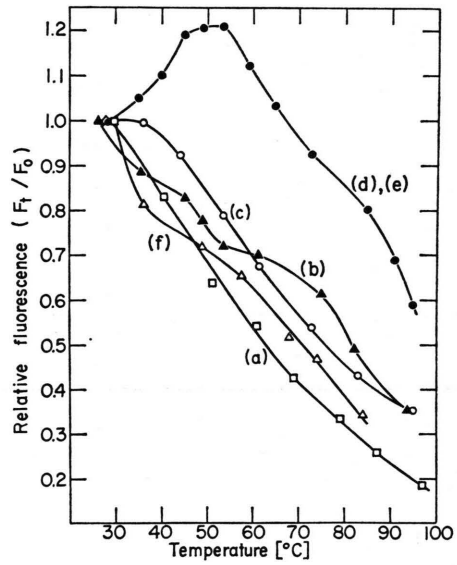

Fig. 3. Thermal quenching of fluorescence from acridine orange (0.5 μg/ml) molecules bound either to DNA or to nucleohistone (dye-to-nucleotide ratio 1/150). The legends to curves (a–f) are the same as in Fig. 2. Curves (d) and (e) were quite coincident and so they are represented by one curve.

Summarizing these results, the denaturation defects in nucleohistone had no resemblance or relevance to the conventional denaturation characteristics obtainable on DNA in solution.

Studies of the DNA-arrangement in nucleohistone – polarization of fluorescence (R) and linear dichroism (D) studies:

The steady values of R and D of oriented nucleohistone are given in Tables II and III. The ratios of these values with the corresponding data on oriented DNA, or DNA-AO complexes (R), give the degree of preferential orientation of DNA in nucleohistone. Rotational diffusion of nucleohistone-AO has been measured prior to flow orientation and afterwards in order that any effect of stretching of the DNA in nucleohistone could be obtained but the P-values in the two occasions were identical. This has proved that the size and shape of nucleohistone were stable under flow. The proportion of histone-to-DNA absorption at 260 nm in the spectrum of native nucleohistone was 3/2:100 (*i. e.* 1.5%). The contribution of histones to polarized absorptions (E_1, E_{11}) at the same wavelength increased to only 2.5 – 4.5%. For this reason, the measured dichroism of nucleohistone has not been corrected for histone absorption. Tables II and III represent direct values of both D and R. The effect of anisotropic light scattering (*i. e.* form dichroism) has been evaluated from direct measurements of E_{11} and E_1 at long wavelengths (*via* range 310 – 440 nm) where neither histones nor DNA absorbs light. Each of E_{11} and E_1 at these wavelengths were similar in magnitudes and so they did not indicate any significant effect of form dichroism. Finally, the contribution of scattered absorption at 257 nm has been checked by the method of linear extrapolation (*i. e.* by plotting log OD *vs* log λ, λ = wavelength where $n = 3.2$ for nucleohistone; $n = 3.8$ for DNA). The contribution of scattered absorption(s) to the total absorption(s) (*viz.* E_{11}, E_1) of nucleohistone at 257 nm was found to be only 10 – 15%. No correction has been introduced into the direct values of D as they would increase only slightly (*e. g.* 1.23 instead of 1.20) on such correction.

Table II. R-values of oriented calf thymus nucleohistone and DNA. Protein-to-DNA ratio of nucleohistone equals 1.52.

	Type of alignment	R-Value [%]
Nucleohistone (water)	streak	+ (12 – 15)
	smear	+ (12 – 15)
	flow	+ (4 – 6)
	(shear 10,000 sec^{-1})	
	fiber	+ (15 – 18)
Calf thymus DNA (0.015 M NaCl, 1 mg/ml)	fiber	+ (60 – 65)
	flow	+ (40 – 45)
	(shear 10,000 sec^{-1})	

Table III. Ultraviolet dichroic ratios (*i. e.* ratios of perpendicular-to-parallel absorptions) of calf thymus whole nucleohistone and salted nucleohistone.

Histones were not removed in columns 5 and 6.

Wavelength [nm]	*D i. e.* (E_1/E_{11}) of streak	*D i. e.* (E_1/E_{11}) of fiber	*D i. e.* (E_1/E_{11}) of flow [q 10,000 sec^{-1}] Water	1 M NaCl (Salted)	2.5 M NaCl (Salted)
257	1.16	1.26	1.07	1.30	1.45
280	1.01	1.00	0.99	1.10	1.25
314	1.01	1.00	0.95	0.70	0.93
366	0.93	0.95	0.80	0.75	0.80
437	1.02	1.03	0.75	0.80	0.87

Dichroic ratios of DNA

	λ [nm]	D(flow) *i. e.* E_1/E_{11}	D(fiber) *i. e.* E_1/E_{11}
Calf thymus DNA 1 mg/ml (0.015 M NaCl)	257	1.83 ($\overline{q}$ 10,000 sec^{-1})	5.00
	280	1.82 ($\overline{q}$ 10,000 sec^{-1})	4.90

Briefly, the results in Tables II and III give the following information: 1. The dye-complexes of both whole nucleohistone and DNA exhibit the same direction of maximum polarization, *i. e.* perpendicular to the direction of alignment; 2. the magnitude of linear polarization of fluorescence (range 5 – 17%) of nucleohistone-AO complex is much smaller than that of DNA-AO complex; 3. the R- and D-(257 nm) values of nucleohistone (or its complex with AO) indicate quite similar but an appreciable amount of oriented DNA along the long axis of whole nucleohistone. The lack of full DNA-orientation in nucleohistone could have several explanations, such as supercoiling of DNA [1, 4]; presence of irregular [14] or regular DNA coils with extended DNA [31], flat packing of DNA in a random DNA-arrangement etc. Further studies have been undertaken to eliminate some of these possibilities.

The whole nucleohistone has been dehistonized stepwise by adding different amounts of NaCl salt

to whole nucleohistone suspended in water, and the change in R- and D-values has been studied. Particularly, the direction of maximum polarization of fluorescence has been watched. This is because if the DNA in nucleohistone had a supercoiled configuration of a regular higher order, then, as the DNA extends by uncoiling, the maximum polarization of fluorescence would reverse its direction (*i. e.* R will change its sign from + to − corresponding to the next lower order of DNA-coiling. Tables III (last two columns) and IV show the results (*i. e.*

Table IV. R-values of oriented calf thymus nucleohistone (protein-to-DNA ratio equals 1.52) measured by flow orientation at a shear (q) of 10 000 sec^{-1}. Salt solution was added directly into nucleohistone solution. Histones were not removed.

Salt molarity	Average R-value [%]
0 (dist. water)	+ 5
0.015 M NaCl	+ 5
0.15 M NaCl	+ 7
1 M NaCl	+12
2.5 M NaCl	+16

R- and D-values) when histones dissociated from DNA have not been removed from the suspension. In this situation exchange [38] of histones would take place and most perhaps also some re-arrangement of DNA would occur, particularly in the high salt. For a given salt concentration, the orientation achieved by the two methods (*i. e.* R and D) was similar. But the orientation obtained in 1 M NaCl was less than that measured in 2.5 M NaCl. Rotational diffusion (P) studies of the same samples have indicated an opposite trend, *i. e.* P was larger in 1 M NaCl than in 2.5 M NaCl. This clearly suggest that a definite physical phase separation occurred in dissociated nucleohistone in 2.5 M NaCl. Since native DNA also showed a similar property, it is imperative that the DNA molecules formed linear (*i. e.* sidewise) small aggregates in the presence of excess counter ions. Structural breakdown of nucleohistone is not probable for the same reason. In 1 M NaCl nucleohistone was still in dissolved phase and its extended configuration had the behaviour of a polymacroion in solution, just as a flexible DNA polymer whose length induces a considerable degree of disorientation at high shear [39].

In Fig. 4 the histones have been removed by selective precipitation [9] and thus the possibility of DNA rearrangement has been eliminated. Both R- (Fig. 4) and D-values (not shown) increase and remain positive in sign as the protein-to-DNA ratio of nucleohistone is diminished. For a dehistonized material of very low histone content (*e. g.* P/D equals 0.2), the R-value is about 31% (plateau region in Fig. 4). Electron microscopy of sonicated nucleohistone or reconstituted nucleohistone (*i. e.* from sonicated DNA and total histones) under similar conditions may explore the various steps of uncoiling (or coiling) of DNA involved in the curve in Fig. 4. Summarizing these results, since no change in the direction of maximum polarization has occurred and the R-value of fully dehistonized nucleohistone represents an uncoiled DNA, it is concluded that the DNA in the starting nucleohistone never had a sigle supercoiled or superhelical configuration (contrast ref. 1, 4).

Analysis of the Results and Discussion

As Shih and Fasman [7] have claimed, if we attribute the hyperchromicity of nucleohistone to base-tilt, the estimated base-tilt (see p. 437, ref. 31, for eqn.) would be about 18°; however, the tilting of the bases has to be dispersed on the DNA length in order to provide long range neighbor interaction [40] for dichroism (D) and also to provide spaces for dye-intercalation (*i. e.* for R).

If we assume uniform intercalation along the length of DNA then each dye should occupy a space every 75 base pairs (dye-to-nucleotide ratio 1/150). Since R and D give the same degree of orientation of base pairs the base tilt would be dispersed (*i. e.* separated) by at least 75 base-pairs or 250 Å in length. Such long range defects in DNA subunits may only be caused by histones although the basic amino acid residues of these proteins stabilize the DNA structure by neutralizing the negative phosphates on DNA.

Various authors [36, 42, 43] have applied Perrin's [41] and Weber's [34, 35] theory of polarization of fluorescence to DNA-protein complexes and to nucleic acid-dye complexes. This theory has been improved here in order to estimate the extended length of DNA in nucleohistone.

According to Weber [34, 35] the partial polarization (P) of fluorescence of a dye-complex at temperature T, for excitation with polarized light vibrating perpendicular to the direction of light propagation, is

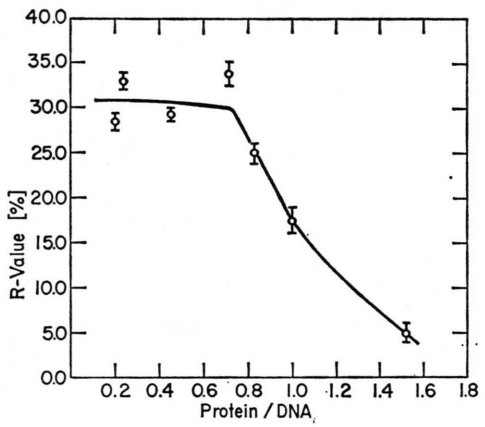

Fig. 4. Variation of the degree of linear polarization of fluorescence (R) with protein-to-DNA ratios of nucleohistone-acridine orange complexes: dye-to-nucleotide ratio equals 1/150 and the dye concentration at 0.5 μg/ml.

related to the rotational relaxation time (ϱ) of the macromolecular complex, life-time (τ) of the excited state and the polarization (P_0) of the same complex in a rigid media (*i. e.* at absolute zero, when $T/\eta = 0$). The relationship (Perrin's law [41]) is given as follows:

$$\frac{1}{P} - \frac{1}{3} \simeq \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\varrho_0}\right) \qquad (1)$$

where

$$\varrho_0 = \frac{3\eta V}{kT} \cdots \qquad (1\,a)$$

for a spherical molecular complex. For ellipsoidal and cylindrical molecular complexes, ϱ_0 is related to the simple harmonic means (ϱ_h's) of the rotational relaxation times along three principal directions of the macromolecules. Thus

$$\varrho_h = \frac{\varrho_0}{r(e)} \qquad (1\,b),\ \text{for a ellipsoid, and}$$

$$\varrho_h = \frac{2}{3}\,\varrho_0 \cdot \frac{1}{r(e)} \qquad (1\,c),\ \text{for a cylinder.}$$

ϱ_0 (or ϱ_h) is a function of the coefficient of viscosity (η), and temperature (T° absolute) of the medium, and of the shape and size ($r(e)$) of the macromolecule. The factor $r(e)$ depends on the axial ratio ($a/b = e$) of the macromolecule. For a cylindrical or prolate ellipsoidal molecule [44] of e greater than 50:

$$r(e) = \frac{3}{e^2}\,[\ln 2e - 0.5]. \qquad (2)$$

The Perrin's plots (*i. e.* $1/P$ *vs* T/η) were curved (convex) toward the axis of T/η for either DNA-AO and nucleohistone-AO complexes (Fig. 5). The curvature in these plots has been related to DNA-breathing in another report [45]. The slight variation of each slope in the temperature range $37 - 64\,^\circ$C has been ignored so that a straight line can be obtained and extrapolated to the origin of T/η axis (η remaining constant). The intercepts on the $1/P$ axis give the P_0-values for the two dye complexes. These are 0.5 and .33 for DNA-AO and nucleohistone-AO, respectively. A theory due to Jablonski [46] shows that for polarizations observed with polarized excitation,

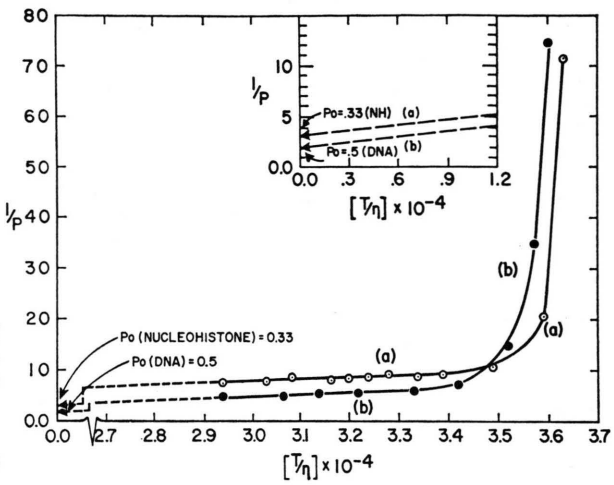

Fig. 5. Perrin's plot ($1/P$ *vs* T/η) for DNA−AO in 0.15 M NaCl (curve b) and nucleohistone-AO (protein/DNA ratio = 1.52) complex (curve a) in water.

$$P_0 = \frac{3\cos^2\Theta - 1}{3 + \cos^2\Theta} \qquad (3)$$

where Θ is the angle between the directions of the absorption and emission oscillators. The limiting polarization (P_0) is given by this theory, as follows:

$$-\frac{1}{3} \leqq P_0 \leqq \frac{1}{2}\,, \quad 0 \leqq \Theta \leqq \frac{\pi}{2}\,.$$

In the present case Θ's for DNA-AO and nucleohistone-AO are 0°, 30° (degrees) respectively. For DNA-AO at room temperature, $P = 0.25$ and at absolute zero $P_0 = 0.5$. Then, from Eqn (1),

$$\left(\frac{\tau}{\varrho_0}\right)_{\text{DNA-AO}} = \frac{2}{5}\ ^*.$$

Similarly for nucleohistone-AO (NH-AO), $P = 0.1$ at room temperature and $P_0 = 0.33$ at absolute zero. So,

$$\left(\frac{\tau}{\varrho_0}\right)_{\text{NH-AO}} = \frac{7}{8} \quad **$$

where ϱ_0-values represent the rotational relaxation times of equivalent spheres of DNA-AO and nucleohistone-AO (NH-AO). Assuming that the life time in the excited state (τ) is similar for the two complexes at room temperature, we have,

$$\varrho_{0\,\text{NH-AO}} \cong \tfrac{1}{2}\,\varrho_{0\,\text{DNA-AO}} \cdot$$

The function $r(e)$ for the DNA of width 20 Å (assumption) and molecular weight of approximately 10^8 daltons was 4.96×10^{-8}. This gave us a longest rotational relaxation time (ϱ_h) for the DNA at about 0.3 sec (N. B. $\eta = .01$ poise, $T = 300$ Å, $(\tau K)/V = .55 \times 10^{-4}$ ergs·sec per c.c. per degree centigrade). Similar values for other DNA's have been reported [47]. The longest rotational relaxation time of nucleohistone was, then, about 0.15 sec. Thus from the P-values of nucleohistone in water and in 1 M NaCl (Table I) and from Eqn (1) and from Fig. 5, it follows that:

$$r(e)_{\text{NH-AO(water)}} \cong 6.6 \times 10^{-8}\,.$$

Then from Eqn (2), axial ratio (e) of nucleohistone $\cong 2.17 \times 10^4$. The axial ratio of DNA of nucleohistone (molecular weight 10^8 daltons) was about 2.5×10^4. However, the contour length of the DNA in nucleohistone was not clearly known. Therefore, the polarizations in Eqn (1) have been related to the molecular lengths of the DNA (L_{DNA}) and the end-to-end distance of nucleohistone (L_{NH}) in the following.

For a randomly labeled cylindrical molecule, the relationship (Eqn (1)) between polarization and the length of the molecule (can be modified as follows):

$$P^{-1} = L^{-1}\,C\left(P_0^{-1} - \frac{1}{3}\right) + P_0^{-1} \qquad (4)$$

where P is the polarization at any temperature, P_0 is the limiting polarization corresponding to $T/\eta \to 0$, L is the length of the molecule. C is given by:

$$\frac{R\,T\,\tau}{\eta\,m\,\bar{v}}$$

where R is the gas constant (*i. e.* Avagadro's No x Boltzmann Constant), T is absolute scale temperature, τ is the time of the excited state of the dye molecule, η is the coefficient of viscosity of the medium, m is the mass per unit length of the molecule and $\bar{v}$ is the partial specific volume of the molecule in suspension.

On substituting the P_0-values of nucleohistone-AO and DNA-AO in the previous equation and assuming that the viscosities, the partial specific volumes (*viz.* 0.68 cm³/g, ref. 10) and τ are similar for dilute solutions of nucleohistone and dehistonized nucleohistone, we get:

$$\frac{L_{\text{DNA-AO}}}{L_{\text{NH-AO}}} \cong 0.4\left(\frac{P_{\text{DNA-AO}}}{P_{\text{NH-AO}}}\right)\left(\frac{m_{\text{NH}}}{m_{\text{DNA}}}\right) \qquad (5)$$

where the suffixes 'NH' and 'DNA' refer to whole nucleohistone and DNA (or dehistonized nucleohistone). Substituting $m_{\text{NH}} = \frac{5}{2}\,m_{\text{DNA}}$ ($\because$ protein/DNA) = 1.52, $P_{\text{DNA}} = 0.17$ (nucleohistone in 1 M NaCl) and $P_{\text{NH}} = 0.10$, we have

$$L_{\text{DNA}} = 1.7\,L_{\text{NH}}\,.$$

The DNA of nucleohistone fiber is thus extended in 1 M NaCl by a factor of 1.7 over the length of nucleohistone. If we substitute the maximum P-value of histone-free DNA (*viz.* 0.25) in the previous equation, L_{DNA} would be about 2.5 times longer than the length (L_{NH}) of the nucleohistone fiber. It is mentionable that the histones which are involved in the folding of DNA in nucleohistone are highly dissociable in 1 M NaCl. Perhaps these histones, as according to Ohlenbusch [48], are involved in the broadening of the melting profile of full nucleohistone. It is also known that these histones are slightly lysine rich [7, 35].

Thus the rotational relaxation time and the axial ratio of nucleohistone which are not much different from that of the free DNA and finally the elongation in 1 M NaCl suggest that the DNA molecule is continuous in the basic molecule of nucleohistone. The length of the total DNA is about twice longer than the end-to-end distance of the nucleohistone molecule. In that case any folding of DNA on its own length would be partial.

The convergence of the parallel beam of light encountered in the microscope lens (*viz.* objective

*, ** For either DNA—AO and NH—AO complexes, τ is close to ϱ_0 but is always less than ϱ_0. Thus τ is intermediate between the rotational relaxation times (10^{-9} sec or less) of free dye and that of bound dye. Therefore there will be partial polarization of fluorescence of bound dye.

and condenser) reduces the actual dichroism or polarized fluorescence of the DNA in nucleohistone. The decrease in the dichroic ratio (or R-value) is determined by the numerical aperture (NA's) of the lenses [31]. In the present optical dichroism system the lenses have been selected so that their numerical aperatures (*viz.* 0.56 for both objective and condenser) do not introduce any significant correction in the observed dichroic ratios. The correction would be about $+1\%$ for the measured dichroic ratios of nucleohistone in the range $1.07-1.26$. The relationship between R and the numerical aperture of the lenses is given by [31].

$$R = f(\mathrm{NA})\, R_0 . \tag{6}$$

In the polarized fluorescence microscope used in this study the objective numerical aperture was 1.32 and the condenser aperture was 0.57. The correction to be added to the measured R-values would be $+2\%$ to $+6\%$ depending upon the magnitude of R in the observed range $+(5-17\%)$. In other words, the corrected R_0-values of fiber oriented nucleohistone would be $+23\%$. The lack in hypochromism of any DNA-containing specimen has been previously related to the loss of dichroism [31] and to the loss of polarized fluorescence, as indicated in Eqns (7) and (8):

$$D_{\mathrm{obs}} = D_0 \left(\frac{1-h}{1+h}\right) \tag{7}$$

$$R_{\mathrm{obs}} = R_0 \left(\frac{1+h}{1-h}\right) - \frac{2h}{1-h} \tag{8}$$

where D_{obs}, R_{obs} are observed values; D_0, R_0 are the real values and h is the percentage hyperchromicity of the DNA in nucleohistone. The percentage hyperchromicity of the DNA in nucleohistone is 15% (*i.e.* $h = .15$) corresponding to 1.08 of the relative rise in the DNA-absorption at 260 nm. If we substitute the previous ranges of D_{obs} and R_{obs} (*viz.* $D = 1.07-1.27$, $R = +(7-23\%)$ on the left hand of Eqns (7) and (8), the the ranges of the real values would be: $D_0 = 1.45-1.69$, $R = +(31-41\%)$. These correct estimates of R- and D-values of oriented nucleohistone (or complex) indicate that about one third of the total DNA should be aligned along the long axis of nucleohistone. The total DNA cannot be fully constrained into an irregularly folded or random coiled configuration. From calculations (Eqn (14), ref. 31) shown in a previous report [31], it can be proved that the maximal amount of oriented DNA which would occur as a result of flat packing across an irregular or random coil, is not more than one fifth of the total DNA in nucleohistone when the dichroic ratio(s) is assumed to be 1.45. A mixed coil(s) which consists of irregular 'helices' (*i.e.* coils) and oriented (straight) DNA is the basic feature of the total populations of nucleohistone studied here. Unless we know for sure that nucleohistones in solution and chromatins in cells in exponential culture have different classes of configuration depending upon the active and inactive states of the genome, the optical anisotropic results would represent the majority of these classes. Bram and Ris's [14] model is relevant in this connection, since the DNA may be partly folded on its own length just like sRNA to produce an 80 Å fiber [1]. Alternatively, the DNA packing may involve separated 'coiled regions' along the length of the same DNA molecule [2]. Since the proportion of the straight lengths of the total 'coiled' and 'extended' DNA regions is 7:3 and the average packing ratio in nucleohistone is about 2:1 (*i.e.* contour length of DNA: end-to-end distance of nucleohistone fiber), a packing ratio of about 4:1 (*i.e.* DNA contour length: straight length of each coiled part) is expected in each 'coiled region'. This model was briefly discussed in an earlier report [25]. Several recent lines [49–51] of evidence have been known since the submission of this manuscript, which seem to support that the basic nucleohistone molecule mostly contains 'extended' and 'coiled' regions in linear arrays. Incidentally, this arrangement brings to our attention a similar model which Busch [12] proposed much earlier.

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