Low resolution models of self-assembled histone fibers from X-ray diffraction studies

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ABSTRACT

X-ray diffraction data from self-assembled histone fibers are presented
for three systems: H4, H3-H4, and the four core histones H2A, H2B, H3 and
H4. These data have been obtained under conditions of high ionic strength
and high protein concentration which are thought to promote histone confor-
mation similar to that found in intact chromatin. The low angle equatorial
scattering ($R < .05$ $Å^{-1}$) is analysed, and, with additional constraints imposed
by electron microscopy data, four low resolution fibrillar models are derived.
Two features common to all the possible models are a maximum outer diameter
of approximately 60 Å and a subfibril diameter of approximately 25 Å. It is
the interference of the protein subfibrils across a central region of low
electron-density - a $10_oÅ$ "hole" - which gives rise to the characteristic
diffraction peak at 36 Å. Possible relationships of the models of the
histone fibers to the structure of the histone component of chromatin are
suggested.

INTRODUCTION

Recently, a low resolution model of the chromatin "core" particle, con-
sisting of 140 base pairs of DNA and eight histones, has been derived from
a combination of single crystal X-ray diffraction and electron microscopy
data[1]. The particle is described as a flat cylinder 110 Å in diameter and
57 Å in height. A similar shape and dimensions were found to be consistent
with the low angle neutron scattering from core particles in solution[2,3].
The neutron experiments furthermore determined that the bulk of the histone
proteins occupies the central region of the particle to a radius of appro-
ximately 32 Å and that the DNA is supercoiled on the outside. Some conclu-
sions may be drawn concerning the conformation of the DNA. Presumably the
strong 28 Å periodicity apparent in the crystal data[1] corresponds to the
pitch of the DNA superhelix wound about the histone core. The radius of
the superhelix must be about 45 Å which, for 140 base pairs of B form DNA
corresponds to somewhat less than two turns. On the other hand, with regard

to the arrangement or symmetry properties of the histones, very little may
be concluded.

In parallel with work on chromatin and the nucleosomal subunits, the
histone proteins have been subjected to intensive structural study in isola-
tion from DNA. The primary sequences of histones from many species have
been determined and show a remarkable degree of evolutionary conservation[4].
The conformational behaviour and interactions among the various histones
have been investigated using a variety of spectroscopic techniques[5] and
X-ray diffraction[6]. However, the question of under what conditions histone
secondary and tertiary conformation and patterns of association would be
similar to those in intact chromatin remained, to a large extent, unanswered.
No simple criterion, such as activity in the case of enzymes, could be cited.
Therefore, the direct applicability of these results to chromatin was, at
best, uncertain. Recently, however, evidence has been growing that under
conditions of high salt, the core histones - H2A, H2B, H3 and H4 - complex
and adopt a conformation very similar to that which they possess in the
intact nucleosome. Weintraub et al.[7] have shown that in solutions containing
NaCl at ionic strengths greater than 0.8M, trypsin digestion of the inner
histones produces the same limit digest pattern as intact chromatin, albeit
with different kinetics. (This result is independent of whether salt or
acid is used for extraction of the histones from the nuclei). Comparative
Raman spectroscopy of nucleosome core particles and of core histones in 2M
NaCl at a concentration of 90mg/ml indicate that most of the native protein
secondary structure is retained in the latter and that no changes in bonding
of either cysteines or tyrosines has taken place[8].

It has further been shown that high ionic strength, in addition to
promoting the correct folding and complexing of the inner histone proteins,
also promotes well defined and reversible higher order association phenome-
na[9,10]. The exact association pathway depends on whether the system consists
of purified single histones, pairs of histones or all four core histones.
However, in all cases, the end products appear in the electron microscope
as long fibers or arrays of fibers. Optical diffraction data from H4
fibers[11], fibers built from pairs of histones or all four core histones[12]
are consistent with a helical structure having a 330 Å repeat and a subunit
spacing of 27 Å. This is commensurate with the symmetry of the nucleosome
crystals. Finch et al. have pointed out that, in these crystals, the histone
core regions must be in contact along the nucleosome flat faces and that
the arrangement of histones may be similar to that in the histone fibers[1].

We have therefore undertaken a series of fiber X-ray diffraction experiments both to complement and extend the optical diffraction work and also to clarify the fibers' possible relationship to chromatin structure. In this communication we present low resolution equatorial X-ray data from fibers of histone H4, the pair H3-H4 and all four core histones - H2A, H2B, H3 and H4 - under conditions of high protein concentration and high ionic strength. Simple model structures, whose equatorial Fourier transforms are consistent with these data, are described and analogies to the arrangement of histones in chromatin are suggested.

MATERIALS AND METHODS

(a) Preparation of histone specimens: Techniques for the preparation of solutions of single histones[9] and pairs of histones[10] have already been described. In the case of H3-H4, it has been shown by crosslinking experiments that these procedures result in the formation of fibers built from heterodimers[12]. A solution of the four core histones was prepared as follows: Lyophilized calf thymus whole histone, extracted from washed nucleoprotein with H_2SO_4[13] was dissolved in 10^{-2}M HCl. The solution was dialysed extensively at 5°C in boiled dialysis tubing against sodium cacodylate buffer, pH 7.0, 10^{-2}M. At this stage, the solution was brought to the desired ionic strength by stepwise dialysis at 5°C against cacodylate buffer containing NaCl. The solutions were left to equilibrate at each ionic strength for at least 12 hours (I=0.05, 0.15, 0.3, 0.75, 1.0, etc.). The histone fibers were pelleted by centrifugation; H1 remained in the supernatant. The specimens of H3-H4 contained H3 and H4 in approximately 1:1 molar ratio (Figure 1a); those of the core histones contained H2A:H2B:H3:H4 in an approximately 1:1:1:1 molar ratio (Figure 1b) as determined by electrophoresis on SDS-containing 15% polyacrylamide slab gels, according to Laemmli[14]. Stained gels were scanned using a Joyce-Loebl microdensitometer, or a Gilford scanner.

(b) Preparation of X-ray specimens: Gels were obtained from concentrated histone solution. Fibers were prepared using a method similar to that of Marvin et al.[15]. Thin films were made by allowing a drop of concentrated histone solution to dry down on a microscope slide and then lifting the film with a razor blade. The film was then cemented to a specially constructed holder for examination by X-ray.

(c) Diffraction methods: The X-ray diffraction experiments were performed
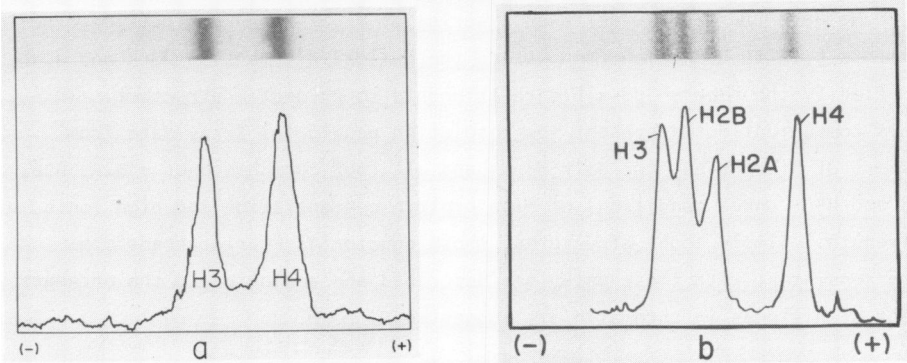
<u>Figure 1</u>. SDS-polyacrylamide gel electrophoresis of aliquots taken from X-ray specimens and the associated densitometer tracings. (a) H3-H4; (b) H2A-H2B-H3-H4.

on a Searle camera using Franks optics[16]. The specimen to film distance was determined by calibration against the NaCl powder diffraction ring at 2.86 Å. The camera was filled with helium which had first been bubbled through a saturated salt solution[17]. The nominal relative humidity (abbreviation, r.h.) inside the camera was monitored using a hygrometer.

X-rays were generated by the Elliott rotating anode generator GX6 operating at approximately 35kV and 25ma with a 200μ focussing cup and Cu target.

(d) <u>Measurement of intensities and positions on X-ray film</u>: Intensities were measured by scanning along the equator with a Joyce-Loebl double beam microdensitometer. At closely spaced points along the scan, the measured height was multiplied by the corresponding reciprocal space radius R in order to correct for specimen disorientation. R was determined by overlaying the scan with a reciprocal space grid based on the calibration ring. In theory, a polarization correction should also be applied, but it was negligible for the small angle region under study.

Measured intensities are probably accurate to no more than ±20% due to uncertainties in the choice of baseline. In the case indicated, an approximate background correction was determined by scanning in the meridional direction and this background was then subtracted from the equatorial data before the disorientation correction was made. The effect of baseline errors on Fourier transforms from continuous diffraction data has been discussed by Wachtel *et al.*[18]. Errors in assigning positions due to the non-linearity of the densitometer table drive and choice of the origin are ±.0025 Å^{-1}. Whole film position measurements were made on a 3.5 times

enlargement of the film.

(e) <u>Electron microscopy</u>: For the solution studies a drop of the specimen solution was placed upon a carbon-coated holey grid and washed with a few drops of buffer and then with a few drops of uranyl acetate. The excess liquid was withdrawn with the edge of a filter paper. For the thin section studies, an oriented fiber of H4 was first fixed by immersion in 2.5% glu-taraldehyde solution in cacodylate buffer (10^{-2}M, pH 7.0, I=0.05 (NaCl)) for 15 minutes at room temperature followed by one hour at 4°C. The fiber was washed twice for 15 minutes in the cacodylate buffer, left overnight at the same buffer at 4°C, and then post fixed in 1% OsO_4 in the above buffer for 35 minutes at 4°C. The fiber was dehydrated through increasing concentrations of ethanol and then propylene oxide. The fiber, laid in a mold perpendicular to the mold face was embedded in Epon 812 medium hardness[19] and cured for 48 hours at 60°C. Sections cut to grey interference color were stained with both lead citrate and uranyl acetate. Details of the methods are described by Pease[20]. In all cases, electron microscopy was performed with a Philips EM300 electron microscope operated at 80kV.

(f) <u>Model calculations</u>: The cylindrically averaged equatorial transforms of fibrillar model structures were calculated using standard procedures,[18,21,22] and then inverted to obtain the radial density distributions[23]. In each case the model profile was compared with the corresponding radial density distri-bution calculated from the data according to the method described in Ref. 18. The sample interval was 0.01 $\overset{o}{A}^{-1}$, sufficiently fine to give a meaningful density profile to 50 $\overset{o}{A}$[24]. The appropriate extrapolation for the value at R=0.01 $\overset{o}{A}^{-1}$ was performed when necessary.

RESULTS

Structural studies. Examination by electron microscopy of a solution containing the four core histones - H2A, H2B, H3, H4 - prepared as described in the previous section and before centrifugation, reveals the existence of long, well-defined fibrous structures (Figure 2). In morphology, these fibers are very similar to the fibers of single histones and pairs of his-tones observed previously[9,10,11]. They have a diameter between 40 $\overset{o}{A}$ and 80 $\overset{o}{A}$, and appear to be composed of at least two protein strands or subfibrils.

Upon centrifugation, the histone solution acquired a gel-like character. The X-ray diffraction pattern from an unoriented highly birefringent gel of the four core histones in high salt is shown in Figure 3. The main features are a strong and diffuse reflection centered at 10 $\overset{o}{A}$, the relatively sharp
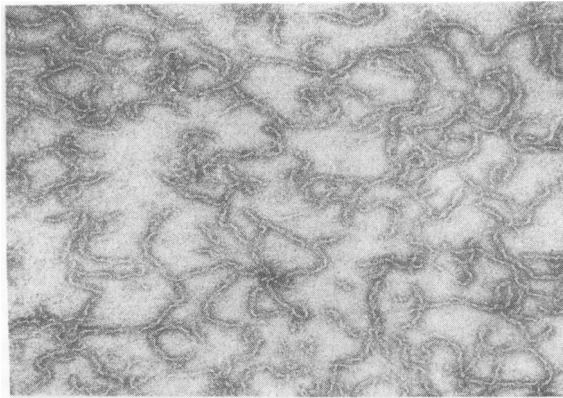
Figure 2. Electron micrograph of assembled fibers of the four core histones - H2A,H2B,H3,H4 - in 10 mM cacodylate buffer, pH 7.0, I=1.0, with 1mM spermine, photographed over a hole in the carbon substrate. (magnification: x 102,750.).

ring at 4.6 Å, a weak ring at 5.1 Å and a low angle ring at ∿36 Å. The first two reflections were also reported by Zubay and Wilkins[6]. It is not clear if the 36 Å reflection is related in any way to the diffuse ring they occasionally observed between 22 Å and 31 Å. The rings at 4.6 Å and 5.1 Å are superimposed upon diffuse scattering centered at 4 Å and the 36 Å ring appears as a shoulder on diffuse scattering extending out from the origin. The intensity of this fine structure is moisture and/or protein-concentration dependent. At the lowest humidities, the three rings are most distinct. As the sample becomes more dilute, first the 36 Å reflection weakens and subse-
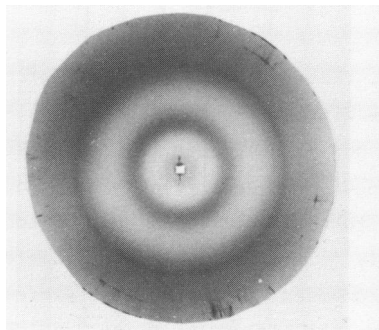
Figure 3. X-ray diffraction pattern from a non-oriented high ionic strength (I>1.5) gel of the core histones - H2A,H2B,H3,H4. r.h.∿27%. Specimen to film distance 5.3cm, enlargement x 0.75. (film 347).

quently the 4.6 $\overset{o}{A}$ and 5.1 $\overset{o}{A}$ reflections are replaced by more diffuse scatter. Both of these effects appear to be reversible.

We have also obtained diffraction patterns from high ionic strength gels of H4 and H3-H4. They are, in all detail, qualitatively similar to Figure 3.

It has furthermore proved possible to obtain partially oriented gels and oriented films and fibers of H3-H4 and H4. In the diffraction patterns of these specimens, all the features described above are present, but exhibit either a meridional or equatorial preference. An example of the low angle diffraction from a partially oriented gel of H4 is shown in Figure 4. Relative to the direction of orientation, the 36 $\overset{o}{A}$ reflection and the continuous scatter visible to the low angle side of it are equatorial. The same is true for oriented H3-H4. However, the degree of orientation is still not sufficiently high to permit the indexing of a system of layer lines. A discussion of the higher angle diffraction from oriented histone specimens will be presented in a subsequent communication[25].

Corrected low angle equatorial amplitudes |F| obtained from oriented and partially oriented specimens of H4 and H3-H4 are shown in Figure 5. The data are scaled together on an arbitrary scale. Because of possible interference from backstop scattering, measurements inside 0.015 $\overset{o-1}{A}$ were not included. The minimum centered at $\sim$.045 $\overset{o-1}{A}$ appears as a true zero of the transform. The situation at 0.02 $\overset{o-1}{A}$ is less certain and this feature may be either a minimum or a zero. The similarity of the H4 and H3-H4 data implies that at least to 20 $\overset{o}{A}$ resolution and with cylindrical averaging, the details of the assembled H4 and H3-H4 structures are alike in axial projec-
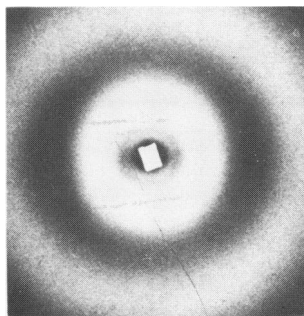

Figure 4. Low angle region of an X-ray diffraction pattern from a semi-oriented gel specimen of H4. r.h.$\sim$48%. Specimen to film distance 4.7cm, enlargement x 1.80 (film 206).

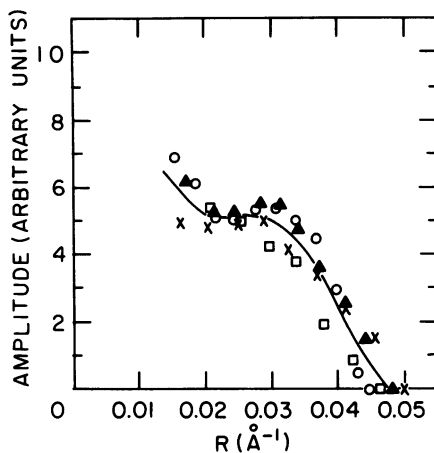**Figure 5.** Sampled amplitudes |F| from continuous equatorial diffraction: (Δ) H4 gel, (film 206); (x) H3-H4 gel, (film 317B); (o) H3-H4 gel (film 310); (□) H4 thin film with background correction (film 204). r.h. <50%. All intensities were measured by densitometry, corrected for disorientation and placed on an arbitrary common scale.

tion. It should be pointed out that in both cases, the scattering profile arises from structural details contrasted against a background of water or water plus side chains.

Further structural information may be obtained from electron microscopy of thin transverse sections of a macroscopic fiber specimen of histone H4. These sections reveal an irregular array of electron dense centers embedded in unstained epoxy resin. (Figure 6). The average diameter of the dots is 27±9 Å with center to center distance 64±9 Å. The latter figure is considered to be a more reliable measure of the true diameter of the dots due to arti- facts resulting from staining. Because the section is approximately 500 Å thick and the stain penetrates to some depth, what one observes is effecti- vely the axial projection of the electron density distribution of the histone fibers. This data is therefore analogous to the equatorial data from X-ray diffraction experiments.

Models. Three different types of data may therefore be used as const- raints on the choice of low resolution model structures for histone fibers. Firstly, under conditions of high salt, H4, H3-H4, and the four core histones - H2A, H2B, H3, H4 - appear in the electron microscope as individual fibrous structures, each comprised of an even number of protein strands and having diameter 40-80 Å [9,10,11]. The separate strands are not smaller than appro-
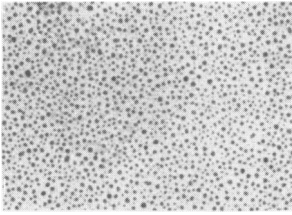
Figure 6. Electron micrograph of a cross-section of a fibre specimen of histone H4. The electron dense regions appear as black dots. The section is approximately 500 Å thick. (magnification x 102,750).

ximately 20 Å in diameter. Second, the thin section microscopy described above places an upper bound of approximately 70 Å on the diameter of H4 fibers. Lastly, a model with this overall morphology must have an equatorial transform which is in reasonably good agreement with the X-ray data.

We have built trial models consisting of two, four and six strands, symmetrically arranged. Each strand was approximated by a uniform density cylinder, and the cylinder radius was varied from 11 Å to 14 Å. Both parallel arrays and plectonemically coiled bundles of strands were investigated, comprising a total of over 150 trial models. In the parallel array, the axis of each strand is parallel to the axis of every other strand, and all are parallel to the axis of the array and equidistant from it. In the case of the plectonemic arrangement, all strands coil around a common axis and are equidistant from it.

The models which best obey the criteria set down are drawn in Figure 7 along with the corresponding cylindrically averaged radial electron density profile. As is apparent, either a two strand or four strand model can explain the X-ray data reasonably well. The two strand plectonemic model is similar to the one suggested by Hyde and Walker on the basis of symmetry arguments[26]. In all cases, the strand diameter is about 25 Å. This is what might be expected if each strand consists of a linear array of the globular regions of histone monomers[26]. The outer diameter is about 60 Å, consistent with both the measurements on thin sections and electron microscopy of histone fibers from solution. The strands or subfibrils are not uniformly close packed, and it is the mutual interference across an internal region of low electron density which gives rise to the distinct equatorial diffraction feature at 36 Å. This ~10 Å "hole" must represent a region of low density side chains or, less likely, water. It is not possible at present to conclusively rule out all but one model. The fact that the H4 fibers appear ribbon-like would favor the two strand models, at least for H4[11]. Consideration of the likely zeroes of the measured scattering profile favors the
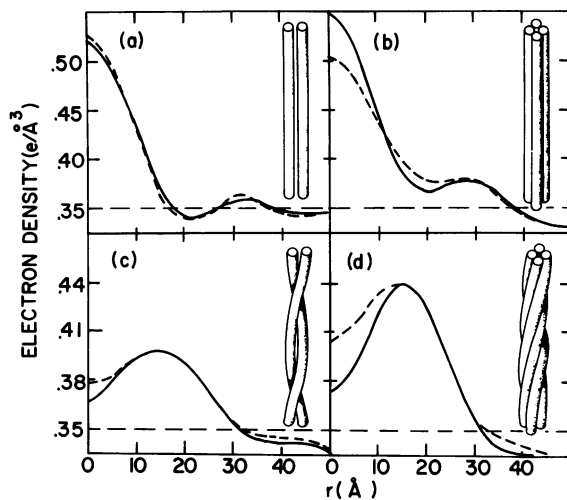
Figure 7. Radial electron density distributions calculated for cylindrically averaged model structures (———): (a) two parallel strands of radius 12 Å, interstrand spacing 34 Å; (b) four parallel strands of radius 12 Å, interstrand spacing either 24 Å or 34 Å; (c) two plectonemically coiled strands of radius 13 Å, supercoil radius 17 Å; (d) four plectonemically coiled strands of radius 12 Å, supercoil radius 17 Å. In each case, a comparable distribution is calculated from the equatorial data (----). Background electron density (water plus 2M NaCl) is indicated by the dashed line. Electron density below background is due in part to effects of truncation (24).

parallel strand models.

DISCUSSION

    X-ray studies of gels, fibers and thin films of histone H4, H3-H4 pairs and the four inner histones H2A, H2B, H3 and H4 at high salt result in markedly similar diffraction patterns. In the region between 75 Å and 10 Å, where information is present concerning the arrangement of histone monomers in higher order structures, we have observed one characteristic ring at a spacing of ∼36 Å. The intensity of the ring relative to the diffracted intensity at 10 Å decreases with increasing water content. In our studies on oriented and partially oriented specimens of H4 and H3-H4, the reflection has been shown to be a prominent shoulder in the continuous equatorial scattering profile. The difficulty in observing other reflections in this region, as seen by optical diffraction from electron micrographs of histone fibers[11], may be due to a combination of weak intensity and an insufficient degree of orientation. Recently, meridional reflections at orders of $(\frac{1}{28})$ Å^{-1}

have been observed in the X-ray diffraction from one specimen of core histones in sodium acetate buffer[27].

Electron microscopy studies predict the assembly of histones into long fibers under the conditions present in the X-ray specimens. Using the dimensions of the fibers seen in the electron microscope as constraints, simple models consisting of arrays of straight or coiled uniform density cylinders were built. The cylindrically averaged equatorial transforms were then compared with the low resolution equatorial data. Four models - two two-strand and two four-strand - have reasonably good transforms; the dominant structural features of the cylindrically averaged radial density profiles are reproduced in the profiles calculated from the X-ray diffraction data as described above. We present these fibrillar structures as low resolution models for assembled fibers of H4 and H3-H4. Although we cannot conclusively rule out all but one model, some bias in favor of two strands derives from the results of electron microscopy and optical diffraction[11]. Since equatorial data reflects structure in axial projection, nothing may be deduced concerning axial periodicity. However, the strand diameter is consistent with each strand being composed of a tightly packed linear assembly of the globular regions of histone monomers. In all cases, the strands are arranged symmetrically about a 10 Å diameter region of low electron density. Although no oriented data is yet available for fibers composed of all four core histones, it is likely that, on the basis of the disoriented pattern and the similarity of the structures observed in the e.m., a related model is appropriate also for them. The similar distribution of polar and nonpolar residues and the extensive sequence homologies found among the four histones render such structural similarity not unreasonable[28].

The experiments described here have been performed under conditions which have been shown to promote a folding and complex formation of the four inner histones that is "native" to chromatin. High ionic strength and protein concentration are prerequisites for the integrity of the histone octamer[29]. On this basis, what may be concluded concerning a relationship between the fibrillar models and the histone core in chromatin or its nucleosomal subunits? Furthermore, is there any independent evidence to suggest that such a relationship exists at all?

To address the second question first, it has been found that preparations of nuclei[30], nucleohistone[31], nucleosomes[32], and the reconstituted H3-H4-DNA complex[33] all show as a feature of the continuous scattering

profile, an X-ray or neutron diffraction ring at 35-37 Å. The strength of the 37 Å equatorial reflection from the crystals of core particles indicates that this ring has a strong, if not predominant, equatorial component[2]. Furthermore, this ring dominates the neutron scattering profile of core particles and nucleosomes in D_2O solution[2,3,32]. Under these conditions, the contribution of the protein is enhanced relative to that from the DNA.

We would therefore like to suggest that relationships do exist between the fibrillar models and the histone component of chromatin - specifically, between the four strand models and the arrangement of all four core histones, and between the two strand models and the arrangement of H3-H4. The 60 Å maximum diameter of the models is consistent with the maximum dimensions cited for the histone core[2,3]. Considerations of linear density[1,26] would favor one or other of the four strand models as a prototype for the histone core of 100 Å chromatin fibers, in which the nucleosome subunits are in contact[34]. This would be the situation demanded also by the crystal packing[1]. The histone octameric core of nucleosomes[35] or core particles may be considered as a truncated four-strand fiber, the height being not greater than 57 Å. Such a truncation would, however, have no effect on the shape of the equatorial transform in the case of the parallel array. In the case of the plectonemic model, the structure could, depending on the pitch of the coiling, degenerate into an approximately parallel array of short protein strands.

On the other hand, the H3-H4 complex with DNA may be described by one of the two strand models. It has been shown that H3 and H4 alone are sufficient to coil DNA into a nucleosome-like structure[36]. The resulting complex contains a tetramer of these histones per 140 base pairs of DNA[37], i.e. half as much protein as native chromatin. Nevertheless, on the basis of our models, we would expect that the characteristic chromatin diffraction ring at 37 Å would also be present in the low angle scattering from such a complex, as has been observed[33].

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