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Superstructures of Wet Inactive Chromatin and the Chromosome Surface

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Superpacking of chromatin and the surface features of metaphase chromosomes have been studied by SiO replication of wet, unstained, and unfixed specimens in an exceedingly thin ($\leqslant 1$ nm) aqueous layer, keeping them wet. Hydrophilic Formvar substrates allow controlled thinning of the aqueous layer covering the wet specimens. Whole mounts of chromatin and chromosomes were prepared by applying a microsurface spreading method to swollen nuclei and mitotic cells at metaphase.

The highest level of nucleosome folding of the inactive chromatin in chicken erythrocytes and rat liver nuclei is basically a second-order superhelical organization (width 150–200 nm, pitch distance 50–150 nm) of the elementary nucleosome filament. In unfavorable environments (as determined by ionic agents, fixative, and dehydrating agents) this superstructure collapses into chains of superbeads and beads. Formalin (10%) apparently attacks at discrete sites of chromatin, which are then separated into superbeads. The latter consist of 4–6 nucleosomes and seemingly correspond to successive turns of an original solenoidal coil (width 30–35 nm), which forms the superhelical organization. When this organization is unfolded, eg, in 1–2 mM EDTA, DNAse-sensitive filaments (diameter 1.7 nm) are seen to be wrapped around the nucleosomes.

The wet chromosomes in each metaphase spread are held to each other by smooth microtubular fibers, 20–30 nm in diameter. Before they enter into a chromsome, these fibers branch into 9–13 protofilaments, each 5 nm wide. The chromosome surface contains a dense distribution of subunits about 10–25 nm in diameter. This size distribution corresponds to that of nucleosomes and their superbeads. Distinct from this beaded chromosome surface are several smooth, 23–30-nm-diameter fibers, which are longitudinal at the centromere and seem to continue into the chromatid structure. The surface replicas of dried chromosomes do not show these features, which are revealed only in wet chromosomes.

Key words: **wet replicas, microsurface spreading, DNA, subunits, superbeads, supercoils, fibers, chromosome bridges**

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In the basic chromatin fibril a "bead and bridge"-like pattern has recently been revealed by various techniques, particularly whole-mount electron microscopy [1–5] and partial nuclease digestion [6–12] of eukaryotic nuclei and of isolated chromatin. The complexes of DNA and about four classes of histones (H_{2A}, H_{2B}, H_3, and H_4) occur as spherical particles – nu-bodies [1, 4, 5], nucleosomes [6], or PS particles [2, 3] – about 8–15 nm in diameter, connected by small stretches of DNA. The general consensus is that H_1 (or H_5) histones may cause supercoiling of the chromatin particles into polynucleosomes. The chromatin superstructure, its packing within each chromosome, and the arrangement of the chromosome complement within the confines of the nuclear envelope are still largely unknown.

Various models of polynucleosome structure have been tested [13], including linear string, flexible string, and two-, four-, and six-contact helices. Contact helices (eg, solenoidal) are apparently the favored model as the result of more recent evidence from freeze-etch replication of Mg^{2+}-induced solenoidal coils of nucleosome strands, each containing about 40 nucleosomes [14]. Those coiled fibers are about 3 nucleosomes wide (ie, diameter 30–35 nm) and have about 6 nucleosomes per turn of the nucleosome helix.

Bak et al [15] have shown from thin-section studies that the compact chromatid structure is a hierachy of helices. Conceivably the higher-order structure(s) of the Finch and Klug-type fiber [14] could be found in whole-mount preparations of interphase chromatin. Thin-section [16], surface-spreading [17–20], and x-ray diffraction [19] studies have also indicated that the primary strand of chromatin is a slightly knobby or smooth fiber, 20–30 nm in diameter. The Finch and Klug-type fiber, which has a similar width, appears to be the analog of this knobby fiber, but the relationship has not been studied thoroughly. Current biochemical [9, 21] and neutron diffraction studies [22–24] emphasize that nucleosomes are wrapped by the DNA molecule. Direct visualization of the DNA molecule on the surfaces of the nucleosomes will be necessary to support this view.

The neutron diffraction studies [22–24] and numerous physicochemical and direct electron microscopic [25] studies indicate that nucleoprotein structure is influenced by salt concentration and by its hydrated state. The supercoiled structures of chromatin and chromosomes having characteristic small-angle x-ray diffraction rings are always lost or altered by the denaturing effect of dehydration in critical-point drying and by surface tensional collapse in air-drying [26–28]. Microtubules and actin filaments often dissociate or degrade in the critical-point drying solvents [29, 30]. It is therefore extremely desirable that for electron microscopy the structures of chromatin and chromosomes, particularly the chromatin superstructure, be studied with nondenatured preparations.

As a preliminary step to this goal, wet, unstained, and unfixed chromatins and chromosomes have been examined by an application of the wet replication technique [31–33]. The physical aspects of this new wet replica technique are established, including the penetration of a replicating vapor through a water vapor barrier [33], the replication of water droplets and water surfaces [31, 32], and the ability to replicate shapes [31, 33]. Coarser details up to 20 nm (eg, bacterial pili) have been shown in surface replicas of standard biological specimens, such as bacteria, spores, human red blood cells, and mouse fibroblasts [31, 33]. The chromatin has been spread onto electron microscopic grids by suitable methods employing various agents (eg, ethylenediamine tetraacetic acid [EDTA], $MgCl_2$, and Photo-Flo [Kodak]) that facilitate studies of the organization of chromatin [1–5, 14].

Observation of these variously treated chromatins has revealed new characteristics of the chromatin superstructure. In addition, direct electron microscopic evidence has been

obtained to prove the inevitable tendency of the chromatin superstructure for a transition into a "beads-on-a-string" configuration when the bound water is removed by drying or by strong fixation. The wet replication technique has also yielded new information about the chromosome surface structure. This work has been presented in preliminary form on several occasions [34–37].

MATERIALS AND METHODS

Wet Chromatin: Preparation, Microsurface Spreading, and Wet Replication

Fresh chicken erythrocyte and rat liver nuclei were isolated and then purified, basically according to the method of Jackson [38]. In the five stock preparations (6–10 ml) the concentration of nuclei was about 4×10^7 per ml. The nuclei in 1 ml aliquots were completely digestible by about 125 units of micrococcal nuclease (Miles Labs. Inc., Elkhart, Indiana) in about 10 min. At pH 11.6 the alkali-digested nuclei represented mainly the DNA spectrum at the shorter UV wavelengths, with a maximum absorption at 260 nm.

Colorimetric tests [39–41] using calf thymus DNA, bovine serum albumin, and yeast RNA as standards have shown that the nuclear spreads used for electron microscopy had full complements of DNA, protein, and RNA. For example, chicken erythrocyte nuclei contained 23% DNA, about 75% protein, and 2–3% RNA. These tests proved that the materials on electron microscopic grids were chromatin, with very little or no ribonucleoprotein contamination.

For electron microscopy the nuclei, recovered after pelleting from high sucrose concentrations, were washed in 10 mM sodium-EDTA, pH 7.2, and diluted about 200-fold with 0.002 M sodium-EDTA [4]. The nuclei were allowed to swell for 15 min with intermittent shaking at 0–4°C; swelling was checked under a phase microscope. Within about 10–15 min most swollen nuclei settled to the bottom of the tube, where they formed a loosely packed pellet. The supernatant was carefully withdrawn and discarded, and 20 μl of the loosely packed nuclei (10^7 per ml) were pipetted and then touched to a small convex surface of water containing a desired hypophase – eg, 1–2 mM EDTA (pH 7.2), 0.2–1 mM $MgCl_2$, or dilute Photo-Flo (three drops of Kodak Photo-Flo 200 per 50 ml of 1 mM EDTA, pH 7.0). This microsurface spreading was performed in a clean, translucent, plastic test-tube cap (diameter 0.5–1 cm, height 1 cm), partly submerged in an ice-water mixture (4°C). Further swelling, osmotic burst of nuclei, and spreading of chromatin occurred at the air–water interface and were monitored with a binocular or an inverted microscope. This is a modification of the microsurface spreading procedure introduced by Parsons [42] for investigations of swollen mitochondria.

Formvar-supported 400-mesh grids, or grids containing 5-nm-carbon-coated Formvar (0.5% in ethylene dichloride), were glow-discharged (30 sec at 0.3 Pa of residual air pressure) just prior to use. Glow discharge etches the grid substrate and makes it hydrophilic [43]. As soon as the chromatin spread, the grids were touched horizontally onto the hypophase containing the spread chromatin. The wet specimen grid was then placed in a humidity box full of saturated water vapor (100% relative humidity), and the excess water on the grid was withdrawn by touching the edges of the grid with torn filter paper. The residual aqueous layer above and surrounding the specimen was usually thinned to a minimal thickness ($\leqslant$1 nm). This thinning was done very rapidly (1–2 min) in a flood of light to avoid condensation of water vapor onto the grid.

After the water had been thinned, the wet specimens were replicated with SiO at

equilibrium water vapor pressure (eg, 3199 Pa, 25°C). Details of the wet replication technique have been published [31–33]. The specimen support (Formvar) and the specimen material were digested in ethylene dichloride (solvent for Formvar) and dilute (10 ×) chromosulfuric acid, respectively [31]. Further shadowing of the replicas (Pt-C at a 35° angle) provided high contrast. Any loss of resolution due to postshadowing was compensated by controlling the thickness (3–5 nm) of the SiO replicas. The shadowed replicas were studied in a transmission electron microscope at 80 kV, precalibrated with a carbon grating replica and periodic catalase crystals.

Most biological surfaces are hydrophilic. Their gross surfaces exhibit a small contact angle (17°–20°) to bulk water – eg, flat water or droplets with large radii of curvature [44]. Chromatins and chromosomes are therefore mounted onto hydrophilic substrates to facilitate thinning of water on the wet specimens [33]. In the ideal situation the contact (equilibrium) angles of the specimen and the substrate match each other, and this leads to uniform thinning of the aqueous layer. The etching effect in glow discharge also facilitates firm adsorption of the specimens onto the grid substrate.

A humidity box with a built-in interference microscope is usually employed to assess the thinning problem, but direct measurement of water thickness of $\leqslant 1$ nm is beyond the capacity of an interference microscope. Replication experiments with small solid markers (eg, ferritin, diameter 12 nm; latex spheres, diameter 85 nm and 88 nm; etc.) have essentially proved the reliability of the thinning procedure described above. In principle the water thickness was gauged by comparing the apparent shadow lengths of those markers projecting through any water layer ($\geqslant 1$ nm) with their shadow lengths as dried markers on identical substrate-containing grids. Because the latex aerosols and ferritin particles aggregate particularly with chromatin and chromosomes, they were added to these specimens in some experiments in order to check the approximate sizes of nucleosomes, superbeads, protofilaments, etc. This precaution has partially avoided any discrepancy due to replica thickness (3–5 nm), shadow deposit (~ 1 nm), or water layer thickness (< 1 nm), all of which were similar for the markers and the chromosomes (or chromatins).

Conventional Chromatin Preparations

Fresh chicken erythrocyte and rat liver nuclei were also used for standard types of stained-and-dried and wet wet chromatin preparations, following particularly Olins et al [2, 4]. Briefly, the swollen nuclei were fixed for 30 min in 1% formaldehyde in 0.001 M sodium-EDTA and then centrifuged through 10% formaldehyde (pH 7.0) onto glow-discharged carbon-coated 400-mesh grids. The wet fixed chromatins and unfixed controls were washed in dilute Photo-Flo (three drops per 50 ml of 1 mM EDTA, pH 7.0). Some of the chromatin-containing grids were vacuum-dried and then shadowed with Pt-C unidirectionally at a 35° angle. Other grids were stained in 5 mM aqueous uranyl acetate and then air-dried or studied wet.

Tissue Culture and Chromosome Preparation

Chinese hamster DON and DEDE cells were grown, isolated at near-neutral pH, and spread at 4°C on a small surface of dilute (100 ×) buffer, as described in the previous section. For chromosome isolation, in principle, the method of Wray and Stubblefield [45] was followed up to the hypotonic swelling of cells in a chromosome buffer (1.0 M hexylene glycol, 0.5 mM $CaCl_2$, and 0.1 mM PIPES) at pH 6.8. Various acid and neutral pH buffers,

including the standard acid fixative methanol-acetic acid (3:1), were also used; but the best results, showing clear and intact details of chromosomes in each metaphase spread, were obtained by applying the present microsurface spreading method to swollen cells (10^8/ml) in the hexylene glycol-PIPES buffer. This method also avoids loss of material.

Eight good fresh batches of cultures (five for DEDE cells, three for DON cells) were used for chromosome isolation at a near-neutral pH. Each of these eight chromosome preparations was spread numerous times, for a total of 83 occasions, and studied in detail. The percentage of usable spreads on each grid was about 40%.

Critical-Point Drying and Vacuum Drying of Chromosomes

For critical-point drying, 100-mesh tabbed gold grids (Ted Pella, Tustin, California) containing glow-discharged carbon-coated Formvar were used. The grid tabs were inserted into an annular steel grid holder consisting of two concentric rings centered on a disc. The chromosomes were fixed in 1% glutaraldehyde for 2 h at 4°C in the hexylene glycol-PIPES buffer and dehydrated in whole mounts on grids in a series of ethanol-water mixtures (10, 20, 30, 40, 50, 75, 95 and 100% ethanol). The grid holder was then rinsed in a series of alcohol-Freon 113 mixtures (25, 50, 75, and 100% Freon 113). Each dehydration step and each rinse lasted 2 min. The chromosomes were critical-point dried in Freon 13 in a bomb similar to that of Cohen et al [46].

RESULTS AND EXPLANATION

Superstructures (Larger Than Nucleosomes) of Wet, Unstained, Unfixed, Inactive Chromatin

Clues to a regular organization of a 30–35 nm diameter fiber (F) were obtained by surface-spreading the nuclei on Photo-Flo diluted with 1 mM EDTA (Fig. 1a). The higher-order structure of this fiber is a wiggly, supercoiled organization whose helices or coilings (C_1, C_2, C_3) are shown at a higher magnification in Fig. 1b. These coilings have different orientations owing to the convoluted configuration of F. For example, C_1, C_2, and C_3 are in different planes perpendicular to the local axis represented by F. This feature gives the impression that the superstructure consists of alternating looping and longitudinal orientations of F. The width of the supercoiled organization across the diameter of the coilings is about 120–150 nm. The distances (pitch) between these coilings are in the range of 300–400 nm. In superstructures where the coilings are closely apposed, the organization is up to 0.2 μm wide (not shown).

As a result of loosening, the rotations of the nucleosome strand in F are found in several on-edge (S_1) and flat (S_2) orientations. The on-edge turns are mutually perpendicular, whereas the flat orientations resemble donuts or anchor rings, with holes at their centers. On a first look these rotations of the nucleosome strand appear to be some type of superbeads (or supernucleosomes), whereas F appears to be a loose stack of such superbeads (C_3 in Fig. 1b), where the nucleosomes are highly contiguous and not discernible. A comparison of their perimeter with the width of nucleosome suggests that about 4–6 nucleosomes would be contained in each nucleosome turn or superbead [14, 47, 48]. The DNA packaging ratio in this superstructure is about 1,400, assuming that the ratio in the nucleosome strand (contiguous beads) is about 7 – ie, 200 base pairs of DNA per nucleosome [6, 9, 21].

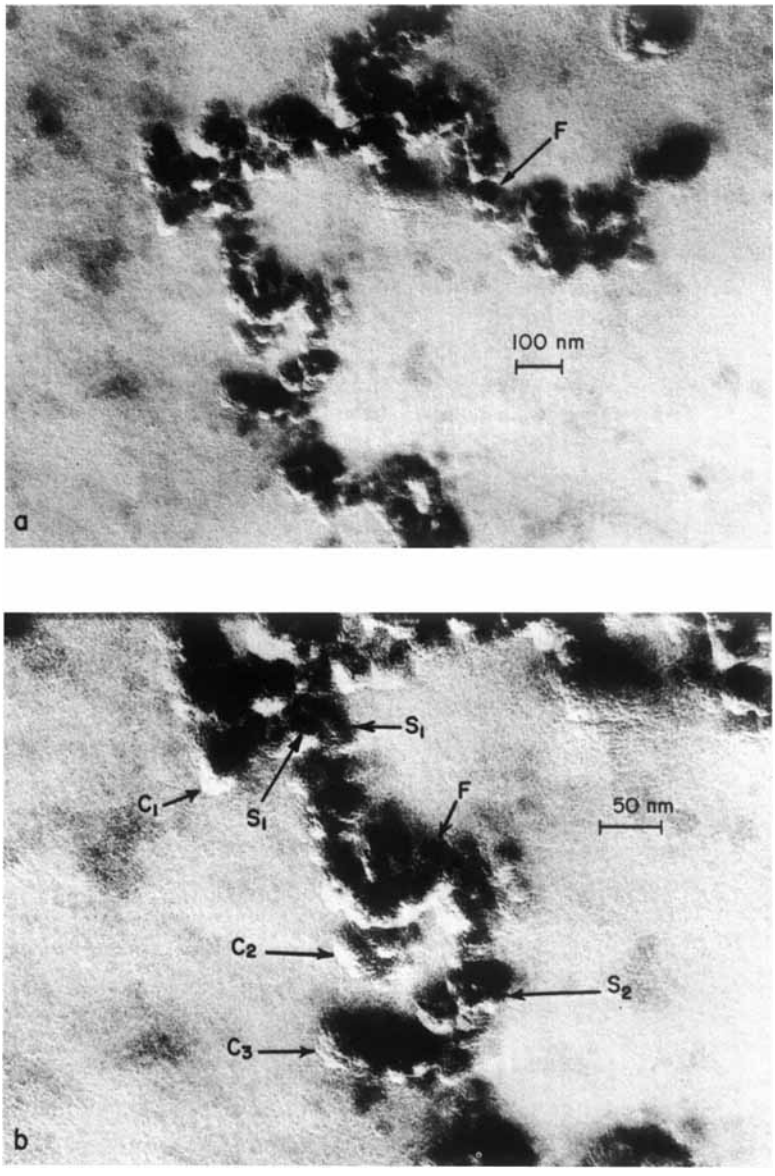


Fig. 1. SiO-replicas (Pt-C shadowed at 35°) of surface-spread chicken erythrocyte chromatin, studied wet after spreading in dilute Photo-Flo (Kodak, solvent 200) in 1 mM EDTA. (a) Wiggly (loosened) superhelical organization of the polynucleosome fiber (F). (b) Higher magnification of a region in (a). C_1, C_2, and C_3 are coilings of the fiber F. Rotations of the nucleosome strand forming the fiber F are indicated by S_1 (on edge) and S_2 (flat). These S structures are called superbeads (or supernucleosomes) in the text.

Effect of EDTA and Mg-Ions

Figure 2a represents the SiO (shadow-cast) replicas of wet chromatin prepared by surface-spreading of chicken erythrocyte nuclei on 1 mM EDTA (pH 7.00). The structure of chromatin is unwound and irregular due to the effect (chelation) of EDTA (marked 2 in Fig. 2a). A decondensed branched structure of the nucleosome strand is also shown (marked 1, upper left corner in Fig. 2a). Nevertheless, structure 2 indicates that the nucleosome strand (arrow "f") had been packed into two higher levels of organization in the original chromatin molecule. This nucleosome strand seems to be packed into a thicker region (nd), whose width for the most part is approximately 25–35 nm, due to its wavy configuration. The nucleosomes (n) are in arrays across this fiber. Finally, structures resembling supertwists or supercoiling are seen. One supertwist is intact (n_T), whereas another (at D) seems to be flattened.

Despite the unwinding effect of EDTA these preparations, as well as fixed and stained but wet chromatins, showed an important result. Filamentous materials about 1.7 nm wide, similar to the width of DNA strands, are seen wrapped around some nucleosomes (at 2). Since the width of DNA is very close to the resolution limit in these experiments, a higher magnification of one region (D) is shown in an inset to illustrate this finding. (See also "Tests for Wet Chromatin Fibers and DNA, Etc," below.)

When $MgCl_2$ (0.2–0.5 mM) is used as hypophase for spreading, a compact, coiled configuration of the fiber F is often seen in which the nucleosomes are no longer visible (Fig. 2b). The nuclei for this preparation had been swollen in EDTA. Note that this fiber F is much smoother than the fiber nd in Figure 2a. Like the chromatin in Figure 1 these preparations were wet, unstained, and unfixed. A comparison of chromatin in Figures 1 and 2 shows that, although the nuclei were preswollen in EDTA in both cases, the final environment on the grid is more determining of chromatin superstructure. The irregular convolutions of the smooth F fiber in Figure 2b are perhaps due to excess Mg^{2+} ions incorporated in the chromatin.

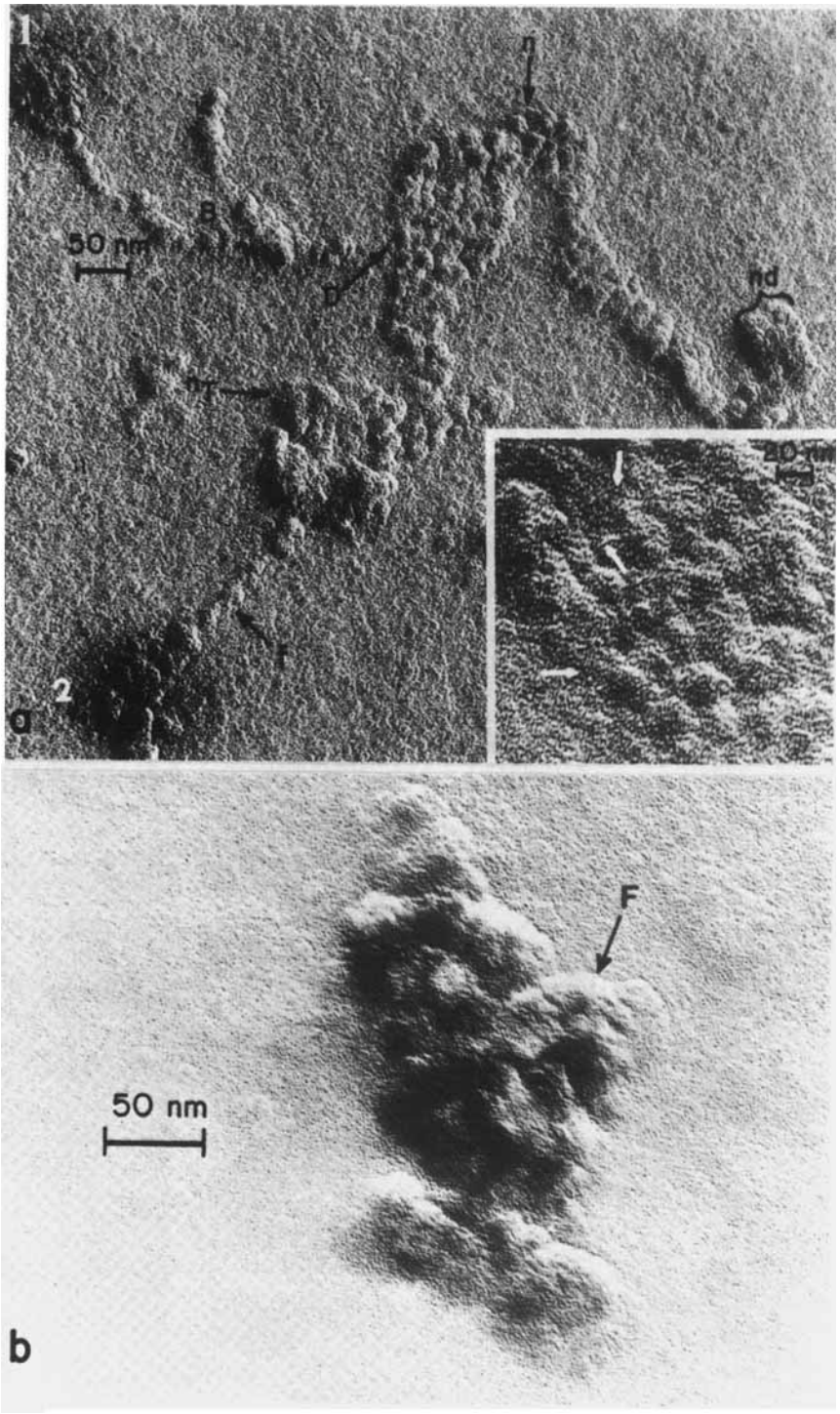
The reader is reminded that the 30–35-nm fiber F has a remarkable analogy with the Finch and Klug fiber [14]. It is understandable why those authors did not see the supercoiling of the F fiber. They used only 40-nucleosome-long strands to make supercoils with Mg^{2+} ions, whereas each superhelical turn of the superstructure in Figure 1 would contain about 30–40 solenoidal rotations of the nucleosome strand.

Size of Wet Nucleosomes and Frequency of Chromatin Studied

Both the shadow-cast replicas and the real size ($\sim$12–14 nm diameter) of the wet nucleosomes (n, Fig. 2a) were a little larger than those of wet whole ferritin molecules (diameter 12.5 nm). A water layer of $\leqslant$1 nm, a replica thickness of 3 nm, and a shadow deposit of 1 nm are taken into account. The water layer in these experiments was clearly less than the diameter of DNA, as the smoothness of the replica also demonstrates.

Effect of Strong Fixation on Chromatin Superstructure

Further evidence of the superbead and supercoiled organization of the fiber F was obtained with wet, fixed chromatin prepared by the centrifugation method of Olins et al [4]. Strong fixation such as 10% formalin apparently collapses the coilings (helices) of F (Fig. 3). About 90% of the materials on the grids had this configuration (see Discussion). The unwound supercoil, as it spills out of the nuclei (N) lysed on the specimen grid, resembles Olympic rings [1]. These rings consist mainly of stretched superbeads (arrows S



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in Fig. 3b). The nucleosomes (n) are seen when the superbeads uncoil into a chain of those subunits. A cluster of nucleosomes is shown by an arrow C.

The area labeled N (for nucleus) in Figure 3a represents only that portion of the nucleus where spilling of chromatin occurred. This spilled chromatin is copious, equivalent to at least 100-μm-long DNA. A continuation of the chromatin from the left of Figure 3a is shown in Figure 3b. It continued as a nucleosome (n) strand in a zigzag orientation up to 4 μm beyond the area shown to the left in Figure 3b (arrows n).

The width of the superbeads (S) is again the same as that of F (25–35 nm), but they are mostly spherical and rarely donut-shaped. When the continuity of the nucleosome strand breaks down, those rings occur as open or closed circles. Each isolated ring (not shown) consists of about 50–60 superbeads. The DNA packaging ratio in each superbead is about 25 [47, 48]. Therefore the ratio in each ring is in the range of 1,250–1,500. (This corresponds to about 56,000 base pairs of DNA or 19-μm-long DNA.) Interestingly, the packaging ratio of each ring corresponds to the DNA packaging ratio of each coiling in Figure 1. Since DNA packaging in the second-order nucleosome superstructure (Fig. 1) is based upon solenoidal packing of nucleosomes, this agreement supports the solenoidal model of Finch and Klug.

The collapse of the chromatin superstructure in Figure 3 was not due to the shearing forces during nuclear explosion. Nuclei under various stages of explosion (ie, spilling of chromatin) indicated that the supercoil actually collapses in nuclei during strong fixation (~10% formalin). The result in Figure 3 suggests that formalin attacks at intervals along the chromatin strand, separated by the observed superbeads.

Tests for Wet Chromatin Fibers and DNA, Etc

To test the superstructure in Figure 1, the specimen grids containing spread chromatin were floated onto droplets of micrococcal nuclease (125 units/ml) in 1 mM $CaCl_2$ for 10 min at 37°C. These experiments were performed three times, each time with several grids. Replicas of these grids revealed beads and superbeads but no regular organization of them. However, a fourth and a fifth experiment with a 10-fold dilution of the enzyme produced slightly longer structures of contiguous beads (diameter 10 nm). These structures contained about 5–55 beads; the majority were 45–55 beads. In most of these structures the beads formed irregular convolutions, but in some of them regular helices of the beads were perceptible. These helices were about 30 nm wide. About 7–9 such helices were found, each containing 4–6 beads per helix.

To test the fibrillar (1.7-nm diameter) structures on nucleosomes of decondensed chromatin (Fig. 2a, inset), grids containing similar preparations were floated onto droplets of DNAse I (25 μg/ml) in 1 mM $MgCl_2$ and incubated (37°C) for 2 h. A similar test was per-

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Fig. 2. SiO replicas (Pt-C shadowed at 35°) of surface-spread chicken erythrocyte chromatin, spread in 1 mM EDTA (pH 7.2), studied wet, unfixed, and unstained. (a) A decondensed chromatin superstructure (No. 2) of a polynucleosome fiber (width 25–35 nm). The nucleosomes (n) have become visible as a result of partial unwinding of this fiber. The field also contains a broken nucleosome strand (No. 1). B = branched structure of the No. 1 strand; f = nucleosome strand of the superstructure (No. 2); nd = polynucleosome strand packed into a thicker fiber (25–35 nm); nT = supertwist of the polynucleosome fiber. The inset is a higher magnification of region D (No. 2); the arrows indicate DNA-sized molecules (diameter 1.7 nm) on several nucleosomes. (b) Spread made in 0.3 mM $MgCl_2$ (pH 7.0) shows a polynucleosome fiber (F) with indistinguishable nucleosomes, smoother than the structure in (a), and irregularly coiled into a compact configuration.

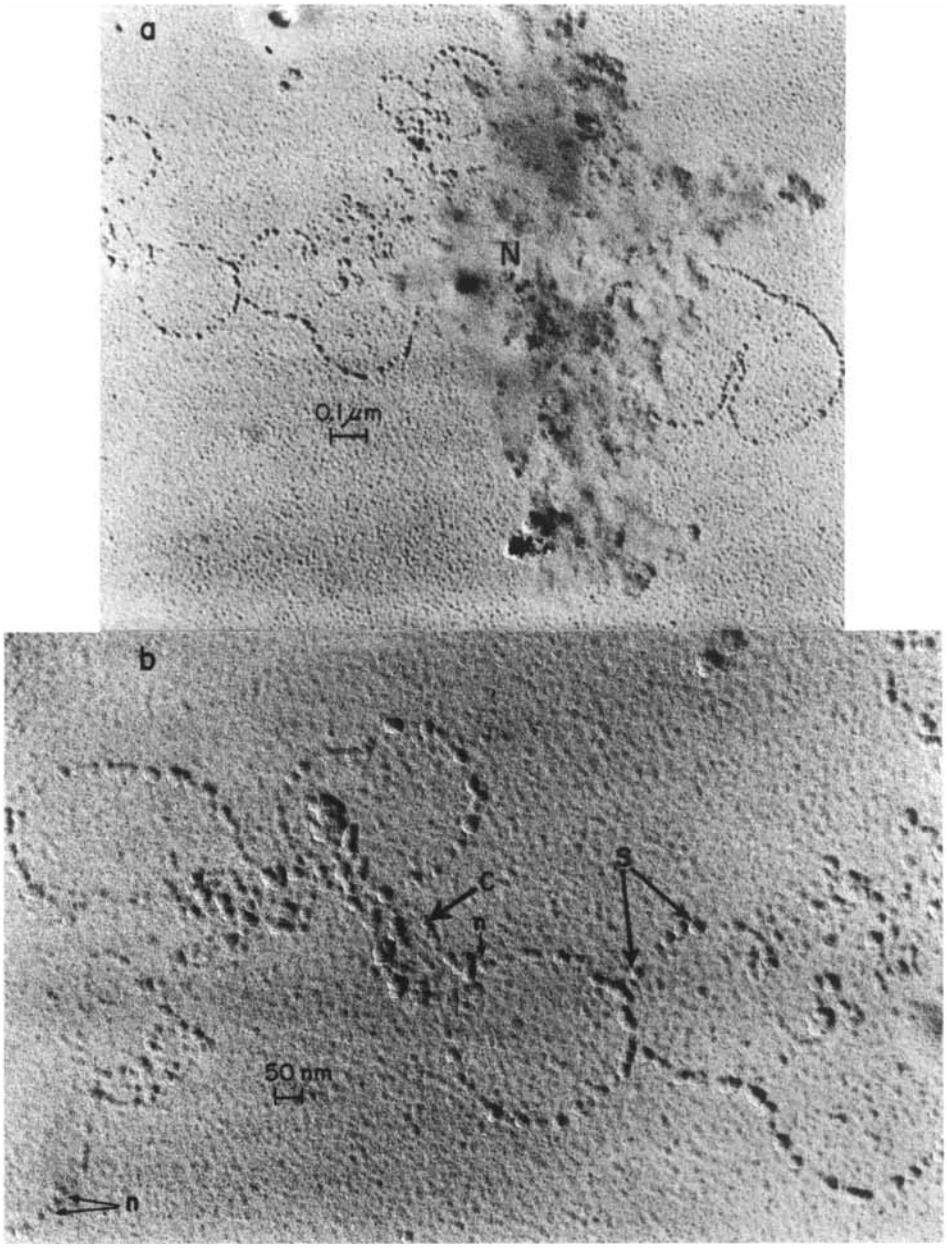

Fig. 3. Collapse of chromatin superstructure. SiO replicas (shadowed at 35°) of centrifuged chicken erythrocyte chromatin, studied wet, fixed (10% formalin, pH 7.0) and Photo-Flo-treated [4]. The chromatin spills out of a nucleus in rings of superbeads and beads. N = collapsed nucleus; C = cluster of nucleosomes; n = nucleosome; S = superbead.

formed with DNAse II (25 μg/ml) in Ca^{2+}- and Mg^{2+}-free Hank's solution. In each case core particles were found as fibers of the same size (30–35 nm) as the decondensed chromatin in Figure 2a, but no DNA-like filaments were seen on these cores.

Further colorimetric tests performed directly on the surface-spread materials (ie, hypophase) confirmed the composition of the fibers on the grids. They were mostly chromatin. These fibers did not disintegrate, as would microtubules, when grids containing fresh (ie, wet) preparations were floated onto droplets of $CaCl_2$ (1–10 mM) for 5–10 min at 4°C.

The polynucleosomal superstructures (Figs. 1 and 2, etc) reported here were strongly predominant (75–80%) with chicken erythrocyte nuclei and slightly predominant (60%) with rat liver nuclei. The rest of the fibers on the grids had a thickness of 25–35 nm, unentangled shape (length 4–40 μm) showing either a smooth configuration or a two-stranded double coil containing side-by-side 10–12-nm-wide filaments. These fibers are also sensitive to nucleases, as grids that contained these materials revealed only nucleosomes after the enzyme treatments. In view of their strikingly different configurations (eg, smooth shape) they are being characterized by definitive experiments using immune labels against DNA and histones.

Effect of Drying on Chromatin Superstructure

The effect of drying on chromatin superstructures was similar to the effect of strong fixation. This was true for air-drying (Fig. 4), vacuum-drying, and critical-point drying with Freon 13 as the transition fluid (not shown).

Stained, fixed, air-dried preparations of chicken erythrocyte nuclei were made by the method of Olins et al [4]. These materials are shown at two different magnifications in Figure 4. Most of the dried chromatin appeared as a configuration of the nucleosome strand stretched into linear arrays of superbeads (s) and beads (n). A very minor population of knobby fibers (20–30 nm) and their aggregates was occasionally seen. Clearly the surface tensional stress in drying collapses most higher-order structures and stretches the nucleosomes (n) apart. These collapsed structures are found if the chromatin preparations are finally dried by any means, regardless of whether treatments such as formaldehyde fixation, Photo-Flo treatment, and positive staining with uranyl acetate have been applied.

This is strong evidence that the higher-order structures of chromatin are sensitive to dehydration, to surface-tensional stress in drying, and to other environmental factors which may remove the bound water from chromatin. The diameter of the dried nucleosomes or subunits is about 9.2 nm in stained materials (Fig. 4) and about 12–15 nm in the shadowed material (not shown).

Wet Chromosome Surface

The wet chromosome replicas (Fig. 5) have in general the following ultrastructural features, which were seen in eight different chromosome preparations, each prepared from a fresh batch of cultures.

The wet chromosome surface contains a dense distribution of subunits, most of which form two major populations by size. If the replica thickness (3 nm), the water thickness ($\leq$1 nm), and the shadow deposit (1 nm) are taken into consideration, the size of the subunits (10–25 nm) agrees with the diameter of nucleosomes and with the rotations, (ie, superbeads) of the polynucleosome fiber. In Figure 5b the subunit shown by n corresponds to the size of a nucleosome. In Figure 5 b and d the features designated K show several larger subunits in arrays of the size of superbeads. The replicas convincingly represented a

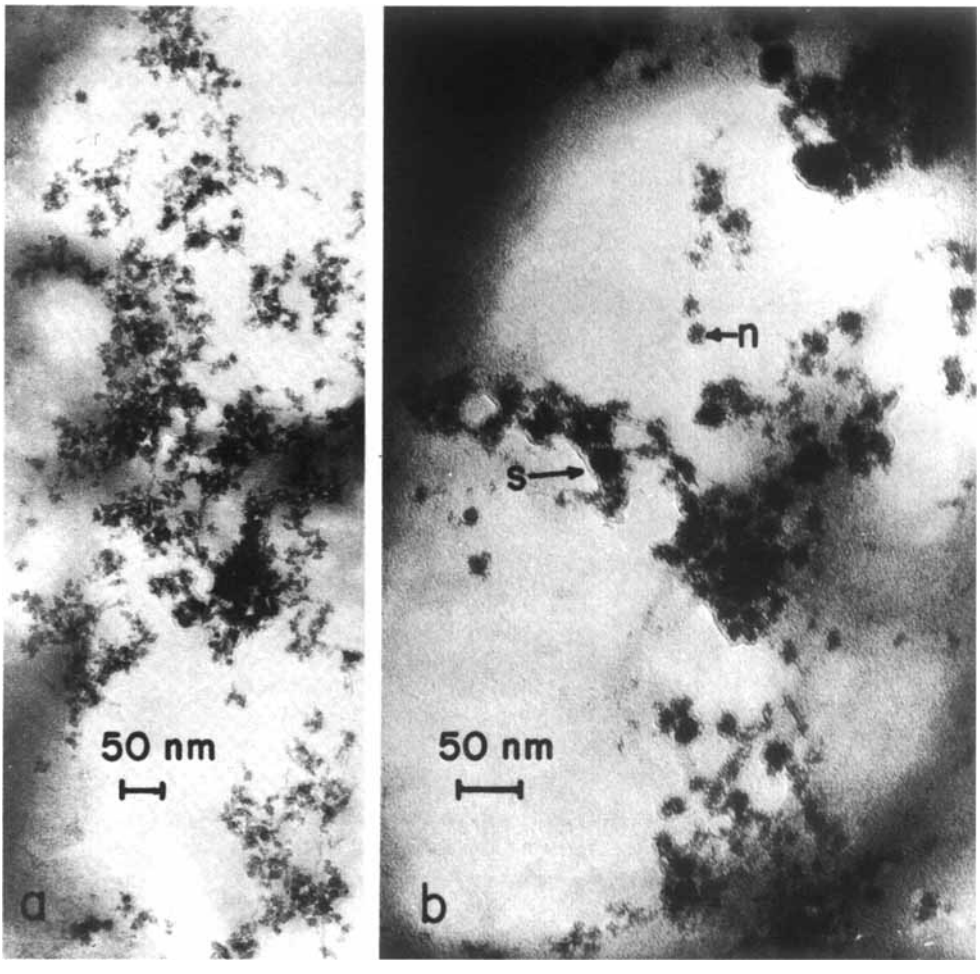

Fig. 4. Chick erythrocyte nuclei fixed in 1% formaldehyde and then centrifuged through 10% formaldehyde onto glow-discharged grids. The grids were washed in Photo-Flo, stained in aqueous uranyl acetate (5 mM), and air-dried [4]. Two magnifications are shown. n = nucleosome; S = superbead.

number of smooth fibers in longitudinal orientations in the centromere (c) region (arrows F in Fig. 5b, d). These fibers seem to penetrate the chromosome arms and are lost from the top view because of the surrounding beaded chromosome surface. These smooth centromeric fibers have never before been reported.

The chromosomes in metaphase spreads are seen oriented at right angles or parallel to each other and are connected at specific regions; ie, the telomere of one chromosome is attached to the telomere or to the nearest centromere region of a neighboring chromosome by parallel bridging fibers (arrow in Fig. 5a). These fibers are generally smooth and about 20 nm in diameter, although some are thicker (up to 35 nm diameter). These fibers and the end-to-end attachments (M in Fig. 6a) between telomeres of successive chromosomes (C_1 and C_2) are microtubules, 20–35 nm in diameter, as they disintegrate on treatment with 1 mM $CaCl_2$. More interestingly, they branch into several 5-nm-diameter protofilaments be-

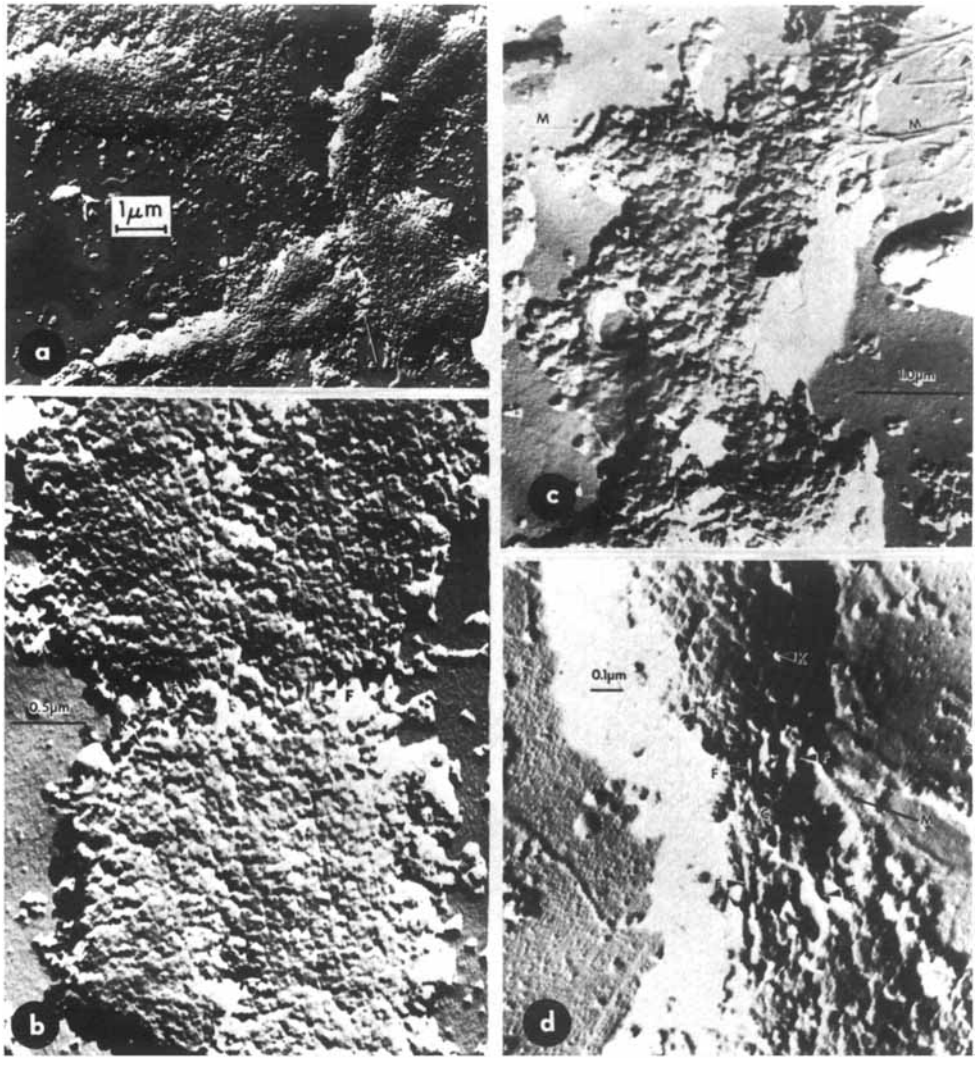

Fig. 5. SiO (Pt-C shadowed, 35°) replicas of wet unstained and unfixed Chinese hamster chromosomes in a metaphase spread.

fore penetrating into a telomere (arrowheads and M in Fig. 5c). Similar long fibers (20-nm diameter) are seen attached just above and below the centromere of some chromosomes (M in Fig. 5d); ie, their attachment to the centromere is at a grazing angle. This particular finding was first reported by Hoskins [49], who performed micromanipulation on the mitotic apparatus.

The smaller chromosome (C_2) in Figure 6a is about 1.8 μm long and metacentric; it may be the X chromosome. The fibers radiating from the telomeric lobe of this chromosome distinguish it from many larger chromosomes, such as C_1, as more microtubules seem to enter or come out of this small chromosome. The materials between the C_1 and C_2 chromosomes in Figure 6a are membranous and not chromosomes.

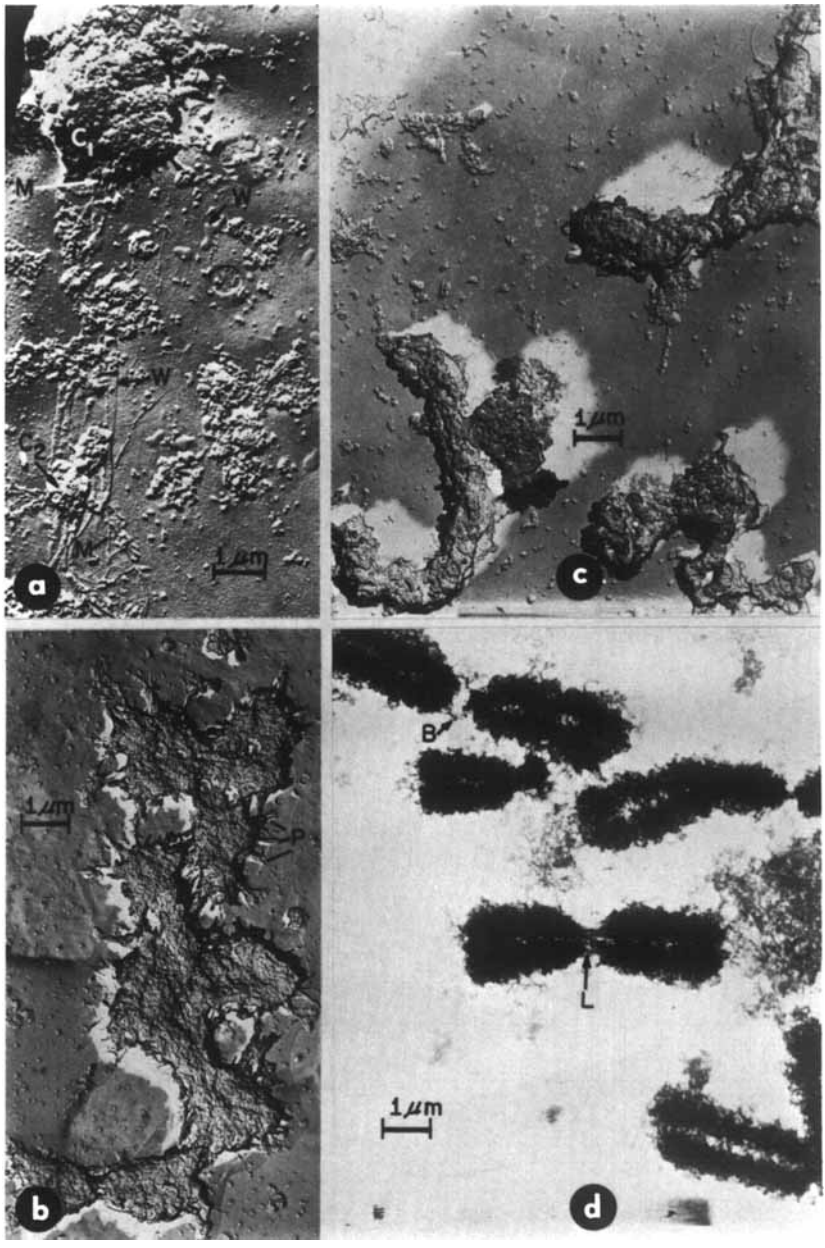

Fig. 6. (a) Replica showing attachment between telomeres of two (C_1 and C_2) wet chromosomes, prepared as in Figure 5. (b) SiO (Pt-C shadowed, 35°) replicas of glutaraldehyde-fixed, critical-point-dried (Freon 113 + 13) Chinese hamster chromosomes. (c) Replicas of chromosomes prepared as for Figure 5, except air-dried. (d) Unfixed, unstained, and air-dried chromosomes observed under a transmission electron microscope.

Figures 5c and 5d particularly show that the water layer (G in Fig. 5d) has been sufficiently thinned by the use of hydrophilic grids for easy resolution of fibrils (5 nm) and fibers (20 nm). On occasion excess water is trapped between parallel fibers or occurs in small patches (W in Fig. 6a). Otherwise the chromosome surface does not retain excess water.

Surface of Dried Chromosomes

The compact shape and surface topography of the subunits (10–25 nm in diameter) are lost, to a greater or lesser degree, on critical-point drying (Fig. 6b) or air-drying (Fig. 6c). Such replicas also differ among themselves. The lateral surface projections seen on critical-point drying (P in Fig. 6b) are spurious because they do not project from the chromosome surface. Similarly, the convolutions of thick (50-nm) fibers seen on air-drying (Fig. 6c) are questionable. Some deleterious effects of chemicals (alcohol, Freon 113, etc) used in critical-point drying are evident in the featureless surface topography of the chromosomes and their adherence to the substrate (Fig. 6b).

The best-known scanning micrographs of critical-point-dried (CO_2) chromosomes [50–53] resemble Figure 6c rather than Figure 6b but are subject to one or the other type of artifacts. These artifacts cannot all be due to buffer-fixative or to poor fixation. Dehydration in general causes a collapse of nucleoprotein fibers and their supercoils in metaphase chromosomes, as is evident from x-ray diffraction studies [27, 28].

Transmission images of the air-dried chromosomes (Fig. 6d) are conventional in appearance, representing primarily the classic condensed chromosomes with tortuous looping and longitudinal fibers [50, 51, 54, 55]. The longitudinal fibers (L; 23-nm diameter) are seen in the centromere region when it is not broken by surface tensional stress (see break B).

DISCUSSION

Thinning of liquid water to a few angstroms was an important achievement, and it has led to the present study. It has potential benefits for replication study of biological specimens in general. Thinning is also helpful in obtaining high-resolution electron diffraction patterns and transmission images of wet and frozen biological specimens because this procedure leaves the specimen wet by its bound water. This approach avoids specimen drying and preparative steps such as fixation and staining.

Various authors have inferred that the higher-order structures in inactive chromatins are damaged by drying and dehydration [4, 26, 27, 56]. The present finding of a transition of the polynucleosome superstructure into unsupercoiled nucleosome filaments on drying (eg, Fig. 4 a and b) and on dehydration by severe fixation (Fig. 3) also supports the x-ray diffraction results [26, 27, 56].

Wet replicas and the drying effect demonstrate that nucleosomes are highly contiguous in wet nucleosome filaments. This resolves a question left open in other reports [3, 57]. The appearance of DNA filaments on the nucleosome surface and their susceptibility to DNAse I and II support a recent neutron diffraction finding that DNA may wrap around the nucleosomes [22–24].

The highest organization of the nucleosome filament in inactive chromatin of chicken erythrocytes and rat liver is a second-order supercoiled structure (ie, a supersolenoid) about 0.15-μm wide (Fig. 1). Nicolini and Kendall [58] have inferred a similar ordered structure from solution studies of chromatin using light scattering, ethidium bromide bind-

ing, circular dichroism, etc. The component which gives rise to the above supercoil is a 30–35-nm-diameter fiber (F). The organization of this fiber is basically a solenoidal (ie, first-order) coil of the nucleosome filament [14]. However, DNA may be exposed at intervals of 4–6 nucleosomes, and it may have small denaturation defects in those regions. Then the solenoidal coil would take the shape of a chain of superbeads under the action of some environmental agents (eg, formalin) or a stretching force (eg, surface tension), which would attack the exposed DNA.

Both models, the solenoid [14, 59–61] and the chain-of-superbeads [47, 48], are thus theoretically relevant to the final second-order superstructure of chromatin. A few recent studies have shown that it is possible to visualize both types of structure from the same chromatin preparations, depending upon the environmental conditions (ionic strength, divalent cations, etc [48, 62]). Stressing the lack of regularity of the solenoidal coiling of the nucleosome filament and taking into account the chain-of-superbeads model, Worcel [63] has given a revised minisolenoid version of his previous solenoid model [59, 61].

The possible explanation might be a repeating biochemical discontinuity in the form of small denaturation defects in the DNA of inactive chromatin. One may suspect that the torsional convolutions of the F-fiber, as it describes the next-higher-order structure, imposes a strain upon the DNA backbone. This may cause partial loss of base-stacking within the framework of the double helix. In fact, the chromatin DNA is slightly (8–10%) hyperchromic in ultraviolet absorption at 260 nm, where DNA has its peak absorption [64, 65]. Circular dichroism, optical rotatory dispersion, and DNA melting studies also suggest that, while histones stabilize the overall DNA-structure, they incorporate into the latter some denaturation defects [65–67]. This conformational feature of the chromatin DNA has been also characterized by applying 1–10% formalin [68], as formalin attacks only denatured regions of DNA and causes a further rise in ultraviolet absorption of DNA.

The repeating subunit (nucleosome) structure of chromatin should give us a new feature to distinguish the basic chromosome fibril from the extrachromosomal processes (eg, microtubules) which assemble onto the chromosomes in mitosis. This feature is readily seen in replicas of wet metaphase chromosomes. In dried chromosomes, on the other hand, the microtubules are hardly distinguishable from the chromosome fibrils (Fig. 6 b–d), and neither critical-point-dried (Fig. 6b) nor air-dried (Fig. 6c) chromosomes show any evidence of nucleosomes in the tortuous looping fibers of the chromosomes (Fig. 6d).

The subunits of the wet metaphase chromosome surface (Fig. 5 b and d) are the size (10–25 nm) of nucleosomes and supernucleosomes (ie, superbeads). It is quite unlikely that they could be anything else. The surface texture of the wet metaphase chromosomes does not immediately indicate any possible folding of the nucleosome filament and its higher-order fiber. While this may be looked upon as unsatisfactory, it suggests that the main chromosome fiber cannot be much thicker than 30 nm. There are other features that are more interesting – eg, the telomeric attachments (M in Fig. 5c) and the rather unbeaded, centromeric, longitudinal fibers (F in Fig. 5 b and d).

The branching of the telomeric attachments (fibers) into several thin fibrils is typical of microtubules, both in the number of separating filaments (about 9–13) and their diameter (5 nm), which is that of the protofilaments constituting a microtubule fiber [69]. These telomeric attachments do not appear to have nucleosome-type subunits. Transmission electron microscopy at high voltages may be helpful in explaining whether (or not) these telomeric extrachromosomal fibers have some connection with the smooth, centromeric, longitudinal fibers (diameter 23–30 nm). The latter are too thick to be

double-stranded DNA. These longitudinal fibers do not disintegrate when chromosomes on the grids are treated with $CaCl_2$ (1–10 mM) at 4°C. Under the same conditions the telomeric and the centromeric attachments dissociate from the chromosomes [70]. Strangely, the telomere-to-telomere and centromere-to-telomere attachments were also seen in acid-fixed (acetic acid:methanol = 1:3) DON and DEDE chromosomes. Paulson and Laemmli [71] suggest that a class of backbone fibers in chromosomes could organize the rest of the chromatin into a looping arrangement. According to them [71] these backbone fibers are made up of nonhistone proteins. The replicas of wet chromatids in G_1 and in G_2 are being examined to study these various problems and the connection of the inactive chromatin structure (Fig. 1) with the chromosome organization.

Direct contact of microtubules with chromatin fibers has been shown in birds [72], in lower eukaryotes [73], and in fungi, eg, yeast [74]. Thin section studies show that in some eukaryotes the microtubules attach to each chromosome at the centromere [70]. The wet replicas show that microtubules (M in Fig. 5d) attach just above and below the centromere at a grazing angle. This finding agrees with two other observations [49, 75]. But this finding may also be slightly inaccurate due to possible alterations in chromosome structure during hypotonic swelling and surface spreading of chromosomes. The spreading is performed immediately after swelling, and it is done in 100-fold diluted buffer at 0°–4°C in order to minimize artifactual aggregation of the spindle remnants and any effect of hexylene glycol on the chromosomes.

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