The Structure and Function of Chromatin

Ciba Foundation Symposium 28 (new series)

1975

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Foreword: histone nomenclature

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Considerable confusion exists between the currently used histone nomenclatures. This situation has arisen because different methods for histone fractionation have been developed which have resulted not only in different nomenclatures but also in different numbers within these nomenclatures being applied to the same histone.

Two nomenclatures have wide usage: (i) that of Butler and his colleagues Johns and Phillips (Johns & Butler 1962; Phillips & Johns 1965; Johns 1969, 1971) who separated histones by selective solvent extraction and chromatography on carboxymethyl-cellulose columns; the lysine-rich histone was called F1, a slightly lysine-rich histone F2b, an intermediate histone F2a2 and the arginine-rich histones F2a1 and F3; (ii) Luck and his co-workers (Luck *et al.* 1958; Satake *et al.* 1960; Rasmussen *et al.* 1962) separated histones into three classes—I, II and III—by column chromatography on Amberlite CG-50 with a gradient of guanidinium chloride. As histone chemistry progressed, the components of each class were separated and this nomenclature was further extended by Macpherson & Murray (1965) and by Bonner and his co-workers (Fambrough *et al.* 1968). The state of confusion is illustrated by the fact that one arginine-rich histone is called F2a1 in one nomenclature and IV in the other nomenclature. These nomenclatures are summarized in the first two columns of Table 1.

In an attempt to resolve the confused state of histone nomenclature, people interested in histone chemistry met informally at the Gordon Conference on *Nuclear Proteins, Chromatin Structure and Gene Regulation* at Beaver Dam, Wisconsin (1972) and, as a result, proposed a new nomenclature based on the amino acid sequences and composition of histones, in which each histone was described by three letters (with the standard one-letter code for amino acids) of the three most abundant amino acids in the order of their abundance. This

TABLE 1
Histone nomenclature

Main histone fractions	*Proposed name*[a]	*Proposed name*[b]	*Gordon conference (1972)*	*Ciba Foundation symposium (1974)*
Lysine-rich histones	F1	Ia Ib	KAP	1 (H1)
Slightly lysine-rich histones	F2a2 F2b	IIb1 IIb2	ALK[c] KSA	2A (H2A) 2B (H2B)
Arginine-rich histones	F3 F2a1	III IV	ARK GRK	3 (H3) 4 (H4)
Other histones				
Unique lysine-rich histone from nucleated erythrocytes	F2c[d]	5[e] or V[f]	KAS	5 (H5)
Lysine-rich histone[g]		T[g]	AKP[g]	6 (H6)

[a] Johns (1969, 1971); Johns & Butler (1962); Phillips & Johns (1965).
[b] Luck *et al.* (1958); Satake *et al.* (1960); Rasmussen *et al.* (1962); Macpherson & Murray (1965); Fambrough *et al.* (1968).
[c] Sautière *et al.* (1974).
[d] Hnilica (1964).
[e] Neelin (1964); Neelin & Butler (1961).
[f] Vidali & Neelin (1968).
[g] Wigle & Dixon (1971), see Dixon *et al.*, this volume, p. 231.

nomenclature is given in the third column of Table 1. Although attempts have been made to have this nomenclature adopted by the IUPAC Nomenclature Committee, it encountered much opposition from some established users of the two nomenclatures in current usage.

This present symposium provided a particularly opportune occasion to discuss further the problem of histone nomenclature since the main protagonists and other major users of the existing nomenclatures were gathered together. Although it was agreed that a single nomenclature was desirable, the one proposed at the Gordon Conference in 1972 did not receive wide acceptance. Its opponents found it difficult to adopt and, therefore, continued to use the existing nomenclatures. Difficulties were also experienced when the second and the third most abundant amino acids were present in almost equal amounts.

The participants in the symposium expressed a general feeling that a new nomenclature should be based in some way on the two nomenclatures at present in wide use. A nomenclature combining elements of these nomenclatures was proposed and is given in the last column of Table 1. As can be seen, it enables easy adaptation by workers already using existing nomenclatures. It

incorporates features of each of the existing nomenclatures and in particular retains the number 4 for the arginine-rich histone, which was the first to be sequenced and for which the sequence conservation was so strikingly demonstrated by DeLange *et al.* (1968, 1969). (Whereas the original proposal was that histones should be called H1, H2A, H2B etc., it may be superfluous to use the prefix H in the text of manuscript, e.g. histone H4, when the form histone 4, etc., will suffice. The prefix could be used [e.g. H4] for describing histones in diagrams and captions.) Because of the high degree of conservation of some histone sequences it seems reasonable to regard the labels in this nomenclature as describing a particular class of histones in most organisms, possibly by the addition of the name of the organism in the description, for example calf histone H4 or pea histone H4, to describe the glycine, arginine-rich histones from these organisms. As other unique histones are found the nomenclature can be extended by the addition of further arabic numerals. Thus, the lysine-rich histone unique to nucleated erythrocytes becomes histone 5, as originally isolated and described by Neelin (1964; Neelin & Butler 1961).

The chemically modified histones may be described by the addition of prefixes, for example 'P,AcH4' to indicate that histone H4 is monophosphorylated and monoacetylated. This can be extended, where necessary, to include the positions of the modifications, as follows: 12,15-P_2H4 or 11,16,18,23-Ac_4H4.

Although not perfect, the proposed terminology had the advantage that it was overwhelmingly accepted by the participants of the Ciba Foundation Symposium who voted 23 in favour with 1 opposed and 1 abstention.

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Editors' note

As the new system of nomenclature was agreed upon only at the end of the symposium, it was decided not to change all the names in the manuscripts (especially as the new system has to be submitted to the IUPAC–IUB Commission on Nomenclature for ratification). In papers in which the old names have been adhered to, the new name for a histone has been given at the first mention in the paper with its definition placed in parentheses after it. Thereafter, the old name is used. This follows the practice recommended by the Biochemical Society and the Chemical Society. In the discussions, to avoid the conflict of different systemic names being used for the same histone, we have adopted the new nomenclature as far as possible, with definitions in terms of the old names where clarity so demands.

We hope that this plurality of names, even though not as satisfactory as the consistent use of the new nomenclature throughout, will facilitate the ready acceptance of the new system.

Introduction

E. L. SMITH

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We have reached a new phase in our understanding of the proteins of the chromosome: the chemistry of the main types of the histones is now reasonably well defined and study of the non-histone proteins has begun. Therefore, in my introductory remarks, it might be worth while to recall briefly one of the major pioneers in this field.

Although it was Miescher who discovered the protamines as protein components associated with the DNA of fish sperm, Albrecht Kossel discovered the histones. Most of us have probably forgotten Kossel's contributions to our knowledge of the chemistry of nucleic acids and proteins. He was among the first to recognize the existence of two different forms of nucleic acids, which languished for a long time under the names of thymus nucleic acid and yeast nucleic acid. Kossel recognized that they differed in the type of sugar they contained but it remained for P.A. Levene and his coworkers to identify these sugars.

Kossel also isolated and identified most of the purines and pyrimidines of nucleic acids—cytosine, uracil, thymine and adenine—but it was Emil Fischer who synthesized most of these. In the study of proteins, apart from his discovery of histones, one of Kossel's major contributions was the discovery of histidine. It is curious that Strasbourg, the city in which Kossel worked, is famous for *pâté de foie gras* and that Kossel initially isolated histones from goose erythrocytes. In those times, when Strasbourg was a German city, the University included among its luminaries at various times, not only Kossel but also Fischer, Hofmeister, Landsteiner and others who contributed so much to our fundamental knowledge of proteins.

Almost one hundred years have passed since the discovery of the histones. It is worth reflecting on how little was known until recently about their chemistry. Now, interest in them has blossomed dramatically. I shall not at-

tempt to anticipate the contributions to this symposium which range from chemical and physical studies to metabolic and biological studies, but I believe that smooth transitions rather than sharp contrasts will emerge from these studies. I suspect that all of us here have masqueraded at times as chemists, biochemists, biologists or cell physiologists but that we actually try to play all these roles simultaneously. We shall need to be all these to understand the fundamentals of the structure and behaviour of the chromosomes and their regulation.

Chromosomal structure as seen by electron microscopy

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Abstract Chromosomes consist of single DNA–histone fibres (with non-histone protein and RNA in the active regions) that are packed by folding or coiling into fibres which can be classified according to size on the basis of electron microscopy. The inactive chromatin of most nuclei consists of fibres about 20 nm thick which unravel under the influence of chelating agents into fibres about 10 nm thick. Further treatment with urea (to break the bonds between histones) unravels this 10 nm fibre into a fibril, 2–4 nm thick, which represents the single DNA double helix with its attached histones.

How is the 2–4 nm fibril compacted to form a 10 nm fibre? X-ray diffraction studies indicate that at least part of the 10 nm fibre is arranged in a regular structure which collapses upon drying. Conventional electron microscopy cannot, therefore, provide an answer to this question because dehydration is unavoidable in the preparation of the specimen. In the near future, we shall use a hydration stage with a 1 MeV microscope to overcome this difficulty but, meanwhile, we have found that a thick layer of negative stain preserves a rather open arrangement in the 20 nm fibre, even when it is air-dried. This technique allows us to demonstrate a loosely coiled 3 nm fibrillar substructure.

The unfolding of the 20 nm fibre by chelating agents suggested a function for cations in chromatin structure. Using potassium pyroantimonate–osmium tetroxide, we have demonstrated the presence of cations in the 20 nm fibres but not in the 10 nm fibre.

The arrangement of 20 nm fibres in chromomeres was studied in polytene chromosomes of *Euplotes* and *Drosophila* with the 1 MeV microscope. Stereomicrographs show branching and complex twisting but no regular supercoiling of the chromatin fibre in chromomeres.

Several reviews of the organization of chromatin and chromosomes have recently been published (Huberman 1973; Stubblefield 1973; Hearst & Botchan 1970; Prescott 1970; Ris & Kubai 1970; Wolfe 1969). This paper constitutes a progress report on attempts to overcome certain limitations in the use of conventional electron microscopy for chromosomal analysis.

The difference in function along chromosomes is one of their most interesting properties and, among the many techniques which provide information on chromosomal organization, only electron microscopy can give direct and specific knowledge about localized regions of chromosomes. Conventional electron microscopy, however, suffers at least two severe limitations in its applicability to the analysis of chromosomes: (*a*) water must be removed from the specimen and (*b*) at 100 keV, only very thin specimens can be usefully resolved to 1–2 nm.

The importance of water in chromatin structure has been demonstrated most clearly by X-ray diffraction patterns of oriented chromatin gels. In the hydrated state, such gels reveal meridional spacings of 11.0, 5.5 and 3.8 nm which disappear when the gel is dried (Pardon *et al.* 1967, 1974). This suggests the presence in chromatin of a regular periodic structure which collapses when water is removed. Pardon *et al.* have suggested that nucleohistone exists as a supercoil about 10.0 nm in diameter with a pitch of 12.0 nm. X-ray diffraction studies alone, however, can neither verify this model nor tell us what proportion of the nucleohistone fibre is in this form and how such regions are arranged in the chromosome. As yet, no such structure has been seen with the electron microscope but this can be reasonably explained by the necessary drying of the specimen.

Obviously, it is important for us to be able to observe *wet* chromatin with such resolution as will reveal the substructure of the nucleohistone fibre. High voltage electron microscopy now promises to bring this goal within reach. Hydration stages which maintain a wet atmosphere are becoming available for these instruments. For instance, Matricardi *et al.* (1972), using a differentially pumped hydration stage in a 200 keV microscope, have obtained electron diffraction patterns extending to 0.2 nm with unfixed wet catalase. However, when the vapour pressure of water was less than 90% of the equilibrium value, diffraction patterns were absent—an indication that the regular structure collapsed during drying. Brownian motion may limit the applicability of the hydration chamber; we shall, therefore, also study the structure of rapidly frozen chromatin observed on a cold stage. While hydration and cold stages are being constructed for the AEI EM-7 high voltage electron microscope at this University, I have been investigating whether embedding chromatin completely in a thick layer of phosphotungstate or uranyl acetate preserves the native structure. Micrographs, obtained with 1 MeV electrons, of such preparations (see later) suggest that this approach will provide interesting new information.

The second major limitation of conventional electron microscopy is that the necessary resolution is obtained only in preparations not exceeding 10–20 nm

in thickness. Because chromosomes are large structures composed of complex arrays of thin filaments, we were unable to study their three-dimensional organization, especially since reconstruction of the structure from thin sections proved impractical. With the 1 MeV electron microscope, however, we can now achieve a resolution of 2–3 nm in 1 μm thick specimens (either isolated organelles or sections of embedded material), and so we shall be able to analyse the arrangement of chromatin fibres in structures of higher order (e.g. chromomeres, heterochromatin and mitotic chromosomes).

Before describing the progress made along these lines, I shall summarize briefly what is known about chromosomal structure with reasonable certainty and point out the major gaps in our understanding. (Further references are given in the reviews mentioned above.)

The conclusion that DNA provides the structural continuity of the chromosome is supported by the observation that deoxyribonuclease breaks chromosomes but ribonuclease and proteinases do not. No evidence has been found for any non-DNA links and a continuous polynucleotide chain seems to be solely responsible for the longitudinal integrity of the chromosome. The much-debated issue of uninemy against multinemy seems to have now been resolved in favour of one DNA double helix per chromosome on the basis of two lines of evidence. (*a*) In yeast, the maximum molecular weight of DNA obtained after gentle lysis agrees with the average DNA content in one chromosome calculated from the number of genetic linkage groups and the total amount of DNA in the haploid unreplicated nucleus (Blamire *et al.* 1972; Petes & Fangman 1972). In *Drosophila*, the largest pieces of DNA obtained by gentle lysis correspond to the total amount of DNA present in the largest chromosomes as measured by ultraviolet cytophotometry (Kavenoff & Zimm 1973). (*b*) The kinetics of reassociation of denatured DNA strongly suggest that a sizeable fraction of the DNA (from 20 to 90%, depending on the organism) consists of unique sequences. This conclusion precludes the presence of more than one identical DNA fibre in a chromosome (Laird 1971).

In most eukaryotes, the length of the DNA is enormous relative to the size of the nucleus and, clearly, the major structural problem for chromosomes is how to pack this DNA into a compact structure though still allowing orderly replication, separation of daughter strands, controlled transcription and genetic recombination. In any nucleus, perhaps 80–90% of the DNA is genetically inactive. In the following discussion I shall restrict my comments to such tightly packed inactive chromatin. The organization of the more unravelled active chromatin, as it is revealed after pretreatment designed to loosen the structure further, has been described by Miller and his co-workers (see Hamkalo *et al.* 1973).

The DNA of eukaryotic chromosomes is combined with an equal mass of histones, and it seems that the compaction of DNA is largely due to its combination with these basic proteins. A full understanding of this packing will result from a knowledge of the properties of histones and the nature of their interactions with DNA and between themselves (as discussed later in this volume). I am concerned here only with the nucleohistone structures.

Three major configurations of the DNA–histone complex are encountered: the *unravelled complex*, the *thin fibre* and the *thick fibre*. Which configuration is found depends entirely on the treatment of chromatin before electron microscopy.

(1) *The unravelled complex.* If isolated chromatin is treated with urea, fibres 3–4 nm thick are observed (Fig. 1) which still retain a full complement of histones (Georgiev *et al.* 1970; Varshavsky *et al.* 1971). Apparently, the links between histones must be disrupted for the nucleohistone fibres to unravel completely. This 3 nm fibre presumably represents the unfolded DNA–histone complex. Studies with models suggest that histones can fit into the major groove of DNA and some direct evidence for this has been obtained (Simpson 1971). Fibres of this size are occasionally found in sections of pellets of isolated chromatin and in nuclei fixed *in situ* (Bram 1972). According to Solari (1971), negative staining of *unfixed* chromatin yields predominantly 3–4 nm fibres. Similar

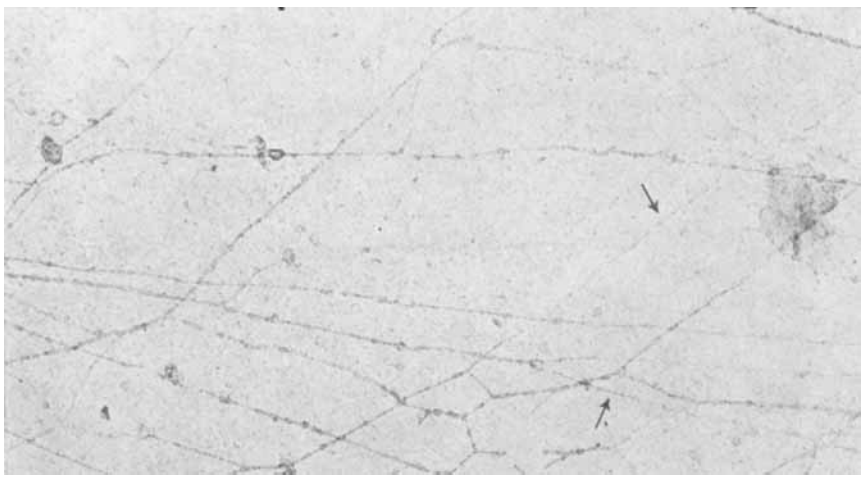

FIG. 1. Chromatin, from newt (*Triturus viridescens*) erythrocytes, spread on 5mM-sodium citrate, picked up on a grid and floated on 8mM-urea for 15 h at 4 °C. The chromatin was then fixed in 4% (v/v) formaldehyde, stained with 7.5% magnesium uranyl acetate and critical-point dried. The thinnest fibres (arrows) are about 3 nm thick. × 88 000.

fibres are present in active chromatin where they form an axial core from which RNA–protein whiskers project (Hamkalo *et al.* 1973).

(2) *The thin chromatin fibre.* Fibres about 10 nm thick are obtained when chromatin is treated with chelating agents (including many buffers commonly used in fixatives, e.g. veronal–acetate, phosphate or dimethylarsinate [cacodylate] [Ris 1968]). Since chelating agents are usually necessary to disperse chromatin, preparations used for biochemical or physical studies generally consist of a tangled mass of these thin fibres. When such preparations are fixed with formaldehyde, dehydrated in ethanol and dried by the critical-point method, the electron micrograph shows knobby fibres about 10 nm thick. Similar structures are obtained if the material is fixed in formaldehyde and negatively stained with uranyl acetate or phosphotungstate, the similarity indicating that neither the dehydration nor the drying is responsible for this appearance. Further, a similar knobby 10 nm fibre is observed when isolated nuclei, cell homogenates or osmotically-fragile cells such as nucleated erythrocytes or yeast spheroplasts (Gray *et al.* 1973) are spread on a Langmuir trough with a chelating agent as hypophase. Thin fibres look alike whether they are obtained from a suspension of isolated chromatin or after spreading on a Langmuir trough and it is unreasonable to suppose, therefore, that the spreading technique has distorted the fibre. This is not surprising if we remember that, on spreading, the nucleohistone fibre is not in the surface film where it might become denatured but is suspended in the chelating agent; the only distortion due to the spreading is therefore in the three-dimensional arrangement of the fibres and not the fibres themselves.

A different kind of thin fibre structure has recently been described by Olins & Olins (1973, 1974) and by Woodcock (1973), who, by using the method developed for active chromatin by Miller & Beatty (1969), find that chromatin consists of strings of spherical particles, 8–10 nm in diameter, connected by fibrils sometimes as thin as 1.5 nm. These observations, although interesting, hardly warrant the conclusion that these particles represent the native structure of chromatin. The conditions in which either the knobby fibres or the spherical particles prevail and how they relate to the conditions in the living nucleus remain to be determined.

How is the thin chromatin fibre related to the unravelled fibre? In essence, we are asking how the DNA is arranged in the thin fibre. Additional information comes from X-ray diffraction studies on oriented chromatin gels (Pardon *et al.* 1974) or on isolated nuclei (Olins & Olins 1972) and from low-angle X-ray scattering by chromatin in solution (Bram & Ris 1971; Bram 1972). Oriented gels and isolated nuclei give reflections at 11.0, 5.5 and 3.8 nm, indicating a regular structure which is found to depend on the hydrated state and is not

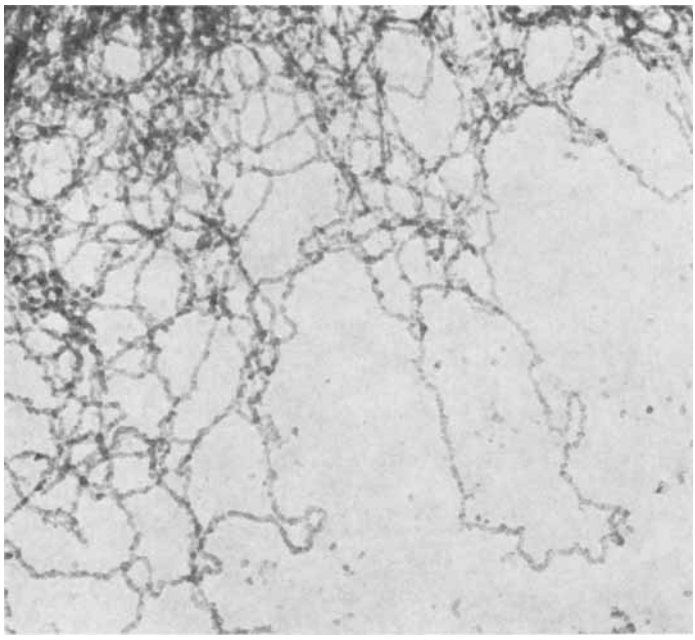

FIG. 2. Chromatin, from frog (*Rana pipiens*) erythrocytes, spread on 0.1mM-Pipes buffer (pH 6.8), fixed in 4% (v/v) formaldehyde, stained in 7.5% magnesium uranyl acetate and air-dried from pentyl acetate. The fibres are about 20 nm thick. × 36 000.

caused by lateral packing of fibres. Fixation in formaldehyde (Pardon *et al.* 1974) or glutaraldehyde (Olins & Olins 1972) preserves this structure. Pardon *et al.* (1967) suggested that these reflections should be obtained from a regular supercoil of the DNA–histone complex having a pitch of 12.0 nm and diameter of 10.0 nm. Low-angle scattering in solution, however, eliminated this possibility for the predominant structure since the calculated mass per unit length is far too large. The lack of strong peaks in the scattering curve also suggests that the packing of DNA is, for the most part, irregular. Since no peak was observed at 8.0 or 10.0 nm, the packed-sphere structure (Olins & Olins 1973, 1974; Woodcock 1973) is unlikely to be a major component in such preparations. This leaves the possibility that the supercoil of 12.0 nm pitch constitutes a minor component of thin fibres or that the reflections are caused by an as yet unconsidered structure. Although conventional electron microscopy cannot resolve this controversy, the high voltage electron microscope with hydration stage may provide the answer in the near future.

(3) *The thick chromatin fibre.* When chromatin is spread on water or on buffers that do not bind metals (Good *et al.* 1966), the fibres are 20–30 nm

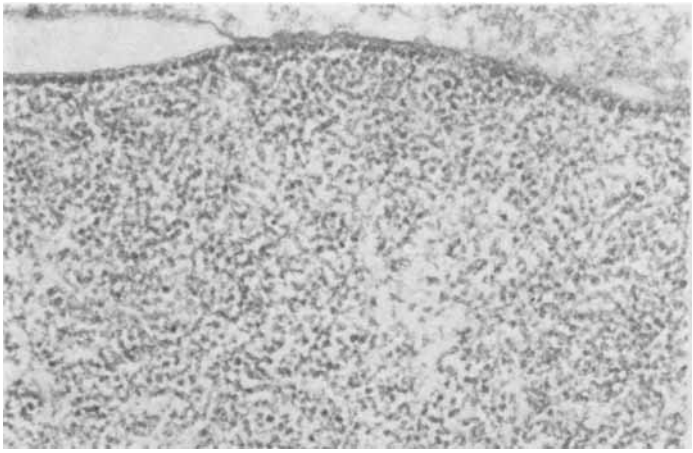

FIG. 3. This section of a nucleus from a frog (*Rana pipiens*) erythrocyte fixed in unbuffered 4% (v/v) formaldehyde, washed in water at 4°C for 18 h, postfixed with 1% unbuffered osmium tetroxide and embedded in epon-araldite. The cross-sections of chromatin fibres are close to 20 nm, the same as in fibres spread on Pipes buffer. × 36 000.

thick (Fig. 2) (see Ris & Kubai 1970). Considerable evidence has accumulated that this thick fibre is the native structure in most nuclei (compare Figs. 2 and 3). In sections, such fibres are best seen if the nuclei are swollen in distilled water after fixation with formaldehyde (Fig. 3). Thick fibres have also been demonstrated in freeze-fractured nuclei (Ris 1969). A characteristic of the thick fibres is the presence of side branches of various length but of similar thickness (Fig. 4) (see also Ris 1967).

The relation between thick and thin fibres still remains unclear. Brief treatment of thick fibres, spread on water, with chelating agents often produces double structures with two side-by-side knobby 10 nm fibres as well as side branches, which may be interpreted as regions where one 10 nm fibre has looped out and folded back on itself (Ris 1967). At least part of the thick fibres might thus be formed when a single thin fibre folds on itself. Whether all thick fibres are constructed in this way is, however, not known. Also, the thin fibre might supercoil or fold, but so far no direct evidence for either process has been published.

The unfolding of thick fibres by chelating agents is reversible. Pooley *et al.* (1973) have demonstrated that addition of magnesium ions to a suspension of 10 nm fibres causes the fibre to thicken to 23 nm. This provides additional evidence that metal ions feature considerably in the control of chromatin structure.

From electron microscopy as well as from other observations it thus ap-

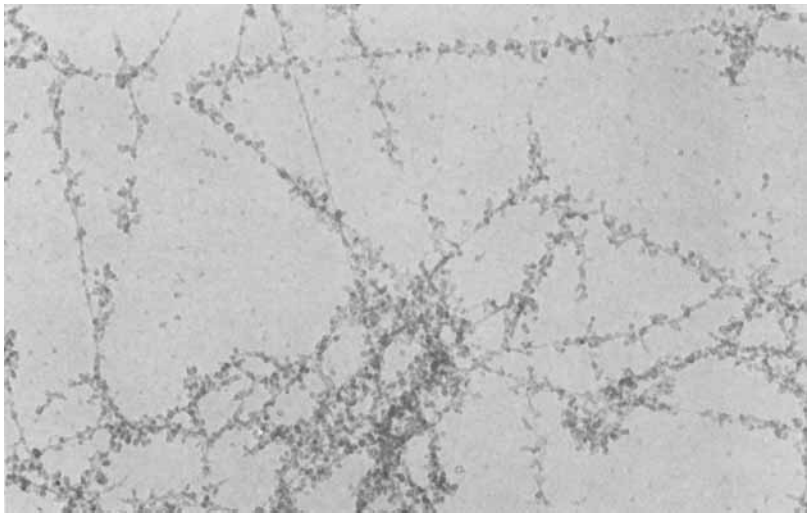

FIG. 4 Chromatin fibres from yeast (*Saccharomyces cerevisiae*). Spheroplasts were spread on water, the film was picked up on a grid, stained in 7.5% magnesium uranyl acetate and dried with the critical-point method. Unstretched fibres are about 20 nm thick. Note the large number of short side branches on these fibres. × 36 000.

pears that the structure of inactive chromatin results from several compactions of one DNA double helix combined with histones and, perhaps, also with minor amounts of non-histone protein and RNA. Most details of this process are still unclear and may vary with external conditions. Even less is known about the arrangement of these fibres in the higher orders of organization such as chromomeres, the different types of heterochromatin, the synaptonemal complex of meiosis and the coiled chromonema of mitotic and meiotic chromosomes.

I shall now describe some of our recent attempts to elucidate these problems, concentrating on the organization of the hydrated nucleohistone fibre, the location of cations in thin and thick fibres and the organization of chromomeres.

THE HYDRATED NUCLEOHISTONE FIBRE

I have already emphasized that, since it has become technically feasible, the investigation of hydrated chromatin by electron microscopy has high priority. Sufficient contrast may be a problem with hydrated specimens and, in addition to positive staining and dark-field viewing, embedding in a solution of uranyl acetate or phosphotungstate will also be explored. While waiting for the hydration stage, I have investigated the results of embedding chromatin in thick

(100 nm) layers of uranyl acetate or phosphotungstate which at 1 MeV still allows high resolution. I had hoped that drying in these salts would preserve the structure of the wet state despite the loss of water.

Erythrocytes of the newt, *Triturus viridescens*, were spread on water or 0.1mM-Pipes buffer (Good *et al.* 1966). The released chromatin was picked up on the formvar side of a carbon-coated filmed grid (400 mesh) which had been made hydrophilic by ion bombardment (Reissig & Orrell 1970) and fixed for five minutes with 4% paraformaldehyde. As an aid in focusing, a suspension of colloidal gold particles (Turkevitch *et al.* 1951) can be added to the grid. A drop of 2% uranyl acetate in water or 2% phosphotungstate solution adjusted to pH 7 with potassium hydroxide is applied to the grid which is then dried in air at room temperature. Staining with phosphotungstate gives the greater contrast, but films are more prone to breakage in the beam. Stereoscopic photographs were obtained at 1 MeV at 40 000 × magnification with a tilt angle of 10°. The thick fibres in the negatively stained preparations in Fig. 5 appear strikingly different from fibres dried with the critical-point method: they are somewhat thicker (about 30 nm) and consist of loosely packed 3 nm subfibrils which have never been seen in material dried by the critical-point method. The stain has obviously penetrated the fibres forming a structureless matrix in which the 3 nm fibrils are embedded. When viewed stereoscopically, the 'noisy' film at the bottom is clearly separated from the glass-like matrix of phosphotungstate surrounding the fibres. In such stereoviews, the arrangement of the 3 nm subfibrils appears complex, for most part irregular and difficult to follow, but in a few locations, there are suggestions of a regular coiled structure with a few gyres at a pitch of about 12 nm (Fig. 5, arrows). The general tendency of the fibrils to run diagonally suggests an irregular coiling or folding of the 3 nm fibre within the thick fibre. Obviously, it is crucial that we obtain similar information about the arrangement of 3 nm fibres in the thin fibre, though the substructure of negatively stained thin fibres has not yet been demonstrable. In any case, it remains to be seen whether the structure seen in thick layers of negative stain resembles the structure in the wet state. But the fact that this method clearly outlines the 3 nm fibril and preserves it as a loosely packed rather than collapsed structure may suggest that we are looking at a less distorted picture than after drying in air or by the critical-point method.

THE LOCATION OF CATIONS IN THE THICK FIBRE

The observation that chelating agents unravel the thick chromatin fibres into thin fibres and that this process is reversible by the addition of bivalent cations suggested that cations, most likely Ca^{2+} and Mg^{2+} (Naora *et al.* 1961), are

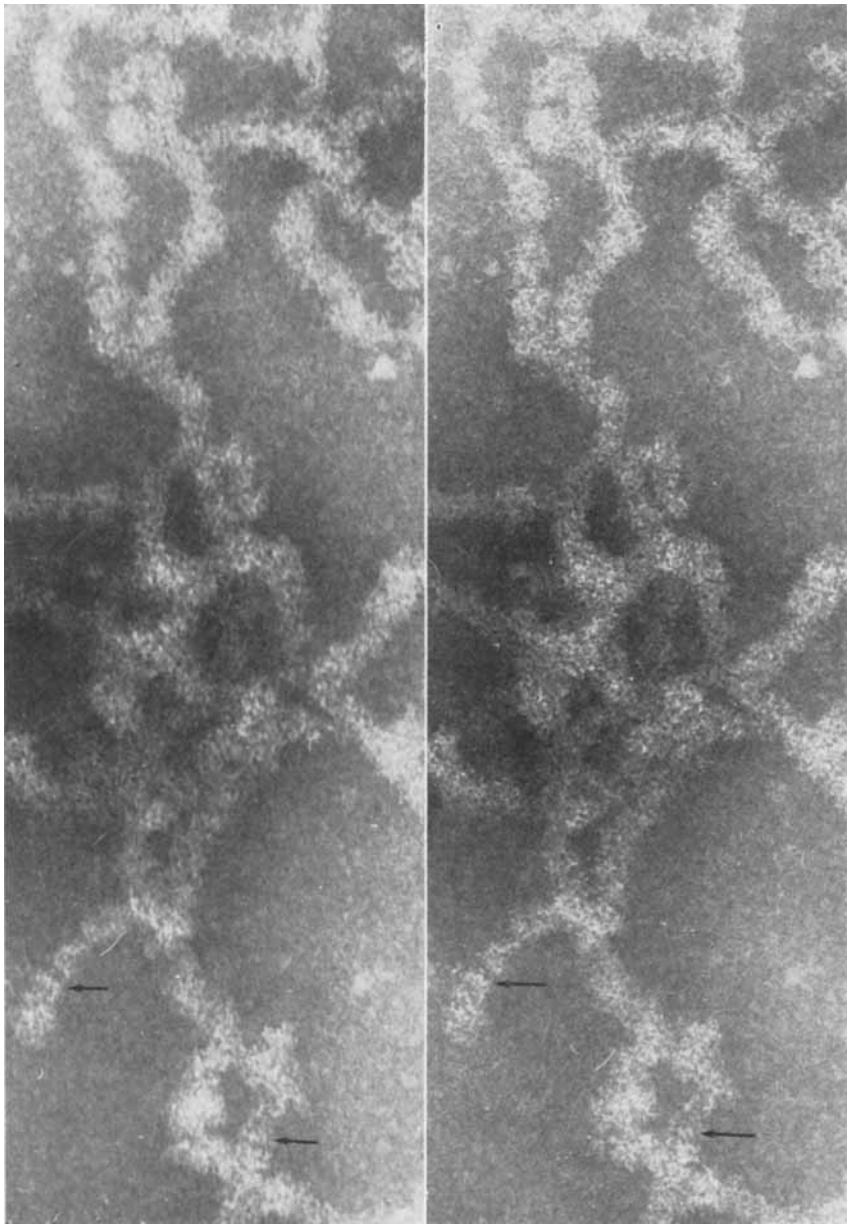

FIG. 5. Chromatin, from newt (*Triturus viridescens*) erythrocytes, spread on water, fixed in 4% formaldehyde, embedded in a thick layer of 2% phosphotungstate (pH 7) and dried in air. Stereomicrograph, tilt $\pm 5°$ from horizontal; 1 MeV. The thin subfibrils constituting the thick chromatin fibres are about 3 nm thick. × 120 000.

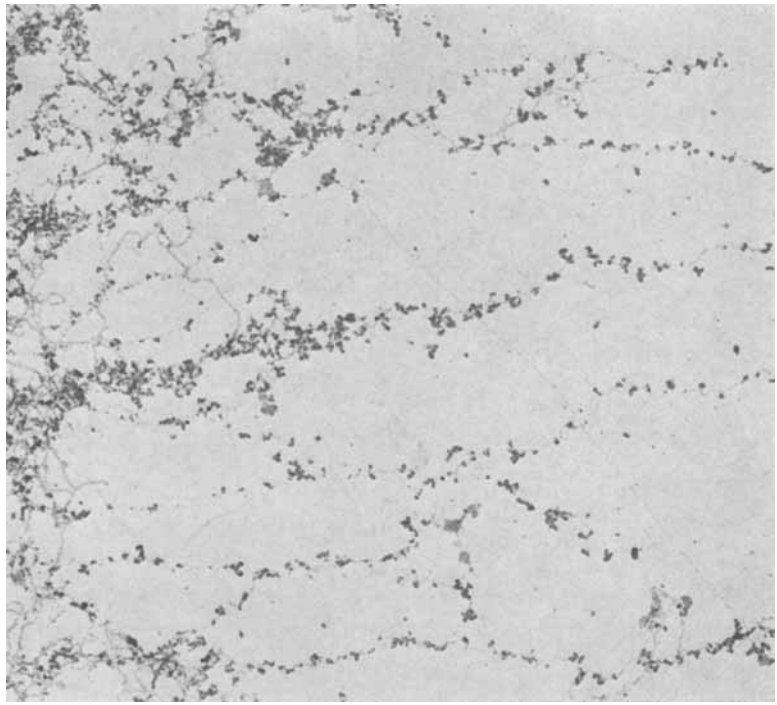

FIG. 6. Chromatin fibres from *Triturus* spread on water, treated for 1 h at 4 °C with potassium pyroantimonate–osmium tetroxide, critical-point dried. The granular precipitate along the fibres indicates the presence of cations (presumably Ca^{2+} and/or Mg^{2+}). × 16 000.

important for chromatin structure. The recent development of methods for visualizing the location of cations in electron micrographs (cf. Klein *et al.* 1972) means we can show directly that such cations are present in the thick fibres, though they are not demonstrable in thin fibres. Clark & Ackerman (1971) demonstrated that treatment of unfixed nuclei with a potassium pyroantimonate–osmium tetroxide reagent resulted in a finely granular precipitate located mainly over condensed chromatin and they implicated calcium bound to nucleohistone in this reaction. I have applied this method to erythrocyte chromatin spread on the following media: (1) water; (2) 0.1mM-Pipes buffer, pH 6.8; (3) 1.5mM-EGTA (ethylene glycol bis[2-aminoethyl]ether tetraacetic acid); (4) 0.1M-phosphate buffer, pH 7.0. The surface film was picked up with a formvar–carbon-coated grid which was then floated on the pyroantimonate-osmium solution for one hour in the cold (Klein *et al.* 1972). The grids were then washed in water, dehydrated in ethanol and dried by the critical-point method. The chromatin fibres spread on water or Pipes buffer (thick fibres)

are coated with a dense granular precipitate (Fig. 6) but the thin fibres of chromatin obtained after spreading on EGTA or phosphate buffer are always completely free of any precipitate. We conclude that cations, presumably Ca^{2+} and Mg^{2+} (Naora *et al.* 1961), are associated with thick fibres but not with thin fibres obtained after treatment with chelating agents. An interesting detail is apparent in Fig. 6: those fibres covered with precipitate are only about 10 nm thick, whereas those which, for unknown reasons, are free of grains are in the 20 nm range. It seems likely that formation of the precipitate effectively removes cations from the nucleohistone and in the process thick fibres are transformed into thin fibres. These results provide direct evidence that cations are involved in chromatin structure.

THE STRUCTURE OF CHROMOMERES

The interphase chromosome shows a characteristic structural differentiation along its length with regions of highly compacted chromatin fibres (chromomeres) alternating with stretches where they are practically straight. This organization is most clearly visible in polytene chromosomes where many chromatids lie parallel so that chromomeres are in register and produce the familiar banding pattern. In material from *Drosophila*, each band corresponds to a specific unit of genetic function (Judd *et al.* 1972). Consequently, it is of considerable interest to know how the chromatin fibres are arranged in chromomere (band) and interchromomere (interband) regions, what properties of DNA, histones and perhaps other components are responsible for this differentiation and how it is related to chromosome function.

Polytene chromosomes are too thick for conventional electron microscopy. Sorsa & Sorsa (1967) have attempted to overcome this limitation by preparing thin sections of the highly stretched chromosomes resulting from squashing after methanol–acetic acid fixation or by treating chromosomes with alkali–urea and spreading on a dilute alkali–urea solution (Derksen & Sorsa 1972). The considerable distortion by these treatments makes the results difficult to interpret.

In our present approach, we use the improved penetrating power in high voltage electron microscopy to analyse minimally distorted chromosomes, either in 0.5–1.0 μm sections of cells fixed in formaldehyde or after gentle squashing of whole fixed cells between slide and coverslip. For successful squashing, acetic acid is required in the fixative. A solution of 4% formaldehyde in 50% acetic acid gives adequate fixation and good separation of chromosomes (Holmquist & Steffensen 1973). The fixation was tested on erythrocytes of the newt, *Triturus viridescens*, for which water-spread chromatin and thin sections