ELECTRON MICROSCOPIC OBSERVATIONS ON THE SUBMICROSCOPIC MORPHOLOGY OF THE MEIOTIC NUCLEUS AND CHROMOSOMES*

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(Received for publication, June 19, 1956)

Since the discovery of the chromosomes and their behavior in mitosis and meiosis, cytologists and cytogeneticists have been largely preoccupied with the nucleus rather than the cytoplasm and have devoted much attention to the problems of reduplication of chromosomes, mutation, and the linear array and function of genes.

On the other hand, investigators concerned with submicroscopic morphology worked mainly on the fine structure of cytoplasm and its organelles (1) and as yet relatively little progress has been achieved on the ultrastructural organization of the nucleus and chromosomes. There is a real need for the application of modern techniques of analytical cytology to the problems of chromosome structure in an effort to settle many points of disagreement in the cytological literature (2). Furthermore, the high resolution recently reached by means of the electron microscope on properly fixed and sectioned material offers the possibility of visualizing *in situ* the nucleoproteins and other macromolecular components which biochemists have been studying *in vitro* after isolation.

From the genetic viewpoint knowledge of the fine structure of chromosomes is of particular importance because of recent evidence which tends to identify the gene with macromolecules of nucleoprotein (3, 4) and to demonstrate, at least for simple genetic systems, that the "one dimensionality and divisibility" of genetic material persist down to its ultimate molecular structure (5).

Early attempts to study nuclear components with the electron microscope consisted of observations on isolated lampbrush chromosomes of amphibian ovocytes (6–8) and on nuclear fragments (9). Some workers reported the presence of fibrillar structures about 500 A in diameter (6) that under certain conditions appeared as paired strands (7). However, this has been contradicted by others who found no evidence of single or paired strands in cross-sections of

* Work supported by a grant of The Rockefeller Foundation.

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the loops of lampbrush chromosomes (10) but found indications of the existence of finer fibrillar components.

The application of more refined methods of specimen preparation for electron microscopy, has greatly improved the changes of resolving the fine structure of the nucleus, but the resolution achieved in most of the electron micrographs published to date has been inadequate to reveal finer details of this structure.

The appearance of high resolution electron micrographs of thin sections of meiotic chromosomes of a grasshopper was described in a preliminary report (De Robertis and Cassarotti, 11). The bulk of the chromosome material was found to consist of a filamentous macromolecular component. Evidence was also presented of a second macromolecular nuclear component occupying the interspaces between the prophase chromosomes. The possible relationship of this component to the activity of the nuceolus was also suggested.

This paper gives a more detailed description of these findings in prophase and metaphase chromosomes of the grasshopper.

It is concluded that the fine structure of the nucleus of the grasshopper spermatocyte is highly complex and undergoes very conspicuous changes in different stages of activity, but nevertheless in all cases a filamentous macromolecular component is recognizable as the basic structure of the chromosomal material.

Techniques

Most of the material was obtained from the testis of the acridid, *Laplatacris dispar*. The testicular follicles were removed and immediately immersed in the fixative. After a few minutes the individual follicles were isolated and removed to fresh fixative for 2 to 6 hours. The fixing mixture was a slight modification of the standard (12) buffered osmium tetroxide at pH 7.4. The solution was prepared by mixing equal parts of a 2 per cent solution of osmium tetroxide, veronal-acetate buffer, and Ringer solution containing 0.5 per cent $CaCl_2$. The final concentration of OsO_4 was thus 0.66 per cent. It was found that the presence of Ca^{++} in the solution insured a better preservation of the macromolecular structure of the chromosomes. The probable explanation of this will be dealt with in the discussion.

After fixation and dehydration each follicle was cut into 3 segments, each one corresponding to approximately one-third of the total length. This was done in order to analyze better the different generations of cells which follow one another in sequence from the rostral end of the follicle, where the spermatogonia lie, to the caudal end where the mature spermatozoa accumulate.

Thin sections were obtained with a special ultramicrotome based on the design of Porter and Blum (13) with provision for thermal instead of mechanical advancement. The thickness of the sections was judged by their interference colors. In general only those giving silver color were used. Extreme thinness of the sections was found to be essential for the demonstration of the filamentous macromolecular component of chromosomes. The supporting film used was in all cases made of formvar. In some instances the embedding material (methacrylate) was removed with xylene and the specimens shadowed with platinum at an angle of 11°.

The electron micrographs were made with an RCA EMU 2C microscope having a well compensated objective pole piece, and small condenser, objective and projector apertures. The micrographs were taken at an original magnification ranging between 8,000 and 12,000 and were enlarged photographically up to 100,000 times or more.

OBSERVATIONS

The Fine Structure of the Spermatocyte Nucleus of the Grasshopper in Prophase

No attempt was made in this work to correlate in every instance the cytological image, as observed with the optical microscope, with that given by the electron microscope. Such a correlation is possible, however, as has been shown by Moses (14) using consecutive thin (for the electron microscope) and thick sections stained by means of the Feulgen reaction (for the light microscope). In a few cases we have made thicker sections and, after removal of the methacrylate, have observed them either with the phase microscope or the ordinary microscope after staining with basic dyes. However, in good electron micrographs of spermatocytes, recognition of the areas occupied by the chromosome material offers no difficulties, particularly when one deals with the advanced stages of prophase (Fig. 1 and Text-fig. 1). The different (leptotene, pachytene, and diplotene) stages can be judged by the dimensions of the chromosome profiles in the section and also by the degree of condensation of the chromosomes. In early stages of prophase the chromosome material possesses a filamentous fine structure but the material is diffuse and there is no distinct separation between the individual chromosomes.

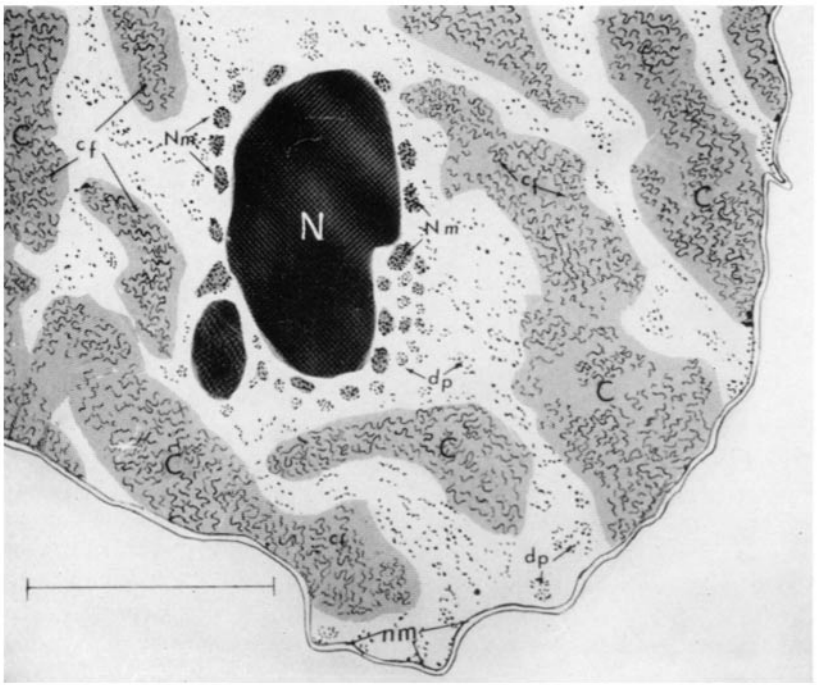
Both in Fig. 1 and in Text-fig. 1 three main regions of the karyoplasm can be recognized: the nucleolus, the interchromosomal regions, and the chromosomes.

1. Nucleolus and Nucleolar Material.—The nucleolus appears as a very dense and irregularly shaped mass that in most cases is compact but in some shows the thread-like structure which has been designated the nucleolonema (15). In advanced stages of prophase the nucleolus is surrounded by numerous round or oval shaped bodies which are of essentially the same electron density and structure as the main nucleolar mass. This *nucleolar material* is interpreted as being derived by fragmentation of the outer portion of the nucleolus. When observed at higher magnification in sections tangential to the nucleolar mass some details of its fine structure are more apparent (Figs. 3 and 4).

The nucleolar bodies are formed of aggregations of dense round particles varying in size from 100 to 250 A. This dense particulate component is more conspicuous in the smallest bodies but it can also be recognized in the larger nucleolar masses and within the nucleolus itself. Careful observation of this material reveals that dense particles apparent in nucleolar masses are also evident in the interchromosomal regions (see Figs. 3 and 4).

2. Interchromosomal Regions and Dense Particles.—The material occupying the space between the chromosomes and corresponding in location to the nuclear sap of classical cytology is represented in Text-fig. 1 and Figs. 1 to 4, 6, and 7. In general, it is of lower electron density than the chromosomes but scattered through it are large numbers of dense particles. In very thin sections and at high resolution (Figs. 2, 6, and 7) practically the only component observed in the interchromosomal areas are dense particles occurring either

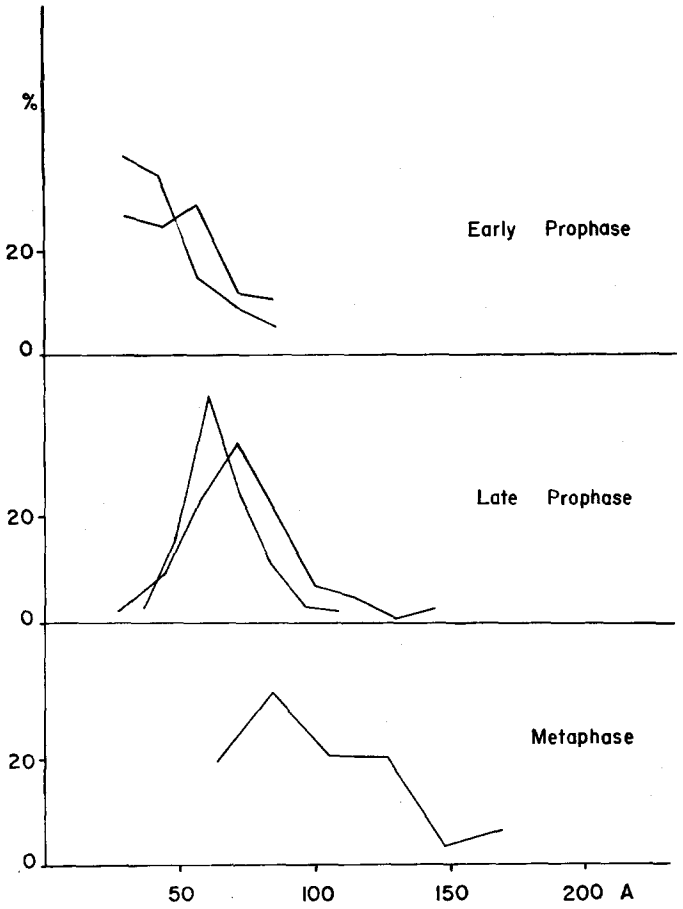
individually or in short chains. The matrix in which they are dispersed appears to be amorphous within the resolutions attained in the present study. As indicated above, the particles are very abundant in the vicinity of the nucleolus and



TEXT-FIG. 1. Diagram showing the main nuclear components of a spermatocyte of the grasshopper in late meiotic prophase: the nucleolus (*N*) surrounded by nucleolar material (*Nm*); the interchromosomal areas containing dense particles (*dp*) and profiles of the chromosomes (shaded masses *c*). The filamentous macromolecular component of the chromosomes is shown as short and longer flexuous threads (*cf*). The representation corresponds to what can be seen in thin sections but actually the filaments may be much longer. The possible disintegration of the nucleolar masses to form dense particles is also indicated. At the bottom left the mark corresponds to one micron.

adjacent nucleolar satellite bodies. Frequently they are closely associated with the nuclear membrane (Figs. 2 and 3) and particles of a similar size and density are found either free in the cytoplasm or adhering to the endoplasmic reticulum. At certain stages of prophase the relationship of the dense particles to the nuclear membrane and the endoplasmic reticulum is very intimate but further discussion of this interesting association is beyond the scope of this paper. Measurements of the diameter of over 200 of the particles show that they vary

in size from 100 to 250 A with a definite peak at 140 A and a mean diameter of about 160 A.



TEXT-FIG. 2. Histograms describing in per cent the distribution of diameters (in A units) of the chromosome filaments of early and late meiotic prophase and of metaphase of the primary spermatocyte of the grasshopper.

3. Filamentous Macromolecular Component of Prophase and Metaphase Chromosomes.—The filamentous macromolecular component of chromosomes is best observed in Fig. 2 and in the further enlargements presented in Figs. 5 to 7 illustrating different chromosome areas. Granules, short and long rods, or long flexuous filaments are seen, the appearance probably depending on how they are oriented with respect to the plane of section. The best interpretation of these electron micrographs and many more of different stages of prophase seems to be

that the entire chromosome is comprised of very fine, irregularly coiled filaments which describe tight gyri and undulations. Because of the thinness of the sections the full length of these filaments cannot be determined, but some of them can be followed without interruption for distances of 1000 to 2000 A or more. In sections from which the methacrylate was removed with xylene and shadowed with platinum, the chromosome areas of the prophase nucleus show filaments of similar size and shape. Metaphase chromosomes show a similar fine structure but the filaments are definitely thicker and much more tightly packed, corresponding to the higher degree of condensation.

Because of the extreme thinness of the filaments measurements of their diameter are difficult to make and the error involved may be very high especially for the thinnest ones. But even considering these shortcomings of mensuration, a study of the histograms reveals some points of interest (Text-fig. 2). The graphs corresponding to the early and late prophase, were made from measurements of a total of 470 filaments in micrographs from four different specimens. The one on metaphase was made on 60 filaments. There is clear evidence that the filaments are thicker at metaphase. In early prophase measurements vary between 28 $\pm$ 7 A and 84 $\pm$ 7 A with a mean of about 47 A. In one specimen 37 per cent of the filaments were of about 28 $\pm$ 7 A. In late prophase the peak is displaced to the right with a mean in the neighborhood of 70 A. In several instances the thicker filaments appear to be double. In metaphase chromosomes the filaments vary between 60 and 170 A with a mean at about 100 A.

It is interesting that neither the prophase nor the metaphase chromosomes show a chromosomal membrane or a central dense structure such as the chromosomal cores described by Moses (16) in the crayfish and confirmed by Fawcett in material from the cat, pigeon, and human (23). The outlines of the chromosomes are irregular and become more sharply defined as the process of condensation progresses.

DISCUSSION

Considerations on Technique.—The poor progress made in the study of the fine structure of the nucleus may be due in part to inadequacies of preparative methods. The filamentous component observed by us in the chromosomes and chromatin material is so extremely fine that its filamentous nature can be demonstrated only in very thin sections and with high resolution electron microscopy. Other problems may be attributable to the fixation. The uptake of osmic tetroxide by the chemical components of the nucleus undoubtedly contributes significantly to the contrast of structures revealed by the electron microscope. In this respect the protein component is probably more important than the nucleic acids because of its greater reactivity with osmium tetroxide

(17, 18). The high P content of DNA and RNA undoubtedly contributes some electron scattering, but the inertness of nucleic acids toward osmium tetroxide (18) is probably an important factor in making more difficult the fixation of nuclear structures, and therefore unsatisfactory their demonstration.

The observation that the filamentous component of chromosomes appears to be better preserved in the fixative containing Ca^{++} is interesting and may prove, upon further investigation, to be quite significant. The possibility that calcium (a major mineral constituent of the nucleus) may play a significant role in maintaining the structural integrity of the nucleus is supported by the fact that it is liberated in stoichiometric amounts during enzymatic depolymerization of DNA (19). Furthermore, in a medium of low ionic strength, the removal of Ca^{++} by citrate or with agents capable of chelating divalent ions produces a dispersion of nucleoprotein from chromosomes, sperm, and interphase nuclei (20, 21). Mazia (21) has recently elaborated a tentative model of organization of the chromosome based mainly on these observations. It is postulated that the chromosome is composed of complex macromolecules of DNA protein held together by bridges of divalent cations, as well as by interactions making for "insolubility" at the moderate ionic strength present in the cell.

Our electron microscope observations are not inconsistent with Mazia's (21) interpretation of the macromolecular structure of chromosomes. However, they are incompatible with the alternative explanation, previously offered by Bernstein and Mazia (20) which depended on the presence of a membrane surrounding the chromosome.

The Nucleolar Material and Dense Particles.—Some details of the structure of the nucleolus and of the associated nucleolar material of the primary spermatocyte of the grasshopper are of special interest. The particulate structure of the nucleolus has previously been observed by Porter (22). In our material this granular structure is especially clear in the numerous nucleolar bodies that may be found surrounding the main nucleolar mass. The transition between the particles observed within the nucleolar bodies and those found scattered throughout the interchromosomal spaces is also very suggestive. From Figs. 2 and 3 and from numerous other electron micrographs the impression is gained that in the spermatocyte the nucleolus is not only made up of a macromolecular particulate component but that it is actively synthesizing similar material which is dispersed to form the nucleolar bodies and the dense particles found in the interchromosomal nuclear matrix. This nuceolar phenomenon observed in the meiotic prophase is even more striking when it is compared with what occurs during spermiogenesis. In the early and late spermatid there is no evidence of a nucleolus or nucleolar material and there are none of the dense particles, as if at this stage, the synthesis of other material unrelated to the filamentous component of chromatin had been suspended.

Fine Structure of Chromosomes.—The observations on prophase and metaphase chromosomes of the spermatocytes of the grasshopper with the electron microscope make it possible to discard some of the common concepts of chromosome structure which have long been a subject of debate in the literature. These include the hypothetical chromosome membrane or calima and the distinction between the chromoneme and the matrix, etc. (for literature see reference 2). In our material the entire chromosome appears as a rather homogeneous mass with a degree of heterogeneity only evident at a macromolecular level. We have not observed the chromosomal cores recently described by Moses (16) as an axial differentiation of the chromosomes, in the spermatocytes of the crayfish. Although this observation has already been confirmed by Fawcett (23) in other favorable material, the constancy and significance of this interesting structure are still obscure. Whatever proves to be the significance of the chromosomal cores it is evident that they represent only a minor part of the total mass of chromosomes. Our observations indicate that the bulk of the chromosome is made of a filamentous component whose dimensions go down to the molecular size of nucleoproteins.

The fact that the thinnest filaments observed in the prophase chromosomes are of the order of 28 ± 7 A suggests that in this case they may represent single deoxyribonucleoprotein molecules. A few of the most pertinent quantitative data on which this interpretation can be based are the following. Indirect determinations have given a width of 40 A for thymonucleohistone (24). In the recent precise x-ray diffraction analysis of deoxyribosenucleoprotamine Feughelman *et al.* (25) find an hexagonal crystal lattice in which $a = 28.7$ A and $c = 34$ A. These values correspond to a diameter of about 24.8 A for the triple coaxial helix of nucleoprotein postulated by the authors. Electron microscope measurements of shadowed specimens of calf thymus deoxyribonucleoprotein have given a diameter of about 25 A (26) for the long filamentous molecule. More information exists about the diameter of the DNA molecule which was measured by a variety of methods (27) including electron microscopy (26, 28). Of particular interest are the observations of Fraser and Williams (29) of fibrillar strands presumed to be the nucleic acid or chromosomal material released from bacterial viruses by a special technique. In shadowed preparations they find that these thin, long DNA threads have a thickness of about 20 A.

The thickening of the chromosome filaments observed during prophase and especially in metaphase is difficult to interpret and nothing can be advanced about the possible mechanisms involved. However, the fact reported in the literature that during the prophase of the primary spermatocyte of the grasshopper there is no change in DNA content (30) tends to rule out the possibility that this size change is due to synthesis and replication of DNA. The thinness of the sections used for the electron microscope precludes the possibility of

obtaining definite data about the actual length of the chromosome filaments. However, it seems evident that their length must be considerable since the continuity of certain filaments can be demonstrated in the section for 1000 to 2000 A or more. This observation is in agreement with the long molecular dimensions attributed to the DNA molecule (27).

The most general conclusion that can probably be derived from the electron microscope observations presented here is that, although the fine structure of the chromosome and chromatin material undergoes profound and complex cyclic rearrangements during meiosis of the grasshopper spermatocyte, in all stages a filamentous macromolecular component can be recognized as the basic structural unit. These findings seem to be in agreement with the concepts of the particulate organization of chromosomes derived mainly from biochemical investigations (21) and may explain the inequality of distribution of parental DNA in chromosome reproduction recently observed by Mazia and Plaut (31) with the use of autoradiography. Furthermore, the presence of filamentous macromolecular units within the nucleus is consistent with the concept that the genes are macromolecules of deoxyribonucleoprotein and that their linear organization persists down to the ultimate molecular structure (5).

SUMMARY

Thin sections of the testicular follicles of the grasshopper *Laplatacris dispar* were studied under the electron microscope. In the primary spermatocytes, during meiotic prophase, three main regions can be recognized within the nucleus: (1) the nucleolus and associated nucleolar material; (2) the interchromosomal regions with the dense particles; and (3) the chromosomes.

The nucleolus is generally compact and is surrounded by nucleolar bodies that comprise aggregations of dense round particles 100 to 250 A in diameter. A continuous transition can be observed between these particles and those found isolated or in short chains in the interchromosomal spaces. Particles of similar size (mean diameter of 160 A) can be found associated with the nuclear membrane and in the cytoplasm.

The chromosomes show different degrees of condensation in different stages of meiotic prophase. The bulk of the chromosome appears to be made of very fine and irregularly coiled filaments of macromolecular dimensions. Their length cannot be determined because of the thinness of the section but some of them can be followed without interruption for about 1000 to 2000 A. The thickness of the chromosome filaments seems to vary with different stages of prophase and in metaphase. In early prophase, filaments vary between 28 $\pm$ 7 A and 84 $\pm$7 A with a mean of 47 A, in late prophase the mean is about 70 A. In metaphase the filaments vary between 60 and 170 A with a mean of about 100

A. Neither the prophase nor the metaphase chromosomes have a membrane or other inhomogeneities.

The finding of a macromolecular filamentous component of chromosomes is discussed in relation to the physicochemical literature on nucleoproteins and nucleic acids and as a result it is suggested that the thinnest chromosome filaments (28 ± 7 A) probably represent single deoxyribonucleoprotein molecules.

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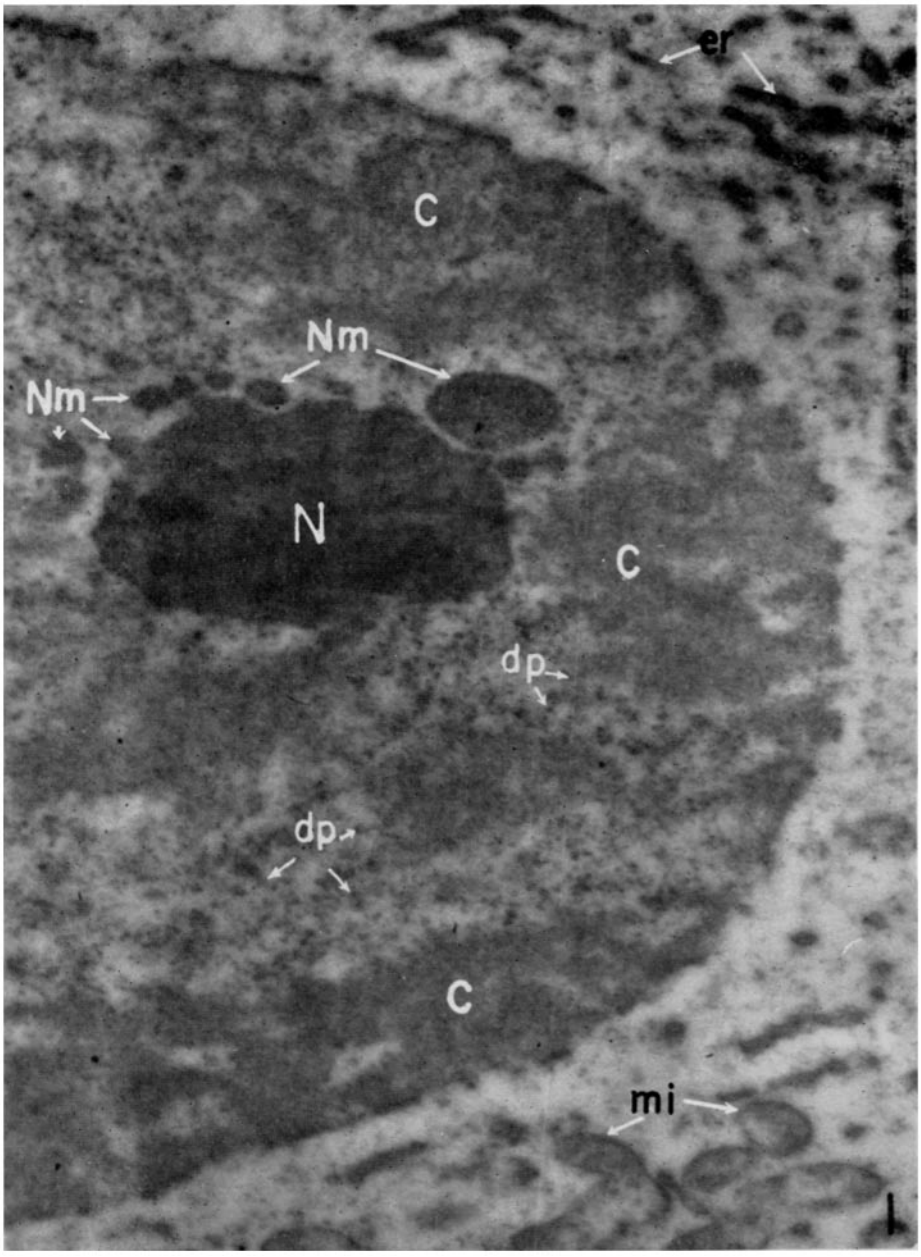
EXPLANATION OF PLATES

c, chromosome.
dp, dense particle.
er, endoplasmic reticulum.
mi, mitochondria.
N, nucleolus.
Nm, nucleolar material.
nm, nuclear membrane.

PLATE 219

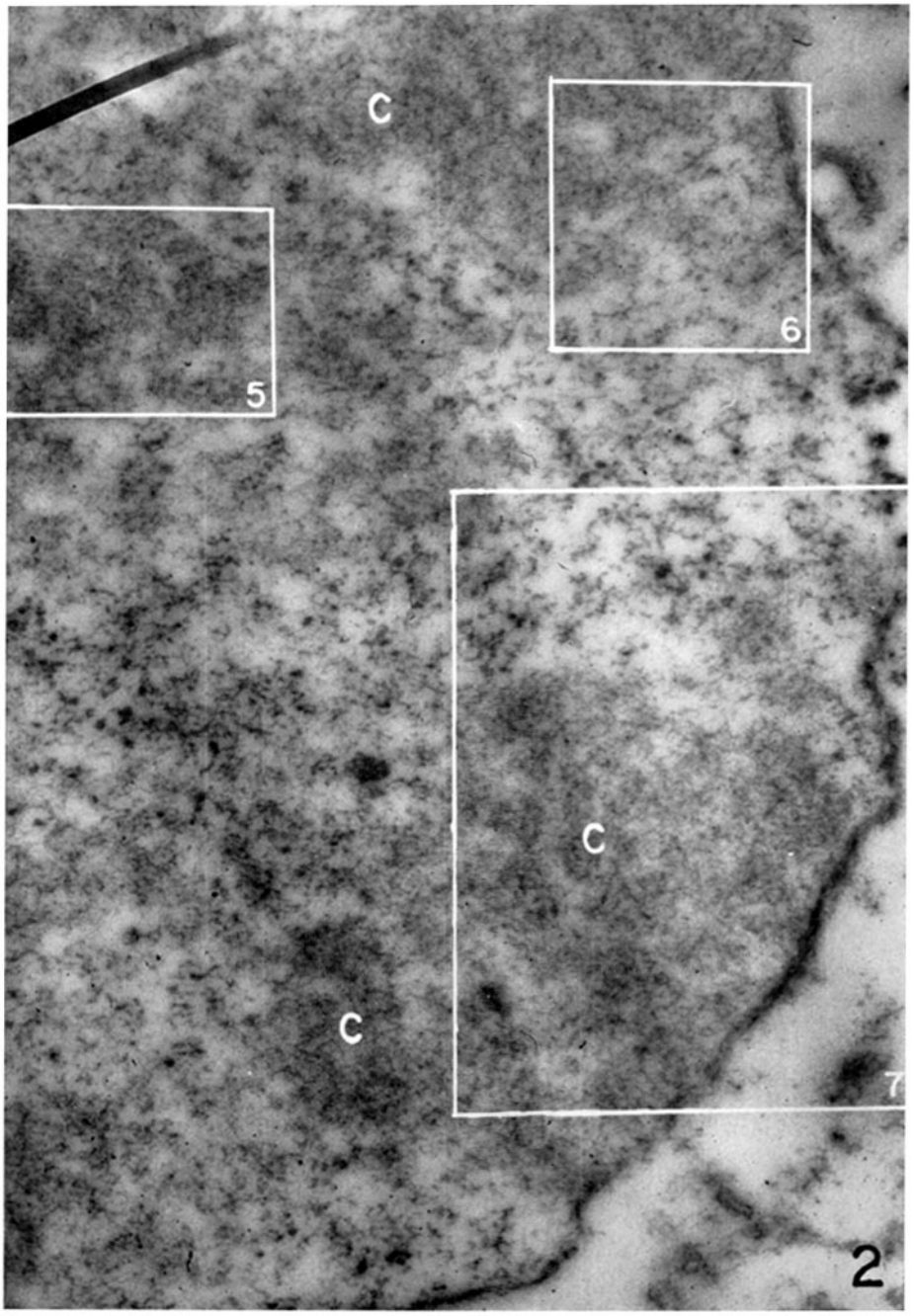
FIG. 1. Electron micrograph of a nucleus of a spermatocyte of the grasshopper *Laplatacris dispar* in advanced meiotic prophase. Within the nuclear area one may observe the dense mass of the nucleolus surrounded by smaller bodies of nucleolar material (*Nm*), the profiles of the chromosomes (*c*), and the interchromosomal areas with the dense particles. In the cytoplasm there are mitochondria (*mi*) and tubular and vesicular elements of the endoplasmic reticulum (*er*). × 34,000.



(De Robertis: Morphology of the nucleus and chromosomes)

PLATE 220

FIG. 2. Electron micrograph of an area of the nucleus of a spermatocyte of *Laplatacris dispar* in advanced meiotic prophase. The double nuclear membrane, the chromosomes (*c*), and the interchromosomal areas filled with the dense particles can be recognized. At this magnification it is already evident that the bulk of the chromosome is made of a fine filamentous material. The insets correspond to the regions of the chromosomes enlarged further in Figs. 5, 6, and 7. × 41,000.



(De Robertis: Morphology of the nucleus and chromosomes)

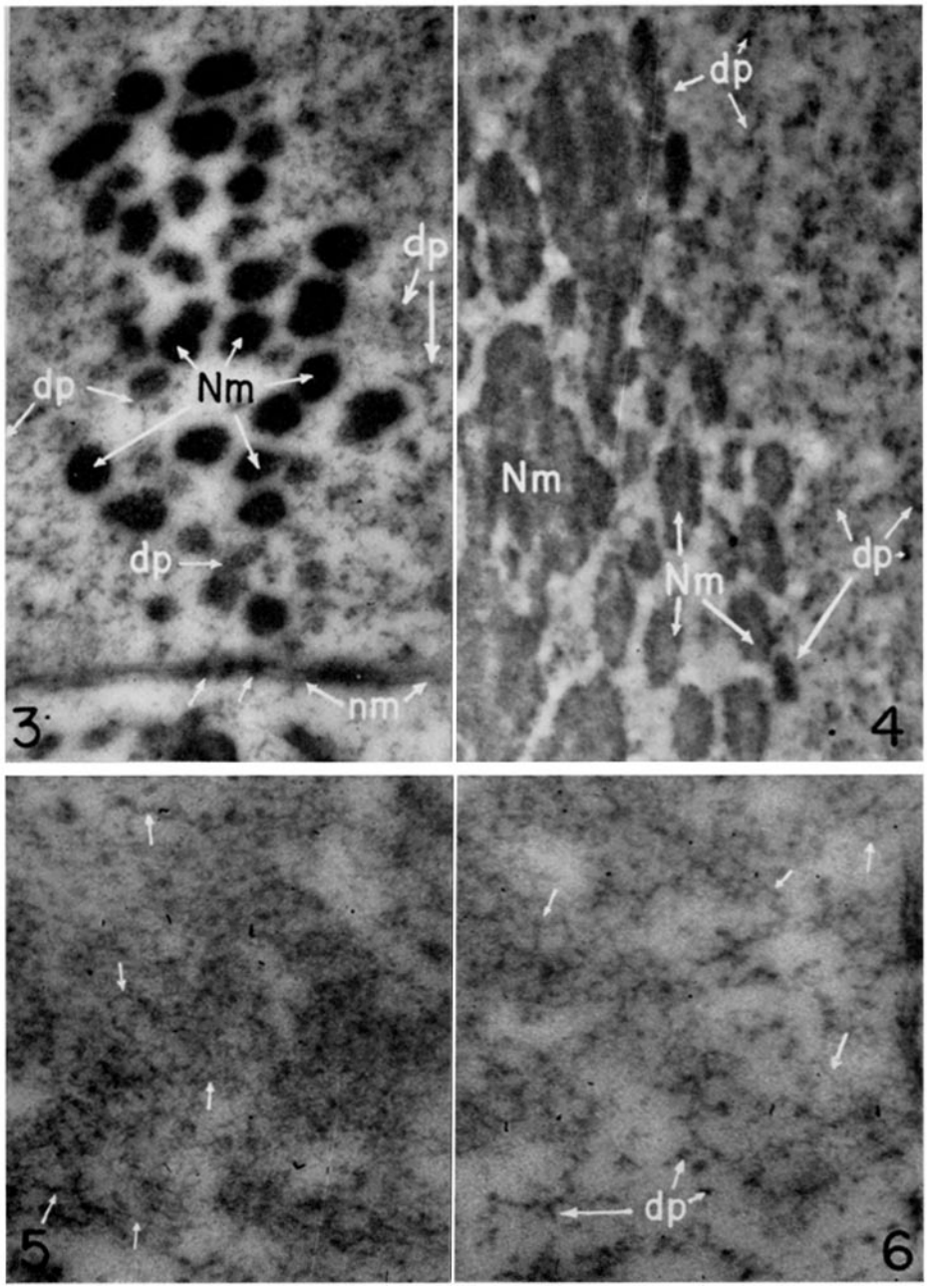
PLATE 221

FIG. 3. The same material as depicted in Fig. 1. The section has been cut tangentially with respect to the nucleolus and shows a group of very dense nucleolar bodies. Near the nuclear membrane (*nm*) these bodies show a granular structure, and what can be interpreted as transitional stages of disintegration of the bodies to form dense particles (*dp*). This macromolecular component is scattered all around the nucleolar material. Regions of the nuclear membrane of increased density, probably related to the particles, are marked with arrows. × 44,600.

FIG. 4. The same description as in Fig. 3. The granular structure of the nucleolar masses is clearly observed. Toward the left the nucleolar bodies become smaller and seem to disintegrate to form the dense particles. × 44,600.

FIG. 5. The same as Fig. 4 at higher magnification. Arrows mark some of the most conspicuous fine flexuous macromolecular filaments of the chromosomes. Some of them are extremely long. × 80,200.

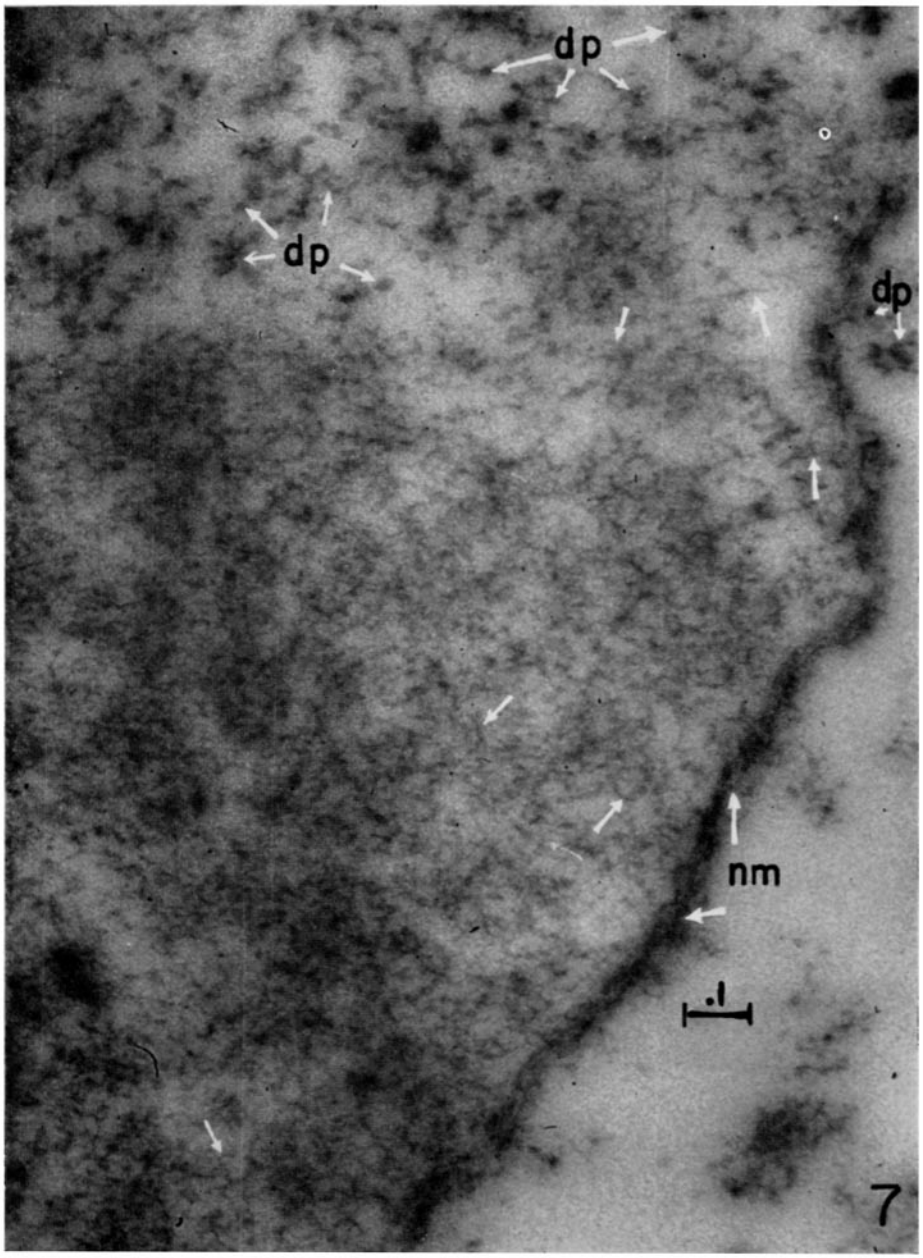
FIG. 6. The same description as for Fig. 5. At the bottom there are dense particles within an interchromosomal space. Some of the chromosome filaments appear to be double. × 80,200.



(De Robertis: Morphology of the nucleus and chromosomes)

PLATE 222

FIG. 7. Enlargement corresponding to the inset of Fig. 2. A chromosome closely associated with the nuclear membrane is shown, and at the top an interchromosomal space with dense particles scattered or forming short chains. Some similar particles are observed in the cytoplasm. Some of the long flexuous filaments of the chromosome are indicated with arrows but it can be seen that the entire mass is made of these filaments. × 82,000.



(De Robertis: Morphology of the nucleus and chromosomes)