STUDIES ON NUCLEI USING CORRELATED CYTOCHEMICAL, LIGHT, AND ELECTRON MICROSCOPE TECHNIQUES*

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PLATES 133 AND 134

Despite the development in recent years of superior means for preserving and studying fine structure in thin sections, our knowledge of nuclear morphology at the electron microscope level has been vastly exceeded by our knowledge of the cytoplasm. The elaborate membranous and granular structures of the cytoplasm have no counterpart in the often homogeneous masses of granular, fibrous, and amorphous material of the nucleus. As a result, most electron microscopists acknowledge that the nucleus appears to be as remarkable for its lack of obvious ordered detail as the cytoplasm is for its richness in it. There are three obstacles that appear to stand in the way of interpreting thin sections of the nucleus in terms of the grosser structures long familiar to the light microscopist: (1) The similar electron-scattering properties among various components lead to poor contrast. The one structure in the nucleus that is generally distinguishable is the nucleolus, and this is by virtue of its greater density to electrons. (2) There are no membranes delimiting nuclear structures, aside from those surrounding the nucleus itself. (3) In thin sections, it is difficult to interpret the third dimension of large structures that have no clear outline. Thus, to achieve an interpretation consistent with the morphology known from light microscope studies it is evident that a transition from the light to the electron image must be carefully monitored by the observer (*e.g.* reference 10). Cytologists who have chosen to study nuclear structure at the electron microscope level have accomplished this in various ways; nevertheless, the difficulties of interpreting the images of single thin sections remain. Moreover, a direct attack on the nucleus is further complicated by our ignorance of changes in content or distribution of such nuclear components as DNA during fixing and embedding procedures currently in use. Indeed, the homogeneous appearance of many nuclei leads to the suspicion that components not acted on by the fixative may actually be lost or redistributed.

The logical approach to clarifying this problem, and one that would at the same time assist in morphologically identifying nuclear components by virtue of their various chemical compositions, is a cytochemical one. Unfortunately, up to this point the direct application of specific electron stains to electron

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microscope studies has met with little success. Thus the present interest is in a method whereby available cytochemical tests may be correlated with fine structure in the electron microscope. By making use of the time worn device of performing various analyses on separate but adjacent serial sections, results on the same region of tissue at serial levels may then be compared and correlated. The well known Linderström-Lang–Holter technique of using one of a series of sections for morphological detail and others for various biochemical analyses is an example. Thus by employing the same principle, as demonstrated in this work, one may cut a thin (*ca.* 500 A) section for electron microscopy and then immediately cut a thick (*ca.* 1 to 2 μ) section for cytochemical analysis in the light microscope. It then only remains to be shown which of the available cytochemical spot tests is suitable for material prepared in the usual way for electron microscopy.

Since RNA, DNA, and protein are the major constituents of the nucleus, tests for these substances are obviously to be explored first. Our preliminary experiences with controlled acidophilia and basophilia after removing methacrylate from the section with organic solvent were not encouraging. It is true that the localization and spectral absorption characteristics of the dyes, nonspecific staining, and interference due to reduced osmium tetroxide could be improved by additional treatment. But variations which apparently reflect fixation gradients and other anomalies make the results of dubious qualitative or quantitative cytochemical significance. The potential usefulness of ultraviolet absorption is enhanced by the virtue of methacrylate as a matrix which is both transparent to ultraviolet light and of similar refractive index to the fixed tissue. However, as pointed out by Davies (3), reduced osmium has considerable absorption in the ultraviolet regions where nucleic acids and protein also absorb and it thus obscures the cytochemical significance of ultraviolet photographs even when taken at several wave lengths. While approximate corrections for the absorption of reduced osmium can be made on the basis of entire cytospectrophotometric curves, such a procedure hardly facilitates a comparison of visible and electron microscope images.[1] On the other hand, the Feulgen reaction for DNA is a cytochemical test, fortunately specific for the one substance characteristic of the nucleus, that seems relatively free of such difficulties. It has long been known that the reaction is positive after osmium tetroxide fixatives (though overfixation produces a brown coloration that tends to obscure the characteristic color of regenerated Schiff reagent). For example, it was used in 1928 (15) to study the nuclear apparatus of OsO_4-fixed protozoa,

[1] Interference of reduced osmium may be lessened by bleaching with an oxidizing agent, but the effect of such an agent on the protein must be reckoned, as must the possibility of removal of substances during treatment. Even though such variables can be assessed, neither basic staining nor ultraviolet absorption alone, without appropriate enzyme or acid extraction, can distinguish between RNA and DNA, an important point in clarifying nuclear morphology.

by da Cunha and Muniz in 1929 (2), and Piekarski in 1937 (12) for similar studies on bacteria, and in 1939 by Bland and Robinow (1) to study inclusion and elementary bodies of vaccinia in cultured cells. Robinow (14) used the Feulgen reaction and modifications of it on OsO_4-fixed methacrylate-imbedded sections ancillary to an electron microscope study of bacterial cytology and there have been two recent reports reaffirming the superiority of buffered osmium tetroxide as a fixative as evidenced by the Feulgen reaction (5, 16). Our experience has been no exception, but we have found one or two minor modifications necessary to produce most satisfactory results (see below).

With this success of the Schiff reagent, the possibility of utilizing the periodic acid–Schiff (PAS) reaction (see reference 7) for 1,2-glycol groups was also examined with encouraging results. The sequential staining of DNA, using a modified Schiff reagent, and of carbohydrate by the usual PAS procedure as described by Himes and Moriber (4) has also been of considerable use. The PAS reaction followed by fast green has proved a most valuable routine stain for light microscope observations in conjunction with electron microscope studies.

This paper will be concerned with the application of the thick and thin adjacent section technique together with the Feulgen reaction to the general problem of preservation of nuclear detail in the electron microscope and to two specific questions raised during some observations on spermatogenesis in the crayfish.

Materials and Methods

Materials.—A variety of animal and plant material has been investigated but those reported here are rat pancreas, grasshopper (*Melanoplus femur-rubrum*), and crayfish (*Cambarus clarkii*)[2] testis.

Fixation.—½ to 1 hour depending on material, in 1 per cent OsO_4 buffered at pHs ranging from 7.4 to 8.4 with veronal acetate buffer, dehydrated, and embedded in butyl methacrylate polymerized at 47°C. as described by Palade (11).

Sectioning.—*Thin sections* were cut on a Porter-Blum microtome (13) and mounted on carbon-coated (Watson (18)) 200 mesh Athene[3] grids. While the clear spaces in such grids are not much larger than the more easily available 150 mesh variety of copper grid, the grid wires are considerably smaller; this results in less obscuration of the field, a factor of considerable importance in studies of serial sections. Immediately adjacent *thick sections*, usually 1 or 2 μ were then cut. The Porter-Blum microtome facilitates this; thin sections can be followed easily by thick ones simply by setting the advance mechanism of the microtome to its maximum, manually holding the arm away from the knife for the appropriate number of passes, and then allowing it to cut. The thick section was transferred by wire loop to a drop of 10 per cent acetone on an albuminized glass slide, heated gently until flattened, and then dried and heated at 60°C. or so for several minutes.

Staining.—Although the Feulgen and PAS reactions will give positive results without removing the methacrylate, the hydrolysis characteristics of the former are apt to vary from tissue to tissue. We have therefore as routine removed the plastic with hot solvent (chloro-

[2] Obtained from Carolina Biological Supply Company, Elon College, North Carolina.

[3] Obtained from Ernest J. Fullam, Inc., P.O.B. 444, Schenectady, New York.

form or acetone at 60°C. for 15 minutes). Slides are then hydrated in a graded alcohol series. To reduce the loss of sections we have taken the precaution of coating slides with celloidin after 100 per cent alcohol. The Feulgen reagent is prepared and the reaction carried out essentially as summarized by Swift (17) with the exceptions that the leuco Schiff reagent is adjusted to pH 2.4, where we have found maximal color to be developed (unpublished experiments), and that hydrolysis with N HCl at 60°C. is for about 20 minutes. Maximal color is not produced until 18 minutes or so of hydrolysis depending on the tissue, and there is no appreciable visible change for times up to 30 minutes. The PAS reaction is carried out as described by McManus (7).

Light and Electron Microscopy.—Electron micrographs were made either with a Philips model EM-100 or RCA EMU-2C fitted with intermediate lens, at magnifications from 3,500 to 5,500 diameters and enlarged photographically after that, or from 1,500 to 2,000 diameters when orientation pictures of whole nuclei were desired. For light microscopy, slides were mounted in refractive index oil most closely matching the specimen (except for phase microscopy, when the oil chosen had an index of approximately 1.4). Feulgen-stained sections were observed and photographed either with tungsten light filtered with a Wratten number 76 filter or equivalent, or with illumination from a monochromator peaked at 546 mμ, near the absorption maximum of the Feulgen-DNA complex (8). Locating the same cells in the electron and light microscopes was facilitated by first making a low power photographic map of the thick section.

OBSERVATIONS

The Distribution of Chromatin in the Electron Image

The first question to be answered by this technique is whether the relatively homogeneous appearance of nuclei in the electron microscope (Figs. 1 and 3) is due to the loss of most or all the DNA during processing. This rather remote possibility is obviated by the fact that the Feulgen nucleal reaction is clearly positive as Figs. 2 and 4 demonstrate.[4] Only cytophotometric determinations of Feulgen dye content in such nuclei, compared with those of similar cells fixed in various ways (5), or biochemical analyses, could show whether small amounts of DNA may be lost. However, the important point here is that significant amounts of DNA (if not all of it) remain.

The second question raised by the apparent nuclear homogeneity is whether the DNA may not be evenly distributed rather than localized in the chromatin structures usually seen in the light microscope. While correlated phase contrast observations may show chromatin-like bodies in the nucleus, this is no evidence of their DNA nature. Figs. 1 and 2 are electron and light micrographs respectively of directly adjacent sections of the same pancreatic acinar cell nucleus. Aside from the nucleolus (*nucl*) and one dense mass (*ch*), there is nothing immediately obvious in Fig. 1 to suggest the characteristic distribution of DNA shown by the Feulgen reaction in Fig. 2. The perinuclear and perinucleolar DNA is clear in Fig. 2, as are the intranuclear clumps of chromatin. *ch* in Fig.

[4] It should be pointed out here that the plasmal reaction is either negative or negligible as indicated by appropriate controls and blanks.

1 can be seen in Fig. 2 to contain DNA. Closer scrutiny of the electron micrograph in areas that are Feulgen-positive reveals possible differences in fine structure, but these studies are not complete and will not be discussed here.

The cursory deceptiveness of the electron image is especially obvious in Figs. 3 and 4. Fig. 3 is a meiotic prophase of a grasshopper primary spermatocyte; the only strikingly evident structure is the nucleolus. Fig. 4 is either the same or a neighboring nucleus (it is immaterial since all cells in this cyst of the testis were in the same stage as evidenced by both electron and light microscopy) in a serial section several microns removed. While the nucleolus of Fig. 3 finds no counterpart in Fig. 4 because the sections are not immediately adjacent, the chromosomal detail (coiling is evident at the arrow) is certainly not to be expected from the electron image. In this instance, as in all others we have so far encountered, Feulgen-positive material is localized in conventional structures as seen in the light microscope, though this may not be reflected in the electron image.

A third question may now be justifiably asked: when dense structures are seen in electron images of nuclei, are they chromatin? In dividing cells the answer seems obvious, but a comparison with the adjacent Feulgen section permits a statement of certainty and in non-dividing cells such a comparison is necessary. Fig. 5 is an electron micrograph of a primary spermatocyte prophase of the crayfish. Structures resembling chromosomes are apparent, as at *chr*, but other masses (as at arrow) are of questionable identity. Fig. 6 is the adjacent Feulgen-stained section. The best correlations can be made where chromosomes pass almost perpendicularly (as at *a*, *b*, *c*, and *d*), obliquely as at *chr*, or in the rare case where the chromosome lies in the plane of section fortuitously cut so as to be included in both sections (as just above *c*). The mass (arrow) is Feulgen-positive and hence represents either heterochromatin or an agglomeration of chromosomes.

Observations on Crayfish Spermatogenesis

1. Structure in Meiotic Chromosomes.[5]—We have recently described long, ordered, laminated "cores" in early meiotic chromosomes similar to those in Fig. 5 (9). These structures consist of a central dense filament about 150 A in diameter surrounded by a less dense region about 250 A wide, much like a lead pencil. The total width of this structure is then 600 to 700 A. In longitudinal sections, one or two parallel lines, more or less continuous and separated by less dense regions, can be seen on either side of the central "pencil." Interpretation of oblique and rare cross-sections indicates that these outside laminae are sometimes concentric, but further observations show that their appearance

[5] I wish to express my gratitude to Dr. Keith Porter for his generous association in this work, and for having brought to my attention the existence of a central structure in these chromosomes.

may vary, possibly depending on the stage. In some cases they have been seen as massings or thickenings of the discontinuous material of the chromosome on opposite sides of the "pencil." Thus, they have sometimes appeared as concentric shells, and at other times as parallel filaments or ribbons bounding the central apparatus on two sides.

Structures of this configuration are not to be predicted from what we now know of chromosomes. Although they are presently of unknown function, these nuclear structures nevertheless represent the first unequivocal ones so far reported below the resolution of light, integral with what appear to be chromosomes. It then becomes important to know positively whether the associated dense masses are indeed chromosomes. In Fig. 7, the same Feulgen-positive areas, *a*, *b*, *c*, and *d*, shown in Figs. 5 and 6, are presented at higher magnification. It is apparent in all (which are interpreted as oblique sections) that the core (*C*, Fig. 7 *c*) consists of the central filament *r* imbedded in less dense material. The bounding laminae vary in distinctness and number but their double structure can best be seen in Fig. 7 *c*. While the resolution of the light microscope is insufficient to make it possible to say that the cores themselves are Feulgen-positive, it is apparent that they are at least embedded in DNA and hence are undoubtedly integral in some way with the linear structure of the prophase chromosome. (It should be noted here that we have so far seen these structures in material fixed at pHs from 7.6 to 8.4. In addition the same or analogous structures have been observed in primary spermatocytes of *Xenopus laevis*, the rat, and grasshopper but have not yet been positively identified in somatic cells.)

In Fig. 8, a chromosome (*chr*) included in the plane of section for about 10 μ of its length, can be seen terminally associated (*at*) with the nuclear envelope (*ne*). There is no evidence of a chromosome membrane. In the region shown at higher magnification in Fig. 8 *a*, the section cuts through the core (*C*); the central filament (*r*), its surrounding less dense material, and some evidence of outer laminae can be seen. However, at the point indicated by the arrow, the outer boundary is broken, and the less dense region is continuous with a diversion that is lost in the material of the chromosome. Such apparent branching is not uncommon; in this case it is clearly associated with a mass of dense material (chromatin) (Fig. 8, *3*) which seems to be one of a number along the chromosome. In this region through the chromosome axis the mass forms part of an alternating series (Fig. 8, *1*, *2*, *3*, *4*, *5*) which can be interpreted as sections through a spiral of chromosomal material winding around the core. The appearance of the chromosome as it passes out of the section, especially at the extreme right is not inconsistent with this idea. On the other hand, the masses may simply represent chromosomal enlargements, possibly comparable to "lampbrush" extensions. In either case, they appear to have an intimate relation to the core.

2. Elaborations of the Nuclear Envelope during Spermiogenesis.—Spermatogenesis in decapod crustacea has been described by light microscopists (see McCroan (6)). But we find that the reports are largely inaccurate with the possible exception of McCroan's, mainly because most of them antedated the Feulgen reaction. Moreover, by virtue of leading to the production of aflagellate sperm, spermiogenesis in the crayfish is highly irregular, and despite the conscientious use of cytological techniques for demonstrating mitochondrial and Golgi structures, the process is confused in the literature and the analogy to that in flagellate spermiogenesis is obscure. In the course of following the evolution of crayfish sperm with the electron microscope, the curious behavior of spermatid nuclei came to light. The following observations serve to illustrate the application of the correlated thick and thin adjacent section technique to another specific problem.

Shortly after the second spermatocyte division which results in a spermatid with elongate nucleus (Fig. 9), the nuclear surface is erupted in outpocketings or blebs (Fig. 9, arrows). Fig. 10 is an enlargement of the area outlined in Fig. 9. The blebs (Figs. 9, arrows, and 10, *nb*) appear to be associated with small vesicles surrounding the nucleus and massed at one side in the cytoplasm (*cv*). Large vesicles (Fig. 10, *v*) appear to be either cross-sections of fingerlike projections of the nuclear surface or vesicles that have pinched off from the blebs; which they actually are could only be decided from a series of serial thin sections. The question of some interest here is whether DNA is involved in these outpocketings and vesicles. Fig. 11 is the adjacent Feulgen section; the arrows here and in Fig. 9 point to corresponding outpocketings. Although the nucleus in Fig. 9 has been compressed in sectioning, the Feulgen-positive projections in Fig. 11 extend well beyond the surface and in places appear discontinuous. It is obvious then that the DNA does follow the nuclear envelope in its diverticulations. These are completely included in the thicker section with a few exceptions where they are either transected or actually represent isolated vesicles. Unless there is some structural mechanism for retaining the DNA in the nucleus, this behavior risks losing DNA into the cytoplasm in what seems to be random fashion. Such apparently wasteful behavior is certainly not to be predicted from current concepts of the importance of DNA to heredity and its constancy in amount with respect to the genome. This seems particularly true in the formation of such a genetically vital tissue as sperm.

However, that DNA is actually under structural control is suggested from observations of a later spermatid stage. The result of the blebbing and vesiculation is a remarkably elaborate system of convoluted membranes (Fig. 12, *cm*) on either side of the nucleus which is now biconcave (Fig. 12). On one side, surrounded by the membranes and in juxtaposition to the nucleus is a large vesicle (*av*) of which the wall, in the region of the nucleus, is lined by a dense granular material (*am*). This vesicle was incorrectly identified by many early workers as

the nucleus; comparison with the adjacent Feulgen section (Fig. 13) shows that it is Feulgen-negative. Fig. 13 is somewhat misleading in that although the dense rim of vacuolar material (*am*) appears Feulgen-positive, actually it is not; its density so alters its refractive index that it scatters light. When this section is observed at 620 mμ where absorption due to the Feulgen complex is negligible, it is the only structure that can be seen. Subsequent experiments have shown it to be PAS-positive and hence it has been tentatively identified as acrosomal material derived from the acrosomal vesicle (*av*).

The outline of the nucleus in Fig. 13 is smooth in comparison to that in Fig. 11. Yet examination of the area outlined in Fig. 12 and presented at higher magnification in Fig. 14, shows the membranes to be continuous, at least in part, with the nuclear envelope (see especially at arrows, Fig. 14). Fig. 15 is an electron micrograph of another similar nucleus; the arrow points to a region where the nuclear envelope extends into the mass of convoluted membranes and returns. If anything, this kind of outpocketing is more extensive than that at the earlier stage, yet there is no evidence of corresponding extensions of DNA. Thus regardless of whether or not DNA may be lost in the early spermatid, as the sperm begins to be formed, the DNA is retained; at this stage it is somewhat protected from such mechanical loss. The mechanism for accomplishing this undoubtedly lies somewhere in the differences in fine structure of nuclei at the two stages, but it is not presently apparent from our studies.

DISCUSSION

The observations described above, while in themselves meriting attention for their biological significance, were presented here mainly to illustrate the application and value of correlated light (cytochemical) and electron microscope studies using adjacent thick and thin sections. This approach, together with the Feulgen reaction, offers a strong means of attack on the hitherto refractory problem of nuclear fine structure; but it should be obvious that its potentialities are far more extensive. Even the usefulness of the Feulgen reaction need not be restricted to the nucleus *per se*; it should, for example, be of interest to pathologists and virologists in studying cellular inclusions. The extent to which such an adjacent stained section technique can provide cytochemical information about the electron image is, of course, limited to the resolution of the light microscope. Analysis of individual smaller structures must await development of specific electron stains. However, it should be remembered that the latter have an inherent disadvantage: they distinguish different substances (and structures) only by virtue of differences in density between the stained structure and its surround. Although such differences may often be striking, more subtle variations are difficult to detect and interpret. It will be hard to equal the differentiation possible with colored stains in the light microscope. Thus, while useful cytochemical tests, such as the Feulgen reaction for DNA already exist, achieving comparable specificity with an electron stain

remains for the future. The suitability of other tests and reactions for such cytochemical analysis requires further investigation; for instance, the techniques of enzyme histochemistry should certainly be explored. But for the moment, those available, the Feulgen and PAS reactions, offer many possibilities in amplifying studies of cell structure, chemical composition, and function with the electron microscope.

SUMMARY

In this paper, a procedure for correlating electron microscope and light microscope cytochemical studies using immediately adjacent serial thin and thick sections has been described and discussed. This technique, combined with the Feulgen reaction for DNA, has been of particular value in framing and answering both general and specific questions about the nucleus. The results may be summarized as follows:—

Apparent nuclear homogeneity in the electron microscope is not due to loss of DNA as evidenced by positive Feulgen reactions in such nuclei.

Arrangement of Feulgen-positive material in chromosomes, heterochromatin, perinuclear and perinucleolar chromatin, etc., is similar to that customarily observed in the light microscope but this is not necessarily reflected in a cursory survey of the electron image.

Careful comparison of light and electron images shows that fine differences in structure are associated with chromatin localization.

Primary spermatocyte prophase chromosomes of crayfish have been positively identified by their Feulgen-positive nature. Core-like axial structures in such chromosomes have been observed (9) and are described further.

A remarkable feature of spermiogenesis in the crayfish is an elaboration of the nuclear envelope of the spermatid accompanying the formation of what becomes a mass of convoluted membranes in the sperm. In the spermatid, perinuclear chromatin follows outpocketings of the nuclear envelope into the cytoplasm. In the early sperm, on the other hand, although the nuclear envelope is continuous with the system of convoluted membranes, the chromatin is distinct from it and is retained in the nucleus proper by some mechanism independent of the nuclear envelope.

None of the above observations was apparent from the electron microscope images alone; they were possible only by virtue of the correlated cytochemical and electron microscope study of adjacent sections.

The successful use of other cytochemical tests, such as the PAS reaction for certain carbohydrates, in such correlated studies is also described.

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EXPLANATION OF PLATES

All material fixed in buffered OsO_4 and embedded in methacrylate. All Feulgen preparations photographed at 546 mμ.

PLATE 133

FIG. 1. Electron micrograph, rat pancreas acinar cell nucleus. *m*, mitochondrion; *ch*, chromatin mass; *nucl*, nucleolus. Approximately × 6,750.

FIG. 2. Adjacent 2 μ section of same nucleus as in Fig. 1, Feulgen preparation. In addition to abbreviations in Fig. 1, *p nuc ch*, perinuclear chromatin; *p nucl ch*, perinucleolar chromatin. Approximately × 6,750.

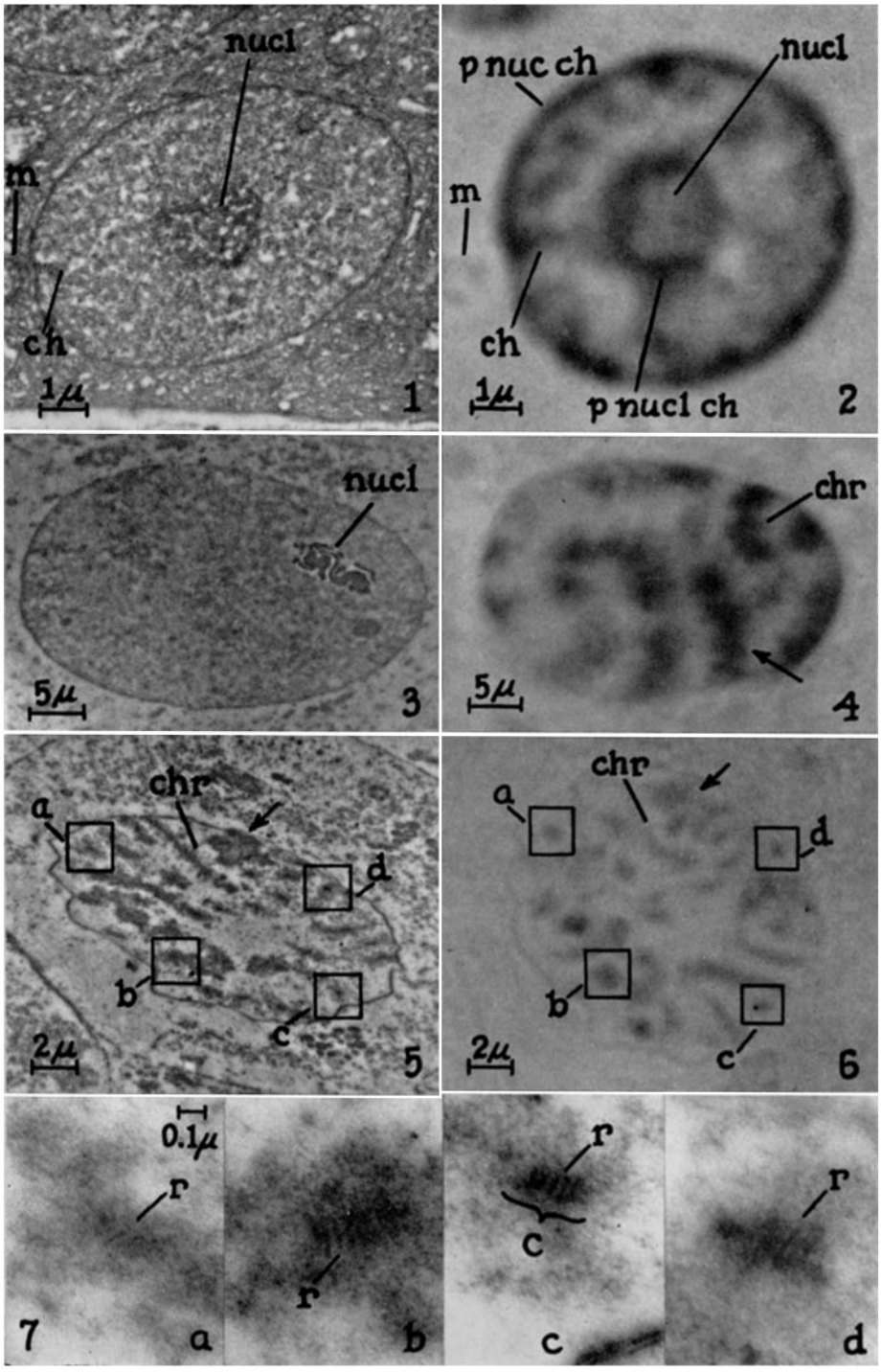
FIG. 3. Electron micrograph, grasshopper primary spermatocyte prophase. *nucl*, nucleolus. Approximately × 1,475.

FIG. 4. 2 μ section, Feulgen preparation of neighboring nucleus to that in Fig. 3, separated by several microns but in the same stage of prophase. *chr*, chromosome; arrow points to apparent coiling. Approximately × 1,500.

FIG. 5. Electron micrograph of crayfish primary spermatocyte prophase. *chr*, chromosome; *a*, *b*, *c*, and *d* are areas similarly designated in Figs. 6 and 7; arrow points to chromatin mass discussed in text. Approximately × 3,140.

FIG. 6. 2 μ section, Feulgen preparation of adjacent section of same nucleus as in Fig. 5. *a*, *b*, *c*, and *d*, are same chromosomes outlined in Fig. 5; arrow points to same chromatin mass as in Fig. 5. Approximately × 3,140.

FIG. 7. *a*, *b*, *c*, *d*, higher magnification of areas outlined in Figs. 5 and 6, showing laminated "cores," *C*; *r*, central dense filament surrounded by less dense region. Approximately × 44,700.



(Moses · Studies on nuclei)

PLATE 134

FIG. 8. Electron micrograph, crayfish primary spermatocyte prophase chromosome. *cy*, cytoplasm; *ne*, nuclear envelope; *nuc*, nucleus; *at*, region of association of chromosome with nuclear envelope; *chr*, chromosome; *1*, *2*, *3*, *4*, *5*, designate chromatin masses presenting aspect of sectioned helix. Outlined area shown in higher magnification in Fig. 8 *a*. Approximately × 11,400.

FIG. 8 *a*. Higher magnification of area outlined in Fig. 8, showing "core," *C*; *r*, central dense filament surrounded by less dense region which, at arrow, is continuous with branch entering chromatin mass *3*. Approximately × 58,500.

FIG. 9. Electron micrograph, crayfish spermatid showing "blebbing" around nuclear periphery especially evident at arrows. *cv*, small cytoplasmic vesicles; *nuc*, nucleus. Outlined area shown enlarged in Fig. 10. Approximately × 3,700.

FIG. 10. Enlargement of area outlined in Fig. 9. *cv*, small cytoplasmic vesicles; *cy*, cytoplasm; *nb*, outpocketing of nuclear envelope; *nuc*, nucleus; *v*, vesicular profile representing either cross-section of outpocketing (*nb*) or vesicle pinched off from such an outpocketing. Approximately × 11,400.

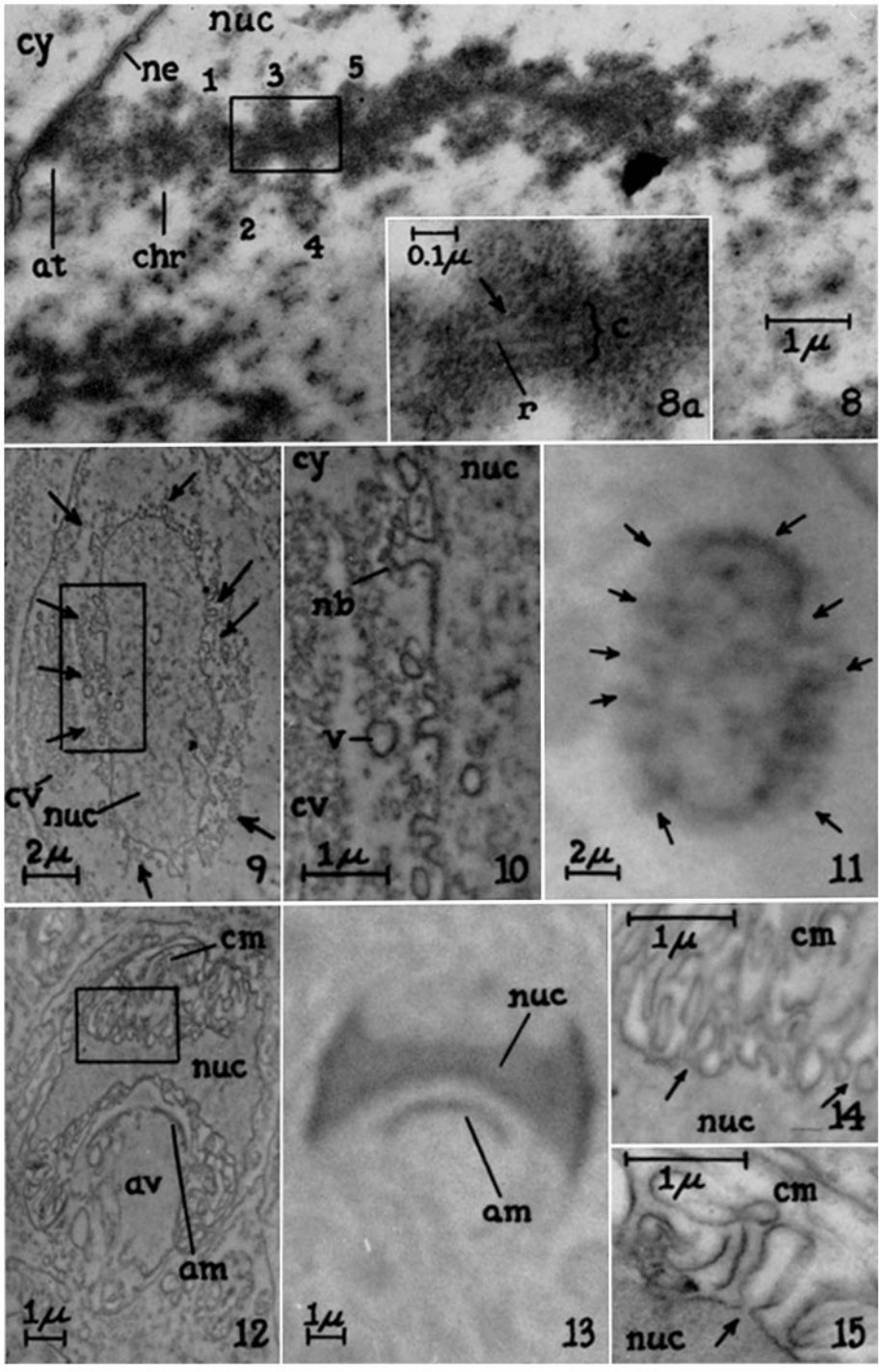
FIG. 11. 2 μ section, Feulgen preparation, adjacent section, same nucleus as in Fig. 9. Arrows point to same areas marked in Fig. 9. Approximately × 3,700.

FIG. 12. Electron micrograph, crayfish late spermatid. *cm*, convoluted membrane system; *nuc*, nucleus; *av*, acrosomal vesicle; *am*, acrosomal material. Outlined area is shown in enlargement as Fig. 14. Approximately × 5,100.

FIG. 13. 2 μ section, Feulgen preparation, adjacent section of same cell as in Fig. 12. *nuc*, nucleus; *am*, acrosomal material. This latter structure is not Feulgen-positive, but is rendered apparent in the photograph only by virtue of its extreme refractility; other experiments have shown this structure to be PAS-positive. Approximately × 5,100.

FIG. 14. Enlargement of area outlined in Fig. 12. *cm*, convoluted membranes; *nuc*, nucleus. Arrows point to regions where nucleus is continuous with space between membranes which appear to be extensions of the nuclear envelope. Approximately × 14,800.

FIG. 15. Electron micrograph of the edge of the nucleus of a crayfish spermatid slightly earlier than that shown in Figs. 12 to 14. *cm*, convoluted membranes; *nuc*, nucleus. Arrow points to region where nuclear envelope bubbles out into the mass of membranes and returns, leaving the nucleus continuous with the space thus formed. Approximately × 17,100.



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