NUCLEAR CHANGES DURING SPERMIOGENESIS IN A PULMONATE SNAIL*

By LIONEL I. REBHUN, Ph.D.

(From the Department of Anatomy, College of Medicine, University of Illinois, Chicago)

Plates 167 to 170

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The diffraction patterns resulting from the application of x-ray techniques to the study of the organization of the sperm head (Sepia, Loligo, and Salmo) are closely similar to those of the sodium salt of DNA (1). This suggests that the DNA of the nucleus of the sperm is organized into a crystalline lattice (1). Polarization optics show that the sperm heads of Loligo (and a wide variety of other anisotropic sperm) are negatively birefringent with respect to the sperm head axis in the living state (2, 3). These facts suggest that in anisotropic sperm heads the DNA is organized into a (imperfect) crystal with the axes of the DNA molecules parallel to the axis of the sperm head (1). One might expect the electron microscope to reveal some semblance of this organization. From published reports of sectioned sperm heads, however, there appears to be only a relatively uniform distribution of small (100 to 150 A) spherical particles within the mature head (4–7). On the other hand reports of spermatids of cat (6), toad (8), and sparrow (9) have shown that at an earlier stage in sperm development there is an aggregation of nuclear material into large (600 to 700 A for cat and toad, and 250 to 450 A for sparrow) particles with a suggestion of an inner structure. There is little electron scattering material between the particles. In the sparrow they are elongated with the axes approximately parallel to the spermatid axis although possibly twisting relative to it. It is suggested by Yasuzumi (9) that helical DNA molecules can be seen within these particles. In the cat (6) and toad (8) there is some suggestion that the large aggregates disappear in the mature sperm. The sectioned mature rat sperm heads obtained by Challice (4) strongly suggest this.

The period during which this aggregation of smaller particles to form larger ones takes place appears to correspond to a period of sperm development during which profound staining changes take place in the head. With methyl green pyronin (or methyl green-acid fuchsin) this involves a change in color from green to red (10, 12) and then back to green again (10). With iron hematoxylin the staining reaction of the head changes from a light grayish purple

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to a deep black and then back to its former hue (10, 11, 13, 15, 16) although in some animals the intense coloration persists (14). Accompanying these staining changes may be changes in the distribution of the staining material from a peripheral location in the head to a central one (14, 16). Recent work indicates that these staining changes (especially those of methyl green) may correspond to changes in the physical state of DNA, the nature of the proteins in the nucleus of the developing sperm, or of their association with DNA (17). The present study, using the pulmonate snail *Otala lactea*, covers the period from spermatid to mature sperm and traces the development and disappearance of an "aggregated" state of the nuclear material during a period in which classical studies show the spermatid to undergo the staining changes mentioned above (10, 11).

Materials and Methods

The pulmonate snail, *Otala lactea*, was used. The snails are obtained in an aestivating condition and may be kept this way in the refrigerator for at least a year and a half and undoubtedly much longer. When material is desired it can be obtained directly from the aestivating snails or from animals brought out of aestivation for several weeks. This is done by removing the membrane secreted across the shell entrance and wetting the animal by leaving it incompletely immersed in a small pool of water. The snails will swell and drown if left completely submerged for too long a period. They can be kept in large glass jars covered with gauze and they will live indefinitely on lettuce. Since they have a tendency to go back into aestivation as soon as the food is gone they must be brought out by wetting them and must be re-fed every other day or so. The snails used in this study were all kept out of aestivation and relatively active for periods of 2 weeks to 3 months before being used.

The ovotestis lies in the visceral hump (37) and may be reached by removing the shell with a blunt scissors. The thin membrane surrounding the visceral hump may then be split and the ovotestis quickly separated from the surrounding tissue to which it is loosely attached. The whole gland is then immersed in cold buffered osmium tetroxide solution and cut into smaller pieces. Since the gland is an alveolar one with no connective tissue binding the alveoli to each other, the fixative has free access to each alveolus. This is especially true if the gland is cut into small pieces and the initial fixation is watched for a moment or two under the dissecting microscope to make sure the alveoli are spread. The pieces are then immersed in an excess of fixative and left in an ice bath in the refrigerator for ½ to 3 hours.

The fixative used is that recommended by Palade (18) but with the pH at about 8.2 (8.1–8.3, the pH of snails' blood) and with sodium chloride added. Since there appears to be little osmotic regulation in these snails (their blood varying from 0.25 per cent to 0.7 per cent equivalent of sodium chloride (38)), this latter is probably not too important, but the final fixative nevertheless contained 0.4 per cent sodium chloride. Subsequently the fixed tissue was washed for about 1 hour in 0.4 per cent Na Cl in distilled water, quickly dehydrated with alcohols, impregnated with a 10 per cent mixture of methyl methacrylate in *n*-butyl methacrylate, and polymerized at 60 to 70 degrees Centigrade (39) either with benzoyl peroxide or luperco at ½ to 1 per cent as catalyst.

The tissue was sectioned with a glass knife on a Porter-Blum microtome (40). Sections were picked up on formvar grids or on carbon grids (19, 20) and studied with an RCA EMU microscope fitted with a 60 micron back focal plane, externally centerable aperture. The microscope was calibrated with a 29,500 line per inch diffraction grating.

Material was also fixed in Carnoy's solution and stained with the Feulgen reagent; and with Flemming's solution without acetic acid and stained with iron-hematoxylin (10, 11). In

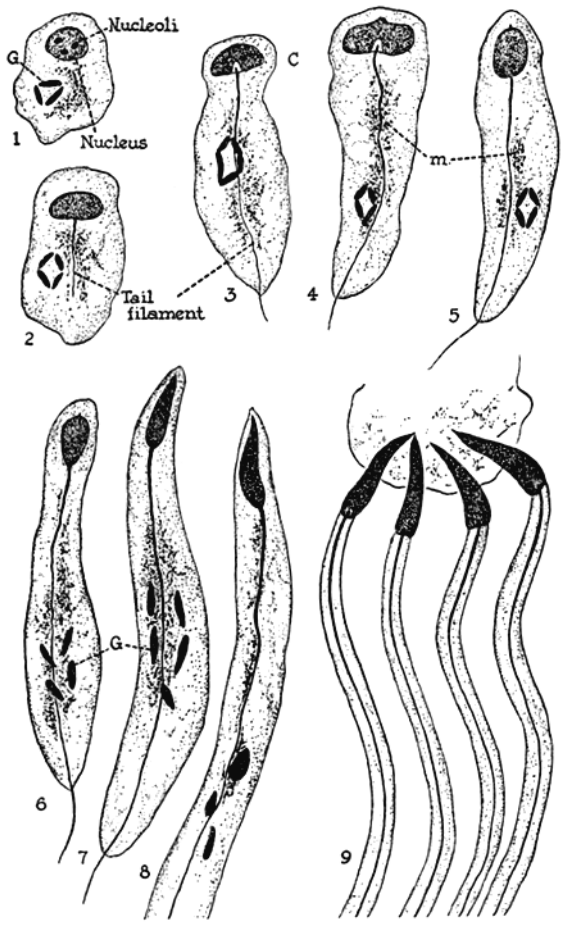
some cases alternate thick and thin sections for light and electron microscopy respectively, were used. The thick sections without staining were examined with a phase microscope or stained with the Feulgen reagent (27) and viewed with a bright field microscope. Living cells were observed with a phase or interference microscope by smearing glands with only body fluids as a medium (for alternate methods see Roque (23) and Nath (22)).

OBSERVATIONS

Resumé of Changes in the Nucleus.—Nuclear changes can be classified into three periods which can be correlated with cytoplasmic events in the development of the sperm. The latter will be discussed at a future date. The first period of nuclear change involves a change in the over-all shape of the spermatid nucleus. It starts as a spherical body and during this first period flattens to a squat figure of revolution with its greatest diameter at right angles to the future axis of the sperm. (See Text-fig. 1-*1* to *4*.) Slight changes take place in the distribution of the material within the nucleus. The second period involves an elongation of the nucleus in a direction perpendicular to that in the previous stage. That is, the axis of revolution now becomes the longest axis of the spermatid head (Text-fig. 1-*5* and *6*). At this time profound reorganizations take place in the nuclear material resulting in the appearance of plates about 60 A thick, 400 A to 1 micron wide, and several microns long (2). These fill the nucleus in regular order and are so arranged that the long dimensions of the plates are parallel to the sperm axis. During the third period of spermatid development the nucleus twists and so loses axial symmetry (Text-fig. 1-*7* to *9*), and at the same time the plates seem to disappear, resulting in a nucleus which appears relatively homogeneous with regard to its contents of scattering material.

DETAILED DESCRIPTIONS

First Period.—The spermatid nucleus is at first approximately spherical. The dense mass seen in the nucleus of the cell in Fig. 1 is a nucleolus. There are two or three present as can be seen from observations of the living spermatids with the phase microscope. The internal structure of this body is difficult to make out in this picture but it appears to consist of tightly packed, dense granules about 150 A in diameter. No evidence of a nucleolonema can be seen in these nucleoli. Other bodies may also appear in the nucleus and are similar to the nucleolus in size except that they are much more irregular in outline and are considerably lighter in electron density. They are similarly constructed of small granules about 150 A in diameter. The rest of the nucleus appears to contain a considerable number of small granules about 100 A in size scattered in a somewhat heterogeneous manner. That is, the granules appear to be clumped into strands rather than to be homogeneously spread through the nucleus. Thin filaments about 60 to 100 A in thickness and less dense than the particles are also found in the nucleus and appear to contribute to this clumping. One can reasonably compare the structure to that of a sponge in which the substance of



TEXT-FIG. 1. Drawings were made from material fixed in Palade's fluid for about ½ hour, smeared on a slide, and observed with the Baker interference microscope. No attempt is made to depict accurately any cellular objects other than the nucleus, the Golgi apparatus, and the tail filament. *G*, Golgi apparatus; *t*, tail filaments; *c*, centriole region; *N*, nucleus; *n*, nucleolus; *a*, acrosome. *1*, early spermatid just after second meiotic division (corresponds to Fig. 1). *2*, spermatid corresponding to that of Fig. 2. *3*, a slightly later spermatid probably between that of Fig. 2 and Fig. 4. *4*, a spermatid corresponding to that of Fig. 4. *5* to *7*, spermatids representing stages in nuclear elongation and corresponding to those in Figs. 3, 5 to 8, 10 to 12. *8*, the pronounced helical twist in the nucleus of this advanced spermatid makes it correspond to that in Fig. 13. *9*, the mature sperm has rid itself of excess cytoplasm. Fig. 9 is a cross-section of a sperm head in this stage.

the sponge is occupied by the granules and filaments and the spaces correspond to the areas more or less devoid of dense material. There are no formed walls around these lighter areas, which are somewhat irregular and are about 400 A

to 700 A across. Soon after spermatid formation the nucleus begins to change its over-all shape and an orientation appears in the cell. The nucleus begins to flatten to form an approximate oblate spheroid the axis of rotation of which coincides with the future axis of the sperm (Text-fig. 1-*1* and *2*). At the same time a shift in relative positions of the nucleus and the main mass of cytoplasm takes place so that the nucleus now occupies the part of the cell which will become the head or anterior end. The tail filaments can be seen in the cytoplasm at this time and an indentation in the base of the head appears, this indentation housing the anterior end of the tail filaments. A further change in nuclear shape occurs (illustrated in Text-fig. 1-*3* and *4*) converting the slightly flattened spheroid to a figure of revolution resembling a flattened top. A conical protuberance which will be tipped by the acrosome contributes to the "top" shape. At about this time, when a polarity has been established in the cell and in the nucleus, two other nuclear events occur. One is a thickening of the nuclear membrane covering the anterior surface of the nucleus and the other a thickening of that covering the posterior hemisphere (Fig. 2). Similar phenomena have been described from studies of light microscope sections of spermatids of other pulmonates (10, 11). The process of thickening and increase in density of the nuclear surface does not cover the entire surface but leaves an equatorial band with a nuclear envelope apparently unchanged. Sections thin enough to determine whether one or both components of the nuclear membrane are involved in the process of thickening have not been obtained. It should be noticed that the interior of the nucleus does not appear to be changed from the picture seen at an earlier time. There are several cytoplasmic events involved with tail sheath formation which are related to the nuclear changes, but they will be detailed at a future date. The one we need to mention here in order to time some future events is that the mitochondria have changed their localization and by the time the events just described have been completed they are clustered about the tail filaments.

The final change within what we define as the first period is the beginning of the elongation of the nucleus in a direction at right angles to the longest diameter of the flattened nucleus. The culmination of this process (which occurs in the period next to be described) is the formation of the familiar elongated sperm head. The process appears to begin with a "softening" of the nucleus so that it becomes somewhat irregular in outline although still maintaining the generally flattened shape it had till now (see also 10). This event appears to be correlated with profound changes which occur in the mitochondria of the cell and with the process by which the mitochondria apply themselves to the tail filaments in the first step in tail sheath formation.

Second Period.—After the "softening" mentioned above, the nucleus begins its axial elongation and at the same time, those changes which we are using to characterize the periods of nuclear change, begin to occur. Specifically in the

region of the proximal centriole the material in the nucleus begins to show some orientation into what appear to be "filaments" which start on the nuclear envelope and turn to run parallel to the axis of the sperm head (Figs. 3 and 4). From the later history of these structures and from some electron micrographs to be discussed below it seems clear that these structures are thin laminae or plates, the linear structures appearing in the micrographs being their profiles. It will be noted in the above figure that the orientation of the nuclear material at this stage is present primarily in the basal region of the nucleus, the material of the anterior region still showing the characteristics described in nuclei in stage 1. It will be noticed that in the region of less striking organization, "empty" regions delimited by dense profiles appear (vacuoles). The cytoplasmic event which is correlated with the appearance of nuclear plates is the beginning of the mitochondrial fusion to form the tail sheath around the tail filaments (that is, the formation of an organized body, not simply a mass of applied mitochondria (24)). The laminae appear throughout the nucleus and the nucleus soon appears as in Fig. 6. It should be noted that the sheets generally show an axial orientation. They are about 60 A thick and are thus about half the thickness of the nuclear dots previously described, although they are about the size of the randomly disbursed threads which accompany the dots.

Fig. 5 shows a slight oblique cross-section of a nucleus judged to be in a stage similar to that of Fig. 6 (which is a diagonal cut whose plane is nearer the sperm axis than perpendicular to it). It will be noticed that in cross-section there is little evidence for any orientation perpendicular to the nuclear envelope and in fact the plate profiles appear randomly oriented. The justification for the description of these objects as plates or sheets comes from micrographs similar to those of the preceding two figures. It can be seen that these plates appear relatively straight when sectioned in a direction approximately parallel to the axis of the sperm but that in cross-section they may appear curled back upon one another to form U-shaped profiles. Arrows point to places where the nuclear envelope can be seen with clarity. It is seen to consist of an inner membrane about 150 A thick and an outer one about 50 A in width, the space separating them being about 100 A wide. In micrographs where the angle of cut is favorable it can be seen that the inner membrane is actually itself double, consisting of two membranes about 50 to 60 A thick separated by a space with similar dimensions. At this stage the compound inner membrane does not appear to form a continuous inner lining to the nucleus, and indeed in places in Figs. 6 and 5 it is seen to be broken and to form gaps. In Fig. 6 the region of the nuclear envelope lining the cavity occupied by the proximal centriole does not possess this three-layered structure although the inner membrane of the, here, double nuclear envelope is thicker than the outer. These statements hold also for later stages as we shall now see.

With further elongation of the nucleus and of the sheets the nucleus takes

on the appearance shown in Fig. 7 (see also Text-fig. 1-*5* and *6*). The axial orientation mentioned previously is now quite pronounced and it can be seen that in several parts of the picture sheet profiles can be traced for at least 1 micron. Areas are then usually encountered in which the sheets are somewhat indistinct and it is difficult to trace the course of a profile through such a region. One would guess that except for this consideration, profiles could be traced for several micra in regions such as that indicated with an arrow in Fig. 7. The section passes approximately parallel to the axis of the sperm but apparently not directly through it. The hollow in the base of the sperm head is occupied by a piece of the proximal centriole apparatus. It will be noted that a thin layer of cytoplasm still surrounds the head.

Figs. 8, 10 to 12 show cross-sections of the head at various levels. In Fig. 8 the section goes through at approximately the level indicated by *A-A* in Fig. 7, and it will be noted that in the interior of the doughnut-shaped cross-section can be seen a part of the proximal centriole region. Within the nucleus itself can be seen the cross-sections of the sheets and it should be noted that these profiles are for the most part perpendicular to the nuclear envelope, at this level. In many places the profiles appear to be hairpin loops starting and ending on the nuclear envelope, either inner or outer, and in some places these loops are contained one within the other (arrow Fig. 8). It can be seen that at the outer boundary of the nucleus the nuclear envelope is doubled and that the inner membrane is three times or so as thick as the outer[1]. The section is unfortunately thick so that relations cannot be seen clearly. However, it would appear that the description given of the nuclear envelope at the previous stage, still holds. In addition to hairpin loops, the plates may run radially from the inner to outer ring and in general the orientation of the plates in the centriole region is radial to the sperm axis.

Fig. 10 is a cross-section of a sperm head at a level somewhat higher than that of the previous section (Fig. 8) and appears to go through the upper end of the centriole (*B-B* Fig. 7). The sheet profiles which start on the inner circle (which of course, is the nuclear envelope lining the pit occupied by the centriole) appear to run perpendicular to it as in the previous picture. In several places profiles appear to attach to the outer boundary of the cross-section and

[1] An additional statement should be made here. The region immediately outside the outer layer of the nuclear envelope appears quite fuzzy in most of these electron micrographs. However, in thin cross-sections, one can see that running over the whole nuclear surface is a set of tubules about 200 A in diameter with centers spaced about 300 A apart (see Fig. 9). This component of the cell continues over the centriole and forms the outermost layer of the tail sheath of the mature sperm, where it will be called the outer rod sheath (24). It is probably homologous to the manchette as seen in the cat sperm by Burgos and Fawcett (6). At any rate, in the regions of the cell containing the nuclei, if the section is not an accurate cross-section, or if it is not thin enough, this mass of parallel tubules forms an area of confusion partially overlapping the nuclear envelope.

these are also approximately perpendicular to the membrane. However, there appear to be regions in which the profiles run parallel to the outer surfaces of the nucleus. In addition in several places between the inner and outer nuclear boundaries the radial orientation so clear in the previous figure is definitely not present. In these regions a group of parallel profiles will split into two or more groups of profiles and join another such group, etc. This process is carried to completion in higher levels of the nucleus, as can be seen in Fig. 11, which is a cross-section of a sperm nucleus at a level similar to that indicated by *C-C* in Fig. 7. It will be seen that some semblance of perpendicularity to the nuclear envelope is maintained by some of the sheets in the nucleus but that others run parallel to it. The interior of the nucleus is now a swirl of sheets and the splitting up of groups of parallel profiles into subgroups, each of which then joins a different subgroup, can be seen to be general. The lengths of the sheet profiles are highly variable as are their shapes. Many appear to end abruptly whereas a small number appear to be arranged in small, closed circles. The lengths of the profiles may be from 400 to 500 A up to a micron if measured along their total meandering course. The course of some of the profiles makes several hairpin turns and it should be noted that although the laminae may show gentle curves, the occurrence of hairpin, 180° turns is quite prevalent. It is partly this which accounts for the fact that despite the irregularity of their paths, the profiles, by and large are uniformly spaced at about 100 A apart (Figs. 8, 10 to 12).

In certain regions, the gaps in the inner nuclear membrane mentioned earlier still persist (Fig. 10), and in some micrographs it appears as if plates may start at the gap edges.

A final consideration is the occurrence of "vacuoles" in the nucleus, that is, light regions bound by thin, dense layers. These were seen in Fig. 3 and can be seen to occur at this later stage in Figs. 10 and 11. Their relation, if any, to the plates is unclear, but they may be related to vacuolization in chromatin during sperm formation as reported for other animals (14, 25). Fig. 12 is a high power view of a cross-section of a sperm head above the centriole and some of the features discussed in the previous descriptions can again be seen.

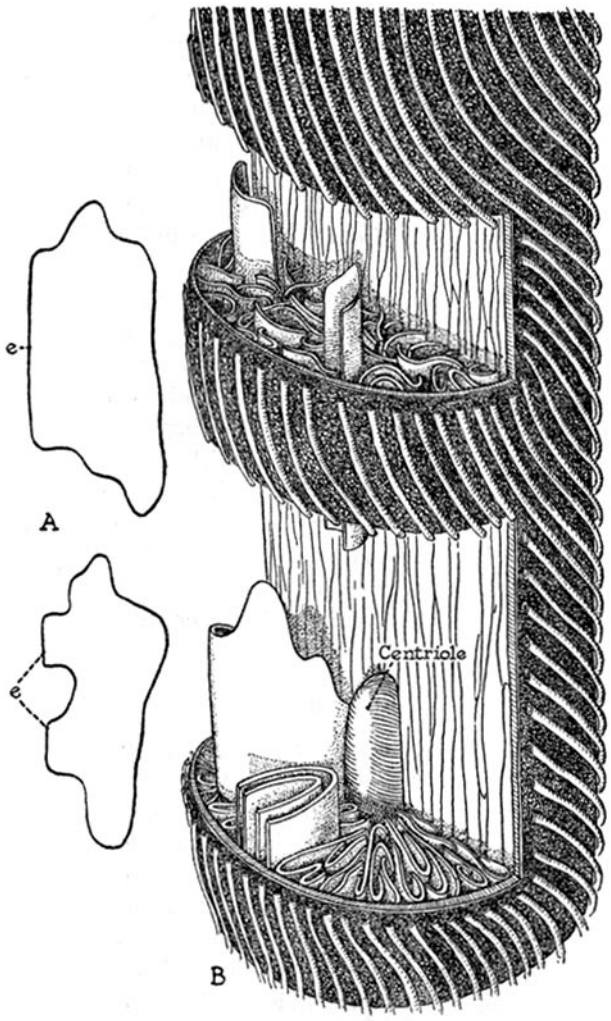
Third Period.—Soon after the great elongation of the sperm head and the appearance of the structures described above the head undergoes a further process leading to the disappearance of the sheets. This process appears to involve a twisting of the nucleus and a thinning and elongation of the anterior ½ micron or so of the head, *i.e.* the acrosome region (Text-fig. 1-*7* to *9*). Fig. 13 shows a longitudinal section including the base of the head. The sheets can still be seen in profile in parts of the head and it will be seen that they have been twisted so that now the profiles appear to be at an angle to the sperm axis. However, since the axis of the sperm head is now in the form of a helix (with a single turn) these plates are probably still parallel to it although ap-

pearing twisted in this section. Further, there are extensive regions in which the sheets can no longer be seen. Where profiles are still visible, they run parallel and can be seen to keep their 100 A spacing. They seem to disappear on either side into homogeneous nuclear regions. The width of the nucleus appears to remain constant during this process, however, and this and the parallel spacing of those plates still remaining visible suggest that material is deposited between the sheets rather than that they fuse. Their apparent absence in the mature sperm probably results from this "filling up" of the spaces between the sheets. The apparent decrease in width of the nucleus in Fig. 13 is due to the fact that the section goes through a nucleus that has (in addition to twisting and elongating as mentioned above) bent in a slight arc along its length and is, therefore, no longer axially symmetrical even in outline.

Fig. 9 is a cross-section of an even later stage and it can be seen that no evidence of the nuclear sheets remain although there are light spaces in the homogeneous nucleus that suggest a linkage with the previous stage. On the outside of the nuclear envelope profiles of tubules about 200 A in diameter can be seen which adhere closely to the nucleus and continue down over the tail sheath (24). It should be noted that the nuclear envelope now appears double with the inner membrane denser than the outer, but single rather than split. This disappearance of the split inner membrane occurs at about the time the plates disappear.

DISCUSSION

We have described certain linear structures appearing in electron micrographs of sections of spermatids of *Otala lactea*. We have explicitly considered that these structures are profiles of thin (about 60 A), flat plates arranged in a specific manner in the nucleus. Cross-sections of the basal regions (*e.g.* Fig. 8) show radially arranged profiles, either stretching across the ring from the nuclear envelope lining the centriole pit to that covering the outside of the nucleus, or starting on one part of the envelope and looping back to the same part, thus resembling a hairpin. In cross-sections taken at higher levels in the nucleus, this radial arrangement becomes less and less clear (see Figs. 10 to 12). It would be of great interest to know whether the profiles seen at higher levels belong to plates which are attached to the nuclear envelope at some other level in the spermatid head. This question cannot be answered without some adequate method of serial sectioning. However, if we use the fact that in basal regions all the profiles are attached to the nuclear envelope and at higher levels some are attached, we may conjecture that the plates in higher regions, though irregularly shaped, are ultimately attached to the nuclear envelope. We can imagine, for example, that they are shaped (when flattened out) like those of Text-fig. 2 *A*. If we then imagine such plates folded parallel to their length (perhaps more than once) and then packed into the spermatid nucleus



TEXT-FIG. 2 *A*. The irregular plates are diagrammatic representations of flattened nuclear plates postulated to exist on the basis of the electron micrographs. They should be thought of as being folded parallel to one of the edges, labelled *e*, and inserted in the nucleus parallel to the sperm axis and attached, along *e*, to the innermost part of the nuclear envelope.

TEXT-FIG. 2 *B*. The lower half of a nucleus of stage two is depicted with slices removed so that the internal arrangement of plates can be seen. It must be stressed that this is a hypothetical model based on random and not serial sections. The plates are more or less radially arranged in the region of the centriole but lose their orientation as we proceed toward the acrosome. The hypothesis discussed in the text requires that each plate can ultimately be traced back to the nuclear membrane. The small tubules surrounding the sperm head represent the rod sheath (to be described at a later date) probably corresponding to the mammalian manchette (6).

parallel to its axis, we arrive at a model such as that in Text-fig. 2 *B*. It is felt that all the micrographs so far obtained of spermatids in this stage can be interpreted as sections of a structure such as depicted in Text-fig. 2 *B*.

While this work was in progress several papers by Grassé *et al.* (45–48) appeared on sperm formation in the pulmonate snail *Helix pomatia*. The micrographs published by Grassé *et al.* resemble in almost all details those published in this article. Grassé *et al.*, however, do not interpret their micrographs in terms of plates, but assume that the linear structures seen in, for example, Figs. 7 or 12, are actually thin filaments, the chromonemata. In order to account for the differences in orientation of these filaments seen in different micrographs they assume a highly complex process of "lateral loop" formation. Specifically, Figs. 5 and 6 represent in their scheme, nuclei in which "short lateral" loops have formed on chromonemata which are attached to the nuclear envelope over the centriole and which, by and large, run parallel to the spermatid axis. The next stage in their scheme is the disappearance of lateral loops and a straightening out of the chromonemata, a stage possibly resembling that of Fig. 7. Finally, they conceive of a period of formation of "long lateral loops" such as would account for Figs. 8, 10 to 12. It is clear that this interpretation is incompatible with that given above in terms of plates. A critical point in the interpretation of Grassé *et al.* is the existence of a stage in which the chromonemata are straight and parallel to the spermatid axis with no side loops. A cross-section of such a system (*e.g. C-C* in Fig. 7) should reveal a nucleus filled with 60 A dots. Grassé *et al.* do publish a micrograph which they claim is in this state. However, in contrast to the rest of their micrographs, this one shows almost nothing else but a circle containing dots and it is not possible to form an independent judgment as to what this object is. We have never, in the many hundreds of spermatids observed and recorded, seen a nucleus in an advanced stage which contained anything but linear profiles of the type described, and this is true no matter what the angle of section. Indeed, a glance at Text-fig. 1-*9* will indicate why this statement may be made with some certainty. Here are depicted four spermatids, at the same developmental stage, embedded in a supporting cell in one of the orientations in which they are found, *i.e.*, with their heads radially arranged (there are usually 50 or so spermatids in such a group). It is clear that of such a group of nuclei a variety of sections may be taken which will include cross-sections, and sections at all angles to the spermatid axis, thus allowing near cross-sections and near longitudinal sections of nuclei in the same stage to be compared. Another orientation of these heads, often seen, is that in which they are arranged with approximately parallel axes, but staggered at different heights in the supporting cell. This again can give cross-sections of nuclei at different levels but in the same developmental stage. Indeed, Figs. 10 and 11 come from such a region. From consideration of such micrographs in which cross-sections show irregular linear

profiles and longitudinal sections, parallel straight ones, we feel that the interpretation of the micrographs in terms of chromonemata, *i.e.* thin filaments, and lateral loops is untenable no matter how attractive such an interpretation may be at first glance, and that a hypothesis in terms of plates, as has been described, is more clearly supported. The work of Gibbons and Bradfield (49) also encourages the interpretation of these structures as plates. They have seen similar components in spermatid nuclei in *Locusta migratoria* and feel that these represent "membranes," *i.e.* plate-like structures (personal communication).

We hesitate, however, to prejudice an ultimate interpretation of these structures in terms of filaments, since it is conceivable that the plates are formed by lateral aggregation of filaments into plate-like structures. At any rate, the earlier plates are smaller than the later ones (*e.g.* Figs. 3 to 6) and whatever the process of plate formation may be, it appears to start on the nuclear envelope overlying the centriole pit and spread distally from there.

Another question that arises concerning the plate-like structures, is their composition. Since the plates are densely packed and all-pervasive in the nucleus and again since they appear to be highly oriented, suggesting a relation to chromosomes, the possibility must be considered that they are composed of DNA.

In a study of alternate sections, a thick one for the Feulgen reaction for the light microscope and a thin one for the electron microscope, Moses (27) showed that some nuclear regions which were Feulgen-positive (and therefore contained DNA) showed no high electron density in the corresponding areas in the electron microscope (grasshopper spermatocytes). On the other hand, in crayfish spermatocytes Feulgen-positive chromosome regions corresponded to dense areas which "looked like" chromosomes in the electron micrographs. It seems clear, therefore, that if Feulgen-positive areas are visualized in the electron microscope, it is not because of their DNA content but because of some other component of the region which can vary in its state in different phases of cell differentiation or activity. That is, DNA neither reacts with osmium tetroxide nor possesses sufficient density of its own to account for the dense regions morphologically resembling chromosomes. We feel that this observation tentatively rules out DNA as being directly responsible for the observed density of the nuclear plates in snail spermatid nuclei.

There is a question whether the plates are visualized because of a native density or because they have reacted with and bound osmium tetroxide. The present work does not answer this, but, because of the fact that the period of plate formation corresponds to a period of profound staining changes in the nucleus (see Introduction), it does not seem unreasonable to assume, tentatively, that the densities observed are due to osmium binding. If this is true, then the observations of Bloch and Godman (30, 31), Gatenby (10, 11), and

Bahr (26) may throw some light on the composition of the plates. Indeed, Bloch and Godman suggest that decreased methyl green binding reflects a change in the DNA-protein complex in the nucleus, with a "release" of histone. If the decreased methyl green binding in spermatid nuclei described by Gatenby (10, 11) reflects the same phenomena (in terms of the DNA-protein complex), then we may suggest that the "released histone" will bind with the osmium tetroxide, since Bahr (26) showed that histones, but not DNA or protamine react strongly by precipitation and color change with osmium tetroxide (if Bahr's *in vitro* experiments can be carried over to living cells). It is clear that some such process of release is needed in our hypothesis if we are to avoid the complications due to the fact that histones occur not only in spermatic tissue but in other cells as well (30–32, 36).

That histone-like substances do occur in Gastropod spermatic tissue was shown by Hultin and Herne (35, see also 29) for *Patella vulgaris* and *Patella coerulea*. The proteins there found are "intermediate" in composition and molecular weight to protamines and histones of vertebrate tissue. We would suggest, therefore, as a tentative hypothesis, that a simplification of snail spermatid nuclear proteins, similar to (though possibly not to the same extent) that occurring in fish sperm nuclei (28, 34), takes place in snail sperm head formation, and that one of the events in this process is the "release" of active osmium binding sites on histone. This process would allow the visualization of the plate-like structures in snail spermatids and may also be involved in the formation of an "aggregated chromatin state" in cat (6), toad (8), and sparrow (9) spermatids. The specific form taken by the (presumably) stained nuclear material would then be a reflection of factors that lead to the crystalline state of nucleoprotein in the mature sperm, and would be expected to be different in isotropic and anisotropic sperm heads (2, 3, 33).

Even though we do not consider the plates to be DNA or DNA-osmium, it is nevertheless difficult to believe that the all-pervasive and regularly organized nuclear plates do not in some way reflect the organization of DNA in the head. From the birefringence data we would, indeed, picture the elongated DNA molecules with the axes parallel to that of the sperm axis (2, 3, 33). In the case at hand we would then picture the DNA-protein in the pulmonate snail somehow organized into flat sheets folded parallel to the sperm axis. An important question would then be whether the DNA lies between the flattened plates or within them. The diameter of a DNA-protein molecule isolated by Bernstein and Mazia (44) was about 250 A and that isolated by Liquier-Milward (43) was 100 to 150 A. If the DNA-protein bears a relation to the nuclear plates in *Otala*, it would seem impossible to put a complex of diameter 250 A into the nucleus in any consistent way. On the other hand, a 100 to 150 A molecule would fit between the nuclear plates in *Otala*, with the plates then forming thin partitions between regions containing DNA. On the other hand, Kahler

and Lloyd (41) found DNA fibers to be about 15 A in diameter (in agreement with the model of Watson and Crick (42)) and DNA-protein fibers about 25 A in diameter. Such a molecule would easily fit inside the 60 A nuclear plates in *Otala spermatid* nuclei.

It is not possible to distinguish between these alternatives with the present observations. If the DNA lies within the sheets, however, then the suggestion can be made that the nucleus can organize its genetic material in sheet-like structures presumably important in template hypotheses of gene action. The speculative nature of the above discussion should be kept clearly in mind.

SUMMARY

Changes in both external form and internal arrangement of nuclear material have been investigated in the differentiation of the sperm of the pulmonate snail, *Otala lactea*. Sperm head differentiation begins with a flattening of the previously spherical nucleus and a thickening of the nuclear envelope covering the anterior and posterior surfaces of that nucleus. Tail filaments can be seen in the cytoplasm at this time. At a slightly later period the mitochondria begin to form the tail filament sheath and at this time the nucleus begins to elongate in a direction parallel to the future axis of the sperm. At the same time the nuclear material begins to orient itself at right angles to the nuclear surface which lines the pit occupied by the centriole. As nuclear elongation proceeds, this orientation of nuclear substance takes on the appearance of 60 A thick sheets bent in a direction parallel to the sperm axis. Soon the sheets fill the entire nucleus. The nucleus then begins to twist along its axis so that it starts to take on the mature, flame-shaped form. At this time the flat sheets begin to disappear and in the mature sperm head they are no longer visible (see Text-fig. 2 *B*).

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EXPLANATION OF PLATES

N, nucleus.	*C*, centriole.
n, nucleolus.	*S*, tail sheath.
m, mitochondria.	*g*, Golgi apparatus.

PLATE 167

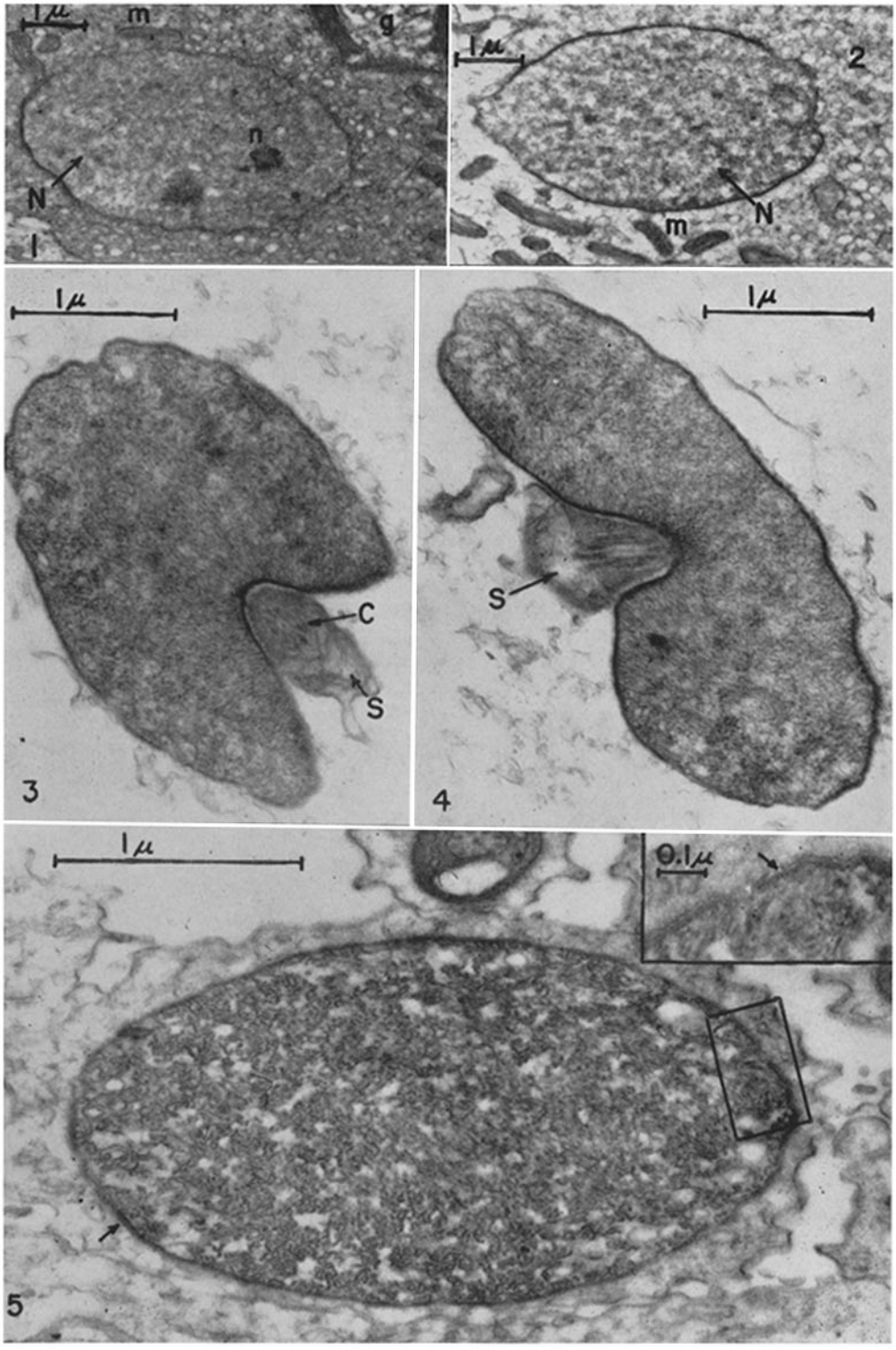
FIG. 1. The nucleus of an early spermatid. The elliptical shape of the nucleus is in part due to section compression. Mitochondria and Golgi apparatus can be seen. × 9,550.

FIG. 2. The nucleus of this spermatid is definitely flattened and shows the thickening of anterior and posterior surfaces characteristic of early nuclear differentiation. An equatorial belt of nuclear surface is untouched by this process. The mitochondria have definitely gathered on the posterior side of the nucleus. Corresponds to Text-fig. 1–*2*. × 9,550.

FIGS. 3 and 4. The nuclei shown here have reached similar stages though the nucleus in Fig. 3 is probably a little more advanced. The main features of nuclear organization at this stage are twofold: one, the material in the nucleus in the region of the centriole begins to show an orientation into lines (in cross-section), approximately perpendicular to the nuclear surface; two, the nucleus begins its axial elongation. The orientation has not yet spread to the distal regions of the nucleus which still, therefore, resemble the nuclei in Figs. 1 and 2. The tail filaments are now beginning to be surrounded by a definite sheath, which is formed first at the centriole and then distally from it. Both figures × 23,900.

FIG. 5. At a somewhat later stage than in Figs. 3 and 4, the oriented material is more widespread in the nucleus. The nucleus shown here is cut in a plane making an angle with the sperm axis so that the over-all shape appears elliptical. It can be seen that short (400 A to 1000 A) "filaments" (60 A to 100 A thick) appear to fill the nucleus. The distinction between osmiophilic and non-osmiophilic regions is becoming quite distinct. In several places, membrane-limited regions seem to exist. × 35,800.

The inset shows a piece of the nuclear surface and nuclear envelopes. This appears to have two main parts, a thin (50 A) outer portion, separated by a space of about 100 A, from a thicker (150 A) inner membrane. From other micrographs, this latter membrane is probably itself double, being composed of two 50 A membranes separated by a 50 A space. × 69,500.



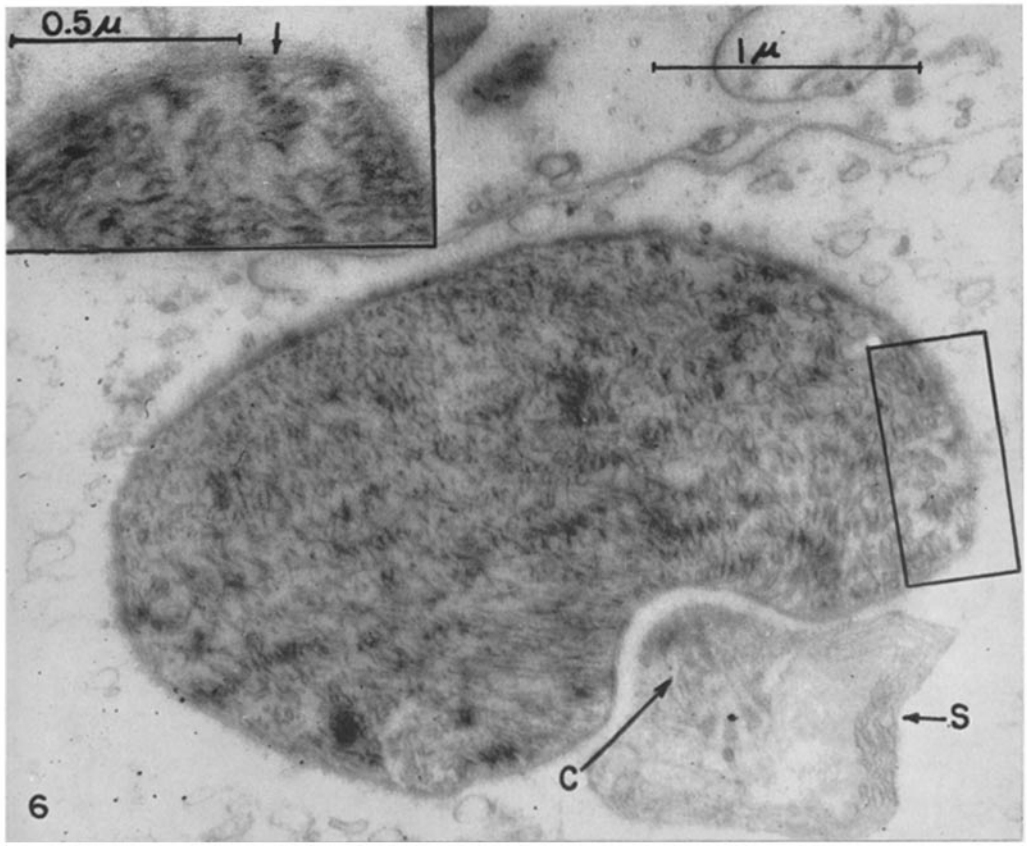
(Rebhun: Nuclear changes during spermiogenesis)

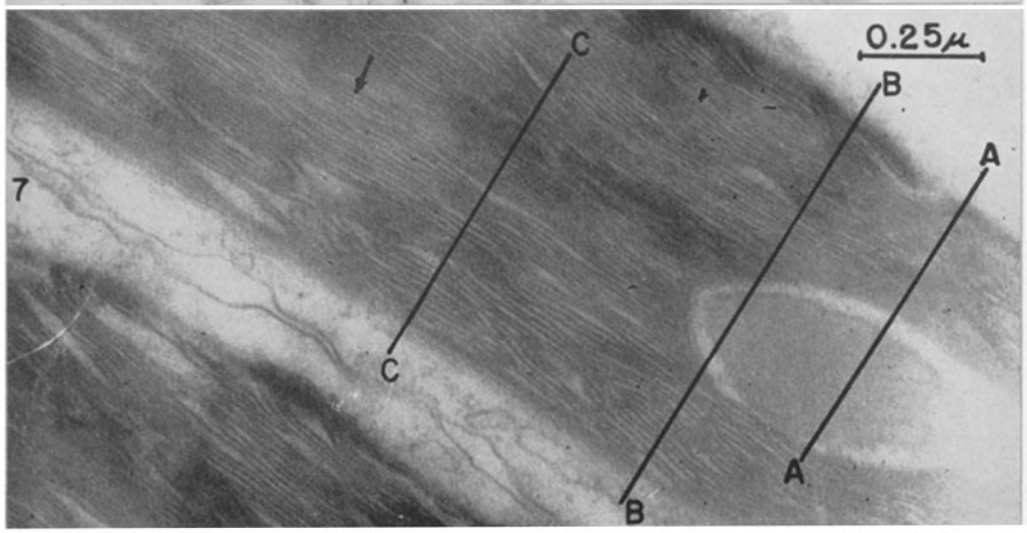
PLATE 168

FIG. 6. The plane of section in this figure appears to be nearer to longitudinal than cross-sectional and should be compared to Fig. 5. The orientation of nuclear material is more complete than in Figs. 3 and 4 and the individual elements appear longer and more extensive in the nucleus. The pronounced orientation around the centriole region is visible. The tail sheath can be seen surrounding the centriole. × 34,400.

Inset: A piece of the nuclear envelope shows considerations identical to those in the inset in Fig. 5. ×59,200.

FIG. 7. Parts of two late spermatid nuclei are shown. The larger piece is a longitudinal section parallel to but not including the spermatid axis. A lateral piece of the proximal centriole is included in the centriole pit. The longitudinal striations (plates) are about 100 A. Part of the thickness of the plate is contributed by the thickness of the section; *i.e.*, thinner sections include less of the plates and they consequently appear thinner. About 60 A is the lower limit of plate thickness that has been found. × 63,100.





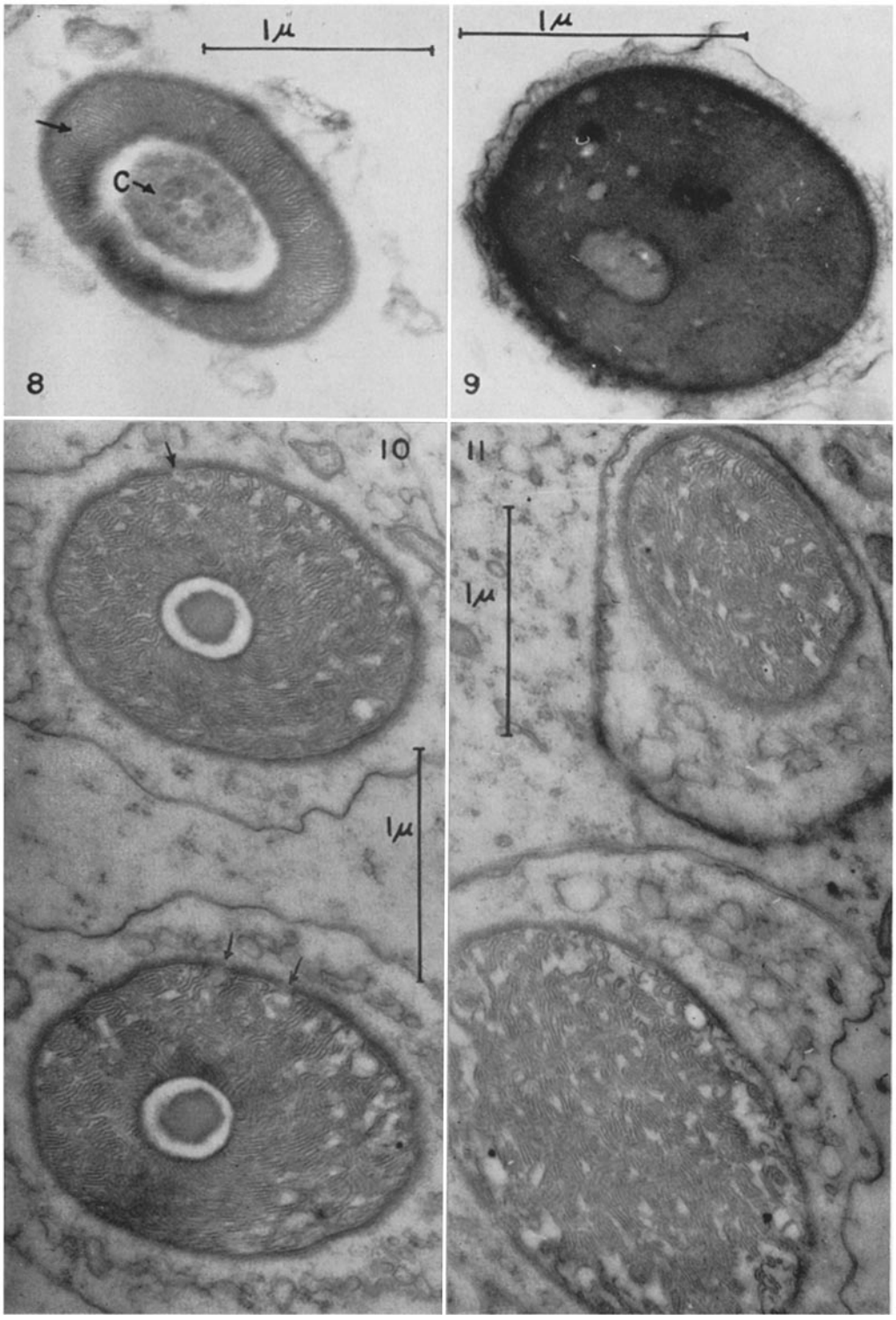
(Rebhun: Nuclear changes during spermiogenesis)

FIG. 8. The plane of section of this micrograph is approximately that of line *A-A* in Fig. 7. The internal space in the "doughnut" is occupied by the proximal centriole and will be described elsewhere. The ring of nuclear material contains cross-sections of nuclear plates oriented in a pronounced radial fashion. Some plates appear to run from the nuclear envelope lining the pit to that lining the outer surface. Many of the profiles are in the shape of hairpin loops starting and ending either on the inner or outer surfaces. In some places one may find one loop within another. The outer nuclear surface can be seen to have the nuclear envelope structure described in Figs. 5 and 6 (arrow). × 34,300.

FIG. 9. The nucleus depicted in cross-section here is a mature one, and it can be seen that the nuclear plates have disappeared although lighter spaces in the interior possibly indicate the remains of these. The nuclear envelope is simply doubled and does now show the triple structure characteristic of the previous stage. The print is purposely made overcontrasty to bring out the 200 A rod sheath cross-sections. Each appears as a tubule with outer wall about 40 to 50 A. About 300 A separates the centers of the rods. They will be discussed at a later date in relation to the tail sheath. × 42,300.

FIG. 10. Two late spermatid heads are shown in cross-sections at about level *B-B* in Fig. 7. The top piece of the centriole (non-filament portion) occupies the center (some shrinkage has undoubtedly contributed the clear space around the centriole). The radial orientation of plate profiles seen in Fig. 8 is still present in the regions adjacent to the centriole. In the regions adjacent to the outer nuclear surface, this radial orientation is no longer clear and profiles may be found running parallel to the membranes. In places the thick inner portion of the nuclear membrane appears broken (arrows). Otherwise it appears to have the same structure indicated in Figs. 5 and 6. The nuclear envelope lining the centriole pit does not appear to show this compound structure (see, for example, Fig. 6), but not enough good micrographs have been obtained to clarify this situation. × 34,900.

FIG. 11. Two late spermatids are sectioned at levels corresponding to that indicated in *C-C*, Fig. 7. The radial orientation of profiles of Figs. 8 and 10 has disappeared, completing this process, begun in Fig. 10. The nuclear envelope shows the same compound structure described in previous figures. In some places, spaces appear to be limited by membranes. The relation of these to plates is not clear. The two cells are partially embedded in a type of supporting or nursing cell. × 34,900.

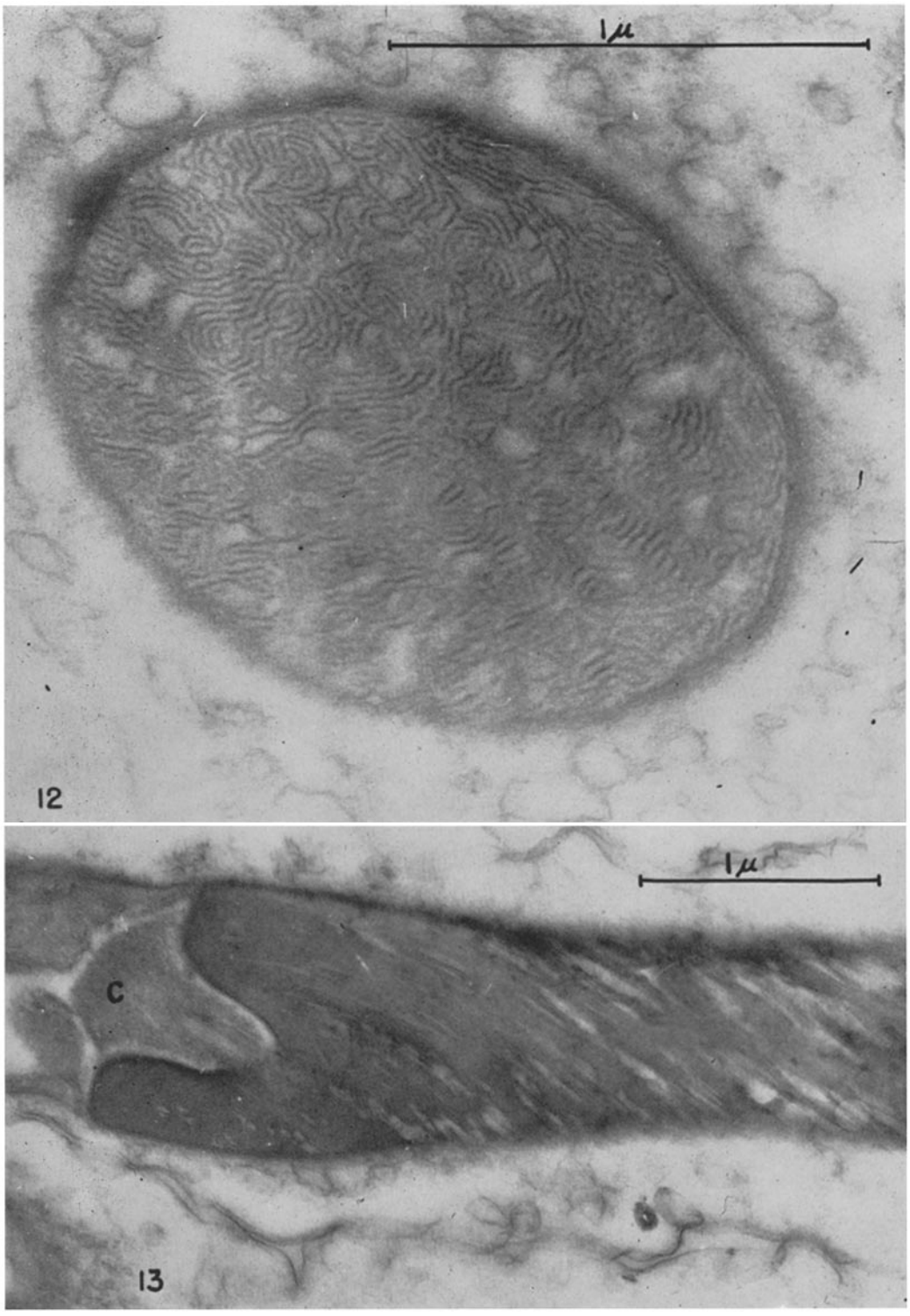


(Rebhun: Nuclear changes during spermiogenesis)

PLATE 170

FIG. 12. The spermatid nucleus is sectioned at a level corresponding to those in the previous figure. Considerations of nuclear envelopes and profile orientation mentioned there, also apply here. Profiles may be parallel to or perpendicular to the nuclear surface. Occasional profiles are circular. Some start and stop with no obvious connection to any other. Some are relatively straight and others loop around. × 75,500.

FIG. 13. The basal portion of a period three spermatid nucleus can be seen. The twist in the plates is clear and it can be seen that most of these have disappeared. In areas devoid of plates a homogeneous region is present. The nuclear envelope of such regions resembles that of Fig. 9 and is not composed as it is in Figs. 5, 6, etc. The proximal centriole indicates the twist that the nucleus has undergone. ×35,800.



(Rebhun: Nuclear changes during spermiogenesis)