THE FINE STRUCTURE AND CHEMICAL COMPOSITION OF NUCLEI DURING SPERMIOGENESIS IN THE HOUSE CRICKET

I. Initial Stages of Differentiation and the Loss of Nonhistone Protein

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ABSTRACT

The early stages of nuclear differentiation in spermatids of the house cricket are described with regard to the fine structural elements and chemical components which occur. Particular attention is given to the loss of nonhistone protein from the nucleus and its relation to chromatin structure. Granular elements about 25 to 80 mμ in diameter, and fibers about 8 mμ in diameter occur in the earliest spermatid nucleus. The fibers are found in diffuse and condensed chromatin while granules are found only in diffuse material. DNA and histone parallel the chromatin fibers in distribution, while nonhistone protein and RNA parallel the granules in distribution. The granules and most of the nonhistone protein are lost, simultaneously, after the early spermatid stage. The protein loss occurs without detectable change in the structure of chromatin fibers. Chromatin fibers first show a structural change in mid spermiogenesis, when they become thicker and very contorted. Unusually thin fibers (about 5 mμ) also appear in mid spermatid nuclei; they are apparently composed of nonhistone protein and free of DNA and histone.

INTRODUCTION

The nuclei of orthopteran and molluscan spermatids have been studied extensively with the electron microscope, since they develop prominent fibrous and lamellar structures which are regularly arranged and more amenable to analysis than the components of somatic nuclei are (1, 2). It is not yet clear what the relation of these unusual spermatid structures is to the structures of somatic nuclei, or how they are formed. Early spermatid nuclei contain fibers similar in appearance to those of somatic chromatin, and it is generally assumed that a transformation of these fibers produces the unusual structures of later stages. It has been suggested that changes in ultrastructure originate from a side-by-side association of these fibers (3, 4), or that the fibers themselves first undergo a change in structure, splitting into subfibers which then associate with each other (5). An almost complete lack of detailed information on the nuclei of early spermatid stages is an obvious source of difficulty in interpretation. Features seen in later stages have been given closer study, since they are more regular and easily examined, but their even-

tual interpretation depends on knowledge of the early events in nuclear differentiation, before regular structures appear.

Change in the protein composition of the nucleus is probably a factor in the development of unusual spermatid structures. Chemical analyses have demonstrated that sperm of some organisms contain little or none of the histone and nonhistone proteins typical of somatic nuclei, their proteins being predominantly protamines or histones that are very rich in arginine (6, 7). Cytochemical studies have demonstrated that basic proteins of unusually high arginine content appear well before sperm maturation (8), and occur in later spermatid nuclei when structure is strikingly unusual (9). Comparable studies of nonhistone proteins have not been reported for any organisms. The occurrence of histone and nonhistone proteins, as well as DNA and RNA, in chromatin fractions isolated from somatic nuclei (10) implies that chromatin fibers may contain both proteins. Ris has suggested that the structure of fibers may depend on their protein, and has concluded that somatic chromatin fibers break down during spermiogenesis into two subfibers, due to the loss of protein, either histone (11) or nonhistone protein (12, 13) or both (5).

The studies reported here resulted from efforts to trace the development of the unusual nuclear structures seen in late cricket spermatids (14, 15), and to correlate the structural changes with protein changes. Study of the nuclear nonhistone protein indicated that it is almost completely lost after the early spermatid stage, and the loss is simultaneous with early morphological changes in the nucleus. Unusual histone was not detectable in early or mid spermatid. The observations reported here describe the initial stages of nuclear differentiation in the spermatid, and illustrate the effect that loss of nonhistone protein has on nuclear structure. It is suggested that this protein occurs in granular elements of the nucleoplasm, rather than in chromatin fibers. The protein loss does not affect the integrity of the fibers or their size. The first detectable change in chromatin fibers is a pronounced thickening, in mid spermatids, and neither splitting nor side-by-side association of fibers appears to be the initial step in differentiation. Unusually thin fibers appear in the mid spermatid, in addition to the thickened chromatin fibers; these thin fibers make up a small nonhistone protein fraction which appears

to be distinct from the large nonhistone protein fraction of the early spermatid.

MATERIALS AND METHODS

Electron Microscopy

Testes of the house cricket *Acheta domestica* (formerly called *Gryllus domesticus*) were fixed in a mixture of 2% osmium tetroxide and 12% dextran buffered to pH 7.2–7.4 with Veronal buffer (16). Fixation was carried out in an ice bath from about 10 min to 1 hr. The testes were then postfixed in 10% neutral formalin overnight in an ice bath. Osmium tetroxide was also used without dextran; no significant, repeatable difference in nuclear structure was observed with the two fixatives. The testes were quickly dehydrated in cold ethanol, beginning with a 70% solution, and embedded in a mixture of *n*-butyl and methyl methacrylate to which 0.1% uranyl nitrate was added to inhibit polymerization damage (17). Sections were stained with lead hydroxide (18), lead citrate (19), or aqueous uranyl acetate (20), and then "sandwiched" with an evaporated carbon film.

Measurements of chromatin fiber thickness were made with a ruler and magnifying lens on micrographs printed at magnifications of 70,400 to 124,000. The measurements were made primarily to compare diameters at different stages. The statistical significance of any differences in the mean diameters can be estimated from the standard errors shown in Table II. How accurate the values are, in absolute units, has not been estimated. Some sources of error that might have contributed to the range of values and influenced the absolute values are obvious. Magnification of the electron microscope was calibrated occasionally but not weekly, and undetected variation in magnification (as great as 10%) probably occurred. In addition, calibrations were not free of error because of imperfections in the calibration grid used (a diffraction grating replica, 28,800 lines/in.). Some magnification errors were probably introduced in making prints. An important source of errors lies in the fibers themselves. The boundaries of fibers could not be identified with certainty, since they are indistinct and irregular; the resolution obtained in most micrographs was 2.0 to 2.5 mμ, and boundaries probably cannot be determined more accurately. Some fraction of the outer indistinct region of the boundary was arbitrarily excluded in making measurements; the diameter measurements indicate the distance between what appeared to be the distinct edges of a fiber. The average error in determining mean diameter should be about the same for different chromatins; means can be compared with each other, therefore, with much more certainty than can be attached to absolute values.
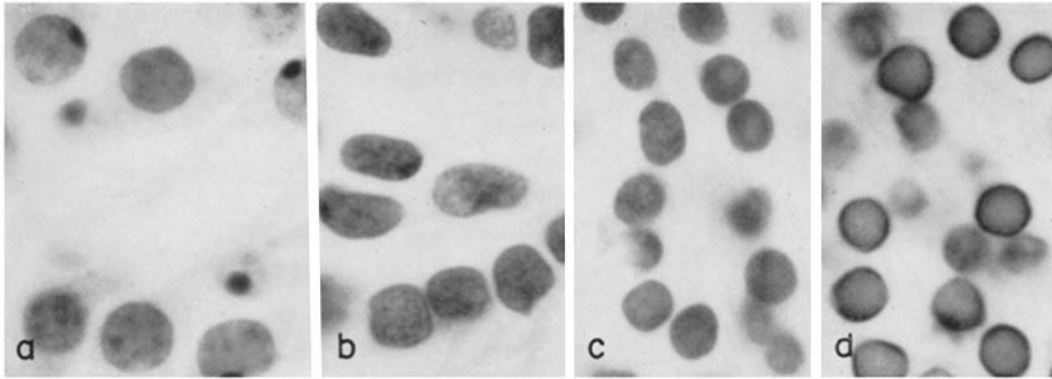
FIGURE 1 Feulgen-stained nuclei. *a* Early spermatid. *b* Crinkled stage. *c* Speckled stage. *d* Shell stage. Note the condensed sex chromosome in several of the early spermatids. It is not yet as diffuse as the rest of the chromatin in the early crinkled nuclei shown in *b*. Stain is restricted to a thin peripheral layer in the shell stage nuclei. Flemming's fixation. × 2200.

Cytochemistry

The testes used for cytochemical studies were frozen-substituted (in isopentane and ethanol, respectively) and postfixed in hot 80% ethanol (21), and embedded in paraffin. The fixation used preserves nuclear and cytoplasmic structures well enough for correlation with the material used in electron microscopy to be feasible.

Proteins were stained by the acid dye fast green. The Alfert-Geschwind procedure of staining at pH 8, after removal of DNA, was used for staining histone specifically (22). The reaction appears to stain frozen-substituted testes with as much specificity as seen with formalin-fixed testes. Pretreatment with nitrous acid, before histone staining, was used to determine the spermatid stage when stainability develops an increased resistance to deamination, indicative of an increased arginine content of the histone (9). Slides were treated with nitrous acid for 30 min (after DNA removal); nitrous acid was made by mixing equal volumes of 10% trichloroacetic acid and 10% sodium nitrite and was replaced with a fresh mixture after 15 min as recommended by Bloch and Hew (9).

Fast green was used at acid pH's to study nonhistone protein. To obtain specific staining of the nonhistone protein, the histone was removed from sections with dilute HCl before staining. The procedure developed for staining nonhistone protein specifically consisted of treating slides with DNase or hot trichloroacetic acid (5% for 15 min at 90°C), then with dilute HCl (0.1 or 0.2 N) for several hours before staining in fast green (at pH 2 or 4.6).

Fast green was used at a concentration of 1 mg/ml. Solutions were adjusted to the desired pH with dilute KOH or HCl, and were not buffered. Slides were stained for 1 hr, then transferred directly to
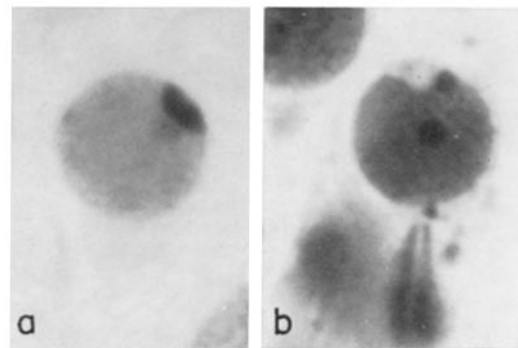

FIGURE 2 Early spermatid. *a* Histone distribution: Alfert-Geschwind stain. *b* Nonhistone protein distribution: fast green staining at acid pH after histone extraction. The X chromosome (at the nuclear periphery) is intensely stained in *a* and unstained in *b*. The diffuse nucleoplasm is stained in both cases. × 3900.

95% ethanol for several minutes, run up to xylene, and mounted.

The basic dye azure B was used to stain RNA as described previously (21). DNA was removed before staining by digestion in DNase. The digestion was carried out at 37°C, for 30 min, with 0.1 mg of DNase per milliliter, in 0.005 M MgSO$_4$, at a pH of 6–6.5.

OBSERVATIONS

Observations are limited here to the differentiation of nuclei which occurs prior to nuclear elongation and the appearance of highly ordered structures in the nucleus. The stages of nuclear differentiation considered have been named for clarity in discussion. Feulgen-stained nuclei of
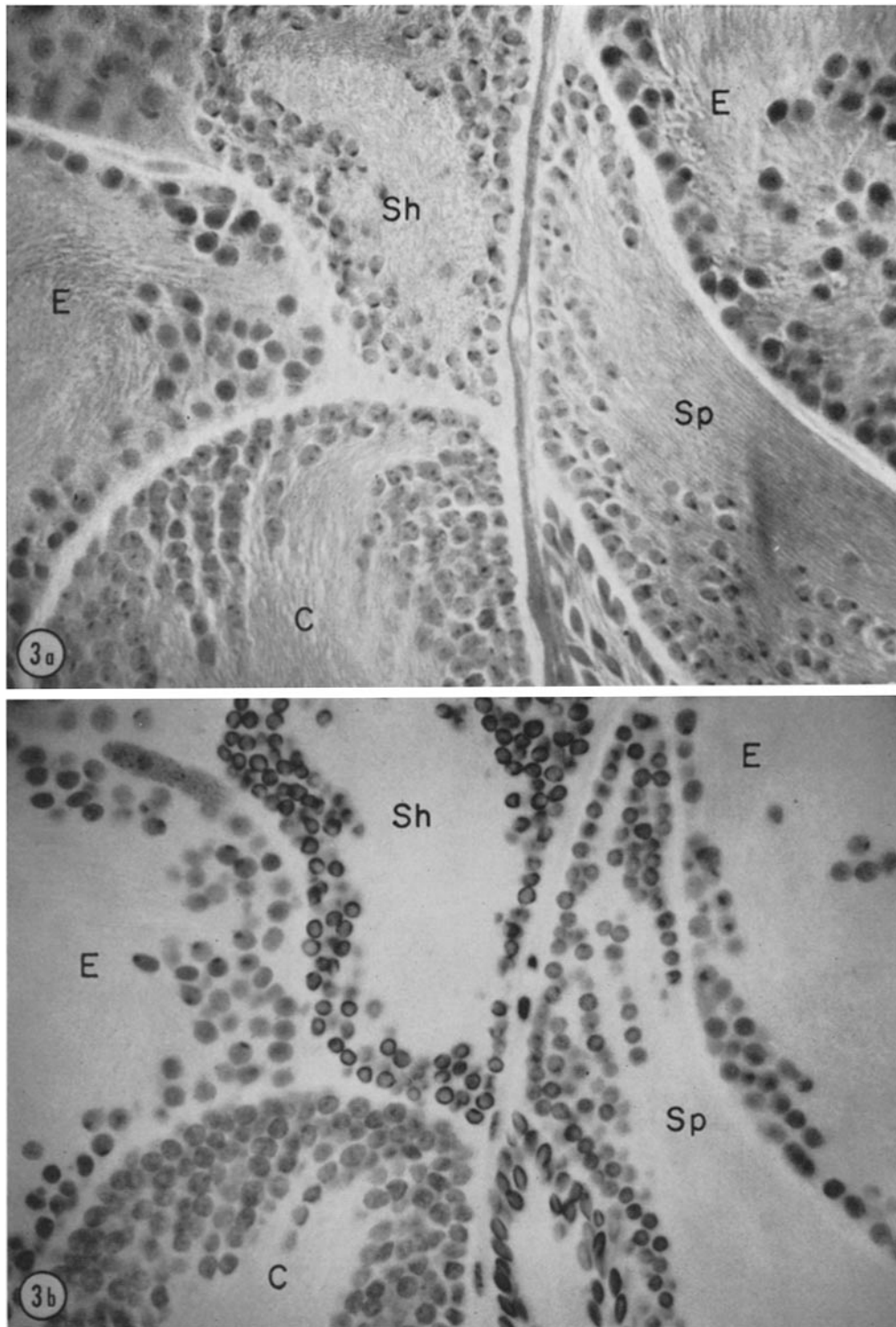
FIGURE 3 Two adjacent sections including the same cysts with spermatids in the various stages of nuclear differentiation. *a* Nonhistone protein stain. *b* Histone stain. Tail structures as well as nuclei are stained in *a*. Early spermatids (*E*), crinkled (*C*), speckled (*Sp*), and shell (*Sh*) stages. × 750.

each stage are shown in Fig. 1, and illustrate differences among the stages in chromatin distribution and in nuclear size and shape. Various Feulgen-neativge nuclear inclusions also serve to distinguish the stages, and are noted in subsequent observations.

The early spermatid stage follows late telophase; at this stage nuclei are larger than at telophase and the chromatin has become diffuse with the exception of the condensed X chromosome, which occurs in half the spermatids. Nuclei decrease in size and become somewhat irregular in shape by the next stage, referred to as the crinkled stage because of the crinkled appearance of the nuclei; the X chromosome becomes diffuse during this stage. The nucleus is still smaller and has re-

TABLE I

Nuclear Volumes (μ^3). Means and Standard Errors

Early telophase	25 ± 2
Late telophase	60 ± 2
Early spermatid stage	95 ± 5
Crinkled stage (early)	52 ± 2
Crinkled stage (late)	34 ± 1
Speckled stage	23 ± 0
Shell stage	19 ± 1

gained its spherical shape at the subsequent stage, the speckled stage; fine patches of material in the nucleus give it a speckled appearance. A striking segregation of material is seen at the next stage, the shell stage, with the chromatin localized at the nuclear periphery in a thin shell. At the early shell stage the nucleus is still fairly spherical and the chromatin layer is not so compact as in the later shell stage. A slight elongation of the nucleus is seen in the late shell stage, and a gap appears in the chromatin layer on one side of the nucleus, adjacent to the centriole and flagellum. (The flagellum is attached on the side, close to the anterior end of the nucleus.)

These stages are readily distinguished by either light or electron microscopy. In a recent electron microscope study of G. domesticus, Schin (15) reported the occurrence of the early spermatid and shell stages, but did not include the intervening transition stages. The peripheral localization of chromatin in shell stage (and later) nuclei was previously described by Schlote and Schin (23). Changes in distribution of nuclear material and in nuclear size prior to nuclear elongation, sim-

ilar to those reported here for the house cricket, were reported for G. assimilis in an early study by Baumgartner (24).
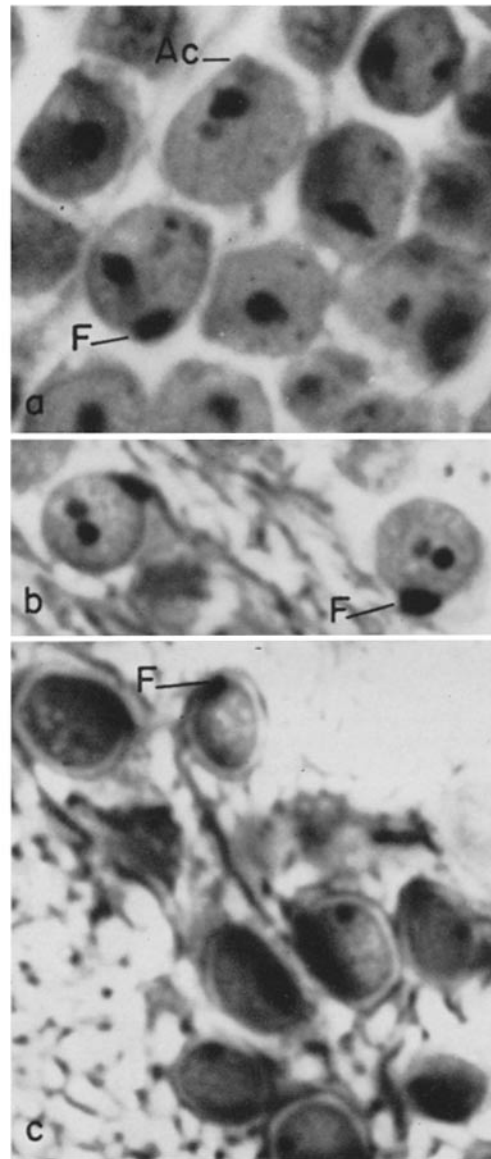

FIGURE 4 Nonhistone protein distribution in (a) crinkled, (b) speckled, and (c) shell stages. Nuclei show lightly stained diffuse material and darker nuclear inclusions. Also visible are stained cytoplasmic structures closely applied to nuclei: the pointed acrosome is at Ac, and material associated with the flagellum at one side of the nucleus is at F. At the periphery of shell stage nuclei a thin, stain-free layer is seen surrounding a stained nuclear interior. × 3900.
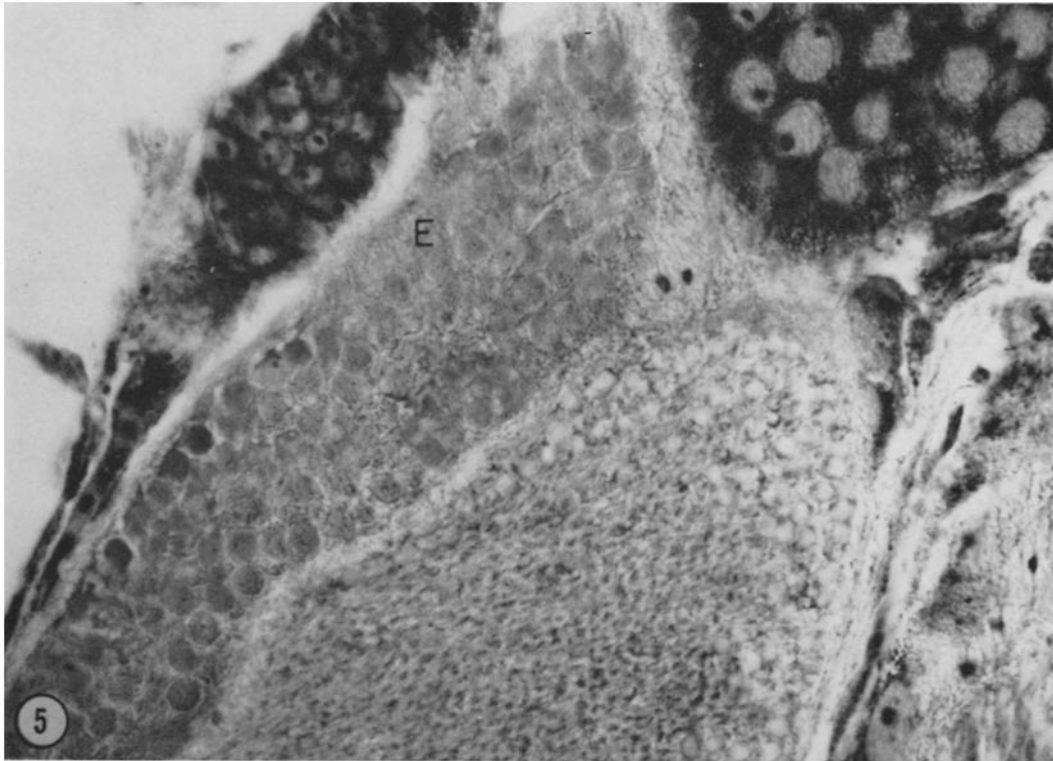
FIGURE 5   Azure B staining of RNA. A cyst of early spermatids (E) with nuclei clearly stained. The adjacent cyst displays empty holes where nuclei would be expected. Spermatogonia and spermatocytes (above spermatids) contain intensely stained nucleoli and cytoplasm, but no obvious nucleoplasmic stain. × 750.

Cytochemical Observations

Fast green staining indicated that protein occurs throughout the nucleus in all the spermatid stages. Histone was consistently found at sites where DNA occurs. Nonhistone protein commonly occurred at the sites where histone and DNA were absent, and was not detectable at sites where they were very concentrated.

The distribution of histone in the early spermatid, shown by the Alfert-Geschwind stain, is illustrated in Fig. 2 *a*. There is a very high concentration of histone in the condensed sex chromosome, and a lower concentration in the rest of the nucleus. Nonhistone protein is found throughout the early spermatid nucleus, with the striking exception of the X chromosome. In nuclei stained specifically for nonhistone protein, as shown in Fig. 2 *b*, the empty area of the X chromosome stands out prominently against the

stained nuclear background. Nonhistone protein occurs in high concentration in several nuclear inclusions: in amorphous material next to the X chromosome and a spherical body, both shown in Fig. 2 *b*, and in a small ring-shaped structure. These inclusions bind only a trace of histone stain, at most.

Loss of nonhistone protein from the nucleus during its early differentiation is evident on comparison of the staining of early spermatids with that in subsequent stages, shown in Fig. 3 *a*. The nucleus of early spermatids shows an intense stain. Nuclei show a much weaker stain at the crinkled stage, and are only slightly stained at the speckled and shell stages. Nuclei decrease markedly in size after the early spermatid stage, as shown in Table I, from a volume of about 100 μ^3 to about 20 μ^3 at the early shell stage. Since a large decrease in nonhistone protein concentration accompanies the 80% decrease in size, considerably less than

20% of the nonhistone protein remains at the shell stage.

Although nuclei at the crinkled stage appear to contain more nonhistone protein stain than speckled and shell stage nuclei, the diffuse material of the nucleus is faintly stained, with about the same intensity, at all these stages. Most of the stain noted in crinkled nuclei is concentrated in nuclear inclusions which appear at this stage. They are irregularly shaped, of various sizes, and scattered throughout the nucleus, as seen in Figs.

3 a and 4 a. The contribution of inclusion bodies to the over-all nuclear stain gradually diminishes. Only two nuclear inclusions (both spherical) occur at the speckled stage and neither is large (Fig. 4 b); at the shell stage there is only one spherical body present (Fig. 4 c).

On close inspection of the shell stage nucleus after staining for nonhistone protein, shown in Fig. 4 c, it is seen that stain is restricted to nonchromatin elements of the nucleus. A thin shell which is free of stain occurs at the periphery
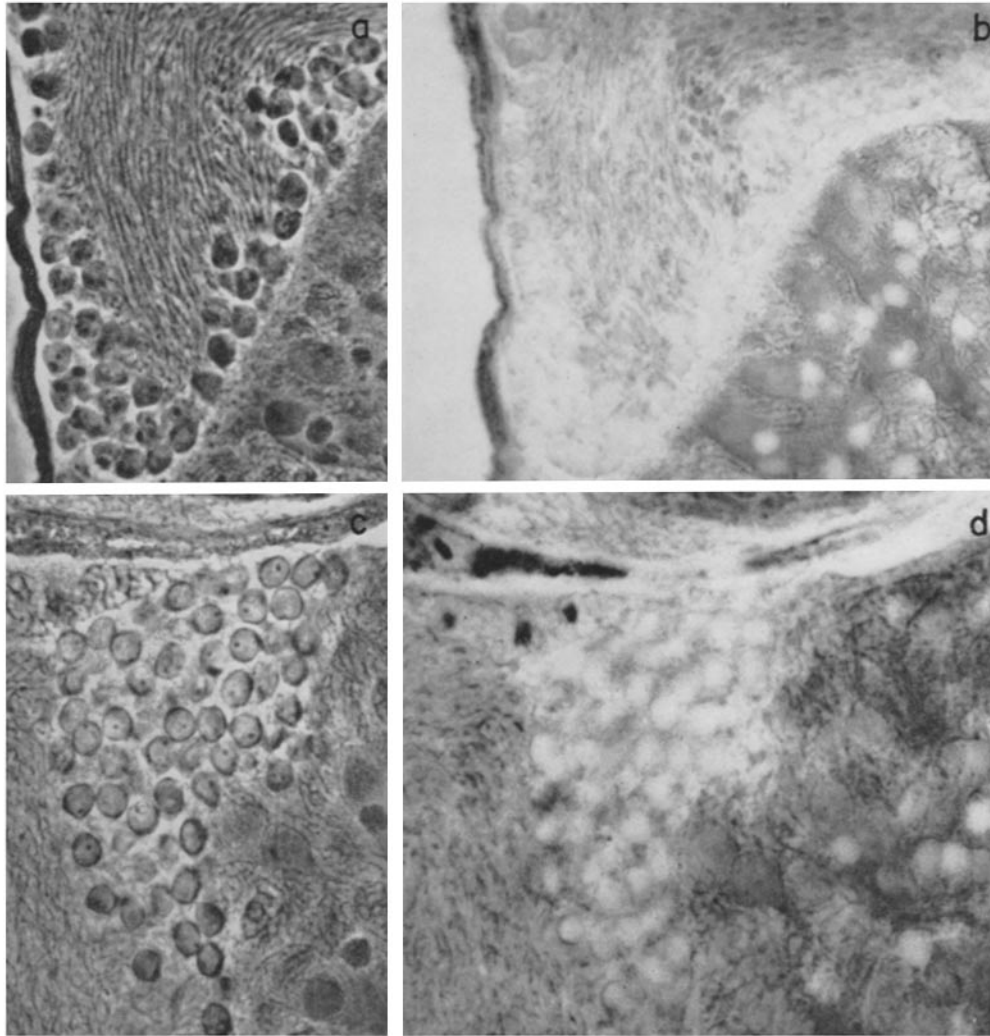

FIGURE 6   Azure B staining of RNA. (a) Phase and (b) bright field views of a cyst of crinkled stage spermatids with their nuclei almost free of stain. An adjacent cyst of early spermatids contains stained nuclei and round, stain-free nebenkerns. × 1000. (c) Phase and (d) bright field views of unstained shell stage nuclei. Some early spermatids occur in an adjacent cyst. × 1200.
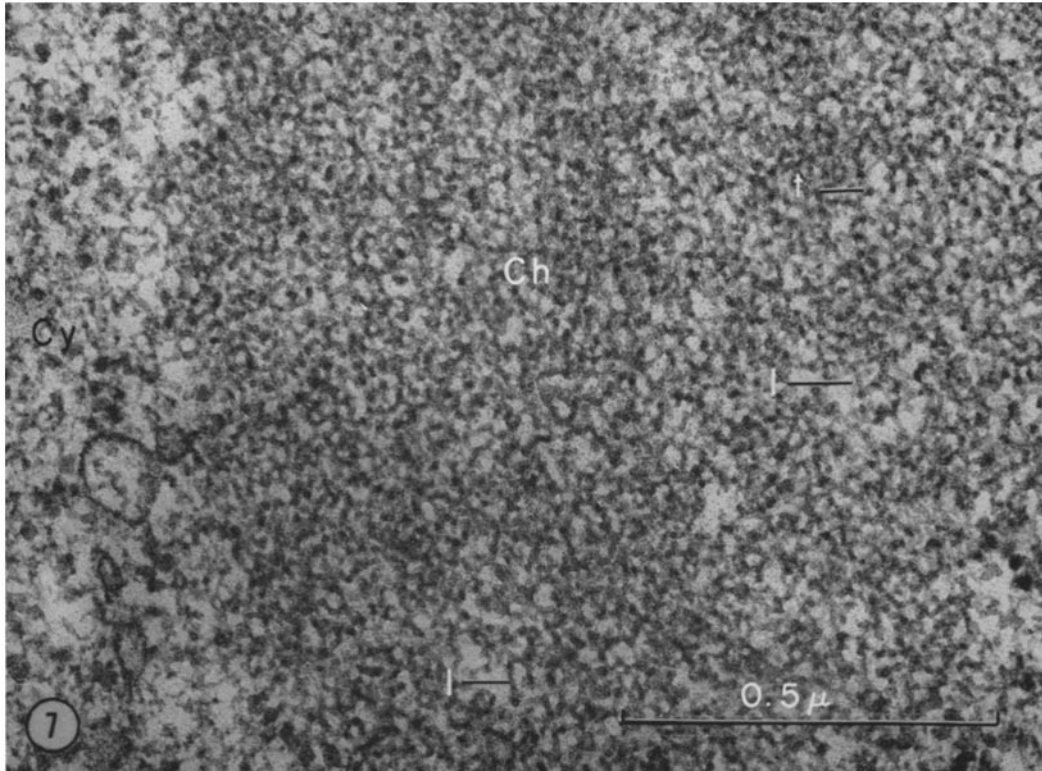
FIGURE 7 A portion of a chromosome (*Ch*) from a cell in one of the meiotic division stages. Longitudinal (*l*) and transverse (*t*) sections through the fibers which make up the substance of the chromosome are indicated. For comparison, a portion of cytoplasm (*Cy*) is included; structures present are granules, vesicles, and fibers. × 98,000.

of the nucleus where chromatin occurs, surrounding a stained interior. (The stain-free layer is very thin, about $\frac{1}{4}\mu$, hence is not apparent at the low magnification of Fig. 3 *a*.) The condensed chromatin of the shell is similar to the condensed X chromosome of the early spermatid (Fig. 2 *b*) in lacking nonhistone protein.

There is no indication of quantitative change in histone during early differentiation. The con-

centration of histone in the nucleus is high at all stages, and becomes higher as nuclear size decreases as expected if the amount per nucleus remains the same (see Fig. 3 *b*). There is a relatively uniform distribution of histone throughout the diffuse material of the nucleus until the shell stage, when it becomes restricted to the periphery of the nucleus where chromatin is localized. The intensity of the histone stain indicates a very high

FIGURE 8 A nucleus in the posttelophase II stage. Distinct regions of two sorts are evident: dense, finely textured chromatin regions (*Ch*), and less dense interchromatin regions (*In*) in which granules (*Gr*) occur. × 34,000.

FIGURE 9 A portion of the nucleus shown in Fig. 8. The chromatin region (*Ch*) is composed of fairly uniform fibers. In the interchromatin region, granules (*Gr*), thin fibers (*f*), and an unidentified body (*U*) are indicated. × 81,000.
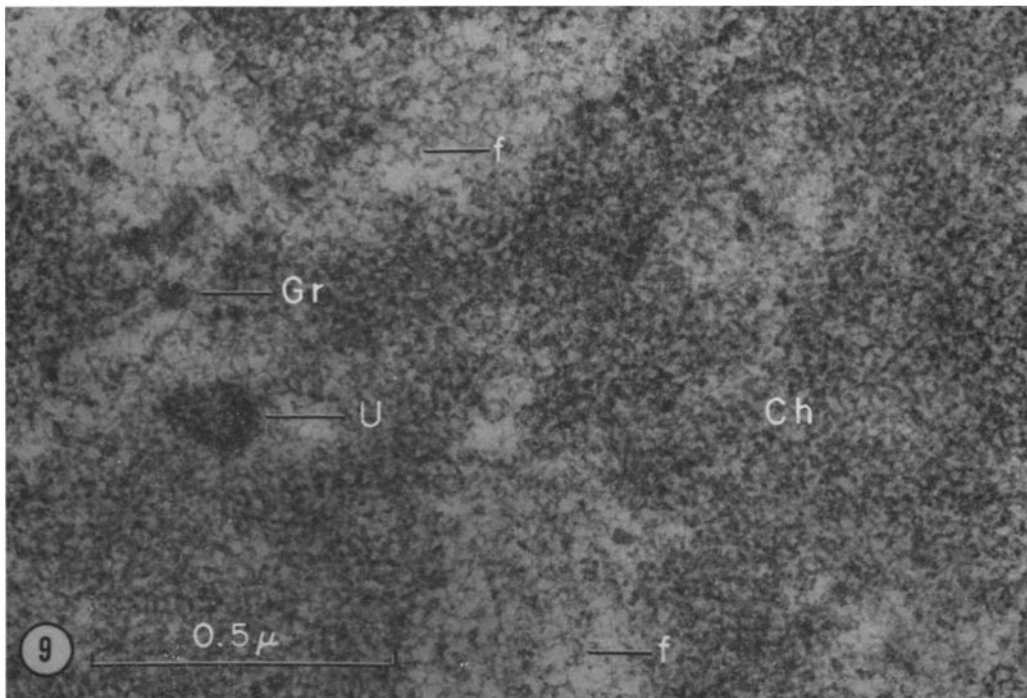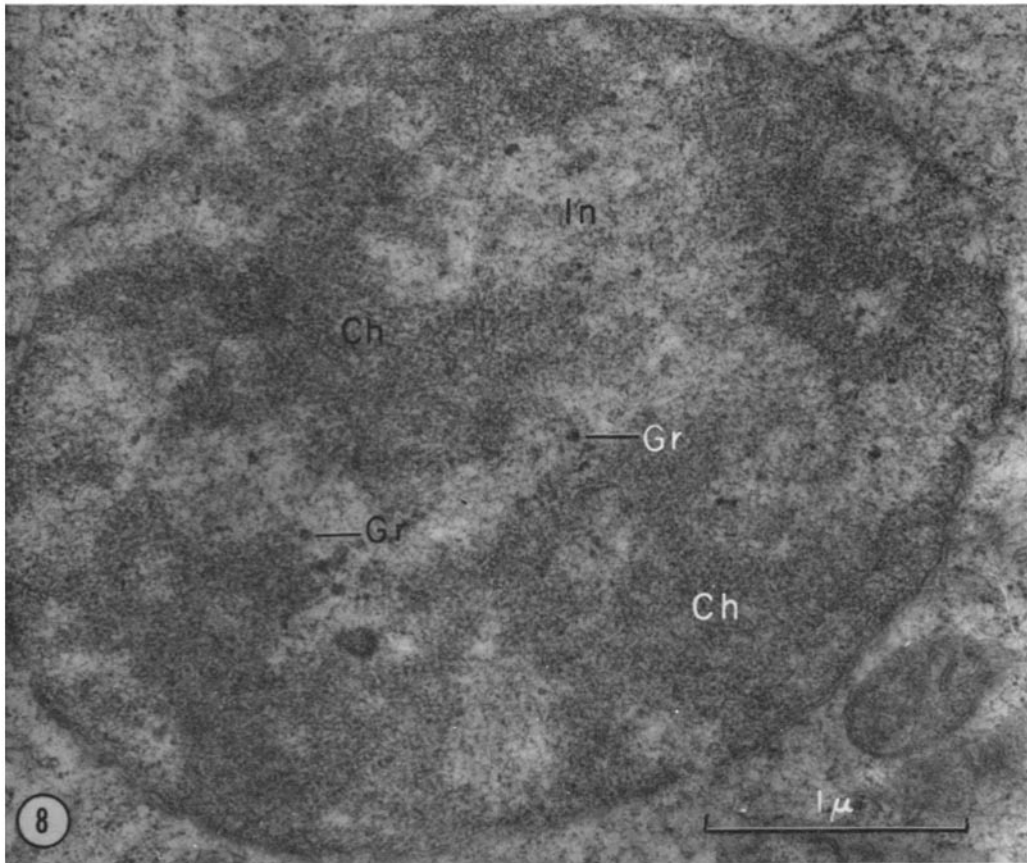
concentration at the nuclear periphery of the shell stage nuclei, as expected if all the histone of the nucleus had become packed into a thin layer. The nuclear inclusions which are prominent in the transition from the early spermatid to the shell stage do not contain detectable histone, and stain-free regions can be found where they occur.

Deamination of sections prior to histone staining did not indicate any increase in the arginine content of histone during early spermiogenesis. Although nuclei in late stages of elongation are stained intensely with the Alfert-Geschwind procedure after deamination, nuclei of early stages bind only negligible amounts of stain. A trace of stain was found in the chromatin of the shell stage, but not of sufficient intensity to indicate more resistance to deamination than is typical of somatic histones or earlier spermatid stages, since the concentration of histone in the shell is many times greater than in earlier stages.

Decreases in nulear RNA during earlier stages are indicated by azure B staining. RNA is readily detectable in early spermatid nuclei, as seen in Fig. 5. Nuclei in the crinkled, speckled, and shell stages bind so little stain that they escape recognition in sections stained for RNA; they are represented by empty regions in the cysts they occupy. The different stages can be distinguished with phase optics (Fig. 6), and a trace of stain is found in crinkled nuclei, apparently due in some part, at least, to the inclusions scattered through the nucleus. In the speckled stage, one of the two inclusion bodies is weakly stained, but otherwise nuclei appear completely free of stain, as do all regions of shell stage nuclei (Fig. 6 d).

Electron Microscopy

Previous electron microscope studies of spermatid nuclei have strongly emphasized their fibrous components, and easily give the impression that fibers are the only ultrastructural components occurring in spermatid nuclei. The fact that most spermatid nuclei also show an unusually uniform chromatin stain further suggests the interpretation that nuclei are pure chromatin and that changes in the chemical composition of nuclei reflect changes in chromatin fibers. Study of cricket nuclei indicated, however, that fibers are not the only structural component present during early spermiogenesis and further, that fibers other than chromatin fibers appear by mid spermiogenesis.

In the early spermatid stage, granular elements as well as fibers are prominent in the diffuse material of the nucleus. Comparison of early spermatids, which have a high nonhistone protein content, with stages after loss of most of the nonhistone protein indicates that granules and proteins are lost simultaneously, implying that the protein occurs in the granule fraction rather than in the fibers of the chromatin. This can be seen in Figs. 10, 12, and 14. In the early spermatid nucleus of Fig. 10, numerous small patches of fine textured material alternate with patches of coarser texture. The finer material, shown at higher magnification in Fig. 11, contains fibers of fairly uniform size. The granules occur in the coarser looking material, shown in Fig. 12. They are spheroidal, although fairly irregular in shape. Their size varies considerably; measurements of 28 granules showed a range from 24 to 86 mμ, with a mean of 41 mμ.

Very few of these granules are seen in the next stage, the crinkled stage, and none are seen in later stages. At the crinkled stage fine textured material, which is composed of fibers as in the previous stage, fills much of the nucleus, as seen in Fig. 14. The nucleoplasm also contains

FIGURE 10 An early spermatid. The condensed sex chromosome (X), and a nuclear inclusion body (IB) are indicated. The nucleoplasm has fine (Ch) and coarse textured (In) regions. A part of an early nebenkern (Nb) is indicated at the lower right. × 16,000.

FIGURE 11 A part of a fine textured region from an early spermatid nucleus. The two clumps of material indicated (Ch) are composed of fibers of the same thickness as chromatin fibers in other cells. ×130,000.

FIGURE 12 A portion of a coarse textured region in an early spermatid nucleus. Numerous granules (Gr) of different sizes are present. Other structures present which have not been identified seem to be fibers, or are irregular in shape. × 100,000.
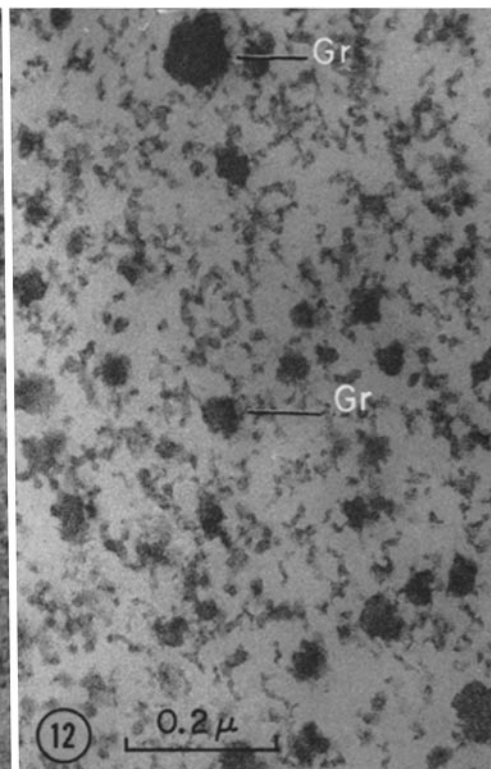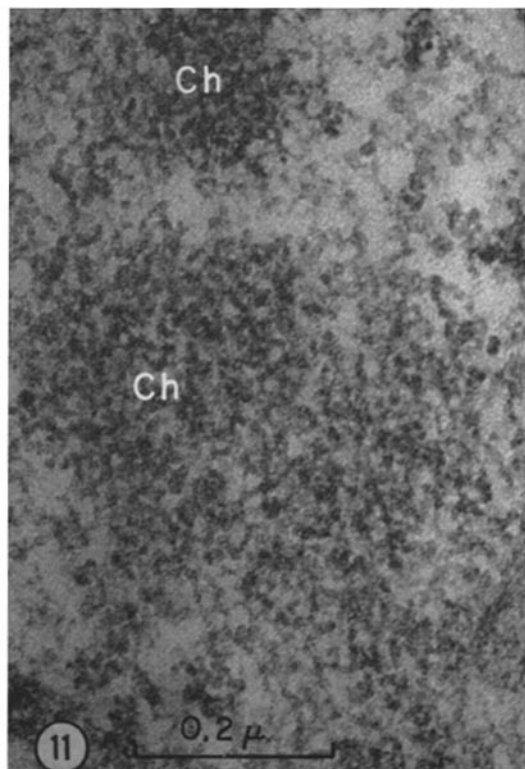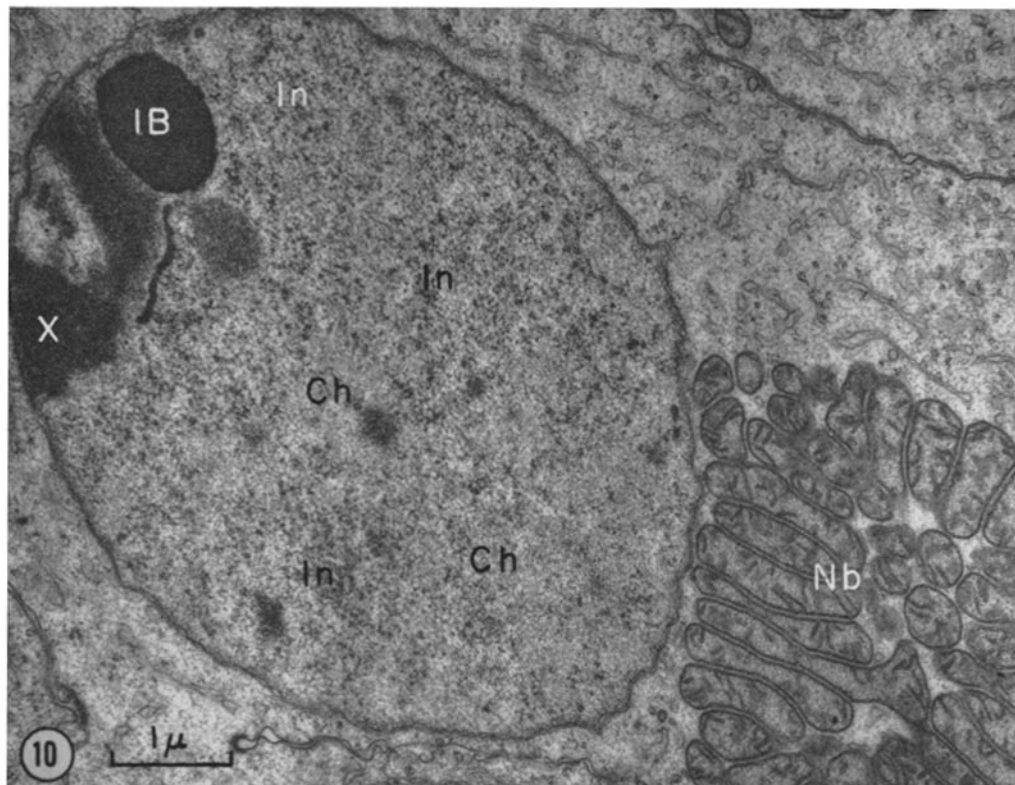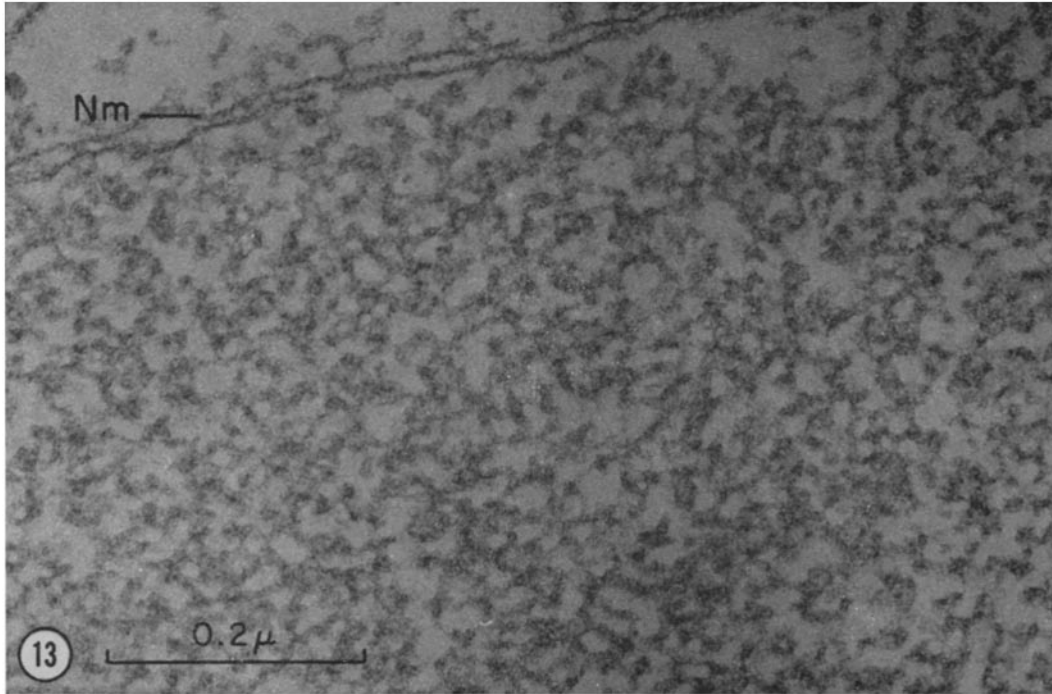
FIGURE 13 A portion of a sex chromosome from an early spermatid. It is composed of fibers with diameters similar to those in other regions of chromatin. A segment of nuclear membrane (*Nm*) is indicated. X 170,000.

some very opaque material in clumps of various sizes, which is not seen at any other stage.

At the speckled stage (Fig. 15) the nucleus appears much more homogeneous than at earlier stages, and the only visible variations in the nucleoplasm are slight differences in the density of small patches. As seen in Fig. 16, which includes regions of higher and lower densities, the material in both cases is composed of fibers, as found for the fine-textured material in earlier stages.

The possibility that nonhistone protein occurs in the fibers of the nucleus, in spite of the implications of the simultaneous loss of granules and protein, was investigated more closely by studying the fibers in detail. Fiber diameters were measured at each stage, with the expectation that if nonhistone protein occurred in them, its loss should have some effect on their size. The range of values (Fig. 17) and the mean diameter found in early, crinkled, and speckled nuclei are surprisingly similar, considering the difficulties of measurement; the mean values found were 8.4,

8.3, and 8.0 mμ, none of which are significantly different from each other, as shown in Table II. The invariable fiber size in these three stages clearly gives no indication that the 8 mμ fibers of early spermatids contained the nonhistone protein fraction which is lost in later stages.

The structure and composition of the condensed sex chromosome of early spermatids are in further agreement with the conclusion that nonhistone protein is associated with the granules rather than the fibers found in diffuse chromatin. As shown in Fig. 2 *b*, the sex chromosome appears to be free of nonhistone protein; and it is constructed solely of fibers with the same diameter as found for fibers of the diffuse chromatin, 8.0 mμ. A sex chromosome is shown in Fig. 13.

The sequence of events leading to the nucleoplasmic structure of the very early spermatid is illustrated in Figs. 7 to 9, showing a chromosome in meiotic division and a nucleus in the growth period between second telophase and the early spermatid of Fig. 10. As frequently observed previously, the chromosome shows no structural ele-

ments except fibers; their mean diameter was found to be 8.2 mμ. The posttelophase nucleus contains large discrete regions made of fibers with a mean diameter of 8.0 mμ, and regions of much lower opacity where a few granules, as well as some fibers, can be seen. The granules have the same general appearance as in the early spermatid nucleus but tend to be smaller; their size ranged from 16 to 32 mμ and averaged 23 mμ in the sample of nine granules measured. Fibers the size of chromosome fibers are not found in the regions of low opacity; the fibers which occur in these regions are relatively thin, their diameter averaging only 3.5 mμ.

Chromatin is localized at the periphery of the nucleus in the shell stage and, as Figs. 19 and 21 show, consists of dense, contorted fibers. The interior of the nucleus contains less contorted fibers than the periphery, and they are fairly thin, with an average diameter of 5.4 mμ in the early shell stage. A small number of the interior fibers was measured in the late shell, and showed a mean of 6.5 mμ, which is not significantly different from the early value $(0.2 > P > 0.1)$. Both of these values are significantly different (Table II) from the means found for chromatin fibers, in every stage: for interior fibers of the early shell stage vs. speckled stage fibers, $P < 0.001$; for in-
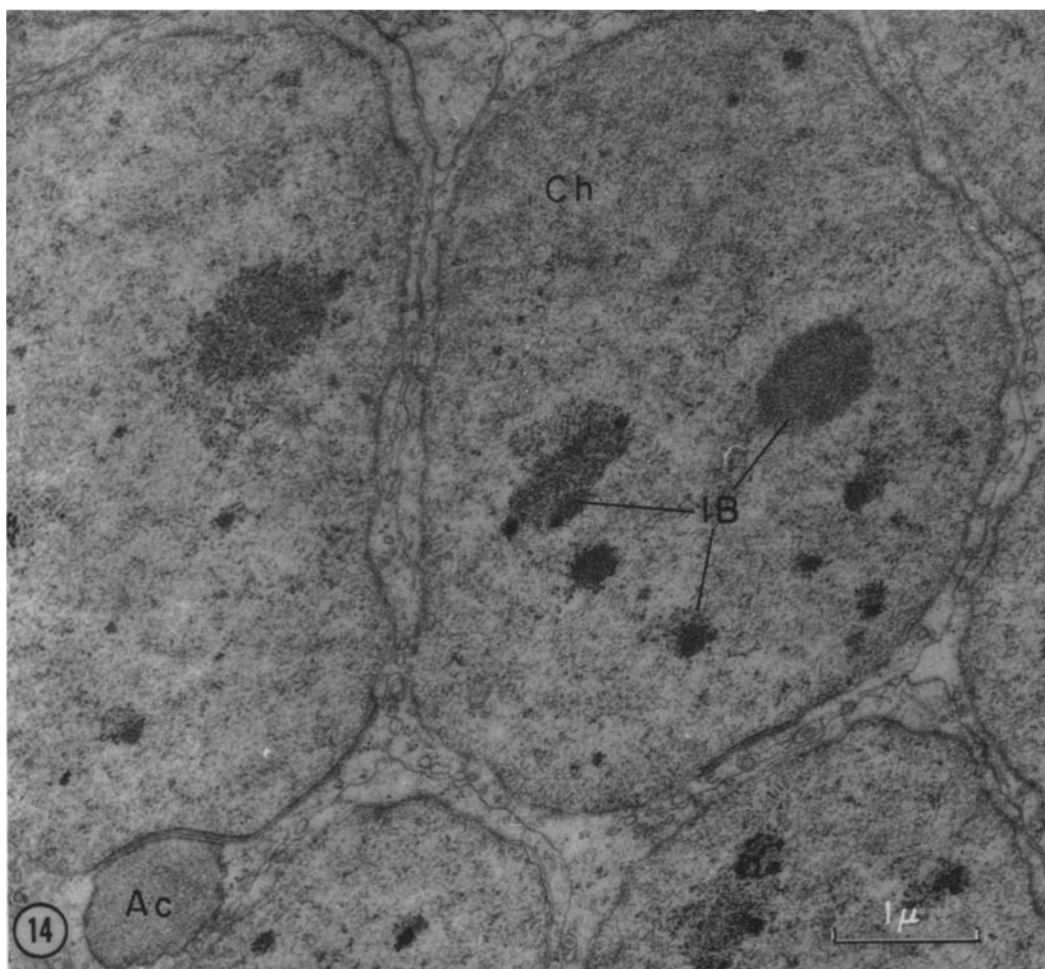

Figure 14   Nuclei in the crinkled stage. Much of the nucleoplasm (Ch) is composed of fibers of the type characteristic of chromatin. Nuclear inclusion bodies (IB) of various sizes also occur. Granules of the type seen in early spermatids are not evident. An acrosome (Ac) in an early stage of development is indicated. $\times$ 19,000.

terior fibers of the late shell stage vs. speckled stage fibers, $P < 0.01$.

The chromatin not only shows a changed localization at the shell stage from that in previous stages; it also shows its first detectable change in fiber morphology. The fibers are much more contorted than in earlier stages, and their diameters are considerably larger. In the early shell stage (Figs. 18 and 19), when the chromatin fibers are in a fairly loose band at the periphery, their mean diameter is 10.9 mμ. A slightly higher value is found in the late shell stage, 13.0 mμ, when the chromatin shell is narrower and appears more opaque (Figs. 20 and 21). In both the early and late shell stages the chromatin fibers were found to have a bimodal distribution of diameters (Fig. 17), in contrast to the unimodal distribution of all other stages. Peaks occured at about 11 mμ and at 17 mμ in both shell stages; the higher mean diameter in the late shell stage reflects primarily a relative increase in the number of fibers in the thicker class.

DISCUSSION

After telophase of the second meiotic division, diffuse material which is identified as chromatin by the Feulgen stain occupies most of the spermatid nucleus. The morphological and chemical characteristics which are shown by this material seem fairly typical of interphase nuclei of somatic cells. Two structural elements are distinguished in it, chromatin fibers and large granules. Both of these elements are dispersed widely through the material, although neither is uniformly distributed.

Recent studies have shown that granular elements such as those found in early spermatids occur in interphase nuclei of various somatic tissues (25–27); the granules are characterized by a relatively large size, irregular shape, and localization in the interchromatin areas of the nucleus, i.e., where chromatin fibers are not highly concentrated. High resolution studies of somatic

nuclei indicate that the granules contain RNA but no DNA (25, 27). The situation seen in cricket spermatids suggests that the granules contain nonhistone protein as well as RNA: they are common only in the early spermatid stage when the diffuse material is rich in nonhistone protein and RNA, and after this stage the granules, nonhistone protein, and RNA all disappear from it at the same time.

It seems unlikely that nonhistone protein occurs in chromatin fibers. The loss of nonhistone protein after the early spermatid stage would be expected to affect fiber structure, and diameter, if the protein occurred in the fibers. Instead, the fibers have the same diameters in the early spermatid and in two stages subsequent to the loss. The condensed X chromosome of the early spermatid provides an example of an extremely high concentration of 8 mμ fibers, with no indication of a nonhistone protein component. In contrast with the nonhistone protein, histone appears to parallel the chromatin fibers in localization and in concentration. Previous studies have clearly demonstrated that DNA is localized in chromatin fibers (25) and have suggested that histone is associated with DNA (22). The indications that chromatin fibers are composed of DNA and histone, and that RNA and nonhistone protein occur in granules, as seen in the early spermatid nucleus, are in support of proposals that this is the situation in other types of nuclei (28, 29).

The earliest stages of nuclear differentiation accomplish the elimination of essentially all components from the nucleus except the chromatin fibers. The fibers begin to change in structure at a time which is well removed from the earlier loss of nuclear components. The only event which is simultaneous with their change in structure is the appearance of thin fibers, composed of nonhistone protein. The sudden occurrence of thin fibers and of thicker chromatin fibers at the shell stage might both be explained if the thin fibers were split off from chromatin fibers in the transition to the shell

FIGURE 15 A nucleus in the speckled stage. Except for a prominent nuclear inclusion body (*IB*) , the nucleoplasm is composed solely of fibers of the chromatin type. A developing acrosome (*Ac*), and a flagellar accessory structure (*FA*) are indicated. × 37,000.

FIGURE 16 A portion of a nucleus from a cell in the speckled stage. The only structural elements present are fibers of a size characteristic of chromatin fibers. × 130,000.
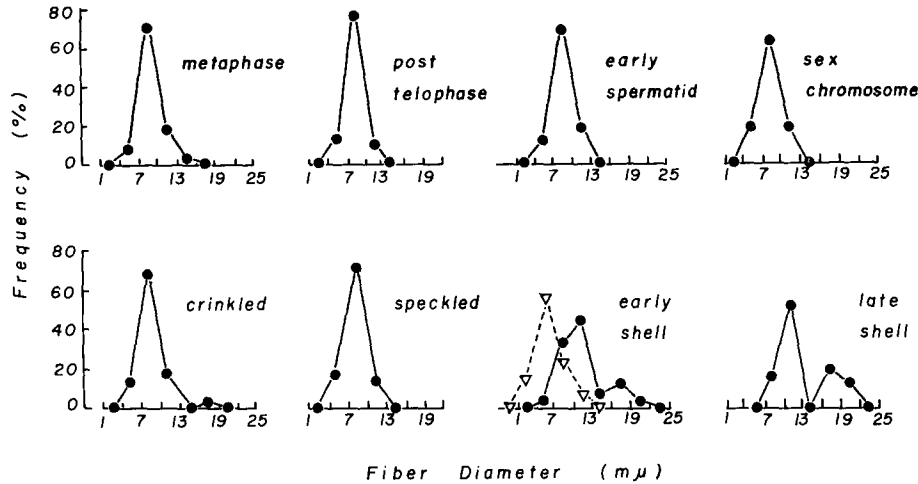
Figure 15. IB, FA, Ac
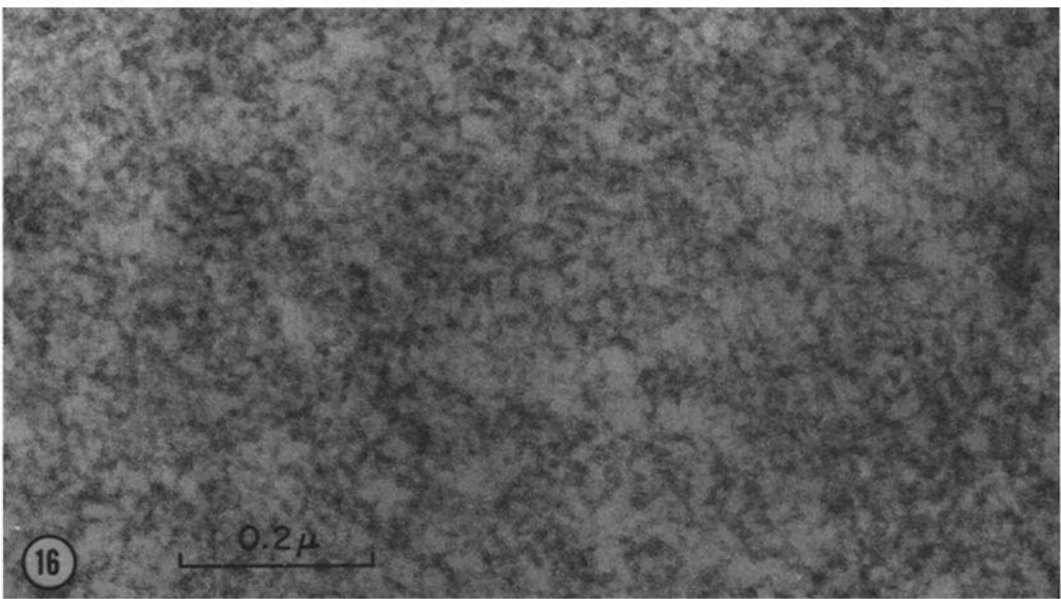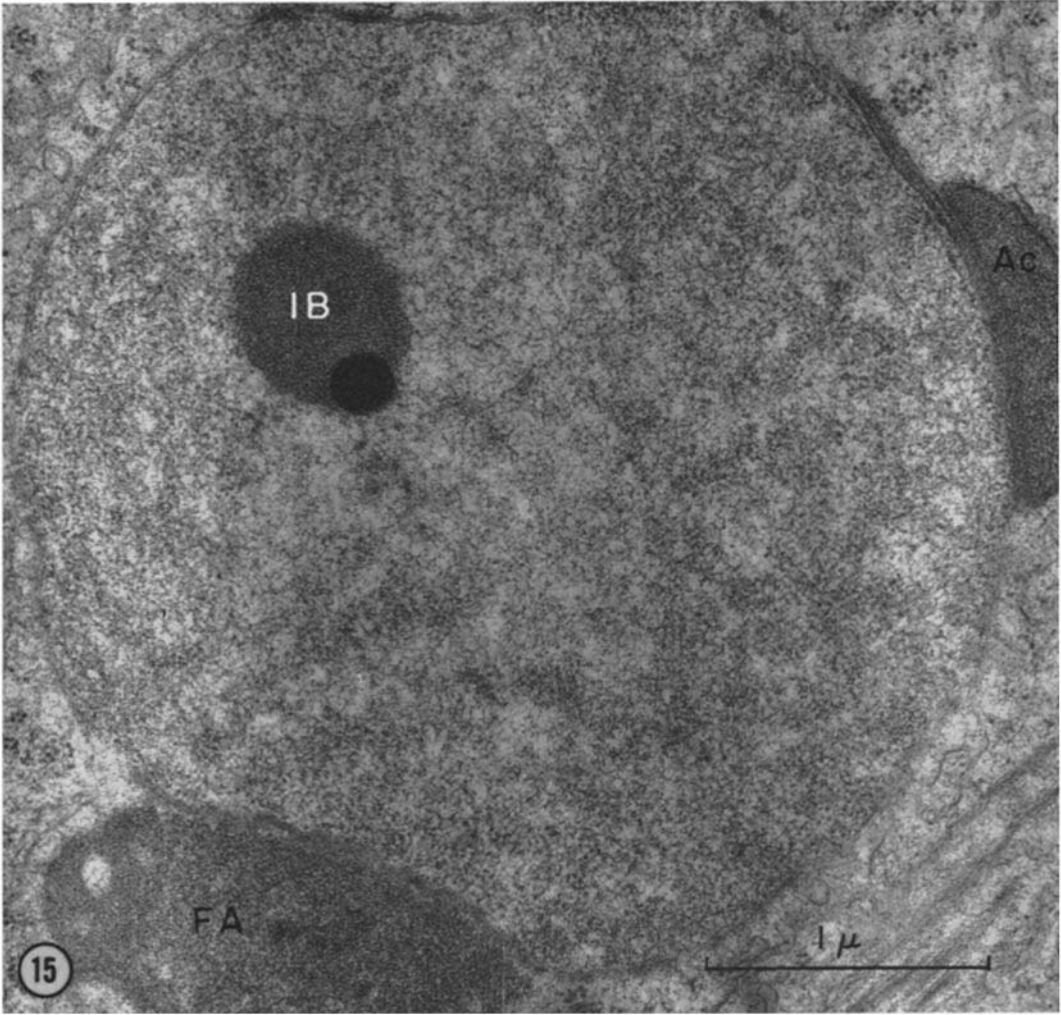
Figure 16.

FIGURE 17 Frequency distributions of measured diameters of chromatin fibers in early spermiogenesis. Diameters of the non-chromatin fibers of the early shell stage are also shown, and are plotted with a broken line.

stage, and their removal caused the morphology of chromatin fibers to change; but this explanation obviously lacks the supporting evidence of the occurrence of nonhistone protein in early chromatin fibers. Formation of the thin fibers by *de novo* synthesis is also compatible with the observations. The appearance of thin fibers in mid spermatid nuclei of grasshoppers has also been reported by Dass and Ris (5), with the suggestion that they are derived from chromatin fibers. Their interpretation was proposed, however, with the assumption that the thin fibers are DNA-histone molecules, which is quite different from the situation found in the cricket.

The thin fibers of the cricket shell stage constitute a nonhistone protein fraction which is very small, especially in comparison with the amount seen in the early spermatid. A small amount of nonhistone protein, concentrated in the nuclear interior, can also be detected in stages of nuclear elongation which occur after the shell stage, sug-

TABLE II

Thickness of Nuclear Fibers in Early Spermiogenesis

Stage	Mean	Standard Error	N
	$m\mu$	$m\mu$	
Metaphase	8.2	0.26	37
Posttelophase II			
Chromatin	8.0	0.18	50
Interchromatin	3.5	0.48	6
Early spermatid			
Diffuse chromatin	8.4	0.26	36
Sex chromatin	8.0	0.25	58
Crinkled stage	8.3	0.19	88
Speckled stage	8.0	0.16	74
Early shell stage			
Chromatin	10.9	0.41	56
Inner fibers	5.4	0.29	54
Late shell stage			
Chromatin	13.0	0.72	25
Inner fibers	6.5	0.79	10

FIGURE 18 A nucleus in the early shell stage. Chromatin (Ch) is localized in a peripheral shell. The non-chromatin material (In) interior to the chromatin is composed of loosely packed, thin fibers. A nuclear inclusion body (IB), developing acrosome (Ac), and flagellar accessory structure (FA) are indicated. × 32,000.

FIGURE 19 A part of the chromatin shell of the cell in Fig. 18. The chromatin is composed of highly contorted fibers (cf), which are thicker, on the average than chromatin fibers in earlier spermatids. A few of the thinner interior fibers (if) and the nuclear inclusion body (IB) are indicated. × 130,000.
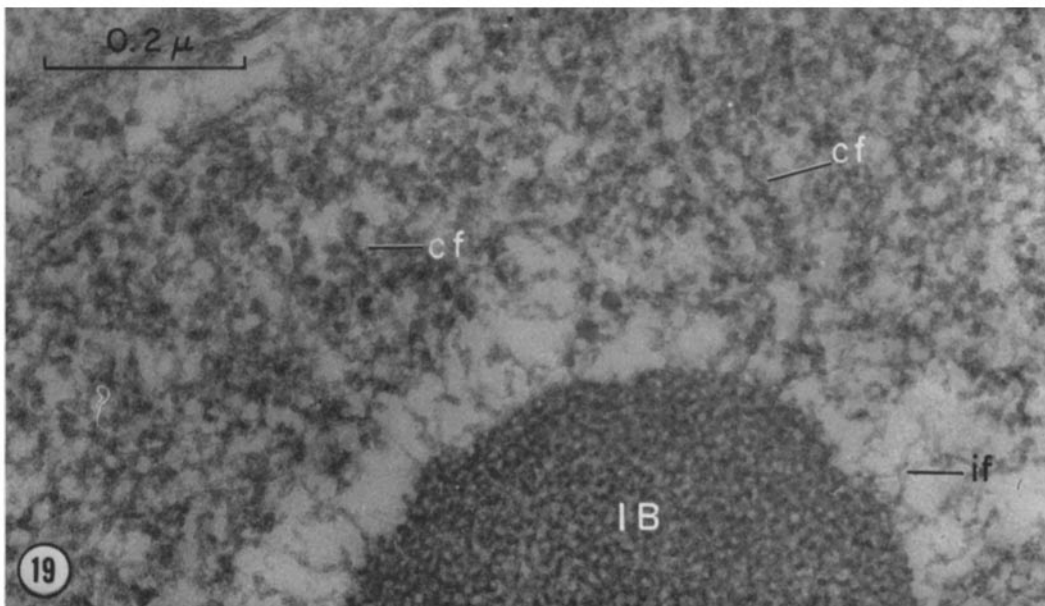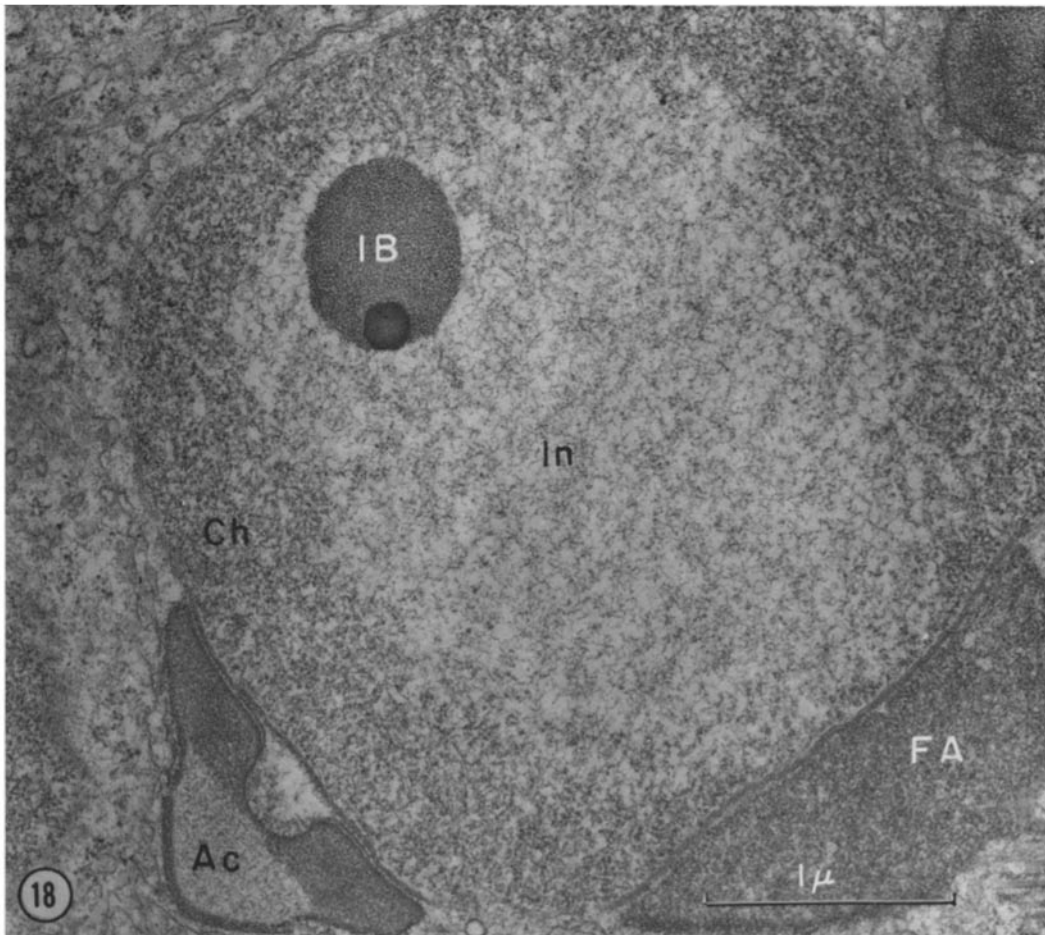
gesting a persistent fraction which is typical of later stages. Since it is characterized by fibrous components and occurrence in stages when strikingly unusual ultrastructure develops, this fraction, as well as chromatin, might provide fibrous elements for the larger structures found in late nuclei. The existence of this fibrous nonhistone protein in the nucleus obviously illustrates the risk in assuming that all fibrous structures found in nuclei of mid and late spermatids are either chromatin fibers or their DNA-containing derivatives.

The chromatin of the shell stage is extremely condensed and composed solely of fibers, and in these two respects is comparable to the sex chromatin of the early spermatid. There were no visible staining differences between these two kinds of chromatin, although the fibers are much thicker at the shell stage and it might be suspected that a chemical change in fibers should accompany their structural change. The observations suggest that the change in fiber structure may result from an extrinsic factor, such as unusual nuclear environment. The failure to detect any chemical change may simply reflect a lack of sensitivity in the methods used, however, and slight changes are not ruled out. It is clear, nonetheless, that the prominent increases in the arginine content of histone, which are detectable by the methods used here in spermatids of other organisms (9, 30, 31), are restricted in the cricket to stages later than those considered here; the replacement of histone by protamine is one of the latest events in cricket spermiogenesis. Neither of these chemical events appears to be correlated with the initial change in fiber structure during spermiogenesis.

The chromatin fibers occur in two size classes at the shell stage; the two distinct modes are especially clear in the distribution curves for the later shell stage, at about 11 and 17 mμ (see Fig. 18). Only one class of chromatin fiber, with a mean of about 16 mμ, is seen at the stage following the shell stage (unpublished observation). The 16 mμ diameter is about twice the diameter seen in early spermatid stages, which seems consistent with the idea proposed from other studies, that chromatin fibers associate with each other during spermiogenesis. Simple association of early fibers to produce 16 to 17 mμ fibers does not, however, explain the occurrence of the 11 mμ class of fibers at the shell stage. These fibers are already considerably thicker than the fibers of earlier stages, and apparently have undergone a part of the thickening process. Gradual thickening of the chromatin fibers seems indicated by their occurrence, and may be more easily explained by increased coiling of the chromatin fiber. The contorted appearance of shell stage fibers also suggests that increased coiling may be involved in the diameter increases. Indications similar to those noted in other organisms (3, 4), that fibers may associate with each other, are also apparent in the cricket, but at stages later than the shell stage. Chromatin fibers become straight and oriented after the shell stage (14, 15), and larger aggregates of chromatin are formed subsequently, apparently at their expense. These straight fibers which give rise to aggregates are not equivalent to the fibers of the early spermatid, however, because of the intervening fiber thickening which arises at the shell stage.

Interpretation of fiber diameters in terms of the structure of chromatin fibers has been a subject of some conjecture. Fiber diameters of about 100 A appear to be typical of somatic nuclei in many tissues and organisms, and it has been suggested that chromatin fibers may contain two DNA-histone molecules of about 40 A diameter

FIGURE 20   A nucleus in the late shell stage. The chromatin forms an electron-opaque peripheral shell which lines the inside of the nuclear membrane everywhere except at the part adjacent to the flagellar accessory structure (FA). The interior is composed of thin fibers similar to those of the early shell stage. The centriole (Ce), part of the flagellum (Fl), and the developing acrosome (Ac) are indicated. × 32,000.

FIGURE 21   A portion of the chromatin shell from a cell in the late shell stage. The chromatin is composed of contorted fibers, somewhat thicker, on the average, than those in the early shell stage. Some of the thinner interior fibers (if) and a part of a nuclear inclusion body (IB) are indicated. × 130,000.
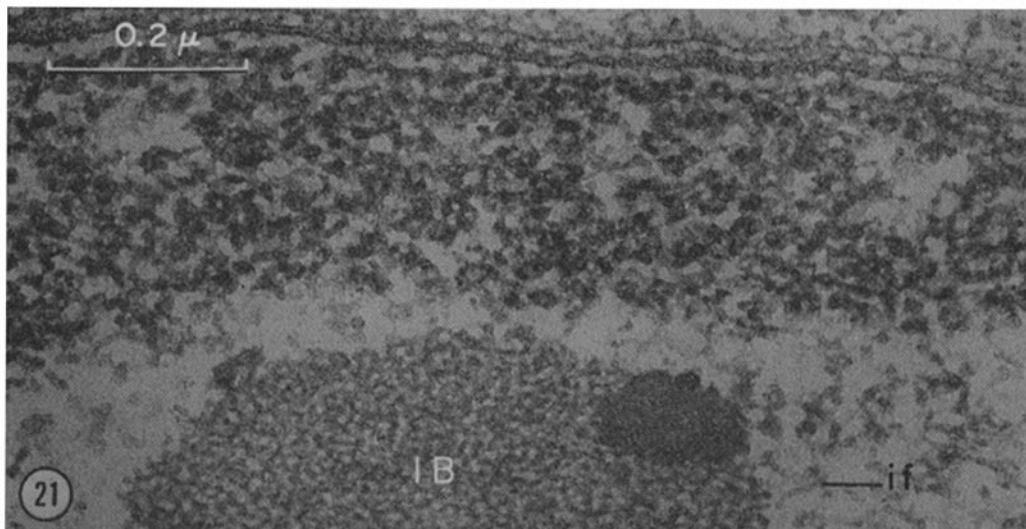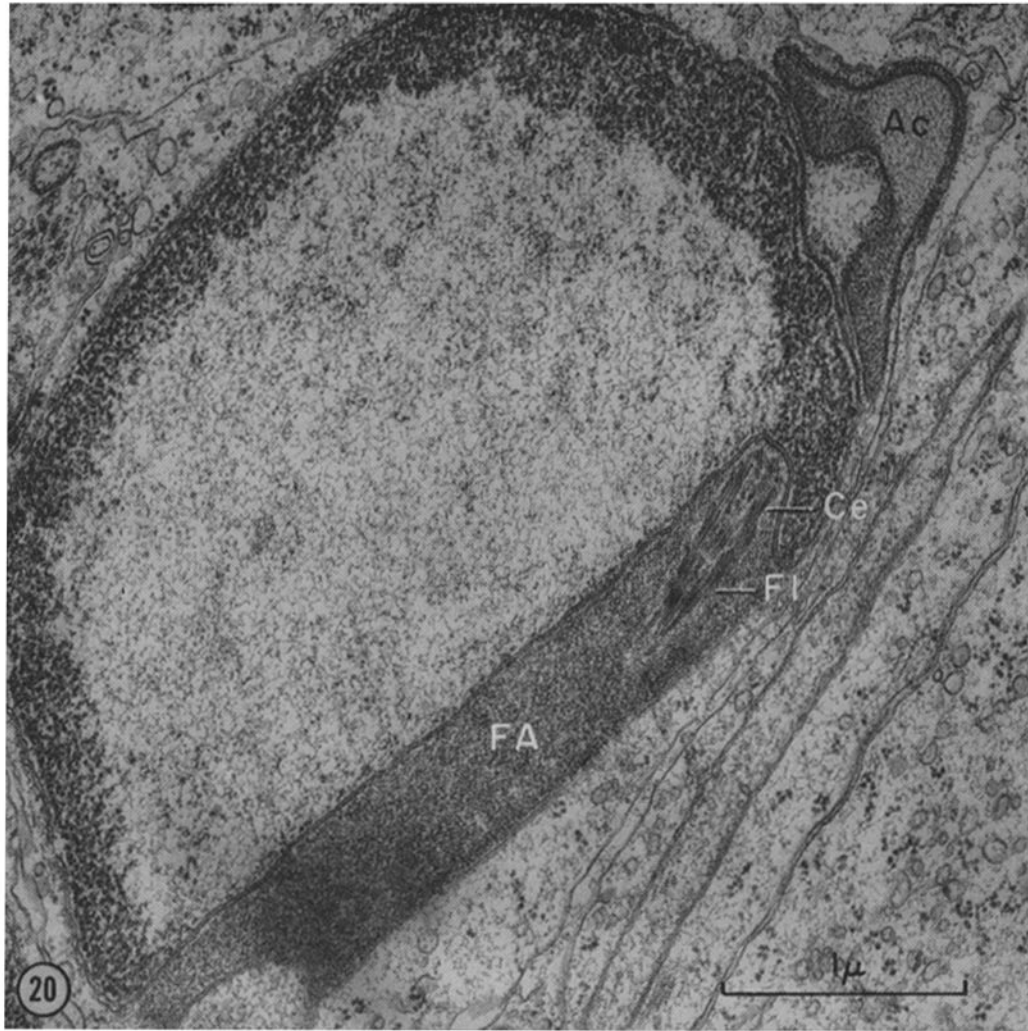
(11). Fiber diameters as thin as 50 A have been found (25, 28), however, and do not support this interpretation. Except for an early study by de Robertis (32), no previous reports have provided information on the actual distribution of sizes which is found in a random sample of chromatin fibers. The measurements on cricket fibers revealed a surprisingly large range of sizes, and indicate that no one value can be said to adequately describe chromatin fibers or to have any particular validity for structural interpretation. The statistical significance of the mean diameters is obvious, but diameters as small as 4 mμ were commonly found in all chromatin samples with mean diameters of about 8 mμ, and a continuous range of sizes up to about 16 mμ. The smallest fiber sizes would seem significant for structural interpretation, if it could be assumed that there is a discrete class of small fibers, of uniform size, and that larger fibers represent aggregates of two or more small fibers. These assumptions are not consistent with the frequency distributions, however. The curves (excluding the shell stage) are unimodal and indicate only one class of fiber, of very variable size, in each kind of chromatin. The curves are approximately normal, with no suggestion that aggregation of small fibers increased the size range, or that discrete size classes of any kind occur. How the various sized fibers of a sample differ from each other is not clear, and there is no obvious criterion for deciding which of the sizes best represents well preserved chromatin fibers.

Some of the variation in size observed in cricket can be attributed to measuring errors, but an extensive range in size is found in single micrographs where measuring errors are minimal. It seems likely that a large part of the variation may be produced by preparative procedures, rather than being typical of fibers in vivo; the fiber sizes must be relatively independent of in vivo conditions, since the same sizes occurred in six different kinds of chromatin, under a variety of conditions, during early spermiogenesis. The data seem consistent with the interpretation that preparative procedures consistently produce the same average effect on fiber size, although not affecting all individual fibers of a sample to the same degree. If such a situation exists, comparison between samples should be valid to detect structural change, but the significance of the actual sizes observed is obscure.

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