EVIDENCE FOR THE CYTOPLASMIC SYNTHESIS OF NUCLEAR HISTONE DURING SPERMIOGENESIS IN THE GRASSHOPPER *CHORTOPHAGA VIRIDIFASCIATA* (DE GEER)

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ABSTRACT

Histone synthesis during spermiogenesis in the grasshopper *Chortophaga viridifasciata* was studied using autoradiographic and cytochemical methods. It was found that meiosis is followed by a cessation of RNA synthesis, an elimination of RNA from the nucleus, and, during the cytoplasmic sloughing accompanying the initial cytoplasmic elongation, a loss of most of the RNA from the cell. The initial phase of cell elongation results in a long spermatid headed by a spherical RNA-less nucleus bounded by a thin RNA-containing layer of cytoplasm. Subsequent nuclear elongation is accompanied by a replacement of the typical histones by others rich in arginine. This replacement is the result of synthesis of new protein. Incorporation of arginine is first seen to occur in the thin cytoplasmic layer surrounding the nucleus. This layer was shown by staining and electron microscopy to contain aggregations of ribosome-like particles. These observations support the conclusion that the histone is synthesized in association with the RNA granules in the cytoplasm, then migrates into the nucleus where it combines with the DNA.

INTRODUCTION

The syntheses of histones and protamines are of interest because of the interaction of these substances with DNA. The parallel increases in the staining of both histones and DNA during the "S period" of interphase prior to cell division led to the early suggestion that during chromosome replication both syntheses are associated (1, 9, 19). However, a more reasonable expectation based upon recent concepts of protein synthesis would be that the syntheses are not connected, that the DNA itself plays no direct role in histone synthesis, that those genes which are involved participate only indirectly, and that synthesis of histones and complexing with DNA are distinct events, separated both spatially and temporally.

Early evidence for an independent synthesis of histones was in fact seen in studies of spermiogenesis in organisms in which a typical histone complement is replaced by a protamine or by histones very rich in arginine (2, 10). In the latter work, a histone synthesis was shown to occur several weeks after premeiotic DNA replication, and at least several weeks prior to fertilization and subsequent replication. Nevertheless the proteins always appeared to be associated with DNA.

Later studies of spermiogenesis in the grasshopper indicated that this organism held promise in permitting a separation of the site of synthesis of histone from the DNA with which it would ultimately complex. The reason for the choice of

organisms is that in the grasshopper the transformation of the nucleus occurs late in spermatid development, when the cell has been stripped of most of its cytoplasm, and the nucleus has voided its RNA. These circumstances facilitate the identification of the synthetic process leading to the nuclear transformation and its localization within the cytoplasm.

MATERIALS AND METHODS

Nymphs of the grasshopper *Chortophaga viridifasciata* (de Geer) were used throughout the experiments. These were collected in Austin, Texas, during the months of December through April, and were maintained in screened cages, whose floors were covered with sand, in an otherwise typical laboratory environment. They were fed only lettuce, fresh leaves being supplied every few days. The animals thrived under these conditions, undergoing a succession of moults and attaining the adult stage within a few weeks.

The development of the spermiogenic cells was studied under the phase microscope. The cells were obtained by extruding the contents of individual tubes of the testis into a culture medium described by Shaw (30). Permanent smears were made in the following manner in order to preserve the morphology useful for identification of stages. The tissue was extruded in a drop of 0.25 M sucrose on an albuminized slide. The masses of cells were gently teased apart, the slides inverted over a small beaker containing a few drops of 1 per cent osmium tetroxide, and the material exposed to the vapor for 5 minutes. The drop was then smeared by traction and allowed to dry. The sucrose formed a glass with no apparent crystallization. The dried smears were postfixed for a few minutes in undiluted formalin, and stored in 70 per cent ethanol or otherwise treated. These preparations could be stained and were utilized for the autoradiographic experiments described in Fig. 13.

Cells were stained for DNA using the standard Feulgen method (see reference 33). Where retention of histone was required, hydrolysis was carried out with 1 N trichloracetic acid (TCA) for 25 minutes instead of the usual 1 N HCl for 12 minutes (9). Schiff's reagent prepared in the usual manner, with hydrochloric acid, was employed in preference to the TCA Schiff's because of its greater reliability. Histones were not lost from formalin-fixed material during this staining. The fast green method of Alfert and Geschwind (4) without or following deamination or acetylation procedures was employed for the demonstration of histones and arginine-rich spermatid histones, respectively (2, 10).

The following procedures were used for the double staining of protein-bound arginine and DNA. Formalin-fixed, paraffin-embedded, sectioned material was acetylated for 1 hour at 60°C in acetic anhydride containing 2 per cent glacial acetic acid. These sections were then stained with the Feulgen-naphthol yellow S double stain as described by Deitch (14) (except for the substitution of TCA for HCl during hydrolysis). The ratios of the extinctions at 430 and 570 mμ were determined and used to express relative ratios of protein arginine to DNA. The measurements were made using a microspectrophotometer similar to that described by Pollister (see reference 32). The cytoplasm of the early spermatid absorbs very lightly at 430 mμ, and most of the cytoplasm has been sloughed from all but the earliest spermatid stages, so that the measurements were made on material which is essentially nuclear.

Alternatively, a Sakaguchi/Feulgen combination was used employing modifications of the Sakaguchi test for arginine described by McLeish *et al.* (28) and Deitch (15). The procedure used was as follows: Sections of formalin-fixed, paraffin-embedded tissue were immersed face down (see Deitch for details) in a solution containing 10 drops of 1.5 per cent 2,4 dichloro-α-naphthol in 70 per cent ethanol and 20 drops of 1 per cent NaOCl (commercial Clorox diluted 1 to 5 with water) in 10 ml of saturated $Ba(OH)_2$ for 30 minutes. The sections turned a dark pink. The slide was then flooded to remove the reaction mixture without permitting the scum of $BaCO_3$ to come in contact with the preparation, and immersed for a short time in a 1 per cent solution of urea. The preparation was then hydrolyzed for 25 minutes in TCA at 60°C and stained with the Schiff's reagent as in the usual Feulgen stain. Fig. 1 shows the change in the absorption spectrum of the Sakaguchi-stained nuclei after exposure of the preparation to acid, and the stability of the resulting yellow color during the subsequent steps leading to Feulgen staining. The pink color of the Sakaguchi reaction can be restored by exposure of the slide to alkaline conditions. This is to be avoided, however, since the yellow color developed under acid conditions has an absorption peak which is sufficiently far from the Feulgen peak to permit independent measurement of DNA and protein arginine. The ratios of the extinctions at 440 and 600 mμ were used to express the relative ratios of protein arginine to DNA during spermatid development.

Azure B was used for the identification of RNA (18), and toluidine blue at pH's 4 and 7 was used in the manner of Prescott and Bender (29) for general basophilia. This method was particularly valuable for staining cells through the stripping film in the autoradiographic studies. Vital staining with acridine orange was found to provide the most sensitive test for RNA (von Bertalanffy and Bickis, 6), in so far as this technique was the only one which permitted convincing visualization of RNA in the late sperma-

tids. Concentrations of 1 part in 10,000 in the culture medium results in cells whose nuclei fluoresce a brilliant green, or, in those cells where the chromosomes can be distinguished in the nuclear sap, a yellow intermingled with orange. The cytoplasm generally exhibits an orange fluorescence. Although this stain gave fairly reproducible results, it was not used critically. The interpretation of the results of this staining rests equally upon other evidence. The fluorescence was observed with the Zeiss fluorescence microscope, utilizing UG 2 and 5 and two BG 12 exciter filters which transmit light of wavelengths between 350 and 400 mμ, and barrier filters 47 and 50 which transmit the fluorescent light of wavelengths above 500 mμ.

Material used for electron microscopy, and for autoradiography of thin sections (with the light microscope), was fixed in 1 per cent OsO_4 buffered at pH 7.4 with Veronal, or, alternatively, with 1 per cent OsO_4 in culture medium. The fixed tissue was embedded in a mixture of Epon and Araldite (Mollenhauer, personal communication). Thin sections were cut with the Porter-Blum ultramicrotome. The electron micrographs were made with an RCA EMU 4F. Unless otherwise stated, the grids were stained with a mixture of barium and potassium permanganate (Mollenhauer, personal communication).

Autoradiographic studies were carried out using the method of Taylor and McMaster (34), employing Kodak Ltd. AR-10 Scientific Plates. The slides were exposed for from 2 to 12 months, depending upon need. Labeled material was administered according to the procedure recommended by Lima-de-Faria (26). The animals, usually nymphs, were first anes-

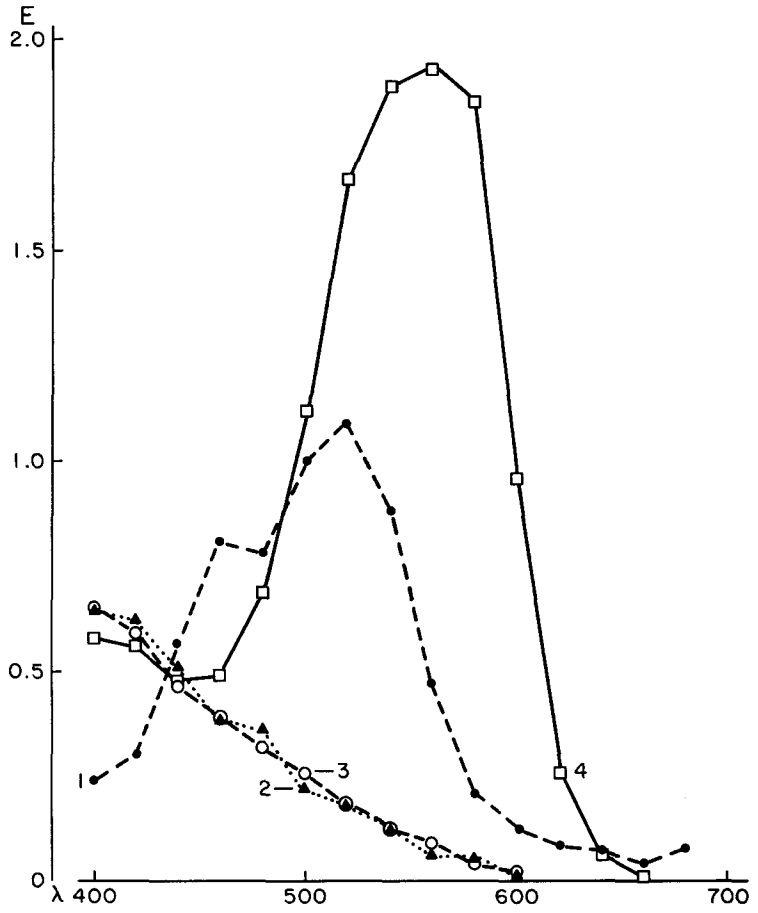


FIGURE 1 Absorption spectra of nuclei stained with the Sakaguchi and the Feulgen reactions. Curve *1:* ●, Sakaguchi stain. Curve *2:* ▲, Sakaguchi stain followed by exposure to cold acid. Curve *3:* ○, Sakaguchi stain followed by acid hydrolysis used for the Feulgen reaction. Curve *4:* □, Sakaguchi/Feulgen stain. A single spermatid nucleus was used for obtaining all four curves.

thetized with CO_2, and then the material was injected at the third segment. Usually from 2 to 10 μl were administered. Unless otherwise stated, the doses given were 1, 10, and 25 μc of H^3-thymidine, H^3-cytidine, or H^3-arginine, respectively. The specific activities of these compounds were 1.9, 1.0, and 0.3 curies/mmole, respectively. The nucleotides were obtained from Schwartz Bioresearch, Mount Vernon, New York, and the arginine from Nichem, Inc., Bethesda, Maryland.

OBSERVATIONS

The morphological changes which developing spermatids of the grasshopper undergo have been described in great detail (13, 21, 25). These changes, shown roughly in Figs. 2 to 7, provide a useful parameter in the study of the metabolism of the spermatid. The sequence can be separated into two phases of elongation. In the first, the bulk of the cytoplasm is sloughed, leaving a spermatid with a long axial filament and a rounded nucleus, both covered by a thin layer of cytoplasm. Some residual cytoplasm of the postmeiotic spermatid is retained in the form of small beads strung intermittently along the axial filament. The second phase involves the nucleus, which changes from a spherical to a filamentous form, having final dimensions similar to those of the tail.

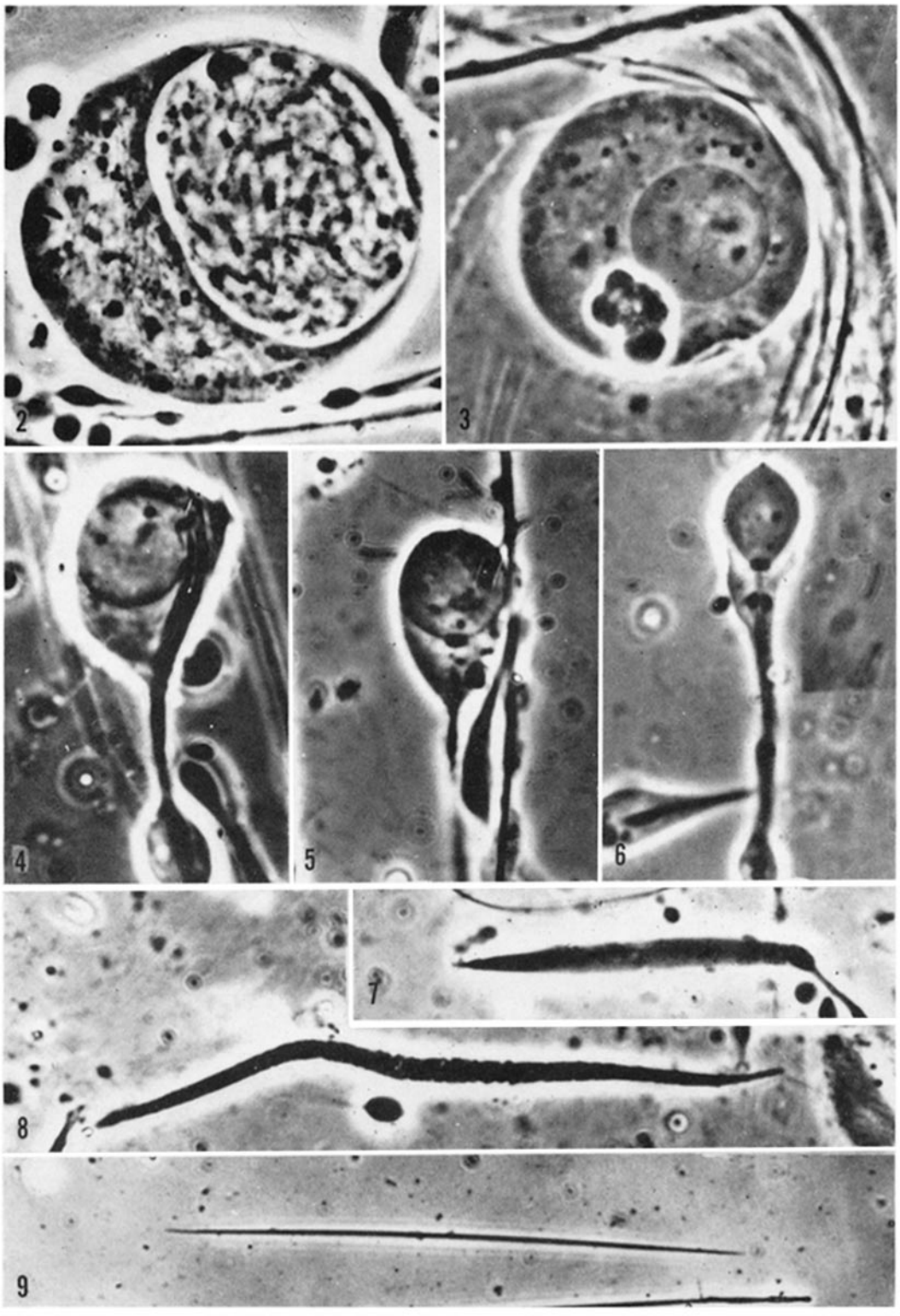
During nuclear elongation the stainability of the histone becomes increasingly resistant to the effect of deamination, indicating replacement of a typical histone by one with a higher arginine-to-lysine ratio (Figs. 10, 11). The presence of a stainable component after deamination is first noted in the early stages of nuclear elongation, when the nucleus has a length-to-width ratio of approximately 2. The initiation of the synthesis of the protein apparently begins while the nucleus is still spherical, however (Alfert, 3). By the time the length-to-width ratio reaches 10 to 20 (stage 5 spermatid), deamination appears to have little effect on staining, the transition being nearly complete. That the fast green staining change reflects synthesis of new protein is supported by the observations of increased ratios of protein arginine to DNA (Fig. 12), arginine-to-tyrosine (3), and a rapid incorporation of labeled arginine into the nucleus (Fig. 13) during these stages. Maximal incorporation occurs when the axial ratio of the nucleus is between 5 and 10.

Attempts to demonstrate RNA with azure B in these actively synthesizing cells gave a puzzling negative result. The nucleus appeared to be devoid of RNA, and only small amounts of basophilia could be detected, primarily along the tail. An attempt was made to test the validity of the following alternatives: nuclear RNA is responsible for protein synthesis but is unstainable, perhaps because of masking; the protein is synthesized in association with the cytoplasmic RNA, then migrates to the nucleus; the histone is synthesized in the nucleus, the process mediated by DNA rather than RNA, or perhaps catalyzed by some mechanism independent of nucleic acids.

Studies on the incorporation of cytidine into RNA showed high incorporation into the early postmeiotic spermatids, primarily into the nucleus. This incorporation is in accord with the high basophilia exhibited by both the nuclei and the cytoplasm of these cells. By the time the first phase of elongation is terminated however, the incorporation has decreased to a very low value. RNA synthesis has virtually stopped by the time histone synthesis begins (Fig. 13).

The time between the decline of RNA synthesis in stage 2 and maximum histone synthesis in stage 5 was estimated by following the course of labeled thymidine, incorporated during premeiotic DNA synthesis, through the spermatid stages. Eighteen days after administration of labeled thymidine, spermatocytes are labeled, but no labeled spermatids are seen. Twenty-five days after administration, label is seen in the early spermatids. The highest frequency of labeling occurs in the class 2 spermatids in which the first phase of elongation is well under way. No label is seen in cells which have begun nuclear elongation. Thirty-two days after administration, label

FIGURES 2 to 9 Fig. 2, a primary spermatocyte. Figs. 3, 4, and 5, successive stages (stages 1, 2, and 3, respectively) of spermatid development, prior to nuclear elongation. Figs. 6, 7, 8, and 9, stages 4, 5, 6, and 8 of spermatid development during nuclear elongation. Figs. 2 to 8, × 1400; Fig. 9, × 650.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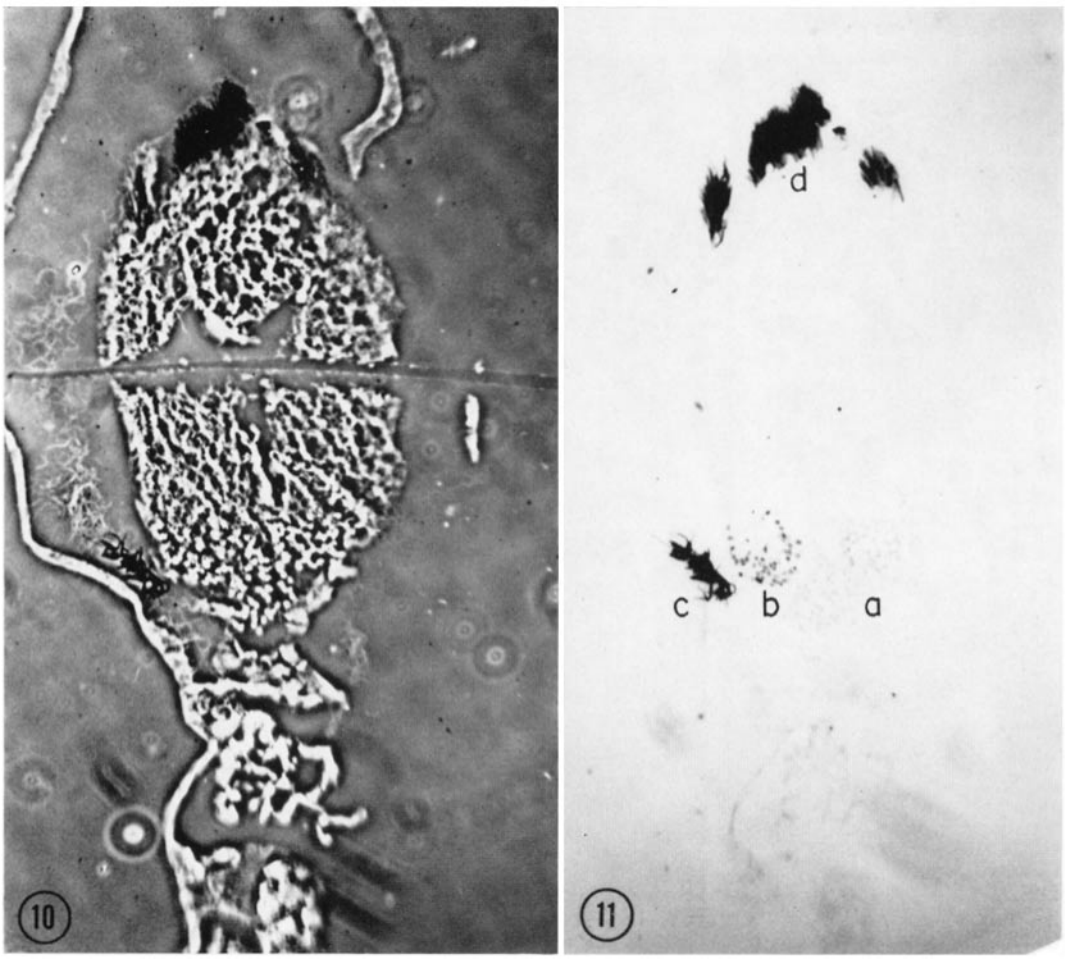


FIGURES 10 and 11 Phase and bright field photomicrographs of tubule of testis stained with fast green after deamination. The letters *a*, *b*, *c*, and *d* indicate changes in staining of the heads during nuclear elongation. The cells in the lower part of Fig. 10 did not exhibit any staining. × 170.

is seen in most of the spermatids, with a higher frequency in the phase which show maximum incorporation of arginine (stage 5). Little labeling is seen in subsequent stages.

Thus the periods of declining RNA synthesis (stages 1 and 2) and maximum histone synthesis (stage 5) are separated by approximately 1 week. This is a maximum figure, and is in accord with a minimum period of 4 days required for the appearance of tritium-labeled cytidine in the stage 5 spermatid (see below). Because of the inordinate exposures required for readable autoradiographs in the case of the thymidine experiments (exposures of 10 to 12 months were used), the data on the timing of the spermiogenic process are very limited.

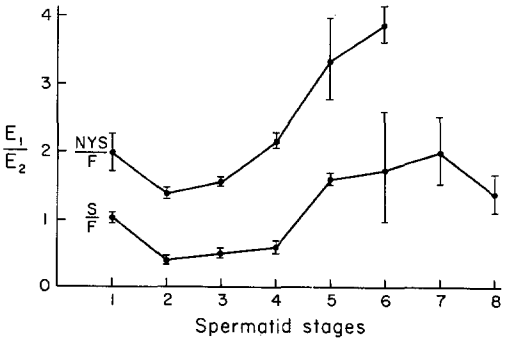


FIGURE 12 Relative ratios of protein arginine to DNA during spermiogenesis as determined from ratios of acetylated naphthol yellow S/Feulgen and Sakaguchi/Feulgen stains. E_1 is extinction of arginine stain, and E_2, of DNA stain.

Acridine orange provided the greatest measure of success in demonstrating basophilia in the stage 5 spermatids. The behavior of the stain was rather unpredictable, occasionally giving ambiguous results. For example, acridine orange concentrations of 1 part in 100,000 usually gave bright green nuclei with faint green cytoplasm. High concentrations of acridine orange yielded completely orange cells. This was attributed to the effects of toxicity (31), for disrupted cells in optimal concentrations often exhibited an over-all orange fluorescence, even the nuclei of the spermatids and sperm cells. Concentrations of 1 part in 10,000, applied to tissues which were carefully handled, gave the following picture which could be fairly faithfully reproduced (Figs. 14 to 17). The chromosomes of the pachytene spermatocyte fluoresce yellow, and can be seen against a background of nuclear material which fluoresces orange as does the cytoplasm. The cytoplasm of the earliest spermatids (stage 1) exhibits a bright orange fluorescence. The nucleus is yellow. A heteropycnotic lump of chromatin, presumably the X chromosome, is bright green, in accord with the view, supported by Hsu (24), that pycnotic chromosomes do not synthesize RNA. The stage 5 spermatids exhibit bright green nuclei, indicative of absence of RNA. The cytoplasm contains numerous scattered granules which fluoresce bright orange. These are most concentrated in the cytoplasmic layer surrounding the nucleus. Because of the erratic behavior of this stain under "unphysiological" conditions, no attempt was made to rely on specific extraction methods to determine whether or not the orange fluorescence in the stage 5 spermatids is indeed attributable to RNA. That RNA is contained in this region is attested to by the presence of ribosomes (see below), and also a very faint RNAse-sensitive azurophilia.

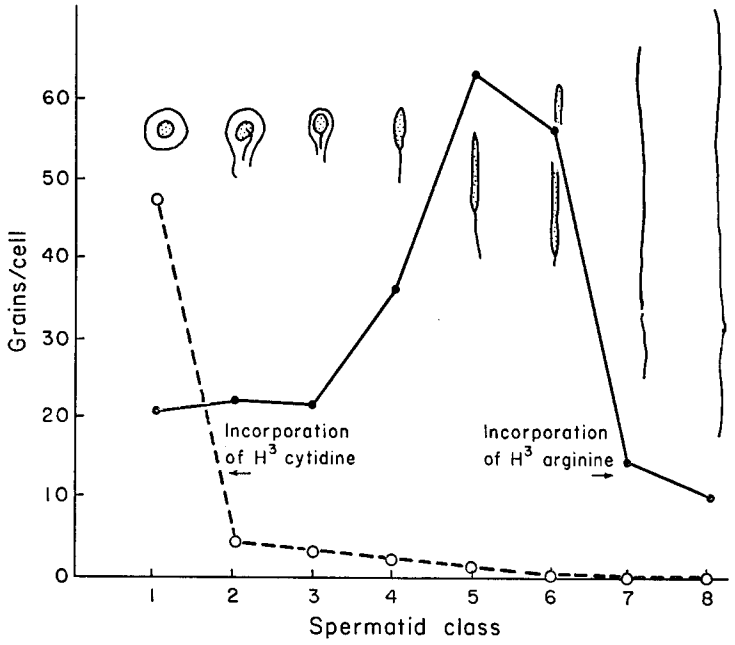
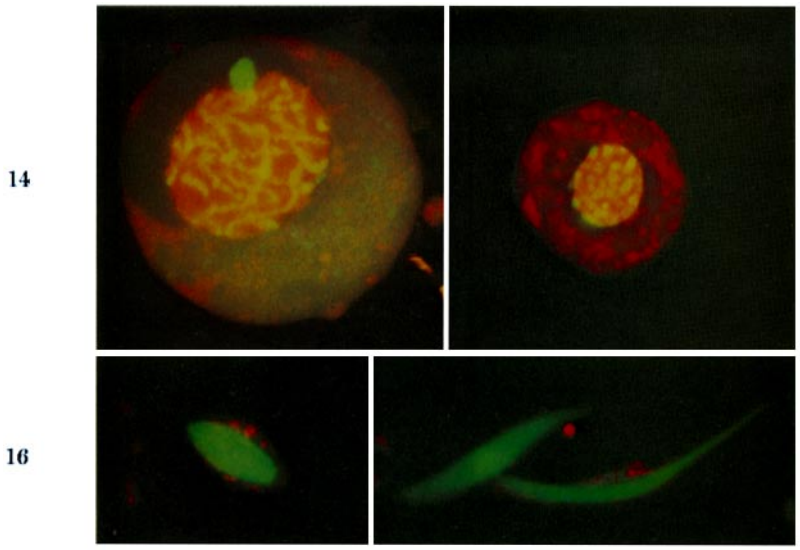


FIGURE 13 Incorporation of cytidine into RNA and of arginine into protein during spermiogenesis. The grains in each case are only those overlying the nucleus. The animals were sacrificed 5 hours after injection of cytidine, 1 day after injection of arginine.

An attempt was made to detect RNA in the stage 4 to 5 spermatids autoradiographically, to determine whether any RNA is to be found in the nucleus, and also to determine the time of synthesis of this cell's RNA. Animals injected with H^3-cytidine were sacrificed at 5 hours and at 1, 4, 7, 14, and 19 days after injection. The pattern of labeling of these spermatids was similar to that

seen in the short-term arginine experiments (see below), most grains overlying the tail and the peripheral rather than the central portion of the head. However, labeling of these cells was seen only at 4 days and thereafter, indicating that synthesis of their RNA occurs during the period of cytoplasmic elongation and perhaps earlier.

taken from animals sacrificed at various times after injection of 25 μc of labeled arginine. In sections of tissue from animals sacrificed up to 3 hours after injection, the grains appear primarily at the periphery of the spermatids (Figs. 18 to 21). When it is possible to discern the nuclear boundary in these preparations, the peripheral grains are seen to



FIGURES 14 to 17 Acridine orange fluorescence of primary spermatocyte, stages 1, 4, and 5 spermatids. × 800.

The presence of basophilia in the perinuclear cytoplasm of the spermatids undergoing the nuclear transformation suggests that the actual synthesis of histone associated with the transformation occurs in the cytoplasm, the newly synthesized histone then migrating into the nucleus where it combines with the DNA. That the *stainable* histone is in association with DNA is clearly indicated by the requirement for removal of DNA by hydrolysis before any histone staining can be effected.

A further autoradiographic study of arginine incorporation was made using 1 μ sections of tissues

overlie the cytoplasm rather than the nucleus. Since grains do not usually occur more than 0.5 μ from some recognizable potential site of incorporation, such as the border of the cell or tissue, (Figs. 18 to 21), the positioning of the grains within this limit reflects the site of the label, and the location of the majority of the grains over the cytoplasm at these short times may be taken as an indication of cytoplasmic synthesis. Some grains can also be seen which overlie the central regions of the head of the spermatids but these are outnumbered by the peripheral grains. In sections of tissue from

FIGURES 18 and 19 Incorporation of labeled arginine into stage 3 and 4 spermatids of grasshoppers sacrificed 1 hour (Fig. 18) and 1 day (Fig. 19) after injection. Note peripheral labeling (arrow). × 2000.

FIGURES 20 and 21 Incorporation of labeled arginine into stage 5 and 6 spermatids of grasshoppers sacrificed 1 hour (Fig. 20) and 1 day (Fig. 21) after injection. Figs. 18 through 21 are phase photomicrographs of 1 μ Araldite-Epon embedded sections. The arrow in Fig. 20 points out a typical example of peripheral labeling. × 2000.

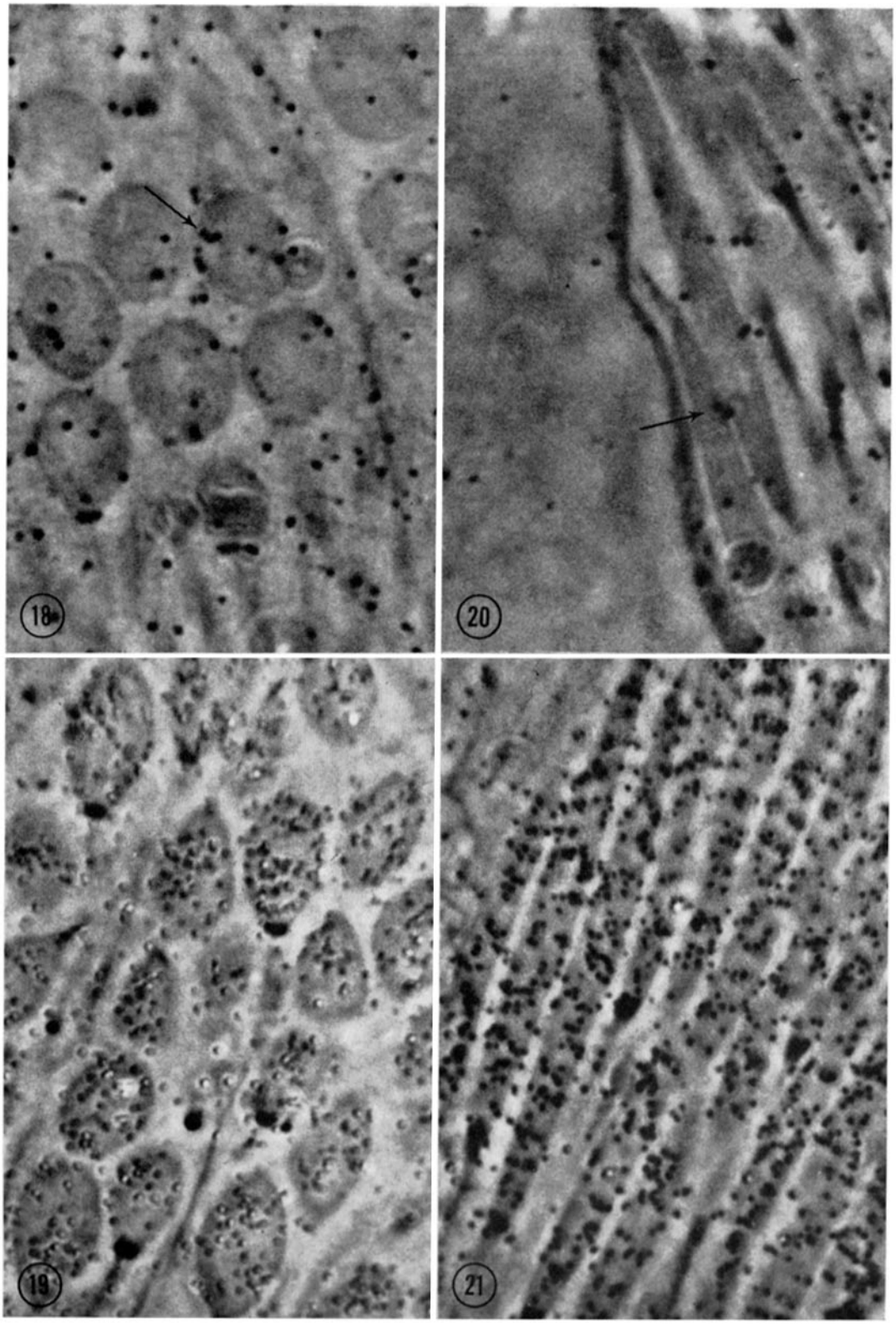

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TABLE I

Relative Amounts of Peripheral Labeling of Spermatid Heads after Injection of Labeled Arginine, and of Labeled Cytidine

Experiment	Peripheral grains
	per cent
H^3-arginine, sacrificed at 1, 2, 5, 15, 30, 45 minutes; 1 and 3 hours	56.2 ± 3.9
H^3-arginine, sacrificed after 1 day	43.2 ± 0.4
H^3-cytidine, sacrificed at 1 and 2 weeks	55.3 ± 5.7

animals sacrificed 1 day after injection, many grains overlie the central areas. These cells are much more highly labeled, most of the increase being attributable to label in the central area.

Since it was usually not possible to distinguish between nucleus and cytoplasm in these thin preparations, grain counts were made scoring grains as peripheral if they touched the cell boundary, and as central if they did not. The values (see Table I) given as per cent of grains which are peripheral, are not to be construed as an estimate of the proportion of the label which is cytoplasmic, for the radioactivity of inner cytoplasmic areas tends to give central grains, and any scattering of the β particles emanating from the thin cortical region will produce grains that overlie the nucleus and the extracellular areas. Nevertheless the values should vary in the same direction as the relative amount of cytoplasmic label. The results are given in Table I.

The close agreement between the values obtained for short term labeling with arginine and long term labeling with cytidine indicates that the arginine is incorporated into protein in the regions containing the RNA. It also is consistent with the view that this synthesis occurs in the cytoplasm; *i.e.*, that the 55 per cent figure reflects essentially cytoplasmic label. The relative decrease in peripheral grains at 1 day after injection of H^3-arginine is significant (2 per cent probability) and reflects an increased central (presumably nuclear) labeling at this time.

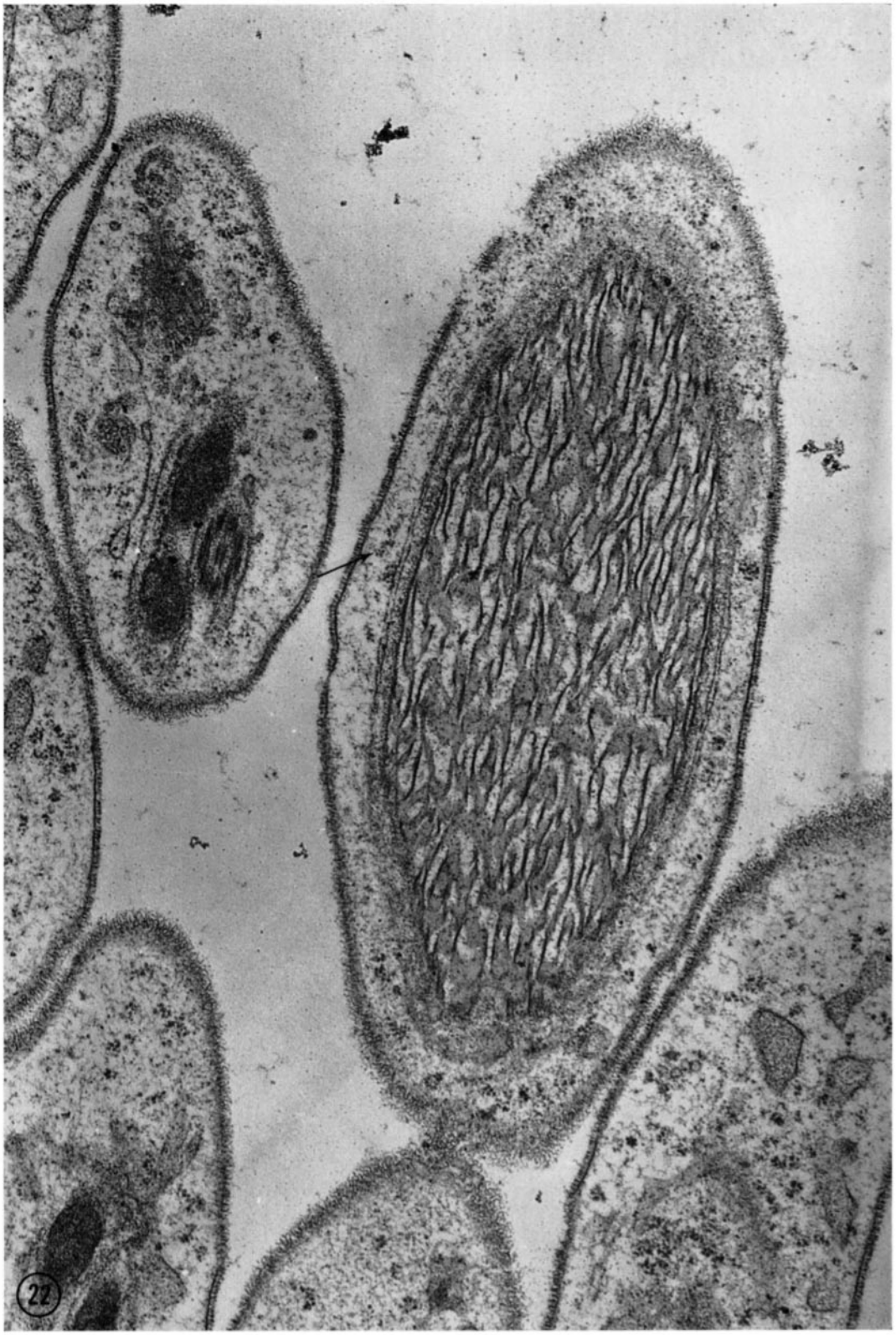
The electron micrographs (Figs. 22 and 23) show the structure of the spermatids undergoing the nuclear transformation. The changes in chromatin structure have been extensively described by others (12, 20, 36). Of particular interest is the thin cytoplasmic layer, within which can be seen elements of endoplasmic reticulum, and other regions which are rich in small granules. These granules, which occur in aggregates, are of a size similar to that of ribosomes, and stain quite strongly with barium permanganate. It is likely that some of these ribosomes are active in histone synthesis. Unfortunately, present preparations are insufficiently radioactive to permit radioautography at the level of the electron microscope.

DISCUSSION

The conclusion that the histone is synthesized in the cytoplasm rests primarily upon three observations, which individually are not compelling but when considered together offer persuasive evidence. These are: (*a*) a peak in arginine incorporation during a period characterized by an increase in protein-bound arginine, indicates synthesis of a new protein; (*b*) the presence of RNA in the cytoplasm and the apparent absence of RNA in the nucleus mark the cytoplasm as the sole site of protein synthesis; and (*c*) the sequence of arginine incorporation, first cytoplasmic then nuclear, suggests that the material synthesized in the cytoplasm serves as a precursor for the nuclear material. An alternative interpretation, that the synthesis of nuclear proteins is independent of the synthesis of cytoplasmic proteins, the latter process utilizing exogenous precursor more rapidly and the former process either being independent of RNA or mediated by a "hidden" RNA, is tenable but seems contrived.

Although histones are commonly considered nuclear proteins (the only unequivocal evidence that a basic protein is indeed a histone is its association with DNA) there are no *a priori* reasons for supposing that they must be synthesized in the nucleus. The abilities of both the nucleus and cyto-

FIGURE 22 Electron micrograph of several spermatids in oblique section. Note polysomes in cytoplasm (arrow), and characteristic disposition of chromatin in plates. × 21,000.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plasm to carry on protein synthesis independently have been amply demonstrated (5, 23). Whether other histone syntheses, such as, for example, those requisite to chromosome replication, are also carried out in the cytoplasm remains to be determined. However, whether in nucleus or cytoplasm, it is likely that the synthesis of histones and their coupling with DNA are distinct and separate events, during chromosome replication as during spermiogenesis.

Independent syntheses of histone and of DNA during the intermitotic period are indicated in a number of recent reports (16, 17, 22, 27, 35, 37). The parallel increases in the staining of histone and DNA during interphase observed in 1955 led, at that time, to the supposition that the two syntheses may be coupled (9). Although this view seemed reasonable then, it does not seem so now, and the old observation is reinterpreted as indicating a complexing of previously synthesized histone with DNA following closely on the heels of DNA replication, the complexing rendering the histone stainable. This idea has recently been advanced by Umaña *et al.* (35). The assumption that the uncombined histone is not rendered visible by the fast green stain, perhaps because of lability or dilution, is supported by the inability of the fast green and related methods to permit detection of the spermatid histones which have been synthesized but have not yet entered the nucleus.

An important idea implicit in the old view of parallel syntheses is that the complex, rather than the DNA alone, is the replicating unit of the chromosome. This view, born of the desirability of invoking hereditary variations in gene expression which are chromosome based, need not be discarded. That histone synthesis may occur independently of DNA synthesis need no more invali-

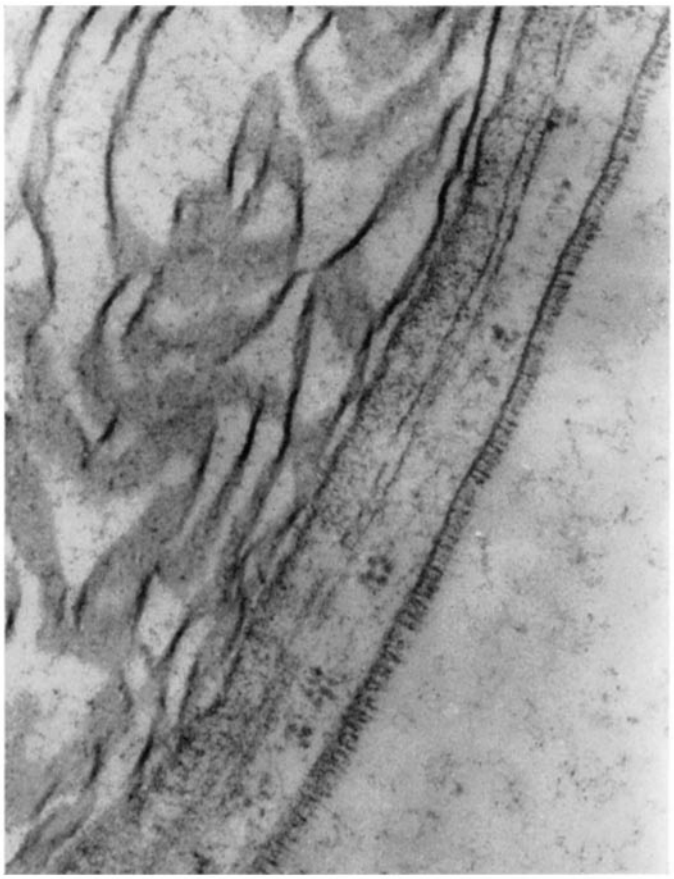

FIGURE 23 Electron micrograph of a portion of the head of a stage 4 to 5 spermatid. × 46,000.

date the concept of the complex as the replicating unit than does the prefabrication of nucleotides in the cytoplasm invalidate the concept of DNA as a self-replicating structure. The important consideration is whether the assembled complex after replication is identical with the original. If it is, the complex may be considered the replicating unit. Whether the complex does in fact replicate faithfully remains a crucial question. Determinacy cannot be the result of specific affinities of DNA's for "their own" histones, for, in view of the ambivalence of DNA's and histones, such affinities could not be very exacting (8).

The conventional synthesis of histones by RNA templates which may be presumed to have their origin in specific genes suggests the existence of a hierarchy of genes, histone-producing and histone-associating, the latter comprising all if not most of the genome. Variation in the association of DNA with histones, such as occurs during spermiogenesis and also during subsequent stages of the life cycle of many organisms (7, 11), reflects a variable influence played by the histone-producing genes on the rest of the genome (8).

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