Schedule of Spermatogenesis in the Pulmonate Snail *Helix aspersa,* with Special Reference to Histone Transition*

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ABSTRACT

The schedule of spermatogenesis is determined from the times necessary for cells labeled with tritium thymidine during premeiotic DNA synthesis to pass through the successive spermatogenic stages. A transition from a typically somatic histone rich in lysine, to a histone rich in arginine is shown to occur during spermatid stages. A later shift to a protamine is observed in the maturing sperm. These changes are characterized by the use of *in situ* staining methods. The transition to an arginine-rich histone is accompanied by incorporation of tritium-labeled arginine, hence reflects synthesis of new protein. Comparison of the timing of arginine and thymidine incorporation, and independent measurements of DNA, show that in contrast to the case of premitotic chromosome duplication, the histone synthesis in the spermatid is unaccompanied by DNA synthesis. During the initial histone change, fine filaments are formed within the nucleus, which aggregate to form lamellae. This fine structure is lost during maturation of the sperm.

INTRODUCTION

Reconciliation of the observation of DNA constancy with the hypothesis of variable gene expression during differentiation requires the operation of factors controlling DNA activity. Whether such control is mediated by other chromosomal components or is the result of direct action of non-chromosomal factors upon DNA is unknown. The works of Briggs and King (7, 21) and of Nanney (27) imply that controlling elements exist within the nucleus. That they may be chromosomal is suggested by the fact that they are hereditary and persist through chromosome reproduction in changing cytoplasmic environments.

That histones or protamines might play a role in regulating genetic expression had been proposed by the Stedmans in 1950 (32) and by Danielli in 1953 (15). However, support for this hypothesis has been slow in coming and, at best, is equivocal.

Its validity would depend, at least, upon the existence of differences in the histones found in different cell types. Of the relatively few studies which have sought to compare histones from various somatic tissues, those of Cruft, Mauritzen, and Stedman (10), demonstrating differences in the occurrence of fractions separable by their electrophoretic mobilities, are in accord with the hypothesis. Conversely, the works of Crampton *et al.* (9) and Vendrely *et al.* (36) stress similarities rather than differences in histones from different tissues. These latter experiments utilize analyses of amino acid composition. Whether these analyses overlook subtle differences remains an important question.

Obvious differences have been found fairly consistently, however, in comparisons of the amino acid content of basic protein derived from sperm and somatic tissues. The classic example is had in the protamines of salmon sperm (25, 22). Protamines, or histones approaching protamine in basicity and amino acid composition, have been obtained from other sources, including the sperms of invertebrates (17, 18), of mammals (12, 37),

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birds (14), and from *Lycopodium* spores (11). (A somatic source of a protamine-like substance, from pupating insects, has also been described (30).)

The question arises as to whether the transition from histone to protamine occurring during sperm development is unique, or whether it represents an extreme change which occurs to a lesser extent in all differentiating cells. Whether or not studies on sperm will have a bearing on the "generalized cell" cannot be predicted at present. Nevertheless, this process in sperm has some interesting features of its own. The histone change, involving synthesis (see below) is unaccompanied by any detectable DNA synthesis. Since the process is not part of chromosome duplication it is reasonable to suppose that it may involve a change in chromosome function. Furthermore, consideration of the fate of the sperm nucleus leads to some interesting suppositions. Assuming a similarity in the histones of the maternal and paternal chromosome complements in the early cleaving egg, one would expect to find either a protamine in the egg chromosomes, to match that of the entering sperm, or alternatively, to find upon fertilization a further transition in the sperm head leading to the formation of a somatic histone. Recent experiments on *Helix* indicate a transition in the male pronucleus to a histone type which resembles that of the egg chromsomes and which persists for a number of cell divisions (6). This histone exhibits properties which distinguish it from both sperm histone and typical adult somatic histones.

Thus the developing sperm cell and its products provide examples of histone differentiation which have important biological implications. Equally important, the sperm affords a system which lends itself readily to analysis. The sperm protamines and the somatic histones are sufficiently different that the two can be distinguished using cytochemical methods (1, 2). Thus Alfert has been able to conclude that in salmon the histone-to-protamine transition occurs during spermatid differentiation (1). It will be shown that in *Helix* a similar transition also occurs and is accompanied by gross changes in nuclear morphology, which can serve as parameters permitting correlation of the transition with other metabolic events in the cell. The spermatid nuclei retain their characteristic form after isolation from the cell. This, and the fact that the sperm head is predominantly nucleus, permits an autoradiographic approach to be used with

confidence that nuclear incorporation can be distinguished from cytoplasmic incorporation.

The purposes of the present experiments are the following: (*a*) to establish the timing of the spermatogenic stages in order to facilitate control in later labeling experiments, (*b*) to describe the chemical nature of the change from histone to protamine, and (*c*) to establish whether or not this transition is effected by synthesis of new basic protein. A correlation is also made between the changes in the basic protein and changes in nuclear fine structure such as have been described by others (19, 20, 29, 39).

Materials and Methods

Helix aspersa obtained from the local environs were cultured in laboratory terraria. The animals were fed lettuce sprinkled with chick feed, were given soil in which to lay eggs, and periodic watering. Animals taken during the winter and spring months mated, laid eggs, and otherwise behaved in an apparently normal manner under cultivation.

The animals used for the scheduling of spermatogenesis were injected with 25 μc. of H^3-labeled thymidine, specific activity of 1.9 c. per m$_M$ (Schwartz Biochemicals, Mount Vernon, New York). 22 snails were used (see Table II). The radioactive animals were sacrificed at intervals and samples of ovotestis were fixed in 10 per cent neutral-buffered formalin for paraffin embedding and sectioning, or ovotestes were teased apart, smeared on slides, fixed in osmium tetroxide vapor while still wet, and allowed to dry rapidly. Sections and smears were stained using a modified Feulgen technique (5) and autoradiographed with AR-10 stripping film (Kodak Ltd. of England) following the procedure of Taylor and McMaster (34). The autoradiographs were exposed for 11 to 15 days. In making counts all nuclei whose centers fell within a transect of the slide bounded by lines on an ocular micrometer were considered. In the smears spermatid and sperm nuclei overlaid by 3 or more grains were considered to be labeled. Other nuclei containing 12 or more grains were considered to be labeled, since the premeiotic nuclei have four times the amount of DNA of the postmeiotic stages. The background was negligible in the smears. In the sections containing no autoradiographed spermatids those spermatocyte nuclei overlaid by 5 or more grains were considered to be labeled. These figures were dictated by background and by the decreased sensitivity of the autoradiographic technique when used with the sections as compared with smears. Although smears are generally more reliable for autoradiography, sections were needed for the early samples because of the difficulty in distinguishing early spermatocyte stages in the smears. In the sections which contained

both labeled spermatocytes and spermatids, cells which exhibited 12 and 3 grains, respectively, were considered labeled. The distribution of labeled cells among the various gonial, spermatogenic, and spermiogenic stages was determined at different times after administration of isotope. When labeled postmeiotic stages were present the frequencies of labeled spermatocyte cells were weighed by a factor of four. The times spent in the various spermatogenic stages were found from the times of entry into and exit from the stages. (See below for further details.)

Incorporation of H³-labeled arginine[1] was determined as follows: 10 μc. (specific activity, 100 c./mol) were injected into each of 3 snails and the snails sacrificed at 18 hours, 3 days, and 12 days after injection. Samples of ovotestis were smeared, fixed in osmium tetroxide, stained with the trichloroacetic acid-Feulgen procedure (5), and autoradiographed as described above. Before smearing, however, the tissue was forced through a small pipette several times with mantle fluid obtained from an unlabeled snail. This procedure served to remove many of the spermatid nuclei from the cells and to suspend them in a medium in which the cytoplasmic debris and extracellular fluid were highly diluted. Only free nuclei were considered in these counts, with the exception of mature sperm and very late spermatid stages in which the heads were essentially free of cytoplasm. The various spermatid and sperm stages were scored for the number of grains overlying the nucleus, and the distribution of grains for each stage was determined.

Microphotometric measurements were made using the method of Pollister (see Swift, 33). Smears of ovotestis were dried, fixed in 10 per cent neutral-buffered formalin, rinsed in running tap water for several hours, and stained with the Feulgen reaction. Hydrolysis was carried out for 12 minutes in 1.0 N HCl at 60°C. Drying and formalin fixation resulted in fairly homogeneous distribution of stain. The nuclei were treated as flat ellipses, and the following formula was used:

$$\text{Relative amount of DNA} = d_1 d_2 \log \frac{I_0}{I}$$

in which d_1 and d_2 are the long and short diameters of the nuclei, and I_0 and I, the incident and transmitted light. A wave length of 570 mμ was used. The spade-shaped nuclei presented a semicircular aspect in profile, and the radius of the circle was substituted for one of the diameters in making the calculations. Ten nuclei were measured from each of the stages represented.

MacLeish, Bell, LaCour, and Chayen's modification of the Sakaguchi test was used for detection of protein-bound arginine (23). Histone staining was accomplished in the manner prescribed by Alfert and Geschwind

(4). Two alternative modifications of this procedure were used which stain protamines and presumably labile histones as well as the histones stained by Alfert's method. These were carried out as follows: Materials mounted on slides were hydrolyzed for 6 hours, or overnight, in a saturated aqueous solution of picric acid at 60°C. They were rinsed in water, and then immersed for 1 hour in a 0.1 per cent solution of bromphenol blue at a pH of 2.3. The slides were then rinsed in 95 per cent ethanol made slightly alkaline by stirring 50 ml. with a glass rod which had first been wet with alcohol and then held over a bottle of concentrated aqueous ammonia. Differentiation was effected by storing the slide for 10 to 12 hours in a single change of 95 per cent ethanol. The slides were then dehydrated and mounted. Alternatively, the slides, after the rinsing following picric acid hydrolysis, were stained in a 0.1 per cent solution of eosin Y buffered at pH 8.3 for 3 to 6 hours. Differentiation in distilled water for 5 minutes followed. Occasionally, differentiation in water brought to a pH of 8.3 proved necessary to improve specificity of staining. Bromphenol blue had been used under different conditions by Mazia et al. for cytochemical staining of proteins (24). Under the conditions used here, however, both the picric acid bromphenol blue and the picric acid eosin procedures resulted in the specific staining of nuclear basic proteins, including protamines. Picric acid, as any other acid, hydrolyzes DNA. Short time hydrolysis (½ hour to 1 hour) removes the purines, permitting staining of the apurinic acid with Schiff's reagent (Feulgen reaction). After longer times of hydrolysis (6 to 12 hours) the DNA is completely removed. Staining with Schiff's reagent no longer gives a positive result. The basic groups of the proteins, thus unmasked, are available for binding acid dyes. The essential innovations of these modifications consist of the use of an acid and of dyes which successively form precipitates with protamines as well as histones, and thereby prevent leaching of protamines from the cell during the procedure.

Deamination by the Van Slyke reaction (35) was carried out by immersing slides for 15 minutes each in two freshly prepared solutions containing 5 per cent sodium nitrite and 5 per cent trichloroacetic acid. (This step was performed after removal of DNA by acid hydrolysis.) Acetylation was effected by 1 hour treatment with acetic anhydride at 60°C. (26).

Material used for electron microscopy was fixed in 2 per cent osmium tetroxide buffered at pH 7.4 with phosphate buffer. This was followed by postfixation overnight in 10 per cent neutral-buffered formalin. The material was embedded in methacrylate and sections cut at $\frac{1}{40}$ of a micron.

RESULTS

Schedule of Spermatogenic Changes.—The spermatogenic cells of the ovotestis of *Helix aspersa*

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[1] We wish to thank Dr. J. H. Taylor for providing the H³-arginine.

TABLE I

Stage	Distinguishing features
Gonial 8-cell stage	Cyst contains 8 cells. Small cells, high nucleocytoplasmic ratio.
Gonial 16-cell stage	Cyst contains 16 cells. Morphology similar to above. (Text-fig. 1)
Early primary spermatocyte	32 cells in cyst. Morphology similar to above.
Bouquet stage	Chromosomes organized into a bouquet-like structure. Cell enlarges. Increase in number of mitochondria. Formation of ring-like acroblast. (Text-figs. 2, 3)
Diffuse stage	Dissolution of "bouquet." Further increase in cell size. (Text-figs. 4, 5)
Late primary spermatocyte (diakinesis)	Large size. Approximately 20 μ in diameter. (Text-fig. 6)
Meiosis	
Early spermatid	Similar to late primary spermatocyte, except for size (one-fourth the volume of the latter). Nucleus migrates toward the periphery of the cell and becomes homogeneous. Formation of flagellum. (Text-figs. 7, 8)
Spade spermatid	Depression of proximal side of nucleus at position of centriole. Nucleus assumes shape of an arrow, then of a spade. The nucleus becomes "phase dense." The flagellum is now a prominent part of the cell. (Text-fig. 9)
Round spermatid	Nucleus condenses slightly, is rounded at tip, with a hollowing out "to accommodate" the blepharoblast. (Text-figs. 10, 11)
Long spermatid	Elongation of the head. The nucleus may be rounded at the tip and confined within the cell membrane (long 1), or may be pointed and emerging with the cytoplasm attached at various points along the length of the tail (long 2). (Text-fig. 12)
Ovotestis sperm	Cytoplasm completely sloughed off.
Hermaphroditic duct sperm	Similar to ovotestis sperm.
Spermatophore sperm	Similar to ovotestis sperm.

are organized into cysts. A cyst contains 1 large granular polyploid or polytene nurse cell, and a number of spermatogenic cells. The latter are at a similar stage of development, and presumably arise from a single cell which divides mitotically five times, to yield 32 primary spermatocytes. Subsequently the spermatocytes undergo meiosis to produce 128 spermatids, which develop into mature sperm without further division. An early description of this process was given by Gatenby (16).

The early spermatocytes and late spermatogonial cells are similar morphologically. However, they may be distinguished by taking into account the number of cells within the cysts. Delimitation of later stages of spermatogenesis is simplified by the occurrence of rather dramatic changes in morphology. Table I and Text-figs. 1 to 12 describe a number of stages which were selected because they offered convenient morphological criteria by which the course of spermatogenesis could be described and with which other events might be correlated.

The maturing sperm leave the ovotestis and

enter the hermaphroditic duct where they are stored. Later the sperm are enclosed within a long transparent structure forming the spermatophore, which is transferred to the partner during copulation. The snails are hermaphroditic and mating is reciprocal.

The duration of each stage was estimated by the times necessary for cells, labeled during the periods of DNA synthesis, to pass through the stage. This is shown in Table II and in Text-fig. 13. Each curve in Text-fig. 13 represents the cumulative percentage of the labeled cells which have entered a given stage at different times after administration of the labeled thymidine. The curve on the immediate right represents the cumulative percentage of labeled cells which have entered the subsequent stage, and hence have passed through the given stage. Therefore the area bounded by the "entrance" curve on the left and the "exit" curve on the right provides a measure of the time spent by the cells within the given stage.

Although the set of points at each time is provided by only 1 snail (with the exception of 30 days), each cell stage is represented from 3 to 6
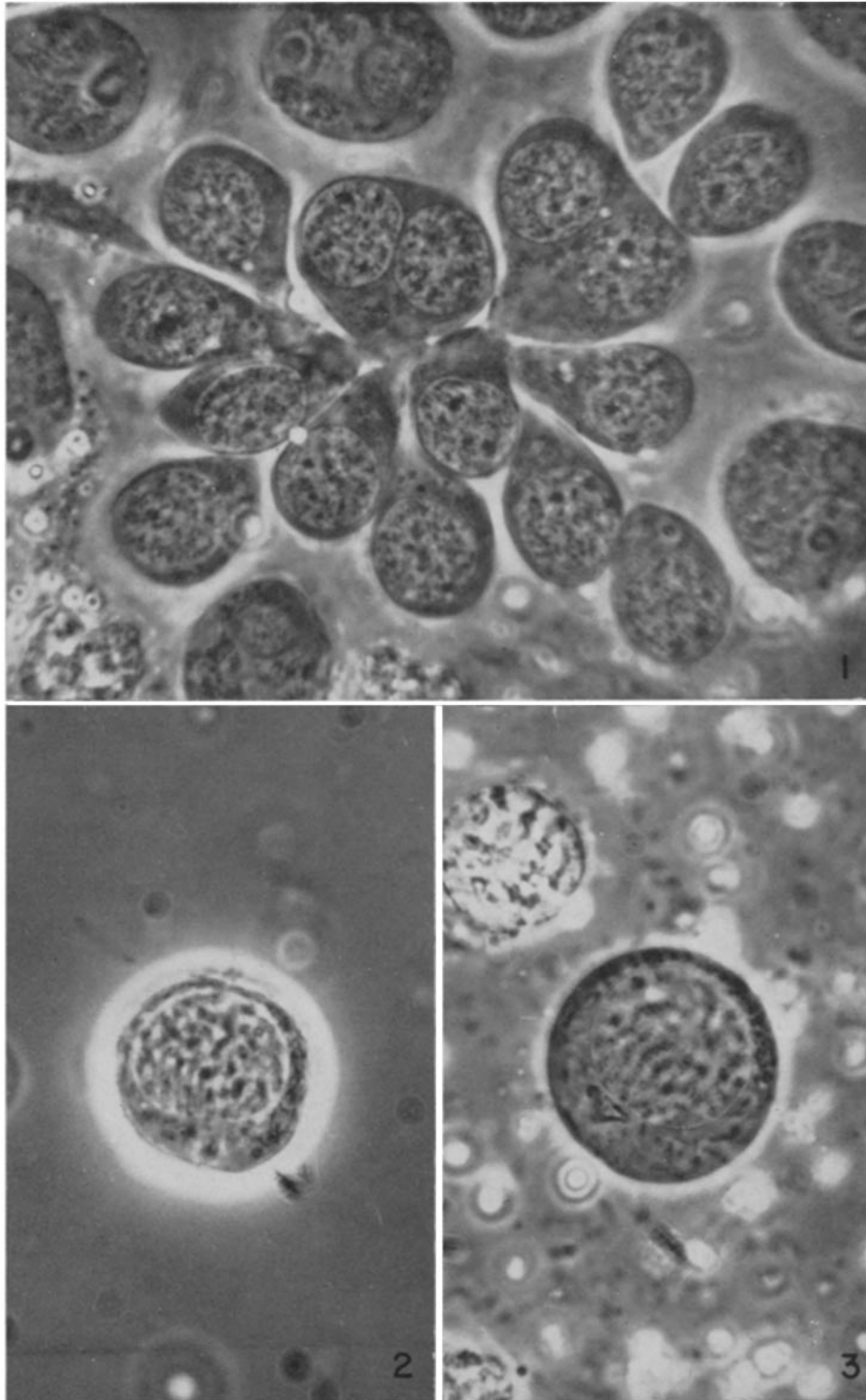
TEXT-FIG. 1. Cyst containing 16 spermatogonia. The large granular nurse cell has been lost during the preparation. Multinucleate cells are frequently encountered in all stages of spermatogenesis. Dark medium phase optics were used. Magnification in Text-figs. 1 to 12: × 1700.
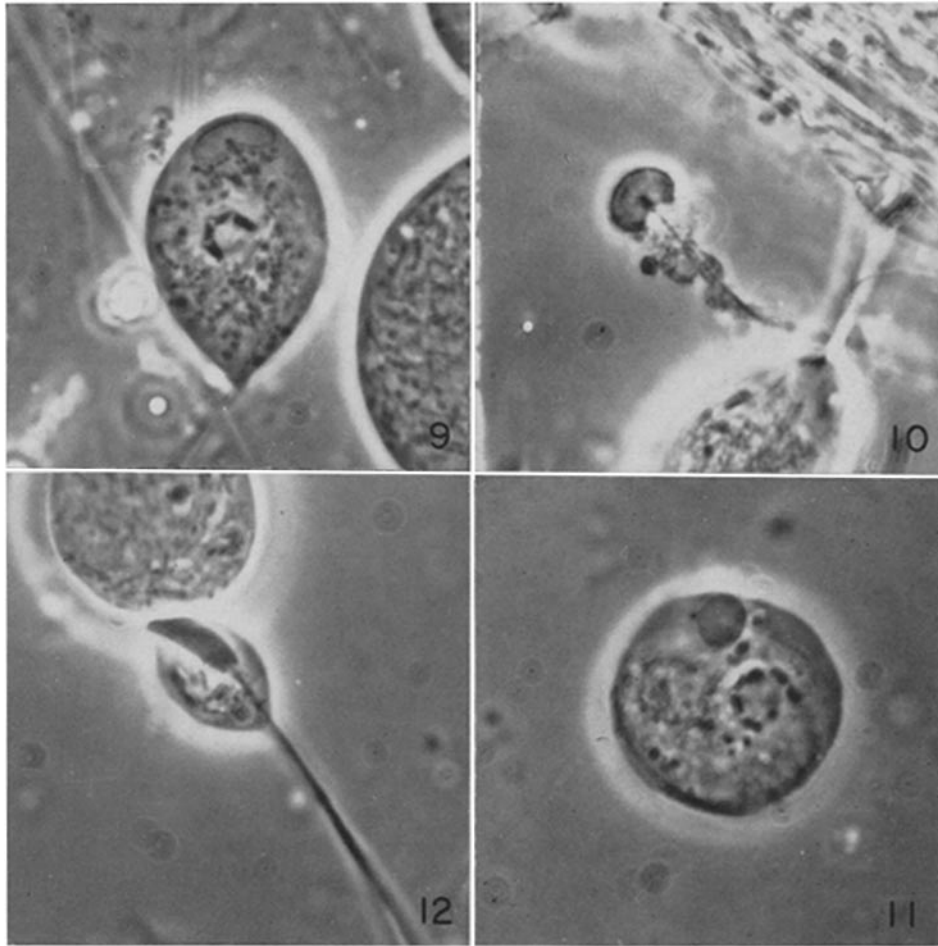
TEXT-FIGS. 2 and 3. Early and later bouquet stages.
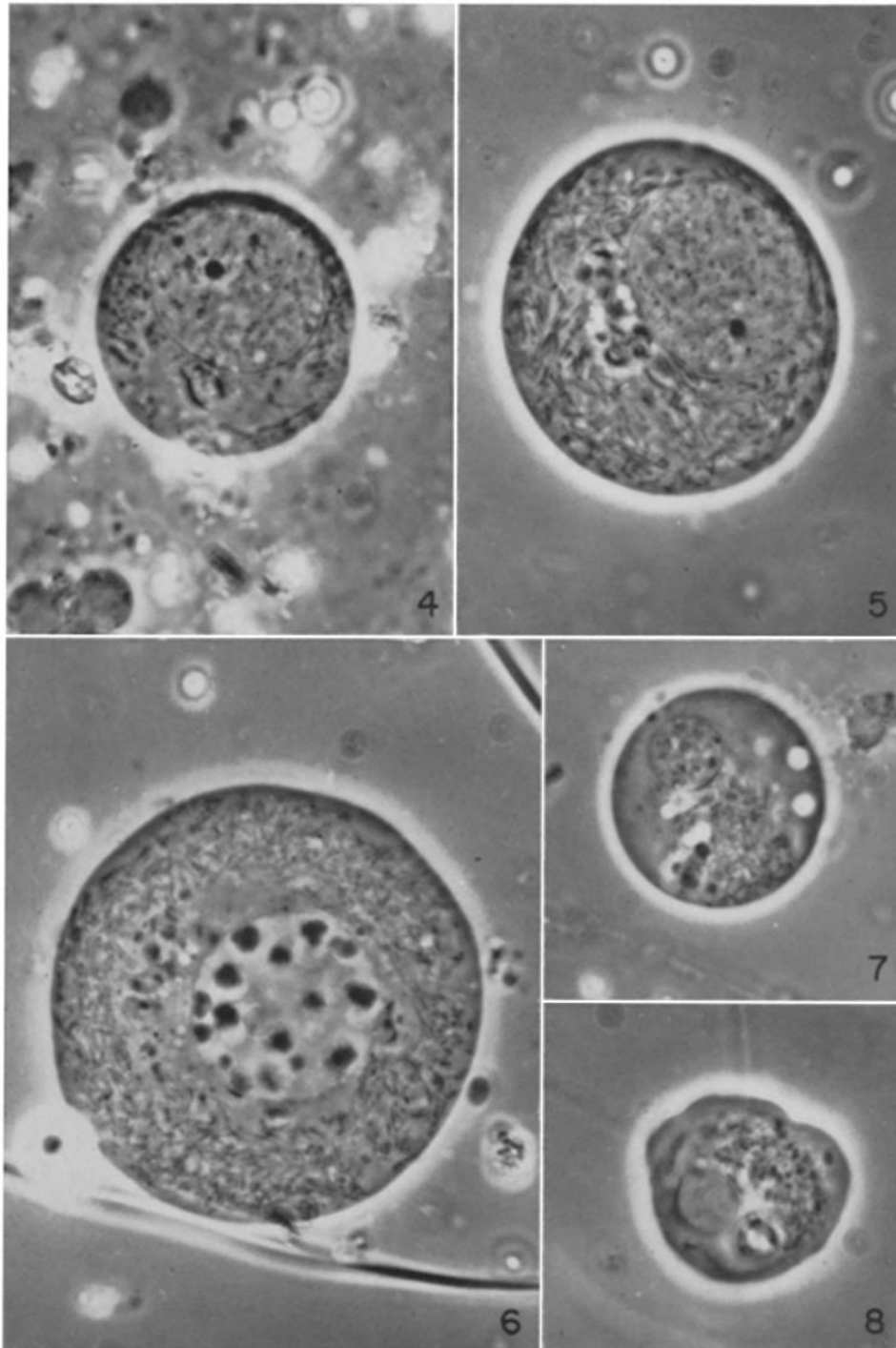
Text-Figs. 4 and 5. Early and later diffuse stages.

Text-Fig. 6. Diakinesis.

Text-Figs. 7 and 8. Two early spermatid stages. Text-fig. 8 is slightly later, as indicated by the nuclear homogeneity.

TEXT-FIG. 9. Early spade stage.

TEXT-FIG. 10. Spermatid nucleus with adhering nebenkern and axial filament, removed from a spermatid in the round stage.

TEXT-FIG. 11. Spermatid, round stage.

TEXT-FIG. 12. Elongated spermatid.

times among the snails used; hence the times spent by the cells within these stages is in effect an average of times obtained using from 3 to 6 snails.

Under conditions in which label is detected with a high efficiency gonial cells which are many generations removed from the primary spermatocyte stage will eventually yield "labeled" primary spermatocytes and spermatids. However, the isotope originally incorporated will be diluted with each intervening cell division. Under the conditions of these experiments the cells which show label as spermatocytes are mainly derived from cells which had taken up label either as early spermatocytes or as gonial cells synthesizing DNA in preparation for the final gonial division. No appreciable labeling was detected in bouquet or in subsequent stages in animals sacrificed 1 day after administration of thymidine. Grain counts made on primary spermatocytes in sections of the 1-day sample gave an average of 11.1 ± 3.9 grains for 57 cells which exhibited an autoradiograph (more than 4 silver grains). The grains were distributed among cells in such a manner that a twofold dilution of label resulting from a single division would have eliminated, at most, 30 per cent of the progeny from the "labeled population," by bringing their grain counts to less than 5. A fourfold

TABLE II

Cumulative Percentages of Cells Incorporating H^3-Labeled Thymidine during Late Gonial and Early Spermatocyte Stages, which have Already Entered into the Various Stages of Spermatogenesis at Different Times after Administration of Isotope

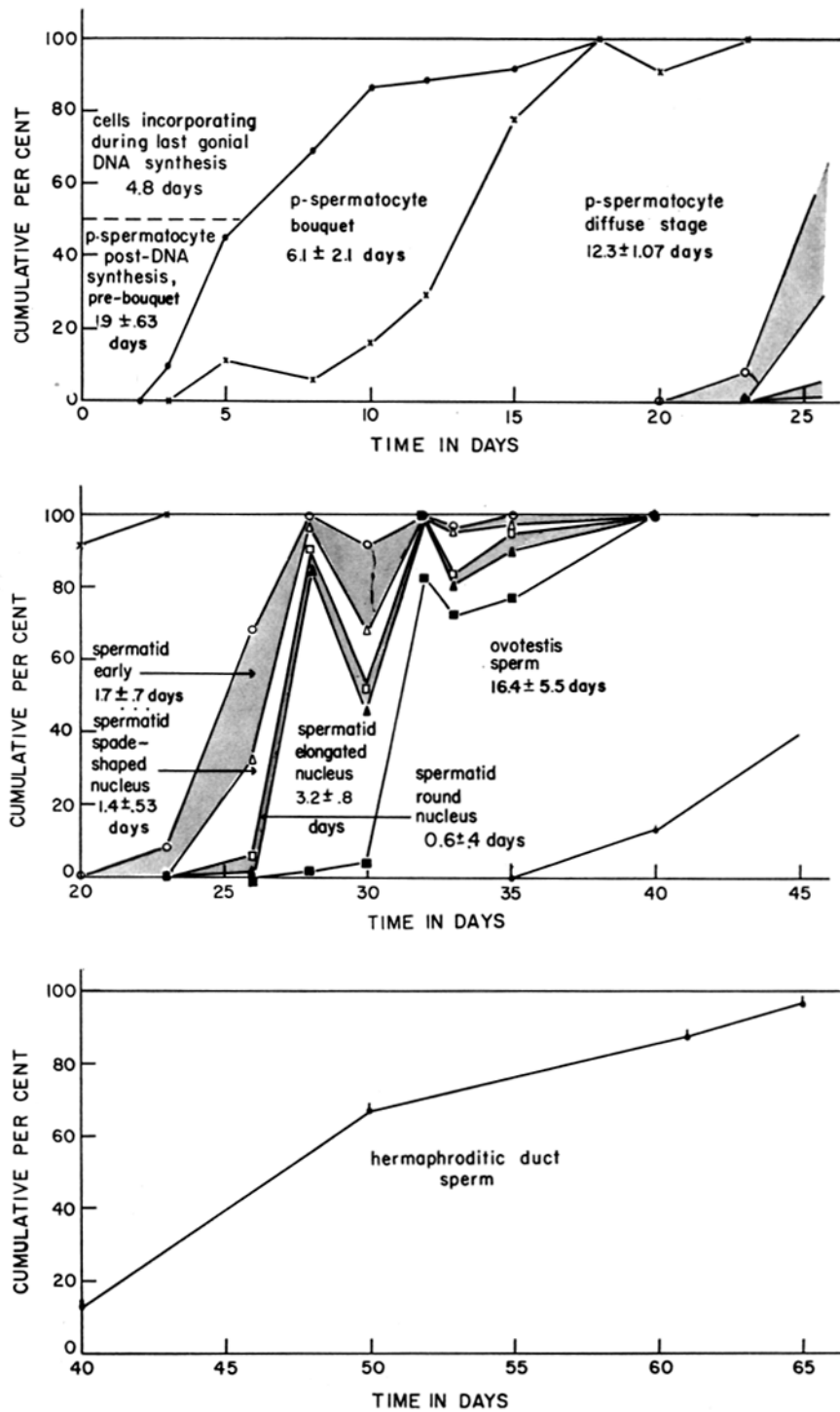|	Time			Stage					
	Pre-bouquet	Bouquet	Diffuse	Early spermatid	Spade-shaped spermatid	Round spermatid	Long spermatid	Ovotestis sperm	Hermaphroditic duct sperm‡
1	100	0							
2	100	0							
3	100	9.8	0						
5	100	45.3	11.3	0					
8	100	68.7	6.2	0					
10	100	86.3	16.4	0					
12	100	88.7	29.9	0.9	0				
15	100	92.0	78.0	0					
18			100	0					
20		100	91	0					
23			100	9.7	0.3	0.3	0.3	0	
23*			100	7.6	0				
23 (average)			100	8.6	0.1	0.1	0.1	0	
26			100	56.4	36.7	8.8	2.0	0	
26*			100	80.0	24.0	5.0	0		
26 (average)			100	68.2	30.3	6.9	1.0	0	
28*				100	97.6	90.2	84.4	2.2	
30*			100	88.2	37.7	5.7	1.9	1.9	
30*				100	98.8	98.8	90.3	6.8	
30* (average)			100	94.1	68.2	52.2	46.1	4.4	
32*				100	100	99.0	98.5	83.5	
33*			100	96.9	95.4	84.0	80.2	72.6	
35*				100	98	95	90	77	
40*								100	13
50*								100	67
61*								100	87
65*								100	97

* Smeared preparations.

‡ Exit of sperm from the ovotestis into the hermaphroditic duct was estimated by the loss of labeled sperm from the ovotestes. At 35 days after administration approximately 15 per cent of the mature sperm and long spermatids were labeled; at 40 days, 13 per cent; at 50 days, 5 per cent; at 61 days, 2 per cent; at 65 days, 0.5 per cent.

dilution resulting from 2 divisions would have eliminated, at most, 96 per cent of the progeny from the labeled population. (The smears yielded grain counts of 2 to 3 times those of the sections. The minimum number of grains over a cell necessary for its consideration as an "incorporator" was raised accordingly.) In effect, most of the cells derived from gonial cells incorporating thymidine prior to the next to the last gonial division are excluded from the counts. Variation in the amounts of detectable labeling among the progeny of gonial incorporators in different snails

may be considerable, and will contribute to the error.

Sources of Error.—The main sources of error in these determinations arise through variations among the snails in (a) the rate of the spermatogenic process, (b) the amounts of label incorporated, and (c) the amounts of detectable labeling.

The 28-day snail illustrates the nature of the error arising through variation in the spermatogenic rate. The process appears to have been accelerated in this snail. More of the later stages are labeled than would be expected. (See Table II, Text-fig. 13.) The results are (a) an apparent

TEXT-FIG. 13. Time course of spermatogenesis. Ordinate: Cumulative percentages of labeled cells which have entered into the various stages of spermatogenesis. Entry into bouquet, ●; diffuse stage, ✕; early spermatid, ○; spade-shaped spermatid, △; round spermatid, □; elongated spermatid, ▲; ovotestis sperm, ■; and hermaphroditic duct sperm, ♦. Abscissa: Time since administration of label to the organism. The time spent in each stage is represented by the area bounded on the left by the "entrance curve," and on the right by the "exit curve." The values given were determined by measurement of these areas. The time spent between spermatocyte DNA synthesis and bouquet was estimated by assuming that half the labeled spermatocyte cells were spermatocytes when synthesizing DNA and the other half were in the final gonial stage.

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underestimate of the duration of the early spermatid stage, exit from which had virtually gone to completion, (b) an overestimate of the duration of the elongated spermatid stage, entry into which had begun prematurely, and (c) relatively unaffected durations of the spade and round stages. In the spade and round stages, the error due to early entry and early exit compensate, and the duration as determined by measurement of area between the curves is unaffected. A "late" snail would result, conversely, in an overestimate of the duration of early stages and an underestimate of later stages.

Variations in incorporation and assessment of incorporation result in variations in the number of gonial cells which give rise to spermatogenic cells with detectable label. A highly labeled snail would appear to be "late," since an increased number of gonial cell generations contributing labeled spermatogenic cells would result in a relatively high amount of label in the early stages. A snail in which label is detected with a higher efficiency (for example, through thinner sectioning) would similarly appear to be "late," because of detection of label among the progeny of gonial cells further than one division removed from the spermatocyte stage.

A further error may stem from the mistaken identity of any stage, especially of transition stages.

The standard error, determined for each stage by measuring the span between entry and exit curves at 9 different cumulative percentage levels, varies between 11 per cent in the case of the diffuse stage and 68 per cent for the round spermatid stage.

Histone Transition.—Two changes are seen in the deoxyribonucleohistone of the developing sperm. The first, occurring in the spermatid, is a change from a typical somatic histone to one which is rich in arginine.

Text-figs. 14 and 15 show the same section stained successively with the TCA-Feulgen procedure for DNA, and, following deamination with nitrous acid, Alfert and Geschwind's alkaline fast green method for histone. Deamination, which removes the epsilon amino groups of lysine, results in the loss of stainability of typical histones, which owe much of their basicity to lysine. The staining of histones which have a high arginine-to-lysine ratio is relatively unaffected by deamination, and the lack of effect on the staining of sperm cells

indicates the presence of such an arginine-rich histone in these cells.

The transition from a typical histone to an arginine-rich histone can be followed by comparison of the effect of lysine blocking on the acid dye binding in different stages of spermiogenesis (Text-figs. 16 to 26). The picric acid bromphenol blue procedure was used on these preparations. Alkaline fast green after trichloroacetic acid hydrolysis yields similar results with the exception of the more mature sperms (see below). Deamination invariably causes complete loss of staining in the somatic cells, the spermatocytes, and the early spermatids (Text-fig. 15), a partial loss of staining in the spade and round spermatids (Text-figs. 17 and 22, 18 and 23), and little or no loss of staining in later spermatids and early maturing sperm (Text-figs. 19 and 24, 20, and 25). The transition apparently begins in the spade stage and is completed by the time the spermatid reaches the elongated stage. The change in the ratio of arginine-to-lysine during the transition is accompanied by an increase in the absolute concentration of protein-bound arginine as was indicated by the Sakaguchi test.

This transition is followed by a further alteration in the deoxyribonucleohistone complex which is shown by an increasing lability of both DNA and the protein to acid hydrolysis. Staining of some ovotestes sperm and all sperm from the hermaphroditic duct by the fast-green method results in a low intensity of staining and a "precipitation" of the staining material on the nuclear membrane. The fully developed spermatophore sperm do not stain with the alkaline fast green method. In this respect the mature sperm of *Helix* behave as though they contain a protamine. Protamines are leached from the cell during hydrolysis with trichloroacetic acid. The histones of the spermatophore sperm (and protamines as well) can be demonstrated by staining with eosin Y at a pH of 8.3 after removal of DNA by picric acid hydrolysis. In this procedure the otherwise labile histone or protamine is always maintained in an insoluble state as a part of a precipitated complex. The dye bromphenol blue has an advantage over eosin of a high extinction at a wave length to which the eye is very sensitive.

Text-figs. 20 and 25 show the effect of acetylation on bromphenol blue staining of the spermatophore sperm. This preparation went unhydrolyzed to avoid the precipitation or coagulation effect
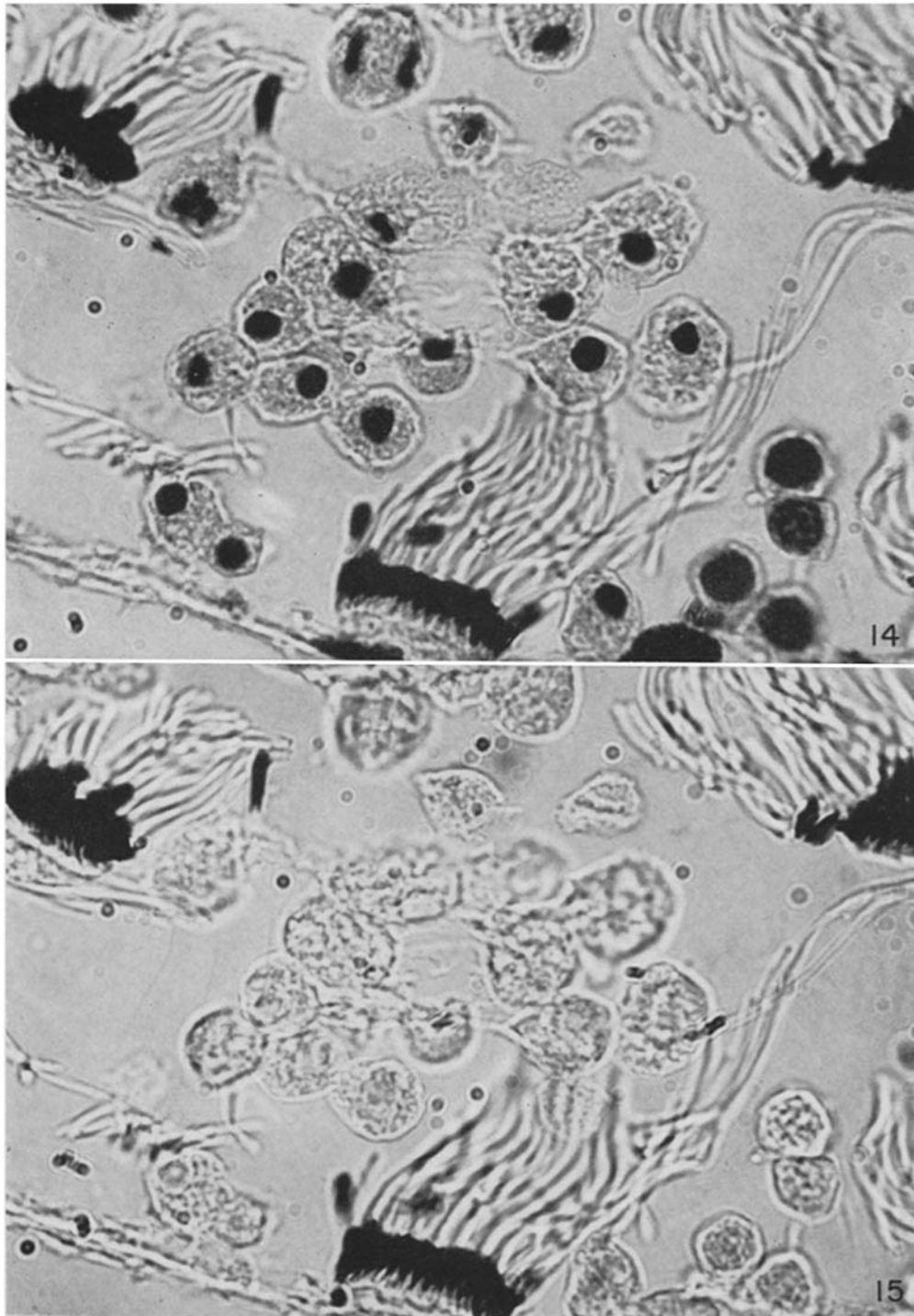
Text-Figs. 14 and 15. Section successively stained with the TCA-Feulgen procedure for DNA (14), then with the fast-green method after deamination for arginine-rich histones (15). Note loss of histone staining in all nuclei but those of the clumped sperm heads. The cells in the center are early spermatids which have not yet gained the high arginine histone. Magnification: × 1,350. Green and red filters used for Text-figs. 14 and 15 respectively.
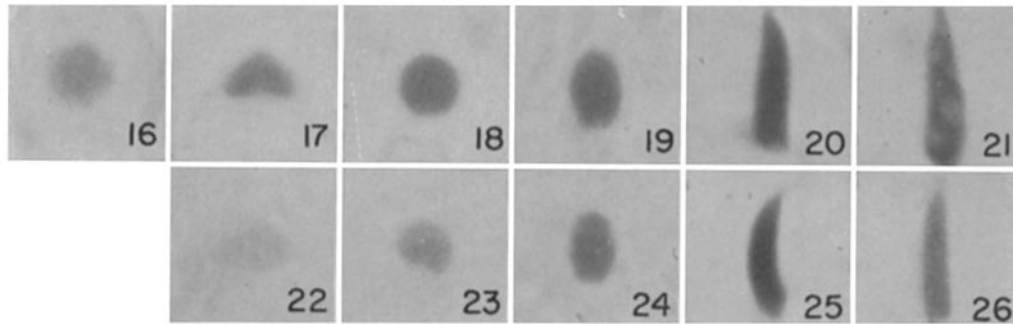
TEXT-FIGS. 16 to 21. Picric acid–bromphenol blue stain demonstrating histones and/or protamine in the early, spade, round, long, spermatids, ovotestis sperm, and spermatophore sperm respectively. Magnification in these and Text-figs. 22 to 26: × 3,200. The sperm in Text-fig. 21 was unhydrolyzed.

TEXT-FIGS. 22 to 26. Picric acid–bromphenol blue stain after deamination (22 to 25) and acetylation (26) demonstrating high arginine histones and protamine in spade, round, and long spermatids, ovotestis sperm, and spermatophore sperm. The sperm in Text-fig. 26 was unhydrolyzed.

produced by picric acid on these cells. (Acid dye-staining without hydrolysis proved possible in these cells and in the post-transition spermatids.) The photographs are therefore not directly comparable with those preceding them in the series. However it can be seen that blocking of lysine exerts relatively little effect on the staining of the mature sperm.

To summarize: The transition to an arginine-rich histone occurs during the late spermatid stage. An increasing lability of the histone to acid hydrolysis begins in the ovotestis sperm and culminates in the mature spermatophore sperm. An arginine-rich protein is maintained, suggesting the presence of a protamine in the mature sperm.
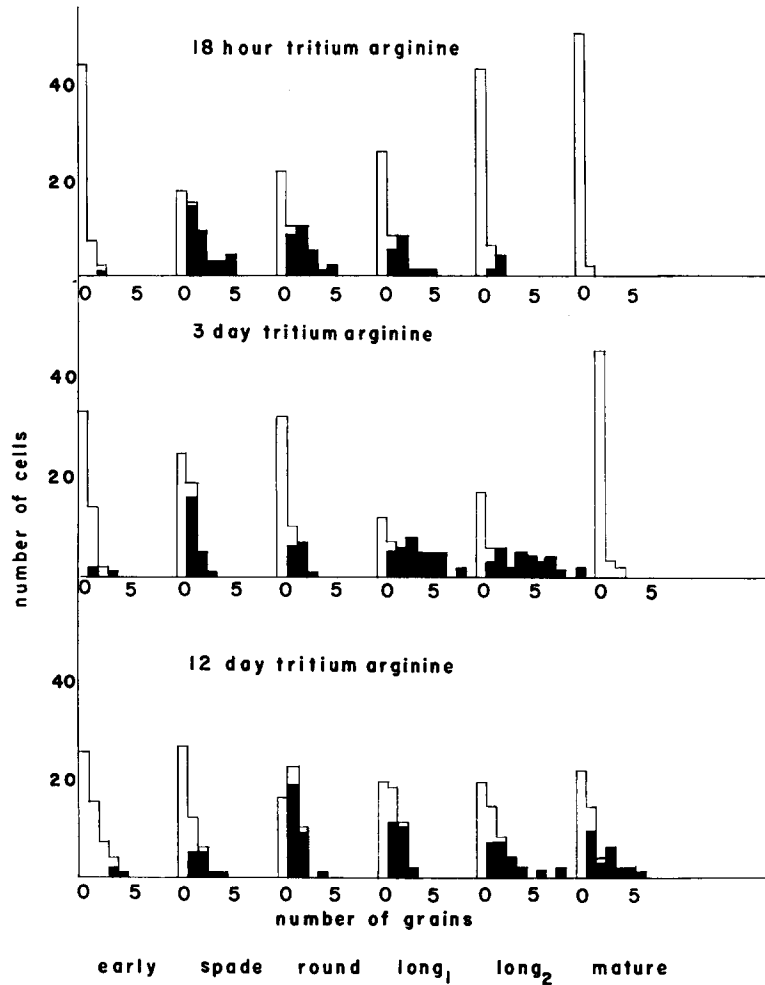
Incorporation of Tritium-Labeled Arginine.—As indicated in Text-fig. 27, tritium-labeled arginine appears primarily in the spade, the round, and the early elongated spermatid nuclei 18 hours after administration of isotope. Only those nuclei were scored which were either freed from the cell (see Text-fig. 10) or "emergent," insuring the nuclear origin of the autoradiograph. An "emergent" nucleus is one which projects from the body of the cell, permitting distinction to be made between nuclear and cytoplasmic label. At 3 days after administration most label is found in the early elongated and late elongated spermatid nuclei. Most of the late elongated nuclei were "emergent." At 12 days after administration label is found in the maturing sperm as well (Text-figs. 28, 29).

Incorporation of arginine into the spermatid nucleus begins at approximately that stage in

which the histone transition is first seen, the early spade stage.

Determination of DNA in Spermatid Nuclei.—The relative amounts of DNA in spermatid stages were determined to ascertain whether or not the protein synthesis could be correlated with synthesis of DNA. The results are shown in Table III. There is no evidence of DNA synthesis in the spermatid nuclei. These findings are in accord with the lack of immediate incorporation of thymidine into these stages. The discrepancy between the haploid value of approximately 6 and the expected value of 8 (based upon the diploid and twice-diploid values of 16 and 32 respectively) probably owes to the increasing effect of scattered light as a source of error in the measurements of the small dense spermatid nuclei. The sperm nuclei were too small to measure with the equipment available.

Fine Structure of the Spermatid Nucleus.—A number of electron microscopic studies of spermatid development have demonstrated the formation of filamentous and lamellar structures within the nucleus (19, 20, 29, 39). Figs. 1 to 5 show several stages in the spermatid development. It is seen that the spade-stage nucleus contains fine filaments, roughly 120 to 150 A in diameter (Fig. 2). The elongating spermatid contains folded convoluted lamellar structures oriented parallel to the long axis of the sperm head. The late spermatid (Fig. 5), the hermaphroditic duct, and the spermatophore sperm are relatively homogeneous with little evidence of structure. The formation of the filaments and their aggregation into ribbons

TEXT-FIG. 27. Incorporation of tritium-labeled arginine into spermatid nuclei. Ordinates: Number of nuclei. Abscissas: Number of grains overlying nuclei removed from the different stages. The unshaded bars represent nuclei with no overyling grains, and the nuclei with grains which are attributable to background. The expected frequency of nuclei having x grains due to background was calculated from the frequency of nuclei having zero grains, assuming a Poisson distribution of background grains. The shaded regions represent the nuclei showing significant label.

or lamellae parallels the transition of the somatic histone to an arginine-rich histone, as judged by the correspondence of the stages in which the two events occur.

Table IV summarizes the events occurring in the development of the sperm cells.

DISCUSSION

Time Sequence.—The timing of spermatogenesis, from premeiotic DNA synthesis in the primary spermatocyte to maturation of the sperm, resembles that seen in the mouse by Oakberg (28)

and Claremont and Leblond (8). Spermatogenesis in the mouse has a duration of 24 to 27 days; one-third to one-half of this time is spent in the spermiogenic stages. According to Sirlin and Edwards (31) label incorporated into DNA during spermatogenesis does not appear in the ejaculated sperm until 30 to 35 days after administration of label. In *Helix aspersa* the time between completion of development in the ovotestis and "ejaculation" is extended, but in its grosser aspects the course of the process in the two organisms is similar.

These time studies are of interest chiefly because
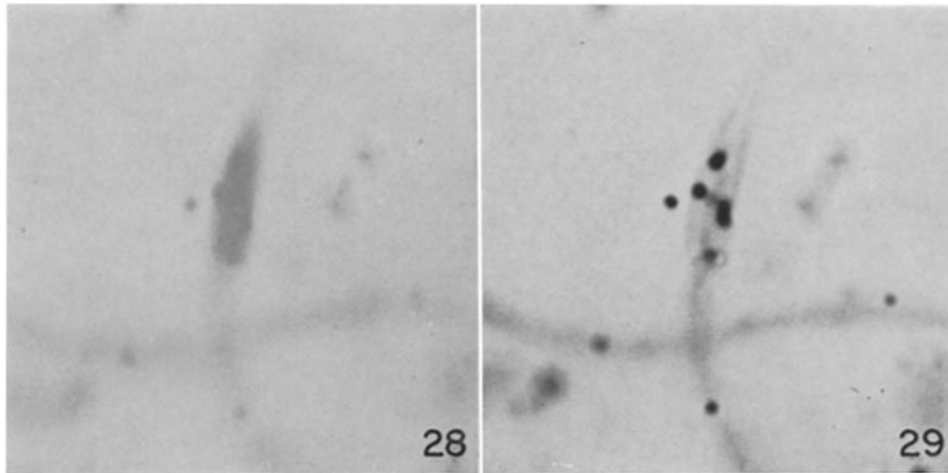
Text-Figs. 28 and 29. Photographs of a TCA-Feulgen–stained tritiated-arginine-labeled sperm head and its autoradiograph. Magnification: × 4,300.

TABLE III

Comparison of the Relative Amounts of DNA in Some of the Spermatogenic Stages of Helix

Stage	Presumed gonial	Primary spermatocytes	Early spermatids	Spade spermatids	Round spermatids	Long spermatids
Amount DNA	16.1 ± 3.6	32.7 ± 3.8	7.8 ± 1.5	5.9 ± 1.4	5.6 ± 1.0	5.8 ± 0. 7

TABLE IV

Summary of Events Occurring during Spermatogenesis

Stage	Type of histone	Incorporation of arginine	Fine structure
Premeiotic	"Somatic type"		Typical interphase nucleus
Early spermatid	"Somatic type"	−	Typical interphase nucleus
Spade spermatid	Some high arginine	+	Filaments
Round spermatid	High arginine	+	Lamellae
Long spermatid	High arginine	±	Lamellae
Ovotestis sperm	High arginine, some cells slightly labile	−	Homogeneous, dense
Hermaphroditic duct sperm	High arginine, increasingly labile		Homogeneous, dense
Spermatophore sperm	High arginine, very labile (protamine)		Homogeneous, dense

they will facilitate labeling experiments in further studies of histone metabolism. Two pertinent questions involve the fate of the early spermatid histone during the transition to the high arginine histone, and the fate of the latter histone and protamine during subsequent stages, including fertilization.

Histone Transition.—The relative inability of nitrous acid treatment to affect the acid dye-binding ability of the histone of late spermatids and mature sperm reflects the low contribution of lysine to the over-all basicity of sperm histones. This effect had already been described by Alfert for guinea pig sperm (2) and salmon sperm and spermatids (1), using acetylation to block lysine. The nuclear basic proteins of *Helix aspersa* spermatids can then be tentatively described as arginine-rich histones, and may be protamines or, more probably, proteins similar to those described by Hultin and Herne (18) as intermediate between histone and protamine. They are probably higher in arginine content than the high arginine histones obtained from calf thymus by Daly and Mirsky (13). These latter histones, which contain approximately equimolar ratios of arginine and lysine, bind appreciably less fast-green after nitrous acid treatment (6).

It is evident from the present studies that transition from a relatively lysine-rich to an arginine-rich histone is accompanied by incorpora-

tion of arginine into the nucleus. Coincidence of these two processes suggests that the transition involves synthesis of a new protein. This interpretation is supported by the increase in protein arginine demonstrable by the Sakaguchi test. The histogram of the 12-day sample is a less convincing demonstration of a high amount of label within the sperm cells at this time than is the fact that labeled sperm (Text-figs. 28, 29) are present in this slide and absent in the 18-hour and 3-day labeling experiments. The low frequency of labeled cells here as compared with the labeling of earlier stages at earlier times owes to the "dilution" of the labeled sperm in a large pool of unlabeled sperm.

The significance of the lability of the maturing sperm histone is obscure. The fact that procedures which result in the precipitation of protamines as well as histones (picric acid hydrolysis of DNA followed by staining with bromphenol blue, or with eosin at alkaline pH's) permit staining of these cells indicates that the inability of the cells to stain with the alkaline fast green method is an expression of solubility of the basic protein during the latter staining procedure. That this lability may be used to characterize a protamine was indicated in Alfert's studies on salmon (1), and is supported by recent work with the squid, *Loligo opalescens* (Bloch, unpublished). When ripe the *Loligo* testis is full of sperm and spermatids. Only sperm are found in the duct leading from the testis and in the spermatophores. Chromatography of the basic nuclear protein hydrolysates permitted characterization of the protein of the duct and spermatophore sperm as a true protamine, and the bulk of that of the testis sperm as histones rich in arginine. The protamine contains mainly arginine and relatively few other amino acids. The histone has an almost full complement of amino acids, including lysine. The protein of the duct and spermatophore sperm is, accordingly, acid labile. It is demonstrable by staining with eosin at a pH of 8.3 after picric acid hydrolysis. It is lost, however, during the alkaline fast green procedure for histones. The testis, on the other hand, contains both sperms which are labile and spermatids which are stable to trichloroacetic acid hydrolysis. Both types of cells stain with eosin after picric acid regardless of whether or not lysine is blocked. Thus in *Loligo* acid lability of the sperm is clearly associated with the presence of a protamine. Acid stability of the testis spermatids, and perhaps

sperm also, is apparently associated with the presence of an arginine-rich histone, although the possibility must be recognized that this histone may be, in fact, a mixture of protamine from the labile sperm and protamines plus other stabilizing proteins, perhaps histones, from the stable spermatid fraction. The latter interpretation was offered by Alfert to explain the stability and, at the same time, the lack of effect of acetylation upon the fast-green staining of late salmon spermatids (1). These were thought to contain a protamine and a protein which stabilized the chromatin.

Nuclear Fine Structure.—The changes in the fine structure in the nucleus are similar to those described by Grassé for *Helix pomatia* (19) and Rebhun for *Otala lactea* (29). Rebhun's suggestion that the changes might parallel the transition from histone to protamine is confirmed by our results. Initiation of the formation of filaments occurs during the spade stage, coinciding with arginine incorporation and alteration in the staining properties. By the early elongated stage, when the transition is essentially complete, the nucleus is filled with electron-dense convoluted lamellae arranged parallel to the long axis of the sperm head. The thickness of the lamellae is roughly that of the filaments seen in the spade stage, thus supporting the contention of Kaye that the plates are formed by aggregation of the filaments (20). Further development results in transformation of the lamellar structure and formation of a homogeneous electron-dense nucleus. This form prevails in the hermaphroditic duct sperm and the spermatophore sperm, and therefore characterizes the sperm head containing the acid labile deoxyribonucleoprotein.

General Considerations.—The significance of the histone transition remains elusive. It is apparently a fairly general phenomenon in spermiogenesis, and sperm appear to be unique among cells if only in the extremity of the change. A clue might be had in a peculiar property of germ cells. While there is no reason to suspect that the developmental history of spermatogenic tissue in *Helix* is fundamentally different from that of any other tissue, its developmental future is unique. Insofar as differentiation of a cell is a progressive limitation of its developmental potencies, the sperm, as the precursor of the male pronucleus of the fertilized egg, presents a nucleus in which the general trend in development is reversed.

Briggs and King's experiments demonstrating

the loss of totipotency among nuclei during early development had prompted Stern (see discussion in 21) to suggest the opposite experiment—determination of the ability of earlier spermatid nuclei to effect successful development—to find out when the sperm nucleus gains totipotency or whether it had ever lost it. Unlike many other organisms, however, sperm maturation in *Rana* is unaccompanied by an apparent histone transition. (Carp is also exceptional in this respect (38).) The ability of frog sperm nuclei to bind fast-green is abolished by nitrous acid, as is that of somatic nuclei (Bloch and Hew, unpublished).

Nevertheless, a relationship between histone transition in sperm and a "return to totipotency" cannot be ruled out. It is of interest that the transition in sperm, when it does occur, is variable in its timing among different organisms and that alterations, as demonstrated by changes in staining properties, continue after fertilization. In *Helix* the transition to an arginine-rich histone occurs in the spermatid, while the acid labile condition (a protamine) obtains after the sperm is mature from a morphological standpoint. In guinea pig and rat the initial transition occurs relatively late, being observed in the morphologically mature sperm. In mouse this protein remains stable to acid up to the point of fertilization, after which it becomes non-stainable with fast-green, to resume staining only during mitosis or during interphase later in development (3). Alfert attributes this staining change to a masking of histones rather than to a further transition or to acid lability, owing to the fact that mitotic chromosomes do stain. In *Helix* a change occurs after fertilization which is not similarly attributable to masking, but which does argue for a further transition (Bloch and Hew, 6).

Gametogenesis is but one stage in a sequence embracing gametogenesis, fertilization, and cleavage. Sperm formation is an intermediate step in this process. At present, generalizations regarding the role or the universality of the histone transition usually occurring in sperm awaits analysis of continued development after fertilization. This is essential to determine the extent to which variations among organisms, such as is seen in the exceptional behavior of frog and carp, might be explained by variations in the timing of the transitions.

SUMMARY

The spermatogenic process in *Helix aspersa* is resolved into a number of morphologically defined stages. Their durations, as determined from the times needed for cells labeled with tritiated thymidine to pass through the stages, are as follows: spermatocyte, after DNA synthesis and before the bouquet stage, 1.9 days; bouquet stage, 6.1 days; diffuse and division stages, 12.3 days; early spermatid, 1.7 days; spade spermatid, 1.4 days; round spermatid, 0.6 days; long spermatid, 3.2 days; ovotestis sperm, 16.4 days. The times spent by the sperm in the hermaphroditic duct and in the spermatophore are unknown.

In the spermatids with spade-shaped and rounded nuclei a transition occurs in which a histone rich in arginine replaces, or is added to, a typically somatic histone in which lysine predominates. This transition is accompanied by an incorporation of labeled arginine within the nucleus, and appears to reflect a synthesis of new histone. The later appearance of labeled arginine in subsequent stages follows a course similar to that seen with the labeled thymidine. The transition is coincident with the formation and aggregation of fine electron-dense filaments within the nucleus.

The histone of the maturing sperm, in the hermaphroditic duct and the spermatophore, becomes increasingly acid labile. Its behavior suggests a transition to a true protamine. In these sperm the filamentous and lamellar structure of the nucleus has given way to an electron-dense substance which is diffusely disposed throughout the nucleus.

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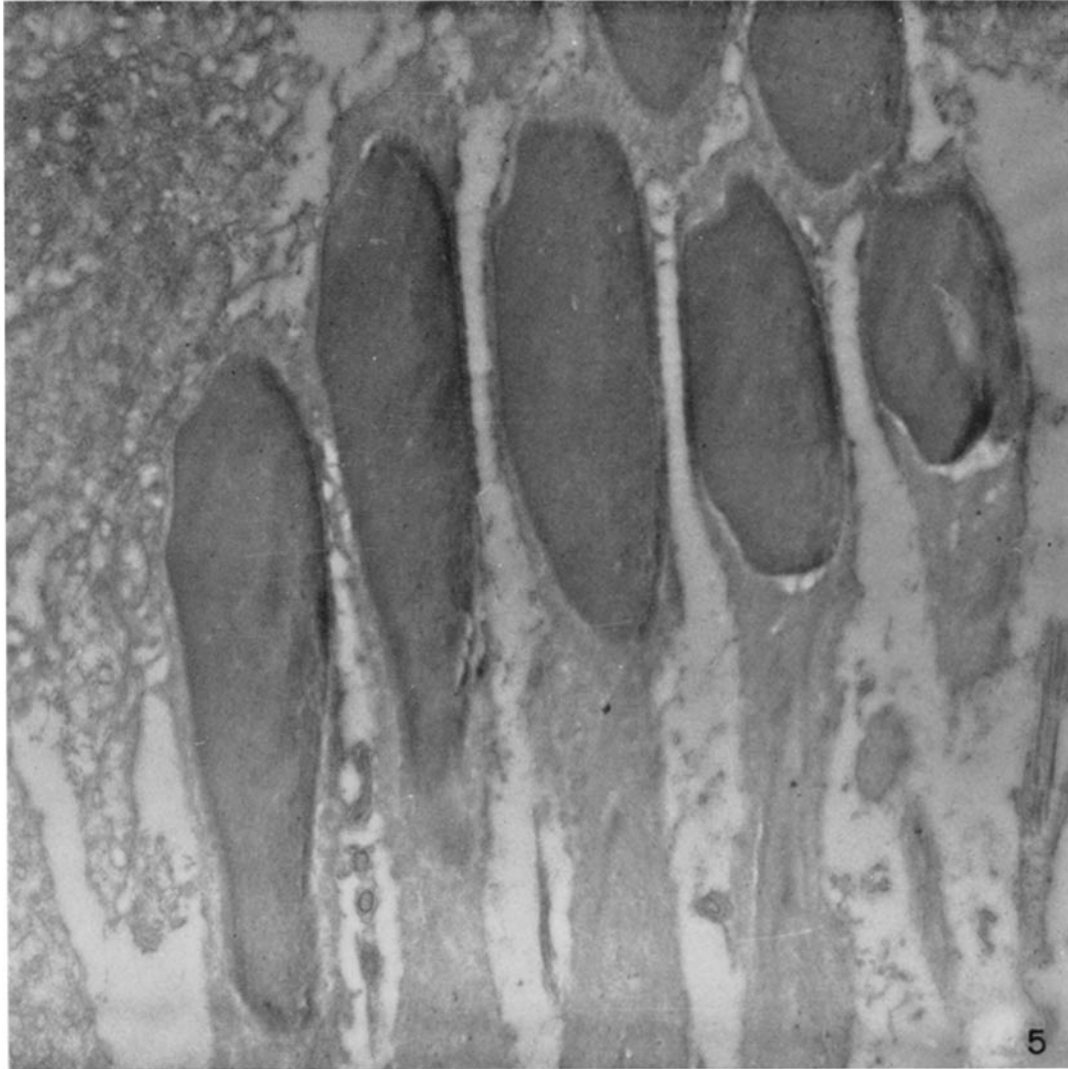EXPLANATION OF PLATES

PLATE 284

FIGS. 1 to 4. Electron micrographs of early, spade and elongated spermatids. Magnification: 17,200.
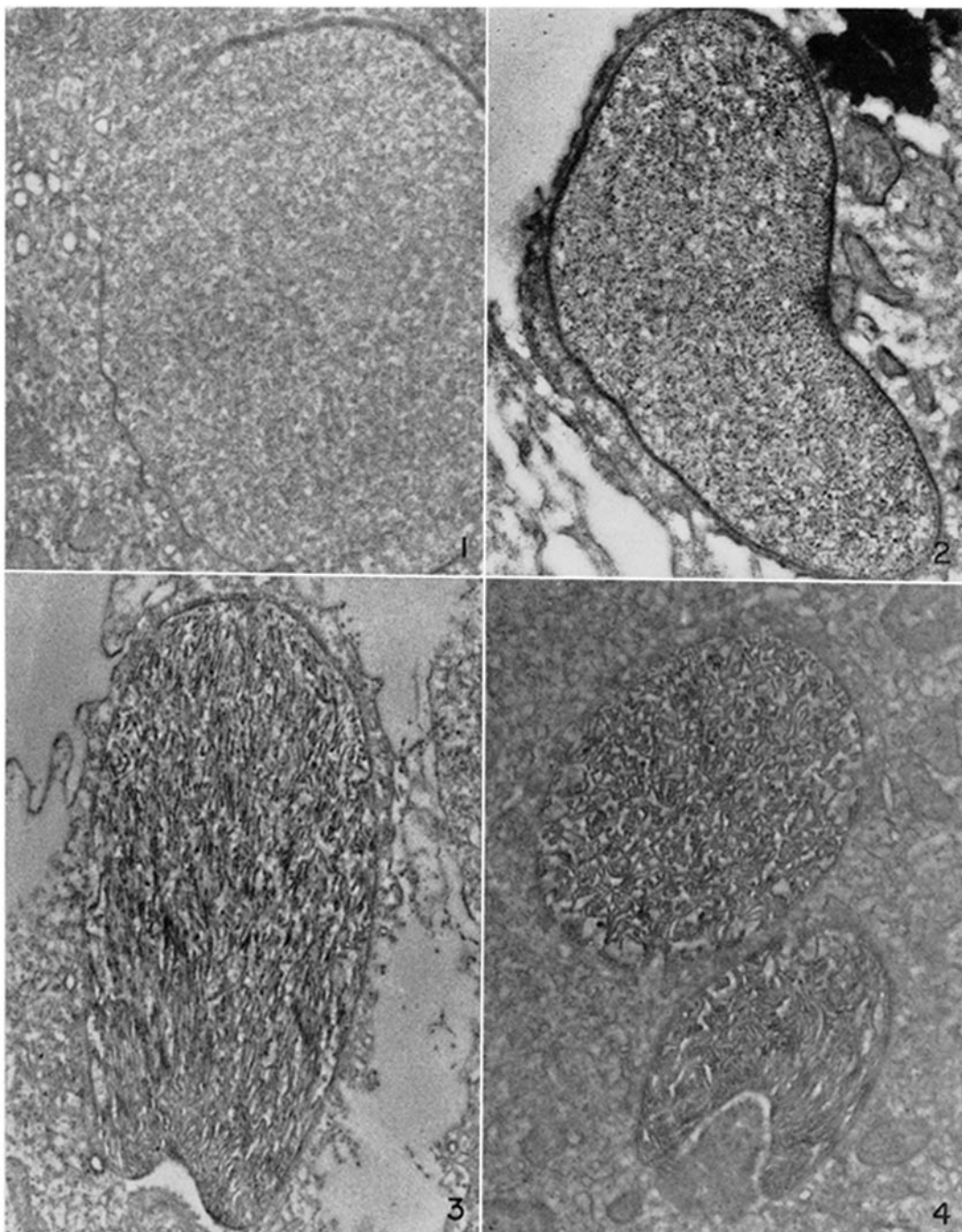
(Bloch and Hew: Spermatogenesis and histone transition)

PLATE 285

FIG. 5. Electron micrograph of maturing ovotestis sperm. Magnification: $\times$ 16,800.

(Bloch and Hew: Spermatogenesis and histone transition)