A KINETIC STUDY OF DNA AND BASIC PROTEIN METABOLISM DURING SPERMATOGENESIS IN THE SAND CRAB, *EMERITA ANALOGA*

JACK C. VAUGHN and LILLIAN A. THOMSON

From the Department of Zoology, Miami University, Oxford, Ohio 45056

ABSTRACT

Cytochemical and radioautographic techniques define and confirm a staging scheme for developing spermatids of the decapod crab, *Emerita analoga*. Quantitative photometric data demonstrate that developing spermatids lose a significant proportion of their nuclear proteins, as evidenced by diminishing binding of fluorodinitrobenzene. Photometric results also show that much (but not all) of the spermatid nuclear protein loss is in somatic-type histone, as evidenced by a dramatic fall in the histone/DNA ratio of these cells during a period in which nuclear DNA content remains constant. By the end of spermiogenesis, the sperm nuclear histone and protamine content is approximately zero, whereas some nonbasic protein persists. Loss of spermatid nuclear somatic-type histone is not accompanied by synthesis of gamete-type histone (e.g. protamine or arginine-rich histone), showing that the processes of displacement and synthesis of nuclear basic proteins during histone transition are not subject to obligatory coupling. Labeling studies suggest that nonbasic acrosomal proteins (presumably partly enzymes) are synthesized in the cytoplasm, after which they move into the acrosome. Stainable basic proteins accumulate in the acrosome during precisely the period of nuclear somatic histone loss, suggesting nuclear-cytoplasmic transfer.

INTRODUCTION

Numerous recent reports have appeared relating the state of DNA-histone (or DNA-protamine) interaction to the repression of DNA-dependent RNA synthesis in eucaryotes (Huang and Bonner, 1962; Pogo et al., 1966; Skalka et al., 1966. For reviews see Georgiev, 1969; Phillips, 1971). Histones apparently behave like nonspecific repressors at the transcriptional level, maintaining the genes in a permanently turned-off state unless activated by other agents, which lead to acetylation, methylation, or phosphorylation of the basic proteins and restoration of gene activity. The correlation of net positive charge of the DNA-associated basic proteins with degree of gene repression (Skalka et al., 1966) and the generality that during animal spermatogenesis somatic-type histones are replaced by gamete-type histones or protamines, which have a very high net positive charge (for review see Bloch, 1966 b), have led to a common belief (of uncertain origin) that the appearance of these gamete-type nuclear basic proteins is in some way related to the turning off and continued repression of sperm genes. This presumed genetic function has been postulated to involve the restoration of the genome to a state devoid of the developmental history of the individual (Olins et al., 1968).

We have recently reported that mature spermatophore sperm of the sand crab *E. analoga* do not contain nuclear basic proteins (histones or protamines) as defined by standard cytochemical tests (Vaughn, 1968 a, 1968 b; Vaughn et al., 1969). These sperm do, however, contain abundant acro-

somal basic proteins: lysine-rich proteins (in the walls of the acrosomal tubule, or "capsule") and arginine-rich nonprotamine basic proteins (in the apical cap, or "ears"), as first reported for this species by Bloch (1966 *a*, 1966 *b*). General absence of histones and protamines in mature crustacean sperm nuclei has also been reported for the hermit crab *Eupagurus bernhardus* (Chevaillier, 1966, 1968), various species of the crab genus *Cancer* (Langreth, 1969), and for the crab *Libinia emarginata* (Vaughn and Hinsch, 1970, 1971). If nuclear basic proteins are important agents in gamete gene repression, then unique repressor mechanisms may be operating for crustacean sperm, whose genome is not transcribing for RNA synthesis, as shown by lack of uptake of uridine-³H (Vaughn and Thomson, unpublished). A study of the metabolism of the basic proteins in these interesting and unique cells should be of interest at this time, and may contribute to an eventual understanding of the mechanism of gene repression in gametes.

The relationship between nuclear somatic-type histone loss and the appearance of the acrosomal basic proteins is also not yet clear in decapod crustacea. Chevaillier (1966) hypothesized that the acrosomal basic proteins in *Eupagurus* sperm may in part represent discarded somatic-type nuclear histones, for which he proposed the name "decapodine" (1967). Bloch (1966 *b*), studying *E. analoga*, observed that the acrosomal basic proteins are first seen in a cytoplasmic vacuole in the early spermatid, that the spermatid nuclear histone stained typically at this time, and hypothesized that the vacuolar basic proteins may be synthesized *in situ* and stored in the ctyoplasmic acrosome. This interpretation was also favored by Langreth (1969), who observed that nuclei in mature *Cancer* sperm cells contain very fine chromatin fibrils (resembling those in bacteria, which lack histones, [Raaf and Bonner, 1968]). She observed that this fibril morphology (and also loss of nuclear histone stainability) develop concomitantly near the end of spermiogenesis, and she found no morphological evidence for a gradual conversion of thicker fibrils, characteristic of those containing histones, into the fine fibrils, but rather a more-or-less rapid change near the end of spermiogenesis. Nuclear histone stainability became more faint shortly before the change in fibril morphology, but did not fall to zero until the fibril transition occurred. Because nuclear histones continued to stain at the time that acrosomal basic proteins were first cytochemically

demonstrable, she concluded that it is unlikely that the acrosomal basic proteins have a nuclear origin.

The purpose of this paper is to present qualitative and quantitative data bearing on the synthesis and kinetics of nuclear and acrosomal basic proteins during spermiogenesis, which will clarify the relationship between the nuclear histones and the origin of the acrosomal basic proteins present in mature sperm of the sand crab.

MATERIALS AND METHODS

Tissue Preparation and Cytochemistry

Sand crabs (*E. analoga*) were collected on the beach at Venice, Calif. during the summer. The testes and also the spermatophore sperm were processed as described elsewhere (Vaughn and Locy, 1969), using sea water-formaldehyde fixation. DNA was stained by the Feulgen method (Feulgen and Rossenbeck, 1924) as modified by Bloch and Godman (1955) for retention of histones. Histones were stained with the pH 8.1 fast green method (Alfert and Geschwind, 1953) as modified by Bloch (1966 *b*) or the picric acid–bromphenol blue method (Bloch and Hew, 1960 *b*). Lysine residues were occasionally blocked by deamination (Van Slyke, 1911) or acetylation (Monné and Slautterback, 1951), in order to test for presence of high protein arginine/lysine ratios. For lysine blockage, the modifications described by Bloch (1966 *b*) were employed. Staging of spermiogenesis was done on cells stained by the Feulgen-alkaline fast green method (Vaughn, 1966) which results in the DNA staining deep magenta and the stable non-DNA-associated basic proteins staining green.

Microspectrophotometry

The relative amount of Feulgen-stained DNA in photographically mapped nuclei was determined by using the standard plug method of Swift (1950) on a Brinkmann microspectrophotometric apparatus (Brinkmann Instruments Inc., Westbury, N.Y.), as described elsewhere (Vaughn and Locy, 1969). For determination of relative nuclear histone content, the identical alkaline fast green–stained cells were relocated, after removal of nucleic acids (5% trichloroacetic acid, 90°C, 15 min), and an optical plug, equal in diameter to that used for DNA determination, was advanced through the center of each cell nucleus. To minimize error in returning the plug to the near-identical position, the largest allowable plug was used in each case, and many nuclei were measured. Histone content was determined by absorption readings taken at 626 ± 5 mμ, and DNA content by absorption readings at 570 ± 6 mμ. All slides used for de-

termination of histone/DNA ratio were stained together. Toad liver sections were run as controls. Relative dye content was expressed as $E \cdot r^2$ in each case, where E is the measured absorbance at the appropriate wavelength and r is the radius of the nucleus in question (Swift, 1950).

Protein-bound nuclear lysine + tyrosine was determined simultaneously with nuclear DNA content, using the 1-fluoro-2,4-dinitrobenzene–Feulgen (FDNB-Feulgen) double-staining technique (Bloch, MacQuigg, and Brack, personal communication, 1964; Bloch, 1966 b; as utilized by Vaughn, 1966). Lysine + tyrosine values were calculated from the absorption readings taken at 402 ± 5 mμ, using equations described elsewhere (Vaughn, 1966), employing the crab haploid DNA value and Feulgen extinction coefficient derived in our laboratory (Vaughn and Locy, 1969), and the extinction coefficient for FDNB (1.84×10^{-7} moles/EA) derived by Bloch, MacQuigg, and Brack. All slides used for determination of protein-bound lysine + tyrosine were stained together. Staining was preceded by a 15-second dip in boiling water to remove bound formaldehyde (Bloch, 1966 b).

Radioautography

Tritiated thymidine (New England Nuclear Corp., Boston, Mass., 6.7 Ci/mmole, 0.5 mCi/ml, batch No. 340-57-1) was used as a precursor of DNA; tritiated lysine (same source, 8.7 Ci/mmole, 0.5 mCi/ml, batch No. 334-214) was used as a protein precursor. Isotopes were diluted with "instant ocean" sea water (Aquarium Systems, Inc., Eastlake, Ohio) so that each animal received a subcarapace injection of 1.0 μCi of the appropriate isotope in 6 μl of solution. The crabs were maintained in the laboratory aquaria on a diet of yeast. Testes were removed and fixed at times ranging from 1 to 29 days. Tissue sections 3 μ in thickness were mounted on gelatinized slides and Feulgen stained, using trichloroacetic acid hydrolysis instead of hydrochloric acid in order to prevent loss of histone-like proteins (Bloch and Godman, 1955). These slides were then covered with Kodak AR 10 stripping film, and processed as previously described (Vaughn, 1966). After exposure (usually for 4–6 months) and development, sections were stained through the emulsion with pH 8.1 fast green (Alfert and Geschwind, 1953), but without acid hydrolysis, and mounted for observation. Staining with Feulgen and alkaline fast green facilitated stage recognition.

RESULTS

Cytochemistry of Staging

On the basis of observation of sections containing meiotic divisions and the resulting early sper-

matids, "stage 1" in spermatid maturation was easily recognized. Microspectrophotometric data (Vaughn and Locy, 1969) have further identified these cells by establishing their DNA content to be one fourth that of primary spermatocytes. The nucleus in these early spermatids is nearly spherical and the cells show relatively little cytoplasmic polarity; the cytoplasm does not bind alkaline fast green (Fig. 1). The chromatin is tightly packed just after the last meiotic division, but soon assumes a vacuolated appearance, with three or more large "vacuoles." These regions may be similar to those described by Moses (1961) in electron micrographs of developing crayfish spermatids, in which the early spermatid nucleus contains numerous trapped membranes, which displace the chromatin, giving the nucleus a "vesiculate" appearance. Stage 2 spermatid nuclei appear little changed; the stage is characterized by the appearance of dull gray-green staining in the cytoplasm (Fig. 2). By stage 3, the nucleus has begun to flatten somewhat on the cytoplasm: the latter binds more alkaline fast green at this stage (Fig. 3). Appearance of the intensely staining acrosomal apical cap in the cytoplasm distal to the nucleus (Figs. 4, 5) marks stage 4. The cytoplasmic basic protein (within the capsule) also stains more intensely, and is nearly spherical during this stage. The nucleus is still somewhat flattened. As the capsule begins to elongate, it assumes an elliptical outline, and is intensely stained with alkaline fast green. This is designated stage 5 (Fig. 6). The capsule continues to elongate, and has become a short, thick rod by stage 6 (Fig. 7). By this time, the nuclear volume has noticeably decreased and the nucleus has reestablished a nearly spherical symmetry; the nuclear vacuoles are now much reduced. Considerable elongation of the capsule marks stage 7. Early in this stage the nucleus is spherical, but by late stage 7 it becomes somewhat elongated (Fig. 8). This "elongation" of the nucleus is artifactual, however, as living testis sperm observed by phase contrast microscopy have spherical nuclei. Stage 8 spermatids are recognized by failure of the fixative to preserve their nuclear integrity, so that their nuclei are often largely destroyed during specimen preparation. Stage 9 sperm (Fig. 9) are found in the paired spermatophores, and are devoid of cytochemically definable nuclear histones and protamines. The cells shown in Fig. 9 were prepared by a dry-ice squash method described elsewhere
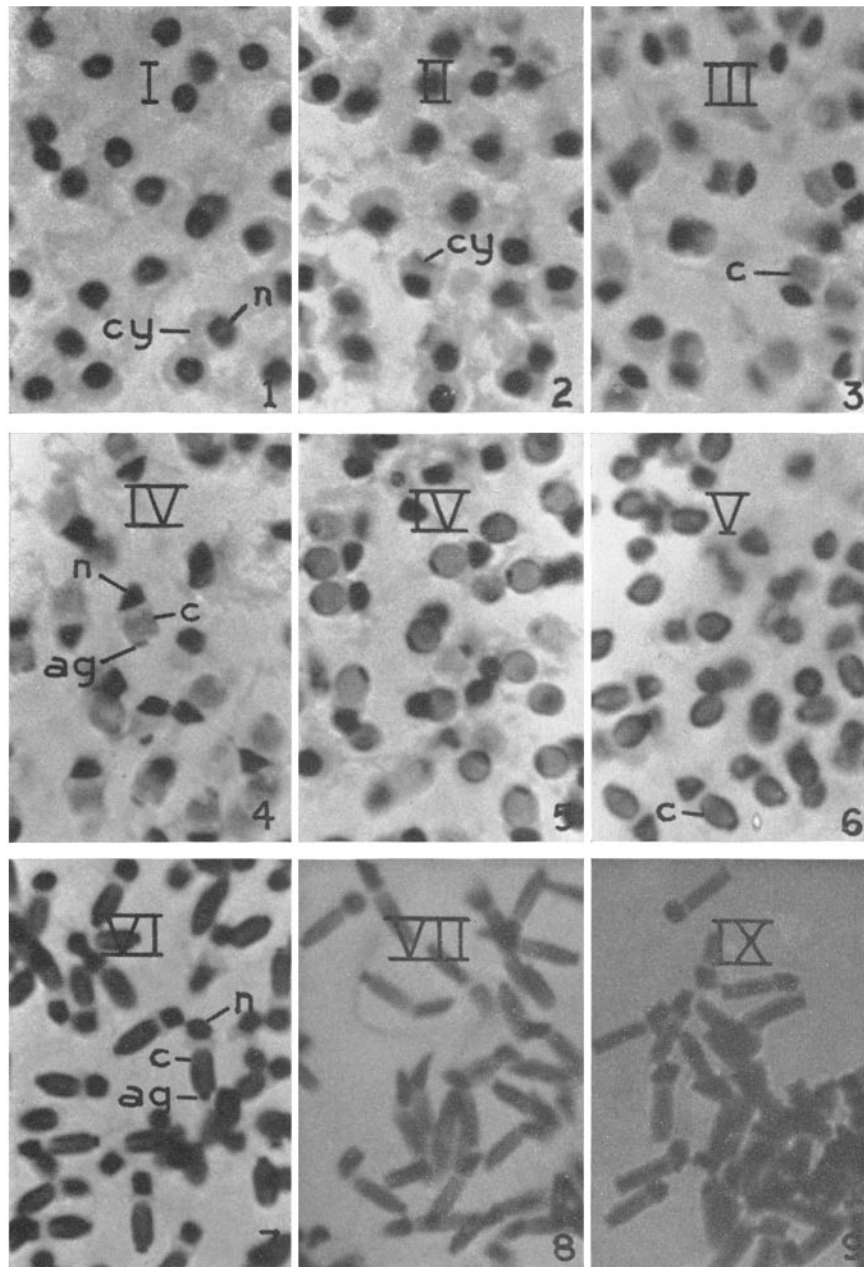
FIGURES 1–9 Sectioned crab testis lobules are shown at different stages of spermiogenesis in Figs. 1–8. Fig. 9 shows a squash preparation of spermatophore sperm. Roman numerals denote the (arbitrary) stages in spermiogenesis. DNA is stained red; non-DNA–associated basic proteins are stained bright green. Kodak high speed Ektachrome film, Feulgen-alkaline fast green technique, formaldehyde fixation. *n*, nucleus; *cy*, cytoplasm; *c*, acrosomal capsule; *ag*, acrosomal apical cap. × 1250.

(Vaughn, 1968 _b_), which preserves the delicate nuclear structure.

Thymidine Timing of Staging

The sequence of developmental stages which has been presented has been checked by following thymidine-^{3}H–labeled cells through spermatogenesis. Although incomplete, the data from this study (Table I and Figs. 10–13) show that the proposed sequence is probably correct, and give some information as to the time required for spermiogenesis to be completed. These data show that although

TABLE I

Thymidine-^{3}H Timing of Crab Spermatogenesis

Days after injection*	1	3	5	6	8	10	11	16	23	29
									Spermatids	
Stage labeled				Spermatocytes				1	4	7

* Days after injection of tritiated thymidine on which the indicated stage first becomes labeled.
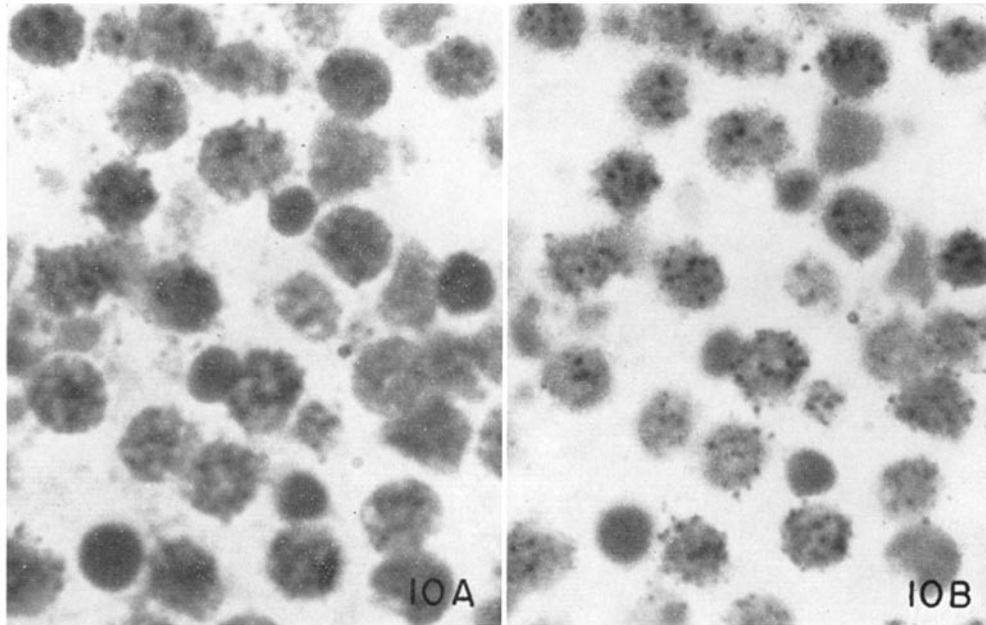
FIGURES 10–13   Thymidine-^{3}H labeling of sectioned crab testis lobules is shown at different stages of spermiogenesis, following various periods of "chase" after initial exposure to the isotope. The micrographs are presented in pairs, focused at the level of the nuclei and at the level of the silver grains. Sections were stained by the Feulgen technique before application of emulsion, and stained through the emulsion with alkaline fast green after development, to show acrosomal structures. _n_, nucleus; _cy_, cytoplasm; _c_, acrosomal capsule; _ag_, acrosomal apical cap. × 1750.

FIGURES 10 _a_ and 10 _b_   Primary spermatocytes, 3-hr chase.

FIGURES 11 _a_ and 11 _b_   Stage 1 spermatids, 16-day chase.

FIGURES 12 _a_ and 12 _b_   Stage 4 spermatids, 23-day chase.

FIGURES 13 _a_ and 13 _b_   Stage 7 spermatids, 29-day chase.
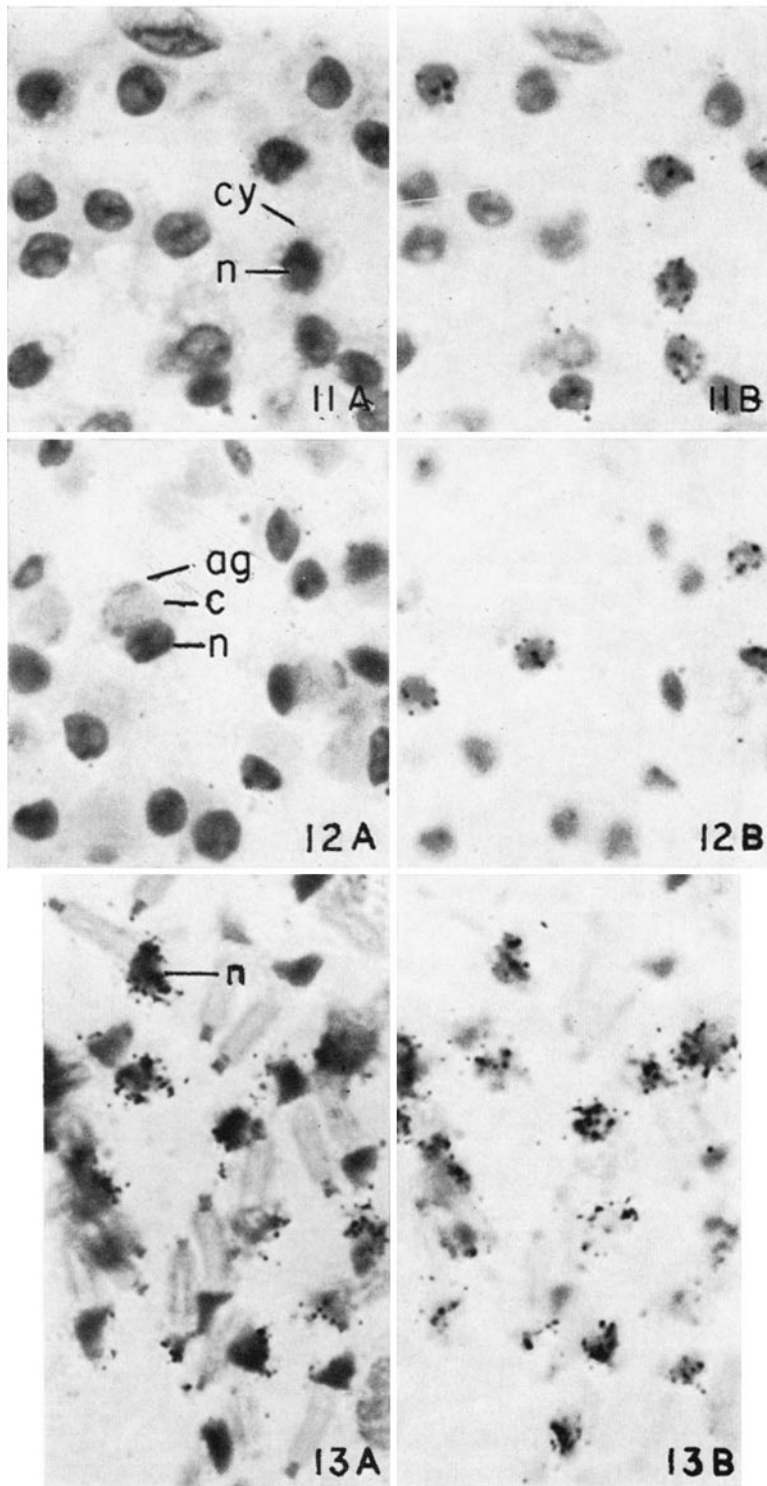
See legend under Figs. 10 a–10 b. × 1750.

TABLE II

FDNB-Feulgen Double-Stain Data on Spermatid Nuclei*

Stage of spermiogenesis	Number of nuclei measured	Mean ratio: E_{402}/E_{570}	Moles nuclear lysine + tyrosine ($\times 10^{-14}$)‡
Stage 1 spermatid (early)	31	0.64 ± 0.02	0.60 ± 0.02
Stage 2 spermatid (early)	28	0.52 ± 0.02	0.49 ± 0.02
Stage 3 spermatid	31	0.52 ± 0.02	0.49 ± 0.02
Stage 4 spermatid (acrosome)	30	0.40 ± 0.02	0.37 ± 0.02
Stage 5 spermatid (elongating)	28	0.32 ± 0.02	0.30 ± 0.02
Stage 6 spermatid (elongating)	32	0.24 ± 0.01	0.22 ± 0.01
Stage 9 spermatozoa	30	0.12 ± 0.01	0.11 ± 0.01

* Standard errors are given.

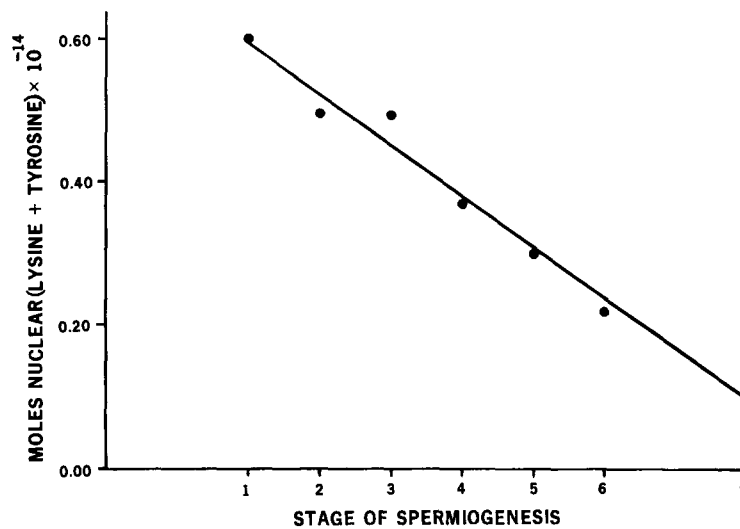‡ Calculated from formulas cited in Materials and Methods.

FIGURE 14   Absolute nuclear protein content, as reflected by absorbance of fluorodinitrobenzene-stained spermatids, is plotted against stage of development for various stages in spermiogenesis. Each point represents the mean value for readings on at least 28 different nuclei.

primary spermatocyte nuclei become labeled after 25 min, stage 1 spermatids do not receive this label until 16 days after the last premeiotic DNA synthesis, stage 4 until 23 days, and stage 7 until 29 days. These periods represent the time by which the first few cells have progressed this far, and are accordingly characterized by low percentages of labeled cells.

Developing Sperm Nuclear Total Protein Content

The data given in Table II and Fig. 14 show that the sperm nuclear protein content, as measured by bound fluorodinitrobenzene, falls drama-

tically during spermiogenesis. Values for spermatid nuclear protein lysine + tyrosine range from about 0.60×10^{-14} moles (stage 1) to 0.11×10^{-14} moles (stage 9, spermatophore sperm).

Developing Sperm Nuclear Histone Content

The data given in Table III and plotted in Fig. 15 as frequency distribution curves show that the distributions of DNA and histone, with regard to cell class (primary spermatocytes and early stage 2 spermatids), are similar, as would be expected. Such a comparison does not, however, prove that the histone content closely follows the DNA content for *individual* nuclei, a point which must be

TABLE III

*Relative Amounts of Nuclear DNA and Histone in Crab Testis Spermatogenic Cells**

Stage of spermatogenesis	Number of nuclei measured	E_{570}	E_{626}	DNA $(E_{570} \cdot r^2)$	Histone $(E_{626} \cdot r^2)$	Histone/DNA
Experiment I						
Primary spermatocyte	54	1.15 ± 0.04	0.97 ± 0.04	8.0 ± 0.04	6.7 ± 0.04	0.84 ± 0.02
Stage 1 spermatid	42	0.63 ± 0.01	0.37 ± 0.01	1.7 ± 0.01	1.0 ± 0.01	0.59 ± 0.01
Stage 2 spermatid	57	0.74 ± 0.01	0.50 ± 0.01	1.7 ± 0.01	1.2 ± 0.01	0.68 ± 0.01
Stage 5 spermatid	23	0.66 ± 0.01	0.12 ± 0.01	—‡	—‡	0.18 ± 0.01
Stage 6 spermatid	31	0.88 ± 0.02	0.11 ± 0.01	1.6 ± 0.02	0.2 ± 0.01	0.13 ± 0.01
Experiment II						
Primary spermatocyte	30	1.10 ± 0.03	0.48 ± 0.02	7.6 ± 0.03	3.3 ± 0.02	0.43 ± 0.01
Stage 1 spermatid	15	0.87 ± 0.01	0.28 ± 0.01	2.3 ± 0.01	0.8 ± 0.01	0.33 ± 0.01
Stage 1 spermatid	30	0.90 ± 0.01	0.26 ± 0.01	2.4 ± 0.01	0.7 ± 0.01	0.29 ± 0.01
Stage 2 spermatid	34	0.96 ± 0.01	0.28 ± 0.01	2.3 ± 0.01	0.7 ± 0.01	0.29 ± 0.01
Stage 3 spermatid	28	1.15 ± 0.02	0.19 ± 0.01	—‡	—‡	0.17 ± 0.01
Stage 4 spermatid	33	1.11 ± 0.01	0.11 ± 0.01	—‡	—‡	0.09 ± 0.01
Stage 6 spermatid	35	1.11 ± 0.03	0.11 ± 0.03	2.2 ± 0.03	0.2 ± 0.03	0.09 ± 0.02
Stage 6 spermatid	35	1.08 ± 0.03	0.09 ± 0.01	2.1 ± 0.03	0.2 ± 0.01	0.08 ± 0.01
Stage 6 spermatid (late)	36	1.12 ± 0.02	0.06 ± 0.02	2.1 ± 0.02	0.1 ± 0.02	0.05 ± 0.02

Interexperimental values cannot be compared, although it can be seen that when relative histone content, as measured by bound fast green, falls by half (spermatocytes) between Experiment I and Experiment II, this also occurs for spermatids, so that the two sets of data generate similar curves.

* Standard errors are given.

‡ Owing to nonspherical nuclear morphology, these cells could not be utilized for calculation of DNA or histone content.
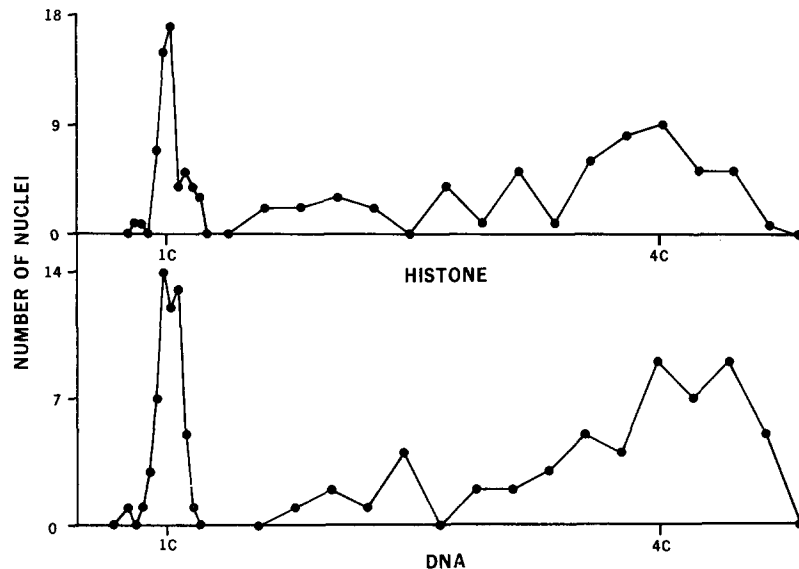

FIGURE 15 Frequency distribution curves for relative amount of DNA (lower curve) and histone (upper curve) in crab stage 2 spermatids and primary spermatocytes.
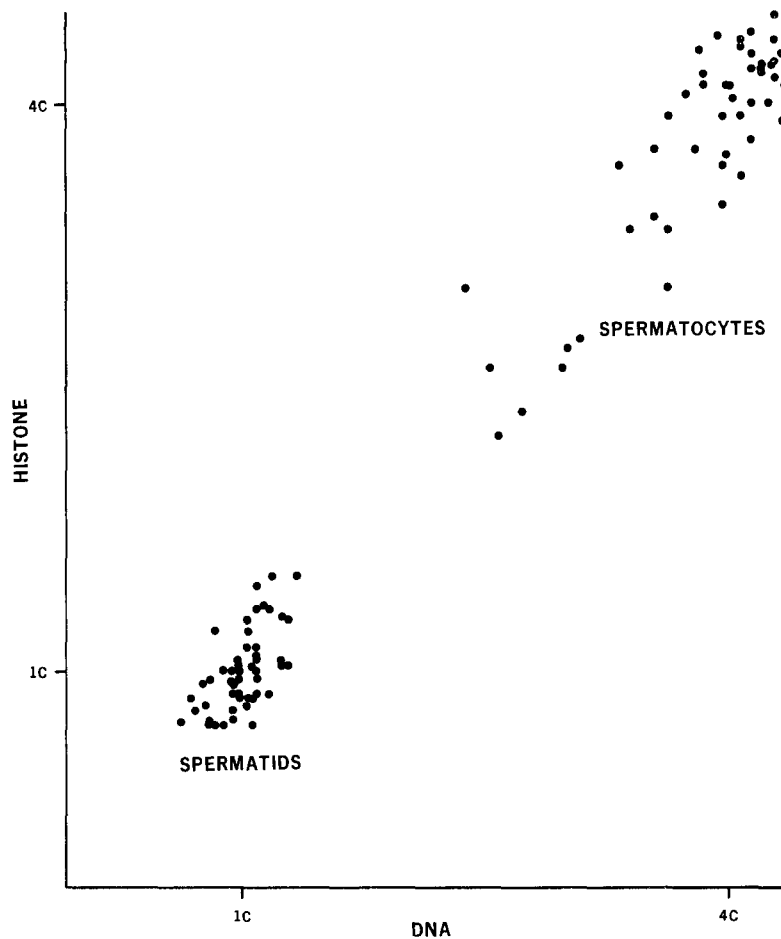
FIGURE 16   Relative amounts of DNA plotted against relative amounts of histone for identical cells in stage 2 spermatids and primary spermatocytes. Logarithmic scales.

demonstrated for typical nuclei before exceptions can be analyzed. That histone content does closely follow DNA content for individual nuclei is indicated when the DNA content is plotted against the histone content for the identical nuclei (Fig. 16). Bloch and Godman (1955) found similar results in typical nuclei of growing mammalian cells. Among both early spermatids and spermatocytes, most cell sections which contain the x-ploid amount of DNA also contain the x-ploid amount of histone. This correspondence between DNA and histone content in spermatids is lost following stage 2 of spermiogenesis, for, although DNA content remains relatively constant, histone content rapidly diminishes, as reflected by the histone/DNA ratio (Table III and Figs. 17–18), which remains constant only during spermatocyte growth and stages 1–2 of spermiogenesis. It is evident that developing

spermatids lose considerable stainable nuclear somatic-type histone, especially between stages 2 and 4.

Evidence for the final drop in spermatid nuclear histone content following stage 6 is qualitative, and based upon observation of the mature testis sperm (stage 8) and the spermatophore sperm (stage 9), whose nuclei do not bind alkaline fast green. Quantitative readings on large numbers of testis stage 8 spermatids and spermatophore sperm, stained with the alkaline fast green procedure, were not possible for technical reasons (discussed later).

Acrosomal Protein Synthesis

After injection with lysine-^{3}H and a 25-min chase, newly synthesized protein is present
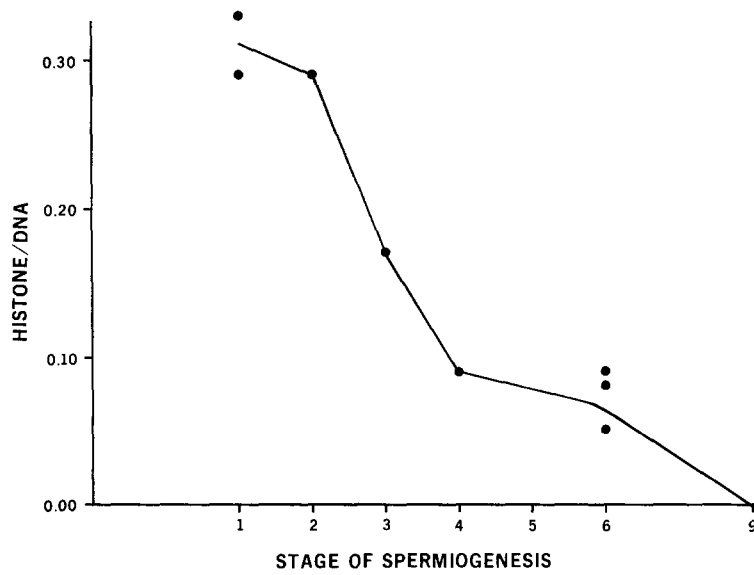
FIGURE 17 Ratio of relative amounts of histone/DNA, as reflected by absorbance of the same nuclei successively stained with the Feulgen method for DNA followed by the alkaline fast green method for histone, is plotted against the various stages in spermiogenesis.
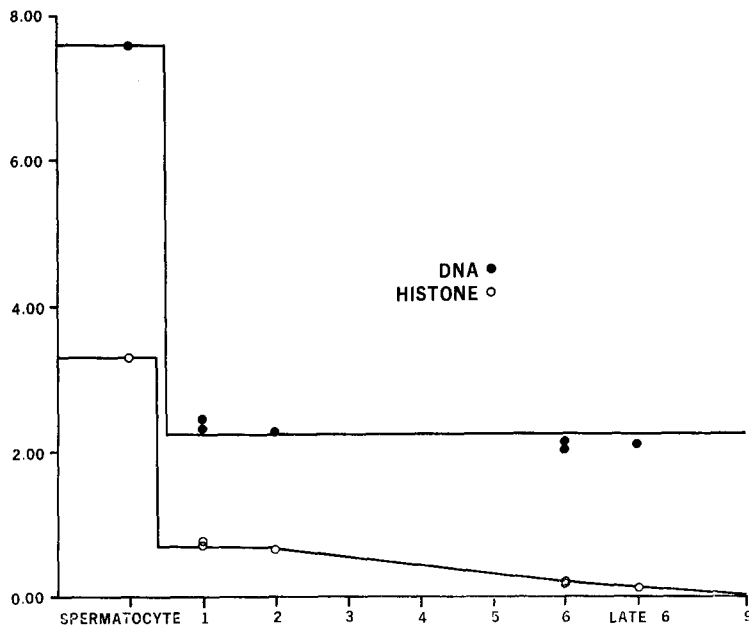

FIGURE 18 Relative DNA content and relative histone content are plotted individually against stage of development for crab spermatocytes and spermatids.

throughout the cytoplasm within stage 1 and 2 spermatids, which contain little stainable acrosomal basic protein. Few grains appear over the nuclei of these cells (Fig. 19). By stage 4, which is a time of considerable increase in acrosomal basic protein content, two classes of labeling patterns are commonly seen following a 25-min chase: labeling about the edges of the developing acro-

some, and labeling throughout the acrosome (Fig. 20). A similar picture is seen for stage 6 spermatids (Fig. 21), although less protein is now being synthesized. Stage 7 spermatids are labeled sparsely, predominantly over the cytoplasm at the edges of the capsule, as is also true for stage 8 spermatids (Fig. 22).

The results of a 24-hr chase clarify the meaning of these data. Stage 4 spermatids are now heavily labeled throughout the acrosomal capsule, suggesting that newly synthesized protein has moved in from the cytoplasm (Fig. 23). Similarly, stage 7 spermatid capsules have become labeled (Fig. 24), presumably due to movement of newly synthesized proteins into the capsule. Stage 8 spermatids continue to show only occasional cytoplasmic labeling (Fig. 25); insufficient chase time has elapsed to allow for movement of the label into their acrosome (or the protein synthesized in stage 8 sperm cytoplasm is nonacrosomal).

Label does chase into stage 8 spermatid acrosomes following longer chase times (Fig. 26), although the minimum chase time has not been determined. It is interesting that no label chases into the mature sperm *nuclei* in these experiments (Fig. 26). This is likely due to the large scale loss of nuclear proteins during spermiogenesis, a loss which is not balanced by new nuclear protein synthesis.

DISCUSSION

The testes of the sand crab are especially suitable for studies involving quantitative photometry, in which readings of many nuclei at each stage of development must be made, in that all of the hundreds of cells occurring in a given testis lobule are at almost precisely the same stage of development. Furthermore, a number of easily recognizable "stages" in spermatid development permit study of nuclear protein metabolism during cell differentiation. Spermatid nuclei remain spherical during spermiogenesis, which is helpful when making quantitative photometric readings using the plug method. Finally, nuclear contents undergo little condensation, so that packing effects on the quantity of dye bound, such as have been reported to occur in maturing bull sperm (Gledhill et al., 1966), are minimized. The effects of crab sperm nuclear histone depletion upon Feulgen photometry have recently been reported (Vaughn and Locy, 1969), and it has been found that this is of no consequence to quantitative Feulgen photometry in this system.

Staging of Spermiogenesis

Staging of spermiogenesis by staining acrosomal polysaccharides with the periodic acid–Schiff method has been used with various species of rodents by Leblond and Clermont (1952 a, 1952 b) and has served to facilitate further detailed studies on protein (including histone) and RNA metabolism during gamete development (Monesi, 1964 a, 1964 b; Monesi, 1965; Vaughn, 1966). During studies on crab histone metabolism, we found it necessary to develop a staging scheme which would serve not only for staging spermatids which had been subjected to various cytochemical procedures (by using adjacent sections), but which would also be useful for determining stage of development by staining sections through developed radioautographic emulsion.

Although the sequence of stages, as presented, appeared intuitively obvious, we have also obtained objective support for the sequence by partially timing spermiogenesis, using thymidine-³H. This simple procedure has been very effectively used to time spermiogenesis in snail (Bloch and Hew, 1960 a), fly (Chandley and Bateman, 1962), rat (Clermont et al., 1959), mouse (Nebel et al., 1961; Monesi 1962), and rabbit (Amann et al., 1965; Swierstra and Foote, 1965). Using this method, the time required for a cell to develop from the last premeiotic DNA synthesis to a given stage in spermiogenesis is given by the time required for labeled DNA to appear in that spermatid after administration of label; the duration of each stage may be estimated from the time necessary for cells, labeled during this last DNA synthesis, to pass through the stage. Only one or two animals were used to determine each time in the present study, which is a possible source of error should the rate of the spermatogenic process vary between organisms. Although the data here reported are only preliminary, they indicate that the sequence of stages is probably correct, and that a nearly mature (stage 7) sperm cell is produced about 29 days after the last premeiotic DNA synthesis (Figs. 13 a, b). This last premeiotic DNA synthesis occurs in large primary spermatocytes (Figs. 10 a, b), whose DNA content at the completion of synthesis (11.9 × 10⁻¹² g) has been measured photometrically, and found to be four times the haploid value, thus confirming their identity (Vaughn and Locy, 1969).

These staging and preliminary timing experiments have facilitated further radioautographic
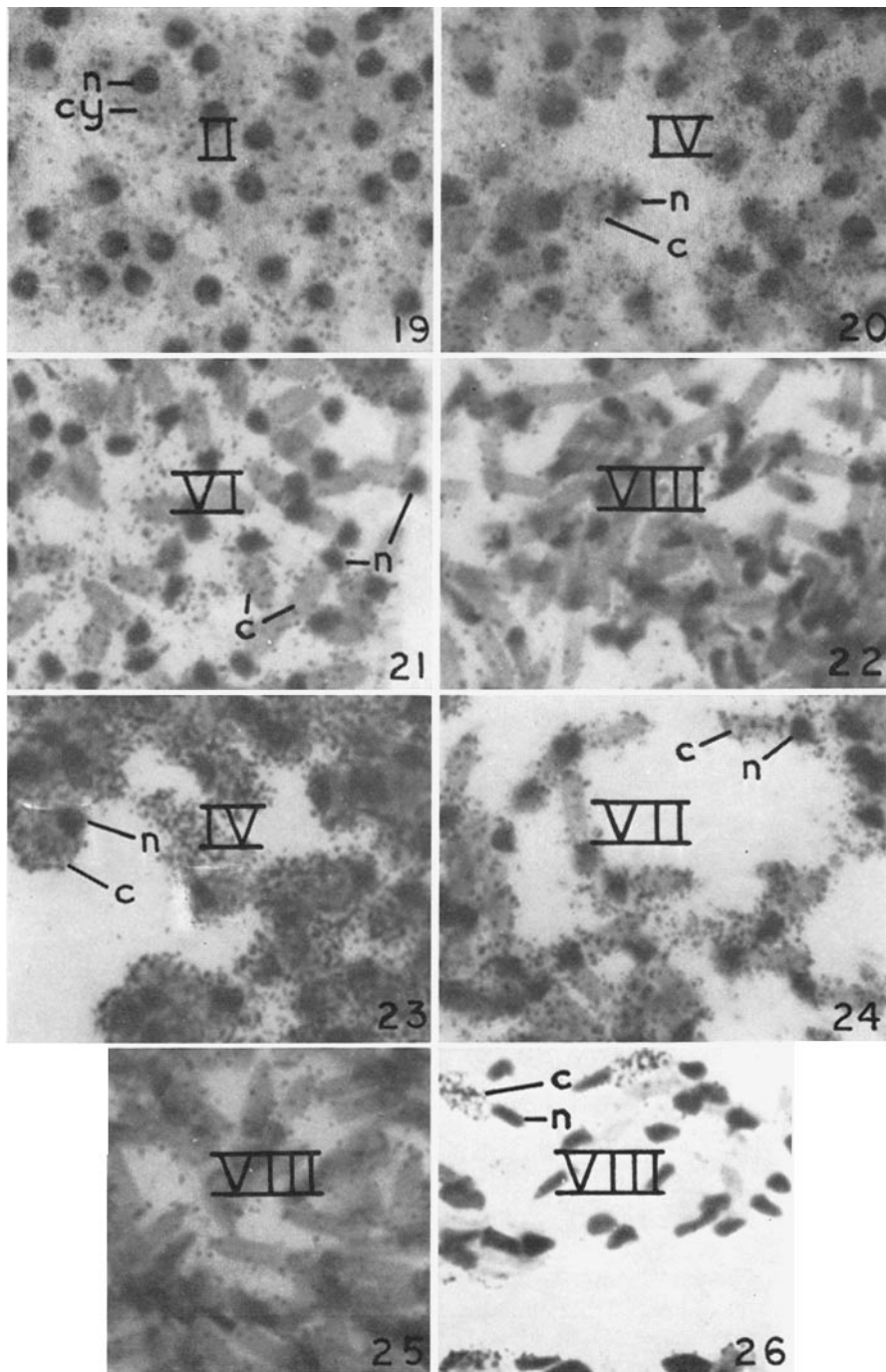
FIGURES 19–26   Lysine-^{3}H labeling of sectioned crab testis lobules is shown at different stages of spermiogenesis, after various "chase" periods following an isotope injection. Sections were stained by the Feulgen technique before application of emulsion, and stained through the emulsion with alkaline fast green after development. All sections of a given chase time are from the same slide, so that grain numbers can be related to quantity of new protein synthesis. Roman numerals denote the stages in spermiogenesis. n, nucleus; cy, cytoplasm; c, acrosomal capsule. $\times$ 1200.

FIGURES 19–22   25-min chase.

FIGURES 23–25   1-day (24-hr) chase.

FIGURE 26   18-day chase.

kinetic studies and photometric analyses of histone metabolism.

Nuclear Protein Changes in Spermiogenesis

The data which have been presented suggest a gradual but continuous loss of nuclear protein-bound lysine + tyrosine throughout crab spermiogenesis. It is evident that stage 9 (spermatophore) sperm contain a low level of protein, a result which contrasts with the conclusions reached by Langreth (1969) for *Cancer* sperm, and by Chevaillier (1966) for *Eupagurus* sperm, in which absence of detectable binding of acid fast green, FDNB, and mercuric bromphenol blue by mature sperm nuclei suggested complete absence of proteins. However, no quantitative measurements were attempted in these studies, and very faint staining could easily have been overlooked. Bloch (1966 *a*, 1966 *b*) has reported that *Emerita* sperm nuclei contain roughly equal amounts of lysine and arginine, as determined cytophotometrically, although the source of these cells (testis or spermatophore) is not given. Direct chemical analysis of isolated sperm chromatin from the spider crab *Libinia emarginata* (Vaughn and Hinsch, 1970, 1971) has also shown that protein is associated with this sperm DNA, although this protein is highly acidic.

Loss of sperm nuclear protein, including histone, has been reported to occur during spermiogenesis in rats (Vaughn, 1966) and in the crab *Eupagurus* (Chevaillier, 1966). In rats, where quantitative measurements were made, the loss is only partly accounted for by loss of nuclear somatic-type histones, the remaining protein loss being due to nonhistone proteins, which are discarded before the histones begin to leave the nucleus. Furthermore, in rats, accompanying the loss of nuclear somatic-type histone is a concomitant gain in gamete-type histones (arginine-rich histone), which may be synthesized in the cytoplasm, as has been reported for grasshopper (Bloch and Brack, 1964) and trout (Ling et al. 1969), but questioned for mouse (Monesi, 1964 *a*), where the synthesis may be nuclear. This exchange of somatic-type histone for basic proteins typical of gametes is of nearly general occurrence in animals, and has also been reported to occur in a few lower plants (for reviews see Bloch, 1966 *b*, 1969). Data presented in the present paper suggest that the spermatid nuclear protein loss during stages 1–2 (Fig. 14) is nonhistone protein, since histone loss lags until after stage 2 (Fig. 17). Much of the crab nuclear

protein loss is due to somatic-type histone, and is most marked during stages 2–4 (Fig. 17). Histone loss is halted during stages 4–6, and accelerates again during stages late 6–9 (Table III, Fig. 17).

The observations that the spherical nuclear morphology of stage 6 testis sperm is easily fixed, whereas stage 7 sperm are more difficult to preserve in formaldehyde (these nuclei always appear elongate in sectioned material, despite the fact that sperm with elongate nuclei are never seen when fresh testis squashes are examined by phase contrast microscopy), that testis stage 8 sperm nuclei appear exploded and degraded in tissue sections (as do sperm nuclei of sectioned spermatophores), and that stage 8 testis sperm and also freeze-fixed spermatophore sperm do not bind histone stains, suggest that these latter nuclei have suddenly lost protein (largely histone, as they no longer bind histone stains). This contention is supported by the data given in Table II and in Fig. 14, which show a large fall in total nuclear protein content between stages late 6 and 9. Loss of histone protein is presumed to be the explanation. In this regard, the mechanism of action of aldehydes as fixatives in morphological studies is believed to be by the formation of methylene bridges between two neighboring protein amino groups (Bowes and Cater, 1965). Brutlag et al., (1969) have recently shown that formaldehyde also joins chromosomal proteins and DNA tightly together, thus affording a further basis for the mechanism of action of aldehydes in fixation. The exploded crab sperm nuclei were not measurable for these reasons, although qualitative visual examination showed lack of binding of alkaline fast green or bromphenol blue among early, well-fixed spermatophore sperm as well. These qualitative observations are recorded as a curve extrapolation in Figs. 17 and 18.

Acrosomal Protein Synthesis

In cytochemical and radioautographic studies of spermatogenesis in the echiuroid worm *Urechis caupo*, it has been reported that stainable acrosomal basic proteins appear (Das et al., 1967), and that their appearance is correlated with uptake of labeled amino acids (Das, 1968), suggesting that new synthesis of acrosomal basic proteins is occurring here.

Our findings suggest that crab acrosomal proteins are not synthesized *in situ*, but rather that they may have a dual origin. Certain of these proteins are synthesized within the surrounding cytoplasm
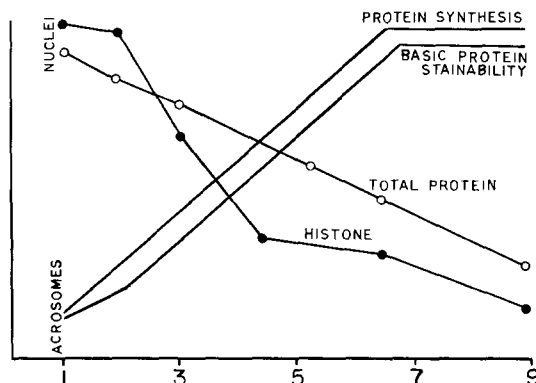
FIGURE 27 Schematic summary of spermatid nuclear and acrosomal protein changes during development, expressed as a function of stage of spermiogenesis. The curves for nuclear protein content are quantitative, whereas those for acrosomal protein are qualitative estimates. An apparent cause-effect relationship exists between loss of nuclear histones and increase of acrosomal basic protein content; acrosomal nonbasic proteins (presumably enzymes in part) are synthesized in cytoplasm and then move into the acrosome. New synthesis of all acrosomal basic proteins appears unlikely, since acid-extractable basic proteins from total sperm (which contain no stainable nuclear basic proteins) move like somatic-type histones in electrophoresis. These proteins come from the acrosome (Vaughn, et al., 1969), and originally were nuclear.

during at least spermiogenic stages 3–6, although cytoplasmic protein synthesis is also going on during stages 1–2, which may also be acrosomal. This conclusion is drawn from labeled lysine incorporation patterns, showing initial labeling about the edges of the acrosome, which is soon chased into the acrosome. The possibility that the crab acrosomal *basic* proteins are not among the proteins being newly synthesized, but instead represent sloughed nuclear somatic histones, has been hypothesized by Chevaillier (1966). This hypothesis was given support when it was observed that acid-extractable basic proteins from *Emerita* spermatophore sperm move like somatic-type histones during electrophoresis (Vaughn et al., 1969). Since the sperm nuclei contain no stainable histones, these "histones" presumably came from the acrosome. However, the possible existence of masked sperm nuclear histones was not ruled out. The kinetic data in the present paper (summarized in Fig. 27) support the idea that displaced nuclear somatic-type histones move to the acrosome during spermiogenesis, although this conclusion is only tentative.

REFERENCES

ALFERT, M., and I. I. GESCHWIND. 1953. A selective staining method for the basic proteins of cell nuclei. *Proc. Nat. Acad. Sci. U.S.A.* 39:991.

AMANN, R. P., H. H. KOEFOED-JOHNSEN, and H. LEVI. 1965. Excretion pattern of labelled spermatozoa and the timing of spermatozoa formation and epididymal transit in rabbits injected with thymidine-^{3}H. *J. Reprod. Fert.* 10:169.

BLOCH, D. P. 1966 a. Histone differentiation and nuclear activity. *Chromosoma.* 19:317.

BLOCH, D. P. 1966 b. Cytochemistry of the histones. *Protoplasmatol. Handb. Protoplasmaforsch.* 5(3d):1.

BLOCH, D. P. 1969. A catalog of sperm histones. *Genetics.* 61 (Suppl.):93.

BLOCH, D. P., and S. D. BRACK. 1964. Evidence for the cytoplasmic synthesis of nuclear histone during spermiogenesis in the grasshopper *Chortophaga viridifasciata* (De Geer). *J. Cell Biol.* 22:327.

BLOCH, D. P., and G. C. GODMAN. 1955. A microphotometric study of the syntheses of desoxyribonucleic acid and nuclear histone. *J. Biophys. Biochem. Cytol.* 1:17.

BLOCH, D. P., and H. Y. C. HEW. 1960 a. Schedule of spermatogenesis in the pulmonate snail *Helix aspersa*, with special reference to histone transition. *J. Biophys. Biochem. Cytol.* 7:515.

BLOCH, D. P., and H. Y. C. HEW. 1960 b. Changes in nuclear histones during fertilization, and early embryonic development in the pulmonate snail *Helix aspersa. J. Biophys. Biochem. Cytol.* 8:69.

BOWES, J. A., and C. W. CATER. 1965. Crosslinking of collagen. *J. Appl. Chem.* 15:296.

BRUTLAG, D., C. SCHLEHUBER, and J. BONNER. 1969.

Properties of formaldehyde-treated nucleohistone. *Biochemistry*. 8:3214.

CHANDLEY, A. C., and BATEMAN, A. J. 1962. Timing of spermatogenesis in *Drosophila melanogaster* using tritiated thymidine. *Nature (London)*. 193:299.

CHEVAILLIER, P. 1966. Contribution a l'étude du complexe ADN-histone dans le spermatozoide du Pagure *Eupagurus bernhardus* L. (Crustace Decapode). *J. Microsc.* 5:739.

CHEVAILLIER, P. 1967. Mise en évidence et étude cytochimique d'une proteine basique extranucleaire dans les spermatozoides des Crustaces Decapodes. *J. Cell Biol.* 32:547.

CHEVAILLIER, P. 1968. Etude cytochimique ultrastructurale des nucleoproteines dans le spermatozoide du Pagure *Eupagurus bernhardus* L. (Crustace Decapode). *J. Microsc.* 7:107.

CLERMONT, Y., C. P. LEBLOND, and B. MESSIER. 1959. Durée du cycle de l'épithelium seminal du rat. *Arch. Anat. Microsc. Morphol. Exp.* 48 (Suppl.): 37.

DAS, N. K. 1968. Developmental features and synthetic patterns of male germ cells of *Urechis caupo*. *Wilhelm Roux' Arch. Entwicklungsmech. Organismen.* 161:325.

DAS, N. K., J. MICOU-EASTWOOD, and M. ALFERT. 1967. Cytochemical and biochemical properties of basic proteins of *Urechis* acrosomes. *J. Cell Biol.* 35:455.

FEULGEN, R., and H. ROSSENBECK. 1924. Mikroskopisch-chemischer Nachweis einer Nucleinsaure vom Typus der Thymonnuclein-saure und die darauf beruhende elektive Farbung von Zellkernen in mikroskopischen Praparaten. *Z. Phys. Chem.* 135:203.

GEORGIEV, G. P. 1969. Histones and the control of gene action. *Annu. Rev. Genet.* 3:155.

GLEDHILL, B. L., M. P. GLEDHILL, R. RIGLER, and N. R. RINGERTZ. 1966. Changes in deoxyribonucleoprotein during spermiogenesis in the bull. *Exp. Cell Res.* 41:652.

HUANG, R. C., and J. BONNER. 1962. Histone, a suppressor of chromosomal RNA synthesis. *Proc. Nat. Acad. Sci. U.S.A.* 48:1216.

LANGRETH, S. G. 1969. Spermiogenesis in *Cancer* crabs. *J. Cell Biol.* 43:575.

LEBLOND, C. P., and Y. CLERMONT. 1952 a. Spermiogenesis of rat, mouse, hamster, and guinea pig as revealed by the "periodic acid-fuchsin sulfurous acid" technique. *Amer. J. Anat.* 90:167.

LEBLOND, C. P., and Y. CLERMONT. 1952 b. Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Ann. N.Y. Acad. Sci.* 55:548.

LING, V., J. R. TREVITHICK, and G. H. DIXON. 1969. The biosynthesis of protamine in trout testis. I. Intracellular site of synthesis. *Can. J. Biochem.* 47: 51.

MONESI, V. 1962. Autoradiographic study of DNA synthesis and the cell cycle in spermatogonia and spermatocytes of mouse testis using tritiated thymidine. *J. Cell Biol.* 14:1.

MONESI, V. 1964 a. Autoradiographic evidence of a nuclear histone synthesis during mouse spermiogenesis in the absence of detectable quantities of nuclear ribonucleic acid. *Exp. Cell Res.* 36:683.

MONESI, V. 1964 b. Ribonucleic acid synthesis during mitosis and meiosis in the mouse testis. *J. Cell Biol.* 22:521.

MONESI, V. 1965. Synthetic activities during spermatogenesis in the mouse. *Exp. Cell Res.* 39:197.

MONNÉ, L., and D. B. SLAUTTERBACK. 1951. The disappearance of protoplasmic acidophilia upon deamination. *Ark. Zool.* 1 (Ser. 2):455.

MOSES, M. J. 1961. Spermiogenesis in the crayfish (*Procambarus clarkii*) II. Description of stages. *J. Biophys. Biochem. Cytol.* 10:301.

NEBEL, B. R., A. P. AMAROSE, and E. M. HACKETT. 1961. Calendar of gametogenic development in the prepuberal male mouse. *Science (Washington).* 134:832.

OLINS, D. E., A. L. OLINS, and P. H. VON HIPPEL. 1968. On the structure and stability of DNA-protamine and DNA-polypeptide complexes. *J. Mol. Biol.* 33:265.

PHILLIPS, D. M. P. (Ed.) 1971. *Histones and Nucleohistones*. Plenum Press, London.

POGO, B., V. G. ALLFREY, and A. E. MIRSKY. 1966. RNA synthesis and histone acetylation during the course of gene activation in lymphocytes. *Proc. Nat. Acad. Sci. U.S.A.* 55:805.

RAAF, J., and J. BONNER. 1968. Properties of *Escherichia coli* deoxyribonucleoprotein as compared with the deoxyribonucleoprotein of higher organisms. *Arch. Biochem. Biophys.* 125:567.

SKALKA, A., A. FOWLER, and J. HURWITZ. 1966. The effect of histones on enzymatic synthesis of RNA. *J. Biol. Chem.* 241:588.

SWIERSTRA, E. E., and R. H. FOOTE. 1965. Duration of spermatogenesis and spermatozoan transport in the rabbit based on cytological changes, DNA synthesis and labelling with tritiated thymidine. *Amer. J. Anat.* 116:401.

SWIFT, H. 1950. The desoxyribose nucleic acid content of animal nuclei. *Physiol. Zool.* 23:169.

VAN SLYKE, D. 1911. A method for the quantitative determination of aliphatic amino groups. *J. Biol. Chem.* 9:185.

VAUGHN, J. C. 1966. The relationship of the "*sphere chromatophile*" to the fate of displaced histones following histone transition in rat spermiogenesis. *J. Cell Biol.* 31:257.

VAUGHN, J. C. 1968 a. Histone metabolism in the crab *Emerita analoga*: Spermiogenesis and early embryogenesis. *J. Cell Biol.* 39 (2, Pt. 2):138 a. (Abstr.)

VAUGHN, J. C. 1968 b. Changing nuclear histone

patterns during development. I. Fertilization and early cleavage in the crab *Emerita analoga*. *J Histochem. Cytochem.* **16:**473.

VAUGHN, J. C., J. CHAITOFF, R. DeLEON, C. GARLAND, and L. THOMSON. 1969. Changing nuclear histone patterns during development. II. Isolation and partial characterization of "decapodine" from sperm cells of the crab *Emerita analoga*. *Exp. Cell Res.* **54:**362.

VAUGHN, J. C., and G. W. HINSCH. 1970. Fractionation and characterization of nuclear and acrosomal components of the sperm of the spider crab, *Libinia emarginata*. *Amer. Zool.* **10:**523.

VAUGHN, J. C., and G. W. HINSCH. 1971. Isolation and characterization of chromatin and DNA from the sperm of the spider crab, *Libinia emarginata*. *J. Cell Sci.* In press.

VAUGHN, J. C., and R. D. LOCY. 1969. Changing nuclear histone patterns during development. III. The deoxyribonucleic acid content of spermatogenic cells in the crab *Emerita analoga*. *J. Histochem. Cytochem.* **17:**591.