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

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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 cytoplasmic 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, [Raf 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**

1. 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 Loeb, 1902) 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 Haw, 1960 b). Lysine residues were occasionally blocked by deamination (Van Slyke, 1911) or acetylation (Monné and Slatteback, 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. Staining 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.

2. 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 nearly 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 to-
etgether. Test 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 ap-
propriate wavelength and $r$ is the radius of the nucleus 
in question (Swift, 1950).

Protein-bound nuclear lysine + tyrosine was de-
termined 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 $a$; as utilized by Vaughn, 1966).
Lysine + tyrosine values were calculated from the 
absorption readings taken at 402 ± 5 nm, using 
equations described elsewhere (Vaughn, 1966), 
employing the crab haploid DNA value and Feulgen 
extension coefficient derived in our laboratory 
(Vaughn and Locy, 1969), and the extinction coeffi-
cient for FDNB (1.84 $\times$ 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 pre-
cursor. 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 solu-
tion. The crabs were maintained in the laboratory 
aquaria on a diet of yeast. Tissues were removed and 
fixed at times ranging from 1 to 29 days. Tissue sec-
tions 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 contain-
ing 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 chroma-
tin, 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 re-
established a nearly spherical symmetry; the nu-
clear vacuoles are now much reduced. Consider-
able 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 cyto-
chemically definable nuclear histones and prot-
amines. The cells shown in Fig. 9 were prepared by 
a dry-ice squash method described elsewhere

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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. X 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 spermatogenesis to be completed. These data show that although

| Days after injection* | 1 | 3 | 5 | 6 | 8 | 10 | 11 | 16 | 23 | 29 |
|-----------------------|---|---|---|---|---|----|----|----|----|----|
| Stage labeled         |   |   |   |   |   |    |    |    |    |    |
| Spermatids            |   | 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. X 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. × 1730.
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 dramatically during spermiogenesis. Values for spermatid nuclear protein lysine + tyrosine range from about $0.60 \times 10^{-14}$ moles (stage 1) to $0.11 \times 10^{-14}$ 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

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### Table II

FDNB-Feulgen Double-Stain Data on Spermatid Nuclei*

| Stage of spermiogenesis | Number of nuclei measured | Mean ratio: E$_{402}$/E$_{450}$ | Moles nuclear lysine + tyrosine ($X 10^{-14}$)‡ |
|-------------------------|---------------------------|-------------------------------|-----------------------------------------------|
| Stage 1 spermatid (early) | 31                         | 0.64 $\pm$ 0.02               | 0.60 $\pm$ 0.02                               |
| Stage 2 spermatid (early) | 28                         | 0.52 $\pm$ 0.02               | 0.49 $\pm$ 0.02                               |
| Stage 3 spermatid        | 31                         | 0.52 $\pm$ 0.02               | 0.49 $\pm$ 0.02                               |
| Stage 4 spermatid (acrosome) | 30                       | 0.40 $\pm$ 0.02               | 0.37 $\pm$ 0.02                               |
| Stage 5 spermatid (elongating) | 28                   | 0.32 $\pm$ 0.02               | 0.30 $\pm$ 0.02                               |
| Stage 6 spermatid (elongating) | 32                   | 0.24 $\pm$ 0.01               | 0.22 $\pm$ 0.01                               |
| Stage 9 spermatozoa      | 30                         | 0.12 $\pm$ 0.01               | 0.11 $\pm$ 0.01                               |

* Standard errors are given.

‡ Calculated from formulas cited in Materials and Methods.
TABLE III

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

| Stage of spermatogenesis | Number of nuclei measured | \(E_{1C}\) | \(E_{4C}\) | DNA (\(E_{4C}\)-r\(^{-1}\)) | Histone (\(E_{4C}\)-r\(^{-1}\)) | 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 | -- -- | -- -- | -- -- |
| Stage 6 spermatid        | 31                        | 0.88 ± 0.02 | 0.11 ± 0.01 | 1.5 ± 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 | -- -- | -- -- | -- -- |
| Stage 4 spermatid        | 33                        | 1.11 ± 0.01 | 0.11 ± 0.01 | -- -- | -- -- | -- -- |
| Stage 5 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 (late) | 35                        | 1.08 ± 0.03 | 0.09 ± 0.01 | 2.1 ± 0.03 | 0.2 ± 0.01 | 0.08 ± 0.01 |

* Standard errors are given.

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

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.

FIGURE 15 Frequency distribution curves for relative amount of DNA (lower curve) and histone (upper curve) in crab stage 2 spermatids and primary spermatocytes.

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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 nucleus 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-1H. 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 (Aman 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 inspermiogenesis 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-3H 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. × 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 (1966a, 1966b) has reported that Emeritia 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, 1964a), 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, 1966b, b). 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 I 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 echinoid 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.

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.

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