brought about by insect depredations. A dipterous insect which deposits its egg in the ovary of *Opuntia Lindheimeri* invariably causes a reversion to the vegetative condition. The ovules become atrophied, the funiculi (?) develop into short, cylindrical, curved and twisted structures and the ovary remains green. So far does the ovary change to the vegetative condition that it very commonly gives rise to joints, so that we have joints developing from imperfect but good-sized fruits." Griffiths (1906) also reported that an insect caused sterility in ovaries of *O. versicolor* and that occasionally new stem joints grew from the sterile ovaries of this species.

Polyembryony was reported in *Opuntia vulgaris* Gray (= *O. Compressa* (Salisb.) Macbr.) by Ganong in 1898. He reported that "when the seeds are planted, from many, perhaps a half, of them more than one seedling comes up . . . ." In our germination studies, only 1.2% of the seeds planted in 1970 that germinated had double embryos.

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A CYTOCHEMICAL STUDY OF BASIC PROTEINS IN SPERMATOGENIC CELLS OF *DROSOPHILA VIRILIS*

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ABSTRACT

By means of cytochemical techniques, the basic proteins of the spermatogenic cells of *Drosophila virilis* were studied. The spermatogonia, spermatocytes, and spermatids were found to possess a lysine-rich histone in association with DNA. A transition from the lysine-rich histone to a histone rich in arginine was shown to occur during the final stages of maturation of the sperm. There was no subsequent shift to a protamine in the mature sperm.

Basic proteins occurred in the cytoplasm of spermatogonial through spermatid stages. These cytoplasmic basic proteins were rich in the basic amino acid lysine and gave evidence of being bound to RNA. They are believed to represent structural proteins of ribosomes.

INTRODUCTION

During the terminal stages of spermatogenesis in many organisms, there is a transition from the somatic type histones to histones rich in the basic amino acid arginine. This change is sometimes followed by a further alteration to a protamine in the spermatozoa. The time of the histone transition is constant in any one species, but variable among different species (Bloch, 1969). McMaster-Kaye and Kaye (1976) have shown that the basic proteins of mature sperm from the house cricket are electrophoretically heterogeneous. Although the function of these sperm histones is unknown, it is most frequently suggested that they are involved in gene repression and packaging the DNA into the spermatozoa. However, Bloch (1969), Subirana (1970), and

Vaughn and Hinsch (1972) have questioned these hypotheses.

This study was undertaken to describe the nature of the histone transitions occurring in *Drosophila virilis* during spermatogenesis.

MATERIALS AND METHODS

The *D. virilis* stock (University of Texas strain 1801.1) used in this study was maintained on standard banana-agar-malt-yeast medium at a temperature of 20 to 21° C. All flies used in the experiments were collected within one hour after eclosion and placed on fresh food. Males were fixed and embedded at 2.5 to 5 hours and 12 hours after emergence and daily thereafter for 8 days. Seven- and 14-day-old female flies were also fixed and embedded for studying the spermatozoa contained within the spermathecae.

The flies were etherized and the abdomens were quickly removed and opened in the mid-ventral line. The abdomens were then fixed in 10% neutral buffered formalin for 8 hours or 5% phosphate buffered glutaraldehyde at pH 7.2 for 1.5 hours. After washing and dehydration, the tissue was embedded in 56-57° C Paraplast and sectioned at 10 microns.

The presence of histones was demonstrated using both the alkaline fast green procedure of Alfert and Geschwind (1953) and the bromophenol blue procedure of Bloch and Hew (1960). To distinguish between arginine-rich and lysine-rich histones, the deamination procedure of Van Slyke (1911) and the acetylation method of Moone and Slautterback (1951) were used to selectively eliminate staining due to the amino-groups of lysine.

RNA was selectively removed from some sections prior to staining by treating them with 10% perchloric acid at 4° C for 12 hours (Kasten, 1965).

RESULTS

All germ cell nuclei exhibited staining after extraction of the DNA with either trichloroacetic or picric acid (Table 1). However, when the staining was performed without prior acid hydrolysis to remove the DNA, no staining occurred in any spermatogenic cells. Deamination or acetylation prior to staining resulted in a complete loss of nuclear stainability in all spermatogenic stages up to the maturing sperm (Fig. 1, stages 1 and 2). In addition, only a small portion of the sperm bundles in the adult testis continued to stain following deamination or acetylation, whereas numerous ones had stained in those sections not subjected to lysine blockage. The mature sperm bundles were unaffected by either deamination or acetylation and continued to stain intensely as expected if they contained histone rich in arginine (Fig. 1, stages 3 and 4). Sperm contained within the seminal vesicle of the male and within the spermathecae of the female also continued to stain intensely with alkaline fast green or bromophenol blue after deamination or acetylation (Fig. 1, stage 5). The lack of effect of deamination or acetylation on the stainability of the maturing sperm and the mature sperm indicated that they contained arginine-rich histone.

Nucleolar staining with bromophenol blue occurred in those spermatogenic cells which had been previously hydrolyzed with picric acid (Fig. 1, stage 6), but was absent following deamination or acetylation. No nucleolar staining occurred in any spermatogenic cells stained with alkaline fast green and constituted the major difference observed between alkaline fast green

TABLE 1: *Summary of nuclear basic protein staining.*

Spermatogenic Cell	Fast Green	Fast Green after Deamination or Acetylation	Bromophenol Blue	Bromophenol Blue after Deamination or Acetylation
Spermatogonia	+	−	+	−
Primary Spermatocytes	+	−	+	−
Secondary Spermatocytes	+	−	+	−
Spermatids	+	−	+	−
Spermatozoa, Immature	+	−	+	−
Spermatozoa, Maturing	+	+	+	+
Spermatozoa, Mature				
Testis	+	+	+	+
Seminal Vesicle	+	+	+	+
Spermatheca	+	+	+	+

and bromophenol blue when staining for nuclear basic proteins.

Spermatogenic cells stained with alkaline fast green or bromophenol blue after removal of both nucleic acids with trichloroacetic acid or picric acid showed an intense cytoplasmic staining (Fig. 1, stage 6). The staining was most intense in the spermatogonia and the spermatocytes and decreased in intensity in the spermatids.

When the sections were deaminated or acetylated prior to staining, both alkaline fast green and bromophenol blue stainability was completely abolished in the cytoplasm of all spermatogenic stages. The lack of staining after lysine blockage indicated that the cytoplasmic basic proteins were rich in the basic amino acid lysine. Sections which received no trichloroacetic acid or picric acid treatment prior to staining completely lacked cytoplasmic staining.

When only the RNA was removed from the sections by cold perchloric acid, the cytoplasmic staining was essentially similar to that obtained when both nucleic acids were extracted by hot trichloroacetic acid or picric acid treatment, but was somewhat less intense (Fig. 1, stages 7 and 8). The spermatogonia and spermatocytes were again found to stain more intensely, but cytoplasmic staining in the spermatid stages was very light to absent. Sections stained without removal of the RNA were completely unstained. Following removal of the RNA with perchloric acid, the nucleolus remained unstained with alkaline fast green, but stained when bromophenol blue was used.
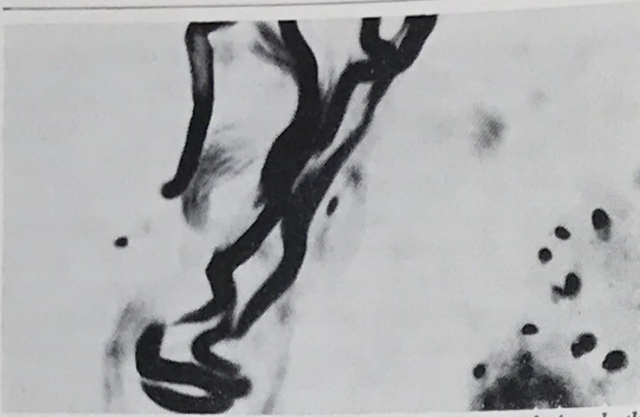
FIG. 1, Stage 1: *Maturing sperm bundles stained for both lysine-rich and arginine-rich histones. Formalin fixation. Alkaline fast green. (x 970).*

FIG. 1, Stage 2: *Maturing sperm bundles stained for arginine-rich histones after deamination with nitrous acid. Formalin fixation. Alkaline fast green. (x 970).*
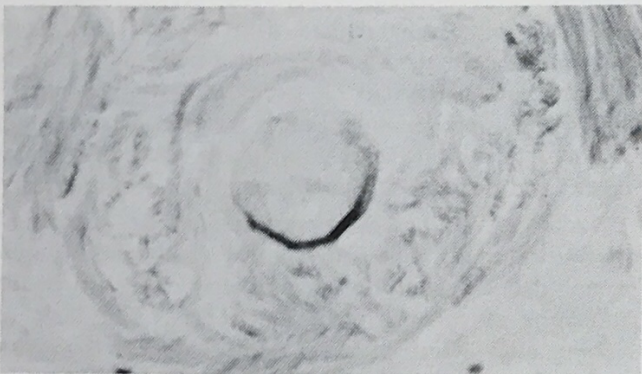

FIG. 1, Stage 3: *Mature sperm bundle stained for both lysine-rich and arginine-rich histones. Formalin fixation. Bromophenol blue. (x 970).*
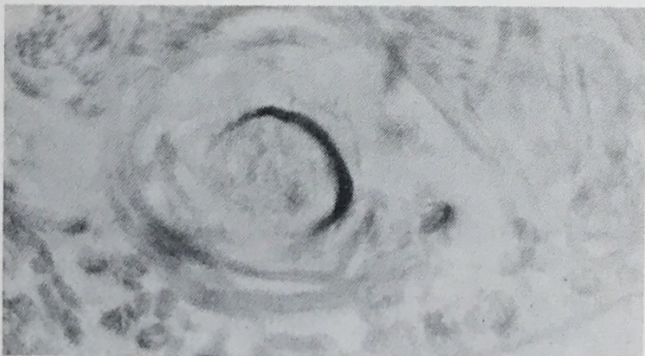

FIG. 1, Stage 4: *Mature sperm bundle stained for arginine-rich histones after deamination with nitrous acid. Formalin fixation. Bromophenol blue. (x 970).*
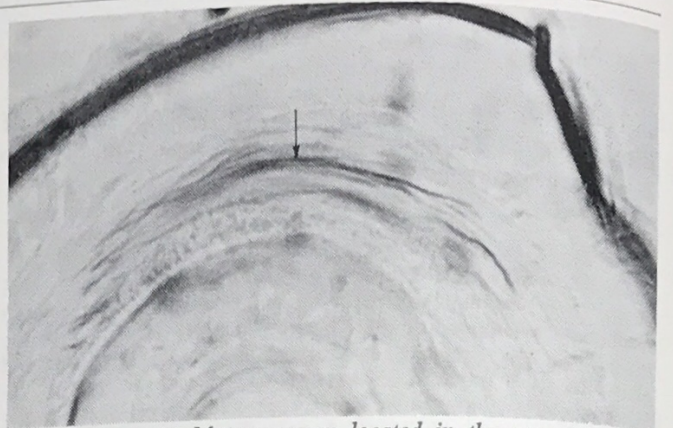

FIG. 1, Stage 5: *Mature sperm located in the spermatheca of a 7-day old female fly stained for arginine-rich histones (arrow) after deamination with nitrous acid. Formalin fixation. Alkaline fast green. (x 970).*
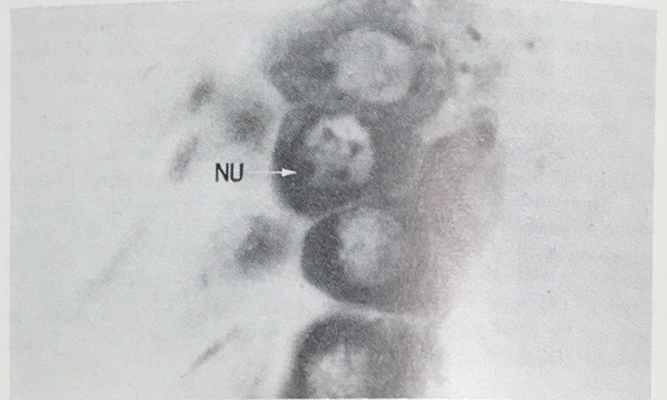

FIG. 1, Stage 6: *Advanced growth primary spermatocytes showing histone distribution and nucleolar (NU) staining. Formalin fixation. Bromophenol blue. (x 970).*
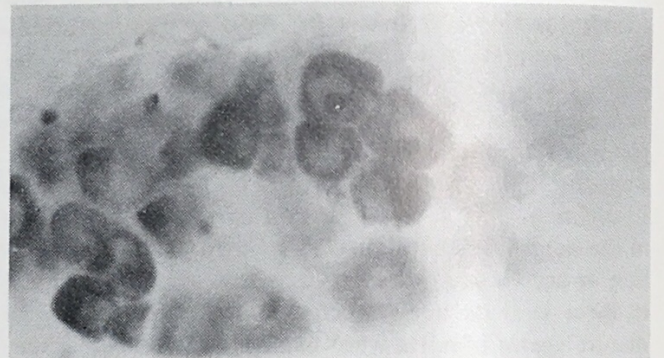

FIG. 1, Stage 7: *Primary spermatocytes stained for cytoplasmic basic proteins after removal of RNA. Formalin fixation. Bromophenol blue. (x 970).*
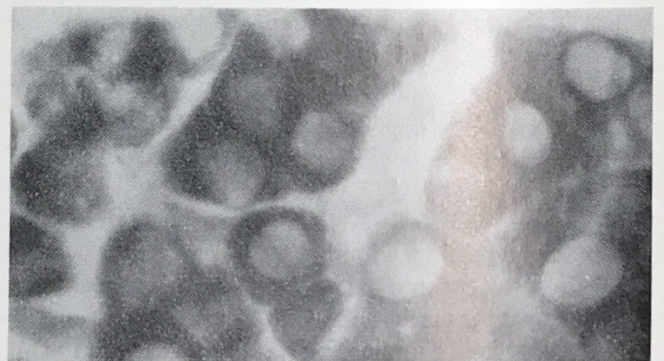

FIG. 1, Stage 8: *Primary spermatocytes showing the presence of cytoplasmic basic proteins. Formalin fixation. Alkaline fast green. (x 970).*

DISCUSSION

The failure of all spermatogenic stages earlier than the maturing spermatozoa to stain following lysine blockage with nitrous acid or acetic anhydride indicated that these early stages possessed the normal complement of somatic histones, whereas the maturing and the mature sperm possessed histone rich in the basic amino acid arginine. Light microscopy revealed no detectable differences between the immature and the maturing spermatozoa; however, they did differ in the type of basic protein found in association with DNA. The immature sperm still possessed a lysine-rich histone, whereas the transition to an arginine-rich histone had occurred in the maturing sperm. This seems to indicate that the histone transition occurred rather abruptly during the process of sperm maturation in *D. virilis,* just as had been reported for *D. melanogaster* (Das et al., 1964). Furthermore, since the spermatozoa contained within the spermathecae of the female stained just as intensely as did those in the male testis, the basic protein of the mature spermatozoa was a histone and not a protamine. Since protamines are soluble in TCA, they would have been removed during hydrolysis and the sperm would not have stained (Alfert, 1956). Consequently, no shift to a protamine occurred in the mature sperm. According to the criteria of Bloch (1969), the sperm of *D. virilis* can be placed into the mouse and grasshopper category. This type of sperm contains basic proteins that are very rich in arginine but are more complex than the protamines.

Although the reason for the histone transition during spermatogenesis is unknown, it has been suggested that the more basic sperm histones are involved in gene repression. Marushige and Dixon (1969) reported a decrease in template activity of chromatin which paralleled the replacement of histones by protamines during sperm maturation in the trout testis. However, Olivieri and Olivieri (1965) and Gould-Somero and Holland (1974) have reported that no transcription occurs in *D. melanogaster* beyond the spermatocyte stage. Thus, the genes of *Drosophila* are already repressed before the histone transition occurs. In addition, Vaughn and associates (Vaughan et al., 1969; Vaughn & Hinsch, 1970, 1972) have found that the sperm DNA of many decapod crustaceans has no basic proteins associated with it. Subirana (1970) has also reported that virtually no change in histones occurs during sperm maturation in the sea cucumber, and Verma (1972) has demonstrated that the histones of the honeybee sperm are similar to those of somatic cells. Although the gamete-type basic proteins may function to inhibit gene activity, it may not be their most essential role since gene repression can occur in sperm in the absence of any basic proteins or without replacement of the somatic-type histones by more basic gamete-type proteins. Bloch (1969) has pointed out that the basic nuclear proteins of sperm are highly "nonconservative" in contrast to the somatic histones and that a wide variation in basic sperm proteins occurs among the different classes of

organisms. Vaughn and Hinsch (1972) have suggested that there may exist a common mechanism of gene repression in all sperm that is not directly related to the type of histone or protamine present in the spermatozoa.

The fact that bromophenol blue was found consistently to stain the nucleolus of spermatocytes and spermatids contrasted sharply with the lack of nucleolar staining in similar spermatogenic stages following treatment with alkaline fast green. Bloch (1966) suggested that the difference in staining behavior might be due to differences in the sensitivities of the dyes. When Gifford and Dengler (1966) tested the effect of various chemical fixatives upon alkaline fast green stainability of histones in plant tissues, they found a lack of nucleolar staining with all chemical fixatives. However, they found the nucleolus to stain readily with alkaline fast green in freeze-dried tissue. Nucleolar staining by alkaline fast green has been reported also in the ovarian nurse cells (Davenport & Davenport, 1966b) and the salivary gland cells (Horn & Ward, 1957) of *D. virilis.* Studzinski (1965), using zinc to demonstrate nucleolar basic proteins, has shown that the basic proteins of the nucleolus are not in firm combination with other cellular components. In addition, Whitfield et al. (1964) demonstrated the presence of a trichloroacetic acid extractable nucleolar histone in rat thymocytes. Trichloroacetic acid extraction might, therefore, remove any basic proteins in association with the nucleolus of *D. virilis.*

The cytoplasm of the spermatogenic cells of *D. virilis* is characterized by the presence of basic proteins which are detectable with cytochemical procedures. The occurrence of such cytoplasmic basic proteins has been reported in the oocytes of molluscs (Davenport & Davenport, 1965a), insects (Davenport & Davenport, 1966b), echinoderms (Davenport & Davenport, 1966a), ascidians (Davenport & Davenport, 1965b), and amphibians (Horn, 1962). Extranuclear basic proteins have been reported in the spermatogenic cells of only a limited number of species. The sperm capsule of several decapod crustaceans was found to contain basic proteins (Vaughn et al., 1969; Langreth, 1969). However, these capsular basic proteins represented sloughed nuclear histones. Vaughn (1966) observed basic proteins rich in lysine in the sphere chromatophile of the rat sperm. The basic proteins of the sphere chromatophile were shown to represent histones sloughed from the spermatid nucleus at the time of transition to an arginine-rich histone. In addition, Das et al. (1967) described arginine- and lysine-rich proteins in the acrosome of *Urechis caupo.* However, the cytoplasmic basic proteins observed in *D. virilis* were not found in the acrosome of the spermatozoa, but were present in the cytoplasm of spermatogonia through spermatids. These cytoplasmic basic proteins do not represent sloughed nuclear proteins, since the transition from a lysine-rich to an arginine-rich nuclear histone does not occur until the latter stages of spermiogenesis.

The use of hot trichloracetic acid or picric acid to

extract both nucleic acids or perchloric acid to remove only RNA indicated that the cytoplasmic staining can in large part be attributed to basic proteins associated with RNA. The cytoplasmic basic proteins of oocytes (Davenport & Davenport, 1965a, 1965b, 1966a) and mouse fibroblasts (Whitefield & Youdale, 1965) have been shown to be associated with RNA as a nucleoprotein complex. Since the cytoplasmic staining was completely abolished by deamination or acetylation, the basic proteins of the cytoplasm of the spermatogenic cells appear to be rich in the basic amino acid lysine. The cytochemically detectable basic proteins of the cytoplasm may represent structural proteins of the ribosomes. Several biochemical investigations have indicated that the ribosomes of somatic cells are rich in the basic amino acids arginine and lysine (Crampton & Petermann, 1960; Wang, 1962; Lindsay, 1966). These ribosomal proteins are similar to nuclear histones in isoelectric point, composition, and molecular weight.

Summary

During spermatogenesis in *D. virilis*, the transition from a lysine-rich to an arginine-rich histone occurred during the process of sperm maturation. There was no subsequent shift to a protamine in the mature sperm.

Basic proteins were demonstrable in the nucleolus with the bromophenol blue technique, but not with the alkaline fast green procedure. These nucleolar proteins were probably labile and thus extracted during hydrolysis with trichloroacetic acid.

Basic proteins occurred in the cytoplasm of spermatogonial through spermatid stages. These cytoplasmic basic proteins appeared to be associated with RNA and may represent structural proteins of ribosomes.

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