

# SYNTHETIC ACTIVITIES DURING SPERMATOGENESIS IN THE LOCUST

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## ABSTRACT

Isolated testes of the locust *Schistocerca gregaria* were immersed in solutions of tritiated thymidine, cytidine, uridine, or arginine for short periods to study nucleic acid and protein synthesis during spermatogenesis. DNA synthesis in this tissue is completed prior to initiation of meiosis. Protein synthesis continues throughout the whole meiotic cycle as well as during spermatid development. Meiotic cells, except those in metaphase through early telophase, and early spermatids are also actively synthesizing RNA. The heteropycnotic X-chromosome does not produce RNA at any stage of spermatogenesis. The rates of protein and particularly RNA synthesis decrease as chromosome condensation progresses. Depression of RNA synthesis, however, is not always accompanied by cytologically detectable condensation of chromatin, since very little or no RNA is synthesized in spermatids in which chromatin condensation has barely begun.

Most of the earlier studies on synthetic activities in meiotic tissues were concerned with DNA synthesis. With a few exceptions (Sparrow *et al.*, 1952; Ansley, 1957; Wimber and Prensky, 1963), it has been shown in most such studies that DNA synthesis is completed before the onset of meiotic prophase (see Swift, 1950; Taylor, 1958 and 1959; Lima-de-Faria and Borum, 1962; Monesi, 1962). RNA and protein synthesis during meiosis in plants has been studied by Taylor (1958 and 1959) by labeling cells for about one day with  $P^{32}$ , orotic acid- $C^{14}$ , cytidine- $H^3$ , or glycine- $C^{14}$ . He (1959) detected labeled RNA in the cytoplasm as well as in the nuclei of microsporocytes up to leptotene. Cells at later stages of prophase incorporate RNA precursors, at a reduced rate, only in the nuclei. The synthesis of protein, as judged by uptake of glycine- $C^{14}$ , also occurs during meiotic prophase, but the rate decreases considerably as prophase progresses. These results from studies on RNA and protein synthesis were obtained, for the most part, by the use of relatively unspecific precursors and after long exposure of cells to the precursors.

The present paper deals with a quantitative radioautographic study of RNA and protein synthesis during spermatogenesis in the locust after cells had been labeled for a short time with the tritiated RNA precursor uridine or cytidine, or with the tritiated amino acid arginine. In addition, the period of DNA synthesis in the locust testis has been checked by using thymidine or cytidine labeled with tritium. While our study was in progress, Henderson (1963) published a brief note presenting qualitative observations of RNA synthesis in the locust. Our current findings on RNA synthesis corroborate and extend those of Henderson.

## MATERIAL AND METHODS

Tubules of the desert locust (*Schistocerca gregaria*) testis were incubated, after dissecting away most of the fat, in Hoyle's solution with added thymidine-methyl- $H^3$  (250  $\mu\text{c}/\text{ml}$ ; sp. act. 6.7 c/mm), cytidine- $H^3$  (250  $\mu\text{c}/\text{ml}$ ; sp. act. 1.0 c/mm), uridine- $H^3$  (250  $\mu\text{c}/\text{ml}$ ; sp. act. 1.25 c/mm), or L-arginine- $H^3$ -HCl (125  $\mu\text{c}/\text{ml}$ ; sp. act. 1.05 c/mm). Samples were collected at various times from 3 minutes to 2 hours.

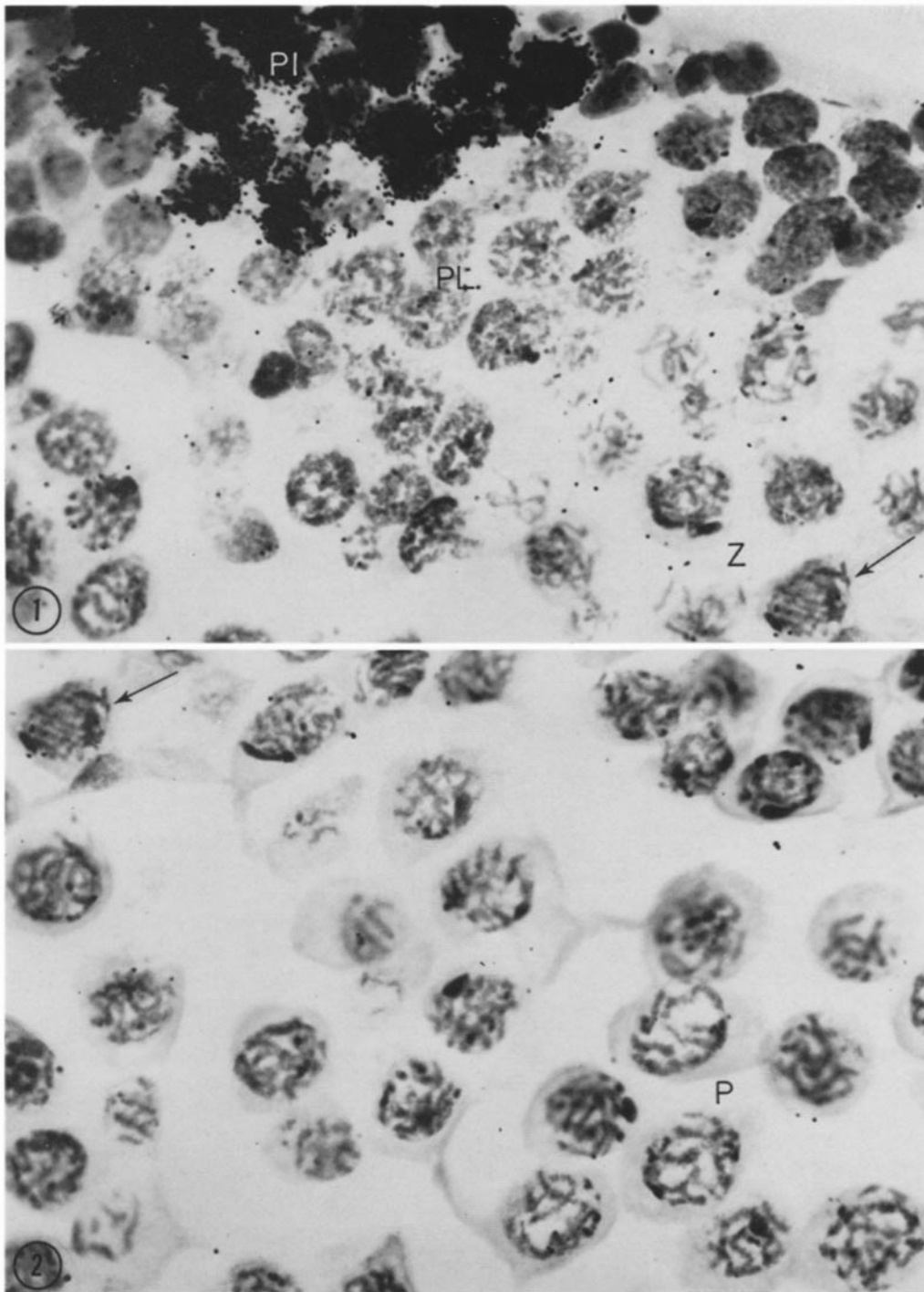


FIGURE 1 Incorporation of thymidine- $H^3$  ( $250 \mu\text{c}/\text{ml}$ ) for 2 hours in the premeiotic interphase (*PI*) nuclei in locust testis. Note that thymidine- $H^3$  is not incorporated into preleptotene (*PL*) and zygotene (*Z*) cells.

FIGURE 2 Absence of incorporation of thymidine- $H^3$  in pachytene (*P*) cells.

Figs. 1 and 2 were obtained from different parts of the same tubule; the arrows point to the same cell. The radioautographs of Feulgen-stained sections ( $5 \mu$  thick) were exposed for 7 days. Cells fixed in acetic-alcohol.  $\times 1240$ .

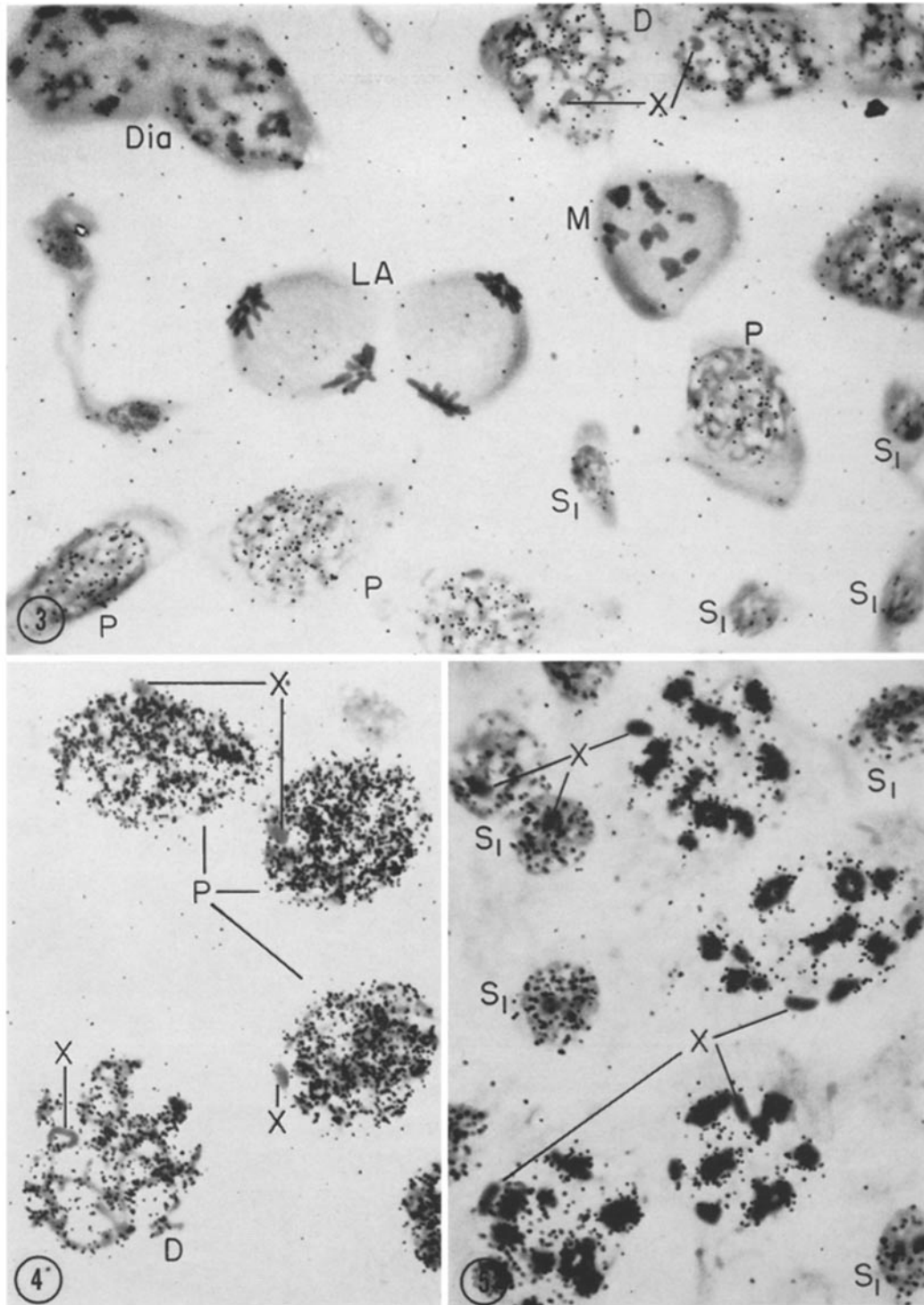


FIGURE 3 Incorporation of uridine- $H^3$  ( $250 \mu\text{c}/\text{ml}$ ) for 12 minutes into RNA of meiotic prophase and early spermatids only. *P*, pachytene; *D*, early diplotene; *Dia*, diakinesis; *M*, metaphase I; *LA*, late anaphase I; *S*<sub>1</sub>, early spermatids; *X*, sex chromosome.  $\times 660$ .

FIGURES 4 AND 5 Differential labeling of autosomes and the X-chromosome in meiotic prophase cells which were exposed for 2 hours to cytidine- $H^3$  ( $250 \mu\text{c}/\text{ml}$ ). Both figures show heavily labeled autosomes and the unlabeled X-chromosome. *P*, pachytene; *D*, early diplotene; *S*<sub>1</sub>, early spermatids. Autoradiographs in Figs. 3 to 5 were exposed for 21, 5, and 2 days, respectively. Acetic alcohol-fixed cells were stained with hematoxylin.  $\times 950$ .

Tubules exposed to thymidine- $H^3$ , cytidine- $H^3$ , or uridine- $H^3$  were fixed in acetic-alcohol, while tubules exposed to arginine- $H^3$  were fixed in 10 per cent neutral formalin. Squashes or paraffin sections (2 or 5  $\mu$  thick) were made and radioautographs prepared by dipping the slides in liquid emulsion (Eastman Kodak, NTB2). Some slides were digested with DNase, RNase, or both enzymes, prior to application of emulsion. Samples in which labeling of DNA or protein was to be detected were stained with the Feulgen reaction before coating with emulsion, while samples in which RNA labeling was to be studied were stained with Harris' alum hematoxylin through the processed emulsion. No appreciable loss of label from proteins occurred when formalin-fixed samples were subjected to the Feulgen staining procedure (see also Mattingly, 1963). Feulgen-stained cells were counterstained with fast green through the processed emulsion in order to make cell outlines visible.

The rates of RNA and protein synthesis were determined by counting silver grains over sectioned (5  $\mu$  in thickness) or squashed cells labeled for 30 minutes with uridine- $H^3$  and arginine- $H^3$ , respectively. Counts were expressed per nuclear section or per cell area and per unit area (100  $\mu^2$ ) after correcting for the background grains which were usually few. Measurements of nuclear or cell area were made on camera lucida drawings. Although the tubules were immersed in Hoyle's solution containing the precursors from 3 minutes to 2 hours, active synthesis of nucleic acids and proteins in most of the tubules continued through the entire period. Following a 2-hour exposure to labeled precursors, appreciable movement of cells from one stage to another did not occur since no difference in the pattern of labeling of various cell stages was seen between samples collected at 3 to 6 minutes and at 2 hours.

There are some uncertainties in the identification of various meiotic stages, especially various stages of

prophase, in the radioautographs. Such difficulties were overcome by examining alternate sections stained with the Feulgen reaction and fast green and not subjected to radioautography. Following formalin fixation, however, it was difficult to distinguish between cells in leptotene and zygotene stages even in preparations not coated with emulsion. These cells, therefore, were grouped as "early prophase" (see Table II).

## RESULTS

Some of the premeiotic interphase nuclei become labeled following exposure of tubules to thymidine- $H^3$  for 30 minutes to 2 hours (Fig. 1). Likewise, tubules immersed in cytidine- $H^3$  for 1 and 2 hours, then treated with RNase and followed by Feulgen staining prior to coating with emulsion, show the same labeling pattern. That these nuclei, labeled with thymidine- $H^3$  or cytidine- $H^3$ , had been in the process of DNA synthesis is evident from the virtual absence of radioactivity following DNase digestion. No incorporation of thymidine- $H^3$  or cytidine- $H^3$  into DNA of meiotic prophase nuclei is seen even after exposing the emulsion for a period sufficient to produce a heavy grain density over DNA-synthesizing premeiotic interphase nuclei (Figs. 1 and 2), thus confirming the results of most earlier studies (see Swift, 1950; Plaut, 1953; Taylor, 1958 and 1959; Lima-de-Faria and Borum, 1962; Monesi, 1962). In the radioautographic preparations of tubules exposed up to 2 hours to another batch of thymidine- $H^3$ , an over-all labeling of many cells, including those in prophase, occurred. This labeling, however, was not confined to nucleic acids, since treatment of cells with DNase or RNase followed by the acid hydrolysis used in Feulgen staining did not appreciably affect

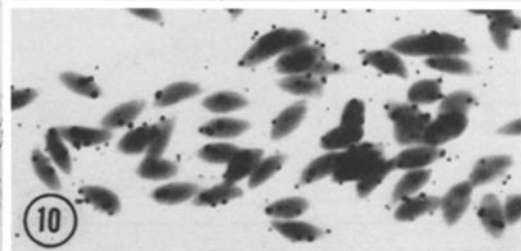
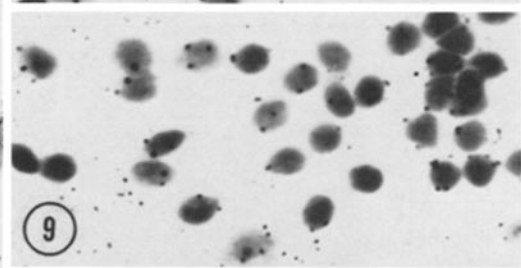
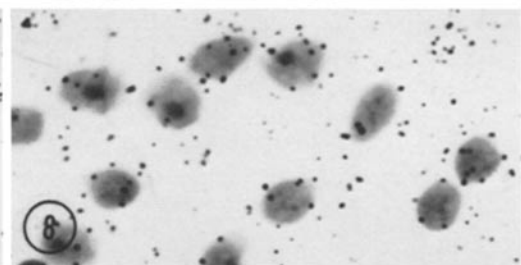
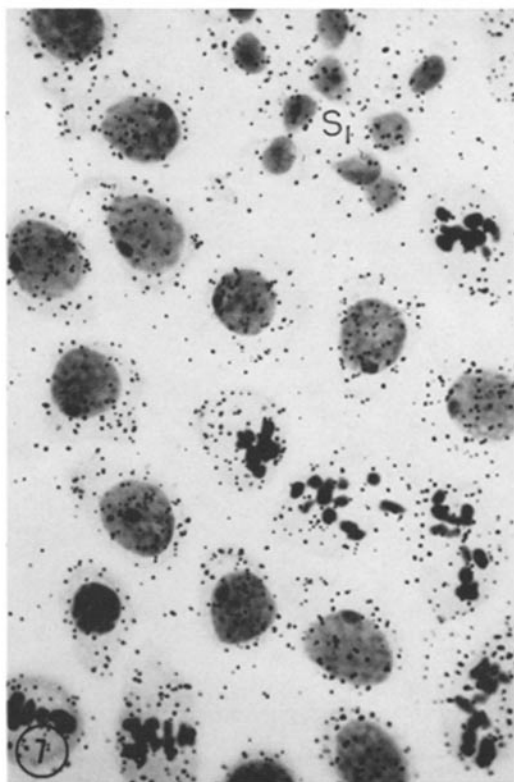
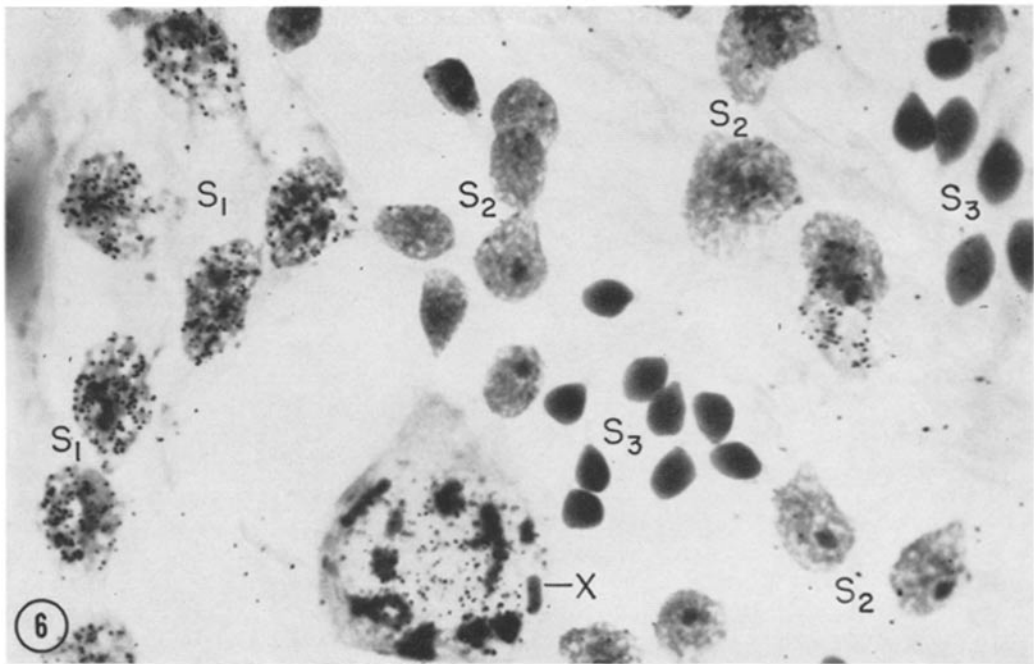
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FIGURE 6 Incorporation of cytidine- $H^3$  (250  $\mu\text{c}/\text{ml}$ ) for 2 hours into RNA of spermatids.  $S_1$ , early spermatids;  $S_2$ , spermatids in which nuclear condensation has barely begun;  $S_3$ , condensing spermatids. Note that only early spermatids are labeled. Radioautographs of acetic alcohol-fixed squashed cells were exposed for 2 days and stained with hematoxylin.  $\times 950$ .

FIGURE 7 Incorporation of arginine- $H^3$  into proteins of all meiotic cells and early spermatids ( $S_1$ ). Note that both cytoplasm and nucleus are labeled.  $\times 630$ .

FIGURES 8 TO 10 Incorporation of arginine- $H^3$  in spermatids at different stages of development. Note that early spermatids (Fig. 8) are more active than condensing (Fig. 9) and elongating (Fig. 10) spermatids.  $\times 1040$ .

Cells in Figs. 7 to 10 were exposed to 125  $\mu\text{c}/\text{ml}$  of arginine- $H^3$  for 30 minutes. All radioautographs of formalin-fixed and Feulgen-stained squash preparations were exposed for 30 days.



this radioactivity. Such non-specific labeling may indicate the presence of labeled impurities in a particular thymidine- $H^3$  sample (see also Nigon and Gillot, 1964).

All spermatocytes, except those in first and second metaphase, anaphase, and early telophase, are labeled after exposing cells to uridine- $H^3$  for 3 to 30 minutes or to cytidine- $H^3$  for 1 to 2 hours (Figs. 3 and 4), corroborating the recent observations of Henderson (1963). During these exposure times, labeling is confined to nuclei only (Figs. 3 and 4). Nucleolar labeling is detected only after exposing the emulsion for a relatively long time. Digestion with RNase reveals that all the radioactivity present in the cells following an exposure of 3 to 30 minutes to uridine- $H^3$  is limited to RNA; as mentioned above, however, cytidine- $H^3$  is incorporated not only into RNA but also into DNA of some premeiotic nuclei during 1- and 2-hour exposure times.

In tubules exposed to uridine- $H^3$  or cytidine- $H^3$  for 3 minutes to 2 hours, early spermatid nuclei are found to be labeled (Figs. 3, 5, and 6,  $S_1$ ). The incorporation of these RNA precursors, however, virtually stops in spermatids in which nuclear condensation has barely begun (Fig. 6,  $S_2$ ). The condensing and elongating spermatids are inactive with respect to uptake of RNA precursors (Fig. 6,  $S_3$ ).

In the present study it has been found, similar to the observations of Henderson (1963), that in all stages of meiosis as well as in early spermatids

the heteropycnotic X-chromosome, in contrast to the autosomes, does not incorporate uridine- $H^3$  or cytidine- $H^3$  (Figs. 3 to 5). When the radioautographs of squashed preparations or 2- $\mu$ -thick sections of tubules, supplied with uridine- $H^3$  for 30 minutes and cytidine- $H^3$  for up to 2 hours, were exposed for the length of time necessary to produce a high grain density over labeled nuclei, the X-chromosome stands out clearly as unlabeled among the autosomes (Figs. 4 to 6).

Table I presents the quantitative data on incorporation of uridine for 30 minutes, expressed as total number of grains per section of nucleus and also as grains per unit area. While the total radioactivity of the nucleus cannot be determined from the 5- $\mu$  sections, it is proportional to the observed number of grains per unit area. The enlargement of the nucleus from preleptotene to zygotene with the accompanying increase in chromosomal surface in contact with the emulsion will cause an overestimation of grains produced by  $H^3$  per nuclear section; this would explain the lack of difference in grains per section from preleptotene to zygotene. A change in activity from preleptotene to zygotene is, however, apparent on a per unit area basis. Both expressions (columns 4 and 5) show similar trends in stages beyond zygotene: a considerable decrease in incorporation from pachytene through diakinesis and complete absence of incorporation in metaphase and anaphase. While incorporation is resumed at interkinesis and at the end of the second meiotic di-

TABLE I  
*Incorporation of Uridine- $H^3$  (250  $\mu\text{C}/\text{ml}$ ) for 30 Minutes During Spermatogenesis in the Locust*

Cell stage	No. of nuclear sections	Mean area of sections $\mu^2$	Mean No. grains per section	Mean No. grains per 100 $\mu^2$ of section	Grains per 100 $\mu^2$ in % of preleptotene
Preleptotene	30	45 $\pm$ 1.2	64.6 $\pm$ 3.3	143.7 $\pm$ 7.8	100
Leptotene	15	67 $\pm$ 2.1	61.1 $\pm$ 4.2	91.4 $\pm$ 5.7	64
Zygotene	30	89 $\pm$ 2.8	63.7 $\pm$ 3.0	72.0 $\pm$ 2.8	50
Pachytene	45	115 $\pm$ 2.8	21.5 $\pm$ 1.3	19.1 $\pm$ 1.1	13
Diplotene	38	114 $\pm$ 3.4	13.4 $\pm$ 0.7	12.0 $\pm$ 0.7	8
Diakinesis	30	100 $\pm$ 4.2	9.2 $\pm$ 0.9	9.8 $\pm$ 1.2	7
Metaphase I	30		0		
Anaphase I	30		0		
Interkinesis	15	52 $\pm$ 3.1	11.5 $\pm$ 1.8	21.3 $\pm$ 2.6	15
Prophase II	9	56 $\pm$ 3.5	5.2 $\pm$ 0.6	9.1 $\pm$ 0.9	6
Early spermatid	30	55 $\pm$ 1.3	5.0 $\pm$ 0.4	9.0 $\pm$ 0.7	6

Radioautographs of 5- $\mu$ -thick sections were exposed for 5 and 11 days. The values from both radioautographs were pooled after making adjustment (to 5 days) for the difference in exposure time; the average grain ratios between different cell stages were similar after the two exposure periods.

vision, the rate of incorporation does not again equal that of preleptotene and early prophase.

In contrast to RNA precursors, arginine- $H^3$  is incorporated into proteins of both cytoplasm and nucleus of cells at all stages of meiosis, during 15 and 30 minutes' exposure (Fig. 7). In these samples, all spermatids were also labeled (Figs. 7 to 10). Whether or not there is a differential incorporation of arginine- $H^3$  in the autosomes and the X-chromosomes cannot be determined in the present study, because of heavy cytoplasmic labeling.

The quantitative data presented in Table II of the 30-minute incorporation of arginine- $H^3$ , expressed per cell and per unit area, were obtained from formalin-fixed squash preparations. Compar-

tively small and in some cases insignificant (Taylor, 1960; Prescott and Bender, 1962; Konrad, 1963). Our earlier results (unpublished) on protein synthesis during mitosis in onion root tip cells show a decrease of about 30 per cent in the rate of incorporation of leucine- $C^{14}$  or histidine- $H^3$  into proteins from early prophase to metaphase-anaphase cells. Metaphase and anaphase onion root tip cells have been found to be inactive with respect to RNA synthesis (Das, 1963). Similarly, during spermatogenesis in locust the rate of protein synthesis, as judged by incorporation of arginine- $H^3$ , decreases slightly in metaphase, anaphase, and early telophase cells which do not synthesize RNA. In the absence of new RNA synthesis, protein synthesis continues, although at a

TABLE II  
*Incorporation of Arginine- $H^3$  (125  $\mu\text{C}/\text{ml}$ ) for 30 Minutes During Spermatogenesis in the Locust*

Cell stage	No. of cells	Mean cell area $\mu^2$	Mean No. grains per cell	Mean No. grains per 100 $\mu^2$ of cell	Grains per 100 $\mu^2$ in % of early prophase
Early prophase	42	284 $\pm$ 6.0	22.8 $\pm$ 0.9	8.1 $\pm$ 0.4	100
Diakinesis	21	280 $\pm$ 8.8	18.3 $\pm$ 1.2	6.5 $\pm$ 0.4	80
Metaphase I	34	284 $\pm$ 6.0	15.3 $\pm$ 0.7	5.4 $\pm$ 0.3	67
Anaphase I	20	304 $\pm$ 7.2	18.5 $\pm$ 1.4	6.1 $\pm$ 0.3	75
Early spermatid	50	136 $\pm$ 3.2	11.2 $\pm$ 0.4	8.2 $\pm$ 0.3	101
Condensing spermatid	25	56 $\pm$ 1.2	3.2 $\pm$ 0.3	5.6 $\pm$ 0.4	69
Elongating spermatid	25	40 $\pm$ 1.2	2.4 $\pm$ 0.2	5.9 $\pm$ 0.5	73

Radioautographs of squashed cells were exposed for 7, 10, and 30 days. The last exposure time was used only for counting grains over spermatids. All values were pooled after making adjustment (to 7 days) for the difference in exposure time; the average grain ratios between different cell stages were similar after the three exposure periods.

ing these with the data of Table I, it can be seen that arginine- $H^3$  incorporation occurs at all stages, with a maximum drop of only about 30 per cent during division (also compare Figs. 3 and 7). On a per unit area basis, the rate of incorporation of arginine- $H^3$  in the early spermatids is similar to that of early prophase. As the spermatid nucleus becomes condensed and starts to elongate, however, the rate decreases again (see also Figs. 8 to 10). At a still later stage of elongation, spermatids continue to incorporate arginine- $H^3$  at a slow rate.

#### DISCUSSION

Recent radioautographic studies reveal that although RNA synthesis decreases considerably during mitosis, the drop in protein synthesis is rela-

reduced rate, as the development of spermatids progresses. The RNA produced earlier, therefore, sustains the protein synthesis in these cells. According to Bloch (1963), the incorporation of arginine- $H^3$  in the elongating spermatids may be indicative of synthesis of an arginine-rich protein, since the transition of histone to a more basic, arginine-rich protein takes place most rapidly at this stage.

Almost all the RNA synthesized in meiotic nuclei of the locust, in contrast to mitotic nuclei generally, is chromosomal in origin, since RNA labeling in the two small nucleoli of early prophase cells is inconspicuous. Furthermore, RNA is synthesized in the late prophase cells in which nucleoli are not seen. According to Watkins (1962) the increase in chromosomal mass at diakinesis in

grasshopper may be due to the RNA which is synthesized at this stage. Further experiments in which locusts were sacrificed 1 and 2 days following administration of cytidine- $H^3$  show the presence of labeled RNA in the cytoplasm as well as in the nuclei of meiotic cells. Presumably, the labeled RNA has been transferred from the nucleus to the cytoplasm. It appears likely, therefore, that at least some of this RNA supports protein synthesis in later meiotic cells in which RNA is not produced (see above).

Earlier studies on RNA synthesis during mitosis in animal and plant cells have demonstrated that the condensed mitotic chromosomes fail to support RNA synthesis (see Taylor, 1960; Prescott and Bender, 1962; Das, 1963). It is evident from the present study as well as from the studies of Taylor (1958 and 1959) and Henderson (1963) that the rate of RNA synthesis, as judged by incorporation of labeled RNA precursors, decreases during meiosis as chromosome contraction progresses. In contrast to late mitotic prophase, however, RNA synthesis continues, although at a much reduced rate, in the contracted bivalents at the diplotene and diakinesis stages. According to Ris (1945), chromosomes at these stages exhibit lampbrush structure and one may, therefore, expect them to be active in RNA synthesis (*cf.* Gall and Callan, 1962). The disappearance of the lampbrush structure, concomitant with the dissolution of the nuclear membrane before metaphase, coincides with the cessation of RNA synthesis. In the second meiotic division, RNA synthesis occurs only in those stages in which chromosome coiling is somewhat relaxed.

The failure of the X-chromosome to synthesize RNA at any stage of meiosis may also be interpreted to result from chromosome condensation, since the X-chromosome remains compact throughout the whole meiotic cycle and does not exhibit the lampbrush structure of the autosomes. Some recent studies (Hsu, 1962; Frenster *et al.*,

1963) reveal that the condensed heterochromatin in interphase cells is also less active in RNA synthesis than dispersed euchromatin. It should be mentioned in this connection that it was recognized in earlier studies that genes in condensed chromosomes are relatively inactive or repressed (see Cooper, 1959). Frenster *et al.* (1963) consider the possibility of histone being the repressor of RNA synthesis in condensed chromatin (see also Huang and Bonner, 1962; Allfrey *et al.*, 1963).

The depression of RNA synthesis, however, is not always or necessarily accompanied by cytologically detectable condensation of chromatin. The early spermatid nuclei of the locust, which have the cytological appearance of interphase nuclei, *i.e.*, diffuse chromatin, exhibit greatly reduced RNA synthesis per unit area when compared to premeiotic nuclei. This may indicate that only few genes remain active immediately after meiosis. At a slightly later stage, RNA synthesis ends suddenly in spermatids in which nuclear condensation has barely begun. It is noteworthy that these latter spermatids are in the process of rearranging their DNA-protein complex (see Alfert, 1959), whereby the typical histone is replaced by a more arginine-rich protein.

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*Note added in proof:* While this manuscript was in press, Henderson published more extensive observations, similar to ours, on RNA synthesis during spermatogenesis in locusts and grasshopper (*Chromosoma*, 1964, 15, 345). The following studies on similar problems have since been published also: BLOCH, D. P. and BRACK, S. D., *J. Cell Biol.*, 1964, 22, 327; MONESI, V., *J. Cell Biol.*, 1964, 22, 521; MUCKENTHALER, F. A., *Exp. Cell Research*, 1964, 35, 531; WATKINS, M. J., *Exp. Cell Research*, 1964, 36, 14.

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