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STUDIES ON THE RATE OF SPERMATOGENESIS IN *DROSOPHILA*

EFFECTS OF X-RAYS AND STREPTONIGRIN

By

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With 2 Figures in the Text

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Attempts to determine the rate of maturation of spermatogenous cells in the *Drosophila* testis are not new. COOPER (1950) evaluated the histological information then available and provided a rough time schedule. KHISHIN (1955) constructed a more refined time scale by making a comparative cytological study of testes from larvae and pupae and determining — by an analysis of X-ray induced mutations — the time required for cells of different types to become functionally mature spermatozoa. GAY and WEINGART (GAY, 1963) used the more direct method of following through spermatogenesis those cells whose chromosomes had been tagged with tritiated thymidine at the time of DNA replication in the spermatogonia and spermatocytes of larval testis. It was thus possible to construct a time scale in which the rates of spermatogenesis in control and X-ray irradiated males could be compared (KAUFMANN and GAY, 1963). The H^3-labeling chronometric method was also utilized by CHANDLEY and BATEMAN who injected males with the isotope and took samples of testes every 24 hours for staining by the Feulgen method and subsequent production of radioautographs (CHANDLEY and BATEMAN, 1963).

The necessity of agreeing upon an accurate time schedule has arisen because the literature is replete with papers whose conclusions are based partially on the assumption that sampling on a particular day after irradiation will yield primarily sperms that were treated at a certain stage of development. Many of these papers deal with the phenomenon of differential radiosensitivity and the mechanism whereby it operates during spermatogenesis in *Drosophila*. Strangio reviews some recent papers dealing with this problem (STRANGIO, 1961). After a consideration of this representative sample of reports by LÜNING, BATEMAN, IVES, and SÄVHAGEN (quoted by STRANGIO) it can be seen that their interpretations are based on the underlying assumption that the rate of progress of cells through spermatogenesis is essentially the same for immature and mature testes, and for control and treated germ cells. A suggestion that this assumption might not be valid because of differences in the rate of sperm release between control and treated flies and between flies treated in different ways was made by AUERBACH (1958). Furthermore, on the basis of indications of a possible 24 hour time lag induced by X-rays in the larval testes, radiation must be suspect as a rate-determining factor (KAUFMANN and GAY, 1963). Two questions, therefore, seemed to be in need of further consideration: (1) Can a schedule derived from

data about the time required for mutations to appear in functionally mature spermatozoa, when induced by X-rays in specific types of cells of the immature testes, have validity in determining the rate of progress of cells of a similar type through spermatogenesis when the mature testis is irradiated? and (2) is the rate of spermatogenesis the same in an X-rayed or chemically treated testes as it is in an untreated testis, so that similar time scales will be applicable? This report essays to answer these questions.

In addition, an attempt was made to elucidate the mechanism of any radiation induced retardation that might occur. Streptonigrin is an extract of *Streptomyces flocculus* which has been reported to inhibit net DNA synthesis in bacteria (LEVINE and BORTHWICK, 1963). If the radiation-induced retardation were due to an inhibition of DNA synthesis at some stage of spermatogenesis, then the schedules for X-rayed and streptonigrin-treated germ cells should resemble each other more closely than they resemble that of the control, or untreated, gametes.

With these various objectives in mind, adult testes were either irradiated with X-rays or injected with streptonigrin. The rate of spermatogenesis in each of these groups, refined by sampling at 24 hour intervals, was then compared to that of an untreated, control group. The latter group also provided a check for precision in the currently accepted general time scale.

Material and Methods

Two experiments, designated A and B were run during the past year. They were identical in design except where noted below. There were 51 males in the control group of experiment A, and 43 males that were given 1000 roentgens of X-rays. In experiment B, there were 97 males in the control group, 112 irradiated males, and 32 that were injected with streptonigrin.

1. Stocks: Swedish-b^9 stocks of *Drosophila melanogaster* were used. (The numerical superscript refers to the ninth subculture during the past 25 years from single pairs whose F1 progeny were examined cytologically to determine the absence of gross chromosomal aberrations.) Flies were grown on cornmeal-agar-molasses media seeded with yeast and having Tegosept M as mold inhibitor (DEMEREC and KAUFMANN, 1964) and were maintained at a temperature of 25 ± 0.5^0C.

2. Method of isotope incorporation: Composition of the solution that was fed to the flies: Experiment A: 0.11 cc of tritiated thymidine, S.A. = 6.7 c/m mole, was micropipetted into 0.5 ml of a sterile, 10% sugar-water solution. Experiment B: 0.13 cc of tritiated thymidine, S.A. = 6.7 c/m mole, was micropipetted into 0.5 ml of a sterile, 10% sugar-water solution. The chosen concentration of isotope was similar to that found by GAY and WEINGART (personal communication) to effect incorporation of H^3-thymidine into the testes when larvae were fed. The choice of dose in a particular experiment must be made largely through a process of trial and error (BOYD, 1955). Following BOYD's premise that the correct dose in a specific instance is that which produces definitive radioautographs without doing appreciable damage to the organism, the concentration selected was found to be satisfactory. No evidence of lethality or sterility was found.

The sugar-water method of isotope incorporation through ingestion was a slight modification of that used to effect P^{32} incorporation into *Drosophila* males (OFTEDAL, 1959). In the experiment reported here, thin wooden sticks about 2 cm wide by 5 cm long, to which 1×3 cm strips of filter paper were affixed by paraffin drops, were placed in vials and moistened with the radioactive solution. In experiment A, approximately 5 drops were placed on each piece of filter paper. In experiment B, the filter papers were checked periodically and kept continually moist. The latter procedure was found to yield the best radioautographs.

Males were collected at eclosion, held for 32 hours, and starved for 13 hours. Then, at age 45 hours post-eclosion, the males were placed in vials containing the isotope solution

and allowed to feed for three hours. From 30—50 males ware placed in each of the vials depending on the total sample size.

3. X-ray treatment: As soon as possible after the completion of the three hour feeding treatment to effect isotope incorporation, when the males were 48 hours old, they were etherized, divided into groups of 25, and placed in perforated gelatin capsules for irradiation. 1000 r were administered by a 250 kvp, 15 milliampere X-ray machine. 0.5 mm Cu and 1.0 mm Al filters were used. The dose was measured by a Victoreen ion chamber. In experiment A, the target-skin distance was 19 cm and the dose rate was 953 r/min. Experiment B males were irradiated at two different times. Group 1 had a TSD of 22.3 cm and a dose rate of 740 r/min. Group 2 had a TSD of 22.4 cm and a dose rate of 725 r/min.

4. Streptonigrin injection: Streptonigrin was obtained from Dr. MAIMON COHEN of the Department of Human Genetics, University of Michigan. The source and method of dilution of the material has previously been described (COHEN, SHAW, and CRAIG, 1963). The concentration employed in this report was 10 mg/ml. Males were injected between the fourth and fifth sternites by use of a glass needle connected to a tuberculin hypodermic syringe by means of a piece of rubber catheter tubing. The injections were made immediately after the isotope had been incorporated by feeding.

5. Brood method: At 24 hour intervals after the cessation of the tritiated thymidine-sugar water treatment, i.e. at 48 + x (24) hours after eclosion of the males (where x = brood number), for a total period of 216 hours, or 9 days, samples of three males were taken from each experimental group. Each of the remaining males was given 2—3 virgin females. In experiment A each male and his mates had an individual vial. In experiment B, mass cultures were employed which consisted of about 15—20 males plus 30—60 females per bottle. Fresh females were presented each day at the same time that males were removed for sampling of the testes. In experiment A, fresh females were additionally provided at 6 hours post-treatment.

6. Fixing, embedding, and staining schedule: The testes were fixed in Carnoy's solution (1 part acetic acid: 3 parts ethyl alcohol), dehydrated through a standard alcohol series, cleared in xylene, and embedded in paraplast (obtained from the A. H. Thomas Co.). The embedded testes were then sectioned at 5 microns and stained by the Feulgen procedure. The time of hydrolysis was 12 min.

7. Radioautographs and histological examination: Kodak AR-10 radioautographic stripping film was put over the slides which had originally been "subbed" with gelatin as an adhesive (BOYD, 1955). Samples were developed at weekly intervals to determine the optimum time of exposure. Subsequently, the slides obtained in experiment A were developed at 6 weeks, and those in experiment B at 4 weeks.

The slides were then examined microscopically with the oil immersion objective and, in some cases, with the aid of phase contrast optics. The photographs reproduced herein are representative samples of the appearance of the label at various stages of spermatogenesis. No distinction was made between primary and secondary spermatogonia because of the difficulty of accurately distinguishing between the two kinds of cells in the apex of the adult testes. Other classifications were generally made in accord with the outline of morphological criteria for the identification of stages of spermatogenesis as presented by COOPER (COOPER, 1950).

Results

The results are summarized in Tables 1 and 2. Table 1 lists all the types of cells that were found labelled on a particular day. This portrayal of the range of possible overlap per day should be beneficial to investigators employing refined brood techniques. Table 2, listing the oldest cell type found labelled on each day, must be consulted in order to see if any discrepancies in the rate of sperm maturation exist among the three experimental groups.

Several interesting observations may be made after a perusal of the two tables. A comparison of the results for control and irradiated males in Table 2 reveals indications of an X-ray induced lag. The time tables are identical for

Table 1

Days post-treatment	Control	X-rayed	Streptonigrin treated	*
1	spermatogonia, primary spermatocytes	spermatogonia, primary spermatocytes	spermatogonia, primary spermatocytes	9
2	primary spermatocytes, secondary spermatocytes	primary spermatocytes, a few secondary spermatocytes	primary spermatocytes	8
3	early spermatids	early spermatids	primary spermatocytes, secondary spermatocytes, early spermatids	7
4	late spermatids	early spermatids	early spermatids, late spermatids	6
5	late spermatids, early sperm, a few mature sperm	early spermatids, late spermatids	late spermatids, early sperm, a few mature sperm	5
6	late spermatids, early perm, mature sperm	late spermatids, early sperm, a few mature sperm	(no sample)	4
7	early sperm, mature sperm	late spermatids, early sperm, mature sperm	early sperm, mature sperm	3
8	mature sperm	early sperm, mature sperm	(no sample)	2
9	mature sperm	mature sperm	(no sample)	1

* The number of the brood that will yield gametes that were treated in this stage of development.

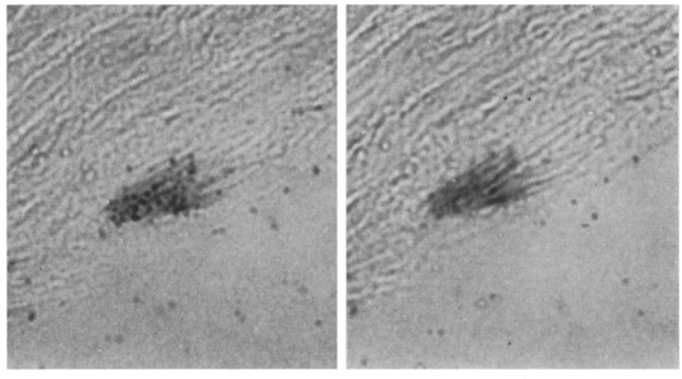
Table 2. *Oldest cell found labelled at each day*

Days post-treatment	Control	X-rayed	Streptonigrin treated	*
1	primary spermatocytes	primary spermatocytes	primary spermatocytes	9
2	secondary spermatocytes	secondary spermatocytes	primary spermatocytes	8
3	early spermatids	early spermatids	early spermatids	7
4	late spermatids	early spermatids	late spermatids	6
5	mature sperm	late spermatids	mature sperm	5
6	mature sperm	mature sperm	(no sample)	4
7	mature sperm	mature sperm	mature sperm	3
8	mature sperm	mature sperm	(no sample)	2
9	mature sperm	mature sperm	(no sample)	1

* The number of the brood that will yield gametes that were treated in this stage of development.

the first three broods. However, late spermatids appear in brood four of the control group, but not until brood five in the irradiated. Mature sperm appear in brood five of the control, but not until brood six of the X-rayed. Moreover, Table 1 exhibits a persistent pattern of a 24 hour delay in cell maturation between control and irradiated males after the third brood.

There is a difference between the types of spermatogenous cells found labelled in the second broods of the control and streptonigrin treated groups of males.



a b

Fig. 1a and b. Pattern of incorporation of H^3-thymidine into DNA of sperm heads from an irradiated adult 216 hours after the administration of X-rays (brood 9). a Shows Feulgen stained tissue. b Shows silver grains in the overlying stripping film

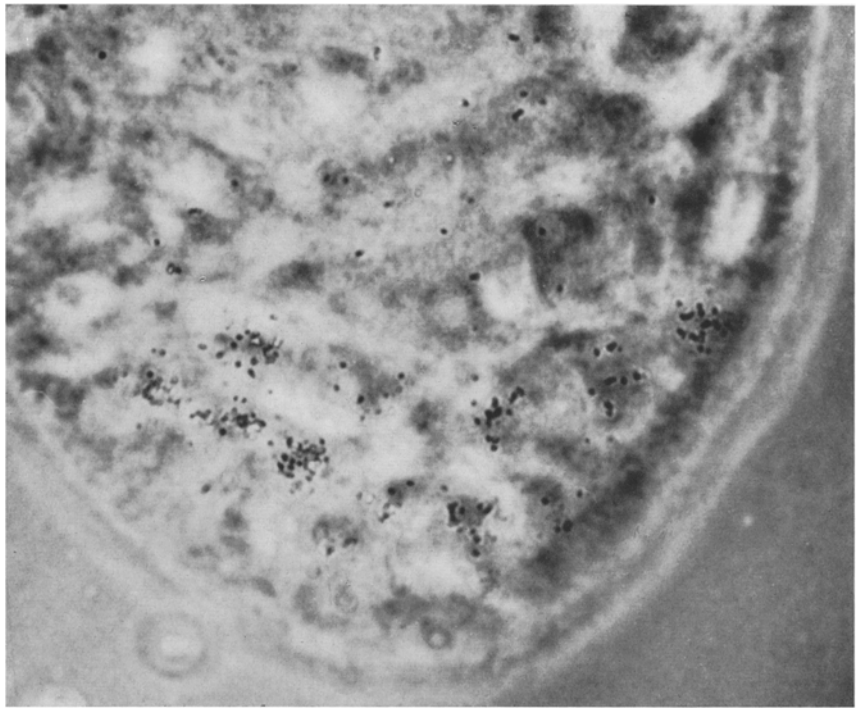


Fig. 2. Pattern of incorporation of H^3-thymidine into the nuclei of spermatogonia at the apex of the coiled testes taken from an untreated male 24 hours after the completion of isotope feeding

Table 1 presents a suggestion also that primary spermatocytes persist longer in the streptonigrin treated samples than in the controls.

Another noteworthy fact is that the rate of spermatogenesis in the adult male *Drosophila* appears to vary somewhat from that reported for injected larvae (KAUFMANN and GAY, 1963). If comparisons are made between the two

time schedules using hours after tritiated thymidine treatment as a baseline, the progress of cells through spermatogenesis seems to be about 24 hours faster in the adult testis as opposed to that of the immature testis. Moreover, closer inspection reveals that the acceleration occurs in the interval from isotope incorporation to the appearance of the label in primary spermatocytes (at 48 hours post-treatment in the larvae; at 24 hours post-treatment in the adult).

Discussion

A knowledge of the rate at which cells progress through spermatogenesis in *Drosophila* under varying experimental conditions is essential to the proper use of the brood technique for detecting patterns of differential radiosensitivity among developing male gametes. This knowledge is also essential for an understanding of the mechanisms responsible for variation in response of cells to radiation during different stages of gametogenesis. Auerbach championed the method of testing successive broods among the progeny of chemically treated flies in determining the effect of the treatment on cells in different stages of development (AUERBACH, 1949). She was able to provide indirect information about the rate of spermatogenesis by correlating types of genetic damage induced preferentially in certain broods to the stages which would be expected on genetic theory to respond to insults in that manner (AUERBACH, 1954; SONBATI and AUERBACH, 1960). Bateman refined the brood method by sampling at 24 hour intervals and was able to trace thereby the time of dominant lethal induction by irradiation (BATEMAN, 1956). Other uses which have been made of the brood procedure have been discussed by CHANDLEY and BATEMAN in the preface to their own work which involved the use of a multipurpose stock (CHANDLEY and BATEMAN, 1960).

The incentive to study differential radiosensitivity springs from two hopes. Such probes into the spectrum of responses of the genetic material during the course of gametogenesis might help to fit a piece into the unsolved puzzle of how to translate the Watson-Crick model into terms explaining the operation and organization of DNA at the chromosomal level. A further general interest of radiation genetics, that of protecting man from transmissible consequences of both chronic and acute radiation insults, can also justify such studies (OSTER, 1963). Ultimately, the damage must be understood on a biochemical level. To attain this end, investigations must descend from a consideration of the response of the organism to that of the cell, and, finally, to the intracellular reaction pathways. "In reaching meaningful interpretations we must specify the original sites of ionization or activation, trace the subsequent distribution of energy, define the conditions that lead to amplification or diminution of chromosomal damage, and identify precisely the nature of the mutational response" (KAUFMANN and GAY, 1963).

What implications and conclusions can be gleaned from the experiment reported here? Support for the suggestion that X-rays effect a rate change in the progress of cells through spermatogenesis (KAUFMANN and GAY, 1963) may be found in the comparison between the control and irradiated adult males. As much as a 24 hour X-ray induced lag may exist in damaged cells. This indicates that the delay reported by KAUFMANN and GAY may not be merely a result

of the immaturity of the testes they used. Two sets of evidence — one for larval and one for adult *Drosophila* — point to a retardation of the rate of gametogenesis induced by X-rays. Certainly, this question would benefit from further investigation, especially from the construction of a dose effect curve. In the mean time, however, investigators who wish to employ a time table which was constructed for unirradiated males to present conclusions on irradiated adults must be cognizant of the possibility that differences do exist between the chronometry of irradiated and untreated testes, between mature and immature males. It may be of interest to reevaluate some of the earlier experiments that employed three day broods in the light of the time tables presented here.

It was not possible to demonstrate that the schedules of male gametogenesis in the X-rayed and in the streptonigrin treated groups were more similar than were either of these groups to the control. Thus, no evidence for the theory that irradiation causes a decrease in gametic development by the inhibition of DNA synthesis can be put forward. However, a comparison of the time tables for the control and the chemically treated groups shows the two not to be identical. Secondary spermatocytes do not appear until the third brood in the streptonigrin treated males, and then occur together with persistent primary spermatocytes. No primary spermatocytes appear in the third brood of the control group. Only early spermatids and secondary spermatocytes appear in the second brood. After the third brood, the schedules coincide.

A comparison of the temporal progress of normal spermatogenesis in the untreated adult male with that reported for the developing male (KAUFMANN and GAY, 1963) indicates that the rate in the mature testes may be initially faster than that operative in the immature testes by as much as 24 hours. A comparison with the sequence reported for adults (CHANDLEY and BATEMAN, 1962) is more difficult to make because they only divided the maturation period into four stages: spermatogonia, spermatocytes, spermatids, and sperm. Furthermore, their descriptions of the morphological criteria used to identify these divisions suggest that their terminology is not strictly comparable to that set forth in this report. If the terminology is the same, then the two time schedules for the adults are not identical, since labelled sperm bundles appear at a later day in the males they used than in those described in Table 1. Of course, the possibility always exists that this reflects a peculiarity of stocks or variations in experimental procedures.

Summary

1. The rate of spermatogenesis in adult *Drosophila melanogaster* was measured by use of tritiated thymidine as a radioactive tracer. Three experimental groups existed: control, irradiated, and streptonigrin-treated males.

2. A refined time schedule for normal spermatogenesis is presented and compared to schedules prepared by other investigators.

3. Evidence for a decrease in the rate of spermatogenesis induced by X-rays and by streptonigrin is tabulated.

4. The importance of this line of investigation to studies on the differential radiosensitivity of male gametes is discussed.

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