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Spermatogenesis of the silkworm and its bearing on radiation induced sterility. I

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INTRODUCTION

The silkworm as a material for research on mutagenesis

Differences in radiation sensitivity and mutability of germ cells at various stages in maturation have been investigated and discussed by a number of workers (see reviews by Glass (1956), Muller (1958) for *Drosophila*; Russell (1954), Hertwig (1957), Russell, Russell and Oakberg (1958) for mice; Tazima (1959, 1961) for the silkworm).

Most of the investigations dealing with this subject have been carried out in the mouse and adult *Drosophila*, where germ cells in different phases exist together in the testis and the response pattern has been determined by testing successive brood at definite intervals after irradiation (Hertwig 1938a, Bateman 1956a, b, 1958, Friesen 1937, Lüning 1952, Auerbach 1954, Alexander *et al.* 1955, 1959, Ives 1960). However, it is not easy to determine exactly which type of cells are being tested when the adults have been irradiated, and this has occasionally given rise to conflicting interpretations as seen in the discussions by Bateman (1956b) and Auerbach and Sirlin (1956), and Auerbach (1957), Sirlin and Edwards (1957) and Bateman (1957).

The use of the silkworm has an advantage in this respect because the majority of germ cells develop almost simultaneously in the testis during the larval stage of the silkworm. Tazima (1958b, 1959, 1961) has made use of this for investigating the changes in radiation induced mutation rates during different stages of gametogenesis in this insect. Only few studies which are comparable to this have been done in *Drosophila* larvae (Khishin 1955a, Alexander 1954, 1960). A precise analysis of the relation between the stage of spermatogenesis and the developmental phases of the silkworm, and knowledge of the duration of the successive stages of spermatogenesis is indispensable for the

interpretation of the results of irradiation experiments. The results of such an analysis are presented in this paper.

Cytological basis of radiation induced sterility in animals

It is now well established that the period of temporary sterility observed at definite intervals after irradiation of adult male mice and *Drosophila* is the result of destruction of late spermatogonia (and/or the youngest spermatocytes) by radiation (Hertwig 1938b, 1957, Russell 1954, Russell, Russell and Oakberg 1958; Friesen 1937, Welshons and Russell 1957, Ives 1960). However, a special problem was raised by Tazima's (1958a) finding that an extremely sensitive stage to the sterilizing effect of X-rays occurs early in the fifth instar of the male. Since it is known that by this time most of the germ cells have already differentiated into spermatocytes, these results seemed to suggest that in the silkworm spermatocytes are the most sensitive stage to radiation damage (Tazima 1958a, Kogure and Nakajima 1958). This suggested the possibility of a species specificity in radiation response of spermatogenic cells in animals.

Careful investigation of the stage distribution of spermatogenic cells in this insect confirmed that the stage of excessive sterility corresponds to the late prophase of the primary spermatocytes, but secondary spermatogonia are also easily destroyed by moderate doses of X-rays. Yet, in spite of this fact, the sterility due to the destruction of spermatogonia can hardly be recognizable in the silkworm. To solve this seeming contradiction, regenerative process of the irradiated germ cells were studied.

These studies have made it possible to establish a consistent explanation of results obtained for silkworm, *Drosophila* and mouse, concerning the cause of radiation induced sterility in these animals.

Estimation of mutation rates induced in spermatogonia

The genetic material of the human male is carried in spermatogonia during most of the life cycle; the length of time occupied by meiosis and spermiogenesis is not accurately known but it is unlikely to exceed a year and may well be less. Therefore, in a human population exposed to chronic irradiation, spermatogonia form the majority of treated cells and it is their sensitivity which in the main determines the genetical effectiveness of the exposure.

It has been well established that in *Drosophila* sperm radiation induced mutation rates do not depend on radiation intensity (Lea 1954, Muller 1954). This is true also for mouse spermatozoa (Russell,

Russell and Kelly 1958). However, Russell, Russell and Kelly (1958) found that in mouse spermatogonia chronic γ-irradiation is mutagenically less effective than acute X-ray irradiation. In view of the importance of the problem for the estimation of genetic hazard of radiations to man it has become of great concern whether the same principle holds true in other animals than the mouse.

In addition to the advantage mentioned above, i.e., the synchronous development of germ cells, the silkworm has one further great advantage for the estimation of mutation rates in that very large numbers of individuals can easily be examined by using egg color mutants, such as *pe* and *re*, as specific markers (Tazima 1956, 1958b, 1959, 1961). Thus, statistically more reliable data can be obtained on this material more easily than on mice or *Drosophila*.

Since spermatogenesis of the silkworm does not take place in the adult, the methods for inferring the stage of the germ cells during treatment are different from those used for mice and adult *Drosophila*. In these animals the sterile period has been used as a marker for separating genetic changes induced in spermatogonia from those induced in later germ cell stages (Russell 1951, Russell, Russell and Kelly 1958, Friesen 1937, Auerbach 1954, Alexander *et al.* 1955, 1959, Ives 1960).

In the silkworm, the correlation between the developmental stage of the whole animal and the types of germ cell present in the testis can be used instead. Thus, it was found that irradiation should be given before the middle of the second instar in order to sample sperm irradiated as spermatogonia. That the irradiated spermatogonia actually develop into functional spermatozoa was shown histologically. Using this method, the genetic effects of acute and chronic irradiations on spermatogonia and oögonia of the silkworm were investigated and the results were reported elsewhere (Tazima, Kondo and Sado, 1961).

LITERATURE REVIEW

1. Spermatogenesis in the silkworm

Many investigations have been carried out on spermatogenesis in the silkworm (Verson 1889, 1891, 1894, Grüneberg 1903, Toyama 1894, 1909, Tichomirow 1898, Kawaguchi 1928, Machida 1929, 1935). The number of chromosomes has been studied by Toyama (1894, 1909), Yatsu (1913), Katsuki (1918), Oguma (1919) and Kawaguchi (1928), the structure of the testis by Toyama (1894, 1909) and Ômura (1936), the intra- and extra-testicular* behaviour of the spermatozoa by Ômura (1936, 1938a)

* According to the suggestion of Dr. C. Auerbach, the term 'extra-testicular' will be used instead of 'post-testicular' proposed by Ômura (1936, 1938a).

and the embryonic development of the gonads by Toyama (1902, 1909) and Miya (1958, 1959). The following is a summary of these investigations.

(a) *Structure of the testis.*

The testes are paired, kidney-shaped organs and lie immediately under the dorsal skin of the eighth segment where the star spots are seen. Each testis consists of four testicular follicles, which are covered with a common envelope, membrana communis, and each is placed facing the other with the concave side, on both side of the dorsal vessel. From the proximal end of each testicular follicle one ductulus efferens testis arises. Four ductuli efferentes testis unite to form a ductus efferens. The testicular follicle is partitioned from its ductulus efferens testis by the membrana basilaris. Detailed descriptions of the structure of the testis and those of the extra-testicular organs were given by Ômura (1936).

(b) *Differentiation of germ cells during embryonic stage.*

Embryonic development of the gonads was first described briefly by Toyama (1902, 1909) and has been thoroughly investigated more recently by Miya (1958, 1959). The determination of the germ cells of the silkworm is brought about through the entrance of some cleavage nuclei into the definitive region of the periplasm i.e., the germinal cytoplasm, and is completed at the time of blastoderm formation. The germinal cytoplasm is located on the ventral side at a distance from the posterior pole of about one third the whole embryonic length. The multiplication of the primordial germ cells occurs for the first time during blastoderm formation and ceases at the time of its completion. The determined germ cells make a group and are clearly distinguished from the cells of the germ band. Owing to the elongation of the germ band, the germ cells are distributed over several segments. Usually germ cells are observed most frequently in the 6–8th segments; sometimes they are recognizable not only in thoracic segments but in the gnathal one of the head.

At 60 hours after egg laying, gonad formation is induced by segmental genital ridges which have originated from the ventral wall of the coelomic sacs in the 6–9th segments. As a result, germ cells which happen to be distributed to other segments than the above four can not be included by genital ridges and are then excluded from gonad formation. In the 90 hours old embryos, gonad formation is already completed. The number of primordial germ cells participating in the formation of gonad varies with the strain used, namely, from 18 to 47 cells per one gonad.

A full description of the embryonic development of the gonad can be found in the papers by Miya (1958, 1959).

(c) *The development of male germ cells.*

In the embryonic stage, each testis consists of only one follicle in which a few primordial germ cells are observed. When the larva is about to hatch three depressions appear inside the follicular wall. They gradually deepen until four cavities are formed. At the blind end of each cavity, there appears a large cell, which is termed 'apical cell.'

Apical cells. In the history of the studies on spermatogenesis of the silkworm, apical cells come first, because they have been the main subject of investigation during 1889–1909. There have been some conflicting opinions as to the origin and function of apical cells. Some workers considered that they are formed from epithelial cells of the gonad (Toyama 1894), but others insisted that they are derived from the primordial germ cells (Verson 1889, 1891, 1894, Grüneberg 1903, Toyama 1909). Recently, Miya (1959) reported that apical cells might be deformed germ cells. As to the function of these cells, Verson (1889, 1891, 1894) considered that they are progenitors of the germ cells, but Toyama (1894) concluded that they are not germ cells but are supporting cells which connect all the younger genital elements (spermatogonia) with the wall of the testicular follicle and probably nourish them. This view has been generally accepted by later investigators (Tichomirow 1898, Grüneberg 1903).

Spermatogonia. Spermatogonia are situated near the blind end of each testicular follicle and are arranged concentrically around the apical cell. In the newly hatched larva, only small numbers of germ cells are present in the testis and these gradually increase with the development of the larva. The cytoplasm of the youngest spermatogonia (primary spermatogonia) is connected with that of the apical cell by a spine-like process. The more advanced spermatogonia (secondary spermatogonia) are contained in a common cyst, in which, according to Kawaguchi (1928), six synchronous divisions may occur resulting in the production of 64 (2^6) primary spermatocytes.

Spermatocytes. In the first part of the spermatocyte stage, the size of the cell is small due to the preceding repeated mitoses of the spermatogonia. Early spermatocytes in the resting stage are similar in appearance to spermatogonia. They then enter into the growing stage and their nuclei undergo remarkable changes (meiotic prophase) and enter into the maturation division (metaphase I). It is noteworthy that in the silkworm two stages between pachytene and diakinesis have been described as secondary contraction- and diffuse-stages (Kawaguchi 1928); they correspond to the diplotene stage in other organisms.

The number of chromosomes of the silkworm has also been a point of some disagreements. Toyama (1894) and Katsuki (1918) reported that 28 chromosomes can be counted in diploid cells (spermatogonia) and 14 in haploid ones. On the contrary, Yatsu (1913) counted 2n=50–60 in spermatogonia and n−28 in the first maturation division. This number was confirmed again later by Oguma (1919), Kawaguchi (1928) and many other investigators.

Spermatids. The processes occurring during the spermatid stage are more complicated than in the earlier stages. This stage begins with the end of the second maturation division and terminates at the time of completion of spermatozoa. Spermiogenesis of the silkworm was studied in detail by Machida (1935).

Eupyrene and apyrene spermatozoa. In the silkworm, as in other Lepidoptera, two types of spermatozoa are always distinguishable in pupal testes. They are eupyrene and apyrene spermatozoa. The difference between these two types of spermatozoa will be seen in Plate 12, Fig. 19. As seen in the plate, bundles of spermatozoa are contained in cysts. In case of eupyrene spermatozoa bundle, a mass of nuclei, or heads of spermatozoa, are seen in the anterior end of the bundle (eup); while in apyrene spermatozoa cysts the nuclei are granular in shape and are situated in the central part (apy). The nature of these two types of spermatozoa was investigated experimentally by Machida (1929). He showed that spermatocytes which have terminated the maturation divisions during the larval stage develop into eupyrene spermatozoa, those that completed them in the early pupal stage produce both types of spermatozoa, and those that completed them in the late pupal period transform into apyrene spermatozoa. He also found that apyrene spermatozoa degenerate usually in the distal portion of the testicular follicle. Machida (1929) and Ômura (1936) considered that apyrene spermatozoa cannot be observed in the extra-testicular organs of this insect except in very rare cases. However, Iriki (1941) stated that they can be observed in the extra-testicular organs of males as well as in the bursa copulatrix and the receptaculum seminis of females after copulation. According to him, eupyrene spermatozoa observed in the female reproductive organs are thicker and larger but less active than the apyrene spermatozoa, which are thin, small and highly active. A similar view had been presented earlier by Tsukaguchi and Kurotsu (1922).

These facts should be kept in mind in the interpretation of the results of irradiation experiments.

(d) *Intra- and extra-testicular behaviour of spermatozoa.*

In the testis bundles of spermatozoa are contained in cysts. Each bundle passes out into the ductulus efferens through the membrana

basilaris in a particular and complex manner each perforating its own pathway by its own action. In the course of passing out, the sheath of the bundle is cast away and left in the lobulus. The passing out of the spermatozoa begins in the late pupal stage about 3 days before emergence, and the majority of the spermatozoa migrate into the extra-testicular organs during the pupal stage. No formation of new spermatozoa in the lobulus accompanies this migration. Consequently the testis becomes smaller with age. Spermatozoa gain activity only in the course of migration. Full maturity of the spermatozoa is attained during their passage from the testis into the ductus deferens.

Ômura (1938) carried out careful investigations on the structure and function of each part of the extra-testicular organs of males. He showed that in the extra-testicular system, no secretion other than the prostatic one is required for the induction of normal activity in fully matured spermatozoa.

2. Radiation effects on spermatogenesis

The effects of irradiation on spermatogenesis have been studied by means of fertility tests, microscopical study of sperm, histological study of the testes, and induction of mutations. This part of the literature review consists of two sections, namely, (a) histological and cytological effects on spermatogenesis and (b) effects on fertility of males.

(a) *Histological and cytological effects on spermatogenesis.*

The effects of X-irradiation on the testis were first reported by Albers-Schöneberg as early as 1903. He found that daily delivery of X-rays sterilized the testes of five rabbits and six guinea pigs without affecting their sexual potency. The first fairly complete study of the X-irradiated testes were done by Bergonie and Tribondeau (1904). Since then the voluminous papers on this problem have been published by a number of workers. As to the histological results, there have been considerable disagreements among authors. Some workers reported that spermatocytes or spermatids are the most sensitive to radiation, but many others concluded that late spermatogonia are the most sensitive. These studies have been reviewed by Russell (1954), Kaufmann (1954), Casarett and Casarett (1957a) and Hertwig (1957).

In mammals, there is now a good agreement that late spermatogonia are extremely sensitive, and that spermatocytes, spermatids and sperm are more resistant to radiation (Russell 1954, Oakberg 1955).

Oakberg (1955) calculated an LD_{50} of 20–25 r for intermediate and type B spermatogonia. This statement would be much more useful if LD_{50}'s for source of the other stages were given for comparison. In the irradiated testis, reduction in numbers is found first in spermatogonia and the later germ cell stages disappear successively in the order of spermatogenesis (Hertwig 1938b). It is generally accepted that the disappearance of these stages is attributed to the failure of replacement by the depleted spermatogonia (Russell 1954). Degeneration of spermatogonia in the mouse occurs primarily as damaged cells reach the late interphase or early prophase of their first post-irradiation division; a few cells may undergo one or more divisions before degeneration (Oakberg 1955). Type A spermatogonia show heterogeneous radiosensitivity (Oakberg 1955, Jones 1960) and a considerable number of this type of cells escapes from the radiation damage and repopulates the germinal epithelium (Hertwig 1938b, Oakberg 1955).

A much debated question is whether necrosis or inhibition of mitosis is the important factor in spermatogonial depletion after irradiation. Oakberg (1955, 1959) insisted that depletion of spermatogonia in the mouse irradiated with doses ranging from 25 r to 600 r of acute X- or γ-rays can be explained by cell death (necrosis) without postulating a mitotic inhibition of type A spermatogonia. On the contrary, Bryan and Gowen (1956, 1958) and Jones (1960), working with rats, concluded that this depletion is brought about through the inhibition of mitosis with cell death playing only a minor role. Casarett and Casarett (1957b) proposed a cooperation of several factors. They stated that in the rat depletion of spermatogonia after acute irradiation was brought about largely by inhibition of mitosis of type A spermatogonia and normal differentiation of type B spermatogonia, to some extent by spermatogonial death and by inhibition of mitosis of type B spermatogonia and possibly by premature or increased rate of differentiation of spermatogonia. Jones (1960) suggested that gross quantitative differences may exist between the rat and the mouse in the response of spermatogonia to irradiation.

Spermatocytes are more resistant to radiation than late spermatogonia, but several investigators described various forms of abnormal sperm derived from irradiated spermatocytes in mice (Schaefer 1939) and in rats (Casarett and Casarett 1957a). Bateman (1958) also stated that in mice sperm from primary spermatocytes irradiated with 500r are incapacitated in some subtle way while 200 r does not seem to have this effect. Recently, Oakberg and DiMinno (1960) studied the radiation sensitivity of mouse primary spermatocytes and found that there is an inverse relation between cell-killing and chromosome breakage: pre-leptotene was most sensitive and diakinesis-metaphase I

most resistant to the induction of cell death, whereas pre-leptotene and leptotene were most resistant and metaphase I most sensitive to chromosome breakage. Furthermore, they found that while 500r does not kill all products of spermatocytes, it could reduce the number of functional sperm to such low numbers that fertility was impaired. Thus, it is clear that, while destruction of spermatogonia is the major cause of the sterile period, irradiation of spermatocytes may contribute to it through causing functional oligospermy. Cells irradiated as primary spermatocytes may give rise to morphologically abnormal spermatids and spermatozoa (Schaefer 1939, Oakberg 1955, Oakberg and DiMinno 1960, Casarett and Casarett 1957a).

Spermatids and spermatozoa that had been formed before irradiation are much more radiation resistant than earlier stages (Oakberg 1955).

In insects, the cytological effects of irradiation on spermatogenesis have been intensively investigated in grasshoppers because of the ease with which cytological work can be carried out. The first report on this subject was published by Mohr (1919), who showed that earliest spermatocytes of the grasshopper were the most sensitive stage to radium-irradiation while other types of germ cells were fairly resistant. However, White (1935) found that X-rays induced pycnosis in the spermatogonia but not in the spermatocytes. Eker (1937) and Cocchi and Uggeri (1944) also reported that in the same insect the late, or the secondary, spermatogonia are extremely sensitive to the killing effect of radiation, but the primordial or the youngest spermatogonia were highly resistant. Although Cocchi and Uggeri (1944) observed that some early spermatocytes became pycnotic after irradiation they considered that these degenerating cells were irradiated as cells in the telophase of the last spermatogonial mitosis which, according to them, was the most sensitive. They found that cells at late gonial stages were drastically impaired by irradiation with a dose as low as 25 r and most of them degenerated after 100 r. Spermatocytes and spermatids are 600 times and 240 times, respectively, more resistant than the late secondary spermatogonia. Primordial spermatogonia and sperm were far more resistant than the spermatids.

Chromosome aberrations induced by irradiation of spermatocytes have also been investigated by a number of workers in this material (White 1937, Creighton 1941, Ohnuki 1958). It is suggested that cells carrying these aberrations will not give rise to sperm capable of fertilizing eggs (White 1937, Ohnuki 1958).

In *Drosophila*, histological observations were made on the testes of irradiated adult males with similar results to those obtained with mice and grasshoppers (Friesen 1937, Welshons and Russell 1957).

To summarize, it is now evident that in mice, rats, grasshoppers, *Drosophila* very similar pictures have emerged: preferential destruction of late spermatogonia; chromosome abnormalities leading to non-functional sperm in spermatocytes; relatively high resistance to killing (not to chromosome breakage and mutation) in post-meiotic stages.

(b) *Effects on fertility of males.*

It has long been recognized that in rodents a period of fertility immediately following irradiation is succeeded by an interval of temporary sterility after which fertility is restored. It is also known that the length of the initial fertile period and that of the temporary sterile period vary depending on the species, the radiation doses and other biological factors. Sperm used in the matings during the initial period of fertility following irradiation were mature spermatozoa at the time of treatment; sperm utilized during the later part of this period were irradiated as spermatids or spermatocytes. Based on the histological findings that the late spermatogonia are destroyed by moderately high doses of irradiation which do not destroy the other germ cell stages (Hertwig 1938b, Oakberg 1955), the period of temporary sterility is taken to correspond to the interval in which replacement of the destroyed germ cells from younger ones has not yet been completed. Consequently, sperm used in the matings of the post-sterile period are considered to have been spermatogonia at the time of irradiation. The sterile period is thus used as a marker for separating genetic changes induced in spermatogonia from those induced in later germ cell stages (Russell 1951, Russell, Russell and Kelly 1958). As will be seen below, the same principle has been applied also to irradiated males of adult *Drosophila* (Friesen 1937, Auerbach 1954, Alexander *et al.* 1955, 1959).

In insects, the effects of X-rays on the fertility of males have been investigated by a number of *Drosophila* workers, in connection with genetical problems (Friesen 1937, Lüning 1952, Auerbach 1954, Bateman 1956a, Ives 1960). In *D. melanogaster*, it has been shown repeatedly that a period of lowest fertility occurs about 7–9 days after irradiaton of adult males. In *D. virilis*, the period of pre-sterile fertility continues for 17–19 days, when the temporary sterility occurs as a result of aspermy (Alexander *et al.* 1955, 1959). Some workers considered that this low fertility may be the result of dominant lethals (Lüning 1952, Bateman 1956a), but others showed that fertilization rarely occurs during this period (Friesen 1937, Auerbach 1954, Kaplan 1958). There have been some conflicting opinions among these authors concerning the cell stage which corresponds to the sterile period. Some workers considered that this period corresponds to irradiated late spermatogonia and/or youngest spermatocytes (Friesen 1937), while

some others attributed sterility to the destruction of meiotic stages (Demerec and Kaufmann 1941) or to dominant lethals in spermatids (Lüning 1952, Fritz-Niggli 1955). Auerbach (1954) insists that the rate of sperm utilization is a very important factor in the determination of the cell stages in successive broods.

There are two accepted causes of reduced fertility in irradiated animals (Friesen 1937, Hertwig 1938a, b, Auerbach 1954, Russell 1954, Bateman 1958): (a) lack of fertilization due to destruction of late spermatogonia and—at higher doses—spermatocytes, (b) inability to produce progeny as a result of dominant lethal mutations, with peak frequency in spermatids.

3. Radiation effects on silkworm germ cells

In the silkworm, a sterilizing effect of X-rays was reported first by Katsuki (1925). The histological changes of the testes after X-irradiation were briefly described by Obata (1934) and Hayashi (1935). However, these studies were not sufficient for a clear understanding of the general effects of irradiation on spermatogenesis in this insect. The fact that the silkworms were exposed to repeated doses of X-rays obscures the primary effects of the radiation on germ cells.

Recently, Tazima (1958a, 1959) found an extremely sensitive stage to the sterilizing effect of X-rays early in the fifth instar of the male, suggesting a higher sensitivity of spermatocytes than of spermotogonia. Soon after the present author started the work to be reported here, Kogure and Nakajima (1958) published their extensive work on the same problem. They stated that early prophase of the primary spermatocyte is most sensitive to irradiation; however, cells irradiated at this stage are not killed but transformed into apyrene spermatozoa. They concluded that this may be the main cause of radiation induced sterility in the silkworm male. They further stated that spermatogonia are more resistant to irradiation than spermatocytes. On the contrary, the preliminary experiments of the present author showed clearly that the secondary spermatogonia, especially at later stages, are extremely sensitive to radiation while other types of cells are much more resistant (Sado 1959b). He also found that spermatocytes at early meiotic prophase are more resistant than those at late meiotic stages (Sado 1961a). Regeneration of irradiated spermatogonia was also investigated by him (Sado 1959b) and will be presented here in full detail. In spite of these apparent disagreements, the same explanation may be possible that irradiation of silkworm larvae at early fifth instar causes the functional oligospermy of the treated individuals as a result of reduction in number of functional spermatozoa (Sado 1960c, 1961c).

MATERIAL AND METHODS

A wild type strain (C 108) was used as material. Male larvae at various stages were exposed to X-rays. The conditions of irradiation were 180 kvp, 25 ma, with 1.0 mm Al filter; target distance, 50 cm in air; dose-rate, 147.7 r/min.; total doses, 500 r, 1000 r and 2000 r. In a few experiments, much higher doses ranging from 5000 r to 15000 r of acute X-rays were also given to the larvae just before hatching and to the late pupae on the day before emergence. The irradiated stages varied with the purpose of the experiment. Irradiated individuals were divided into two groups, one for testing fertility after emergence and the other for histological examination.

Histological observations were made on material that had been fixed (Bouin's fluid) and sectioned (7–10μ) by the usual paraffin method. Sections were stained with Delafield's hematoxylin and eosin. Doses and intervals between irradiation and fixation are given in each part of the experimental results.

The fate of irradiated germ cells in the seminal vesicles of adult male moths and in the reproductive tracts of females after mating was studied with Feulgen smears and on the living materials. In order to make the Feulgen smear preparations, contents of the organs to be examined were smeared on a slide-glass, fixed with Carnoy's fluid for 10–30 minutes, and stained with the usual Feulgen procedures.

In order to estimate the reduction in number of spermatozoa produced by the irradiated insects, total numbers of eupyrene and apyrene spermatozoa bundles were counted on serial sections. According to Machida's (1929) method, testes were fixed with Bouin's fluid, embedded in paraffin, sectioned at 20 μ and stained with Delafield's hematoxylin. On such preparations a mass of nuclei of each type of spermatozoa bundle can easily be discriminated from other part of a bundle. Thus, total number of spermatozoa bundles in each testis was scored on a serial section only at the portion where nuclei are observed. In this method, possible source of error is that single mass of nuclei may be scored twice or more. Therefore, the total number scored by this method was corrected by the equation, $N=K\times A$, where N and A represent scored and real number respectively, while K is a proportionality constant dependent on the size and form of the mass of nuclei of a bundle and the thickness of the sectioned material. According to Machida (1929), K is about 2.0 for the eupyrene spermatozoa bundle and 3.0 for the apyrene one, when the counting is made for 20μ thick preparations.

RESULTS

I. Spermatogenesis in the silkworm

1. *Development during the larval stage.*

The testis of a newly hatched larva of C 108 strain as well as of other races contains only a mass of primordial germ cells. Three days after hatching, primary and secondary spermatogonia* are differentiated but spermatocytes do not yet appear. The primary spermatocytes at the resting stage can hardly be distinguished from the latest spermatogonia. Differentiation of spermatogonia into primary spermatocytes occurs first in the middle of the second instar, 6 days after hatching. Since primary spermatocytes in synapsis are situated very close to the latest secondary spermatogonia, the duration of development from the resting stage to synapsis of the primary spermatocytes must be very short. As the larva grows in the third instar, differentiation of primary spermatocytes from spermatogonia occurs more frequently. Pachytene spermatocytes appear in the late third instar (10–11 days after hatching). At the beginning of the fourth instar (13–14 days after hatching), a majority of cells in the testis are in synapsis and pachytene stages of the primary spermatocytes. At this time spermatogonia have become much less numerous than spermatocytes. In the late fourth instar (16 days after hatching) the most advanced spermatocytes enter into the second contraction- and diffuse-stages. The meiotic prophase persists until the larvae reach the fourth molting or the very early fifth stage (17–18 days after hatching), when cells in diakinesis and in metaphase I are observed for the first time. Within a day after metaphase I, secondary spermatocytes and the first spermatids appear in the testis. This suggests that the duration between metaphase I and the formation of the first spermatid is very short. Around the end of the fifth instar (24 days after hatching), fully formed eupyrene spermatozoa appear in the testis. Apyrene spermatozoa make their appearance in the early pupal stage (31–32 days after hatching, or 1–2 days after pupation) and thereafter increased progressively in number (see Tables 5, 6 and Fig. 3). The time table of the first appearance of the cells at the successive stages of spermatogenesis is summarized in Table 1. The majority of the cells develop about two or more days later than indicated in the table. The relation between the development of spermatogenic cells and the

* In this paper, spermatogonia that locate very closely around the apical cells are termed 'primary spermatogonia'; their cytoplasm is connected with that of the apical cell by a spine-like process. Spermatogonia that are covered with a common envelope, the gonocyst, are described as 'secondary spermatogonia.'

developmental stages of the silkworm is illustrated schematically in Fig. 1.

Table 1. Development of spermatogenic cells during larval life of the silkworm.

Days after hatching	Larval stage	Stage of the most advanced germ cells
6 days	II-2	Spermatocyte I, synaptic stage
10-11	III-3	Spermatocyte I, pachytene stage
16	IV-3	Spermatocyte I, second contraction and diffuse stage
17-18	4th molt — V-1	Spermatocyte I, diakinesis and metaphase I
18-19	V-2	Secondary spermatocytes and earliest stage of spermatids
24	V-6	Fully formed eupyrene spermatozoa

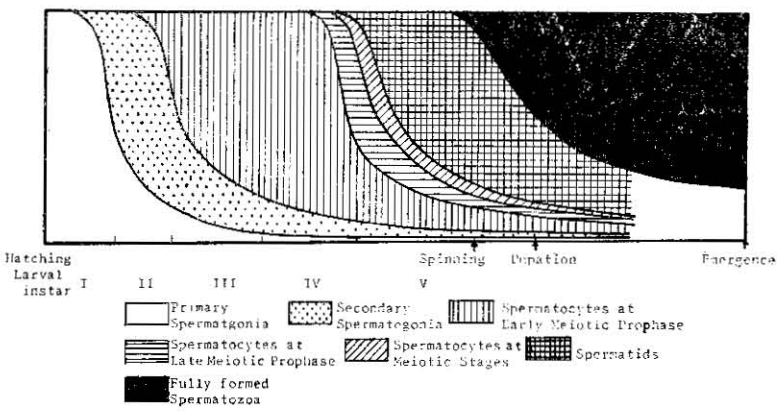


Fig. 1. Schematic illustration of spermatogenesis in relation to the developmental stages of the silkworm male.

2. *Duration of spermatogenesis.*

From the foregoing histological observations, the approximate duration of each stage of spermatogenesis in the silkworm has been estimated (Table 2).

It will be seen that the meiotic prophase of the primary spermatocytes takes about 10 days, while the late meiotic stages proceed within a short time. It takes about a week from the beginning of spermiogenesis to the completion of eupyrene spermatozoa. The estimated values agree well with those obtained from the observation of

the regenerated germ cells after acute X-irradiation, as will be seen later.

Table 2. Approximate length of duration of each stage of spermatogenesis in the silkworm.

Stage of spermatogenesis	Duration
Spermatogonia	a few days
Spermatocytes	
Meiotic prophase I	10–11 days
Metaphase I–Anaphase II	within 1 day
Spermatids	5–6 days
Spermatozoa	4–15 days*
Total spermatogenesis	about 20 days

* In silkworms, spermatozoa are stored in the testis until a few days before ejaculation. Therefore, the duration of storage varies from 4 days to 15 days.

3. *Types of spermatozoa.*

The reproductive tracts of female moths mated with normal males usually contain two types of spermatozoa. One type is thicker, longer and much less active than the other, which is small and highly active. Some workers considered that the former type might be derived from the eupyrene spermatozoa and the latter from the apyrene ones (Tsukaguchi and Kurotsu 1922), but others assumed that apyrene spermatozoa, since they cannot be observed in the extra-testicular organs of males, degenerate within the testis (Machida 1929, Ômura 1936). This assumption lost weight by the observation of Iriki (1941), who found both apyrene and eupyrene spermatozoa in the extra-testicular organs of male moths as well as in the reproductive tracts of females after copulation. These two types of spermatozoa were separated by centrifugation. When eupyrene spermatozoa alone were introduced by artificial insemination into the bursa copulatrix of female moths, they could not reach the receptaculum seminis. From this experiment, Iriki concluded that eupyrene spermatozoa are carried from the bursa copulatrix into receptaculum seminis by highly active apyrene spermatozoa. If this conclusion is correct, apyrene spermatozoa are necessary for the normal process of fertilization.

In view of the significance of this question for the nature of radiation-induced sterility the seminal vesicles of newly emerged moths were examined in Feulgen smear preparations. If, according to Iriki's view, the small active spermatozoa in the bursa copulatrix are derived

from apyrene spermatozoa, the latter should be present in the seminal vesicles.

Preliminary tests showed that the nuclei of apyrene spermatozoa in the testis stain deeply with Feulgen-Schiff reagent (Plate 12, Fig. 17, apy).

18 such preparations of seminal vesicles were examined and about 15,000 spermatozoa bundles were scored. However, no apyrene spermatozoa were found in any of these preparations (Plate 9, Fig. 1). This observation does not support the view that the smaller and more active spermatozoa in the bursa copulatrix are derived from apyrene spermatozoa and confirms the earlier observation by Machida (1929) and Ômura (1936).

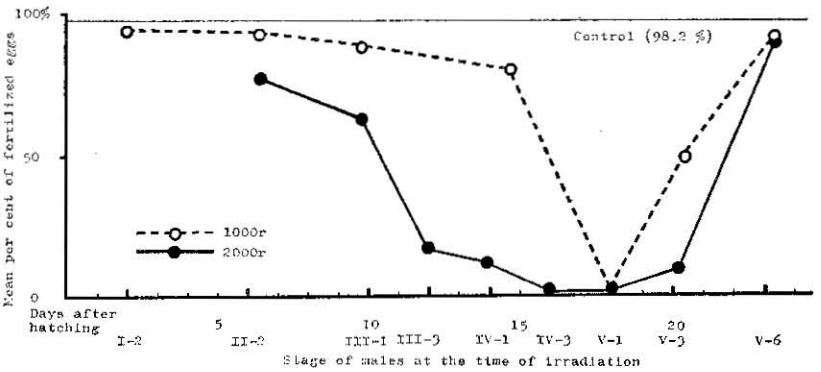


Fig. 2. Fertility of males irradiated at different developmental stages.

II. Radiation response of spermatogenic cells

1. *Effect of X-rays on the fertility of males.*

Although it was already known that the male larvae in the early fifth instar are most sensitive to the sterilizing effect of radiation (Tazima 1958a, 1959, Kogure and Nakajima 1958), a further test was carried out by the author in order to compare the results of fertility tests with histological and cytological observations. The data on fertility are given in Table 3 and are shown graphically in Fig. 2. They are consistent with those reported by Tazima (1958a, 1959) in showing the extreme sensitivity of the germ cells in early fifth instar. When animals were exposed to a higher dose, 2000 r, the sterilizing effect of X-rays was very severe and extended toward the younger larval stages, but treatment before the early third instar gave good fertility even after exposure to 2000 r.

Males from the same two irradiated series were used for the histological and cytological studies reported below.

Table 3. Relation between the irradiated stages of males and the per cent of fertilized eggs in F_1* (Exp. 592, 593).

Dose	1000 r					2000 r				
Irradiated stages	No. of treated males	No. of eggs scored	No. of fert. eggs	No. of unfert. eggs	% of fert. eggs	No. of treated males	No. of eggs scored	No. of fert. eggs	No. of unfert. eggs	% of fert. eggs
I-2	11	5,738	5,485	253	95.6	—	—	—	—	—
II-2	19	8,893	8,208	685	92.3	6	1,735	1,350	385	77.8
III-1	13	5,387	4,891	686	90.8	25	8,681	5,425	3,256	62.5
III-3	—	—	—	—	—	20	5,526	967	4,559	17.5
IV-1	—	—	—	—	—	21	2,979	408	2,571	13.7
IV-2	14	5,646	4,998	648	88.5	—	—	—	—	—
IV-3	—	—	—	—	—	16	719	0	719	0.0
V-1	30	2,018	17	2,001	0.8	13	581	0	518	0.0
V-3	18	6,581	3,211	3,370	48.8	17	2,109	214	1,895	10.1
V-6	23	10,937	10,182	754	93.1	22	9,782	8,843	919	90.4
Control	11	6,351	6,236	115	98.2		—			

* All pigmented eggs are scored as fertilized and all unpigmented ones as unfertilized. Contribution of unpigmented eggs that have been fertilized but died before pigmentation to percentage of fertilized eggs might be small, if any. The reason for this will be discussed in the continuing paper (section III).

2. Histological and cytological effects of X-rays on spermatogenesis and the nature of radiation-induced sterility of the male silkworm

1) *Radiation response of spermatogonia.*

(a) *Degeneration of spermatogonia after irradiation.*

As the testis of the early fifth instar contains various cell types ranging from primary spermatogonia to young spermatids (Fig. 1), the relative sensitivity of these cells to X-rays can be determined by irradiating the larvae at this stage. Testes from irradiated males were fixed at definite intervals after irradiation and were examined histologically.

Six hours after the irradiation a few necrotic cells were observed in the gonial region; these increased in number with time after irradiation up to the period of 24 to 48 hours (Plate 9, Fig 2). The first sign of degeneration was observed in the late secondary spermatogonia but, in very few instances, necrosis of the youngest spermatocytes was also observed. About 60 to 70 per cent of the secondary spermatogonia, especially at the later stages, were necrotic 24 hours after exposure to 1000r (Plate 9, Fig. 3). At 48 hours, most of the secondary spermatogonia were degenerating (Plate 9, Fig. 4). Thereafter, these dead cells disappeared rapidly from the irradiated testis, greatly reducing the number of spermatogonia. Thus, on the third day after exposure to 1000 r or more, secondary spermatogonia were rarely observed but apical cells and primary spermatogonia still survived without showing any cytological abnormalities (Plate 9, Fig. 5a, Plate 10, Figs. 5b, 5c, 5d).

Quite similar results were obtained when larvae were irradiated at younger stages (Plate 10, Figs 6, 7, Plate 11, Fig. 9).

(b) *Regeneration of spermatogonia after irradiation.*

Five or more days after irradiation with 1000 r, regeneration of spermatogonia occurred in the depleted gonial region. The fate of the regenerated germ cells varied with the stage of the irradiated larvae.

(i) *Irradiation during the early fifth instar.* When larvae were treated on the 2nd day of the fifth instar, regeneration of spermatogonia occurred 7 days after treatment, when the irradiated individuals had matured to the spinning stage. Differentiation of regenerated spermatogonia into spermatocytes was observed first in the middle pupal stage but they never transformed into eupyrene or apyrene spermatozoa.

(ii) *Irradiation during the fourth instar.* Similarly, when animals were irradiated on the 2nd day of the fourth instar, regeneration of spermatogonia was observed 7 days after treatment, namely on the 4th day of the fifth instar. On the 11th day after the exposure,

when the exposed insects attained maturity, the regenerated spermatogonia increased in number and some germ cells had differentiated into the synaptic stage of the primary spermatocytes (Plate 11, Fig. 8). However, these regenerated germ cells did not differentiate into eupyrene spermatozoa but transformed only into apyrene ones. The fate of the regenerated germ cells was the same when larvae of this stage were irradiated with 2000 r.

(iii) *Irradiation during the second instar.* Table 4 summarizes results of a histological study of regeneration of spermatogonia in animals irradiated with 1000 r on the 2nd day of the second instar. Three days after irradiation, the testicular follicles were almost empty

Table 4. Development of regenerated germ cells after irradiation with 1000 r on the 2nd day of the second instar.

Days after irradiation	Development of regenerated germ cells
5–8 days	Regeneration of spermatogonia begins
8–9 „	Differentiation of spermatocyte I (synaptic stage)
17–18 „	Meiotic metaphase I
24 „	First appearance of fully formed eupyrene spermatozoa

and degenerating cellular debris was observed, but a few primary spermatogonia were found unchanged (Plate 11, Fig. 9). The repopulation of spermatogonia commenced 5–6 days after exposure, and the differentiation of these cells into spermatocytes was observed on the 8th or 9th day. Many spermatocytes derived from the regenerated spermatogonia were in the synaptic and pachytene stages 13 days after exposure, namely, on the 2nd day of the fifth stage (Plate 11, Fig. 10). Spermatocytes at various meiotic stages and early spermatids were observed on the 18th day after treatment, when the irradiated larva attained maturity (Plate 11, Figs. 11, 12). 24 days after irradiation, i.e., on the 2nd day of pupation, fully formed eupyrene spermatozoa were observed, and these increased in number thereafter. Fertility tests showed that they are functional (see Fig. 2).

From Table 4, the time required for the successive stages in spermatogenesis can be estimated in the way described in Section I–2. This results in estimates of 3, 10 and 6 days for the spermatogonial stage, meiotic prophase and the spermatid stage respectively. It is also found that even after irradiation with 2000 r, regenerated spermatogonia developed into functional spermatozoa. It is a noteworthy fact that irradiation with 1000 r of primary spermatogonia scarcely

affects the time required for each stage in the subsequent development of the treated germ cells. This agrees with the findings that in mice doses as high as 1000 r have no retarding effect on meiosis or on spermiogenesis of cells irradiated as spermatids, and that maturation of spermatids arising from cells irradiated as spermatocytes is not retarded by 100 r, although it may be retarded by higher doses (Oakberg 1957).

(iv) *Irradiation during the first instar.* When silkworms were irradiated with 1000 r during early in the first instar, there was hardly any observable degeneration of spermatogonia. However, the development of germ cells was somewhat retarded as compared to that of the unirradiated larvae. The first appearance of meiotic metaphase I was observed two days later than in the control. Apparently, primordial germ cells and primary spermatogonia, which at this stage form the bulk of the irradiated germ cells, are not killed but retarded from mitosis for a few days.

A few tests with much higher doses were also carried out. Larvae just before hatching were irradiated with 5000 r or 10000 r. Development of germ cells was also observed in these cases, but the fertilities of the treated insects could not be tested because all of them died before emergence. From this experiment, it may be concluded that the primordial germ cells are extremely resistant to X-rays.

(v) *Production of giant cysts from regenerated spermatogonia.* Even after irradiation during early larval stages the regenerated spermatogonia do not always develop into normal eupyrene and apyrene spermatozoa. Usually, 64 primary spermatocytes are counted in one cyst (Kawaguchi 1928). As a result of the maturation division, one primary spermatocyte produces 4 spermatids or spermatozoa. Thus normally one cyst gives rise to 256 spermatozoa. In the irradiated testes, giant cysts containing hundreds of spermatogonia, spermatocytes or spermatids developed sometimes from regenerated spermatogonia. Plate 11, Fig. 13 shows some of those abnormal cysts. Serial cross sections showed that one of them contained at least 798 primary spermatocytes; if all of these had been able of further development, this cyst would have given rise to at least 3192 spermatids. Two other giant cysts are also shown in Plate 11, Fig. 13, but their cells were not counted. In general, it is not easy to count the number of spermatids in these abnormal cysts because a considerable part of the spermatids are degenerating. It is very likely that most of the germ cells contained in these abnormal cysts will not develop into normal spermatozoa.

2) *Sensitivity and time of degeneration of spermatocytes irradiated during meiosis.*

The hypersensitivity of secondary spermatogonia shown in the

foregoing section does not account satisfactorily for the excessive sterility of insects irradiated in the early fifth instar because at that time most of the germ cells have already differentiated into spermatocytes. Therefore, the effects of X-rays on the spermatocytes were investigated.

In the silkworm, spermatocytes, especially at meiotic prophase I, are a very long lived cell type. As described in Section I·1, the majority of germ cells contained in the testis of the early fourth instar are spermatocytes in the synaptic and pachytene stages,* while spermatocytes at late prophase† prevail in the early fifth instar (Fig. 1). The effects of X-rays on the spermatocytes at early and late meiotic stages were investigated by irradiating larvae of these two stages with 1000 r or 2000 r.

Most, if not all, of the spermatocytes irradiated with 1000 r at early meiotic prophase did not show any degenerative figures before or after meiosis and developed into eupyrene spermatozoa. A testicular follicle shown in Plate 12, Fig. 14 contains metamorphosing spermatids and fully formed eupyrene spermatozoa that had developed from spermatocytes treated in early meiotic prophase. Fertility data (Fig. 2) confirm that these spermatozoa were functional. However, after irradiation with 2000 r, degeneration occurred later on in the spermiogenic stage and structurally abnormal spermatozoa were produced (Plate 12, Figs. 15, 16). From this experiment, it is clear that spermatocytes at early meiotic prophase are fairly resistant to X-rays.

Spermatocytes treated with 1000 r at the late meiotic stages also completed maturation divisions without showing any necrotic figures. However, 5 days after the treatment, spermatids with necrotic nuclei were frequently observed (Plate 12, Fig. 15). Furthermore, eupyrene spermatozoa with structurally abnormal heads (nuclei) appeared later on in the pupal period, 10 days or more after exposure (Plate 12, Fig. 16). Since the spermatocytes at early meiotic prophase are less affected by this dose, there can be no doubt that most of these abnormalities were derived from cells irradiated at late meiotic stages, especially late prophase.

When the primary spermatocytes of the grasshopper or the mouse are exposed to X-rays, various types of chromosome aberrations are observed immediately or at a certain interval after irradiation (White 1937, Kashiwabara 1957, Ohnuki 1958, Oakberg and DiMinno, 1960). It

* Hereafter, the term 'early meiotic prophase' will be applied to both these stages.

† Hereafter, it will be subsumed under the term 'late meiotic stages,' since a few spermatocytes at meta- and telophase and even at early post-meiotic stages are also found at this time.

has been suggested that the cells which carry those aberrations will not give rise to sperm capable of fertilizing eggs. It seems likely that also in the silkworm the majority of the abnormal spermatids and spermatozoa derived from cells in which X-rays had induced gross chromosomal disturbances. Actually chromosome aberrations have been observed in some of the irradiated testes (Plate 12, Fig. 18). However, the detection of such aberration is not easy in the silkworm because the chromosomes are too small and numerous for cytological analysis.

3) *Radiation response of spermatids and mature spermatozoa.*

As the testis at late fifth instar larva contains germ cells at various stages in spermiogenesis, this might be most favorable material for examining radiation sensitivity of spermatids of the silkworm. Male larvae on the 6th day of the fifth instar were irradiated with 2000 r and their testes were examined histologically 3, 5 and 10 days after the treatment. No detectable changes were observed in cells irradiated as spermatids.

In order to know radiation sensitivity of mature spermatozoa, male insects were irradiated with 5000 r, 10000 r and 15000 r on the last day of the pupal stage, i.e., one day before emergence. 20 hours after the treatment, spermatozoa in the seminal vesicles of the newly emerged moths were examined microscopically with the Feulgen smear preparations. Any abnormal figures were not found among 20 such preparations.

From these observations, it can be said that spermatids and spermatozoa, particularly the latter, are highly resistant to radiation. However, it might be presumed that radiation damage to these cells appears after the fertilization (Tazima, 1961).

4) *Reduction in the number of sperm produced by irradiated males.*

Since it was found that spermatocytes treated at late meiotic stages degenerate later on during the spermatid stage or transform into inviable sperm, it can be expected that the number of sperm produced by the treated individuals is reduced. Therefore, a quantitative histological study of the eupyrene and apyrene spermatozoa bundles (Plate 12, Fig. 19) was carried out in the testes of males which had been exposed to 1000 r on the 2nd day of the fifth instar. The results are presented in Tables 5 and 6.

From Table 5, it will be seen that in the irradiated testes, only a few bundles of apyrene spermatozoa were found, while many more such bundles were observed in the non-irradiated ones throughout the pupal period. This effect of radiation, however, can have no bearing on the induced sterility, since, as stated earlier, apyrene spermatozoa do appear to play no rôle in fertilization even under normal conditions.

Table 5. Number of apyrene spermatozoa bundles in the testes at various intervals after irradiation (Exp. 601).

Stage observed	No. of testes examined	Obsd. no. of apyrene sperm bundles	Corrected no. of apyrene sperm bundles K=3.0†	Corrected no. of apyrene sperm bundles per testis
5-9 Control	8	0	0	0
(7 days*) Irradiated	8	0	0	0
P-1 Control	8	41	13.4	1.7
(10 days*) Irradiated	8	54	17.9	2.2
P-3 Control	8	11,204	3,731.3	466.4
(12 days*) Irradiated	8	4,026	1,338.8	167.3
P-5 Control	8	38,741	11,580.3	1,447.5
(14 days*) Irradiated	8	218	72.7	9.1
P-7 Control	7	35,555	11,851.6	1,693.1
(16 days*) Irradiated	8	312	100.6	12.6
P-9 Control	6	37,348	12,449.4	2,074.9
(18 days*) Irradiated	6	108	36.0	6.0

* Days after irradiation.

† K is a constant calculated from the size of a nuclear mass of spermatozoa bundle (on an average, that of apyrene spermatozoa is 28μ in diameter and 50μ in length) and thickness of the sections (20μ). —Machida (1929)

Table 6. Number of eupyrene spermatozoa bundles in the testes at various intervals after irradiation (Exp. 601).

Stage observed	No. of testes examined	Obsd. no. of eupyrene sperm bundles	Corrected no. of eupyrene sperm bundles K=2.0†	Corrected no. of eupyrene sperm bundles per testis
5-9 Control	8	3,285	1,642.5	205.3
(7 days*) Irradiated	8	3,071	1,535.5	191.9
P-1 Control	8	11,940	5,820.0	725.0
(10 days*) Irradiated	8	9,836	4,918.0	614.8
P-3 Control	8	22,948	11,475.5	1,433.4
(12 days*) Irradiated	8	16,762	8,381.0	1,047.6
P-5 Control	8	28,906	14,453.0	1,806.6
(14 days*) Irradiated	8	21,391	10,197.0	1,274.6
P-7 Control	7	26,888	13,494.0	1,927.8
(16 days*) Irradiated	8	21,398	10,693.5	1,336.7
P-9 Control	6	27,314	13,657.0	2,276.2
(18 days*) Irradiated	6	17,599	8,799.5	1,466.6

* Days after irradiation.

† K is a constant calculated from the size of a nuclear mass of spermatozoa bundle (on an average, that of eupyrene spermatozoa is 20μ in diameter and 22μ in length) and thickness of the sections (20μ). —Machida (1929)

As regards the eupyrene spermatozoa, Table 6 shows that the testis of irradiated individuals contained, at the 5–9 and P–1 stages, as many bundles of this type of spermatozoa as the non-irradiated testis. Since, at the time of treatment, the testis contained a considerable number of post-meiotic cells this result is considered to suggest that the observed eupyrene spermatozoa might have been derived from cells which were irradiated as early spermatids. After the P–3 stage, the number of eupyrene spermatozoa bundles per testis was markedly reduced in irradiated animals, and a considerable proportion of the sperm bundles was structurally abnormal (Plate 12, Figs. 16, 20). The proportion of these abnormal bundles to the total counted is shown in Table 7. It will be seen that the proportion of normal spermatozoa bundles decreased with the growth of the pupa up to the P–7 stage (16 days after the exposure). After this stage a slight increase was observed. Early spermatocytes presented at the time of irradiation may be responsible for this increase.

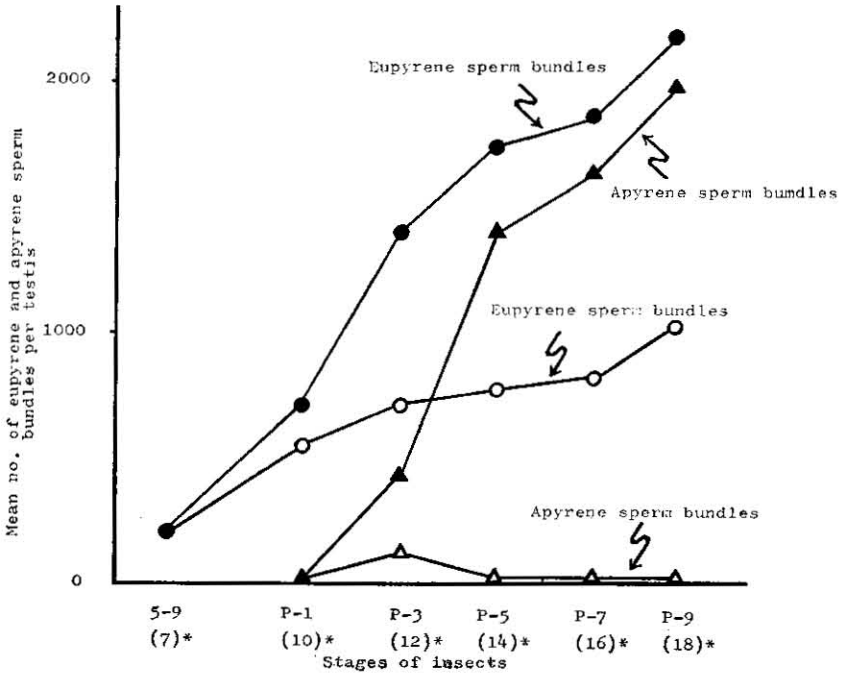


Fig. 3. Number of eupyrene and apyrene sperm bundles in the testes of irradiated and non-irradiated insects (Exp. 601). Black: Non-irradiated group. * Days after irradiation.

In order to compare the number of structurally normal eupyrene spermatozoa bundles in irradiated and non-irradiated testes, the pro-

portion of abnormal bundles in the irradiated group was subtracted from the total given in Table 6. The calculated number of morphologically normal eupyrene spermatozoa bundles and apyrene ones are shown graphically in Fig. 3.

Table 7. Proportion of normal eupyrene spermatozoa bundles to the total examined in the testes of irradiated insects (Exp. 601).

Stage observed	No. of normal eupyrene sperm bundles	No. of abnormal eupyrene sperm bundles	Total no. of eupyrene sperm bundles examined	Ratio of normal eupyrene sperm bundles to the total examined
5-9	603	—	603	1.00
P-1	1,626	72	1,698	0.96
P-3	1,134	507	1,641	0.70
P-5	798	584	1,382	0.58
P-7	703	555	1,258	0.56
P-9	985	471	1,456	0.68

5) *Types and numbers of spermatozoa observed in the reproductive organs of females mated with irradiated males.*

(a) *Types of spermatozoa.*

If the highly active sperm observed in the female reproductive organs were actually derived from apyrene spermatozoa, as Iriki (1941) considered, no or very few active sperm should be found in the bursa copulatrix of females mated with males irradiated in the early fifth instar, for as shown in the foregoing section, only very few apyrene spermatozoa are produced in the testes of such males.

25 bursae copulatrices containing sperm were examined. Both active and non-motile sperm were found in all of them.

From this observation it may be considered that both active and non-motile sperm observed in the reproductive organs of females after copulation are derived from the eupyrene spermatozoa, though the reason why such a differentiation appears among eupyrene spermatozoa was not known.

(b) *Numbers of spermatozoa.*

Ômura (1938b) showed that sperm is ejaculated first into the bursa copulatrix, from there it is transferred to the receptaculum seminis and finally enters the egg. The amount of sperm in the bursa copulatrix and the receptaculum seminis of adult females mated

with irradiated males was examined, and the results are summarized semiquantitatively in Table 8. In all females mated with non-irradiated males large quantities of sperm were present in both bursa copulatrix and receptaculum seminis. On the contrary, the receptaculum seminis of females mated to males that had become sterile after irradiation

Table 8. Amount of sperm observed after oviposition in the reproductive tracts of females mated with irradiated males and the percentage of fertilized eggs.

Irradiated stage and dose	No. of individuals	Amount of sperm in		Per cent of fertilized eggs
		bursa copulatrix	receptaculum seminis	
592 IV-2	1	+++	++	88.9%
(1000 r)	2	++	++	73.2
	3	++	+	86.3
	4	+++	++	95.1
	5	++	++	90.7
592 IV-2	1	++	−	0.0
(2000 r)	2	++	?	0.0
	3	+	−	0.0
	4	++	+	19.3
	5	++	−	0.0
592 V-2	1	++	+	5.3
(1000 r)	2	+	±	0.0
	3	++	+	10.0
	4	++	±	0.0
	5	−	−	0.0
	6	++	−	0.0
	7	+	−	0.0
602 V-2	1	±	−	0.0
(1000 r)	2	+	−	0.0
	3	−	−	0.0
	4	++	−	0.0
	5	−	−	0.0
	6	+	−	0.0
592 Cont.	1	+++++	++++	96.5
	2	+++++	++++	99.1
	3	++++	++++	97.6
	4	++++	++++	98.4
	5	+++++	+++	92.3
	6	+++++	++++	95.3

with 1000r or 2000r contained very few or no spermatozoa, even though a considerable number of sperm was found in the bursa copulatrix. It seems that most of the spermatozoa in the irradiated group were no longer functional.

In summary, it may be concluded that irradiation of larvae in the early fifth instar causes firstly, a reduction in number of spermatozoa produced and secondly, the formation of non-functional sperm, resulting in the functional oligospermy of the treated insects.

Explanation of Plate 9

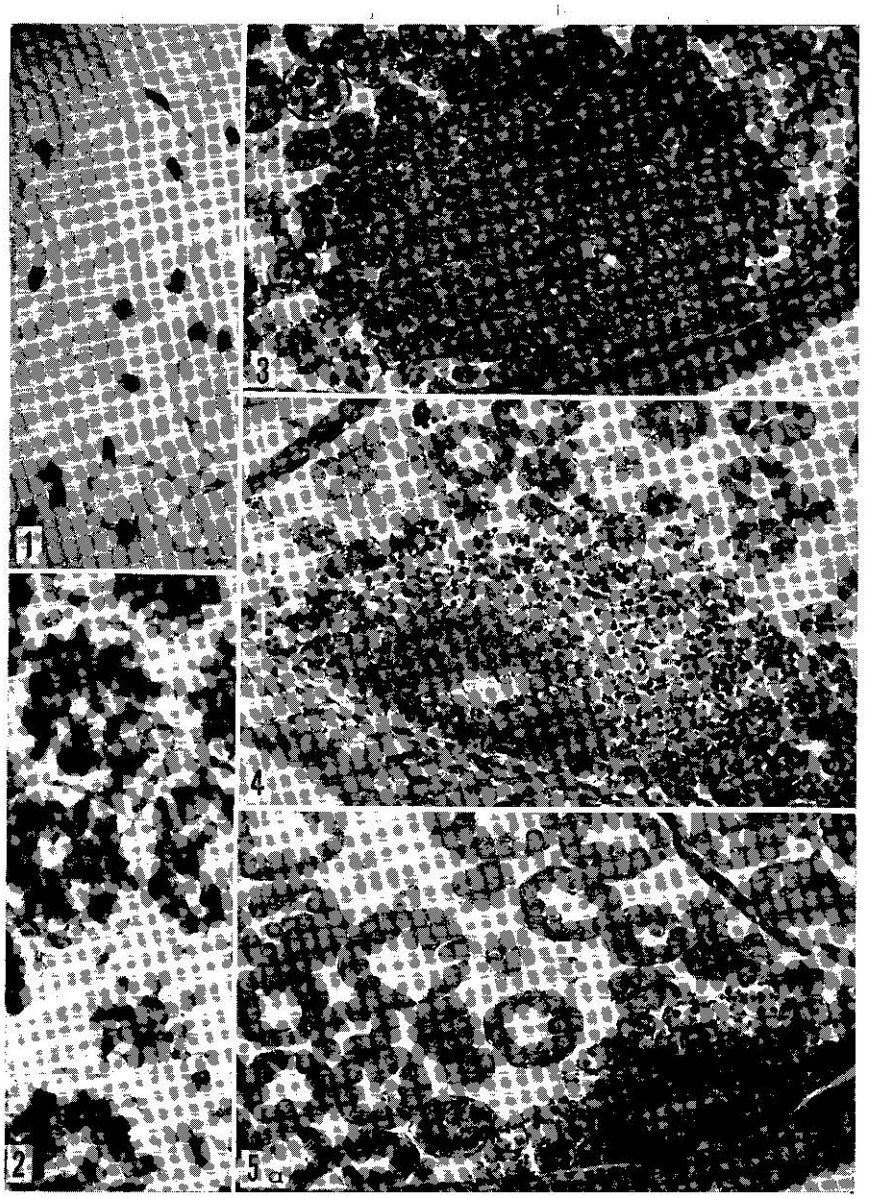
Fig. 1. Bundles of eupyrene spermatozoa in a seminal vesicle of a newly emerged male moth. Note that nuclei of apyrene spermatozoa are not observed. Feulgen smear preparation. (×126)

Fig. 2. Necrosis of the secondary spermatogonia. 24 hours after irradiation with 1000 r. Spermatocytes in the synaptic stage are normal in appearance. (×756)

Fig. 3. Gonial region one day after irradiation with 1000 r. (×252)

Fig. 4. Gonial region two days after irradiation with 1000 r. (×252)

Fig. 5a. Depleted gonial region three days after irradiation with 500 r on the second day of the fifth instar. Apical cells and primary spermatogonia are not killed. (×252)



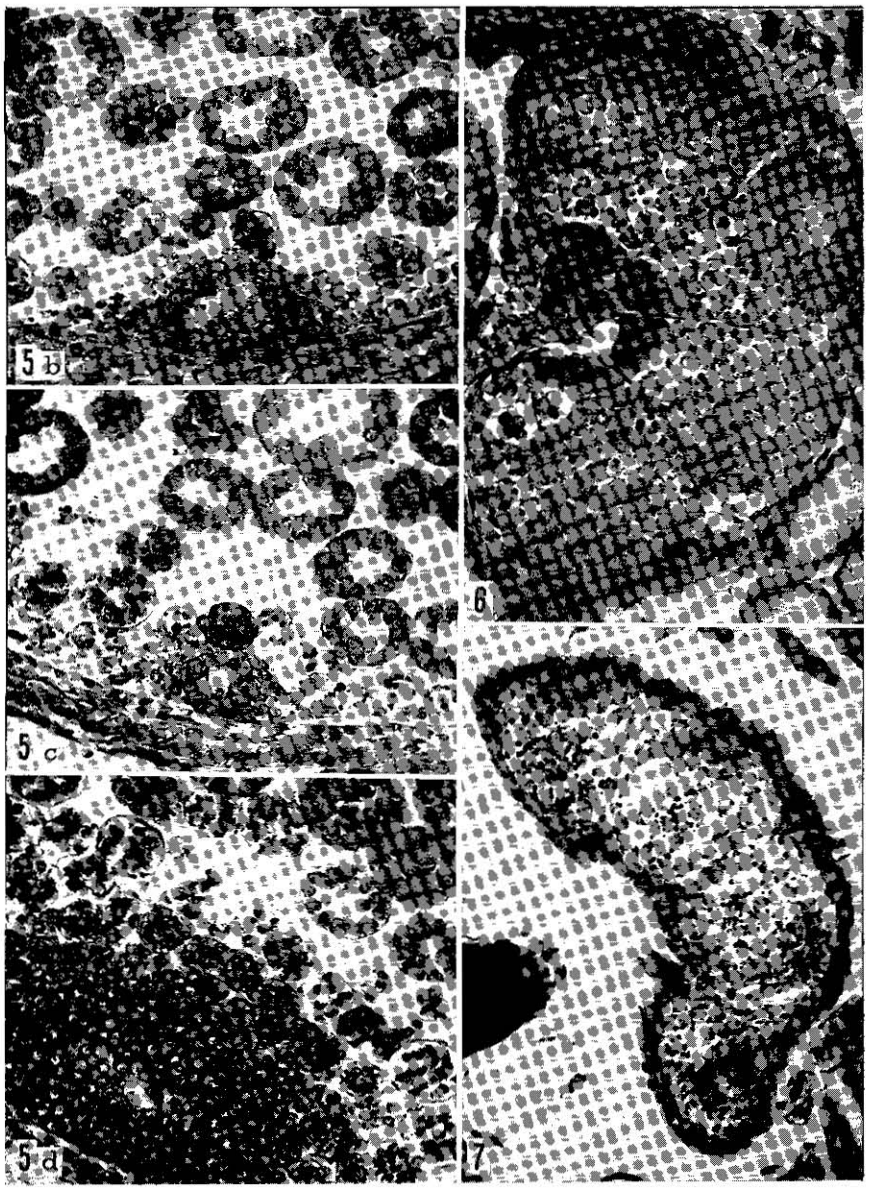
Spermatogenesis of the silkworm. I

Explanation of Plate 10

Figs. 5b, 5c, 5d. Depleted gonial region three days after irradiation with (b) 1000 r and (c) 2000 r on the second day of the fifth instar. Apical cells and primary spermatogonia are not killed. (d) Control. (×252)

Fig. 6. Testis of a larva on the third day of the second instar after exposure to 1000 r on the previous day (24 hours before). See necrosis of the secondary spermatogonia. (×252)

Fig. 7. Testis of a larva on the first day of the third instar that had been exposed to 1000 r three day earlier. (×126)



Spermatogenesis of the silkworm. I

Explanation of Plate 11

Fig. 8. Regenerated gonial region on the 11th day after irradiation with 1000 r on the 2nd day of the fourth instar. Some regenerated germ cells have differentiated into primary spermatocytes. (×126)

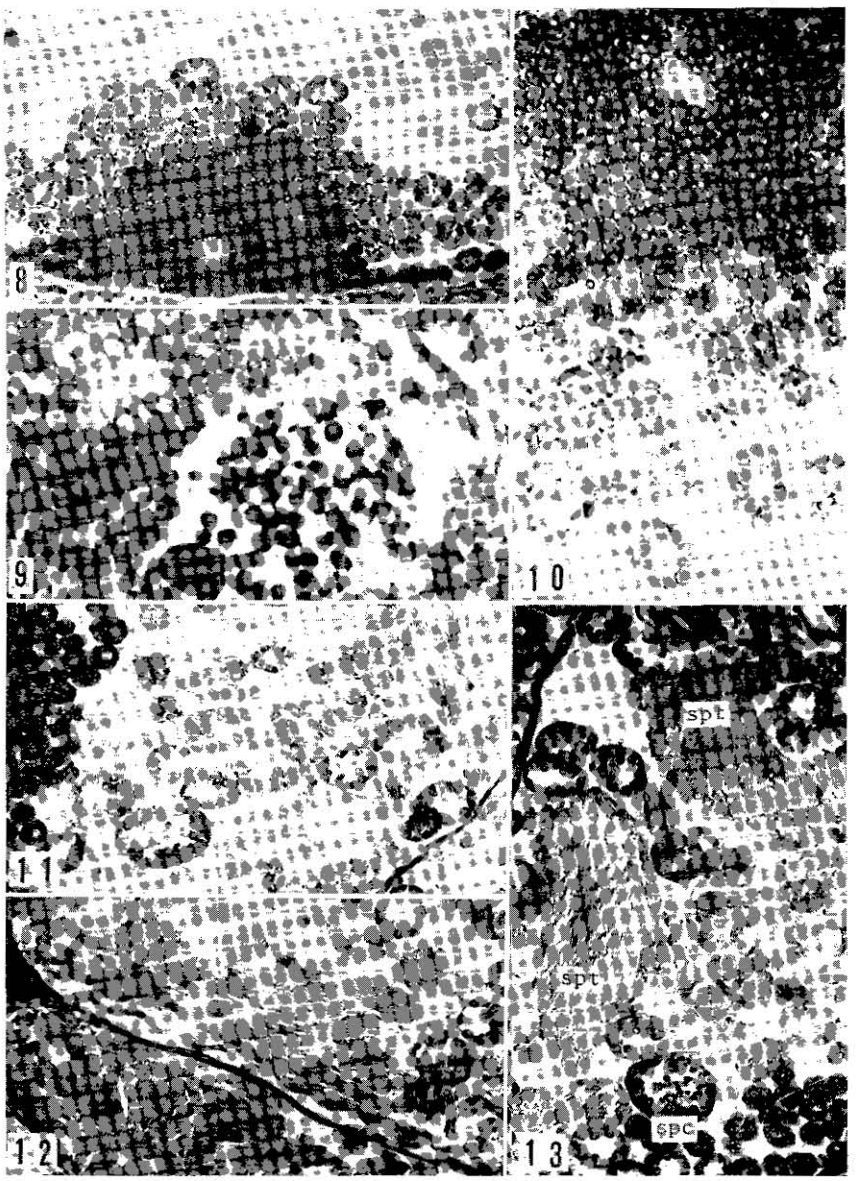
Fig. 9. Gonial region of the same testis as shown in Plate 10, Fig. 7. Apical cell and primary spermatogonia have survived unchanged. (×800)

Fig. 10. Regenerated germ cells observed 13 days after exposure to 1000 r on the 2nd day of the second instar. Spermatocytes in synaptic and pachytene stages have already differentiated. (×252)

Fig. 11. Part of a testicular follicle, showing spermatocytes in various stages of meiosis, ahd spermatids that have developed from the regenerated spermatogonia. 18 days after irradiation with 1000 r on the 2nd day of the second instar. (×126)

Fig. 12. Another part of the testicular follicle shown in Plate 11. Fig. 11. (×126)

Fig. 13. Giant cysts of primary spermatocytes (spc) and spermatids (spt) developed from regenerated spermatogonia. Note that some cells are degenerating. (×126)



Spermatogenesis of the silkworm. I

Explanation of Plate 12

Fig. 14. Testicular follicle showing regenerated gonial region and fully formed eupyrene spermatozoa that are derived from cells irradiated in early meiotic stages. 11 days after exposure to 1000 r on the 2nd day of the fourth instar. (×126)

Fig. 15. Necrotic nuclei of spermatids that are derived from irradiated spermatocytes. (×252)

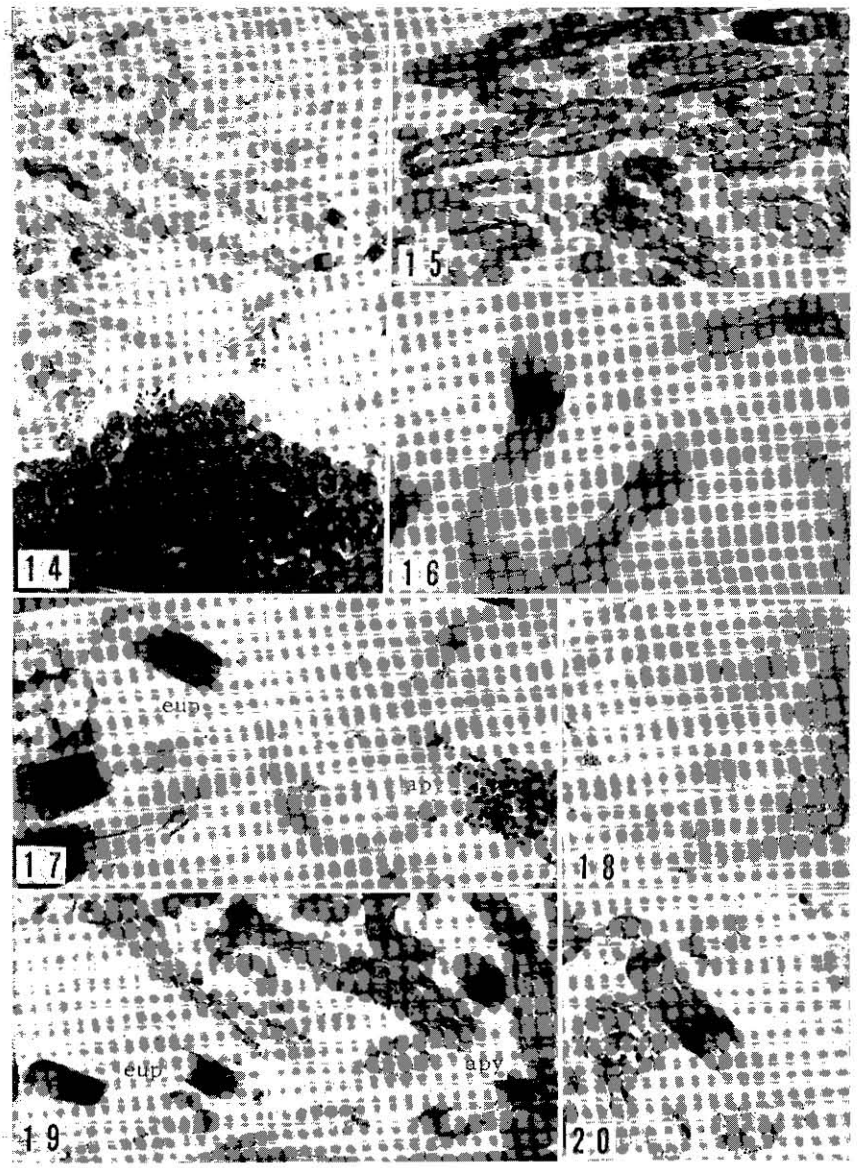
Fig. 16. Bundles of eupyrene spermatozoa with structurally abnormal heads (nuclei) from irradiated spermatocytes. Note that the number of spermatozoa on one cyst is much less than in Plate 12, Fig. 17. Feulgen smear preparations. (×504)

Fig. 17. Bundles of eupyrene spermatozoa without structural abnormality in a non-irradiated testis. Note that the nuclei of apyrene spermatozoa are deeply stained. Feulgen smear preparations. (×504)

Fig. 18. Chromosome bridges observed three days after irradiation in early fifth instar. (×1100)

Fig. 19. Bundles of eupyrene (eup) and apyrene (apy) spermatozoa in a testis of a non-irradiated pupa. 20μ thick section. (×150)

Fig. 20. Bundles of eupyrene spermatozoa with structurally abnormal heads (nuclei) from irradiated spermatocytes. 20μ thick section. (×200)



Spermatogenesis of the silkworm. I