Effect of Aminopterin on Oogenesis of the Silkworm, *Bombyx mori* L. (Lepidoptera : Bombycidae)¹

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Aminopterin at the concentration of 50 μ g had no effect on oviposition of the female silkworm when injected in the fifth larval instar, but their fecundity and fertility decreased when treated after the spinning age. The most drastic effect was shown at the beginning of the pupal age. However, if injected at a later period, the fecundity and fertility increased again. Effectiveness of the antimetabolite varied with races. In histopathological detection of treated ovaries, the oocyte with follicle cells which were just differentiating and arranging in a line around the egg chamber (the beginning of vitellogenesis) showed severe damage, and degeneration occurred both in the nurse and follicle cells concurrently. On the other hand, when injected at about pupation time a change of voltinism was observed.

INTRODUCTION

The inhibitory effect of folic acid antagonist, aminopterin, on the ovarian development has been studied in various insect species. According to HUENNEKENS (1963) and PERRY and MILLER (1965), aminopterin blocks the enzyme dihydrofolic acid reductase and inhibits the supply of tetrahydrofolic acid which is essential for synthesis of nucleic acid. Species used in the experiments reported to date were restricted in most cases to Diptera (AKOV, 1967; CRYSTAL, 1964; GOLDSMITH and FRANK, 1952; LABRECQUE et al., 1960; MITLIN et al., 1954, 1957), and in some instances to Hymenoptera (GROSCH, 1963; ZIELINSKA and GRZELAKOWSKA, 1965) and Hemiptera (PATCHIN and DAVEY, 1968) and the mode of its action varied with the species and developmental stages of the insects. Accordingly, further investigations are required on other species to ascertain the exact effect of folic acid antagonist on insect oogenesis. As little is known in Lepidoptera, in this respect, we believe that the silkworm presents a particularly good subject for investigations as to aminopterin's mode of action and as to the exact stage at which aminopterin is most effective, since germ cell development of this insect occurs almost synchronously with growth.

This paper presents the results of investigations conducted to determine the effect of injected aminopterin on the reproduction of female silkworm in various stages of their development.

MATERIALS AND METHODS

In each experimental series, 20 individuals of three races, Konjiki, N.106×

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Daizo and pure Daizo, were used. Aminopterine² dissolved in 0.1 per cent potassium bicarbonate was injected into body cavity of the insects with the dose of 50 μg (0.025 ml), at various stages from the beginning of the fifth larval instar to emergence as adults. Equivalent amounts of 0.1 per cent potassium bicarbonate without aminopterine were injected into the same stages and used as controls. After injection, the insects were kept at 23°C throughout the remainder of their life cycle. Upon emergence, each adult was allowed to mate with fertile males and eggs were collected. Two weeks after oviposition the number of eggs laid was counted and percentages of unfertilized eggs were calculated in each of the experimental series. In the calculation, after ascertaining a hatching of non-diapause eggs, pigmented eggs were regarded as fertilized and non-pigmented (unhatched) eggs as unfertilized. At the same time histopathological examination was performed using the Konjiki race of insects that were treated on the first day of pupal age, a stage which appeared to be very sensitive to the drug. At 24 hour intervals after the treatment the ovaries were removed, fixed in BOVIN's fluid and embedded in paraffin. Sections were cut at 7 μ and stained with MAYER's acid-hemalaun.

RESULTS

Effect of Aminopterine on Fecundity and Fertility

The 0.1 per cent potassium bicarbonate had no effect on egg development and change of voltinism. At the concentration of aminopterine used in this experiment the mortality was approximately 10-20 per cent in each series except for those treated at prepupal stage where approximately 50 per cent of treated insects died because of failure to pupate.

Fig. 1 Changes in fecundity of females injected with 50 μg of aminopterine at different developmental stages.

—●— Konjiki, —○— N. 106 x Daizo, —▼— Daizo.

V1-V5 : 1st day-5th day of 5th instar, SP : spinning age, PP : prepupa (1 day before pupation), P1-P9 : 1st day-9th day of pupal age, M : moth.

² Aminopterine (4-aminopteroyl glutamic acid) used in this experiment was from SIGMA chemical Co., U.S.A.

Fig. 2 Changes in percentage of unfertilized eggs from females injected with 50 μ g of aminopterin at different developmental stages.

—●— Konjiki, —○— N. 106 x Daizo, —▼— Daizo,
 ...●... No egg was laid.

Abbreviations for injected stages are the same as those indicated in Fig. 1.

To evaluate the effect of aminopterin on oogenesis, the number of eggs laid and percentages of unfertilized eggs were examined in each of the experimental series treated at various stages of their development. The results obtained are shown in Figs. 1 and 2. From the results, no apparent effect in adult fecundity and fertility was noted in the insects treated at the larval stage. However, when the treatment was made at the start of the spinning age, the number of eggs laid by the adults was reduced considerably, and the minimum number of eggs were laid by insects treated at the beginning of pupal stage. When the treatments were carried out at later periods fecundity and fertility increased again. The effectiveness of aminopterin was markedly different in each race, but the trends in sterility and number of eggs laid were approximately the same. N. 106 x Daizo and pure Daizo races were fairly resistant to aminopterin whereas the Konjiki race was extremely sensitive and oviposition was completely absent when they were treated on the second day of pupal period. To determine if any eggs remained in the body cavity of the females which did not oviposit the moths were dissected and examined. The number of eggs found in the body were very few and immature, indicating the degeneration of the oocytes.

Histopathological Observation on the Treated Ovary

To determine the effect of antimetabolite on ovary, histopathological examinations were conducted on the most susceptible race (Konjiki) of insects. The insects were treated on the first day of pupa. When the test was initiated the four ovarioles were still enveloped by an ovarian membrane and the majority of the ovarioles were filled with developing oocytes. In these oocytes the follicle cells were just differentiating and arranging in a line cylindrically around the egg chamber. Nurse cells were in full activity supplying nutritional substance into the egg chamber. In the distal parts of each ovariole, germarium and developing young oocytes were observed.

Twenty-four hours later no pronounced difference was observed between the treated ovary and that of the control. However 48-72 hours after treatment the

Fig. 3 Histopathological changes in ovary injected with aminopterin on the 1st day of pupa. Nurse cells 48 hr (B) and 72 hr (C) after injection and the control (A) showing the clumped chromatin and degenerating process ($\times 600$). Follicle cells 72 hr (E) after injection and the control (D) exhibiting disorganizing process ($\times 600$). An ovariole 96 hr (G) after injection and the control (F) showing degenerating oocyte ($\times 100$). *n* : nurse cell, *e* : egg cell, *f* : follicle cell.

ovarioles had broken through the membrane and elongated into the body cavity in the untreated ovary, whereas the elongation of ovarioles was much less in the treated ovary. In the control ovary, egg chamber grew rapidly and nurse cells became gradually smaller and the follicle cells arranged in a line around the egg chamber. On the contrary, in the treated ovary 48 hours after the treatment, the chromatin of the nurse cells was clumped and follicle cells began to disorganize showing pycnosis of the nuclei. Seventy-two hours after injection, the chromatin in the nurse cells coagulated in masses and the cell boundaries disappeared (Fig. 3. B, C). Concurrently, follicle cells began to disorganize (Fig. 3. E). The young oocytes situated at the distal end of each ovariole, however, appeared still normal. Ninety-six hours after treatment with aminopterin the nurse cells had masses or

clumped chromatin and follicle cells had completely collapsed. The chromatin in the nucleus of the egg chamber also began to clump. It was no longer possible for clear distinctions to be made between egg and nurse chambers (Fig. 3. G). Almost all the oocytes in the ovary showed degeneration and young oocyte at distal parts of ovarioles appeared to be affected, showing developmental arrest.

Effect of Aminopterin on the Change of Voltinism

When aminopterin was injected at around the time of pupation, females that had been destined to produce diapause eggs laid non-diapause eggs. Fig. 4 shows the percentages of non-diapause eggs from females treated at various stages of

Fig. 4 Changes in percentage of non-diapause eggs from females injected with 50 μ g of aminopterin at different developmental stages.
 —●— Konjiki, —○— N. 106 x Daizo, —▼— Daizo.
 Abbreviations for injected stages are the same as those indicated in Fig. 1.

their development. As seen from the results, no change of voltinism was obtained when the larvae were injected with the drug during the fifth larval instar. However when injected about the time the insect reached the spinning age, the percentage of non-diapause eggs increased gradually and reached a maximum two days after pupation. After that, the number of non-diapause eggs decreased again and none was oviposited from females injected at the end of pupal age. Such trends were shown on the three races used in this experiment, though the percentages varied with races.

DISCUSSION

In *Drosophila* (KING and SANG, 1959; GOLDSMITH and FRANK, 1952) and in the housefly (LABRECQUE et al., 1960; MITLIN et al., 1954, 1957), aminopterin inhibited the ovarian development markedly when offered in the diet. However, in the silkworm *Bombyx mori*, no effect was obtained when the aminopterin was injected in the fifth instar larva. The chemical was most effective when the insect was treated at the beginning of pupal age, in which a marked reduction of the fecundity and fertility was noted. Such stage difference in effectiveness of the antagonist may be due to the difference in stages of gonadal development at the time of treatment. Furthermore, it may be related partly with the nutritional condition, namely the folic acid and other substances necessary for their normal growth can

be sufficiently supplied from the mulberry leaves in the larva, while no supply of the substances is newly obtained after spinning.

In histopathological observation on treated ovaries in *Drosophila* (KING and SANG, 1959), it was reported that the nurse cells are particularly sensitive to aminopterin. In *Rhodnius*, however, the first tissues to show histological changes following aminopterin treatment are the follicle cells (PATCHIN and DAVEY, 1968). To explain such difference, they suggested that *Rhodnius* has telotrophic ovaries in which the nurse cells play a role only in the earliest stage of oogenesis, whereas Diptera have polytrophic ovaries in which nurse cells play a more important role in the development of oocyte, especially in the vitellogenesis.

In the silkworm, *Bombyx mori*, abnormalities occurred concurrently both in nurse and follicle cells and no discrimination of the damage was observable. According to cytological investigations on vitellogenesis of the silkworm (MACHIDA, 1940; OTSUKI, 1965) the yolk was produced from both the ground substance from nurse cells and those secreted from follicle cells. The secretory activity of follicle cells commenced when cells began to arrange cylindrically in a line around the egg chamber at the beginning of the pupal age, which closely corresponded with the time when the effect was the most drastic. It may therefore be concluded that the most sensitive stage to aminopterin in the silkworm oogenesis was the beginning of the vitellogenesis, in which the differentiation of follicle cells and nutritional supply from nurse cells occur most actively.

On the other hand, when the pupae destined to lay diapause eggs were treated with aminopterin at pupation time the moths produced non-diapause eggs. Regarding the change of voltinism in *B. mori* by injection of chemicals, some experiments have already been reported (HASEGAWA, 1943; YOSHITAKE, 1954; MOROHOSHI and OKUMA, 1968). Considering from the results obtained in this experiment, however, it may be suggested that there is some relation between the change of voltinism and metabolic inhibition of nucleic acid synthesis of nurse and follicle cells in the vitellogenesis, though the experimental evidence for such assumption is not sufficient in the present experiment. Furthermore, it seems likely that when the ovarian damage is severe, but not to the extent of complete degeneration, unfertilized eggs are laid and if the damage is slight, the eggs become non-diapause.

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