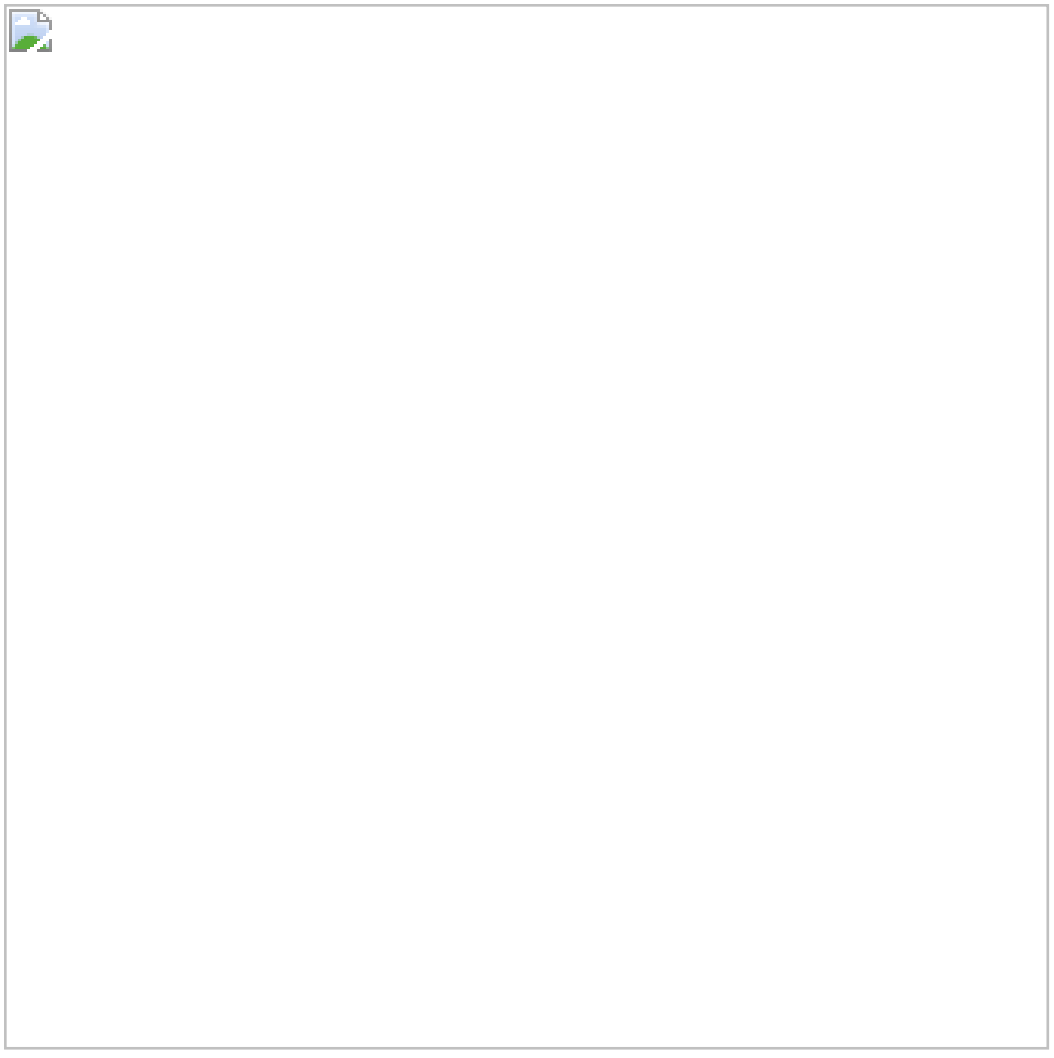

DNA Synthesis of Wing Disc Cells and the Effects of Mitomycin C and X Ray Irradiation on the Wing Development of Bombyx mori

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DNA Synthesis of Wing Disc Cells and the Effects of Mitomycin C and X Ray Irradiation on the Wing Development of *Bombyx mori*

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ABSTRACT—The rate of DNA synthesis of wing discs and the effects of X ray irradiation and mitomycin C injection were observed during last larval and early pupal stage of *Bombyx mori*. The rate of DNA synthesis in wing discs of *Bombyx mori* was estimated by means of the incorporation of ^{3}H-thymidine during the fifth larval stage. Mitomycin C injection and X ray irradiation were performed during the fifth larval instar and pupal stage to investigate the effects of inhibition of DNA synthesis. The rates of ^{3}H-thymidine incorporation were high on days 0, 1, 2, 4, 5, 7, 8 and 10 of the fifth larval instar. Wings of eclosed adults were affected by injections of mitomycin C between the 7th day of the fifth larval instar and the second day of the pupal stage. Exposing the insects to X rays during the fifth larval instar affected wing and scale formation, and at earlier stages, it had a more deleterious effect. Irradiation at 24–36 hours after pupation inhibited wing expansion and scale formation most among pupal stages. The results suggested that the peaks of ^{3}H-thymidine incorporation during the fifth larval instar and the pupal stage have specific significance for wing development.

INTRODUCTION

Löbbecke [16] has described a short S-phase in the prepupal stage, as well as a short G2-phase in the early stage of the last larval instar and in the prepupal stage in *Ephestia* wing discs. Lafont *et al.* [13] reported a high rate of DNA synthesis in the early stage of the last larval instar and in the prepupal stage in *Pieris* wing discs. Kawasaki and Iwashita [8] have found two peaks of mitosis in the wing discs of *Bombyx mori* during the fifth larval instar. Thus, DNA synthesis may be stage specific and have specific significance in the development of the lapidopteran wing discs during the last larval instar.

Lafont *et al.* [14] have noted two peaks of DNA synthesis in *Pieris* in the pupal stage, and Kawasaki *et al.* [9] reported two types of DNA synthesis in *Bombyx* wings during the pupal stage; one occurred before mitosis for differentiation of scale and socket forming cells and the other was for polyploidy of scale cell nuclei. Thus, DNA synthesis is also stage specific in lepidopteran pupal wings.

Wing development after X ray irradiation is inhibited during the larval stage [17]. Mitomycin C injected into insects around pupation inhibits adult wing formation [5, 19–21]. However, the relationship between the DNA synthesis of wing disc cells and the imaginal wing formation has not been examined in detail. The author demonstrated that DNA synthesis is elevated in wing discs at a specific stage and its significance for imaginal wing development is discussed.

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MATERIALS AND METHODS

Animals

Bombyx mori hybrids N124×C124, were reared under a 12L-12D photo period at 25°C. Under these conditions the fifth larval stage lasted 11 days. Cocoon spinning and tracheole migration of the wing disc began on day 7. Cuticle deposition and evagination of wing discs occurred on day 10. In this report, day 0 designates the 24 hr after molt from the fourth to the fifth instar.

Incorporation of ^{3}H-thymidine in the wing discs during the fifth larval instar

Wing discs of the fifth larval instar were dissected and incubated for 4 hours in Grace's insect medium (GIBCO) containing 0.5 μCi of methyl-^{3}H-thymidine (sp. act. 40–60 Ci/mmol, Amersham). The discs were rinsed with phosphate buffered saline (PBS: NaCl, 8 g; $Na_2NPO_4 \cdot 12H_2O$, 1.15 g; $NaH_2PO_4 2H_2O$, 0.2 g in 1 liter) and stored at −20°C until use. Incorporation was estimated as follows. Discs labeled with ^{3}H were homogenized in PBS, then an equal volume of 10% trichloroacetic acid (TCA) was added. Tissue homogenates were passed through a Millipore filter (0.45 μm), which was rinsed three times with 10 ml cold TCA. Tissue homogenates together with the filter were dried under a halogen lamp, then placed in the bottom of a counting vial containing liquid scintillation fluid [toluene: 2,5-diphenyloxazole: 1,4-bis-2-(5-phenyloxyazole)-benzene; 1L; 4 g: 0.1 g] and counted in a liquid scintillation counter (Aloka; LSG-900).

The DNA contents were estimated as follows. Wing discs were homogenized in PBS, then an equal volume of 10% TCA was added. Precipitates were extracted with ethanol-ether (1:1), then centrifuged at 2,000 g for 10 min. Precipitates were extracted with 5% TCA at 90°C for 20 min and the supernatant was obtained by centrifugation. DNA was estimated in the supernatant using diphenylamine according to Burton [1].

Injection of mitomycin C

Mitomycin C was dissolved in distilled water, then 2.5, 5.0 and 10.0 μg/g were injected through the intersegmental membrane of the

second dorsal abdominal segments into larvae and pupae from the beginning of the fifth larval instar until adult emergence.

X ray irradiation

Insects were irradiated with 5,000 or 10,000 Röentgens of X rays filtered through 0.5 mm Al, using a Hitachi X ray system (M13R-1505).

Autoradiographic study

After an incubation in Grace's insect medium, the wing discs were fixed in Carnoy's fixative for 2 hr. The tissues were then dehydrated and embedded in paraffin. Paraffin sections of 5 μm were prepared, and the slides were coated with autoradiographic emulsion (Sakura NR-M2). After exposure for 2 weeks the auto radiograms were developed.

DNase and RNase digestion

After removing the paraffin, the sectioned wing tussues were digested with DNase I (Boehringer Mannheim) and RNase A (Boehringer Mannheim). The concentration of both enzymes was 20 μg/ml in PBS. The reaction proceeded at 37°C for 1 hr, after which it was stopped with distilled water.

RESULTS

Incorporation of ^{3}H-thymidine

Incorporation of ^{3}H-thymidine was estimated from 4 series of 4 discs each and the DNA content was estimated from 10 discs at the same stage as shown in Figure 1. The rate of ^{3}H-thymidine incorporation was low on days 3, 6 and 9 and early on the 10th day.

Wing tissues treated with DNase contained no dense granules (Fig. 2a), whereas those exposed to RNase (Fig. 2b)

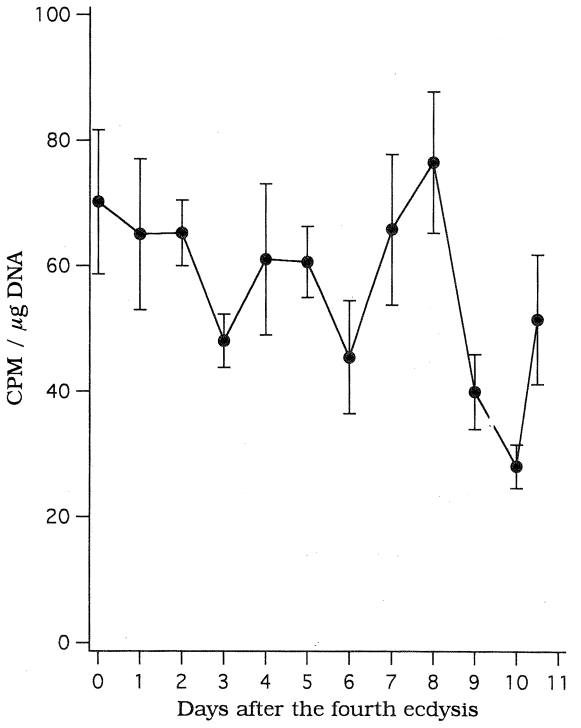


FIG. 1. ^{3}H-thymidine incorporation during the fifth larval instar. Each datum indicates mean value±S.D. in 4 samples from 4 series. The amount of incorporation at day 3 was significantly smaller than that of day 2 ($P<0.01$; Student's t-test). On day 6 it was significantly less than that on days 5 ($P<0.05$) and 7 ($P<0.01$), and it was significantly smaller on early day 10 than late on day 10 ($p<0.01$).

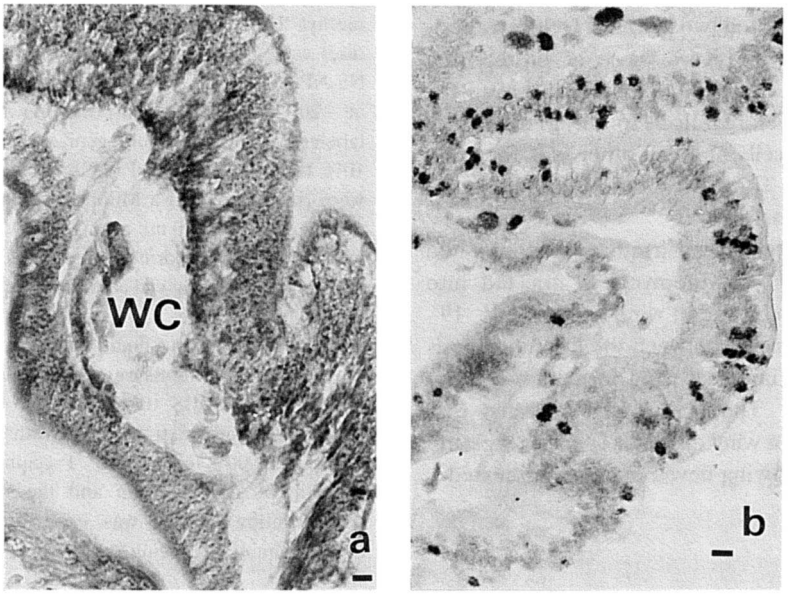


FIG. 2. Wing tissues after autoradiography. Bars equal 10 μm. The wing disc tissue at stage S2. Deparaffined tissues were digested with DNase. Granules disappeared from the tissues. WC; wing cavity. (b) The wing tissue retains the granules after digestion with RNase. The tissue is from the same stage as Figure 2a.

TABLE 1. Effects of mitomycin C injection on wing formation

*Stage of injection	Number of insects tested	Dose of mitomycin C (μg/g weight)																	
		2.5						5.0						10.0					
		N	A	B	C	I	L	N	A	B	C	I	L	N	A	B	C	I	L
D7	25					22	3					2	23					3	22
D8	25			7		6	12			5	2	6	12				3	9	13
D9	25			7		10	8				9	8	8				10	9	6
D10	25			13		12					12	13					18	7	
P0	10		10								10						10		
P1	10		5	5					4	6						3	7		
P2	10	10						3	7							9	1		

*Day of the fifth larval (D) and pupal (P) stage. N, Normal wings; A, Wings are normal except they have few scales as are shown in Fig. 3a; B, Wings do not expand and have a few scales as are seen in Fig. 3c; C, Wings do not expand and have no scales as seen Fig. 3d. L, insects died in larval form; I, intermediate of larvae and pupae.

and PBS without enzyme incorporated ^{3}H-thymidine into the nuclei. These results indicated that the ^{3}H-thymidine incorporation reflected the amount of DNA synthesis in wing disc cells.

Injection of mitomycin C

Mitomycin C was injected from the beginning of the fifth larval instar until adult eclosion. There were no inhibitory effects on wing formation when injected before the spinning stage (day 7) or after day 3 of the pupal stage. The effects on the wing formation by mitomycin C injection are summarized in Table 1. During the affected stage, the earlier the injection, the more obvious were the effects on insects or wing formation. Wing formation was inhibited markedly by a high dose of mitomycin C. Most larvae receiving 5.0 or 10.0 μg/g weight of mitomycin C on day 7 died in larval form. Those receiving 2.5 μg/g weight of mitomycin C on day 7 died in intermediate form of larva and pupa. Injections on days 8 and 9 also brought about larval and intermediate type. Adults that received 2.5 and 5.0 μg/g weight of mitomycin C on days 8 and 9 had abnormal wings that did not expand and there were a few scales (type B, Table 1; Fig. 3c). The wings did not expand and had no scales after injections of 5.0 and 10.0 μg/g weight of mitomycin C (type C, Table 1; Fig. 3d). Injection on day 10 brought about the intermediate type, type B (2.5 μg) and type C (5.0 and 10.0 μg). High doses of mitomycin C injection after pupation also inhibited wing elongation and scale formation, and the younger the stage at which mitomycin C was given, the more wing formation was affected (Table 1). An injection of 2.5 μg/g on day 0 induced adults with expanded wings but few scales (type A in Table 1, Fig. 3a). However higher doses markedly affected the expansion and scale formation (Fig. 3d).

Tritiated thymidine incorporation was ihibited by mitomycin C injection from the beginning of the spinning stage to day 1 of the pupal stage (Fig. 4a), but was not affected during the feeding period (Fig. 4b) and after day 2 of the pupal stage (Fig. 4c).

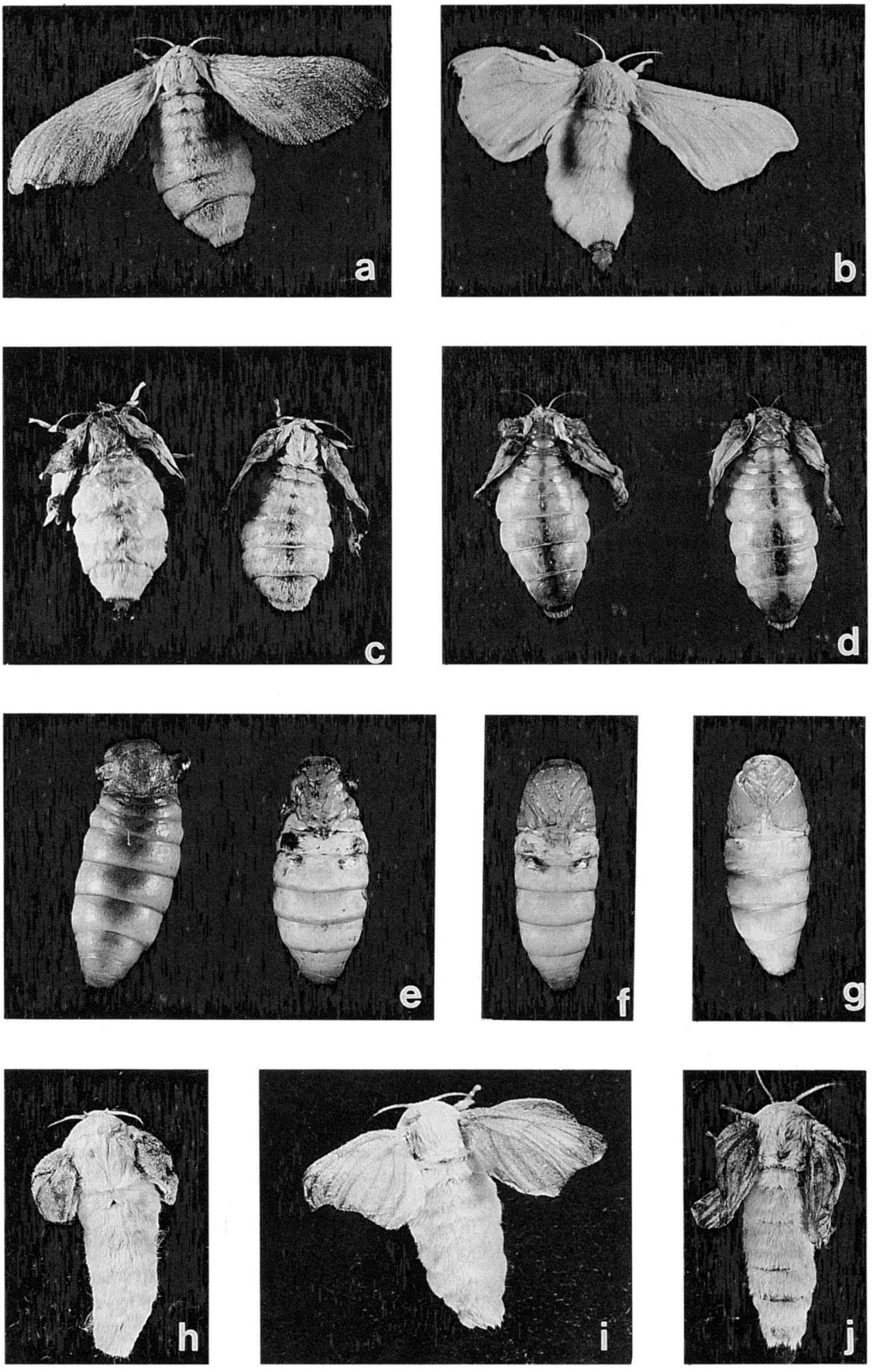
X ray irradiation

The wing defects resulting from 10,000 Röentgens of X ray irradiation during the fifth larval instar are presented in Fig. 5. Wing areas of eclosed adults were measured after being irradiated during the fifth larval stage. Defects were obvious before day 4 (Figs. 3e, f, h). As shown in Figures 3e and 3f, the cuticle area covering the wings is small and emerged adults have small wings with few scales (Fig. 3h). Irradiation with 5,000 Röentgens on days 0 and 1 of the fifth larval instar reduced wing size to about 20%. Irradiation between days 5–8 reduced the wing size by about 50% (Figs. 3g, i), as well as the number of scales. However the effects of irradiation on the size and scale formation of the wings decreased in accordance with time, and wings were normal following irradiation on day 9. Irradiation on day 10 affected wing expansion and scale formation. Scale formation was extremely inhibited, and scales and sockets were hardly observed (Fig. 3j).

Irradiation after pupation affected wing expansion as well as scale formation and the most sensitive stage was 24–36 hr after pupation as indicated in Table 2 and Figure 3d. Irradiation with 10,000 Röentgens inhibited adult wing morphogenesis more than 5,000 Röentgens. Irradiation between 0 and 60 hr after pupation brought about adults having normal shaped wings with few scales (type A in Table 2, Fig 3a) or wings that did not expand and had few scales (type B in Table 2, Fig 3c). The incorporation of ^{3}H-thymidine was inhibited by X ray irradiation (Fig. 6a) compared with insects that were not treated (Fig. 6b).

DISCUSSION

As Henke and Pohley [6] described, several mitotic events occur in the lepidopteran insect wing disc during the last instar. Synchronization of the cell cycle in the wing cells is supposed to be controlled by JH and ecdysteroid in hemolymph as described below. Kawasaki and Iwashita [8] identified two peaks of mitosis in the wing discs of *Bombyx*



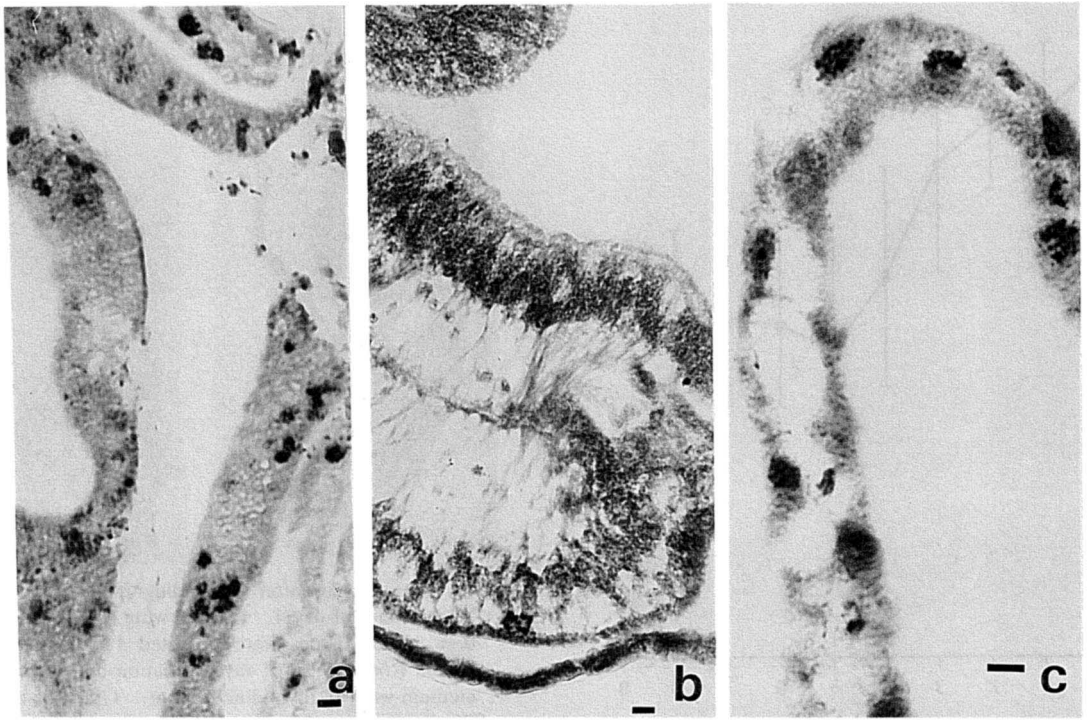


FIG. 4. Wing tissues after autoradiography. Bars equal 10 μm. (a) The wing tissue after injecting 10 μg/g of mitomycin C on day 4 of the fifth larval instar. Tissues were dissected 4 hr after injection and incubated in Grace medium for 4 hr. Granules were found in the wing tissue. (b) The wing tissue of the S2 stage after mitomycin C treatment. Granules were not present over the tissue. (c) The wing tissue of the P2 stage. Dense granules are scattered over the scale cell nuclei.

mori during the fifth larval instar. One was on days 3, 4 and 5 and the other, on days 8 and 9.

During the fifth larval instar, there were 4 phases of elevated DNA synthesis (Fig. 1). The phase observed on days 4 and 5 seemed to correspond to the first mitotic peak. DNA synthesis at this stage would presumably be induced by the disappearance of JH as Kurushima and Ohtaki [12] reported and as Cherbas *et al.* [3] found in *Drosophila* Kc cells. Wing discs which had gone through DNA synthesis at this phase responded to an injection of 20-hydroxyecdysone with evagination and pupal cuticle deposition [2]. Wing disc cells will be committed to the pupal type and have the capacity to respond to ecdysteroid of high concentration after experiencing this cycle of DNA synthesis. The third phase of increased DNA synthesis was on days 7 and 8 and this phase is supposed to be accelerated by the increased ecdysteroid level as we reported [9]. The DNA synthesis at this stage seems to correspond to the second peak of mitosis. Cuticle formation and evagination occur after this DNA synthesis [8].

The first period of DNA synthesis was between days 0 and 2. Wing disc cells at this stage would not yet be committed to the pupal type, as reported by Kurushima and Ohtaki [12]. The DNA synthesis of this stage occurring in the presence of JH [18] may differ from that without JH as Cherbas *et al.* reported [3], and JH arrests the cells not to be committed to pupal type. The last increase of DNA synthesis was on late day 10, shortly before pupation, after the ecdysteroid titer decreased [10]. Wing disc cells are supposed to be arrested in G2-phase after this DNA synthesis, as Kawasaki and Iwashita [8] found no mitotic figures at this stage. It is supposed that this G2 arrest of disc cells before pupation is similar to that in *Drosophila* [4] and *Calliphora* wing discs [21].

As shown in Fig. 4, the earlier X ray irradiation was applied to the fifth larval instar, the greater was the wing size reduction. This indicated that DNA synthesis during the fifth larval stage was blocked by X rays and was not resumed

FIG. 3. (a-d) Eclosed adults that had received mitomycin C around pupation. (a) Injection of 2.5 μg/g body weight of mitomycin C 0–12 hours after pupation. Wings are normal except they have few scales (grade A in Table 1 and 2). (b) An eclosed control adult that received no mitomycin C. (c) Eclosed adults that had received 2.5 μg/g body weight of mitomycin C on day 9. A few scales are located on the wings and abdomen (grade B in Table 1 and 2). (d) Eclosed adults that had received 10 μg/g weight mitomycin C during 0–12 hr after pupation. No scales are evident (grade C in Table 1 and 2). (e-j) Pupae and adults that received an X ray dose of 10 KR. (e) Ecdysed pupae irradiated on day 1 of the fifth larval instar. Wing growth was extremely affected. (f) An ecdysed pupae irradiated on day 3 of the fifth larval instar. Wing growth was inhibited about 70%. (g) An ecdysed pupa irradiated on day 6 of the fifth larval instar. Wing growth was affected slightly. (h) An eclosed adult irradiated on day 2 of the fifth larval instar. Wing growth and scale formation on the wings were affected. (i) An eclosed adult irradiated on day 6 of the fifth larval instar. Wing growth and scale formation were slightly affected. (j) An eclosed adult irradiated on day 10 of the fifth larval instar. Scale formation and wing expansion were affected.

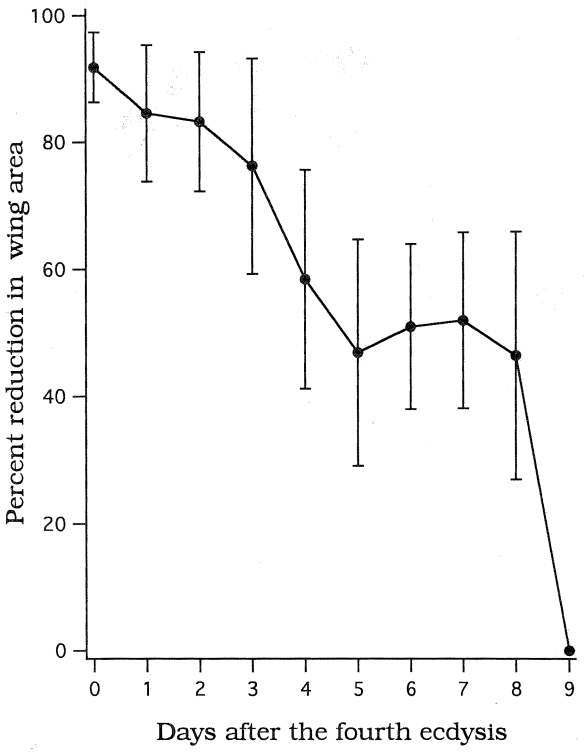


FIG. 5. The fore wings of eclosed adults were measured after the larvae were irradiated on various days during the fifth larval instar. Each point indicates a mean value±S.D. from 8–16 individuals.

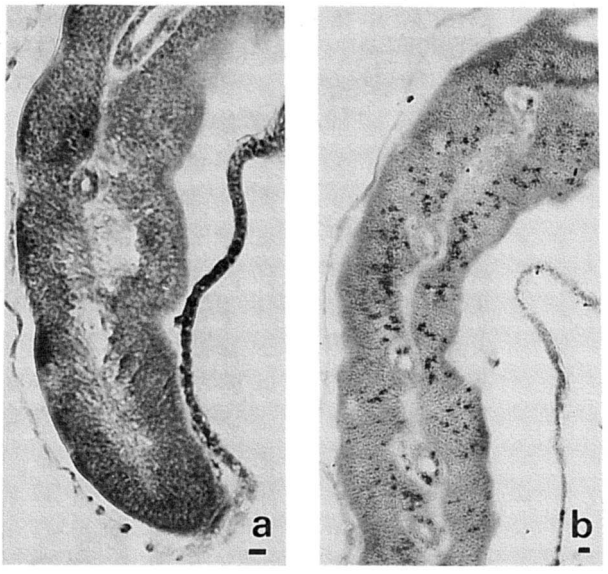


FIG. 6. Wing tissues after autoradiography. Bars equal 10 μm. Wing tissues of day 4. (a) The wing tissue after X ray irradiation. Wing tissues were dissected 4 hours after exposure to 10,000 Röentgen of X ray irradiation and incubated in Grace medium with ^{3}H-thymidine for 4 hr. Granules were not found. (b) Wing tissues from a control, untreated individual. The tissue was from the same stage as (a), and dense granules are evident.

thereafter, and that wing disc cells do not enter the next cycle. This suggested that disc cells that just entered the DNA synthetic stage did not proliferate after X ray irradiation and resulted in the reduction of wing size. These results agree with the report of Meyer *et al.* [16]. They observed wing reduction by X ray irradiation and they stated that cell proliferation was inhibited by X ray irradiation. The effect of X ray irradiation was greatest at 24–36 hours of pupal development. This result was in good agreement with the high rate of DNA synthesis observed by Kawasaki *et al.* [9] during 20–36 hours after pupation.

There was no effect on the eclosed adults by mitomycin C when injected before the spinning stage and the DNA synthesis during these periods was not inhibited (Fig. 2e). This may be because mitomycin C was excreted from the insect body during feeding. Many larvae that received mitomycin C during the cocoon spinning stage could not pupate but remained as larvae or in intermediate forms of larvae and pupae. Organs of the endocrine system would probably be affected by mitomycin C. High doses of mitomycin C inhibited scale formation, wing expansion and apolysis of the pupal cuticle when injected on day 9 or 10. Neither scales nor sockets were observed in the scaleless area. I inferred that the injection of mitomycin C at this stage affected the prepupal DNA synthesis described above. From these results and X ray irradiation that inhibited scale formation but had no effect on the wing size (Fig. 3j) because of the completion of wing size on day 9 (Fig. 5), the DNA synthesis on day 10 is assumed to be that of the scale stem cell.

TABLE 2. Effects of X ray irradiation on wing formation

Time of irradiation (Hours after pupation)	Number of insects tested per dose	5KR				10KR			
		N	A	B	C	N	A	B	C
0–12	10	2	6	2			4	6	
12–24	10	5	3	2			5	5	
24–36	10		7	3			1	3	6
36–48	10	6	4				2	8	
48–60	10	7	3				4	6	
60–72	10	10				10			

N, A, B and C are defined in Table 1.

Wing formation was extremely sensitive to mitomycin C on day 0 of the pupal stage and developmental inhibition became lighter as the stage of injection progressed during pupal stage. Mitomycin C injected on the day of pupal ecdysis probably inhibited the DNA synthesis observed during 20–36 hours after pupation [9]. These results were in accordance with the findings reported by Socha and Sehnal [20] in *Tenebrio*. They identified morphological abnormalities after an injection of mitomycin C 24–40 hr after pupation, when DNA synthesis and mitoses occurred. Krishnakumaran *et al.* [10] noted the inhibited of scale formation in *Antheraea* pupa after injecting mitomycin C. Lawrence [15] has also reported that new hair development is inhibited in *Oncopeltus* by mitomycin C. These authrs observed that mitomycin C injected during periods when high DNA synthesis occurring in wing discs caused heavy defects. Guerra *et al.* [5] have reported that bristle formation is inhibited in the imaginal abdomen of *Drosophila* by mitomycin C when a large number of mitoses were proceeding. The rate of DNA synthesis is high in the *Bombyx* wing tissue during the 20–36 hr, after pupation [9], and this phase of DNA synthesis corresponds well to the mitoses of scale cells, socket forming cells and wing epidermal cells as observed by Kawasaki and Iwashita [7]. I inferred that wing development was affected because the injection of mitomycin C into the early pupal stage inhibited DNA synthesis and the corresponding motoses. Thus, I demonstrated that an injection of mitomycin C at the stage when DNA synthesis of wing disc cells was high in the larval and pupal stages, affected adult wing morphogenesis.

Mitomycin C injection had no effect on wing formation when injected after day 3 of the pupal stage when scale formation had already begun [9]. Scale cell specific ^{3}H-thymidine incorporatin was not inhibited by mitomycin C (Fig. 2g). Schaere [19] has observed defective bristles and sockets in *Phormia* caused by injections of mitomycin C and stated that mitomycin C affected polytenization but not differential division. The results of this study however indicated that mitomycin C did not affect polyploidization.

Thus, DNA synthesis during the fifth larval and the early pupal stage has significance for adult wing development and its inhibition caused different kinds of defects on wing formation depending on the stage of inhibition. The control of the wing disc cell cycle by insect hormones will be resolved near future.

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