To the Graduate Council:

I am submitting herewith a thesis written by Melvin J. Ely, Jr. entitled "The Effects of X-Irradiation on Eclosion, Egg Hatchability, and Larval Survival of the Tobacco Hornworm, Manduca Sexta." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Radiation Biology.

Arthur M. Jungreis, Major Professor

We have read this thesis and
recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

THE EFFECTS OF X-IRRADIATION ON ECLOSION, EGG HATCHABILITY, AND LARVAL SURVIVAL OF THE TOBACCO HORNWORM, *MANDUCA SEXTA*

A Thesis

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Degree

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ABSTRACT

The effects of X-irradiation upon Manduca sexta egg hatchability and larval survival were studied using LD_{50}'s. Radiosensitivity declined with age in developing eggs (LD_{50} of 9.4 kR in 2.5-3 day eggs contrasted to 18.2 kR in 4-4.5 day eggs) and in larvae. The dose-response patterns obtained agree with those previously reported for other insects.

Control of eclosion in Manduca sexta (lab reared at 23-25°C on LD 18:6) was also investigated by utilizing the ED_{50} (X-ray exposure required to prevent eclosion in 50% of the irradiated animals) throughout pupal-adult development as a measure of radiation sensitivity. An initial period (Day 0-6) of nearly constant radiosensitivity (ED_{50} range: 13.0-14.4 kR) was followed by a brief period of increased radiosensitivity between Day 6.5-7.75 (ED_{50}: 8.23 kR). Thereafter a pronounced decrease in radiosensitivity was noted through the day of eclosion (Day 8: ED_{50} = 19 kR; Day 22: ED_{50} = 75 kR).

The initial period of radiosensitivity occurs concomitant with the presence of high endogenous levels of ecdysone, with a maximum observed on Day 7. Therefore, the association between hemolymph ecdysone levels and maximum radiosensitivity was studied. Animals were administered β-ecdysone on Days 1, 4, and 12 and irradiated at various times postinjection. Significant increases in radiosensitivity were noted in hormone supplemented pharate adults. Thus, radiosensitivity exhibited by Manduca sexta on Days 0-7 is in part dependent upon the titer of ecdysone in hemolymph.

The role of the brain as a radiosensitive region was investigated in Day 10 animals by selectively transplanting irradiated and nonirradiated brains into irradiated and nonirradiated animals. In nonirradiated animals, brain exchange or severance of the connectives to the brain resulted in only 45% eclosion success, while placing an ED_{100} irradiated brain in the abdomen of an intact, nonirradiated animal failed to prevent eclosion. Using ED_{100} animals, brains transplanted to the heads of animals debrained following irradiation or into abdomens of intact animals could not induce eclosion. Since the radiation-induced inhibition of eclosion cannot be completely explained in terms of brain damage alone, the presence of a radiosensitive component in addition to the brain is proposed.

Selective shielding of Day 10 animals X-irradiated at an ED_{100} level demonstrated the absence of radiosensitive regions in the abdomen and their presence in both the head and thorax.

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PART I

EFFECTS OF X-IRRADIATION ON EGG HATCHABILITY AND LARVAL SURVIVAL IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*

CHAPTER I

INTRODUCTION

Traditionally, insects have been employed extensively in studies utilizing radiation both to obtain insights into fundamental questions regarding insects, and as experimental tools to investigate principles of general biological interest. One of the earliest observations of the genetic effects of ionizing radiation in insects dealt with the induction of dominant lethal mutations in the sperm of Drosophila melanogaster (Meigen) (Muller, 1927). More exhaustive studies dealing with nongenetic effects of radiation on development have been completed and include comparisons of susceptibility to radiation at various stages of the insect life cycle (Mavor, 1927; Whiting et al., 1931; Halberstaedter et al., 1943; Wharton et al., 1959).

The earliest reported study of this subject was that of Mavor (1927), who demonstrated changes in radiosensitivity with age in D. melanogaster by determining the $LD_{50/5}$ levels (exposure required to kill 50% of animals within 5 days) for eggs, larvae, pupae, and imagoes. Mavor's observations that the egg and larval stages are relatively radiosensitive when compared to pupal and adult stages were corrobated by other investigators working with Habrobracon larvae (Hymenoptera) (Whiting et al., 1931), Calliphora erythrocephala and Culex pipiens (Diptera) (Halberstaedter et al., 1943), and Periplaneta americana (Orthoptera) (Wharton et al., 1959). These early studies were subsequently amplified by survival studies

on a wide range of insect species (see reviews by Hilchey, 1957; Grosch, 1962, 1973; and O'Brien and Wolfe, 1964).

Following the introduction and refinement of successful methods for continuous laboratory rearing (Waldbauer et al., 1964; Hoffman and Dickerson, 1968; Yamamota, 1969; Bell and Joachim, 1976), the tobacco hornworm, Manduca sexta (Lepidoptera), has become a popular subject for physiological and neuroendocrine studies. Irradiation data on this insect, however, have been limited mainly to the effects of sterilizing dosages applied to pharate adults and adults in attempts to suppress tobacco hornworm populations utilizing the sterile male technique (Cantelo et al., 1972; Snow et al., 1974).

As an adjunct to the eclosion studies described in Part II of this thesis, the effects of X-irradiation on egg hatchability and larval survival in M. sexta were investigated both (1) to determine whether the dose-response patterns were similar to those observed in other insect species, and (2) to provide a more complete profile of radiation sensitivity for this species.

CHAPTER II

MATERIALS AND METHODS

Experimental Animals

A colony of tobacco hornworms, M. sexta, has been maintained in this laboratory for over a year (10+ generations) with regular inclusion of eggs from wild type tobacco hornworms. Animals were initially derived from a colony maintained by Dr. Lynn M. Riddiford, University of Washington, Seattle. Larvae are individually reared in plastic containers on a wheat germ-casein diet, similar to that of Yamamoto (1969) and modified after Riddiford (personal communication) and Bell and Joachim (1976), under a photoperiod regimen of 18L:6D (photophase 0530-2330) at 23-25°C.

Approximately 50-75 adult moths are kept in a two cubic meter mating cage containing a food source (25% honey or sucrose solution) and a tobacco plant, the latter serving as an oviposition site. Eggs were collected daily, placed in 120 ml plastic dishes with a one cubic inch piece of synthetic diet (25-30 eggs per dish), and allowed to hatch.

Larvae were transferred at 3-4 days posthatch to individual 120 ml plastic containers with a cube of diet for continuation of larval development.

Four to five days after the fourth to fifth instar larval-larval ecdysis, the mature fifth instar larva (wet weight = 9-11 grams) terminates feeding, purges material from its gut, and begins tunneling through the diet remaining in the rearing dish. A pink ommochrome

pigment is deposited along the dorsum (Nijhout et al., 1974a) and the dorsal vessel becomes visible through the cuticle. These changes mark the transformation of the feeding larva into a wandering larva. It is at this time that the animal, in its natural environment, would leave the host plant upon which it is feeding, tunnel into the soil, and form a chamber in which to pupate.

Under laboratory rearing conditions, within 24 hours after the appearance of the dorsal pigmentation, wandering larvae were placed into artificial pupation cells, consisting of wooden blocks into which 1-inch by 3-inch holes had been drilled.

Following the larval-pupal apolysis (pink stripe stage), the wandering larva enters a pharate pupal stage (of four days duration at 23-25°C) during which the animal becomes inactive and shrinks in size preliminary to the larval-pupal ecdysis. Blocks were regularly checked at twelve hour intervals (0800 and 2000) and newly ecdysed pupae removed at these times.

Pupation is followed by a developmental period (pharate adult development) during which the pupal-adult transformation occurs. Staging of animals during this developmental period was based upon days after pupation rather than upon specific morphological features. Thus, Day 0 of pupal-adult development was the 24-hour period following pupation. Eclosion was observed to occur between 1600 and 2100 beginning on Day 22 for animals maintained at 23-25°C.

Irradiation Procedure

The X-irradiation source was a General Electric Maxitron 300 X-ray unit operated at 250 kVp and 15 mA with 0.5 mm aluminum filtration added (4.75 mm inherent Be filtration) to reduce tissue attenuation by 95-100%. Exposures were altered by varying the time of exposures and/or target-to-object distance. Dosimetry was carried out under experimental conditions utilizing a 250-R thimble ionization chamber read in a Model 70 Victoreen condenser R-meter.

Eggs were irradiated in two-inch by two-inch uncovered cardboard containers at 2.5-3, 3-3.5, and 4-4.5 days following oviposition with an exposure rate of 2380 R/minute. Following irradiation, groups of 25-30 eggs were transferred to 120 ml plastic containers with a cube of diet and allowed to hatch under laboratory rearing conditions. Egg hatch began on the fifth day after oviposition. Percentage hatch was plotted as a function of exposure for each age group and the LD_{50}'s (X-ray exposures required to prevent hatching in 50% of the eggs) were determined. Data were analyzed using log-probit transformation to make the regression of hatchability on exposure linear.

First instar larvae (1-25 hours posthatch) and second, third, and fourth instar larvae (1-25 hours following appropriate larval-larval ecdyses) were irradiated in three-inch by three-inch cardboard containers, covered with a thin piece of tissue paper, with an exposure rate of 1220 R/minute. Following irradiation, larvae were returned to individual rearing dishes with a cube of diet and allowed to continue development under laboratory rearing conditions. Survival through the remaining larval stages, pupation and eclosion successes were monitored.

CHAPTER III

RESULTS

The effects of X-irradiation on hatchability of Manduca sexta eggs are shown in Figure I-1. LD_{50} values for eggs irradiated 2.5-3, 3-3.5, and 4-4.5 days following oviposition were 9.4 kR, 7.5 kR, and 18.2 kR, respectively. Regression lines A and B of Figure I-1 can be represented by a common line with slope of -2.59 ($F = 1.567$; $F_{.05(2,8)} = 4.46$), as determined by analysis of covariance.

Survival of larvae irradiated during either the first, second, third, or fourth instars are illustrated in Figures I-2, I-3, I-4, and I-5, respectively. Larvae failed to complete development and emerge as adults at exposures of 5 kR and above. All larvae receiving an exposure of 15 kR or more died prior to pupal ecdysis. Mortality rates increased with increasing exposures for each instar.

The survival curves reflect a general decline in radiosensitivity with larval age. This can be seen most clearly by comparing the survival pattern of each instar receiving an exposure of 1 kR (Figure I-6).

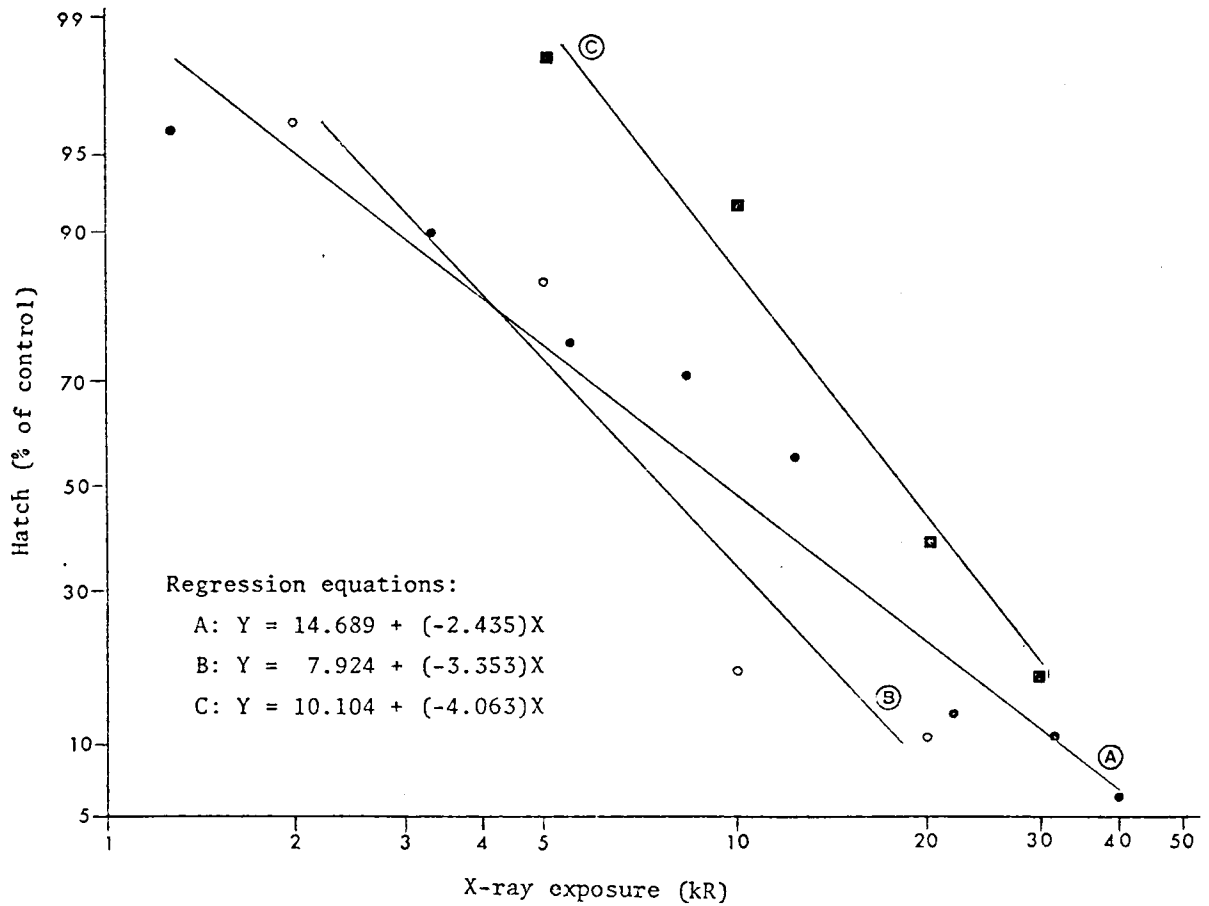


Figure I-1. Hatchability of X-irradiated Manduca sexta eggs. Irradiated (A) 2.5-3.0 days following oviposition, (B) 3.0-3.5 days following oviposition, and (C) 4.0-4.5 days following oviposition. 60-100 eggs per exposure group. Hatch is on probit scale, exposure on log scale. Regression equations are for transformed data.

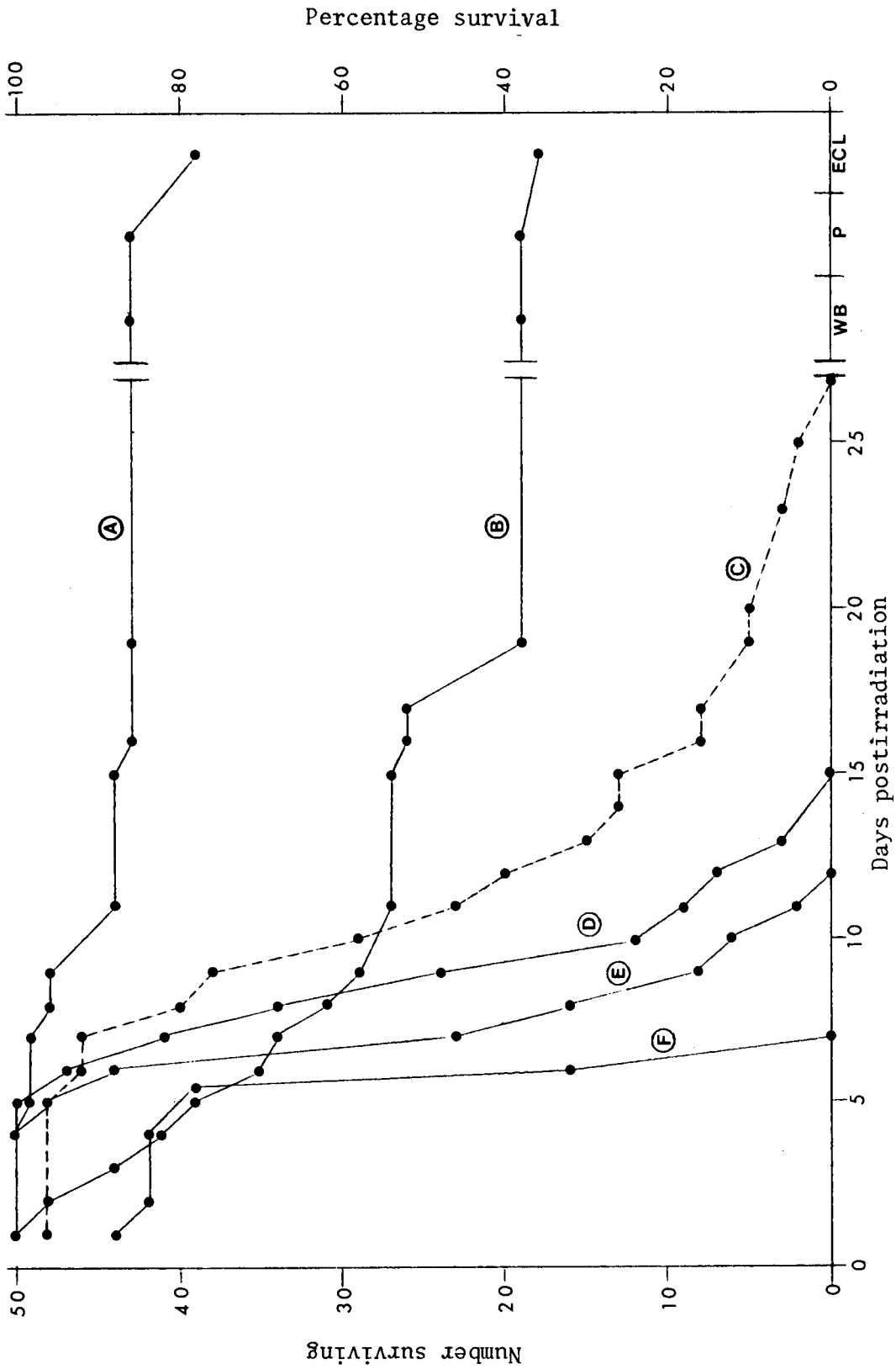


Figure I-2. Survival of Manduca sexta larvae X-irradiated 1-25 hours posthatch. WB = wandering larvae blocked; P = completion of the larval-pupal ecdysis; ECL = eclosed; A = control; B = 1 kR; C = 5 kR; D = 10 kR; E = 15 kR; F = 30 kR.

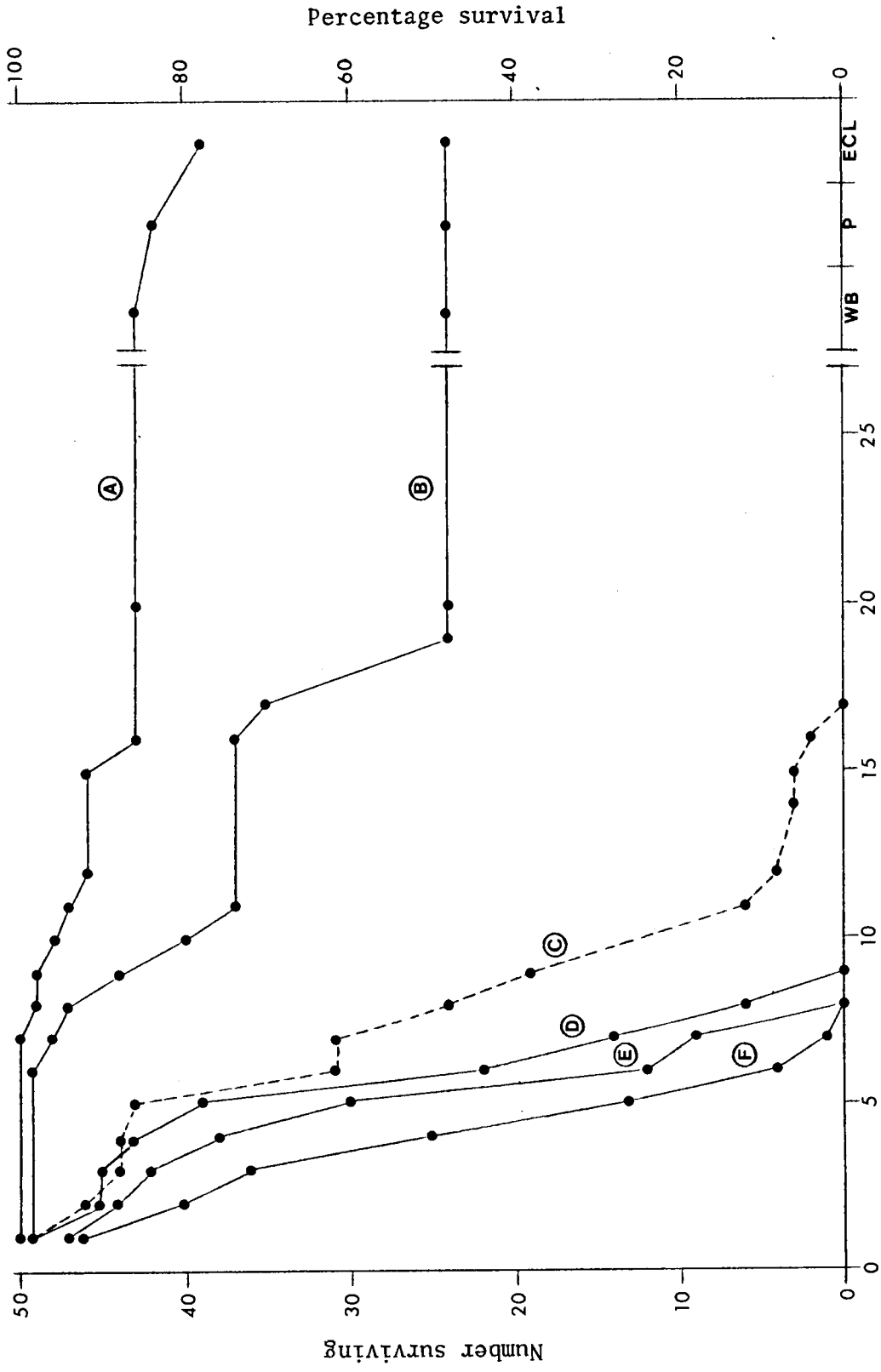


Figure I-3. Survival of Manduca sexta larvae X-irradiated 1-25 hours following ecdysis to second instar. See legend for Figure I-2.

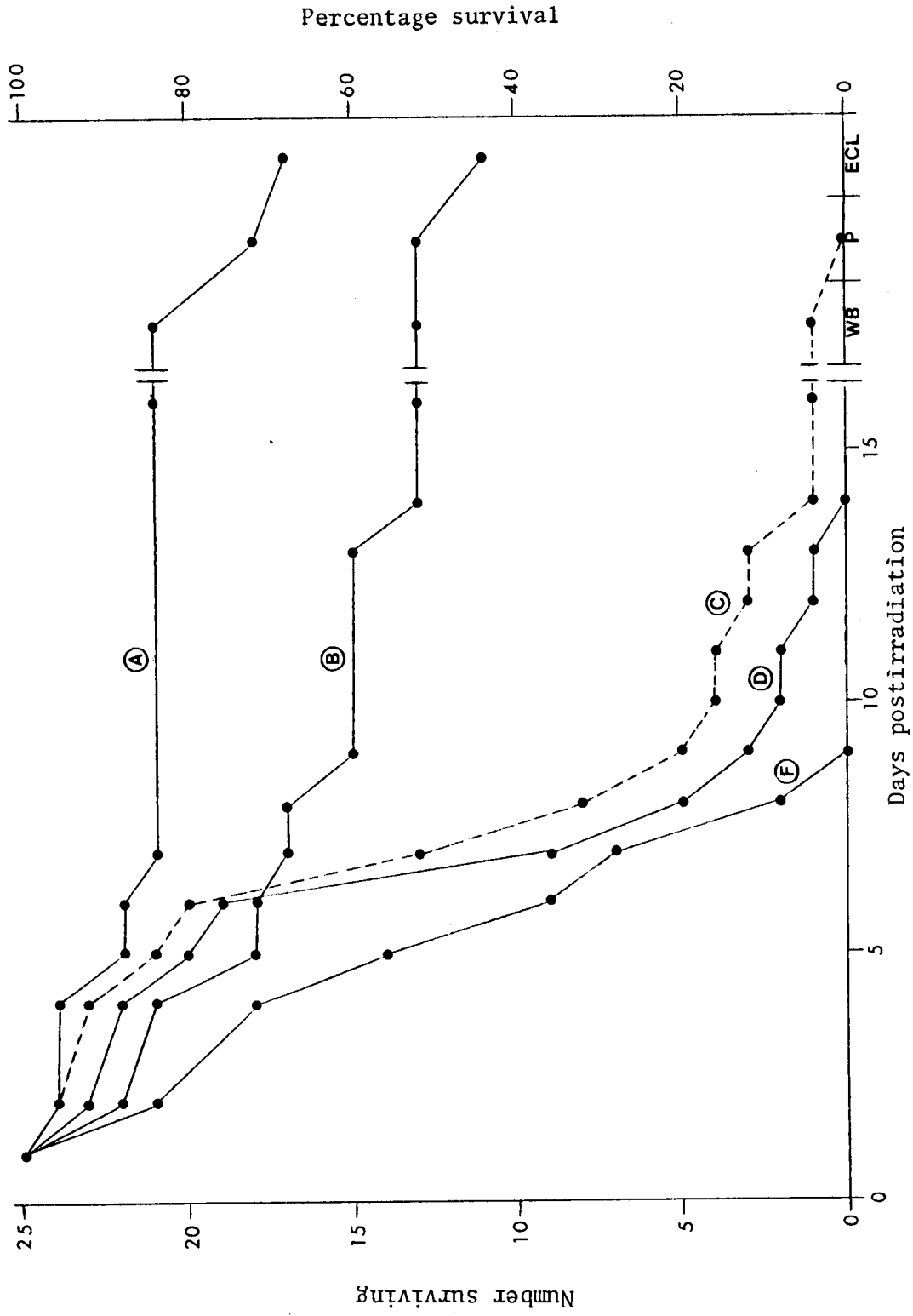


Figure I-4. Survival of Manduca sexta larvae X-irradiated 1-25 hours following ecdysis to third instar. See legend for Figure I-2, page 9.

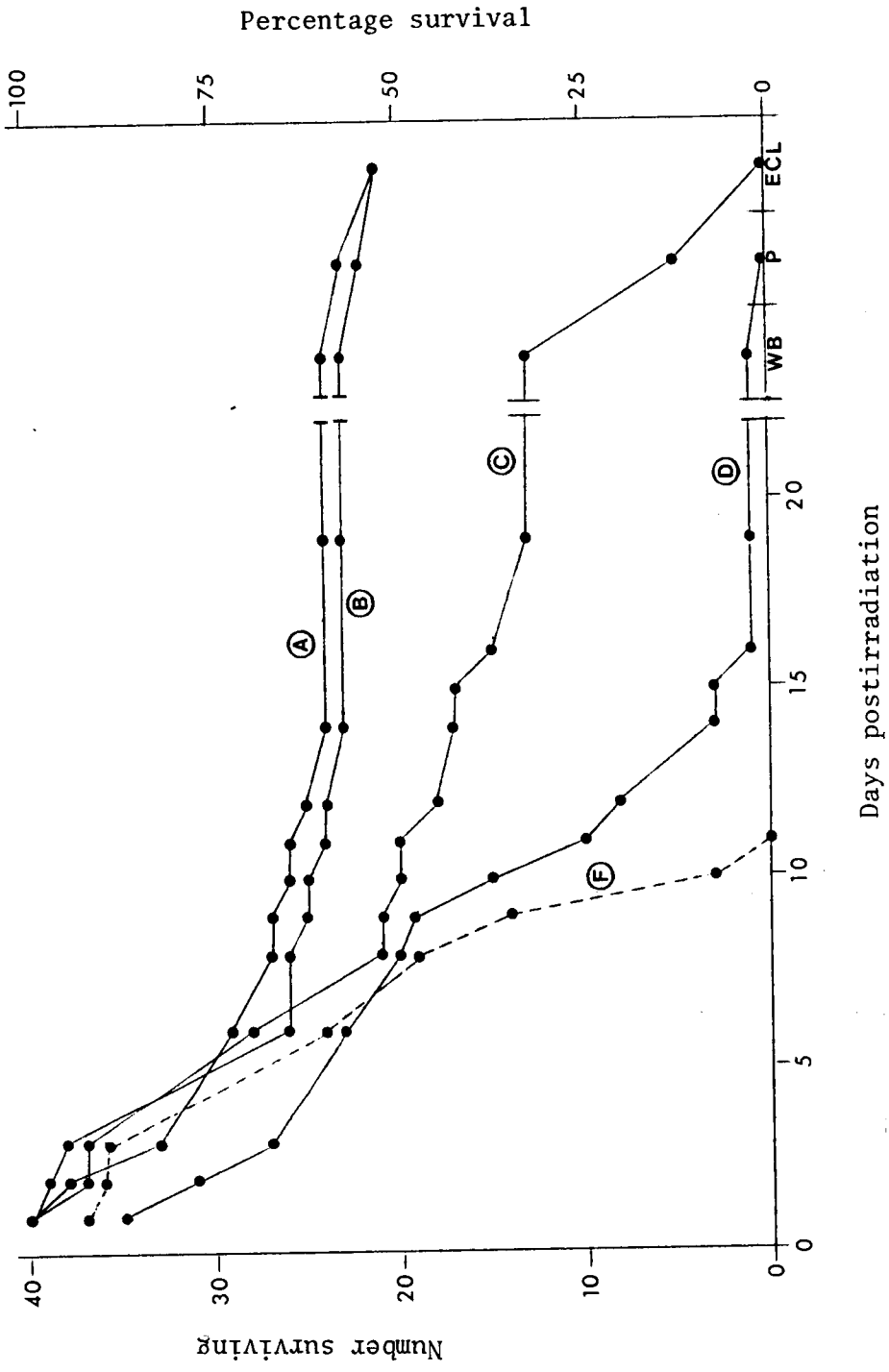


Figure I-5. Survival of Manduca sexta larvae X-irradiated 1-25 hours following ecdysis to fourth instar. See legend for Figure I-2, page 9.

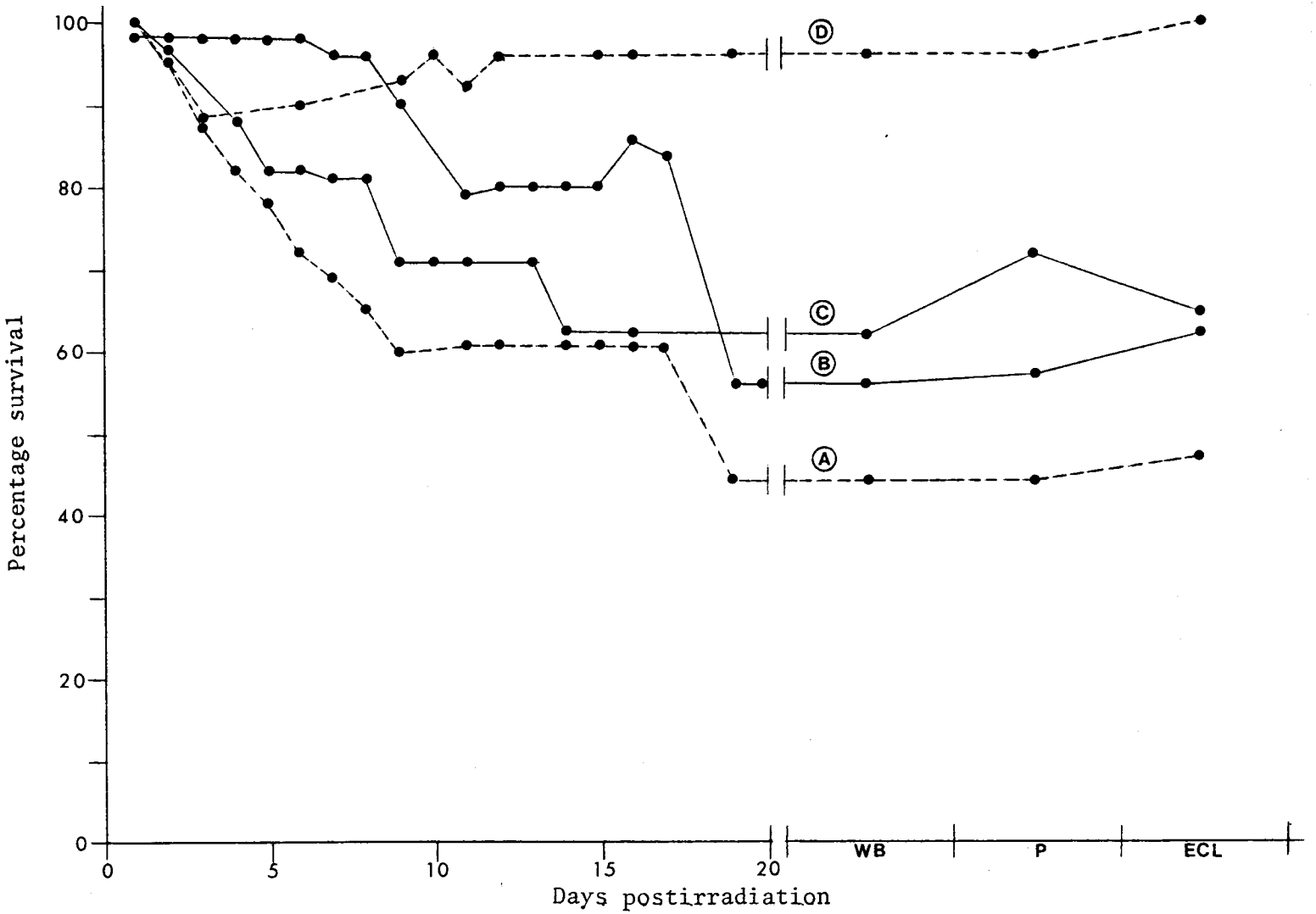


Figure I-6. Survival of Manduca sexta larvae X-irradiated with an exposure of 1 kR. Data taken from Figures I-2 through I-5, pages 8-12. WB = wandering larvae blocked; P = completion of larval-pupal ecdysis; ECL = eclosed; A = first instar; B = second instar; C = third instar; D = fourth instar.

CHAPTER IV

DISCUSSION

The degree of radiosensitivity of insect eggs is dependent upon the stage and rate of development (O'Brien and Wolfe, 1964). A reduced sensitivity to radiation with aging has been reported for eggs of several species of stored-grain insects (Nicholas and Wiant, 1959; Cornwell, 1966), the grasshopper Melanoplus differentialis (Orthoptera) (Evans, 1935), the mosquito Aedes aegypti (Diptera) (Terzian and Stahler, 1958), and the webbing clothes moth Tineola bisselliella (Lepidoptera) (Luscher, 1944).

Variations in sensitivity of D. melanogaster eggs to X-rays has been studied by Henshaw and colleagues (1933, 1935), and by Packard (1935). These investigators noted that the observed variations correlated well with mitotic activity, i.e., high mitotic activity was associated with high radiosensitivity. Murakami and Teulade (1969) and Murakami (1971) reported cyclical changes in radiosensitivity occurring in developing silkworm embryos, Bombyx mori (L.), which correspond to the division cycles of the developmental stages of the silkworm egg. In this species, cells in dividing phases of early cleavage were radiosensitive, while those in interphase and prophase were relatively radioresistant.

The effects of age on observed sensitivity to X-irradiation in M. sexta eggs is in agreement with the changes in susceptibility previously established for a variety of other insect species during embryonic development (Hilchey, 1957; O'Brien and Wolfe, 1964).

The pattern of increasing tolerance to radiation with age as observed in the survival curves of irradiated M. sexta has previously been established for a number of holometabolous insects (Hilchey, 1957; Grosch, 1973). Studies with Bracon hebetor (Clark, 1959) and D. melanogaster (Oster, 1959) provide evidence that larval mortality is ultimately brought about by radiation-induced chromosomal aberrations in somatic tissue. Visible manifestation of these chromosomal effects may not become conspicuous until the next molt, since a brief period of intense mitotic activity precedes the molt.

In conclusion, the dose-response patterns observed in irradiated eggs and larvae of M. sexta are in agreement with those established for other insects on which similar investigations have been reported.

PART II

RADIATION-INDUCED INHIBITION OF ECLOSION IN THE TOBACCO HORNWORM, *MANDUCA SEXTA*

CHAPTER I

INTRODUCTION

Increases in radioresistance in Drosophila melanogaster during pupal-adult development were first observed by Mavor (1927) and have since been documented for a number of holometabolous insects (Grosch, 1962 and 1973; Arena, 1971). Mavor (1927) concluded that ". . . the resistance of the pupae to X-rays begins after all of their important organs have been laid down and that their resistance increases with the growth and differentiation of these organs." Preliminary investigations with Manduca sexta X-irradiated at various intervals during pupal-adult development indicated an increase in radioresistance during this developmental period. Of particular interest in these studies was the observation that X-irradiated animals which failed to eclose were still able to complete metamorphosis.

In the normative scheme of insect development, the sequence of molts and morphological changes characteristic of postembryonic development is controlled by the interaction of hormones produced by components of the neuroendocrine system (see Wyatt, 1972; Doane, 1973). In holometabolous insects, the last major developmental event in the life cycle is eclosion. Hormonal control of eclosion has been demonstrated in at least three species of silkworms, namely Hyalophora cecropia, Antheraea polyphemus and Antheraea pernyi (Truman and Riddiford, 1970). Utilizing "loose-brain" animals and brain homogenates, these investigators concluded that the brain was the site of eclosion hormone production.

Furthermore, the release of hormone is synchronized with photoperiod regimen and acts on the central nervous system to elicit a species-specific pattern of behavior (preeclosion behavior). It is this elicited behavioral response which results in rupture and ecdysis of the pupal cuticle. Preeclosion behavior patterns are programmed in the abdominal ganglia rather than the brain (Truman, 1971; Truman and Sokolove, 1972). Eclosion hormone thus serves to trigger the sequence of abdominal movements necessary for emergence. Utilizing pharate adult A. pernyi in an assay for eclosion hormone activity, Truman (1973) found high concentrations of the hormone in the brains and corpora cardiaca of M. sexta pharate adults on the day of eclosion and provided evidence that the cells in the median neurosecretory cluster of the brain were responsible for eclosion hormone production. Therefore it is postulated that the radiation-induced inhibition of eclosion observed in Manduca results from a disruption of the normal hormonal mechanisms controlling adult emergence.

Studies described in this thesis were undertaken to both analyze in greater detail the effects on adult emergence of X-irradiation of pharate-adults of M. sexta, and to assess the neuroendocrine contribution, if any, to observed changes in radiosensitivity during the pupal-adult transformation exhibited by this species.

CHAPTER II

MATERIALS AND METHODS

Experimental Animals

Tobacco hornworms used in these experiments were derived from an inbred colony maintained by Dr. Lynn M. Riddiford, University of Washington, Seattle. Larvae were individually reared in plastic containers on the diet of Yamamoto (1969) modified after Bell and Joachim (1976) and Riddiford (personal communication) under an 18L:6D photoperiod regimen at 23-25°C according to the methods outlined in Part I of this thesis.

Irradiation Parameters

The X-irradiation source was a General Electric Maxitron 300 X-ray unit operated at 250 kVp and 15 mA with 0.5 mm aluminum filtration added (4.75 mm Be inherent filtration) to reduce tissue attenuation by 95-100%. Exposures were altered by varying the time of exposure and/or the target-to-object distance. Dosimetry, as measured in air, was determined under experimental conditions utilizing either (1) a 250-R thimble ionization chamber read in a Model 70 Victoreen condenser R-meter, or (2) a Model 575 Victoreen Radocon with medium energy (Model 602) probe.

Animals receiving whole-body or partial-body exposures (in the case of shielding studies) were positioned on a styrofoam platform, 2.5 cm in thickness, during irradiation. Following treatment, all animals were returned to laboratory rearing conditions.

Determination of ED_{50}'s

Animals were divided into three to five treatment groups (25 animals per group) and a control group (25 animals) on each day of the pupal-adult transformation. Treatment groups were given a series of graded whole-body exposures at an exposure rate of approximately 325 R/minute. The range of exposures to which animals were subjected on any given day during development was based on preliminary investigations of radiosensitivity. The fraction of irradiated animals eclosing relative to nonirradiated controls was plotted as a function of exposure and the ED_{50} (X-ray exposure required to prevent 50% of irradiated animals from emerging) determined for each day of pupal-adult development utilizing regression analysis. In this work, eclosion will be defined as those events associated with the emergence of an imago from the confines of the pupal exuvia following completion of pupal-adult development.

The relative radiosensitivity of animals prior to pupation was determined by irradiating at an exposure rate of 125 R/minute, early fifth instar larvae (1-24 hours after fourth-to-fifth larval-larval ecdysis), wandering larvae (2-24 hours following appearance of dorsal pigmentation), and pharate pupae on each day of the larval-pupal transformation with a series of X-ray exposures between 0.5 kR and 9 kR. The resulting pupation and eclosion successes were then determined.

Body Shielding Experiments

Animals were subjected to partial-body irradiation on the tenth day after pupation (Day 10) at an ED_{100} level of 48 kR (325 R/minute). A lead plate, 1 cm in thickness, was placed over selected body regions.

Exposures in the shielded regions were approximately 1-2% of those measured in the unshielded regions.

Brain Transplantation Experiments

In a selected group of pharate adults anesthetized with water saturated carbon dioxide (for up to 30 minutes), the brains (supra-esophageal ganglia) were surgically manipulated following procedures slightly modified after Williams (1959). [See Eaton (1974) and Eaton Dickens (1974) for descriptions of the topography of the brain and central nervous system of M. sexta.]

A section of cuticle (approximately 2.5 mm by 2.5 mm) was removed from the dorsal region of the head, and the brain (or brain-corpora cardiaca complexes) either removed or left in place after severing the major nerve trunks leading to the brain. Surgical procedures were carried out with the aid of a dissecting microscope on both nonirradiated animals, and on animals which had just been irradiated with an ED_{100} exposure of 48 kR delivered at 325 R/minute. Prior to each group of operations, dissecting instruments were rinsed in 95% ethanol. Following surgery, a few crystals of an equal part mixture of phenylthiourea and streptomycin sulphate were placed in the wound. Sterile physiologic saline (35 mM KCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 200 mM sucrose) was then added to the haemocoel until the level of hemolymph was flush with the surface of the cuticle. The opening was then covered with a plastic window (cut from cellulose acetate cover slips rinsed in 95% ethanol) and sealed in place with hot paraffin wax. Animals were then returned to the insectory. After 24 hours, pharate adults were checked for the presence of trapped air.

If air bubbles were visible through the plastic window, the wound was opened and resealed as described above.

Intact brains were implanted into the abdomens of treated and control animals by cutting a small opening in the next to last abdominal segment and inserting the tissue. Procedures for abdominal cuticle excision and wound closure were similar to those described above for head surgery.

Ecdysone Administration

Anhydrous 20(R)-hydroxyecdysone (β-ecdysone) (Sigma Chemical Corporation) was diluted with sterile 0.1 M KCl saturated with phenylthiourea to give a solution having a final concentration of 1 microgram ecdysone per microliter.

Animals were individually weighed, anesthetized with carbon dioxide, and injected by means of a 25 microliter Hamilton syringe with β-ecdysone through the mesothoracic tergum on Days 1, 4, and 12 of pharate-adult development. The injection site was then sealed with paraffin wax and the animals returned to laboratory rearing conditions. The quantity of β-ecdysone injected was based upon the volume of hemolymph which was taken to be 40% of body weight.

Following administration of ecdysone, animals were irradiated as outlined below:

1. Day 1 animals receiving 4 μg of β-ecdysone per gram of wet weight were X-irradiated at ED_0 with an exposure of 8.23 kR (325 R/minute) at 6, 24, and 48 hours postinjection. One group of animals, injected on Day 1 with 4 μg ecdysone per gram of wet weight, received

a second ecdysone injection (again, 4 µg/gram wet weight) 24 hours after the first. This group was then subjected to an 8.23 kR X-ray exposure (325 R/minute) 24 hours following the second ecdysone administration. Day 1 animals receiving 4 µg ecdysone/gram wet weight without subsequent irradiation served as a control group.

2. Day 12 animals were treated in a manner identical to that used for Day 1 animals with the exception that the ED_0 X-irradiation exposure was 25 kR (325 R/minute).

3. Day 4 animals were treated in a manner similar to Day 1 animals except that at 6, 12, 34, 36, and 48 hours postecdysone injections the animals were irradiated at an ED_0 level.

4. Day 4 animals were X-irradiated with an exposure of 8.23 kR (325 R/minute) 24 hours following administration of 2, 4, 6, 12, and 16 micrograms of ecdysone per gram of wet weight.

Eclosion success was determined for each experimental group and expressed as a percentage of eclosion success observed in noninjected animals irradiated at similar time intervals. Day 4 and Day 12 data were analyzed using single classification analysis of variance. The Student-Newman-Keuls test was employed for a comparison among means (Sokal and Rohlf, 1969).

CHAPTER III

RESULTS

Effects of X-Irradiation on Eclosion

The effect of X-irradiation on eclosion of M. sexta during the pupal-adult transformation was investigated by initially determining the ED_{50} for animals on each day of development and by utilizing the ED_{50} values as a relative measure of radiation sensitivity (Figure II-1). An initial period of nearly constant radiosensitivity was noted between Days 0 and 6 (ED_{50}'s ranged from 13.0 kR on Days 0 and 6 to 14.4 kR on Day 1). This period of constant sensitivity was followed by a pronounced decrease in radiosensitivity through the day of eclosion (Day 8: ED_{50} = 19 kR; Day 22: ED_{50} = 75 kR).

The timing of maximum sensitivity on Day 7 was determined with greater precision by X-irradiating pharate adults at an exposure level of 8.23 kR (the ED_{50} for Day 7) at 6-hour intervals between Day 6 and Day 8. The increasing radiosensitivity begins 6.25-6.50 days following pupation, reaches a maximum level between 7.25 and 7.50 days, and then returns to a level approximating that seen 6.0-6.25 days following pupation (Figure (Figure II-2).

A comparison of eclosion success in animals irradiated at various intervals between the fourth to fifth larval-larval ecdysis and pupation reveals a general decline in radiosensitivity with developmental age within this stadium (Figure II-3). Interpretation of these data must be tempered with information on success during the larval-pupal ecdysis,

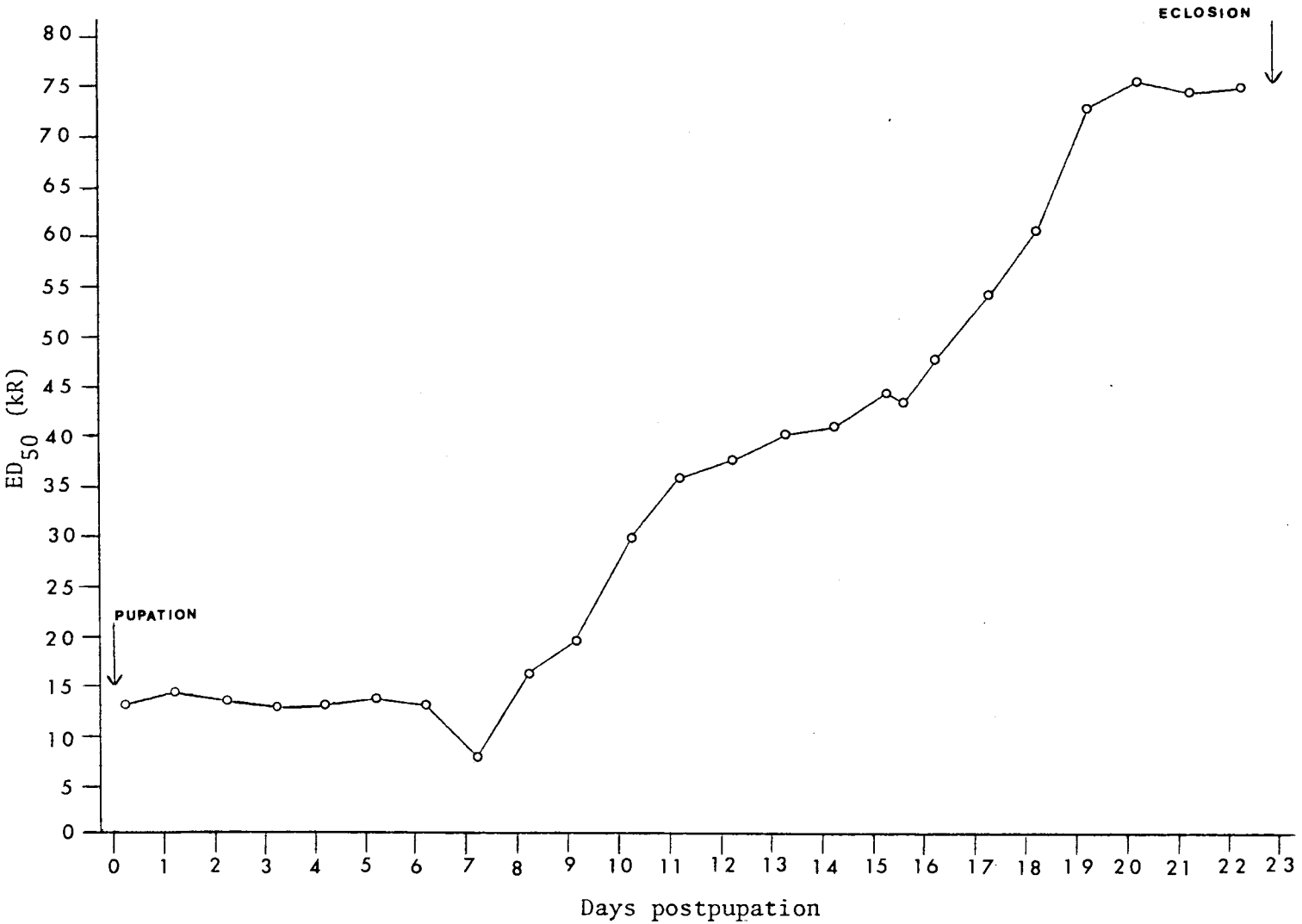


Figure II-1. ED_{50} values (X-ray exposures required to prevent eclosion in 50% of the irradiated animals) during pupal-adult development of Manduca sexta.

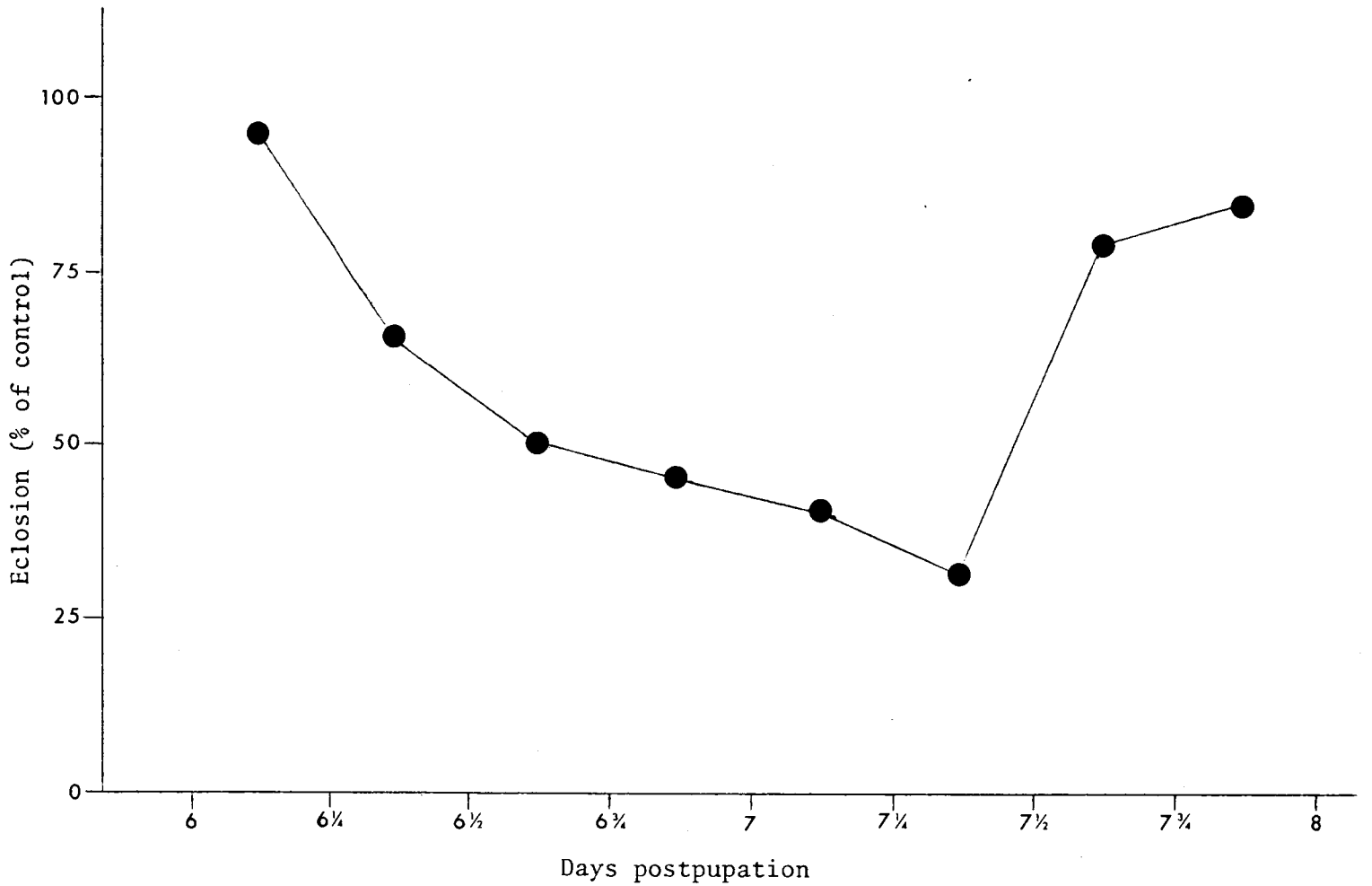


Figure II-2. Eclosion of Manduca sexta X-irradiated during pupal-adult transformation with an exposure of 8.23 kR. 25 animals per exposure group.

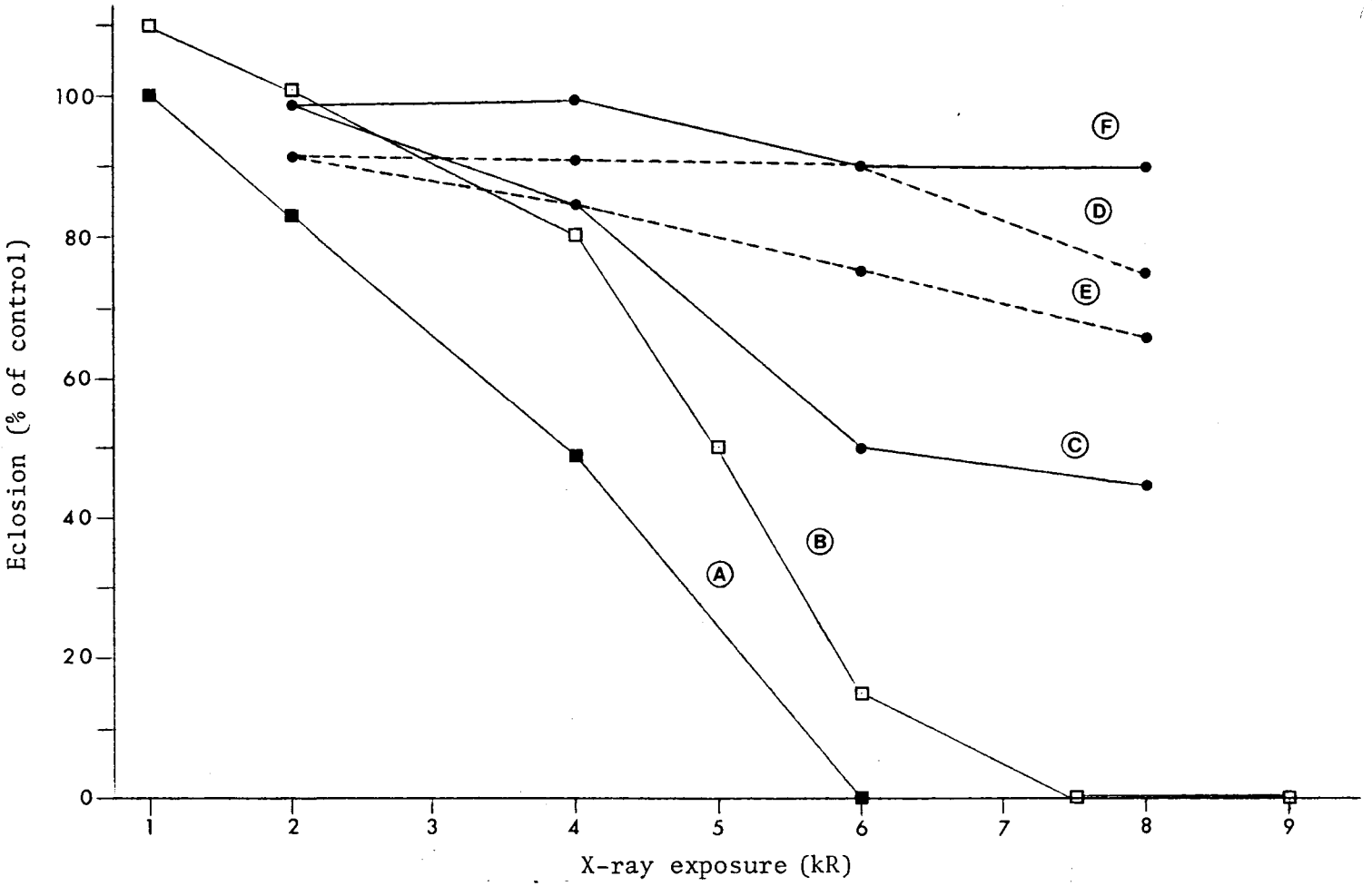


Figure II-3. Eclosion of Manduca sexta X-irradiated at the following developmental stages: (A) 1-24 hours following ecdysis to fifth instar; (B) wandering larvae, 2-24 hours following appearance of dorsal pigmentation; (C) Day-1 pharate pupae; (D) Day-2 pharate pupae; (E) Day-3 pharate pupae; (F) Day-4 pharate pupae. 25 animals per exposure group.

since any attempt to establish a causal relationship between eclosion success in animals irradiated prior to the larval-pupal ecdysis and those irradiated during pupal-adult development is dependent upon the percentage of insects that successfully pupated. For example, the absence of successful eclosion in animals irradiated with an exposure of 6 kR 1-12 hours after the fourth to fifth larval-larval ecdysis can be attributed to the small number of animals (8.3%) that successfully pupated (Figure II-4). In contrast, irradiated pharate pupae all successfully pupated at all exposure levels.

Brain Extirpation/Transplantation Experiments

The role of the brain as a radiosensitive component in animals following the period of maximum radiosensitivity (i.e., Day 7) was investigated following brain extirpation and brain transplantation experiments performed on irradiated (with an ED_{100} exposure of 48 kR) and nonirradiated Day 10 animals.

Debraining of nonirradiated animals resulted in only 3.2% eclosion success (Table II-1). Clearly, the brain is required for successful adult emergence. However, connectives leading from the brain to other regions of the central nervous system must also be intact if eclosion is to occur, since cutting the connectives to the brain of nonirradiated animals resulted in only 45% eclosion success.

Irradiation of Day 10 animals with an exposure of 48 kR (ED_{100} exposure) resulted in 0% eclosion success. Implantation of nonirradiated brains into the abdomens of ED_{100} animals did not improve eclosion success while removal of brains resulted in only 6.7% eclosion success. This

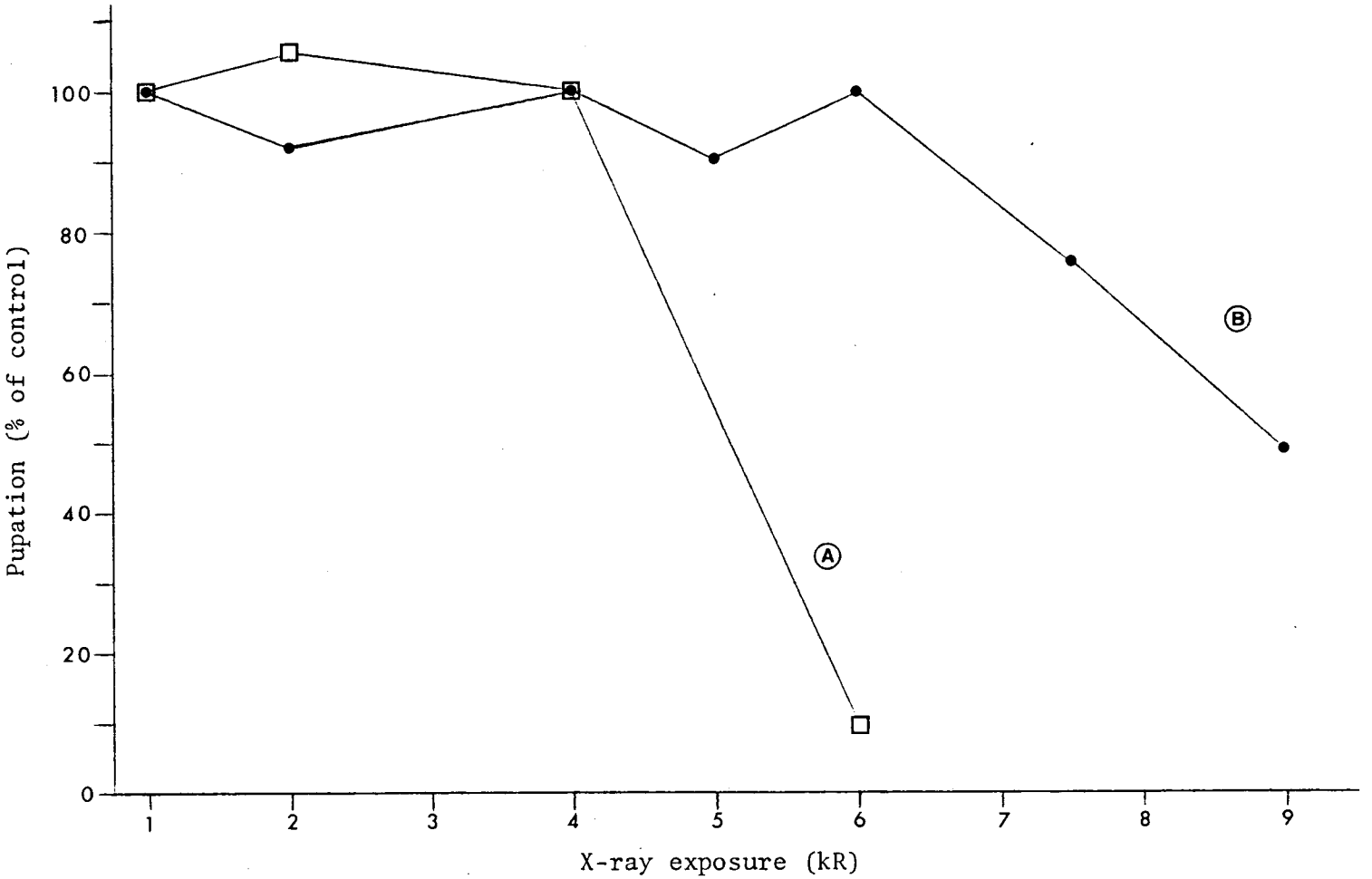


Figure II-4. Pupation of Manduca sexta X-irradiated (A) 1-24 hours following ecdysis to fifth instar, and (B) as wandering larvae, 2-24 hours following appearance of dorsal pigmentation. 25 animals per exposure group.

Table II-1. Eclosion of Manduca sexta following surgical manipulation of the brain and/or X-irradiation with an ED_{100} exposure of 48 kR on Day 10 of pupal-adult development

Group No.	Treatment	Eclosion No.	Eclosion %
NONIRRADIATED ANIMALS:			
1	No surgical manipulation	20/20	100
2	Debrained	1/31	3.2
3	Debrained; nonirradiated brain implanted into head	9/20	45
4	Debrained; ED_{100} brain implanted into head	1/15	6.7
5	ED_{100} brain implanted into abdomen of animal whose own brain intact	15/15	100
6	Nerve connections to the brain severed	9/20	45
ED_{100} IRRADIATED ANIMALS:			
7	No surgical manipulation	0/20	0
8	Debrained following irradiation; nonirradiated brain implanted into head	1/15	6.7
9	Nonirradiated brain implanted into abdomen of animal whose own brain was intact	0/20	0

suggests that radiation damage to the brain alone cannot completely account for the 0% eclosion success observed in Group 7 of Table II-1. However, it is also clear that the brain is "damaged" by irradiation, and that this damage reduces the chance for successful eclosion, as evidenced by comparing the results obtained from Group 3 and Group 4 in Table II-1. Adding nonirradiated brains to debrained, nonirradiated animals results in a 45% eclosion success. Adding brains irradiated at the ED_{100} level to debrained, nonirradiated animals, however, results in only 6.7% eclosion success. The 100% eclosion success observed in Group 5, Table II-1, suggests that the decrease in emergence noted in irradiated animals cannot be attributed to an "active inhibition" of eclosion by the irradiated brain itself.

Effects of Body Shielding on Eclosion Success

Partial body irradiations of Day 10 animals, utilizing an ED_{100} exposure of 48 kR, were performed to identify sensitive and tolerant body regions. Eclosion success following selective shielding is summarized in Table II-2.

Whole body exposure at 48 kR resulted in 0% eclosion success. Shielding of the abdomen alone resulted in 10% emergence while 100% of the animals whose head and thorax were simultaneously shielded during irradiation successfully eclosed.

Comparison of Groups 2 and 3 with Group 5 of Table II-2 indicates that the improved eclosion success observed in these former groups can be attributed to shielding of the thorax (Group 2) or shielding of the head (Group 3). Since shielding of the head alone (Group 4) results in

Table II-2. Eclosion following partial-body X-irradiation of Day 10 *Manduca sexta* with an exposure of 48 kR

Group No.	Body Region Shielded	Eclosion	
		No.	% of Control
1	Head and thorax	20/20	100
2	Thorax and abdomen	18/20	90
3	Head and abdomen	14/20	70
4	Head only	6/20	30
5	Abdomen only	2/20	10
6	No shielding	0/20	0

30% eclosion success, it appears that, of the three body regions shielded, the thorax is the most radiosensitive, with the abdomen being least sensitive (Group 1), and the head region being of intermediate radiosensitivity.

Effects of β-Ecdysone of Eclosion

The possible association between the maximum period of radiosensitivity observed in Day 7 animals and an elevated level of endogenous ecdysone (see Kaplanis et al., 1966) was investigated by injecting Day 1, Day 4, and Day 12 animals with 4 micrograms of β-ecdysone per gram of wet weight and irradiating at specific intervals postinjection with the maximal level of radiation that failed to interfere with eclosion (ED_0 exposure). β-ecdysone has been shown to be the active form of the molting hormone controlling postembryonic growth and development in tobacco hornworms (Kaplanis et al., 1966; King et al., 1974).

Administration of ecdysone to Days 1, 4, and 12 animals, without subsequent irradiation, resulted in mean eclosion successes of 87%, 87%, and 93% respectively (Table II-3). Significant increases in radiosensitivity ($p < 0.05$) were noted in (1) Day 4 animals 12 and 24 hours postinjection, and (2) in Day 12 animals 6 hours postinjection. Irradiation of Day 1 animals following a double-injection routine also increases radiosensitivity, as indicated by 53% eclosion success.

The degree of radiosensitivity observed in Day 4 animals irradiated 24 hours postinjection is clearly dependent upon the quantity of ecdysone administered (Figure II-5). All animals irradiated 24 hours following

Table II-3. Eclosion of Day 1, Day 4, and Day 12 Manduca sexta pharate adults X-irradiated with ED_0 exposures of 8.23 kR, 8.23 kR, and 25 kR, respectively, following injection of 4 micrograms of 20(R)-hydroxyecdysone per gram wet weight. Results are expressed as percentages of noninjected, irradiated controls

Treatments	Eclosion				
		Day 4 Animals		Day 12 Animals	
	Day 1 Animals	Series 1	Series 2	Series 1	Series 2
Injected, nonirradiated	87 (13/15)	80 (12/15)	93 (14/15)	93 (14/15)	93 (14/15)
Irradiated at the following intervals postinjection:					
6 hours	87 (13/15)	60 (12/20)	87 (13/15)	40 (6/15)	53 (8/15)
12 hours	-	30 (6/20)	40 (6/15)	-	-
24 hours	73 (11/15)	47 (7/15)	55 (11/20)	87 (13/15)	100 (15/15)
36 hours	-	72 (13/18)	73 (11/15)	67 (10/15)	40 (6/15)
48 hours	93 (14/15)	100 (15/15)	93 (14/15)	60 (9/15)	47 (7/15)
Irradiated 24 hours following double injection	53 (8/15)	67 (10/15)	60 (9/15)	60 (9/15)	73 (11/15)

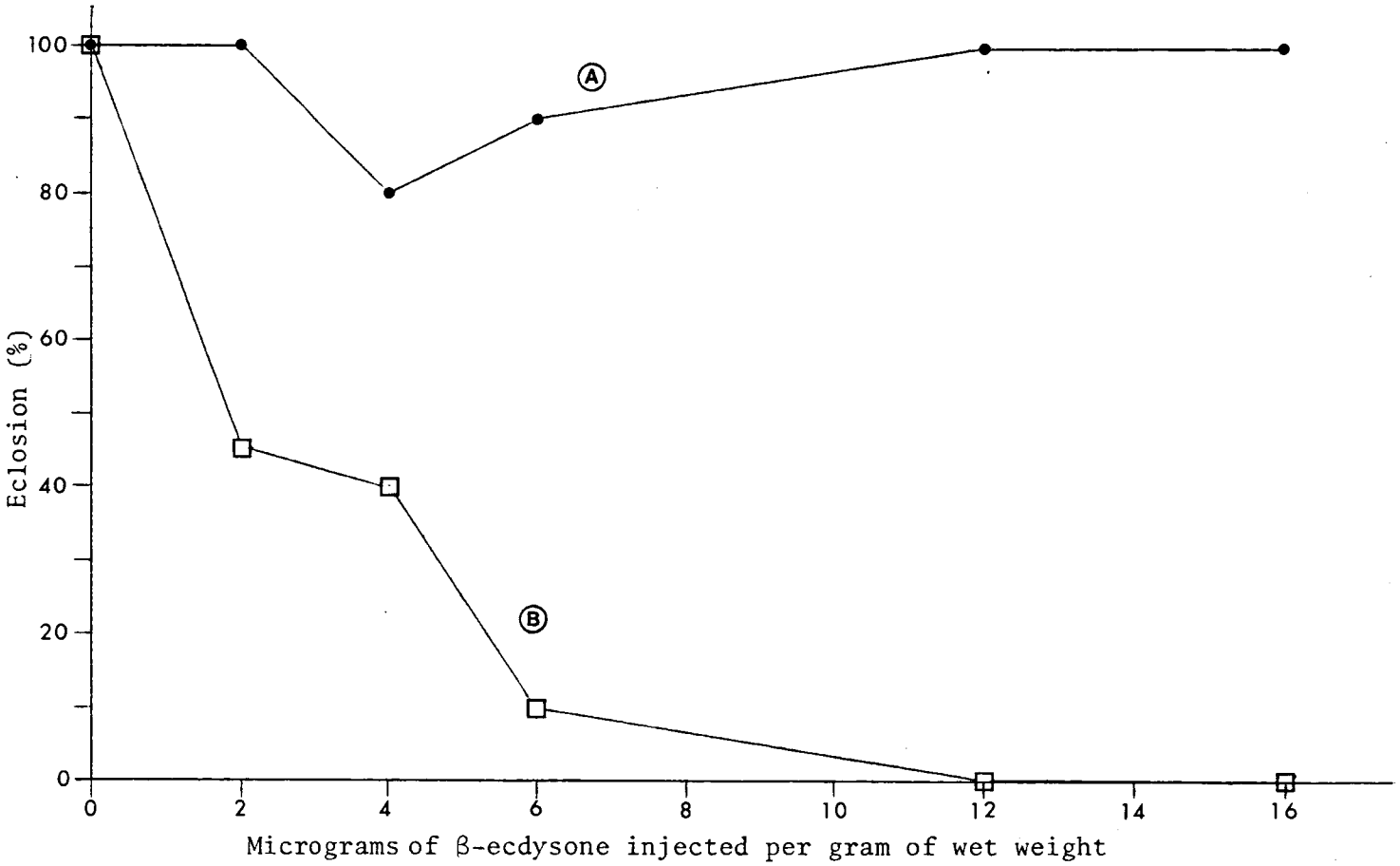


Figure II-5. Elcosion of Manduca sexta following injections of β-ecdysone on Day 4 of pupal-adult transformation. (A) Nonirradiated. (B) X-irradiated with an exposure of 8.23 kR 24 hours postinjection. Twenty animals per treatment group.

injection of 12 and 16 micrograms of ecdysone per gram of wet weight failed to eclose while those receiving identical ecdysone doses but not irradiated exhibited 100% eclosion success.

CHAPTER IV

DISCUSSION

Evaluating the action of X-irradiation of Musca domestica pupae upon eclosion, Rockstein et al. (1965), proposed two possible mechanisms affecting the emergence of irradiated flies, namely (1) premature termination of pupal-adult development, and (2) interference with the actual process of eclosion possibly brought about by damage to the muscle systems involved in the rupture and shedding of the pupal cuticle. These investigators considered the first mechanism to be of minor importance over the exposure range utilized for two reasons, namely that (1) unemerged, irradiated puparia contained fully formed adults, and (2) partial eclosion was observed in some animals. Preliminary investigations with M. sexta indicated that X-irradiation during the pupal-adult transformation could inhibit eclosion without disrupting normal metamorphosis.

In H. cecropia, A. pernyi, and A. polyphemus, specific hormones are involved in eclosion (Truman and Riddiford, 1970). Therefore, my initial hypothesis assumed that the pattern of radiosensitivity (as measured by the ED_{50} values) in M. sexta X-irradiated during the pupal-adult transformation would reflect changes in the radiation sensitivity of some component or phase in hormone production. Were the control of eclosion hormone synthesis and release in M. sexta similar to that of A. pernyi (Truman, 1973), then the observed pattern of radiosensitivity might reflect damage of neurosecretory cells responsible for eclosion hormone

production or even the inactivation of eclosion hormone itself. To date, an assay for eclosion hormone titer in the tobacco hornworm as a function of developmental age has not been reported. Therefore, a direct correlation between radiosensitivity and eclosion hormone synthesis and distribution could not be made.

The titers of ecdysone during pharate-pupal and pharate-adult development have been measured in the tobacco hornworm by several investigators (Kaplanis et al., 1966; Bollenbacher et al., 1975). Employing a biological assay system, Kaplanis et al. (1966) monitored changes in ecdysone levels during pupal-adult development in tobacco hornworms reared at 24 ± 2°C (eclosion on Day 21) and found a single peak of maximum titer 6-8 days following pupal ecdysis. Since this period of maximum ecdysone level coincided with the period of maximum radiosensitivity (Day 7) observed during the determination of ED_{50}'s, the question of a possible relationship between these two variables was raised.

Two models accounting for the observed relationships between ecdysone and eclosion hormones are: (1) synthesis and/or metabolism of ecdysone is interrupted by radiation exposure, and (2) the increased presence of ecdysone renders the animal more sensitive to the effects of radiation. The first model is clearly incorrect, since 93% of irradiated Day 7 animals which failed to eclose completed development. The observed increases in radiosensitivity in Day 1, Day 4, and Day 12 animals injected with β-ecdysone (Table II-3, page 34) provides support for the second model. However, the temporal pattern of response in these groups may be dependent upon both the competence of the animal to respond to

exogenous ecdysone and the titer of endogenous ecdysone at the time of injection and subsequent irradiation. For example, whereas increases in radiosensitivity in Days 4 and 12 animals, respectively, occurred 12 hours and 6 hours postinjection, that in Day 1 animals was elicited only after a double injection of ecdysone (Table II-3, page 34). In this regard, Kaplanis et al. (1966) found that the titer of ecdysone in Day 1 animals is only 60% of that found in Day 4 and Day 12 animals. Since the observed radiosensitivity induced by exogenous ecdysone appears to be dose-dependent, then the total (exogenous and endogenous) quantity of ecdysone present in hemoloymph would be substantially lower in the Day 1 groups receiving single injections relative to Day 4 and Day 12 groups.

The mode of action of ecdysone in effecting the increase in radiosensitivity was not investigated directly. One explanation involves the possible role of ecdysone as a messenger responsible for "turning on" eclosion hormone production in the median neurosecretory cells of the supra-esophageal ganglion. Irradiation of these cells following ecdysone stimulation could interfere with those critical biochemical activities (such as mRNA synthesis) necessary to initially establish the synthetic pathways for eclosion hormone synthesis, storage, and release.

Delay and inhibition of pupation and pupariation are common in X-irradiated larvae of many endopterygote insects such as D. melanogaster (Hussey et al., 1932; Villee, 1946; Bourgin et al., 1956; Horikawa et al., 1960a and 1960b; see also reviews by O'Brien, 1964; Grosch, 1962 and 1973). In these insects, the effect of radiation has been attributed to a lack of ecdysone, since larvae irradiated after ecdysone release exhibit no delay or inhibition of growth, while in larvae irradiated prior to

ecdysone release, the affect could be reversed following ecdysone injection. Working with the fleshfly, Sarcophaga bullata, Sivasubramanian et al. (1974) conjectured that radiation damage to the hormonal mechanism controlling pupation may occur at one or more stages, including (1) synthesis of α-ecdysone, (2) ecdysiotropin-triggered release of α-ecdysone, and (3) conversion of α-ecdysone to β-ecdysone by various body tissues.

Pupation in M. sexta has been shown to be preceded by two periods of ecdysiotropin release, one occurring at the end of the fifth instar phagoperiod just prior to gut evacuation and the second several days later at the initiation of the pharate pupal stage (Truman and Riddiford, 1974a; see also Nijhout et al., 1974a). The two peaks of α-ecdysone secretory activity of Manduca prothoracic glands and of β-ecdysone activity during the fifth larval instar, reported by Bollenbacher et al. (1975), correspond to surges of ecdysone caused by each release of ecdysiotropin.

The temporal organization of ecdysiotropin release and ecdysone synthesis can be related to the relative radiosensitivity exhibited by X-irradiated fifth instar larvae of M. sexta. Expressed in terms of percentage successful pupation, one observes the maximal sensitivity (approximately 8% pupation of 6 kR) during the last larval instar in animals irradiated prior to the first release of ecdysone (i.e., animals irradiated 1-12 hours following ecdysis to the fifth instar), intermediate radiosensitivity (approximately 50% pupation at 9 kR) in wandering larvae irradiated after the first, but before the second ecdysiotropin-stimulated ecdysone peak, and relative radioresistance to pharate pupae

irradiated following the second release of ecdysiotropin, where 100% pupation success was achieved in all groups. Since the second ecdysone peak occurs on Day 2 of the larval-pupal transformation, and since pharate pupae irradiated prior to this rise in ecdysone titer appeared no more radiosensitive over the exposure range utilized than animals irradiated following the second ecdysone release, it is proposed that the decrease in pupation success observed in early fifth and wandering late fifth instar larvae may be related to an indirect effect of X-irradiation upon normal ecdysone metabolism. This effect on ecdysone metabolism can be brought about by radiation-induced inhibition of ecdysiotropin synthesis and/or release rather than a direct affect upon ecdysone synthesis, storage, release, or conversion to an active form.

Eclosion hormone in M. sexta is thought to be synthesized by the median neurosecretory cells of the brain and stored in the corpora cardiaca prior to its release into hemolymph at the time of eclosion (Truman, 1973). Following the initial demonstration that hormone stored in the brain was responsible for eclosion in the silkmoth, H. cecropia, Truman and co-workers (Truman and Riddiford, 1970 and 1974b; Truman, 1971) observed that this hormone was not an absolute requirement for eclosion in this species, since animals debrained during diapause or on days 0-3 of pharate adult development were still able to eclose. However, the coordinated preeclosion behavioral pattern characteristic of this species was observed in only 12% of such surgically manipulated animals. Working with diapausing M. sexta pupae, Judy (1972) reported that animals debrained 20 to 50 days after pupation were still able to terminate

diapause and complete adult development. However, only a few were able to successfully eclose. In contrast to the results of Judy (1972), Wilson et al. (1974) observed a 70% rate of eclosion success in diapausing M. sexta which, following brain removal at selected early, middle, and late pupal ages, were able to terminate diapause and initiate pharate-adult development. To clarify the role of the brain in regulating eclosion in this species, brain extirpation and exchange experiments were carried out in conjunction with selective shielding studies on irradiated and nonirradiated animals at Day 10 of pupal-adult development.

The eclosion success observed in debrained, nonirradiated Day 10 animals was only 3%, again indicating that the brain is required for successful eclosion. This finding is at variance with results obtained with debrained H. cecropia (Truman, 1971) and with M. sexta debrained during diapause by Wilson et al. (1974). Furthermore, the 45% eclosion success seen in animals following simple brain exchange and following severance of the major nerve turnks to the brain indicates that the role of the brain in controlling eclosion cannot be explained entirely in terms of hormonal mechanisms.

The zero level eclosion success observed in Day 10 animals following a 48 kR X-ray exposure is not merely the result of radiation damage to the brain. For example, neither implantation of nonirradiated brains into the heads of animals debrained following irradiation nor implantation of nonirradiated brains into the abdomens of animals whose own brains remained intact significantly improved eclosion success.

Had the brain been the sole target of radiation damage, one would have expected an eclosion success of at least 45% in each of these groups. This is not to say that the brain did not incur damage which in turn decreased the likelihood of a successful emergence. Indeed, the 7% eclosion success observed in nonirradiated, debrained animals into which ED_{100} brains were implanted indicates that radiation damage affecting eclosion does occur to the brain. Were this not the case, a 45% eclosion success would have been expected in this group.

In an attempt to identify radiosensitive body regions without resorting to surgical manipulation, selected irradiations at the ED_{100} level of Day 10 animal body regions were performed. Little enhancement in eclosion success was observed in abdomen-shielded animals. This suggests that any radiation damage in this body region, including damage to the abdominal neurocircuitry and musculature involved in eclosion behavior, does not significantly decrease the chances for successful emergence. Simultaneous shielding of the head and thorax (Table II-2, page 32), on the other hand, resulted in a 100% eclosion success. Furthermore, these data suggest that the relative contribution of shielding the thorax is greater than head shielding in decreasing radiation-induced inhibition of eclosion. Again, radiation damage to the head (including the brain), although a contributory factor, cannot fully account for the decrease in eclosion success observed in these experimental groups.

In conclusion, the pattern of radiosensitivity observed in M. sexta during pupal-adult development reflects specific interference in the

regulation of eclosion. The observed radiosensitivity is, in part, dependent upon the stage in development and decreases markedly just prior to eclosion. It is proposed that the radiosensitivity observed during early development (Days 1-7) is in part dependent upon the endogenous ecdysone titer and may be related to a "switching on" of eclosion hormone synthesis within the brain by rising ecdysone levels. Studies utilizing brain exchange or shielding clearly demonstrate that, although the brain is a radiosensitive region, a second more sensitive component located in the thorax is also present. And lastly, although hormonally controlled, successful eclosion of nonirradiated M. sexta appears to require the presence of an intact brain.

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