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The relationship between insect hormones and cellular degeneration within larval imaginal discs was investigated. It was hypothesized that the molting hormone, ecdysone, or the juvenile hormone, or possibly the hormonal balance between the two hormones, stimulated the process of cellular degeneration in larval tissue.

The larval tissue chosen for these experiments were the dorsal mesothoracic imaginal discs or wing discs of the mutant vestigial of *Drosophila melanogaster*. The discs were removed from late third instar larvae and placed *in vitro* for various time intervals in medium containing beta-ecdysone (4.5 µl/ml) and/or the juvenile hormone (250 mg/25 ml). The discs were then removed from culture, washed, and incubated in a medium containing the substrate succinic acid and the enzyme-specific nitro blue tetrazolium stain. Dying cells did not stain due to the lack of enzyme activity. Following removal from the staining medium, the whole discs were fixed in neutral buffered formilin, then dehydrated in 95% ETOH, and mounted on permanent slides with Euparal. The slides were then examined microscopically. The results observed through examination of the disc slides enabled one to draw the following conclusions. The presence of ecdysone and not the absence or presence of the juvenile hormone stimulates

cellular degeneration. Cell death differentiation and normal differentiation although both are stimulated by ecdysone occur independently of each other. This would indicate that one is dealing with two different types of cellular differentiation in larval tissue, that differentiation which requires a reorganization of cells before completion, and the type of differentiation which occurs with or without cellular reorganization.

AN *IN VITRO* STUDY OF THE EFFECTS OF INSECT HORMONES ON CELL DEGENERATION IN THE WING IMAGINAL DISCS OF THE MUTANT VESTIGIAL OF *DROSOPHILA MELANOGASTER*

by

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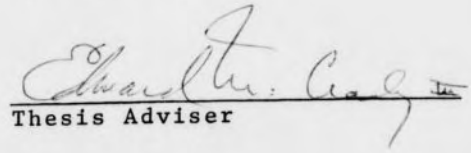

Thesis Adviser

APPROVAL PAGE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

In insect development, certain cells and cell populations are genetically programed for death. Although a cell's fate is determined at a very early stage, cell degeneration does not occur until much later in the course of the developmental process. What causes or triggers cell degeneration and death is not known (Saunders, 1966; Fristrom, 1969).

Past investigations concerning cellular degeneration have dealt primarily with the morphology of the degenerating cell and the overall pattern of cell death with little hypothesizing as to what initiates this process. In culture and in situ, the imaginal discs or adult organ anlagen of Drosophila appear as compact units of undifferentiated cells which exhibit definitive characteristics as to size, shape, and position within the larva. Prior to the late third instar in the imaginal discs of both wild and mutant type of Drosophila melanogaster, cells determined to degenerate do not appear morphologicaly abnormal or different from other disc cells not programed for death (Fristrom, 1968). During the late third instar developmental period and metamorphosis, a disc cell determined to die passes through stages of degeneration involving both nuclear and cytoplasmic changes, while cells not determined to die proceed along normal pathways of differentiation.

The extent and location of degenerating cells in imaginal discs vary according to the particular genotype under consideration. Although cell death is a normal phase in the development of both wild-type and mutant Drosophila, it can best be observed in imaginal discs in which massive cell death occurs. The mutant vestigial is exemplary of this for there is a high degree of cell mortality observed in the wing imaginal discs of this phenotype.

During the late third instar developmental period numerous bodies identified as dying cells have been observed in vestigial wing discs. These degenerating cells seem to be concentrated in the wing blade region of the discs. Only a small number of dying cells were observed in what is termed the thoracic region of the discs. Only a few degenerating cells occurred in wild-type wing discs observed at the same developmental time. (Fristrom, 1969).

Due to this high incidence of cellular degeneration in the vestigial wing discs, definite phenotypic wing modifications are found in the adult insect. Only the basal parts of the wings are present, the wing blade having been reduced to vestiges held at right angles to the body (Lindsley and Grell, 1967).

Based primarily on morphological studies of various mutant and wild-type imaginal discs, Fristrom (1969) has proposed the possibility that cellular degeneration may be

a function of cell type rather than extrinsic conditions which could initiate the process. However, Saunders (1966) suggests that the hormones (molting hormone, ecdysone, and juvenile hormone) that regulate growth and differentiation may also initiate cell death, and thus recognizes cell death as a form of cellular differentiation. Both hypotheses could be valid in that hormones may cause degeneration in cells genetically programed to respond in this fashion.

The intent of this paper is to investigate cell death and its relationship with molting and juvenile hormones. What is the role of hormones in cell death? It has been observed by numerous investigators that ecdysone stimulates normal differentiation, it may also trigger degeneration in those cells determined to die. In addition, it has been demonstrated that if the juvenile hormone is increased at the time of metamorphosis, all cellular differentiation ceases (Chihara et al, 1972). It could be then the lack of juvenile hormone that triggers cell death. This paper will attempt to establish which of these possibilities is the most likely mechanism of hormonal involvement in the onset of cell death in vestigial wing discs.

METHODS AND MATERIALS

The original stocks from which coisogenic experimental stocks were derived were wild-type Sevelen stock, denoted ($S,+^{vg}/S,+^{vg}$) obtained from D. Bodenstein's laboratory at the University of Virginia, Charlottesville, Virginia, and homozygous vestigial stock, denoted (R,vg/R,vg) with Oregon R background from Carolina Biological Supply, Elon, North Carolina. To insure that the genetic background of the two experimental stock differed only at the vestigial locus, the two original stocks ($S+^{vg}/S+^{vg}$ and R,vg/R,vg) were crossed and the resulting progeny allowed to self mate. The resulting wild-type flies were then crossed for six generations and the vestigial offspring were discarded. The eighth cross consisted of single pair matings between wild-type virgin females and wild-type males. It is from this cross that the experimental coisogenic stocks originated. Refer to Table 1 for an outline for the derivation of these experimental stocks. The coisogenic vestigial and wild-type stocks were maintained on David's medium at 25^0C (David, 1962).

Mature adult flies were selected and isolated from stock cultures in glass bottles for egg collecting. Egg laying trays containing David's medium were inserted into these bottles. After a four to six hour period, eggs were harvested from the trays and washed in a 1 to 1.5% solution of sodium

hypochlorite. The eggs were then transferred to sterile bottles containing David's medium and allowed to hatch and develop at 25°C.

Wing discs were obtained from late third instar larvae. The dissection of the larvae was done in sterile Waddington's saline using sterile iridectomy scissors and forceps. The discs were transferred by oral pipetting to the culture medium. All disc transfers were accomplished by oral pipetting using sterile Pasteur pipettes with a bore of approximately 0.1mm.

Cultures of discs in vitro were made in Schneider's medium (Schneider, 1964), to which 11 ml of fetal bovine serum (Microbiological Associates) per 100 ml of medium had been added. The ph of the medium was adjusted to 6.93±.03 by addition of 1.5 ml of N KOH and the medium then sterilized by passage through GS Millipore filters. In experiments dealing with the influence of hormone upon development, wing discs were cultured in the medium as described previously with the addition of 4.5 µg of beta-ecdysone (Schwarz-Mann 909720, lot T-4384) per milliliter of Schneider's medium, alone, and with 250 mg of CalBiochem synthetic juvenile hormone (SJHA), b grade (CalBiochem 420476, lot 025004) to 25 ml of Schneider's medium. Wild-type and vestigial discs were cultured in plain Schneider's medium as controls, in Schneider's medium plus beta-ecdysone and in Schneider's medium plus beta-ecdysone and juvenile hormone in the concentrations previously stated.

TABLE 1

DERIVATION OF EXPERIMENTAL STOCKS

Cross #1: $S,+^{vg}/S,+^{vg}$ X $R,vg/R,vg$

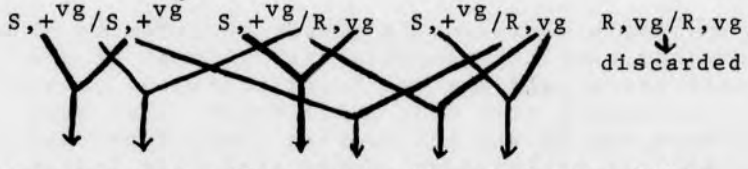
↓

all $S,+^{vg}/R,vg$

↓

Cross #2: Mass mating

↓



Cross #3:

Cross #4: (Crosses 3-7 - Phenotypically wild-type flies were selected each generation and crossed in small groups in normal stock bottles. Vestigial flies were selected against in every generation.)

Cross #5:

Cross #6:

Cross #7:

Cross #8: Single pair matings using virgin females were set-up in shell vials. All flies involved in these crosses were wild-type phenotypically. There were three possible crosses:

a) $S,+^{vg}/S,+^{vg}$ X $S,+^{vg}/S,+^{vg}$

b) $S,+^{vg}/S,+^{vg}$ X $S,+^{vg}/R,vg$

c) $S,+^{vg}/R,vg$ X $S,+^{vg}/R,vg$

After several days of egg deposition, these parents were discarded, and the F-progeny allowed to hatch, emerge and mate randomly with each other, three distinct results were possible in the F_2 generation:

1. The presence of vestigial flies signified that a given generation 8 cross was of the "c" type, and virgin vestigial females were isolated to start the vestigial stock used in the experiments.

2. If the cross was of either type "a" or "b", all progeny would be wild-type in the F_1, but the F_2 would show 1/16 vestigial if the cross at generation 8 was type "b". In this event, stock was discarded.

3. If no flies of vestigial phenotype appeared in the F_2 then it was extremely unlikely that the cross at generation 8 was anything other than type "a". These flies then were placed in continual stock culture for use as the experimental wild-type stock, referred to as "Ore-vg". It is important to note that approximately 100 generations have been produced in this stock by continuous group matings since the original production of the stock, and no vestigial flies have ever appeared.

Discs contained in drops of medium were placed on 24 X 55 mm coverslips treated with Beckman Dessicote. The hanging drops were then suspended over the concavity of a depression slide, ringed with vaseline, and sealed. These *in vitro* slide cultures were incubated in sterile glass petri dishes at 25°C for specific time intervals.

All equipment and instruments used in setting up the experiment *in vitro* were autoclaved prior to the culturing of discs.

In order to investigate the influence of hormones on cell death, it was necessary to devise a method of identifying degenerating cells in the imaginal wing discs of the mutant vestigial and wild-type *Drosophila melanogaster*. This was achieved by utilizing the staining procedure and method of Hammar and Mottet (1971). Cellular degeneration occurring in imaginal discs was demonstrated by means of a staining procedure in which dying or degenerating cells did not stain due to the decreased enzyme activity of succinate dehydrogenase. The substrate, (succinic acid) along with the tetrazolium salt (NBT) were included in the incubation medium. This procedure provides an exact localization of dying cells in the wing discs.

After culture, the discs were transferred to an incubation medium containing the vital stain nitro blue tetrazolium (NBT), and incubated for an average of 2 hours,

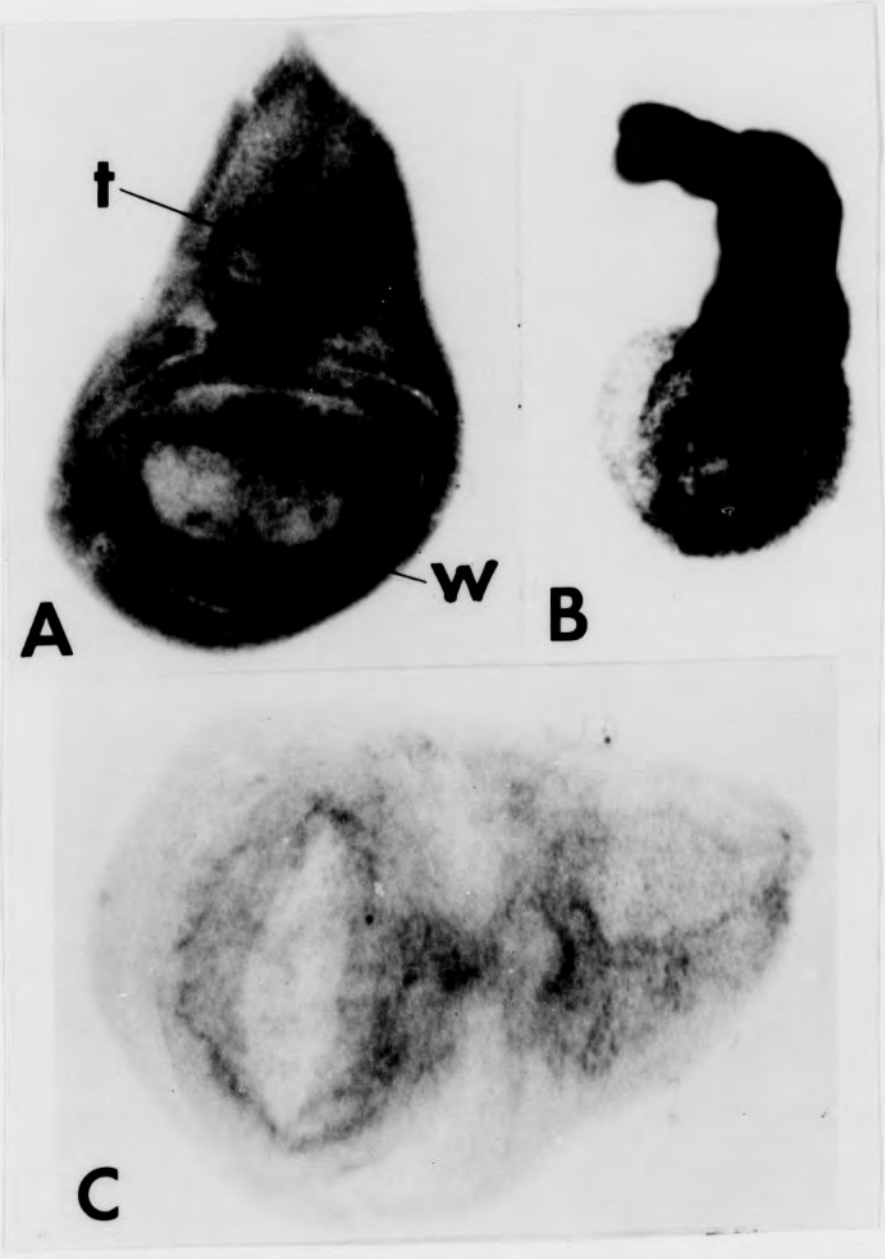
35 minutes. The incubation medium consisted of 1 part NBT (0.5 mg/ml), 1 part 0.2 sodium succinate and 1 part 7.6 pH phosphate buffer (Hammar and Mottet, 1971). The staining procedure and materials used in these experiments followed that of Hammar and Mottet (1971) except for the washes in saline before incubation in stain which were omitted. Following incubation the discs were fixed in neutral buffered formalin for 12 to 16 hours, then dehydrated in 95% ETOH and mounted on permanent slides with Euparal.

RESULTS

Both evaginations and cellular degeneration begin during the late third instar period of development in Drosophila melanogaster. The elevated titre of ecdysone coupled with the reduction in juvenile hormone stimulates evagination, the initial impetus in cellular differentiation. During evagination there is a migration or movement of cells terminated by the outward thrust of the central portion of the disc which ruptures the peripodal membrane. It is important that the degree of evagination be noted for a valid interpretation of these results for evagination is the first visible result of hormonal influence on disc cell differentiation. The discs were graded as to their degree of evagination on a 1 to 4 numerical scale. (Refer to Table 2)

I. Wild-type and vestigial wing discs were removed from late third instar larvae and placed directly into incubation with the NBT stain. Upon extirpation from the larvae, the discs were at various stages of development with evagination already having begun in some anlagen. The average numerical stage of evagination for wild-type discs was 1.9, for vestigial 1.0. Examination of wild-type discs following incubation in the stain revealed an equal distribution of stain in both the wing and thorax area of the discs (Fig. 1).

FIGURE 1



WILD-TYPE IMAGINAL DISCS

a. Wild-type Disc Control. b. Wild-type Disc Cultured In vitro with Ecdysone for Three Days. c. Wild-type Disc Cultured In vitro with Ecdysone and Juvenile Hormone for One Day. t. Presumptive Thoracic Area. w. Presumptive Wing Area.

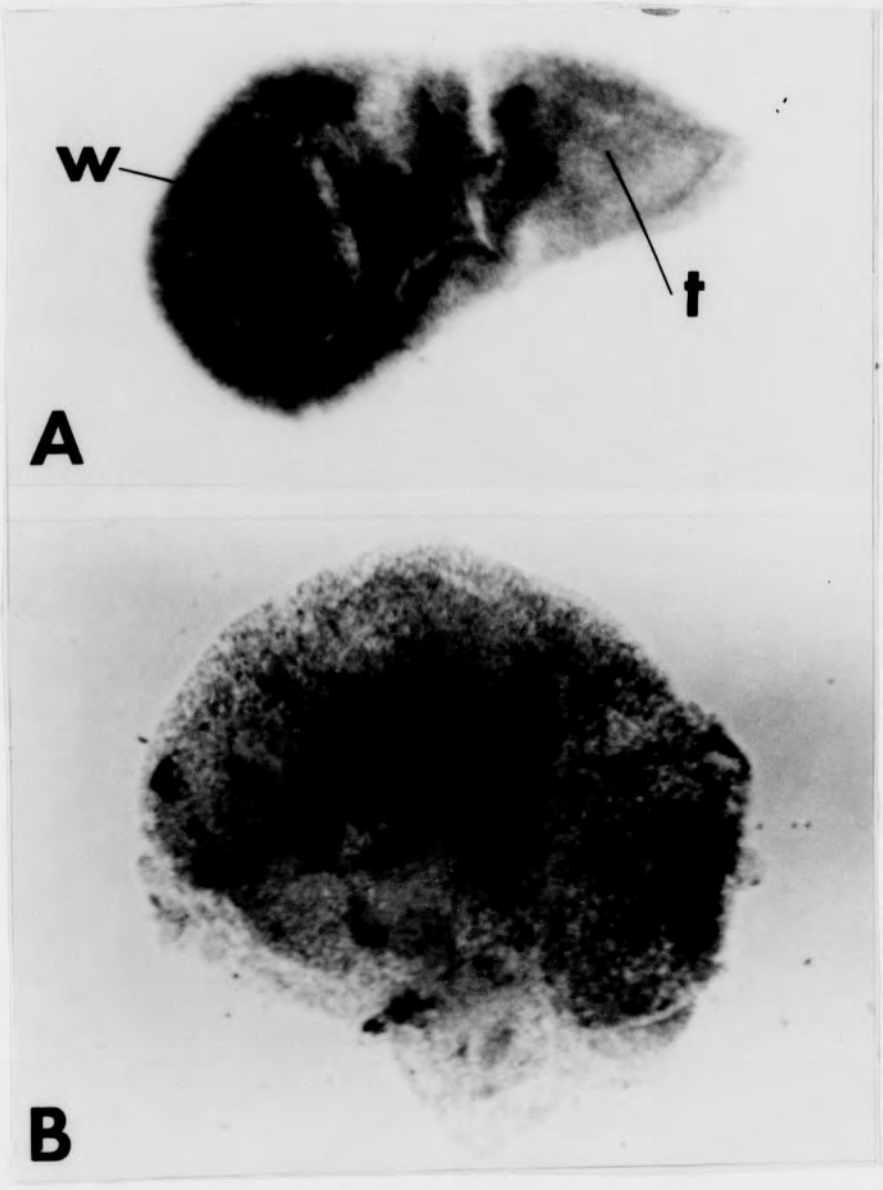
Vestigial discs treated in the same manner demonstrated a mottled distribution of stain. This mottled distribution of stain was peculiar to the vestigial discs. There was no consistant pattern of mottling found in the vestigial discs. Both areas of the discs, wing and thorax, appeared to have taken up the stain. The apparent absence of stain was not limited to either disc region. Patches of cells in both areas, apparently randomly located, stained more and less intensely. Due to the irregularity in mottling, it could not be concluded that cell death had occurred. (Fig. 2).

Wild-type and vestigial discs were removed from third instar larvae and cultured *in vitro* in hormone-free Schneider's medium. All development stopped and the staining results mirrored those recorded for the zero day cultures except for an increase in mottling in the vestigial discs.

II. Third instar wing discs of both wild-type and vestigial discs were cultured in Schneider's medium plus 4.5 µg/ml of beta-ecdysone. The addition of ecdysone to the medium promoted evagination in both disc types. The extent of evagination was greater in the wild-type discs as compared to the vestigial discs. It was noted that the longer the discs remained *in vitro*, the greater the cell loss was from the discs proper.

Examination of stained discs revealed extensive cell death occurring in the vestigial wing discs as evidenced by

FIGURE 2



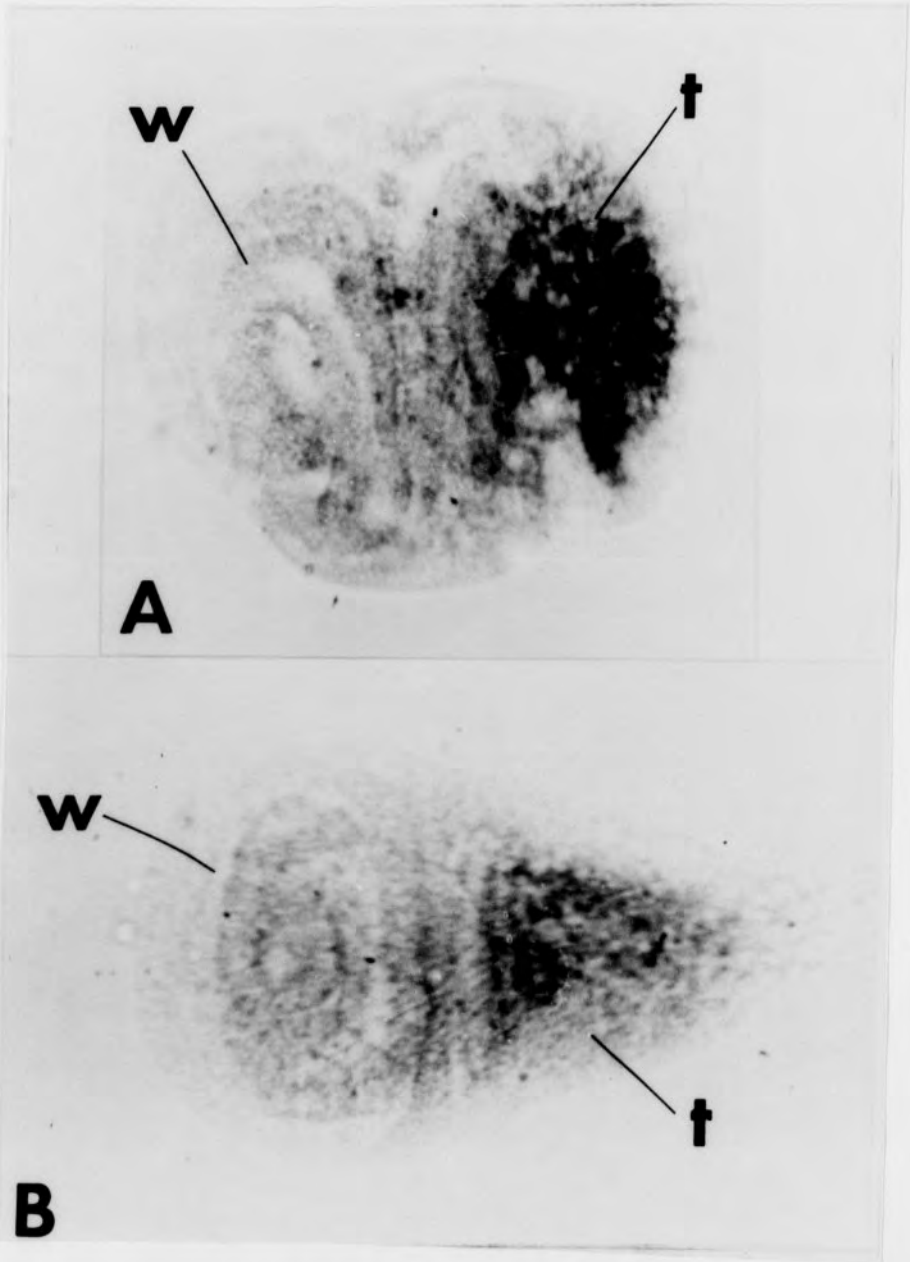
VESTIGIAL WING IMAGINAL DISCS

a. Vestigial Disc Control. b. Vestigial Disc After Culture in Plain Medium for Two Days. t. Presumptive Thoracic Area. w. Presumptive Wing Area.

the clear appearance of cells located in the wing blade portion of the discs (Fig. 3). Wild-type discs appeared uniformly stained throughout both the thorax and wing portion of the disc (Fig. 1).

III. In vitro cultures were set-up demonstrating the effect of both ecdysone and juvenile hormone on the evaginating discs. Again the discs were taken from late third instar larvae and placed in culture. Evagination had begun in some of the discs previous to culturing. The addition of the juvenile hormone halted any further evagination of the discs but did not interfere with cellular death in the vestigial wing discs. Cells that had been programed to die did so, and staining reactions demonstrated this in the majority of vestigial discs (Fig. 3). Wild-type discs showed equal staining in the wing and thorax portion of the discs but, as in vestigial discs, evagination stopped, presumably due to the influence of the juvenile hormone (Fig. 1).

FIGURE 3



VESTIGIAL WING IMAGINAL DISCS

a. Vestigial Disc After Two Days Culture with Ecdysone.
b. Vestigial Disc After Two Days Culture with Ecdysone Plus Juvenile Hormone. t. Presumptive Thoracic Area. w. Presumptive Wing Area.

TABLE 2

CULTURE RESULTS

	Medium Treatment	Number of Discs	Average Degree of Evagination	Stain Distribution T+W+	Stain Distribution T+W-	Time In Vitro
Orvg Disc Culture	Plain (no hormone)	12	1.9	12	0	0
	Plain	1	3*	1	0	3 days
	Ecdysone	11	2.8	11	0	5.5 days
	"	3	3	3	0	1 day
	"	6	4	6	0	2 days
	"	3	3.3	3	0	5 days
	Ecdy.+J.H.	12	2.5*	12	0	1 day
vg Disc Culture	Plain	10	1	10	0	0
	"	4	1	14	0	2 days
	"	7	1.3	7	0	3 days
	Ecdysone	12	1.8	0	12	2 days
	"	7	2.0	3	4	3 days
	Ecdy.+J.H.	15	1.1	5	10	2 days
	"	6	1.3	0	6	3 days

*These discs were at this stage of evagination when put into culture

DISCUSSION

Over a century ago the ring gland, which is now considered the center of hormone production in dipteran larvae, was described by Weisman. It was not until seventy-three years later that the ring gland's function in hormone production was substantiated by the work of Hadorn (1937) and Hadorn and Neel (1938). It was found that there were two primary hormones secreted by the ring gland; ecdysone, commonly referred to as the molting hormone, and the juvenile hormone. Each hormone was found to be secreted by specific cells located in the ring gland; lateral gland cells producing ecdysone and medial gland cells, the juvenile hormone.

The exact time at which the ring gland begins its secretory function has not been determined (Bodenstein, 1950). Bodenstein (1947) has reported that the ring gland loses its ability to promote "growth" in larval discs two days after pupation. It is at this time that the ring gland largely degenerates. Cell degeneration and death in the wing imaginal discs of Drosophila melanogaster, the vestigial mutant, are observed in vivo during the late third instar and early pupal period of development when the ring gland is still actively secreting ecdysone (Fristrom, 1969). At this time the ring gland is no longer actively producing the juvenile hormone (Oberlander and Fulco, 1967).

Both the molting and juvenile hormones are present throughout most of the period of larval development. The juvenile hormone maintains the insect in the larval stage, controlling the larval molts (Wigglesworth, 1964), while ecdysone or the molting hormone provides the "sustained stimulus" for metamorphosis and differentiation of the larva into adult tissues and organs (Oberlander and Fulco, 1961; Oberlander, 1969). The juvenile hormone serves as the qualitative controlling influence throughout development and only in its absence does the larva pupate.

The juvenile hormone titre is relatively high during the larval molts and early instar development, though low during the last instar, and negligible at evagination and pupation (Oberlander and Fulco, 1967; Gilbert and Schneiderman, 1961). At the time of metamorphosis, the ecdysone titre increases , causing the larva to pupate in the absence of the juvenile hormone.

The varying hormone titres in dipteran larvae have not been successfully analyzed. No consistently reliable assays have been developed for dipteran hormones (Chihara et al, 1972). Previous statements referring to hormone titres are based on results obtained through hormone studies of other insects demonstrating complete metamorphosis.

At present, three analogues of the molting hormone ecdysone have been isolated from insects other than diptera,

alpha-ecdysone (Butenandt and Karlson, 1954; Hupper and Hoppe, 1965), beta-ecdysone (Karlson, 1956; Kaplanis et al, 1966; Hoffmeister and Grutzmacher, 1966; Horn et al, 1966; Hampshire and Horn, 1966), and 20,26 dihydroxyecdysone (Thompson et al, 1967). A plant hormone, inokosterone, has also been shown to induce evagination in Drosophila (Mandaron, 1973).

There seems to be some controversy as to which hormone analogue or combination of analogues causes evagination in insect discs. Mandaron (1973) has reported that complete evagination of discs in vitro followed by differentiation can only occur in the presence of alpha-ecdysone. Chihara et al, (1972) reported achieving complete evagination of discs in vitro in the presence of beta-ecdysone which they discerned to be two hundred times stronger in activity than alpha-ecdysone and believed it to be the hormone analogue acting in situ. Mandaron (1973) contends that in the presence of beta-ecdysone evagination is abnormal and incomplete. In vivo investigations into the influence of ecdysone analogues upon disc evagination in Chironomus have demonstrated that both alpha and beta analogues were necessary for complete evagination and differentiation (Clever et al, 1973). Postlethwait and Schneiderman (1970) utilizing cultures in vivo, in which only beta-ecdysone was added, reported complete evagination of Drosophila imaginal discs.

Although there is some debate as to which hormone analogue or analogues mirrors the molting hormone *in situ*, it is generally accepted that ecdysone induces and stimulates evagination and cellular differentiation in *Drosophila melanogaster*. Mandaron (1971) showed by cultures *in vitro* of imaginal discs that such extrinsic factors as a rise in hemolymph pressure, a stimulus from mesenchymal cells, or the direct contact between larval hypoderm and imaginal discs, do not stimulate evagination. Oberlander (1969) demonstrated that ecdysone does not simply trigger evagination, but acts as a "sustaining stimulus" throughout the process. These observations are substantiated further by the results presented in this paper.

Beta-ecdysone was selected as the hormone analogue to be used in the experiments conducted here, mainly because of its availability. A possible detrimental side effect of the beta-ecdysone was noted in imaginal discs remaining *in vitro* with the hormone analogue for a period of more than three days. There was a cessation of evagination and an increase in cell loss from the disc proper. It appeared that possibly the hormone or a hormone by-product could have affected the "cellular cement" of the disc causing this cell loss.

Beta-ecdysone induced evagination in both wild-type and vestigial wing discs. It was also found that ecdysone was required for the continuence of evagination. Discs which had already begun evagination before culturing, stopped evaginating

when placed *in vitro* in a hormone free medium. The degree of evagination varied between the two phenotypes examined. Vestigial discs did not evaginate to the same extent as the wild-type discs. The rate of evagination appeared slower in the vestigial discs. This was possibly due to the slower metabolic rate of the vestigial disc cells. Villee (1946), basing his results on comparative oxygen consumption of vestigial and wild-type discs noted that the metabolic rate of vestigial was half that of wild-type.

Complete evagination and differentiation into adult tissue was not achieved with beta-ecdysone. The extent of evagination in the examined discs (cultured with beta-ecdysone) was sufficient to yield valid results as to the effect of the insect molting hormone on cell death. Cell death occurred in the majority of vestigial discs cultured with ecdysone. Degeneration was not observed in the vestigial wing discs cultured without ecdysone. These results in themselves point to the import of ecdysone in the process of cellular degeneration and suggest that cell death is under hormonal control. Ecdysone seems to stimulate cellular degeneration in those cells determined to die.

Ecdysone stimulates disc evagination and normal cell differentiation through activation of specific genes (Chihara et al, 1972). It is supposed that ecdysone also activates the vestigial gene controlling cellular degeneration.

Villee (1946) has suggested that the mutant gene vestigial produces its effect by altering the rate of some chemical reaction within certain cell disc cells. Cell death could therefore occur due to the interaction of the gene or gene product with a specific cell component. It has been shown in wild-type discs that the addition of ecdysone activates genes and RNA synthesis. This has been substantiated by observation of the puff patterns in polytene chromosomes of Drosophila (Clever, 1966). The exact gene product which interacts with a specific cellular component to cause cellular degeneration is not known. It is thought that the vestigial gene produces its effect by affecting the chemical nature or quantity of a particular enzyme (Villee, 1946).

Since both evagination and cellular degeneration seem induced by the molting hormone, are both processes dependent upon each other? It has been demonstrated that if juvenile hormone is added at the time of evagination, all development ceases. The addition of juvenile hormone (SJHA) inhibits the incorporation of amino acids and nucleosides into macromolecules by blocking their transport into the discs (Fristrom et al, 1969). This may be the mechanism by which evagination is stopped and the action of ecdysone deterred. If evagination and cell death are dependent upon each other then the addition of juvenile hormone at the time of evagination should prevent cellular degeneration and death. If the cell

death is not prevented by the addition of juvenile hormone then it can be assumed that evagination and cellular degeneration occur indepentently of each other, although both are stimulated by ecdysone.

Like the molting hormone, the juvenile hormone has never been isolated from Drosophila or other diptera. It was first isolated in an impure form in the 1950's from the Cecropia moth. A synthetic analogue was developed in 1966 by Law and coworkers. The ability of this "crude synthetic analogue mixture" (SJHA) to inhibit evagination has been observed (Law et al, 1966; Fristrom et al, 1969; Chihara et al, 1972). Chihara reported that the mode of action in vitro of the synthetic hormone differs from the Cercropia hormone isolate. She also questions whether the mode of action of either analogue in vitro mirrors the natural hormonal influence in situ.

Assay of juvenile hormones in vitro has been difficult due to the apparent breakdown and chemical conversions of the hormone by disc cells. In vitro cultures with ecdysone analogues have revealed little breakdown or metabolism of the molting hormone (Chihara, 1972). It has been reported that there is a breakdown and conversion of ecdysone analogues in vivo (Clever et al, 1973).

In a comparison of the various analogues of juvenile hormone, Chihara et al, (1972) found CalBiochem SJHA to ex-

hibit 10% the activity of natural Cecropia hormone. This was based on the effect of the synthetic versus the natural hormone on beta-ecdysone induced evagination. CalBiochem reports that their synthetic hormone demonstrates only 1% the activity of natural Cecropia hormone. In planning the experiments reported on in this paper, the concentration of SJHA used was based on the manufacturer's report of 1% natural activity in order to guarantee adequate concentration of the hormone to be effective, especially since it is a suspension rather than a solution (Chihara et al, 1972; CalBiochem Technical Information).

Discs were cultured with ecdysone and juvenile hormone for one, two, and three day periods. The discs failed to evaginate in vitro when the concentration of juvenile hormone was 10mg/ml and the concentration of ecdysone was 4.5 µg/ml. Without the addition of juvenile hormone the wing discs, both vestigial and wild-type cultured in vitro with ecdysone, began and continued to evaginate. In discs cultured with both ecdysone and juvenile hormone all evagination stopped. These results coincided with past research indicating that the juvenile hormone inhibits evagination. These results also seem to indicate that there was little breakdown or conversion of the juvenile hormone in vitro. If the discs had resumed evagination after a period of time in vitro, metabolism and conversion of the synthetic juvenile hormone would have been suspected.

Cell death was observed as occurring in vestigial discs cultured *in vitro* with both the juvenile hormone and ecdysone. Although evagination was prevented, cell death was not hindered in the presence of the juvenile hormone. Therefore, cell death and evagination are both stimulated by ecdysone but occur independently of each other. They are not inhibited by the same cellular mechanism.

That cell death occurred without evagination in the vestigial wing disc may indicate that we are dealing with two different types of predetermined cellular differentiation. Normal differentiation, i.e. adult wing hair formation, requires the cellular migration of disc cells which occurs during evagination for its completion during pupation. Cell death differentiation does not require this cellular movement since it occurs with or without evagination. Therefore, one might conclude that there are two types of differentiation in discs where cellular degeneration occurs; normal differentiation requiring evagination and death differentiation which does not require cell reorganization.

There were two staining irregularities that consistantly appeared throughout the results. In both wild-type and vestigial cultured wing discs, it was noted that some cells along the outer edge of the distal portion of the wing disc did not appear to take up the stain. These cells, according to the fate map proposed by Murphy (1972),(Fig. 3), are com-

pletely unassociated with wing development and give rise rather to a lateral portion of the adult fly thorax. It is possible that these cells did stain very lightly but due to the transparency of the disc cells in this area, the stain was not detected. The lateral part of the adult fly thorax of the experimental stocks used does not appear malformed. If the thorax were deformed, this would be indicative of possible cell death having occurred in the distal part of the disc. Instead, it seems likely that this absence of stain could be an irregularity in stain diffusion or more simply a problem in optics due to the cellular transparency in this particular distal area.

Another staining irregularity was observed only in the vestigial wing discs. The term chosen to describe this irregularity was the "mottled" effect. Small areas within the vestigial wing disc seemed to stain more intensely than others when the discs were cultured without either hormone. The intensity distribution was irregular and not observed in any specific area exclusively. There was no defined pattern of mottling in any disc; no specific area or cell group consistently appeared more darkly stained than another. Due to the irregularity of this staining pattern, it was concluded that the "mottled" effect was not caused by cellular degeneration. This "mottled" effect was observed in both the wing and thorax area of the vestigial disc. Mottling appeared to

be a phenotypic characteristic peculiar to vestigial discs, enabling a distinction to be made between the stained appearance of wild-type and vestigial wing discs. This failure to demonstrate an even distribution of stain could have been caused by an inability of the stain to diffuse throughout the discs due to varying cellular affinities. It could also have been caused by the slower metabolic rate of the vestigial disc cells. A comparison between vestigial discs treated with ecdysone and vestigial control discs in plain medium demonstrated clearly the difference between mottling and cellular death. Cells in the wing portion of the discs treated with ecdysone demonstrated a complete lack of stain.

The results of this study concerning cell death suggest the following conclusions:

1. The onset of cell death in the wing areas of vestigial wing discs is stimulated by the molting hormone, ecdysone.

2. Both cell death and evagination are induced by ecdysone but occur independently of each other.

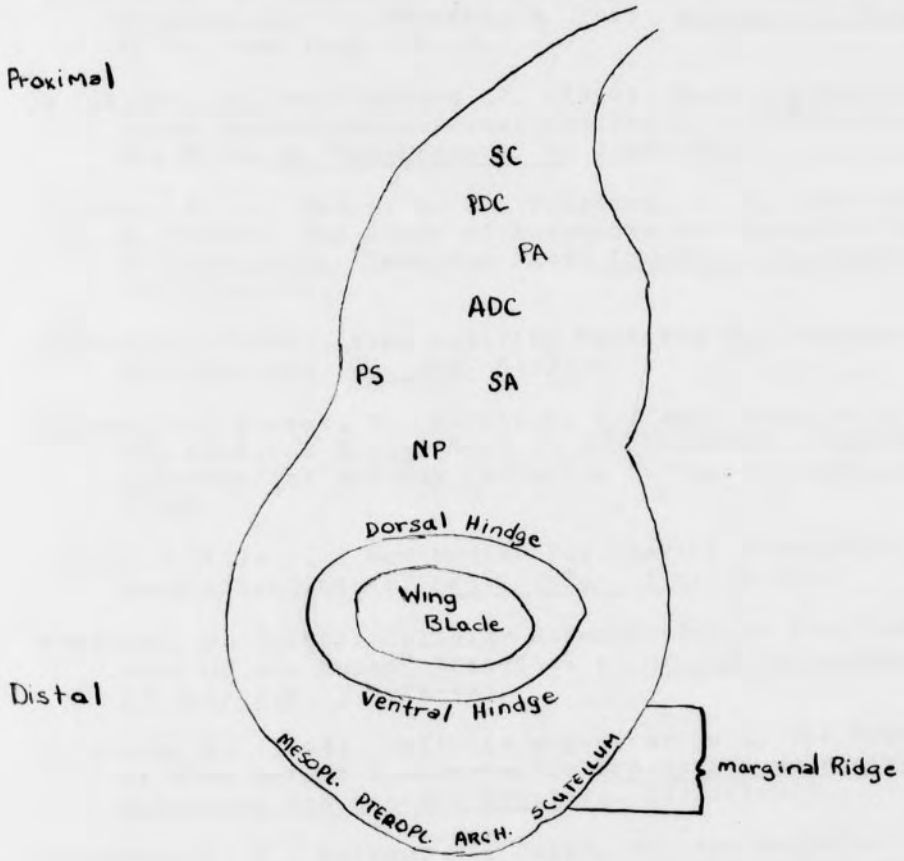
3. Cell death is not inhibited by the presence of, or permitted by the absence of the juvenile hormone.

4. There are, therefore, two types of differentiation occurring in vestigial discs: normal differentiation requiring evagination and cellular reorganization and cell death differentiation which does not require cellular reorganization to occur.

FIGURE 4

"TENTATIVE FATE MAP OF ANLAGEN OF THE MATURE DORSAL MESOTHORACIC DISC"

Murphy (1972)



Macrochaetal Abbreviation:

SC - scutellars
PDC - posterior dorsocentrals
PA - postalars
ADC - anterior dorsocentrals
SA - supraalars
PS - presutural
NP - notopleurals

BIBLIOGRAPHY

Bodenstein, D. (1947). Investigation on the Reproductive System of Drosophila. J. Exp. Zoo. 140:106-152.

Bodenstein, D. (1950). The Postembryonic Development of Drosophila. In: Demerec, M. (ed). Biology of Drosophila. Wiley, New York. Ch. 7.

Butenandt, A., and Karlson, P. (1954). Uber die Isolierung eines Metamorphose-Hormas der Insekten in Kristallisierter Form. Z. Naturforsch. B. 9:389-391.

Chihara, C. J., Petri, W. H., Fristrom, J. W., and King, D. S. (1972). The Assay of Ecdysones and Juvenile Hormones on Drosophila Imaginal Discs In vitro. J. Insect Phy. 18:1115-1123.

Clever, U. (1966). Gene Activity Patterns and Cellular Differentiation. Am. Zoo. 6:33-41.

Clever, U., Clever, I., Storbeck, I., and Young, N. L. (1973). The Apparent Requirement of Two Hormones, α and β Ecdysone for Molting Induction in Insects. Dev. Bio. 31: 47-60.

David, J. (1962). A New Medium for Rearing Drosophila in Axenic Conditions. Dros. Infor. Svc. 36:128.

Fristrom, D. (1968). Cellular Degeneration in Wing Development of the Mutant Vestigial of Drosophila melanogaster. J. Cell Bio. 39:488-491.

Fristrom, D. (1969). Cellular Degeneration in the Production of Some Mutant Phenotypes in Drosophila melanogaster. Molecular and General Genetics. 104:363-379.

Fristrom, J. W., Raikow, R., Petri, W., and Stewart, D. (1969). In vitro Evagination and RNA Synthesis in Imaginal Discs of Drosophila melanogaster. Problems in Biology: RNA in Development. E. W. Hanley, University of Utah Press. 381-401.

Gilbert, L. I., and Schneiderman, J. (1961). The Content of Juvenile Hormone and Lipid in Lepidoptera: Sexual Differences and Developmental Changes. Gen. Comp. Endocrin. 1:453-472.

Hadorn, E. (1937). An Accelerating Effect of Normal "Ring Glands" on Puparium Formation in Lethal Larvae of Drosophila melanogaster. Proc. Nat. Acad. Sci. 23:478-484.

Hadorn, E., and Neel, J. V. (1938). Der Hormonale Einfluss der Ringdruse auf die Puparіumbildung bei Fliegen. Wilhelm Roux Arch, Entwickl. Mech. Org. 138:281-304.

Hammar, S. P., and Mottet, N. K. (1971). Tetrazolium Salt and Electron-Microscopic Studies of Cellular Degeneration and Necrosis in the Interdigital Areas of the Developing Chick Limb. J. Cell Sci. 8:229-251.

Hampshire, F., and Horn, D. H. S. (1966). Structure of Crustecdysone, a Crustacean Moulting Hormone. Chem. Commun. 2:37-38.

Hoffmeister, H., and Grutzmacher, H. F. (1966). Zur Chemie des Ecdysterons. Tetrahedron Lett. 33:4017-4023.

Horn, D. H. S., Middleton, E. J., Wunderlich, J. A. and Hampshire, F. (1966). Identity of the Moulting Hormones of Insects and Crustaceans. Chem. Commun. 11:339-341.

Huber, R., and Hoppe, W. (1965). Zur Chemie des Ecdysons. VII. Die Kristal-und Molekulstrukturanalyse des Insektenverpuppungshormons Ecdyson mit der Automatisierten Faltmolekulmethode. Chem. Ber. 98:2403-2424.

Kaplanis, J. N., Thompson, M. J., Yamamotto, R. T., Robbins, W. E., and Louloudes, S. J. (1966). Ecdysones from the Pupa of the Tobacco Hornworm, Manduca Sexta. Steriods. 8:605-623.

Karlson, P. (1956). Biochemical Studies on Insect Hormones. Vitam. Horm. (New York). 14:228-266.

Law, J. H., Yuan, C., and Williams, C. M. (1966). Synthesis of a Material with High Juvenile Hormone Activity. Proc. Nat. Acad. Sci. 55:576-578.

Lindsley, and Grell. (1967). Drosophila Information.

Mandaron, P. (1971). Sur le mecanisme de L'evagination de disques imaginaux chex la Drosophile. Dev. Bio. 25: 581-605.

Mandaron, P. (1973). Effects of α ecdysone, β ecdysone and Inokosterone on the in vitro Evagination of Drosophila Leg Discs and the Subsequent Differentiation of Imaginal Integumentary Structures. Dev. Bio. 31:101-113.

Murphy, C. (1972). Localization of Primordia within the Dorsal Mesothoracic Disc of Drosophila. J. Exp. Zoo. 179:51-62.

Oberlander, H. (1969). Effects of Ecdysone, Ecdysterone, and Inokosterone on the in vitro Initiation of Metamorphosis of Wing Discs of Galleria mellonella. J. Insect. Phy. 15:297-304.

Oberlander, H., and Fulco, L. (1967). Growth and Partial Metamorphosis of Imaginal Discs of the Greater Wax Moth, Galleria Mellonella in vitro. Nature. 216:1140-1141.

Postlethwait, J. H., and Schneiderman, H. A. (1970). Induction of Metamorphosis by Ecdysone Analogues: Drosophila Imaginal Discs cultured in vivo. Bio. Bul. 138:47-55.

Saunders, John W. (1966). Death in Embryonic Systems. Science. 154:604-612.

Schneider, I. (1964). Differentiation of Larval Drosophila Eye Antennal Discs in vitro. J. Exp. Zoo. 156:91-104.

Thompson, M. J., Laplanis, J. N., Robbins, W. E., and Yamamoto, R. T. (1967). 20-36-Dihydroxecdysone, A New Steriod with Moulting Hormone Activity from the Tobacco Hornworm, Manduca sexta. Chem. Commun. 13:650-653.

Villee, Claude A. (1946). The Genetic Control of Growth Metabolism. Proc. Nat. Acad. Sci. 32:241-245.

Wigglesworth, V. B. (1964). The Hormonal Regulation of Growth and Reproduction in Insects. Advances in Insect Physiology. Academic Press. New York. 248-336.