INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

HORMONAL REGULATION OF PROGRAMMED CELL DEATH IN THE LABIAL GLAND OF THE TOBACCO HORNWORM, *Manduca sexta*

by

REGINALD HALABY

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1997

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

UMI Number: 9732921

Copyright 1997 by
Halaby, Reginald

All rights reserved.

UMI Microform 9732921
Copyright 1997, by UMI Company. All rights reserved.

This microform edition is protected against unauthorized
copying under Title 17, United States Code.

UMI
300 North Zeeb Road
Ann Arbor, MI 48103

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

© 1997

REGINALD HALABY

All Rights Reserved

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

4/29/97
Date

Chair of Examining Committee
Dr. Zahra Zakeri, Queens College

4/30/97
Date

Executive Officer, Dr. Richard L. Chappell

Dr. Jeanne Szalay, Queens College

Dr. Harold Magazine, Queens College

Dr. Richard Lockshin, St. John's University

Dr. Christopher Bazinet, St. John's University

Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Abstract

HORMONAL REGULATION OF PROGRAMMED CELL DEATH IN THE LABIAL GLAND OF THE TOBACCO HORNWORM, *Manduca sexta*

by

Reginald Halaby

Adviser: Dr. Zahra Zakeri

The labial glands of the tobacco hornworm, *Manduca sexta*, is a good model system for studying programmed cell death (PCD). The lysosomal death of the glands, which occurs during the larval to pupal metamorphosis, is predictable, precise, controllable, and nearly synchronous. Cytoplasmic alterations, such as activation of lysosomes, are among the earliest detectable morphological features in dying glands, while nuclear changes are late. We used the following parameters to monitor cell death: morphology by light and electron microscopy; lysosomal enzyme activity; DNA fragmentation; macromolecular synthesis; and energetics. Studies on metabolic parameters of the gland cells during involution revealed that the late modifications in the levels of energy resources and signaling molecules were not likely to trigger PCD. Since the bimodal secretion of 20-hydroxyecdysone (20-HE) coincides with morphologic alterations in dying gland cells and the fact that 20-HE had been by others to induce cell other tissues, we investigated the hormone's role in labial gland PCD. We developed an *in vitro* system to more precisely define

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

the mechanisms that regulate gland cell death. Our *in vitro* experiments demonstrated that glands can be maintained in culture, but 20-HE alone did not cause gland cells to die. Since the internal environment of the animal may be important for 20-HE's regulation of gland PCD, we performed a series of *in vivo* experiments with glands from intact and ligated animals. 20-HE *in vivo* induced impaired mitochondrial respiration, movement and activation of lysosomes, and DNA fragmentation in glands. A single exposure of glands to 20-HE, rather than sustained exposure, was sufficient to induce cell death. Cells from treated glands displayed more shrinkage compared to controls.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Zahra Zakeri, for sharing with me her compassion for practicing good science, her enthusiasm for scientific discovery, and for preparing me for a career as an independent researcher. Dr. Zakeri is also a strong advocate for graduate students.

I am grateful to Dr. Richard Lockshin for sharing his expertise of insect physiology and his sense of humor with me.

I am thankful to the other members of my doctoral thesis committee: Dr. David Smouse, Dr. Harold Magazine, Dr. Christopher Bazinet, and Dr. Jeanne Szalay. Their helpful discussions and time were well appreciated.

I thank my colleagues: Terri Latham, Dr. Harleen Singh Ahuja, Dr. Nicos Karasavvas, Komal Srivastava, Terri Kagan, Yong Zhu, Sara Danzi, Igor Medintz, Rubina Saeed, and Jon King. They helped to create a friendly and cooperative environment.

I wish to thank the Biology Department of Queens College for their support.

I wish to express my sincere thanks to the CUNY MAGNET Program for awarding me the four year predoctoral and dissertation year fellowships. These awards allowed me the opportunity to focus all of my efforts towards completing my research. Thanks to Dr. Pamela Reid and Mr. Matt Schoengood for their participation with the MAGNET Program and their advice.

I am grateful to Mrs. Joan Reid for her helpful guidance and advice.

I am especially thankful to my entire family. In particular, my loving and supportive wife, Claudette, for believing in me and for inspiring me. Thanks to our daughter, Olivia, for her genuine interest in science. I am very grateful to the following people for all their love, encouragement, and the many sacrifices that they have made on my behalf: my grandmother, Yvonne Delva, and my parents, Jean and Myrtha Halaby.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

TABLE OF CONTENTS

PAGE

I. INTRODUCTION -

1. CONCEPTS OF CELL DEATH 2

A. Physiological Cell Death 3
B. Types Of Cell Death 4
C. Cell Death During Metamorphosis 6
D. Labial Gland Of *Manduca* & Cell Death 7
E. Genetic Regulation Of Cell Death 8

2. STEROID HORMONES & CELL DEATH 12

A. Hormonal Regulation Of Cell Death 13
B. Mechanisms Of Steroid Hormone Action 15
C. 20-Hydroxyecdysone Regulation Of Gene Expression 19
D. 20-HE & Metamorphosis In *Manduca* 22

II. RESULTS AND DISCUSSION 24

CHAPTER I. METABOLIC EVENTS DURING PROGRAMMED CELL IN LABIAL GLANDS FROM PUPATING ANIMALS 25

A. Introduction 26
B. Materials And Methods 28
C. Results 36
1. Morphology Of Cell Death In Labial Glands 36
2. Energy Resources Remain Constant During The Early Phase Of Degeneration 36
3. Mitochondrial Functioning Is Intact Until Late 37
4. Localization Of Lysosomes 37
5. Changes In The Levels Of Second Messengers Are Late 38
D. Discussion: Role Of Altered Metabolism In The Death Of Labial Glands 39

CHAPTER II. DEVELOPMENT OF LABIAL GLAND *IN VITRO* SYSTEM 42

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

A. Introduction 43
B. Materials And Methods 44
C. Results 49
1. The Labial Glands Can Be Maintained In Culture 49
2. Effect Of 20-HE On Lysosomal Activity In Cultured Glands 51
D. Discussion: 20-HE Does Not Appear To Directly Trigger Gland Cell Death 53

CHAPTER III. THE RELATIONSHIP BETWEEN 20-HE *IN VIVO* AND GLAND CELL DEATH 57

A. Introduction 58
B. Materials And Methods 61
C. Results 64
1. Effect Of 20-HE On Premetamorphic Glands 64
2. Effect Of 20-HE On Metamorphic Glands 66
3. 20-HE Appears To Trigger Gland PCD 69
D. Discussion: 20-HE Triggers But Does Not Sustain Programmed Cell Death Of Labial Glands 71

CHAPTER IV. POSSIBLE MECHANISMS BY WHICH 20-HE REGULATES GLAND CELL DEATH 77

A. Introduction 78
B. Materials And Methods 79
C. Results 87
1. EcR Expression & Localization 87
2. Microtubules In Degenerating Glands 89
D. Discussion: The 20-HE:EcR Complex May Induce Cell Death In Glands By Acting On The Cytoskeleton 90

CONCLUSIONS 94

III. FIGURES AND FIGURE LEGENDS 96

IV. REFERENCES 129

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

LISTS OF ILLUSTRATIONS

PAGE

Figure 1. Electron Micrographs Of The Gland During Metamorphosis 97

Figure 2. Energy Resources In Glands During Metamorphosis 98

Figure 3. MTT Staining Of Glands During Metamorphosis 99

Figure 4. Histochemical Localization Of Acid Phosphatase In Labial Glands During Metamorphosis 100

Figure 5. Acid Phosphatase Activity In Labial Glands During Metamorphosis 101

Figure 6. Levels Of Second Messengers In Glands During Metamorphosis 102

Figure 7. Histochemical Appearance Of Cultured Labial Glands Stained With MTT 103

Figure 8. Effect Of 20-HE On Protein Synthesis 104

Figure 9. Effect Of Various 20-HE Doses On Gland Protein Synthesis 105

Figure 10. AP Activity In Day -2 Glands Cultured With 2 X 10^{-7} M 20-HE 106

Figure 11. AP Activity In Day -2 Glands Cultured With 3 X 10^{-7} M 20-HE 107

Figure 12. Localization Of Lysosomes In Cultured Metamorphic Glands 108

Figure 13. Effect Of *In Vitro* 20-HE On Metamorphic Glands 109

Figure 14. GalA Activity In Glands During Metamorphosis 110

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 15. GalA Activity In Cultured Glands 111

Figure 16. Effect Of *In Vivo* 20-HE On AP Activity In Premetamorphic Glands 112

Figure 17. Histochemical Appearance Of AP In Untreated Glands From Isolated Abdomens 113

Figure 18. AP Activity In Untreated Glands From Isolated Abdomens 114

Figure 19. Effect Of 20-HE On AP Activity In Glands From Isolated Abdomens 115

Figure 20. Localization Of Lysosomes In Glands That Were Treated *In Vivo* With Or Without 20-HE 116

Figure 21. Effect Of *In Vivo* 20-HE & CHX On AP Activity 117

Figure 22. MTT Reduction In Glands From Isolated Abdomens 118

Figure 23. DNA Fragmentation: Multiple Injections 119

Figure 24. Effect Of A Single Pulse Of *In Vivo* 20-HE On Lysosomes 120

Figure 25. Effect Of Continuous *In Vivo* 20-HE On Lysosomes 121

Figure 26. Evaluation Of Single & Multiple Injections Of 20-HE By DNA Fragmentation & Morphology 122

Figure 27. DNA Fragmentation During Metamorphosis 123

Figure 28. Northern Blot Analysis Of EcR mRNA During Metamorphosis 124

Figure 29. Western Blot Analysis Of EcR During Metamorphosis 125

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 30. Immunocytochemistry Of EcR 126

Figure 31. Examination Of Microtubules In Glands 127

Figure 32. The Effect Of 20-HE On The Cytoskeleton 128

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

PART I. INTRODUCTION

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1. CONCEPTS OF CELL DEATH

A. Physiological Cell Death
    1. Cell Death During Development
    2. Cell Death & Disease
B. Types Of Cell Death
    1. Biochemical & Morphological Characterization Of Cell Death
C. Cell Death During Metamorphosis
    1. Cell Death In *Xenopus*
    2. Cell Death In Insects
D. Labial Gland Of *Manduca* & Cell Death
    1. Programmed Cell Death Of Labial Gland
E. Genetic Regulation Of Cell Death
    1. Cell Death Genes In *Caenorhabditis elegans*
    2. Cysteine Proteases & Cell Death
    3. Inhibitors Of Cell Death
    4. Cell Death Genes In *Drosophila*

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1. CONCEPTS OF CELL DEATH

A. PHYSIOLOGICAL CELL DEATH

1. Cell Death During Development

Cell death is a major aspect of development and homeostasis. Cell death contributes to the sculpting of developing structures in vertebrate and invertebrate embryos. Deletion of interdigital webs in developing limbs (Hammar & Mottet, 1971), development of the fetal intestinal mucosa (Harmon et al., 1984), and retinal development (Penfold & Provis, 1986) all involve cell death. Cell death serves as a major mechanism for the regulation of cell numbers. For example, in the visual system of developing vertebrates, cell death preferentially eliminates neurons that form improper connections (Cowan et al., 1984). In the mammalian embryonic central nervous system, over 1/3 of newly formed cells die (Oppenheim et al., 1982) and during development of *Caenorhabditis elegans* 131 of the 1090 somatic cells die (Ellis & Horvitz, 1986). Some cells seem to die because they have no apparent function, such as the Mullerian duct in male embryos (Price et al., 1977). Cell death can serve as a defense mechanism to remove unwanted and potentially dangerous cells, such as self-reactive lymphocytes (Smith et al., 1989; Schwartz, 1989).

2. Cell Death & Disease

The suppression of cell death increases the susceptibility of an individual to malignancy whereas uncontrolled cell death is associated with degenerative diseases. These include acquired immunodeficiency syndrome (AIDS; Ameison

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

& Capron, 1991; Bonyhadi et al., 1993), cancer (Ling et al., 1993), Huntington's disease (Portera-Caillian et al., 1995), Parkinson's disease (Walkinshaw & Waters, 1995), polycystic kidney disease (Woo, 1995), and Alzheimer's disease (Landfield et al., 1992; Olson, 1993). Many human cancers have mutations or deletions in the tumor suppressor gene, p53 (Levine et al., 1991). The exposure of neurons in Alzheimer's patients to β-amyloid may increase their susceptibility to the cytotoxic effects of excitatory aminoacids (Mattson et al., 1992). Abnormally elevated levels of cell death have been found in the lymph nodes of HIV-infected persons (Muro-Cacho et al., 1995). Acute HIV-1 infection of normal peripheral blood mononuclear cells also induces cell death (Terai et al., 1991). In other neurologic disease, necrotic cell death has been seen in various models of stroke (Linnik et al., 1993; MacManus et al., 1993). It has been shown that certain treatments act by inducing cell death in the affected tissues (Mountz et al., 1994; Macchioni et al., 1994). Indeed a clearer understanding of the regulation of cell death may result in better therapies.

B. TYPES OF CELL DEATH

1. Biochemical & Morphological Characterization Of Cell Death

By morphological criteria, two types of cell death have been described, necrosis and apoptosis. Necrotic cell death is a pathologic form of cell death resulting from acute cellular injury, and is typified by rapid cell swelling and lysis (Wyllie et al., 1980). Apoptotic cell death has features suggestive of self-destruction rather than degeneration seen in necrotic cell death (Trump &

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Berezesky, 1992). Apoptosis is characterized by cell shrinkage, chromatin condensation, systematic DNA cleavage, phagocytosis, and degradation of apoptotic bodies by neighboring cells (Kerr et al., 1972). An important feature of apoptosis is that it results in the elimination of the dying cell without induction of an inflammatory response. Schweichel & Merker (1973) categorized cell death into three biochemically different types. Type I is apoptotic cell death. Type II, lysosomal cell death, is characterized by early lysosomal destruction of the cytoplasm with nuclear degradation being a late event. In type II cell death, a ladder of fragmented DNA may be generated, but only after much of the cytoplasm has already been destroyed (Zakeri et al., 1993). Other laboratories, using pulsed-field gel electrophoresis, have shown that the initial steps seem to be fragmentation of DNA into 300 kb pieces, followed by digestion to sizes of 50-100 kb, before the generation of the ladders (Walker et al., 1993). Type II death is exemplified by the regression of tadpole tail (Weber, 1962), involuting mammary gland (Woessner, 1969), involuting prostate (Sanford et al., 1984), regressing Mullerian duct (Scheib, 1963), and dying insect tissues (Lockshin & Beaulaton, 1974 a,b; Zakeri et al., 1993). Type III, necrotic cell death, is defined by osmotic lysis and occurs in oligodendrocytes that are exposed to nitric oxide (Mitrovic et al., 1995). DNA fragmentation is no longer considered a specific marker for type I death; it can be detected in all three types of cell death (Grasl-Kraupp et al., 1995). Moreover, DNA fragmentation may be the result rather than the cause of cell death (Zakeri et al., 1993).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

C. CELL DEATH DURING METAMORPHOSIS

1. Cell Death In *Xenopus*

Many tissues degenerate during spontaneous metamorphosis in amphibians and insects. There are several tissues in the frog tadpole that undergo substantial or total regression during metamorphosis. The tail and gills undergo total regression (Beckingham Smith & Tata, 1976), while approximately 90% of cells in the small intestine (Ishizuya-Oka & Shimozawa, 1992) and 50% of cells in the nervous system degenerate (Kollros, 1981). PCD of tadpole tail was shown to be accompanied by the activation of lysosomal enzymes (Weber, 1962).

2. Cell Death In Insects

During the course of insect metamorphosis, larval structures degenerate and new adult structures are formed from their remains. In the case of larval muscles, some die during the larval to pupal transition (Weeks & Truman, 1985) and others degenerate after the emergence of the adult (Lockshin & Williams, 1965a,b). Type II cell death of insect tissues is characterized by lysosomal activity, a measure of programmed cell death (PCD), and is exemplified by the following instances: in midgut cells of flies (Radford & Misch, 1971), in *Manduca* intersegmental muscles (ISM; Lockshin & Beaulaton, 1974a), *Drosophila* salivary glands (Lockshin, 1969), and *Galleria* silk glands (Aidells et al., 1971). The delayed degeneration of ISM (Schwartz & Truman, 1983) and neurons (Truman & Schwartz, 1984; Bennett & Truman, 1985; Fahrbach & Truman, 1987) in *Manduca* occurs at the end of adult development.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

D. LABIAL GLAND OF *MANDUCA* & CELL DEATH

1. Programmed Cell Death Of Labial Gland

During the early stages of gland degeneration, levels of energy resources and signaling molecules are adequate. The metabolic collapse of the gland does not occur until day 3 (Halaby et al., 1994). Before metamorphosis, cells of the labial gland display pleiomorphic nuclei, basophilic cytoplasm filled with rough endoplasmic reticulum, and heavily infolded basal membranes (Lockshin & Zakeri, 1994). On the first day of metamorphosis, day 0, we can detect early changes marking the death of these cells. Zakeri et al. (1996) reported that on day 0 there was a sharp decline in total protein synthesis. Translation of isolated poly(A)$^+$ RNA indicates that loss of mRNA is the major component of the fall in protein synthesis (Zakeri & Lockshin, 1994). The pattern of protein synthesis, judged by two-dimensional gels of [^{35}S] methionine incorporation into glands isolated from metamorphosing animals, indicated upregulation or at least persistence of a series of mRNAs that are not seen in premetamorphosing animals. A realignment of the rough endoplasmic reticulum was observed on day 0 (Zakeri et al., 1996). This process may be related to the decrease in protein synthesis.

Another early cytoplasmic change, first detected on day 0, in dying glands was movement and activation of the lysosomal compartment (Halaby et al., 1994; Lockshin & Zakeri, 1994). By day 3, the cytoplasm is replete with autophagic lysosomal vacuoles that presumptively degrade the cytoplasmic constituents (Zakeri et al., 1993). Analysis of lysosomal localization and activity, by measuring

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

acid phosphatase, allowed us to characterize the death of the labial glands as a type II death (Schweichel & Merker, 1973). This type of cell death has been reported in other insect tissues: *Manduca* intersegmental muscle (Lockshin & Beaulaton, 1974a); *Drosophila* salivary glands (Lockshin, 1969); and *Malacosoma* and *Galleria* silk glands (Aidells et al., 1971).

Additional evidence supporting type II death of the labial glands comes from DNA fragmentation studies. DNA content does not drop during the early phases of cell death; nor was an endonucleolytic ladder seen when DNA was examined by ethidium bromide staining or prelabeling with [^{3}H] thymidine (Zakeri et al., 1993). Only by using a more sensitive technique, end labeling of DNA, was DNA fragmentation detected at a very late stage in cell death, day 4. It is important to note that fragmentation does occur in the labial gland, but far too late to determine the fate of the cell.

E. GENETIC REGULATION OF CELL DEATH

1. Cell Death Genes In *Caenorhabditis elegans*

Although the induction of certain genes has been associated with cell death (Quarmby et al., 1987; Buttyan et al., 1988; Schwartz et al., 1990; Piacentini et al., 1991), the relevance of most of the proteins encoded by these genes to the death process remains to be elucidated. In some systems, a requirement for protein synthesis during lysosomal and apoptotic cell death has been established through the use of inhibitors of mRNA and protein synthesis (Tata, 1966; Lockshin, 1969; Munck, 1971; Martin et al., 1988; Weeks et al., 1993). Studies of *Caenorhabditis*

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

elegans were the first to demonstrate that cell death was under direct genetic control, and led to the identification of at least twelve genes that function in nematode PCD. Two of these cell death genes, ced-3 and ced-4, are required for cells to die (Ellis & Horvitz, 1986). A third gene, ced-9, prevents activation of the death pathway in cells that should survive (Hengartner et al., 1992). The molecular characterization of these three genes revealed that ced-3 and ced-9 are homologous to mammalian cell death genes.

2. Cysteine Proteases & Cell Death

The ced-3 protein is a member of the family of cysteine proteases including interleukin-1β-converting enzyme (ICE), which cleaves the inactive 31 kD pro-interleukin-1β cytokine precursor to the active 17 kD form, family of cysteine proteases (Yuan et al., 1993). Unlike other mammalian cysteine proteases, members of the ICE family cleave their substrates following aspartate residues (Lazebnik et al., 1994). The sequence similarity between ced-3 and ICE implied that members of the ICE family should be involved in mediating apoptosis in mammals. Several other observations appear to support this prediction. First, inhibition of ICE family proteases prevents apoptosis (Gagliardini et al., 1994; Nicholson et al., 1995), indicating that, as in *C.elegans*, these proteases are not only sufficient but also necessary for the induction of cell death. Second, an ICE-like protease activity can be detected in apoptotic but not normal cells (Lazebnik et al., 1994; Nicholson et al., 1995). ICE may not represent the only cell death pathway as evidenced by the fact that thymocytes and macrophages from ICE-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

deficient mice undergo apoptosis normally (Li et al., 1995).

3. Inhibitors Of Cell Death

The ced-9 protein belongs to the bcl-2 family of cell death regulators (Hengartner & Horvitz, 1994). This observation, coupled with the fact that overexpression of bcl-2 can prevent PCD in *C.elegans* and partially substitute for ced-9 function (Vaux et al., 1992; Hengartner & Horvitz, 1994), strongly suggests that nematodes and mammals share a common mechanism for cell death (Vaux et al., 1994; Hengartner & Horvitz, 1994). In mammals, some members of the bcl-2 family prevent cell death, whereas others promote it. For instance, bcl-2 (Sentman et al., 1991) and bcl-x_L (Williams & Smith, 1993) prevent cell death , while bcl-x_s and bax promote cell death (Williams & Smith, 1993; Boise et al., 1993) perhaps by forming non-protective dimers or tetramers with bcl-2. The fate of cells may therefore reside in the balance between the opposing apoptotic effects of an ICE/ced-3-like protease and upstream regulatory mechanism involving bcl-2 and its homologues.

The baculovirus p35 gene product has been shown to inhibit PCD in insects (Hay et al., 1994), nematodes (Sugimoto et al., 1994), and mammals (Rabizadeh et al., 1993). The fact that p35 can prevent cell death in these phylogenetically diverse species suggests that its cellular target may be evolutionarily conserved. This hypothesis is supported by the following reports. Cleavage of p35 by ICE (Bump et al., 1995) and by ced-3 (Xue & Horvitz, 1995) is necessary for p35's anti-apoptotic activity. Bump et al. (1995) demonstrated that p35 can stoichiometrically

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

bind to, and inhibit the protease activity of, ICE family members.

4. Cell Death Genes In *Drosophila*

Drosophila has only recently joined *C.elegans* as a model system for the genetic analysis of PCD. Two *Drosophila* cell death genes, reaper and head involution defective (hid), have been isolated (White et al., 1994; Grether et al., 1995). The reaper gene shares limited homology with the death domain (Golstein et al., 1995), a sequence of some 70 amino acids found among members of the TNF receptor superfamily that are critical for triggering apoptosis (Tartaglia et al., 1993). Reaper was originally identified through a chromosome deletion mapping study as a gene that was essential for all of the cell deaths that occur during normal *Drosophila* embryogenesis (White et al., 1994). Deletion of reaper not only delays all developmental cell death, but also suppresses cell death in response to X-irradiation or mutation-induced errors in developmental programming. In all cases of cell death, reaper is induced 1-2 hr prior to the demise of the actual cell. Hid mutants were found to have decreased levels of cell death (Grether et al., 1994). Overexpression of hid promotes apoptotic death, which can be suppressed through expression of baculovirus p35. It is not yet clear how reaper and hid induce PCD.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

2. STEROID HORMONES & CELL DEATH

A. Hormonal Regulation Of Cell Death
  1. Cell Death In Hormone-Dependent Tissues
  2. Hormonal Activation Of Lysosomes During Cell Death
  3. Mechanisms By Which Steroids Induce Cell Death

B. Mechanisms Of Steroid Hormone Action
  1. Nuclear Receptor Activation Upon Binding Of Ligand
  2. Activation Of Gene Expression
  3. Structural & Functional Characterization Of Nuclear Receptors
  4. Protein-Protein Interactions Of Nuclear Receptors
  5. Generation Of Nuclear Receptor Isoforms

C. 20-Hydroxyecdysone Regulation Of Gene Expression
  1. 20-Hydroxyecdysone Acts At The Level Of The Genome
  2. Ecdysone & Vertebrate Nuclear Receptors
  3. Differential Expression Of Ecdysone Receptors

D. 20-HE & Metamorphosis In *Manduca*
  1. Initiation Of Molting
  2. Tissue Responses To 20-HE During Metamorphosis

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

2. STEROID HORMONES & CELL DEATH

A. HORMONAL REGULATION OF CELL DEATH

1. Cell Death In Hormone-Dependent Tissues

Steroid hormones regulate cell death in several systems including the thymus gland (glucocorticoids; Wyllie et al., 1980; Compton & Cidlowski, 1986), prostate (androgens; Sanford et al., 1984; Kyprianou & Isaacs, 1988), and mammary gland (estrogens; Cho-Chung, 1978). The survival of epithelial cells in the ventral prostate depends on testosterone secreted by the testes (Kyprianou & Isaacs, 1988); whereas the survival of cells in the adrenal cortex depends on adrenocorticotropic hormone secreted by the pituitary (Wyllie et al., 1973). Estrogens generally inhibit apoptosis whereas androgens induce apoptosis and progesterone suppresses cell death in uterine epithelial cells (Billig et al., 1993; Rotello et al., 1992). Estrogens have been reported to induce cell survival in MCF-7 cells by upregulating Bcl-2 expression (Teixeira et al., 1995). Thus, it is not surprising that ablation or addition of steroid hormones has been successfully used in a variety of treatments: against leukemia via the glucocorticoid receptor (Helmberg et al., 1995), vitiligo (Hann et al., 1993), and inflammatory bowel diseases with the use of corticosteroids (Hirschfeld & Clearfield, 1995). Steroidal induction of PCD can also lead to pathologies including adrenal hormone activation of Cushing's syndrome (Engelhardt & Weber, 1994) and cumulative glucocorticoid neurotoxicity in Alzheimer's disease (Landfield et al., 1992). The control processes, at the molecular level, involved in hormone-induced actions are

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

still unclear.

2. Hormonal Activation Of Lysosomes During Cell Death

Steroids may induce alterations in membrane functions of lysosomes, in target cells, to mediate secondary changes in the hormone-stimulated target cells. Szego and coworkers (Szego et al., 1971; Szego & Seeler, 1973) argued that an early response of sensitive tissues to estrogenic stimulus was migration of lysosomes to the nuclear membrane and fusion of the lysosomes with the nuclear membrane, allowing lysosomal enzymes to enter the nucleus. There is evidence suggesting that the therapeutic value of the glucocorticoids in arthritis is a reflection of their ability to stabilize lysosomes (Lloyd & Beck, 1969). A pronounced rise, during natural metamorphosis, in lysosomal enzymes has been documented for the labial glands of *Manduca* (Halaby et al., 1994; Zakeri et al., 1996). Lysosomes expand throughout the cytoplasm by moving from the basal surfaces towards the lumen as the labial gland degenerates (Halaby et al., 1994). This movement and activation of lysosomes appears to be triggered by 20-HE (Halaby et al., in preparation). 20-HE has been shown to induce lysosomal activity in dying tissues (Radford & Misch, 1971; Sass et al., 1975; van Pelt-Verkuil, 1979; de Priester et al., 1979). Autophagic vacuoles are predominant structures in regressing tissues of insects (Schin & Clever, 1965; Lockshin & Williams, 1965b; Matsura et al., 1968; Aidells et al., 1971; Lockshin & Beaulaton, 1974a,b; Halaby et al., 1994). Lysosomal autophagocytosis during PCD is not unique to insects. For example, it occurs in involuting decidual cells in mice (Bijovsky &

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Abrahamsohn, 1992) and in the regression of the rat Mullerian duct (Price et al., 1977). Likewise, uterine epithelial cell lysosomes change their intracellular location from basal to apical regions during the estrus cycle (Woessner, 1969).

B. MECHANISMS OF STEROID HORMONE ACTION

1. Nuclear Receptor Activation Upon Binding Of Ligand

The model of steroid hormone action was originally proposed by Jensen et al. (1968) and Gorski et al. (1968) for the estrogen receptor. According to this model steroids (S) passively enter the nucleus where they encounter and bind to the steroid receptor (R) proteins. The SR complex transforms to a DNA-binding form and interacts with cis-acting sequences, hormone response elements (HRE) (Pfahl, 1982; Karin et al., 1984), resulting in the enhanced transcription of target genes (Chandler et al., 1983). The steroid receptors act as ligand-dependent transcription factors.

The binding of the ligand to its cognate receptor has several consequences. In the absence of hormones, steroid hormone receptors are associated with heat shock proteins hsp70 and hsp90 in a large, inactive 8S complex (DeMarzo et al., 1991). In contrast, retinoic acid and thyroid hormone receptors do not interact with heat shock proteins (Dalman et al., 1991). O'Malley et al. (1991) report that ligand binding triggers the release of associated proteins from the receptor and reverses the repression. This simple model, however, has been challenged. For example, steroid-free glucocorticoid receptor can bind its HRE *in vitro* (Willman & Beato, 1986). In addition, a truncated glucocorticoid receptor, which lacks the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

ligand binding domain, is constitutively active (Godowski et al., 1987). Ligand-induced phosphorylation appears to be involved in the activation of the progesterone, vitamin D3, and glucocorticoid receptors (Denner et al., 1990; Brown & DeLuca, 1990; Bodwell et al., 1991). Phosphorylation has been postulated to unmask a transcriptional activation domain or to control nuclear localization of hormone receptors (Moudgil, 1990).

2. Activation Of Gene Expression

After receptor activation and nuclear translocation, the hormone receptor binds to specific HRE located in various gene promoters. These response elements have a palindromic consensus sequence (13-26 base pairs long) consisting of an inverted repeat of half sites separated by a spacer of up to three nucleotides (Beato, 1989). In *Drosophila* directly repeated HRE can also mediate 20-hydroxyecdysone responses (Antoniewski et al., 1996). Zinc finger-like structures in the DNA-binding domain of the hormone receptors mediate specific recognition of HRE (Kumar & Chambon, 1988). Two or more HRE for the glucocorticoid and estrogen receptor have been found in various genes (Muller et al., 1991). The HRE of one steroid receptor can act in synergy with additional copies of their own HRE, or with HRE of other steroids, or with transcription factors. Two different steroid receptors, glucocorticoid and estrogen, have been shown to synergize in the regulation of the chicken vitellogenin II gene transcription (Ankenbauer et al., 1988). Specific transcription factors, such as NF1, Sp1, CP1, and OTF, are capable of cooperating synergistically with steroid receptors (Schule

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

et al., 1988). In the case of OTF1, its cognate recognition site is located next to that of the steroid receptor in the mammary tumor virus (MMTV) promoter (Bruggemeier et al., 1991).

3. Structural & Functional Characterization Of Nuclear Receptors

Members of the steroid receptor superfamily share a common structural motif. These proteins fold into three distinct domains, each mediating distinct functions of the receptor (Loosfelt et al., 1984; Hollenberg et al., 1985; Conneely et al., 1986). The receptors contain an N-terminal variable (transcriptional activation) domain, a central DNA-binding domain of 66 to 68 amino acids, of which 20 residues are invariant, and a C-terminal ligand-binding domain (Spelsberg et al., 1989). The DNA binding-domain, the most highly conserved region among members, is a cysteine-lysine-arginine-rich region that is necessary and sufficient for binding to DNA (Evans, 1988). Eight invariant cysteine residues are believed to coordinate zinc in the formation of two zinc finger-like structures (Miller et al., 1985). The solution structure of the DNA-binding domain has been solved using nuclear magnetic resonance spectroscopy for the estrogen receptor (Schwabe et al., 1990) and the glucocorticoid receptor (Hard et al., 1990). These studies show that the two zinc finger-like motifs actually fold to form a single structural domain unlike the independently folded units of the TFIIIA zinc fingers (Miller et al., 1985). Steroid receptors contain a sequence of basic amino acids near the second zinc finger (between the DNA- and ligand-binding domains) that has been implicated as the nuclear localization signal for several steroid receptors (Picard & Yamamoto,

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1987; Guiochon-Mantel et al., 1989; Green & Chambon, 1991).

4. Protein-Protein Interactions Of Nuclear Receptors

Two types of protein-protein interactions are critical for the modulation of transcription in response to hormones. Type one involves formation of homodimers (Forman & Samuels, 1990). Interactions between receptor dimers at separate HREs have also been reported. Thus, occupation of one HRE site by a progesterone receptor dimer increases the binding affinity of progesterone receptors for the second HRE about 100-fold (Tsai et al., 1989). Type two involves formation of heterodimers. For instance, the retinoic acid α-receptor and the thyroid hormone β-receptor interact cooperatively on a subset of thyroid hormone response elements (Glass et al., 1989). In addition, an isoform of the retinoid X receptor (RXR) serves as a common heterodimeric partner for the vitamin D3, thyroid hormone, and retinoic acid receptor (RAR; Kliewer et al., 1992). A consequence of heterodimer formation is the generation of protein aggregates that display new DNA-binding affinities compared to the individual homodimers.

5. Generation Of Nuclear Receptor Isoforms

Many steroid receptor members themselves give rise to subfamilies by alternative splicing and use of alternative promoters resulting in even greater diversity. Two functionally different progesterone receptor forms are generated by using two distinct transcriptional start sites (Kastner et al., 1990). The three isoforms of the ecdysone receptor arise by alternative splicing (Koelle et al., 1991). The retinoic acid receptors exhibit the highest level of diversity. Two classes of

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

retinoic acid receptors have been identified: RAR α, β, and γ, which bind all-trans retinoic acid (Zelent et al., 1989); and RXR α, β, and γ, which bind the isomer 9-cis retinoic acid (Mangelsdorf et al., 1992). In addition, members of the RAR class give rise to multiple receptor isoforms through differential splicing and promoter usage (Zelent et al., 1991). The existence of such a large number of steroid receptor forms may be involved in generating temporally or spatially restricted hormone responses (Thompson et al., 1987).

C. 20-HYDROXYECDYSONE REGULATION OF GENE EXPRESSION

1. 20-Hydroxyecdysone Acts At The Level Of The Genome

20-HE acts as a temporal signal to coordinate tissue-specific morphogenetic changes in insects (Clever & Karlson, 1960; Ashburner, 1971; Fahrbach, 1992). The rapid effect of 20-HE on puffing activities, discovered by Clever & Karlson (1960), was the first indication that a steroid hormone acts directly at the level of the genome. Ashburner et al. (1974) proposed a model for the activation of the early and late puffs. 20-HE, complexed with its receptor, directly activates early genes while repressing the late genes. The products of the early genes are regulatory proteins responsible for the induction of the late genes, and for the repression of their own expression. The isolation and characterization of three early genes support the Ashburner model: BR-C (Chao & Guild, 1986), E74 (Burtis et al., 1990), and E75 (Segraves & Hogness, 1990). Genetic and molecular studies of BR-C and E74 indicate that these genes are essential for metamorphosis and that they regulate late gene expression (Belyaeva et al., 1980;

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Kiss et al., 1988; Guay & Guild, 1991; Fletcher et al., 1995; Fletcher & Thummel, 1995). These three early genes are all long (up to 100 kb) and complex, contain multiple ecdysone-inducible promoters, and encode site-specific DNA-binding proteins structurally related to transcription factors (Andres & Thummel, 1992).

2. Ecdysone & Vertebrate Nuclear Receptors

The 20-HE receptor, a heterodimeric protein, is encoded by the *Drosophila* ecdysone receptor (EcR; Koelle et al., 1991) and ultraspiracle (USP; Oro et al., 1990) genes. Both EcR and USP belong to the nuclear hormone receptor superfamily (Evans, 1988). This suggests the likely universal nature of these receptors in animals and clearly demonstrates that the receptors evolved prior to the divergence of vertebrates and invertebrates (Mangelsdorf et al., 1995). Indeed, 20-HE may act by mechanisms homologous to those of the vertebrate steroids. When 20-HE binds to the EcR:USP receptor, the complex (Yao et al., 1992) translocates to the nucleus where it may activate or repress expression of genes which contain ecdysone-response elements (EcRE; Riddihough & Pelham, 1987) in their promoter regions. 20-HE responses can be negatively controlled by having seven-up and EcR (Zelhof et al., 1995) or DHR38, the homolog of the vertebrate nerve growth factor-induced-B, and USP (Sutherland et al.. 1995) form inactive heterodimers, preventing activation of the target promoter. The similarity of the sequences of one EcRE to those of hormone response elements (HRE) for estrogen, thyroid, vitamin D3, and retinoic acid receptors suggests that the steroid receptors and their signal transduction mechanisms have been strongly and

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

broadly conserved (Cherbas et al., 1991). Thus, ecdysone may act by mechanisms homologous to those for the other steroids.

3. Differential Expression Of Ecdysone Receptors

20-HE is able to regulate multiple and complex events in a variety of cell types. In *Drosophila*, multiple isoforms of EcR (A, B1, and B2) have been identified (Koelle et al., 1991; Talbot et al., 1993). These proteins differ in their N-termini but contain identical DNA binding- and ligand-binding domains. Likewise, in *Manduca*, EcR-A (Jindra et al., 1996) and EcR-B1 (Fujiwara et al., 1995) have been isolated in the epidermis and wings. This is the first report of conservation of the EcR-B1/EcR-A isoforms among insects. All three EcR isoforms acquire the ability to bind DNA upon heterodimerization with USP (Yao et al., 1992). Although EcR can bind ligand weakly on its own, this binding is greatly stimulated by the addition of USP (Yao et al., 1993). Ligand binding both stabilizes the EcR-USP heterodimer and also increases its affinity for binding to EcRE.

Regulation of the spatiotemporal patterns of EcR expression by 20-HE itself is an additional means of regulating the responsiveness of a particular tissue type. 20-HE has been reported to regulate the expression of EcR isoforms during cell death (Robinow et al., 1993). Prior to metamorphosis in *Drosophila*, larval cells that are destined to die express predominantly EcR-B1, whereas most imaginal cells express EcR-A (Talbot et al., 1993). In adult tissues, the EcR-A isoform is upregulated in neurons that degenerate after emergence (Robinow et al., 1993). Moreover, in *Manduca*, 20-HE regulates the expression of the two EcR isoforms

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

differently (Jindra et al., 1996). EcR is induced directly by 20-HE, providing an autoregulatory loop that increases the level of receptor protein in response to its ligand (Karim & Thummel, 1991). The phenomenon of receptor auto-induction by the hormone itself has also been shown for other members of the nuclear receptor superfamily (Shapiro et al., 1989; Yanita et al., 1990; de The et al., 1990).

D. 20-HE & METAMORPHOSIS IN *MANDUCA*

1. Initiation Of Molting

The larval to pupal molt in *Manduca* is preceded by two peaks of 20-HE secretion from the prothoracic glands. The first peak, which occurs around day -0.5 relative to the start of pupation, does not cause the molting cycle to begin but is required to switch the state of determination, or commitment, of the epidermis (Dominick & Truman, 1984). Before the first peak the epidermis is committed to produce larval characters, while after the peak it is committed to produce pupal characters (Truman et al., 1974; Riddiford, 1978). The second peak of 20-HE, which occurs on day 3, is much larger and is responsible for producing the pupa.

2. Tissue Responses To 20-HE During Metamorphosis

The switchover to pupal commitment during the larval-pupal molt requires 20-HE, but only in the absence of juvenile hormone (Nijhout & Williams, 1974). There are several hormones involved in regulating metamorphosis in *Manduca*. The corpora allata are the glands that produce juvenile hormone (JH), so named since it induces the retention of insects' larval characteristics and prevention of maturation. The brain secretes prothoracicotropic hormone (PTTH), a peptide

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

hormone, only in the absence of JH. JH titers are high at the start of the fifth instar, reaching undetectable levels around day -2 (Nijhout & Williams, 1974). At this time PTTH is released and stimulates the secretion of the commitment peak of 20-hydroxyecdysone (20-HE) on day -0.5.

This reprogramming of the epidermal cells, during the first peak of 20-HE, is accompanied by the loss of mRNAs for larval-specific proteins and the synthesis of new mRNAs and proteins that appear to be required to permanently suppress larval-specific genes (Riddiford, 1982). The first peak of 20-HE also causes dramatic changes in the animal's physiology and behavior. The first and most obvious behavioral response is that the animal stops feeding and voids its gut contents (Dominick & Truman, 1984). The larva then moves away from its food source and searches for an appropriate site for pupation. This activity period is referred to as the wandering phase. Also at this time the aorta becomes visible along the dorsum for the first time which is used to stage the larva as day 0 (the start of the larval to pupal metamorphosis).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

PART II. RESULTS AND DISCUSSION

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER I. METABOLIC EVENTS DURING PROGRAMMED CELL DEATH IN LABIAL GLANDS FROM PUPATING ANIMALS

A. Introduction
B. Materials And Methods
C. Results
  1. Morphology Of Cell Death In Labial Glands
  2. Energy Resources Remain Constant During The Early Phase Of Degeneration
  3. Mitochondrial Functioning Is Intact Until Late
  4. Localization Of Lysosomes
  5. Changes In The Levels Of Second Messengers Are Late
E. Discussion: Role Of Altered Metabolism In The Death Of Labial Glands

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER I. METABOLIC EVENTS DURING PROGRAMMED CELL DEATH IN LABIAL GLANDS FROM PUPATING ANIMALS

INTRODUCTION

The histolysis of insect tissues by programmed cell death (PCD) is well documented (Finlayson 1956; Truman et al. 1992). In *Manduca sexta*, the labial gland (a homolog of the silk gland of *Bombyx mori* and *Drosophila* salivary gland) dies in 5 days during the larva to pupa metamorphosis. The paired epithelial labial gland is a secretory gland approximately 0.2 mm in diameter, consisting of a single layer of giant (1×10^4 μm^3) polyploid cells. It measures up to 17 cm in length, sinuously extending the length of the larva. The gland secretes a viscous, clear, glycoprotein-containing fluid used presumably to solidify the wall of the pupa's burrow. The entire gland dies except for the anterior duct which dedifferentiates into the adult labial gland. The labial gland provides a valuable system to study the mechanisms of PCD since the death of the tissue is nearly synchronous and involves nearly the entire tissue. Thus, a substantial amount of homogeneous dead cells can be studied, uncontaminated by living cells such as blood cells or, in vertebrates, capillary endothelia. Since gland PCD occurs over 5 days, we can isolate glands at different stages of development and sequentially study differences in the levels of cell death.

The gland dies in a well controlled manner in which DNA fragmentation is a late event after substantial cytoplasmic alterations have occurred. Protein synthesis, as measured by incorporation of [^{35}S] methionine into protein, falls

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

rapidly as metamorphosis begins and ultimately resolves to a small number of proteins for which incorporation persists even while the gland deteriorates (Zakeri et al., 1996). The early fall in synthesis is unrelated to the total protein in the gland or the total RNA, both of which remain high during the first two days of metamorphosis (Zakeri et al., 1996). This alteration is protein synthesis, however, appears to be reflective of alterations in the mRNA (Zakeri & Lockshin, 1994). To further investigate the cause of this decline in protein synthesis, we investigated energetics, second messengers, lysosomes, and gland morphology.

A generalized collapse of a cell may be initiated by loss of energy resources (Farber et al. 1990; Trump and Berezesky 1992). We therefore evaluated this possibility using two techniques: 1) reduction of MTT, by living mitochondrial dehydrogenases (Mosmann, 1983), as a measure of mitochondrial function; and 2) measurement of ATP extracted from the glands.

Many transmembrane receptors act indirectly by way of second messengers. These second messengers are small diffusible molecules can activate a wide range of protein kinases in many different kinds of cells. Clearly if intracellular levels of these molecules are altered a cell may be doomed for death. Changes in second messengers often initiate major metabolic changes (Schwartz & Truman 1984). We therefore measured the levels of second messengers during all the stages of metamorphosis in the labial gland to see if these changes could account for the drop in protein synthesis and gland PCD.

We report that changes in ATP levels, mitochondrial function, cyclic

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

nucleotide levels, and inositol triphosphate (IP_3) occurred after the initial commitment to self-destruct was made and protein synthesis began to fall. Energy resources and second messengers fell in concert with the total protein and plummeted only with the morphological collapse of the cell. In contrast, expansion of lysosomes in the cytoplasm occurred early and intensified throughout metamorphosis.

MATERIALS AND METHODS

Rearing Of The Animals

Larvae were fed an Otis modified hornworm diet (BioServ, Cat. # F9666; Frenchtown, NJ). The diet was prepared by bringing 3.2 L of tap water to a full boil for 1 min. While the water was boiling, the media and agar were added into the food container. Once the water had boiled, it was added to the agar/media mixture. Twenty five ml of 37% formaldehyde (Fisher Scientific, Pittsburgh, PA) were added to minimize fungal growth. The contents were mixed for 15 min, using an electric hand blender (Braun), to ensure thorough mixing. The food was allowed to cool to room temperature before being stored at 4°C. Once the food hardened, it was cut into squares. The squares of food were placed into compartmentalized insect trays. The larvae were placed on the food to feed. The food was monitored daily to ensure that it was free from fecal contamination.

Manduca sexta eggs or larvae were purchased from Carolina Biological Supply Company (Burlington, NC). The larvae were kept at 25°C in individual compartments and reared according to the methods of Bell & Joachim (1976).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Most larvae fed for 5 days during the fifth instar before exposing the dorsal vessel (day 0). Day 0 larvae were removed from food and placed in a clean compartment with Kimwipes (Kimberly-Clark, Roswell, GA) to absorb the secretion from the labial gland. Larvae were staged in terms of days before or after the onset of pupation.

Dissection Of The Labial Glands

The larvae were anesthetized on ice for 10 min. The heads were cut off, with a scissors, and a longitudinal incision was made along the ventrum of the larvae. The larvae were laid dorsal side down onto a dissecting plate. The epidermis was pinned down using 26 gauge needles (Becton Dickinson & Co, Lincoln Park, NJ). The right and left labial glands, lateral to the gut, were removed by observation under a dissecting microscope using fine forceps. The common duct, which differentiates into the adult labial gland, was not included in the experiments.

Electron Microscopy

Labial glands were fixed in 2.5% glutaraldehyde in 1X PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.4) at 4°C overnight. Glands were then postfixed in 1% osmium tetroxide in 1X PBS at 4°C for 1 hr, dehydrated in graded ethanols, in propylene oxide, and finally embedded in Spurr resin. Thin sections were cut with a Reichert Ultracut ultramicrotome, collected on copper grids, and stained with uranyl acetate (5% in 70% ethanol) for 15 min and then in lead citrate for 10 min. Observations were done in a JEOL 1200 XII

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

electron microscope.

ATP Measurement

ATP was measured by NADH absorbance using Sigma (St. Louis, MO) kit 366. Labial glands were homogenized in 0.9% NaCl and either used immediately or stored at -20° C. One ml of 12% trichloroacetic acid solution (TCA) was added to 1 ml of supernatant, and incubated on ice for 5 min. The mixture was centrifuged for 5 min at room temperature. The following were pipeted into a 0.3 mg NADH vial in this order: 1 ml of 18 mM 3-phosphoglyceric acid buffer, 1.5 ml dH_2O, and 0.5 ml of supernatant. The vial was capped and inverted several times to dissolve the NADH. The entire contents of the vial were decanted into a cuvet. The initial absorbance was recorded at 340 nm. Four hundred μl of glyceraldehyde 3-phosphate dehydrogenase/3-phosphoglyceric phosphokinase mixture was added to the cuvet and the final absorbance was measured at 340 nm.

MTT Assay

Stock solutions of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; 5 mg/ml; Sigma) were prepared in Grace's medium (Gibco BRL, Grand Island, NY). Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes of living cells (Mosmann, 1983). Glands were incubated in 500 μl of MTT-containing medium, with shaking in the dark, at room temperature for 1 hr and examined by light microscopy (Leitz DMRB; Leica Inc., Deerfield, IL). For more quantitative measurements, following

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

incubation, other glands were rinsed in Grace's medium, the converted dye was solubilized with isopropanol for 30 min. The optical density of the converted dye was measured at 570 nm with background subtraction at 660 nm.

Preparation Of 4% Paraformaldehyde

One gram of paraformaldehyde (PFA; Sigma) was dissolved in 12.5 ml dH_20. The solution was transferred to the fume hood and heat stirred to 65°C. Two and a half μl 10 N NaOH were added and the solution was filtered through a 0.45 μm filter (Nalgene, Rochester, NY). Two and a half ml 10X PBS (1.4 M NaCl, 27 mM KCl, 43 mM $Na_2HPO_4 \cdot 7H_2O$, 14 mM KH_2PO_4, pH 7.4) were added and dH_20 was added to bring the volume to 25 ml. The pH was checked to assure that it was $\leq$ 8. The 4% PFA solution was stored at 4°C for immediate use or at -20°C for longer periods.

Tissue Preparation And Sectioning

A 250 ml Pyrex beaker, filled with 5 ml of isopentane, was chilled by immersing in a Dewar flask filled with liquid N_2. Aluminum foil was molded into 2 cm x 2 cm containers used for freezing the labial glands in Tissue Tek OCT (Miles, Elkhart, IN). The foil containers were placed in the beaker until the glands were frozen. Specimens were stored at -70°C until 5 μm sections were cut onto poly-L-lysine-coated slides using a cryostat (Reichert-Jung Cryocut 1800, Germany).

Preparation Of Coated Slides

Fifty mg of poly-L-lysine (Sigma) were dissolved in 10 ml of 1 M Tris (pH 8) and 990 ml of diethylpyrocarbonate- (DEPC; Sigma) treated water . Slides (Fisher

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Scientific) were sequentially incubated for 30 sec in each of the following: 0.2 M HCl, DEPC-H_2O, and acetone. Slides were allowed to air-dry in the fume hood for 2 hr. Slides were then repeatedly dipped into poly-L-lysine solution for 5 min, covered with foil paper, and dried overnight in the fume hood. Slides were stored at room temperature and used for up to 4 weeks after poly-L-lysine treatment.

Lysosomal Localization & Activity

To localize lysosomal enzymes in the glands, a histochemical assay (Sigma kit 181-A) of AP was used. The p-nitrophenol phosphate (substrate) stock solution was prepared by dissolving a 100 mg capsule in 25 ml dH_2O. The stock solution was dispensed in 0.5 ml aliquots and stored at -20° C. Glands were fixed in 4% PFA at 4° C for 4 hr or overnight, frozen, and sectioned onto poly-L-lysine coated slides. Prior to fixation, 0.6 ml sodium nitrite solution were added to 0.6 ml fast garnet GBC solution, mixed by inversion; allowed to stand 2-4 min; and added to 23 ml dH_2O. Three ml acetate solution and 3 ml naphthol AS-BI phosphoric acid solution were added to the previous solution and mixed well. Slides were fixed in citrate-acetone-formaldehyde solution at room temperature, in a Coplin jar for 30 sec, and rinsed with dH_2O for 1 min. Slides were then incubated for 1 hr at room temperature instead of 37°C (to accommodate insect enzymes) in naphthol AS-BI phosphate and fast garnet stain in acetate buffer. Slides were rinsed with tap H_2O for 2 min, dried for 15 min, counterstained with methylene blue for 1 min, rinsed with dH_2O, and mounted in CrystalMount (Biomeda Corp., Foster City, CA). The presence of acid phosphatase was indicated by distinct red focal precipitates

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

resolved by light microscopy.

To determine the lysosomal enzyme activity in the glands, a biochemical assay (Sigma Procedure No. 104) of AP was also used. The assay was performed by homogenizing glands in 0.5 ml 0.9% NaCl and clarifying the homogenate by centrifugation for 5 min at room temperature. The reaction mixture [0.5 ml p-nitrophenol phosphate (substrate), 0.5 ml 90 mM citrate buffer (pH 4.8), and 0.1 ml of homogenate] was incubated for 30 min at room temperature, and the reaction terminated by the addition of 5 ml of 0.1 N NaOH. In alkali, liberated p-nitrophenol is yellow and can be measured spectrophotometrically at 410 nm. Units of enzyme activity were determined from the calibration curve obtained by measuring the amount of color produced. Since the total protein content of the glands consistently dropped starting at day 0, only total AP levels, rather than AP levels per mg of protein, were measured.

Measurement Of Second Messengers

IP_3 levels were measured using a [^{3}H] radioreceptor assay kit (Dupont NEN, Boston, MA; Cat. # NEK-064). Labial glands were homogenized in 1M trichloroacetic acid (TCA) solution. Centrifugation was performed for 5 min at room temperature. The supernatant was incubated for 15 min at room temperature. TCA was removed from the samples by using a 1,1,2-trichloro-1,2,2-trifluoroethane (TCTFE)/trioctylamine solution. Two ml of TCTFE-trioctylamine was added to the samples for each 1 ml TCA extract, the tubes were vortexed, and incubated for 3 min at room temperature. The top layer, which contained the IP_3,

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

was removed to a clean tube and samples were stored on ice until assayed. The stock solution of receptor/[^{3}H] IP_3 (1 μCi) tracer (RT) was prepared by adding 2.5 ml dH_2O to the vial and storing it at -20° C. RT was diluted 1:15 (v/v) with assay buffer (0.05% sodium azide, 5 mM EDTA, 5 mM EGTA, 50 mM sodium TAPS buffer, pH 8.6). The reaction mixture, 100 μl sample and 400 μl RT, was vortexed and incubated for 1 hr at 4° C. Centrifugation was performed at 4° C for 15 min. The supernatant was decanted as radioactive waste. The pellet was solubilized in 50 μl of 0.15 M NaOH, the tubes were vortexed, incubated for 10 min at room temperature, and counted in a scintillation counter.

The cAMP levels were assessed using a cAMP [^{125}I] radioimmunoassay kit (DuPont NEN, Cat. # NEK-033). Glands were homogenized in 6% TCA. To determine the recovery of cAMP during extraction, 4,000 CPM of [^{3}H]-cAMP marker was added to the TCA extract. The TCA extract was centrifuged at 4° C for 15 min. The supernatant was collected and extracted four times with ether. The ether phase was discarded. The cAMP stock was diluted 1:50 (v/v) with sodium acetate buffer (SAB; pH 6.2). Samples were diluted 1:5 with SAB. Acetylation reagent was prepared by mixing 100 μl of triethylamine and 400 μl SAB. Tracer solution (succinyl cAMP tyrosine methyl ester [^{125}I]) was diluted 1:1 with cAMP carrier serum. One hundred μl antiserum, rabbit anti-cAMP, was added, the tubes were vortexed, and incubated overnight at 4° C. cAMP precipitator, 500 μl, was added to the tubes, they were vortexed, and centrifuged at 4° C for 15 min. The supernatant was decanted as radioactive waste. The

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

tubes were counted in a gamma counter.

cGMP was assayed using a radioimmunoassay kit (DuPont NEN, Cat. # NEX-133). Glands were homogenized in 6% TCA at 4°C. To determine the recovery of cGMP during extraction, 1,500 CPM of [^{3}H]-cGMP was added to the TCA extract. Centrifugation occurred at 4°C for 15 min, the supernatant was extracted four times with ether, and the residue was dissolved in 0.05 M sodium acetate buffer (SAB; pH 6.2). One hundred μl of succinyl cGMP tyrosine methyl ester [^{125}I], containing rabbit serum, was added to all tubes. One hundred μl of antiserum was added to each tube followed by vortexing, and incubation overnight at 4°C. One ml of cold SAB was added, tubes were vortexed, and centrifuged at 4°C for 15 min. The supernatant was discarded as radioactive waste. The tubes were counted with a gamma counter.

Protein Quantification

The protein concentrations from labial gland samples were determined using the Bio-Rad (Hercules, CA) standard protein assay. BSA standards, ranging from 0.2 to 1.4 mg/ml, were prepared with dH_2O. Five μl of Laemmli sample buffer (0.0625 M Tris, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue, pH 6.8) were added to 95 μl of standards. Five μl of sample was added to 95 μl of dH_2O (1:20 dilution). The blank consisted of 5 μl Laemmli sample buffer and 95 μl of dH_2O. The reagent dye was diluted 1:4 with dH_2O, filtered through a 0.45 μm membrane (Gelman Sciences, Ann Arbor, MI), and 5 ml of diluted dye were added to the tubes. After 5 min, the optical density of the dye-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

protein complex was measured at 595 nm. The protein concentration of the samples were measured against the standard curve.

RESULTS

Morphology Of Cell Death In Labial Glands

Morphology is often used as a parameter to monitor cell death. Labial gland cell death displays a distinctly different morphology from that seen in mammalian tissues. Glands from premetamorphosing (day -1) and metamorphosing (day 3) animals were examined by electron microscopy. At day -1, the gland appeared to be healthy with a thick basement membrane located near the lumen (Fig. 1). At day 3, the nuclei are located in basal regions and the cytoplasm is filled with autophagic vacuoles (Fig. 1). Our results suggest the following: 1) the earliest changes observed are in the cytoplasm; 2) there is no evidence for gland cell death that nuclear degradation precedes the collapse of the cell.

Energy Resources Remain Constant During The Early Phase Of Degeneration

Impaired or compromised availability of energy resources to gland cells may result in their death. We, therefore, wanted to measure ATP levels in glands at various stages of metamorphosis. Initial values of ATP (Fig. 2a) were approximately 275 μg/gland on the day prior to metamorphosis (day -1) and peaked to 350 μg/gland on day 0. Total ATP (Fig. 2a, bars) dropped slightly during days 0-2 of metamorphosis, remaining relatively constant as a function of protein (Fig. 2a). By day 3, total ATP levels dropped significantly compared to the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

levels seen at days 0-2 (Fig. 2a).

Mitochondrial Functioning Is Intact Until Late

Since compromising cellular respiration could possibly predispose a cell to die, we monitored mitochondrial function, by biochemical and histochemical MTT assays, in glands at various stages of development. Reduction of MTT peaked on day -1 and thereafter gradually declined during metamorphosis (Fig. 2B). These biochemical observations were confirmed by microscopic examination of glands maintained in culture with MTT (Fig. 3). Day -1 glands stained the most intensely, indicating that the majority of mitochondria were functional, while day 5 glands stained the least intensely (Fig. 3).

Localization Of Lysosomes

Having established that the gland is destroyed by a lysosomal autophagic death (Fig. 1), we wanted to localize these lysosomes at various stages of cell death. Alterations in lysosomal movement and activity may affect the death of the glands. To assess lysosomes, we measured acid phosphatase (AP), a lysosomal marker enzyme, by histochemical and biochemical assays. Prior to metamorphosis, in day -1 glands, lysosomes were identified only along the basal surfaces of these cells (Fig. 4a). During metamorphosis lysosomes migrated through the cytoplasm and increased in quantity. By day 3, the cytoplasmic volume has decreased and virtually the entire mass of the remnant cytoplasm consisted of lysosomes identified as large autophagic vacuoles (Fig. 4b). This condition persisted throughout pupation, the gland was not followed past day 5.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

No lysosome-bearing phagocytes were seen in the vicinity.

We wanted to determine the baseline levels of activity of AP to see if there were any changes during spontaneous metamorphosis. Glands were isolated from animals at various stages and biochemical values of AP were determined. AP activity started to rise as early as day -2 , it leveled off, and peaked at day 3 (Fig. 5). Our biochemical AP data confirms our histochemical AP results during gland cell death. These data suggest that alterations in lysosomes occur early and can be monitored in degenerating glands.

Changes In The Levels Of Second Messengers Are Late

Since fluctuations in the levels of signaling molecules may trigger cell death, we measured the levels of three second messengers during gland cell death. Cyclic AMP remained relatively constant, as a function of protein, during days -1 to 1. A gradual drop in total cAMP (Fig. 6a) reflected the loss of protein; cAMP/μg protein remained relatively steady throughout this period (Fig. 6a). Cyclic AMP fell markedly only when a massive collapse of the gland was evident morphologically, like that observed on day 3.

A radioreceptor assay (Amersham, Arlington Heights, IL) gave exclusively negative values for cGMP. The problem was traced to a dialyzable, heat-stable and non-precipitable component of the extract. Since this problem had been seen before (Weller et al. 1972), we therefore switched to a radioimmunoassay kit (NEN). No interference with the antibody was detected, and this assay was used for further assays. Total cGMP remained rather steady throughout early

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

metamorphosis, even increasing modestly relative to protein (Fig. 6b).

IP_3 underwent a modest but statistically significant fall on day 0 (Fig. 6c), in proportion to loss of protein (Fig. 6c) and gradually declined thereafter. The effect of this drop in IP_3 on gland cell death is currently under investigation.

DISCUSSION

Gland Cell Death Is Not Apoptotic

Programmed cell death can be either apoptotic or lysosomal. The morphology of labial gland cell death is similar to the cell death of *Manduca* intersegmental muscle (Beaulaton & Lockshin, 1977) or *Bombyx* silk gland (Akai, 1984), but differs from mammalian apoptosis in that cytoplasmic destruction is the most prominent early feature. The cytoplasm is destroyed in these systems by lysosomal autophagic vacuoles. The lysosomal enzymes are capable of degrading all macromolecules present in the cytoplasm. Clearly the morphological features found in degenerating labial glands fits that of type II cell death, lysosomal death (Schweichel & Merker, 1973).

Energy Metabolism & Cell Death

Various lines of evidence have implicated altered cellular metabolism in the activation of cell death. Beal (1992) has shown that the delayed onset of neurodegenerative illnesses could be related to the progressive impairment of mitochondrial energy metabolism. In contrast, our results suggest that mitochondrial respiration in the glands does not fail until late during spontaneous metamorphosis. Jurkowitz-Alexander et al. (1992) demonstrated that cell death of

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

a glial cell line was dependent on ATP depletion. Our results conflict with their findings and suggest that ATP levels are adequate until later stages of degeneration. The late changes in the metabolism of the gland, therefore, are not likely to trigger the rapid drop in protein synthesis seen on day 1 (Zakeri et al., 1996) or the death of the gland.

Cellular Signaling & Cell Death

Emerging evidence indicates that cell death is regulated by some of the same signal transduction pathways previously implicated in other physiological cellular responses. McConkey et al. (1992) have found that increases in cAMP can trigger thymocyte apoptosis. Khan et al. (1996) reported that B and T lymphocytes undergoing apoptosis in response to anti-immunoglobulin M antibodies and dexamethasone, respectively, were found to have increased amounts of mRNA for the inositol triphosphate receptor (IP_3R) and increased amounts of IP_3R protein.

We were unable, however, to detect any early and significant alterations in the levels of second messengers in degenerating gland cells. An increase in cGMP levels during degeneration of insect muscle has been reported (Schwartz & Truman, 1984). We did not detect sufficient changes cGMP or in the other second messengers to account for a conversion of metabolism. It may be possible that the time required for dissection might have obtunded or blunted the changes, since levels can change in seconds. The late changes that we found were not likely to shut down total protein synthesis or induce cell death. Signals

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

that promote cell death in one model can suppress cell death in another, indicating that cellular responses are determined by the intrinsic programming of the cell in question.

The Role Of Lysosomes In Gland Cell Death

Lysosomes probably destroy the machinery of protein synthesis in labial glands during cell death. We and others (Sensibar et al. 1990; Jones & Bowen, 1993) confirm that the destruction of the cytoplasm is lysosomal, but the expansion of the lysosomal compartment does not appear to account for the failure of protein synthesis. The shift in the position of lysosomes suggests cytoskeletal alterations, (discussed in Chapter IV). Zakeri et al. (1996) detected early rearrangements of the rough endoplasmic reticulum in the glands at day 0. The activation of lysosomes may account for this change.

We have not yet accounted for the decline in protein synthesis. Energy levels appear to be adequate during early metamorphosis and intracellular second messages do not change enough to invoke any generalized change in state of phosphorylation. Lysosomes play a major role in the final collapse of the cell, but presumptively do not disrupt the rough endoplasmic reticulum rapidly enough to account for the drop (Zakeri et al., 1996). While transcription appears to decrease, other means of inactivation of protein synthesis must be involved. We are currently searching for these possibilities.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER II. DEVELOPMENT OF LABIAL GLAND *IN VITRO* SYSTEM

A. Introduction
B. Materials And Methods
C. Results
1. The Labial Glands Can Be Maintained In Culture
2. Effect Of 20-HE On Lysosomal Activity Of Cultured Glands
E. Discussion: 20-HE Does Not Appear To Directly Trigger Gland Cell Death

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER II. DEVELOPMENT OF LABIAL GLAND *IN VITRO* SYSTEM

INTRODUCTION

Having established the pattern of cell death and specific parameters for its examination in the metamorphosing gland, we were interested in determining if there were any hemolymph factors or hormones required to induce the death of the glands. To do so we set out to develop an *in vitro* system to facilitate manipulation of the gland and measurement of specific cell death parameters and to test mechanistic hypotheses.

An *in vitro* culture system provides a powerful tool to decipher these mechanisms. In primary cell culture it is possible to manipulate external cues, including steroid hormone levels, precisely, and monitor the consequences on cell death with a high degree of resolution. The controlled conditions of cell culture can allow us to follow the sequence of cell death in the gland more precisely. The *in vitro* labial gland system would provide a simpler and controllable system devoid of juvenile hormone or other factors which may interfere with cell death. The proper experimental design of the culture system should make it possible to gain insights that can then be tested *in vivo.* The culture system may enable us to understand the sequence by which the labial glands initiate cell death and how 20-hydroxyecdysone (20-HE) may affect this process.

Since the cells of the labial gland are terminally differentiated cells, we asked what fates these cells have in culture, that is do they: 1) die immediately when placed in culture?; 2) maintain their *in vivo* programming and die over a five day

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

period?; or 3) be re-programmed to die at a different rate than normal, precocious or delayed death? To assess the integrity of glands maintained in culture, we examined protein synthesis and mitochondrial function. Our *in vitro* studies suggest that for cell death activation a requirement of intact organismic relationships may be needed.

MATERIALS & METHODS

Rearing Of Animals

The larvae were reared as described in Chapter I.

In Vitro Culture & Treatment

Dissecting instruments were sterilized by incubating at 160° C, dry heat, for at least 1 hr. Day 0 larvae were surface sterilized with 70% ethanol and dissected on a paraffin dissecting surface that had been sterilized with 70% ethanol. Glands were removed from the animals and were placed in Grace's medium containing antibiotics. Fifty μl of PenStrep [Penicillin-streptomycin solution (PenStrep; Sigma; 10,000 units penicillin and 10 mg streptomycin/ml) was diluted 1:100 with dH_2O] were added to culture dishes containing 5 ml of Grace's medium (Gibco BRL, Grand Island, NY). Glands were incubated at room temperature with gentle shaking or at 37° C in a 95% O_2/5% CO_2 incubator for various times. A PenStrep resistant contaminant was detected after 24 hr of culture in glands that were incubated at room temperature and in the incubator.

Since the gut of the animals contained PenStrep resistant organisms, we had to work hard to prevent contamination and to eliminate them. To determine

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

which antibiotic the contaminant was sensitive to, the following screening was performed. One hundred μl of contaminant were added to 3 ml of Luria-Bertani (LB) Top Agar (0.8 g of Bacto-agar/100 ml LB broth; Difco, Detroit, MI) which was autoclaved prior to use. Sterile Top Agar was stored at 4°C and melted in a microwave oven before use. Top Agar was cooled to 45°C and held molten at this temperature for plating. The following antibiotics were diluted, with dH_2O, to their working concentrations: ampicillin (Sigma; 10 mg/ml) was diluted 1:100, gentamicin (Sigma; 50 mg/ml) was diluted 1:150, and tetracycline (Sigma; 10 mg/ml) was diluted 1:200. Discs were autoclaved and dipped in 95% ethanol prior to dipping into the antibiotic solutions. Two discs per antibiotic were placed onto a lawn of contaminated broth plus Top Agar and incubated at 37°C overnight. Two zones of inhibition, on the contaminated lawn, were observed when the gentamicin-treated discs were used. No zones of inhibition were detected when discs were treated with the other antibiotics. Our results indicated that the microorganisms were sensitive to gentamicin. We therefore chose this antibiotic as our permanent choice for our *in vitro* experiments.

Glands were placed in sterile 60 x 15 mm dishes containing sterile Grace's Media (Gibco BRL, Grand Island, NY) pH 6.5 supplemented with 100 μg/ml gentamicin (Biofluids, Rockville, MD), 2.5 μg/ml amphotericin B (Sigma), and 5% horse serum (Biofluids) and taken to a laminar flow hood (NuAire, Plymouth, MN). Glands were washed three times with Grace's medium. Glands were placed in 35 x 10 mm culture dishes (Corning, Corning, NY) and incubated in Grace's medium

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

alone or in Grace's medium containing 1 μg/ml 20-HE in a 95% O_2/5% CO_2 incubator at 25° C for various times. The medium was changed daily.

Measuring The Energetics By MTT

In some experiments the glands were transferred to Grace's medium containing MTT and incubated for 1 hr in the dark for MTT assays as described in Chapter I.

Biosynthetic Labeling Of Proteins

The glands were removed from culture after the appropriate incubation period and were prepared for protein synthetic analysis. Control and experimentally treated glands were rinsed in sterile complete Grace's medium and transferred to methionine-free Grace's medium supplemented with 250 μl of ^{35}S methionine (240 μCi/ml; > 1,000 Ci/mmol; Amersham, Arlington Heights, IL) in 15 mm multidish wells (Nunc Inc., Naperville, IL). The glands were incubated at room temperature for 1 hr with shaking.

1-D Polyacrylamide Gel Electrophoresis

Glands were prepared for one-dimensional polyacrylamide gel electrophoresis (1-D PAGE) by homogenization in 500 μl of Laemmli sample buffer [Laemmli, 1970; 0.0625 M Tris (Fisher Scientific, Pittsburgh, PA), 2% SDS, 10% glycerol, 5% ß-mercaptoethanol (Sigma), 0.001% bromophenol blue, pH 6.8]. Aliquots of the samples were stored at -70° C for future analysis. Polyacrylamide gels were made from 30% acrylamide (ICN; Cleveland, OH)/0.8% bisacrylamide (Fisher Scientific). The separating gel (1.5 mm thick, 16 cm X 14 cm) consisted

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

of 0.375 M Tris, pH 8.8, 0.1% SDS, and 10-12% acrylamide. The separating gel was polymerized with the addition of 165 μl 10% ammonium persulfate (AP; Bio-Rad, Hercules, CA), and 33 μl of N,N,N', N'-tetramethylethylenediamine (TEMED; Bio-Rad). The separating gels were covered with 1 cm of isobutyl alcohol (J.T. Baker, Phillipsburg, NJ) and allowed to polymerize for 45-60 min. The isobutyl alcohol layer was poured off and the top of the gel was rinsed with dH_2O. The stacking gel consisted of 0.125 M Tris, pH 6.8, 0.1% SDS, and 5% acrylamide. The stacking gel was polymerized by the addition of 50 μl 10% AP and 10 μl TEMED. A 1.5 mm comb was inserted into the stacking gel which was allowed to polymerize for 30-45 min. The comb was removed and the wells were rinsed with Laemmli running buffer (0.025 M Tris, 0.192 M glycine (ICN), 0.1% SDS, pH 8.3). The gel sandwich was attached to the upper chamber of the electrophoresis apparatus (Marsh Biomedical, Rochester, NY) which was filled with running buffer. The samples and Rainbow molecular weight markers (Amersham) were boiled for 5 min prior to loading and sample buffer was added to any empty wells to equalize the volume in all wells. The gel was run at 8 volts/cm until the bromophenol blue dye entered the separating gel. Then the voltage was increased to 15 volts/cm and the gel ran until the dye front was at the end of the gel. Some gels were run overnight at 40 volts.

The gel was stained in 0.05% Coomassie blue (Bio-Rad), 50% methanol, and 10% acetic acid and gently rotated, 40 RPM, at room temperature for 30 min. The gel was destained in several changes of 7% acetic acid and 5% methanol.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

The gel was transferred to EnHance (Du Pont NEN, Boston, MA) for fluorography and incubated with shaking at room temperature for 1 hr.

Gel Drying

The gel was placed on Saran Wrap and a piece of gel blot paper (Schleicher & Schuell, Keene, NH) was placed on top of the damp gel. Another piece of gel blot paper was placed on the drying surface of the gel dryer. The gel blot paper/gel/Saran Wrap sandwich were placed in the dryer. The gel dryer's lid was closed, suction was applied, and the gel was dried for 2 hr at 80° C. The gel was exposed to film at -70° C for appropriate times.

Preparation Of 20-HE Stock Solution

20-hydroxyecdysone (20-HE) stock solutions were prepared by adding 100 μl of 100% ethanol and 900 μl of sterile distilled water to 1 mg 20-HE (Sigma). The 20-HE solution was filtered through a 0.22 μm membrane (Milipore, Bedford, MA) in a laminar flow hood (NuAire Inc., Plymouth, MN). The concentration was checked spectrophotometrically at 240 nm. A 1 μg/μl 20-HE solution has an A_{240} = 26.40 (Meltzer, 1971).

Lysosomal Enzyme Assays

The localization and measurement of acid phosphatase (AP) were performed as described in Chapter I.

The activity of α-galactosidase A (GalA) was measured using the fluorescent substrate 4-methylumbelliferyl-α-D-galactopyranoside (MUG). Glands were homogenized in 0.5 ml of 10 mM sodium phosphate buffer (pH 6.5). The

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

homogenate was clarified by centrifugation at room temperature for 5 min. The reaction mixture (200 μl of 400 mM sodium acetate buffer, pH 4.5, 800 μl of 5mM MUG, and 10 μl of homogenate) was incubated at 37° C for 30 min. The reaction was terminated by the addition of 3.5 ml DAE to 20 μl of the reaction mixture and the fluorescent units of GalA activity were measured using a fluorometer.

Acridine Orange Staining of Whole Mounts

Acridine orange stock solution was prepared with Grace's medium to yield a 5 mg/ml concentration and was stored at -20° C. The stock solution was diluted 1:100 with Grace's medium to yield a final concentration of 50 μg/ml. Glands that had been cultured for various times were incubated, with the diluted acridine orange solution in culture dishes (Corning), for 5 min at room temperature. Glands were placed on Vectabond (Vector Laboratories, Burlingame, CA) coated slides, a 20 X 40 mm coverslip (Corning) was superimposed, and the slides were examined by fluorescence and confocal microscopy using rhodamine filters.

RESULTS

The Labial Glands Can Be Maintained In Culture

We first asked was it possible to maintain the glands in culture. One of our initial difficulties was the maintenance of the gland for more than 24 hr due to PenStrep resistant contaminants. With the addition of gentamicin, amphotericin B (an antifungal), and 5% horse serum to our culture medium, we were able to extend our incubations to 120 hr.

To assess if it was possible for the glands to be functionally viable in the *in*

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

vitro experiments, we measured different parameters that we had examined in the *in vivo* model of gland cell death. One such parameter was the measurement of mitochondrial respiration by the MTT assay. During normal gland development and cell death the mitochondrial functioning is high in glands from pre-metamorphosing animals and slowly declines by D 3 (Figs. 2b,3; Halaby et al., 1994). We initially compared the MTT staining of freshly dissected to that of cultured premetamorphic (days -3 and -1) or metamorphic glands (day 1). Glands were incubated for periods of 6 hr, 18 hr, or 24 hr prior to performing the MTT assay. All glands showed equivalent MTT staining (Fig. 7) with slight variabilities in staining intensity from one section of the gland to another. This was also the case for MTT staining of freshly dissected glands undergoing spontaneous metamorphosis (Fig. 3). This may be due to the folding of the tissue during the assay. Our results indicate that the glands continue to respire normally for up to 24 hr in culture.

Since functionality of the gland appears to be maintained, we asked if there may be differences in protein synthesis that might induce a more subtle alteration. For these experiments, glands were cultured and incubated with [^{35}S] methionine, and equal amounts of protein were separated by 1-D PAGE. We detected no significant changes in the pattern of protein synthesis during different incubation periods.

To determine the effect of 20-HE on protein synthesis, we performed the same experiments with glands treated with or without 20-HE. When

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

premetamorphic glands were exposed *in vitro* to physiological concentrations of 20-HE (1 μg/ml; 2.1 X 10^{-6} M) or to 20-HE *in vivo* for 8 hr, protein incorporation was similar irrespective of the treatment (Fig. 8). When we increased the *in vitro* concentration of 20-HE to 5 X 10^{-5} M, above physiological levels, protein incorporation did not change (Fig. 9). Since we did not detect any significant differences in control or experimental glands by 1-D PAGE, the analysis of protein synthesis was not pursued.

Effect Of 20-HE On Lysosomal Activity In Cultured Glands

To determine if the lysosomal activation seen as early as day 0 during spontaneous metamorphosis was directly or indirectly affected by 20-HE we exposed the gland in culture to exogenous 20-HE. We assayed the progression of cell death by acid phosphatase (AP) activity histochemically and biochemically. There were slight variabilities and inconsistencies in staining intensity with the histochemical assay, depending on the regions that were examined. We therefore performed our investigations on lysosomal activity using the biochemical assay.

We performed studies with glands from premetamorphosing animals to see if 20-HE *in vitro* could induce precocious cell death. In these experiments, glands from different animals served as control and experimental glands. Premetamorphic glands, day -2, were cultured in the presence or absence of physiological levels of 20-HE at this stage of development (0.1 μg/ml, 2.1 X 10^{-7} M) for 8 or 24 hr. There were no statistically significant differences in biochemical AP levels between day -2 control and experimental glands at either incubation period (Fig. 10).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Similar results were obtained from day -2 glands incubated for 24 hr in the presence or absence of slightly higher than physiological levels of 20-HE (0.14 μg 20-HE/ml, 3 X 10^{-7} M; Fig. 11). Although the differences were not significant, there was some degree of variability between control and experimental glands. We concluded from these studies that 20-HE may not affect lysosomal activity in glands at these early stages.

To localize lysosomes, glands were stained with acridine orange which stains lysosomes red-orange. Experiments were performed with D0 glands cultured for 72 hr with or without physiological levels of 20-HE (1 μg/ml). We chose this incubation period in an attempt to mimic the conditions at D3 (maximal AP activity during spontaneous metamorphosis; Fig. 5). Following the culture period, glands were stained with acridine orange. The staining intensity of lysosomes was nearly identical in control and experimental glands (Fig. 12). These experiments suggest that 20-HE does not directly influence the movement of lysosomes seen during spontaneous metamorphosis.

We postulated that perhaps premetamorphic glands were not yet able to respond to 20-HE. Thus, we decided to measure total AP levels from metamorphic glands exposed to 20-HE *in vitro*. Since these glands degenerate during spontaneous metamorphosis, we thought that they might be more suitable for elucidating 20-HE's effects on gland cell death. Glands were isolated from D0 animals and exposed *in vitro* to 20-HE or without hormone for various incubation periods. Sister glands were used as control and experimental glands in the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

metamorphic gland experiments to minimize the variability in histochemical AP results from one animal to another. Glands were exposed to physiological levels of 20-HE (1 μg/ml; 2 X 10^{-6} M) for experiments with metamorphic glands to mimic endogenous hormone levels at this stage. There were no statistically significant differences in AP activity between D0 control and experimental glands at 24 hr, 48 hr, 72 hr, and 120 hr (Fig. 13). There was a significant increase, however, in AP levels at 24 hr compared to levels at 0 hr (Fig. 13). This suggests that the glands may be initially stressed when cultured. We concluded from these experiments that 20-HE may have an indirect effect on lysosomal enzymes.

To determine whether our results on the relationship between 20-HE and lysosomal enzyme activation were specific for acid phosphatase or a more generalized phenomenon, we performed experiments using another lysosomal enzyme, α-galactosidase A (GalA). Mutations in the GalA gene result in the sphingolipidosis called Fabry disease (Desnick & Bishop, 1989). Initially we established GalA activity in glands during spontaneous metamorphosis. GalA levels increased slowly in premetamorphic glands, slightly increased on day 0, and peaked on day 3 (Fig. 14). GalA activity for glands that were cultured at day 2 for 48 hr in the presence or absence of physiological levels of 20-HE (2 X 10^{-6} M) are shown in Fig. 15. 20-HE, *in vitro*, did not induce significant differences in GalA levels between control and experimental glands. These results suggest that 20-HE effects on lysosomes in general may be indirect.

DISCUSSION

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Effect Of Steroids On Lysosomes In Other Systems

Other steroids have been shown, *in vitro*, to reduce the activity of lysosomal enzymes. Kremer et al. (1995) demonstrated that *in vitro* exposure to estrogen by avian osteoclasts caused a reduction in the levels of cathepsin L and β-glucuronidase. Lasnitzki et al. (1965) reported that testosterone-treated prostate glands, in culture, showed less acid phosphatase activity than untreated explants. Moreover, estrogen has been shown to prevent oxidative stress-induced cell death *in vitro* in a mouse neuronal cell line (Behl et al., 1995). These studies lend support to our theory that 20-HE acts indirectly on lysosomes.

20-HE *In Vitro* Does Not Activate Lysosomal Enzymes

The histochemical assay for acid phosphatase (AP) allowed us to localize lysosomal enzymes in the cytoplasm of gland cells. Experiments with this technique, however, revealed that the intensity of the AP staining was variable. Depending on the regions within serial sections that were examined, some cells were highly positive for AP while neighboring cells were not. The histochemical assay also failed to give reproducible results. We therefore preferred the biochemical assay for AP which detects total AP levels for the entire gland, rather than examining one region at a time. This assay was very reproducible and more sensitive and consistent than the histochemical assay.

We chose physiological concentrations of 20-HE in order to mimic endogenous levels of hormone in the hemolymph. The 20-HE endogenous peak titers are 0.07 μg/ml, 1.5 X 10^{-7} M, on day -0.5 and 1.5 μg/ml, 3.1 X 10^{-6} M, on day

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

3 (Bollenbacher et al., 1981). In our hands 20-HE *in vitro* did not alter acid phosphatase (AP) or α-galactosidase A activity. Staining cultured glands with acridine orange also indicated that 20-HE's effect on lysosomal movement may be indirect. Other studies have reported similar results. Caglayan (1990) reported that the activity of AP was not altered in *in vitro* cultures of *Manduca* fat body in response to various 20-HE concentrations and at different incubation periods. Richard et al. (1993) were also unsuccessful in their attempts to demonstrate histolysis *in vitro* of *Drosophila* fat body cells. These results and ours are in contrast to the work of Chinzei (1975). He reported that silk glands of *Bombyx mori* undergo histolysis when exposed to 20-HE *in vitro*. The sustained presence of 20-HE *in vitro* may not be necessary to bring about cell death of target tissues. It has been shown that although 20-HE may directly effect cell death in a variety of tissues *in vitro*, further degradation was independent of the hormone (Chinzei, 1975; Dean, 1978; Zacchary & Hoffmann, 1980). Since we have not demonstrated synthesis of lysosomal enzymes, the increase in enzyme activity in the labial gland, seen *in vivo*, may be attributed to *de novo* enzyme synthesis or to the activation of pre-existing inactive enzymes (van Pelt-Verquil, 1979).

20-HE did not induce precocious death in pre-metamorphic glands or programmed cell death in metamorphic glands, seen *in vivo* during pupation, in our *in vitro* system. Likewise, *in vitro* studies performed with *Drosophila* fat body (Richard et al., 1993) and *Manduca* abdominal motoneurons (Bennett & Truman, 1985) and antennal-lobe neurons (Oland & Hayashi, 1993) demonstrated that 20-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

HE appears to act indirectly.

An alternative interpretation is that our *in vitro* system may not be adequate to promote cell death of the glands. The cells of the labial gland may be stressed in culture, irrespective of 20-HE's presence. Choi & Fahrbach (1995) demonstrated that aqueous extracts of pterothoracic ganglia, applied to cultured ventral nerve cords, were required to induce neuronal death. Our culture system may lack putative hemolymph or other factor(s) responsible for the demise of the labial gland cells, and Grace's medium may not be able to replace these factors.

The *in vitro* effects of 20-HE on labial gland cell death may be explained by the model originally developed by Ashburner et al. (1974). 20-HE induces the expression of early genes that encode transcription factors (Segraves & Hogness, 1990; Burtis et al., 1990; DiBello et al., 1991). These transcription factors also feedback negatively to turn off their own transcription in the continued presence of 20-HE. The continued exposure of cultured glands to 20-HE may repress the expression of death-related genes. The withdrawal of 20-HE may be required to fully activate these genes. To test this hypothesis, we will expose D0 glands to 20-HE *in vitro* for 24 hr, then the glands will be transferred to hormone-free medium for the duration of the incubation period. If 20-HE is a trigger, then a 24 hr pulse may be sufficient to initiate cell death as seen in our ligation experiments (Chapter III).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER III. THE RELATIONSHIP BETWEEN 20-HE *IN VIVO* AND LABIAL GLAND CELL DEATH

A. Introduction
B. Materials And Methods
C. Results
1. Effect Of 20-HE On Premetamorphic Glands
2. Effect Of 20-HE On Metamorphic Glands
3. 20-HE Appears To Trigger Gland PCD
E. Discussion: 20-HE Triggers But Does Not Sustain Programmed Cell Death Of Labial Glands

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER III. THE RELATIONSHIP BETWEEN 20-HE *IN VIVO* AND LABIAL GLAND CELL DEATH

INTRODUCTION

Programmed cell death (PCD) is readily recognizable in insects as larval tissues degenerate in order to make way for adult tissues (Miller, 1950; Finlayson, 1956). PCD of insect tissues is predictable, controllable, and precise. The selective destruction of target tissues during metamorphosis appears to be under hormonal control, namely 20-hydroxyecdysone (20-HE). In most cases rising 20-HE signals the death of larval tissues. The death of neurons in *Manduca sexta* larvae is induced by the prepupal peak of 20-HE on day 3 (Weeks & Truman, 1985). However, delayed degeneration, such as that of the intersegmental muscles (ISM), is controlled by a decline in 20-HE (Schwartz & Truman, 1983; Schwartz & Truman, 1984). The exact mechanisms by which 20-HE induces cell death in such a variety of tissues are as yet unknown.

The labial glands of the tobacco hornworm, *Manduca sexta*, die a lysosomal, autophagic death over a five day period during pupation. Our studies from Chapter I showed that cytoplasmic alterations, increased activity and movement of lysosomes, in degenerating glands coincided with rises in endogenous 20-HE levels. This indicates that the hormone may play a role in the histolysis of the gland (Bollenbacher et al., 1981). Our studies from Chapter II, however, demonstrated that 20-HE was unable to induce cell death in our *in vitro* system. This could be explained by the fact that our culture conditions are lacking

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

a factor(s), which is only found in whole animals, that is necessary to elicit 20-HE's effects on the gland. We, therefore, decided to pursue a line of *in vivo* experiments to elucidate 20-HE's role in labial gland PCD. *Manduca* is particularly well suited for such studies because the blood titers of 20-HE during metamorphosis are known in more detail than for any other insect (Bollenbacher et al., 1981).

Our *in vivo* studies consisted of three approaches. We initially began our studies with glands from premetamorphosing animals. Since these animals have not yet been exposed to high levels of endogenous 20-HE, we wanted to investigate whether exogenous hormone could induce precocious death of the gland. Premetamorphosing animals that are younger than day -2, however, are exposed to high levels of other endogenous juvenile hormone (JH). This hormone antagonizes the effects of 20-HE and promotes larval development in order to prevent premature metamorphosis. To circumvent the influence of JH, we also used prmetamorphosing animals at later stages in development, like day -1, when JH is at undetectable levels (Nijhout & Williams, 1974).

Our second set of studies involved metamorphosing animals. As mentioned in Introduction, these animals have relatively high levels of endogenous 20-HE. It would be very difficult to ascertain if alterations in cell death parameters were due to endogenous or exogenous 20-HE. To facilitate this process, we created essentially 20-HE free abdomens. This was accomplished by ligating animals behind their only known source of 20-HE production, the prothoracic glands

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

located in the thorax, at the first abdominal segment and discarding the body anterior to the ligature. As a result of this surgical manipulation, we could clearly correlate alterations in our established cell death parameters, lysosomal activity and DNA fragmentation, with exogenous 20-HE. We performed two kinds of experiments with isolated abdomens. Initially the abdomens were left untreated (no injections) for various times prior to dissection and analysis of the glands. In other experiments the isolated abdomens were injected with or without 20-HE for various times prior to assaying the glands for cell death.

Our third set of studies also involved using glands from isolated abdomens. We found from the second group of experiments above, that *in vivo* exposure of glands to 20-HE increased the levels of DNA fragmentation and acid phosphatase (AP) activity. The fluctuating nature of 20-HE secretion in the fifth instar of *Manduca* (Bollenbaher et al., 1981), implied that the hormone's effects could be the result of it's actions as either a switch or constant "on" signal. We, therefore, wanted to determine if the hormone was acting to trigger cell death or was it needed continually to maintain cell death. Isolated abdomens were therefore injected with single or multiple injections of vehicle or 20-HE and labial glands were isolated after various incubation periods. We then performed assays corresponding to our cell death parameters. Our conclusions from this data were that 20-HE acts to trigger cell death, since sustained exposure of glands to 20-HE did not induce higher levels of lysosomal movement and activity or nuclear degradation.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

MATERIALS & METHODS

Rearing Of The Animals

Larvae were reared as described in Chapter I.

20-HE Injections

Control and experimental animals that were at the same stage of metamorphosis or that weighed within ± 0.2 g of each other (premetamorphosing animals) were used. Animals were injected, in the abdomen, with 50 μg of 20-HE (1 μg/μl stock; Sigma, St. Louis, MO) or an equal volume of 10% ethanol (the vehicle) using a 30 gauge needle attached to a 50 μl Hamilton syringe. Assuming that hemolymph volume represented 40% of body weight (Cymborowski et al., 1982) and the fact that the molecular weight of 20-HE is 480.6 g/mole, the 20-HE concentration range in premetamorphosing animals was from 3.3×10^{-5} M (D -1) to 1.3×10^{-4} M (D -4). The metabolic half-life of injected ecdysteroids varies between a few minutes to 10 hr during larval development of holometabolous insects depending on the species and the type of ecdysteroid (Koolman. 1982). The glands were dissected after appropriate times and examined for cell death.

Surgical Manipulations

The relatively large size of *Manduca* facilitates surgical and endocrine manipulations *in vivo*. The only known source of 20-HE in *Manduca* is the prothoracic glands which are located in the first thoracic segment. To produce isolated abdomens that were free from this endogenous source of 20-HE, day 0 larvae were ligated between the thorax and abdomen. Day 0 larvae were

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

anesthetized on ice for at least 20 min, ligated around the first abdominal segment, using dental floss, and the anterior body was severed to remove the prothoracic glands. The wounds were sealed with a cyanoacrylate glue (Krazy Glue; Borden, Columbus, OH). Isolated abdomens were left untouched overnight to allow sufficient time for the endogenous 20-HE levels to decline. Starting the next day, the abdomens received single injections, every 24 hr, of either 50 μg 20-HE or an equal volume of 10% ethanol daily prior to dissection of the glands. The 20-HE concentration in isolated abdomens was between 2.2-2.9 X 10^{-5} M. Each injection was placed in a different abdominal segment to avoid excessive damage to one site. In other experiments isolated abdomens received a single injection of 20-HE or vehicle and the labial glands were dissected at the 120th hr.

In some experiments isolated abdomens also received a single injection of cycloheximide (CHX; Sigma), a reversible inhibitor of eucaryotic protein synthesis. One hundred μg of a CHX stock (2 μl of 50 μg/μl stock) were injected, using a Hamilton syringe, in a different abdominal segment than vehicle or 20-HE.

Tissue Preparation & Sectioning

Tissue preparation and sectioning were performed as described in Chapter I with the exception that Vectabond coated slides were used instead of poly-L-lysine coated slides.

Biosynthetic Labeling Of Proteins

Protein synthesis was assayed by incorporation of [^{35}S] methionine into gland proteins as described in Chapter II.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1-D PAGE

Electrophoretic separation of gland proteins was performed as described in Chapter II.

Gel Drying

Gels were dried as described in Chapter II.

MTT Assay

Mitochondrial function was assayed using the MTT assay as described in Chapter I.

DNA Fragmentation Assay

Tissue sections were processed using the ApopTag detection kit (Oncor, Gaithersburg, MD). Slides were treated with target unmasking fluid (TUF; Signet Laboratories, Inc, Dedham, MA) in a Coplin jar at 95°C for 10 min. Slides were then placed at room temperature for 10 min in hot TUF. Slides were washed three times in phosphate buffered saline (1X PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.4) for 5 min at room temperature. The endogenous peroxidase activity was blocked by incubating the slides in 3% H_2O_2 for 5 min at room temperature and washing them twice with 1X PBS for 5 min. After application of equilibration buffer for 5 min, incubation with terminal deoxynucleotidyl transferase (TdT) and digoxigenin-11-dUTP was performed in a moist chamber for 90 min at 37°C, using plastic coverslips. Plastic coverslips were used to ensure more even staining of samples. The incubation was stopped by placing the slides in stop wash buffer for 30 min at 37°C, in a Coplin jar. After

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

washing three times in 1X PBS for 5 min, the slides were exposed to anti-digoxigenin-peroxidase conjugate, using plastic coverslips, for 30 min at room temperature in a moist chamber. Slides were stained in diaminobenzidine (DAB; Research Genetics, Inc., Huntsville, AL) using coverslips for 2 min and counterstained with methylene blue (Sigma) for 1 min in a Coplin jar. Slides were mounted with CrystalMount (Biomeda Corp., Foster City, CA). The red brown DAB color product was observed by light microscopy (Leica Inc., Deerfield, IL).

Lysosomal Enzyme Assays

Histochemical and biochemical acid phosphatase (AP) assays were performed as described in Chapter I and Chapter II, respectively.

In other experiments after *in vivo* exposure to 20-HE, glands were stained with acridine orange and examined by fluorescence and confocal microscopy as described in Chapter II.

RESULTS

Effect Of 20-HE On Premetamorphic Glands

We started our analysis of the relationship between 20-HE and gland cell death with glands from premetamorphosing animals. This was a reasonable starting point to test the hormone's ability to induce premature death in glands. Another advantage of using premetamorphosing animals was that it allowed us to avoid interference from endogenous levels of 20-HE, which are very low at this stage of development. Premetamorphosing animals, days -4, -3, -2, and -1, were injected with or without 20-HE for 8 hr or 24 hr incubation. The state of the gland

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

was assessed by examining differences in protein synthesis and activity of acid phosphatase (AP). For protein synthesis measurement, glands were dissected, transferred to methionine-free Grace's medium, and incubated *in vitro* with ^{35}[S] methionine for 1 hr. Protein was isolated and analyzed by gel electrophoresis. No significant changes in protein synthesis were detected between control and experimental glands at each stage of metamorphosis (Fig. 8). Some alterations in protein synthesis were detected between the stages, unrelated to the addition of 20-HE. This data suggests that 20-HE does not appear to alter protein synthesis at these stages.

Since lysosomal activity is a good and early indicator of the progression of cell death in the gland, we examined the activity of AP. AP activity was assayed by biochemical measurement. Day -4, -3, and -2 animals were injected with vehicle or 20-HE and gland were dissected after 8 hr or 24 hr. There was no significant change in the level of AP activity. We do detect a significant increase in the level of AP activity, however, in day -1 glands after 24 hr (Fig. 16). This result was noteworthy for two reasons: 1) day -1 coincides with the first peak of 20-HE levels; and 2) 20-HE may prematurely induce cell death of the gland an entire day earlier compared to spontaneous metamorphosis. Although 20-HE induced an increase in AP activity in premetamorphic glands, the hormone may not be able to hasten the metabolism of animals at these stages. Premetamorphosing animals that received daily injections of 20-HE started to metamorphose at the same time as control animals.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Effect Of 20-HE On Metamorphic Glands

To determine if 20-HE was necessary and sufficient to induce PCD in the glands, we performed experiments on ligated animals in the absence of exogenous treatments. Animals were ligated at D0 and isolated abdomens were left untreated (no injections) for various times and then their glands were evaluated for cell death by AP assays. The histochemical appearance of lysosomes from these experiments showed that few lysosomes were detected at 24 hr, 48 hr, and 72 hr (Fig. 17). The most intense reaction was detected at 96 hr. By 120 hr, the cytoplasm consisted mainly of remnant nuclei and therefore presumably did not stain as intensely compared to glands at 96 hr (Fig. 17). The biochemical AP values in these untreated glands was approximately similar from 48-120 hr except for a significant decline at 144 hr (Fig. 18).

Having established baseline values of AP, abdomens were injected with one injection daily of either 20-HE or vehicle for various periods. Exposure of labial glands to 20-HE increased the levels of AP activity at 8 hr and 72 hr incubation times only (Fig. 19). The 8 hr increase suggests that the 20-HE injection mimics the first endogenous peak of the hormone on day -0.5. The level of AP activity in 8 hr control glands was significantly lower than that of D0 glands from spontaneously metamorphosing animals (Figs. 5,19). This implies the following: 1) that ligation reduced the endogenous levels of 20-HE to low levels; and that these isolated abdomens provide us with an ideal environment in which to examine the effect of adding exogenous 20-HE on the gland. The result after 72 hr

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

suggests that the exogenous 20-HE mimics the second endogenous peak of the hormone.

Untreated control glands from above had similar levels of AP activity as ethanol treated glands at 48 hr, 72 hr, and 96 hr (Fig. 19), but AP activity in untreated glands was higher than that of glands during spontaneous metamorphosis. This suggests that a background level of cell death occurs in these abdomens presumably due to altered metabolism in the gland cells.

We confirmed the alterations in lysosomal activity by staining glands with acridine orange. Glands from day 3 spontaneously metamorphosing animals were used as a second control. Control (vehicle injected) and experimental glands were isolated from isolated abdomens. Control and experimental glands were incubated in the presence or absence of 20-HE for 24 hr and examined at 72 hr. We chose this incubation period to mimic the hormonal conditions that exist in D3 glands. In day 3 and control glands, the lysosomes were lightly scattered throughout the cytoplasm (Fig. 20a,b). Lysosomes from experimental glands, however, were densely scattered throughout the cytoplasm. Altered morphology occurred in experimental glands, such as shrinkage of the cytoplasm (Fig. 20c). Based on evidence in the literature, these results suggest that 20-HE's ability to induce movement of lysosomes in gland cells may involve the cytoskeleton (see Chapter IV).

We also attempted to localize lysosomes by using immunocytochemistry. We sought out to find antibodies that would specifically label lysosomes. Initially

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

we used a LysoTracker (Molecular Probes, Eugene, OR) fluorescent probe that label acidic organelles in living cells. LysoTracker probes are dyes not antibodies and as a result the staining in gland cells was diffuse. We did not pursue experiments with this dye because of this apparent nonspecific binding. We then decided to use an antibody that was specific for lysosomes. We performed immunocytochemical experiments using a lysosome associated membrane protein (LAMP-2) monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA). This antibody, used originally to immunize mice, was unable to cross react with labial gland sections. Further investigation is warranted in an effort to find additional methods to label lysosomes in dying gland cells.

To determine if 20-HE affects the synthesis of lysosomal enzymes, we used the eucaryotic inhibitor of protein synthesis, cycloheximide (CHX). Glands from isolated abdomens were exposed to either vehicle (10% ethanol), 20-HE, CHX, or CHX plus 20-HE for 24 hr and total AP levels were measured. The AP activity for control, 20-HE treated, and CHX treated glands was approximately equal (Fig. 21). The AP activity for the CHX/20-HE treated glands was 2-fold higher than control or CHX treated glands and 2.5-fold higher than 20-HE treated gland (Fig. 21). These results suggest the following: 1) lysosomal activity can be activated in the absence of protein synthesis; and 2) there may be a synergistic effect between 20-HE and CHX on lysosomal activity in labial glands. Alternatively, there may be proteins, synthesized at this time, that antagonize the effects of 20-HE.

We have previously used the MTT assay to assess cell viability in the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

glands. To determine whether 20-HE alters mitochondrial function during cell death of the gland, isolated abdomens were injected with multiple injections (one injection every 24 hr) of either vehicle or 20-HE over a 72 hr period. Glands were assessed by MTT analysis. We chose this incubation time because it represented a time when maximal cell death of the gland occurs during spontaneous metamorphosis. MTT reduction experiments revealed that experimental glands respired half as well as that of controls (Fig. 22). These results suggest that 20-HE compromises mitochondrial respiration in degenerating gland cells.

To determine if DNA degradation was under the influence of 20-HE, we used the ApopTag kit to identify new 3'-OH DNA ends generated by DNA fragmentation *in situ* in glands from isolated abdomens. One set of isolated abdomens received a single injection every 24 hr of vehicle or 20-HE. The staining of nuclei was more intense in 20-HE treated glands that were exposed to 20-HE for 48 hr and 96 hr (Fig. 23). Hormone treated glands also displayed altered morphology confirming our histochemical AP results.

20-HE Appears To Trigger Gland PCD

To determine if glands needed continuous exposure to 20-HE or if a single pulse of the hormone was sufficient to induce PCD, we performed the following experiments. A single injection of 20-HE or vehicle was administered and cell death assays were performed at 120 hr, five days later. We chose this particular incubation period because during spontaneous metamorphosis the gland dies over a five day interval. A single injection of 20-HE appeared to trigger PCD as

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

demonstrated by the increase in number and movement of lysosomes after 120 hr (Fig. 24). The level of lysosomal activity at 120 hr in glands after a single injection of 20-HE was comparable to that of glands from animals that had received daily injections of 20-HE (Fig. 25).

Having established that 20-HE induced DNA fragmentation in gland cells exposed to daily injections, we investigated if a single pulse of 20-HE could induce gland cells to die. Isolated abdomens were injected once with or without 20-HE and glands were assayed for DNA fragmentation after 120 hr. The staining of labeled nuclei in experimental sections was lower compared to that of controls at 120 hr (Fig. 26). This result may reflect the fact that in hormone treated cells, virtually the entire cytoplasm is destroyed and the gland consists primarily of remnant nuclei (Fig. 26) that may have fewer 3'-OH ends available for the TdT-catalyzed reaction to occur. The nuclei of glands from isolated abdomens that were treated with a single injection of 20-HE and dissected at 120 hr displayed comparable staining compared to controls (Fig. 26). The intensity of staining in experimental glands from animals that received one injection was comparable to that of experimental glands from animals receiving daily injections (Fig. 26) and glands at a later stage of cell death, day 4 (Fig. 27). The morphology in experimental glands, which resembled that of a day 4 or 5 gland, was different from that of the controls. The DNA fragmentation assay, however, did not indicate an that the experimental glands were at an advanced stage of cell death relative to controls.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

DISCUSSION

20-HE Secretion & Cell Death

A number of indirect data exist making it possible to assume that the degeneration of insect tissues occurs under the influence of 20-HE. Van pelt-Verkuil et al. (1979) reported that acid phosphatase (AP) activity could be evoked by transplanting inactive fat body of *Calliphora* into host larvae of a later developmental stage, when 20-HE titers are high. Bodenstein (1943) showed that when a salivary gland and the annular gland, produces 20-HE, of *Drosophila* at the beginning of the third instar were transplanted simultaneously into the abdomen of an adult fly, the salivary gland showed early signs of lysis.

The bimodal secretion of 20-HE during the fifth instar of *Manduca* appears to coincide with the alterations in our parameters for characterizing cell death. We have shown that 20-HE induces AP activity in premetamorphic, day -1, glands. This increase in enzyme activity is simultaneous to the commitment peak, which occurs on day -0.5, when the movement and activation of lysosomes were first detectable (Lockshin & Zakeri, 1994; Zakeri et al., 1996). We have also shown that 20-HE induces AP activity in glands from isolated abdomens at 72 hr incubation periods. The prepupal peak occurs on day 3, when virtually the entire mass of the remnant cytoplasm consisted of lysosomes and vacuoles (Halaby et al., 1994; Zakeri et al., 1993). These observations suggest that 20-HE may play a role in inducing the programmed cell death of labial glands.

Lysosomes And Cell Death

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

The activation of lysosomal enzymes that occurs during the involution of the prostate gland (Helminen et al., 1972), the mammary gland and the uterus (Woessner, 1969) has been shown to be regulated by steroid hormones. 20-HE is believed to trigger the death of insect tissues (Smith & Nijhout, 1983; Fahrbach & Truman, 1987; Robinow et al., 1993; Haas et al., 1995). The induction of lysosomal activity by 20-HE has been reported in other instances of insect PCD (Radford & Misch, 1971; Sass & Kovacs, 1975; Dean, 1978; van Pelt-Verkuil, 1979).

The increase in lysosomal enzyme activity during labial gland degeneration is one of the earliest detectable morphological markers of programmed cell death. It is detected on day 0, the onset of the larval to pupal metamorphosis (Zakeri et al., 1993; Halaby et al., 1994). The movement of lysosomes from basal to apical regions in the cells of the labial gland (Halaby et al., 1994) suggests that lysosomes play a pivotal role in the destruction of the cell. The lysosomal movement is presumably the result of alterations in cytoskeletal components which may be regulated by 20-HE (see Chapter IV).

Our data showed that AP activity in the labial gland was maximal on day 3 during spontaneous metamorphosis (Fig. 5). The labial gland undergoes lysosomal autophagy and the cytoplasm becomes highly vacuolated on day 3. Therefore, the observed increase in the acid hydrolase activity may be associated with this process. A parallel condition has been reported for the mid-gut of *Spodoptera eridania* where the activities of lysosomal enzymes were correlated with the extensive tissue degradation at the end of the larval development (Young,

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

1979).

We have shown that 20-HE induces lysosomal enzyme activity in glands from intact premetamorphosing and ligated animals and lysosomal movement in dying gland cells in isolated abdomens. This suggests that 20-HE can induce a precocious lysosome response in the glands. It is not certain whether the lysosomal enzymes are primarily responsible for initiating the histolytic action or whether they in turn depend upon prior alterations with the target cells. What is clear, however, is that the injection of 20-HE alone into day -1 larvae is sufficient to produce an early lysosome response identical, so far as we can tell, to that which would normally occur at a later time in experimental glands from isolated abdomens (Fig. 19).

The increase in AP activity may be attributed to *de novo* enzyme synthesis, to the activation of pre-existing inactive enzymes, or to changed properties of the enzyme such as altered pH (van Pelt-Verkuil, 1979). An alternative mechanism may involve sequestration of exogenous lysosomal enzymes by a tissue (Collins, 1975). 20-HE may increase the synthesis of hydrolytic enzymes in our experiments as during the larval-pupal metamorphosis there is a coincident increase in protein synthesis with increased lysosomal enzyme activity (Laufer, 1961; Aidells et al., 1971; Smith & Birt, 1972). In tadpole tail resorption, neosynthesis of acid hydrolases has been shown to be regulated by thyroxine (Weber, 1962; Tata, 1966). We showed contrasting results to those of others. Our experiments with cycloheximide (CHX) suggest that 20-HE may be activating pre-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

existing lysosomal enzymes. An alternative possibility me be that proteins which antagonize or block the effects of 20-HE are synthesized. The molecular processes that are responsible for the increase in AP activity during labial gland cell death deserve further investigation.

Our histochemical AP data with untreated glands from isolated abdomens clearly showed that the number of lysosomes remains fairly low for the 24-72 hr (Fig. 17). 20-HE titers are known to increase sharply during this period in *Manduca* (Bollenbacher et al., 1981). Ligation prevents this increase in 20-HE titer in the isolated abdomens. It would therefore seem reasonable to suggest that 20-HE may regulate the amount of lysosomes present during gland cell death. Contrary to our histochemical data, biochemical AP data for untreated glands from isolated abdomens showed a different pattern. AP activity was fairly similar from 48-96 hr (Fig. 18) and declined thereafter. One possible explanation for this discrepancy is that ligation may alter the metabolism in isolated abdomens resulting in increased AP activity that might be 20-HE independent.

Ecdysone As A Trigger

It has been shown that endogenous 20-HE is rapidly inactivated in some insects and that one injection can last only a few hours (Karlson & Bode, 1969; Koolman, 1982). We presume that the synthetic hormone used in our experiments is likewise inactivated and cell degeneration seen 24 hr after the injection may indicate that the hormone has acted to trigger a chain of self-sustaining events leading eventually to the lysosome response and DNA fragmentation.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

This hypothesis seems reasonable in the light of work by Clever (1964) on salivary glands of *Chironomus tentans*, in which cells exposed to 20-HE showed evidence for activation of two gene loci within 3 hr following injection. Chromosomal puffing and RNA synthesis were taken as criteria of gene activity; the puffs subsided after the injected hormone was inactivated. Other gene loci in these chromosomes became active only 2 or 3 days following the single injection of 20-HE. This activation was interpreted as being induced by gene products emanating from the earlier reacting puff regions. We can speculate that 20-HE induces the expression of late genes in the labial gland which then may execute the death program. Support for this theory comes from the model originally proposed by Ashburner et al. (1974). This model states that the 20-HE/receptor complex induces early genes, transcription factors, that in turn activate late genes and repress their own transcription.

Our *in vivo* experiments support the argument that 20-HE triggers rather than directly maintains degeneration. A single exposure to 20-HE induces levels of lysosomal activity and DNA fragmentation comparable to those following continuous exposure to the hormone. In other situations, however, the effects of 20-HE are considered to result from direct modulation of gene expression. 20-HE has been reported to regulate the expression of ecdysone receptor (EcR) isoforms during cell death (Robinow et al., 1993). This suggests that cell-specific responses to 20-HE may be mediated by cell-specific expression of particular EcR isoforms. The endogenous peak of 20-HE during pupation in *Manduca* has been shown to

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

suppress mRNAs for larval-specific proteins while initiating the synthesis of new pupal-specific mRNAs (Riddiford, 1982). Less conclusive arguments are that, in *Manduca,* protein and mRNA synthesis are required for 20-HE-dependent death of identified motoneurons (Weeks et al., 1993; Fahrbach et al., 1994) and of skeletal muscle via the ubiquitin conjugation pathway (Haas et al., 1995). 20-HE may control the synthesis of proteins that participate in the lysis of *Drosophila* salivary gland (Aizenzon & Zhimulev, 1975). In the labial gland, it therefore seems plausible that 20-HE may upregulate the expression of a gene(s) that in turn starts a cascade of events leading to death of the cell. Our results support the notion that 20-HE is a trigger of cell death in the gland and that its continued presence may not be required to kill the cells.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER IV. POSSIBLE MECHANISMS BY WHICH 20-HE REGULATES GLAND CELL DEATH

A. Introduction
B. Materials & Methods
C. Results
1. EcR Expression & Localization
2. Cytoskeletal Rearrangements In Degenerating Glands
D. Discussion: The 20-HE:EcR Complex May Induce Cell Death In Glands By Acting On The Cytoskeleton

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CHAPTER IV. POSSIBLE MECHANISMS BY WHICH 20-HE REGULATES GLAND CELL DEATH

INTRODUCTION

The alterations in our cell death parameters in the labial gland, induced by 20-HE, may be explained by several theories. All of the actions of 20-HE are apparently mediated by the ecdysone receptor (EcR) which when activated by the hormone triggers a cascade of transcription factors that may ultimately direct the death of gland cells. In *Manduca* there exists two isoforms of EcR whose expression patterns change dependent on tissue type and time of development (Jindra et al., 1996). EcR mRNA has been shown to be differentially expressed during metamorphosis in *Drosophila* and *Manduca* (Talbot et al., 1993; Fujiwara et al., 1995). In *Manduca* EcR mRNA isoforms showed developmental fluctuations that correlated with the endogenous 20-HE titer in the epidermis, wing discs, and wings. *Manduca* EcR-B1 and EcR-A mRNAs were directly induced by 20-HE (Jindra et al., 1996). It is of interest to determine if the differences in the levels of cell death in glands at different stages are mediated by a changing combinatorial pattern of different EcR isoforms and, if so, how are they controlled by 20-HE. To investigate whether the triggering of gland cell death by 20-HE may be limited by receptor availability rather than by hormone titers directly, we performed northern and western blotting and immunocytochemical analyses of EcR.

In Chapter II we showed that 20-HE does not directly affect lysosomes in gland cells. The movement of lysosomes from basal to lumenal regions of the

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

gland on day 0, however, indicates that changes in cytoplasmic trafficking may occur (Lockshin & Zakeri, 1994). In particular, we induced an increase in the number and movement of lysosomes in glands that were treated *in vivo* with 20-HE. We have shown in Chapter I that the cytoplasm of dying labial gland cells become filled with autophagic lysosomal vacuoles that destroy the cellular content of these cells. Lysosomes have been shown to migrate by aligning along centrosomal microtubules (Matteoni & Kreis, 1987).

An alternative mechanism responsible for the effects of 20-HE on lysosomes of the gland, might be that the hormone acts on the cytoskeleton to bring about the death of the gland. We showed that 20-HE treated gland cells displayed enhanced cytoplasmic shrinkage compared to control glands (see Chapter III). We initially examined the changes in the microtubules of the labial glands during spontaneous metamorphosis by performing immunofluorescence studies. Having established that there was a reorganization of tubulin during cell death, we wanted to determine if this alteration was regulated by 20-HE. In this Chapter we demonstrate that 20-HE induces cytoplasmic alterations that coincided with changes in the tubulin localization. Little is known, however, about the mechanisms that bring about changes in the cytoskeleton during programmed cell death.

MATERIALS AND METHODS

Animals

Isolated abdomens were used for these experiments as described in

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Chapter III.

RNA isolation

Labial glands were dissected as in Chapter III and dorsal and lateral intersegmental muscles were excised from pupal abdomens. Tissues were immediately frozen in liquid N_2 and stored at -70°C until used for RNA isolation. Total RNA was isolated from the tissues according to Cathala et al. (1983). All solutions used for RNA work contained diethylpyrocarbonate (DEP; Sigma) treated dH_2O. Tissues were weighed on dry ice prior to pulverization in liquid N_2 using a mortar and pestle. The tissues were homogenized in 7 volumes of GTC-lysis buffer (5 M guanidine monothiocyanate, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5, and 8% ß-mercaptoethanol, added just before use). Five to seven volumes of 4 M LiCl were added and samples were incubated at 4°C overnight. The samples were centrifuged at 9,000 RPM for 90 min at 4°C. The pellets were resuspended in 2-3 ml of cold 3 M LiCl, vortexed, and centrifuged at 9,000 RPM for 1 hr at 4°C. The pellets were resuspended in solubilization buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2% SDS). The samples were frozen in a dry-ice-ethanol bath and vortexed until they thawed. The RNA was extracted with phenol/chloroform/isoamyl alcohol 25:24:1 (v:v:v). Extractions were performed by mixing the RNA sample with an equal volume of phenol/chloroform/isoamyl alcohol, vortexing, centrifugation at room temperature, and removal of the upper, aqueous phase to a fresh tube. RNA was precipitated with 0.4 M LiCl and 2 volumes of 100% ethanol. The concentration of RNA was confirmed by

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

spectrophoretic readings at 260 nm. The samples were stored at -20°C until they were used.

Electrophoresis Of RNA

A known concentration of RNA sample was centrifuged for 30 min at 4°C. The RNA pellet was dissolved in sample buffer (1X MOPS [20· mM 4-morpholinepropanesulfonic acid, 1 mM EDTA, 5 mM NaOAc. pH 7]. 50% formamide, 1X formaldehyde) and incubated at 70° C for 5 min. The samples were cooled on ice prior to the addition of 1 μl ethidium bromide (10 mg/ml) and 1 μl of bromophenol blue (10 mg/ml). Samples were loaded onto an 0.8% agarose/2.2 M formaldehyde gel. The gel was run at 30-50 volts for 4-6 hr in 1X MOPS. After electrophoresis, the gel was incubated in 10X SSC (1X SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7) for 1 hr at room temperature. The RNA was visualized by UV illumination of the gel to check for degradation of the RNA.

Northern Transfer

The RNA was transferred overnight to a nylon membrane (Amersham) by capillary blotting in 10X SSC. The blot was crosslinked (Stratagene, La Jolla, CA) by UV light. After the northern transfer, the RNA was visualized by UV illumination of the membrane to ensure that complete transfer had occurred.

Transformations & Plasmid Preparation

A single colony of *E.coli* was used to inoculate 5 ml Luria-Bertani (LB broth; Difco, Detroit, MI) and grown at 37° C overnight. Next morning, 25 ml of LB with 0.5 ml of the overnight culture were grown for 3-5 hr at 37° C with shaking. The

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

mixture was transferred to a clean tube, incubated on ice for 20 min, and centrifuged at 2,500 RPM at room temperature for 15 min. The pellet was resuspended in 25 ml cold, sterile 0.1 M $CaCl_2$ incubated on ice for 30 min. and spun as above. The pellet was resuspended in 3 ml 0.1 M $CaCl_2$, and was stored on ice for 30 min or at 4°C overnight. EcR3 (generous gift from Dr. Lynn Riddiford) plasmid DNA was diluted to 10 ng/μl and 300 μl of competent cells were added to 20 ng DNA in a conical tube. As a control, 300 μl of cells only were added to another conical tube. The conical tubes were incubated on ice for 1 hr, with occasional shaking; heat-shocked at 42°C 90 for sec; 1 ml LB was added; and cells were grown at 37°C for 45 min. Several dilutions were plated onto LB/Amp plates and incubated at 37°C overnight. A single colony was inoculated into 5 ml TB/Amp and incubated at 37°C overnight.

Minipreps Of EcR Plasmid DNA

Five ml of medium were inoculated with a single colony of *E.coli* and grown to saturation at 37°C with shaking. One and a half ml of cells were microcentrifuged for 1 min at room temperature. The pellets were resuspended in 100 μl of ARS I (Alkaline Rapid Screen; 50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8) and incubated on ice for 30 min. Two hundred μl of ARS II (0.2 N NaOH, 1% SDS) were added to the solution and incubated on ice for 5 min. One hundred fifty μl of ARS III (3 M NaOAc, pH 4.8) were added to the solution and incubated on ice for 45 min. The solution was microcentrifuged at 4°C for 15 min and the supernatant was removed to a clean tube. Phenol/chloroform/isoamyl

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

alcohol extraction was performed and the solution was precipitated with ethanol at -70° C for at least 30 min. Microcentrifugation was performed at 4° C for 15 min and the pellet was resuspended in 139 μl dH_2O. RNase, 1 μl, was added and the solution was incubated at 37° C for 10 min. Then 60 μl of 5 M NaCl and 50 μl 1.5 M NaCl/30% polyethylene glycol (PEG) were added and incubated on ice for 30 min. Microcentrifugation occurred at 4° C for 30 min. The pellet was resuspended in 50 μl dH_2O. Five μl were used for a restriction digest.

Large Scale Preparation Of EcR Plasmid DNA

Two hundred fifty ml TB/Amp (3 g tryptone, 6 g yeast extract, 1 ml glycerol, 225 ml dH_2O, and ampicillin) were inoculated with 5 ml of an overnight culture of *E.coli* and incubated overnight at 37° C with shaking. Centrifugation at 5,000 RPM at 4° C for 15 min was followed by resuspending the pellet in 10 ml ARS I and lysozyme and incubating on ice for 30 min. Twenty ml of ARS II were added and incubation was on ice for 5 min; then 15 ml ARS III were added and incubation was on ice for 45 min. The solution was centrifuged at 1,200 RPM at 4° C for 20 min; the supernatant was transferred to clean tubes; and the solution was precipitated with an equal volume of isopropanol and incubated at -70° C for 20 min. Centrifugation was performed at 1,200 RPM 4° C for 20 min. The pellet was resuspended in 7 ml TE (10 mM Tris, 1 mM EDTA, p H 7.4), 4 ml NH_4OAc were added, and the solution was precipitated with ethanol at -70° C for 20 min. Centrifugation was performed at 1,200 RPM at 4° C for 20 min. The pellet was resuspended in 7 ml TE and 10 μl RNase A (10 mg/ml) and was incubated at

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

37°C for 30 min. Three ml of 5 M NaCl and 2.5 ml 30% PEG/1.5 M NaCl were added and incubation occurred at 37°C for 30 min. Centrifugation was performed at 1,200 RPM at 4°C for 20 min; the pellet was dissolved in 560 μl dH_2O, 70 μl 10X proteinase K buffer; 70 μl proteinase K (5 mg/ml) were added; and incubation was performed at 37°C overnight. Seventy μl of 3M NaOAc were added, the solution was phenol/chloroform extracted, and precipitated with ethanol at - 70°C. The pellet was dried in a speed vac and dissolved in 400 μl dH_2O.

Probe Labeling & Hybridization

Blots were prehybridized in 50% formamide, 5X SSC, 5X Denhardt's solution (1X Denhardt's solution = 100 mg Ficoll, 100 mg polyvinylpyrrolidone, and 100 mg BSA in 500 ml dH_2O), 50 mM $NaPO_4$ (pH 7), 500 μg/ml salmon sperm DNA, 0.2% SDS at 42°C for 3-4 hr. To generate the radiolabeled probes, 400-600 ng of the EcR3 cDNA fragment (a gift from Dr. Lynn Riddiford, University of Washington) was labeled with 5 μl of α-^{32}P-dCTP (Amersham; 3,000 Ci/mM; 10 mCi/ml) using the Amersham Multiprime DNA labeling system. The labeled DNA was separated from unincorporated nucleotides on a push column. The amount of radioactivity was measured by placing 1 μl of probe onto a small piece of Whatman paper, adding 10 ml CytoScint (Fisher Scientific, Pittsburgh, PA) and counting counts per minute using a scintillation machine (Beckman, Fullerton, CA). This EcR cDNA probe should detect all isoforms. Pre-hybridization was performed in 10 ml of pre-hybridization buffer (50% formamide, 5X SSC, 5X Denhardt's, $NaPO_4$, 500 μg/ml salmon sperm DNA) for 3 hr at 42°C. Hybridization was

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

performed in 10 ml of hybridization buffer (50% formamide, 5X SSC, 1X Denhardt's, 20 mM $NaPO_4$, 10% dextran sulfate, 100 μg/ml salmon sperm DNA) overnight at 42°C.

Washing RNA Blots

Blots were washed in 2X SSC/0.1% SDS and 1X SSC/0.1% SDS for 15 min each at room temperature; in 1X SSC/0.1% SDS for 15 min, 0.1X SSC/0.1% SDS twice for 20 min, and 0.1X SSC for 30 min each at 65°C. Blots were exposed to film with intensifying screens at -70°C for an appropriate time.

Western Blotting

Equal amounts of protein from labial gland and epidermis samples were loaded onto a 1-D PAGE gel and electrophoresed as described in Chapter II. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) in a semidry blotter (Owl Scientific, Cambridge, MA), using the manufacturer's recommended transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3), for 2 hr at 400 mA. Blots were incubated with blocker (1X PBS-T [137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, 0.1% Tween 20, pH 7.4], 5% Carnation nonfat dry milk) at 4°C overnight. Blots were reacted with an EcR monoclonal antibody (generous gift from Dr. Lynn Riddiford), diluted 1:1000 with blocker, overnight at 4°C. Blots were washed two times for 7 min with 1X PBS. Blots were detected with a horseradish peroxidase-labeled rabbit anti-mouse IgG secondary antibody (Sigma; diluted 1:8,000) with a 1 hr incubation at room temperature. The blots were washed three times in 1X PBS-T for 5 min and once

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

for 5 min in 1X PBS. The ECL kit (Amersham) was used for antigen detection.

Immunocytochemistry

Slides were washed once in 1X PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.4) for 5 min in a Coplin jar. Slides were placed in a moist chamber where EcR monoclonal antibody, diluted 1:1000 in 1X PBS, was applied using plastic coverslips. Incubation was performed overnight at 4° C. The coverslips were removed, slides were washed three times in 1X PBS for 5 min and once in 1% bovine serum albumin (BSA; Sigma) dissolved in 1X PBS. Slides were placed in a moist chamber for application of the secondary antibody (anti-mouse IgG peroxidase conjugated; Sigma), diluted 1:150 in 1% BSA in 1X PBS. Incubation was performed overnight at 4° C. The slides were washed three times in 1X PBS for 5 min, stained with diaminobenzidine (DAB; Research Genetics, Huntsville, AL) for 2 min, and counterstained in methylene blue (Sigma) diluted 1:10 in dH_2O. The slides were mounted in CrystalMount (Biomeda, Foster City, CA) and the reddish-brown DAB color product was observed by light microscopy (Leica Leitz DMRB, Deerfield, IL).

Immunofluorescence Of Tubulin

Labial glands were fixed in 4% paraformaldehyde in PEM buffer (60 mM PIPES, 5 mM EGTA, 5 mM $MgCl_2$, pH 6.9) at 4° C overnight. Glands were frozen and 5 μm sections were cut onto slides as described in Chapter I. The slides were washed once for 5 min each in PEM. Slides were incubated overnight at 4° C with a monoclonal anti-α-tubulin antibody (Amersham, Cat # N 356) diluted 1:100 in 1%

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

bovine serum albumin (BSA) in PEM. Slides were washed three times for 5 min each in PEM and once in 1% BSA in PEM for 5 min. Slides were incubated overnight at room temperature with a FITC-conjugated anti-mouse IgG (Calbiochem, Cat # 401219) diluted 1:200 in 1% BSA in PEM. The slides were washed three times for 5 min each in PEM. Slides were then incubated in the dark for 10 min with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Sigma) to stain the nuclei. Slides were washed once in PEM, mounted in CrystalMount, and observed by fluorescent microscopy.

RESULTS

EcR Expression & Localization

To evaluate the relationship between the effect of 20-HE and cell death in the gland and alterations in expression EcR mRNA, we first examined the expression of EcR mRNA in the gland during spontaneous metamorphosis. Labial glands were collected from animals at various stages, total RNA was isolated, and analyzed by northern blot hybridization using EcR3 DNA as a probe. The EcR3 cDNA probe corresponded to the DNA- and ligand-binding domains of EcR and therefore was able to detect both *Manduca* EcR isoforms. EcR mRNA expression was low in premetamorphic glands, day -2, and increased at day 0, remaining constant until day 3 (Fig. 28). The expression pattern for EcR mRNA, in the gland, coincides with the ecdysteroid titers. At day -2 the ecdysteroid titer is very low, then there is a small rise by day -0.5, and then there is a rise starting on day 0 and peaking by day 3. Our results suggest a correlation between EcR mRNA and 20-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

HE levels in the animal.

The EcR mRNA expression was correlated with the EcR protein by western blot analysis. Ten μg of isolated gland protein from animals at various stages of spontaneous metamorphosis were separated by PAGE. *Manduca* epidermis protein was used as positive control, since the EcR antibody was detected from this tissue (Fujiwara et al., 1995). The separated proteins were transferred to nitrocellulose membrane and reacted with an EcR monoclonal antibody. This antibody detects an epitope in the common region consisting of the DNA-binding and first quarter of the ligand-binding domains and therefore, should detect the two *Manduca* EcR isoforms. The EcR protein was present at all stages examined with little changes in the levels of the protein. The expression of EcR at the level of protein appears to be less dramatic than EcR mRNA expression throughout spontaneous metamorphosis (Fig. 29).

Although there were no significant changes in EcR protein during spontaneous metamorphosis at stages when the gland undergoes cell death, days 0 to 3, it is still possible that there could be changes in localization of EcR protein as gland cell death progressed. To investigate this possibility we used immunocytochemical experiments, initially performed with day -1 glands because this timepoint was a positive control for detection of the EcR antibody from epidermis (Fujiwara et al., 1995). This stage represents a premetamorphic gland and we can detect high levels of EcR protein by western blot at this stage. Positive staining was detected in both nuclear and cytoplasmic sites, with more

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

prominent staining in the nucleus (Fig. 30). By day 4 of metamorphosis, however, we detected a shift from the nucleus to the cytoplasm (Fig. 30b; arrows). This finding suggests a translocation from the nucleus to the cytoplasm as the gland dies. To determine if EcR protein localization is regulated by 20-HE, we injected isolated abdomens with vehicle or 20-HE and glands were examined after 24 hr. We again found prominent staining of the nucleus in gland cells from vehicle injected abdomens (Fig. 30c). In contrast, 20-HE treated glands showed a progression into cell death, by morphology, and more prominent staining of EcR protein in the cytoplasm (Fig. 30d). The nuclei in hormone treated glands was still positive to a degree. This stage, however, is not comparable to day 4 shown in (Fig. 30b) and represents an earlier stage of cell death around day 2 or 3, by morphology. These results suggest that EcR protein may be present in the nucleus at early stages of metamorphosis and then translocates to the cytoplasm at later stages of cell death.

Microtubules In Degenerating Glands

The cytoskeleton has been postulated to play a role in the movement of lysosomes. We sought to determine if alterations in the cytoskeleton occurred in the gland and if these alterations were regulated by 20-HE. Changes in tubulin were investigated in glands from animals undergoing spontaneous metamorphosis and glands from ligated animals treated or not treated with 20-HE. In labial glands from days -1, 3, and 5, comparable staining of fluorescein isothiocyanate (FITC)-labeled tubulin and DAPI were detected (Fig. 31). These results were inconclusive

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

because we could not achieve the level of resolution required to examine rearrangement of the cytoskeleton. To investigate if this observation was under the influence of 20-HE, we examined these changes in glands that had been exposed *in vivo* to 20-HE. Exposure of glands to 20-HE appeared to accelerate cytoplasmic shrinkage and tubulin changes more prominently compared with control glands at 120 hr (Fig. 32).

DISCUSSION

EcR Expression Profile

In order to examine if 20-HE's effect on cell death could be reflected by alterations in transcription or translation of EcR, we needed to determine the pattern of EcR expression during gland metamorphosis and cell death. Our northern blot analysis indicates that EcR mRNA is differentially expressed in labial glands from premetamorphosing and metamorphosing animals. The level of EcR mRNA in metamorphic glands, however, did not change significantly. We did not pursue northern blot analysis on 20-HE treated glands, since EcR mRNA expression was not sufficiently altered during spontaneous metamorphosis to warrant it. Jindra et al. (1996), however, have shown that the two *Manduca* EcR isoforms are induced by 20-HE in larval and pupal wing discs and in epidermis. The induction of EcR mRNA may reflect the change in 20-HE levels and perhaps changes in the levels or regulation of genes that are responsive to 20-HE. The EcR mRNA profile correlates with the endogenous levels of hormone during the fifth instar. Western blot experiments indicated that the EcR protein was expressed

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

at equivalent levels in glands during spontaneous metamorphosis. Immunoblot experiments were performed at least three times and we consistently detected no significant alterations of EcR at the level of protein. Our results are contradicted by those of Jindra et al. (1996). They report that the developmental profile of the EcR protein followed that of the mRNA.

Although the levels of EcR mRNA and protein were relatively unchanged in during the later stages of spontaneous metamorphosis (days 0 to 3), we theorized that there may be changes in the localization of EcR protein during gland cell death. Since EcR is a nuclear receptor, one would expect that EcR is located in the cytoplasm when it is not bound to ligand and then translocates to the nucleus once it has bound ligand. Preliminary immunocytochemistry experiments revealed that anti-EcR staining was mostly nuclear in premetamorphic (day -1) and cytoplasmic staining predominated at later stages (day 4). These results suggest that the receptor may no longer be bound to 20-HE and thus, the receptor is inactivated at this stage. Localization of EcR protein did not appear to be affected by 20-HE treatment of glands from ligated abdomens. Unfortunately we could not confirm these observations in many other stages due to the shortage of EcR antibody.

Rearrangement Of Tubulin Cytoskeleton

Various lines of evidence indicate that injury to cytoskeletal components can initiate cell death. Kruman et al. (1992) found that colchicine and cytochalasin B, agents that disrupt the cytoskeleton, can activate apoptosis in thymoma cells.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Geiger et al. (1982) similarly demonstrated apoptotic cytolysis of T-lymphocytes initiated by injury to microtubules, while Kolber et al. (1990) have shown that cellular DNA fragmentation can be induced in a number of cell lines by agents that perturb microfilaments.

Lysosomes have been shown to move along microtubule tracks (Matteoni & Kreis, 1987). We were interested in determining if there were changes in the microtubule network of gland cells during spontaneous metamorphosis. Such changes might account for the movement of lysosomes from basal to lumenal regions of gland cells during cell death. Others have reported that 20-HE affects the distribution of tubulin (Berger et al., 1980). Birkenbeil et al. (1979) demonstrated electron microscopically that 20-HE was present in the microtubules of the prothoracic gland of *Galleria mellonella*.

Our results showed that the tubulin cytoskeleton was altered during spontaneous cell death of the labial glands. We detected more reorganization of tubulin in 20-HE treated glands compared with controls. We were not able to capture high resolution images of this event due to some equipment problems with our fluorescence microscope. We had to capture the images on a confocal microscope, but we could not reconstruct serial sections of the glands. Using fluorescence microscopy we detected the following: tubulin organization was intact in day -1 glands; it began to change by day 3; and on day 5 most of the tubulin and cytoplasm were destroyed. Our results are confirmed by Jochová et al. (1997). They detected changes in microtubule and actin filament network in

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

salivary glands of *Drosophila* during spontaneous metamorphosis.

We were also unable to ascertain the role of 20-HE in the cytoskeletal alterations observed in the glands. Since *in vitro* exposure of glands to 20-HE did not induce cell death, we did not pursue *in vitro* experiments using microtubule disrupting agents like colchicine. Moreover, *in vivo* administration of colchicine would probably kill the larvae. We can, however, speculate that 20-HE does affect the cytoskeleton from our histochemical AP data (Figs. 24,25), acridine orange (Fig. 20), and DNA fragmentation (Figs. 23,26) experiments. These results showed altered morphological features in 20-HE treated glands. We could not determine with certainty if the cytoskeletal modifications were caused by 20-HE or other possibilities.

In particular, it is unclear whether such alterations are an unavoidable implication of cell volume changes or whether they result from early modifications of cytoskeletal elements. Disturbances of the cytoskeletal organization could be caused by other events including modifications in protein phosphorylation/dephosphorylation, Ca^{2+} overload, protease activation, or protein-protein interactions (Maro & Bornens, 1982; Yano et al., 1995; Gregorio et al.. 1993; Lavie et al., 1985; Dyer & Benjamins, 1989). The possibility that cytoskeletal alterations play a relevant role in the programmed cell death of labial glands deserves further consideration.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

CONCLUSIONS

Programmed cell death (PCD) is a prominent feature during development. The characterization of this type of death can be either apoptotic or lysosomal. We have established the use of several parameters to monitor the progression of cell death, during spontaneous metamorphosis, in the labial glands of the tobacco hornworm, *Manduca sexta*. We showed that the cell death of the glands is a type II or lysosomal death. In addition, we showed that changes in metabolic parameters and cell signaling occur too late to presage the demise of the glands.

The PCD of tissues during metamorphosis has been presumed to be under endocrine regulation. In this study we tested this hypothesis by investigating the role of 20-hydroxyecdysone (20-HE) in the induction of cell death in the labial glands. We developed an *in vitro* organ culture sytem to determine if the glands would undergo spontaneous metamorphosis in culture. This system was also used to study the effect of 20-HE and gland cell death without interference of systemic factors. We showed that in our culture system, 20-HE did not directly affect lysosomes nor did it kill gland cells. This suggested that the intact animal may be required in order to see the effects of 20-HE on labial glands.

We initiated our *in vivo* studies by asking if 20-HE could induce precocious cell death in glands from premetamorphosing animals. We showed that although exogenously added 20-HE could induce lysosomal enzyme activity at this stage, the hormone did not sustain cell death that was comparable to glands from later stages. We then asked if 20-HE could induce cell death in glands from

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

metamorphosing animals. We showed that 20-HE triggered gland cells to die at this stage by inducing movement and activity of lysosomes. In addition. we showed that exposure of glands to a single pulse of 20-HE was as effective as multiple pulses for the propagation of cell death signaling. These results support the hypothesis that 20-HE is sufficient to trigger gland cell death, but may not be needed to sustain cell death.

The effect of 20-HE on the movement of lysosomes suggested that the cytoskeleton may be involved. We investigated the hypothesis that 20-HE may trigger lysosomal autophagic death in glands by modifying components of the cytoskeleton. We showed that a rearrangement of the tubulin cytoskeleton occurred during spontaneous metamorphosis. In addition, we showed that 20-HE triggered alterations in the tubulin cytoskeleton of dying gland cells. Further investigation is warranted to how 20-HE, and steroid hormones in general, affect lysosomes.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

PART III. FIGURE AND FIGURE LEGENDS

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

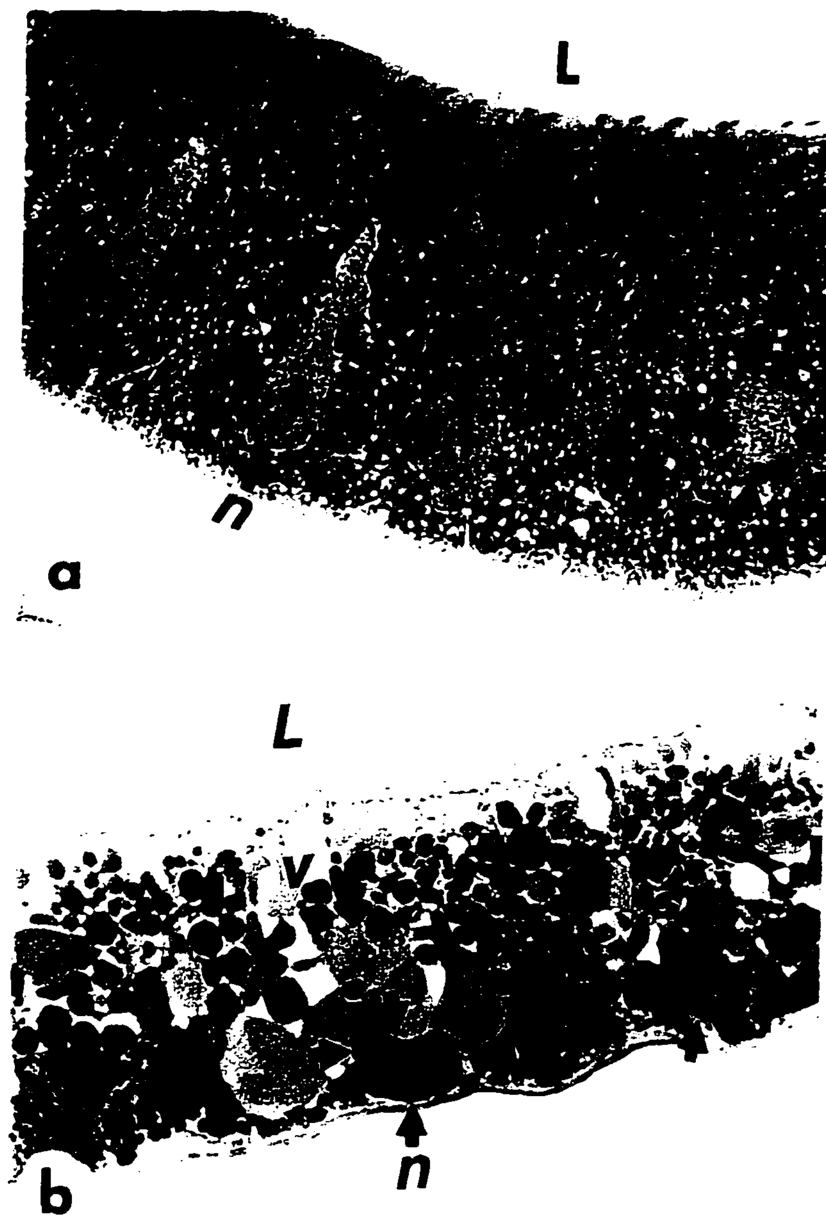

Figure 1. Electron Micrographs Of The Gland During Metamorphosis. (a) Day -1 gland. (b) Day 3 gland. Before degeneration begins on day -1, the nuclei (n; delineated by arrows) are somewhat basal and there is a thick basement membrane at the lumenal (L) surface. On day 3 the cytoplasm is virtually destroyed by autophagic vacuoles and the nuclei are pushed to the basal surface. Magnification: a and b 592X. Microscope: compound.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

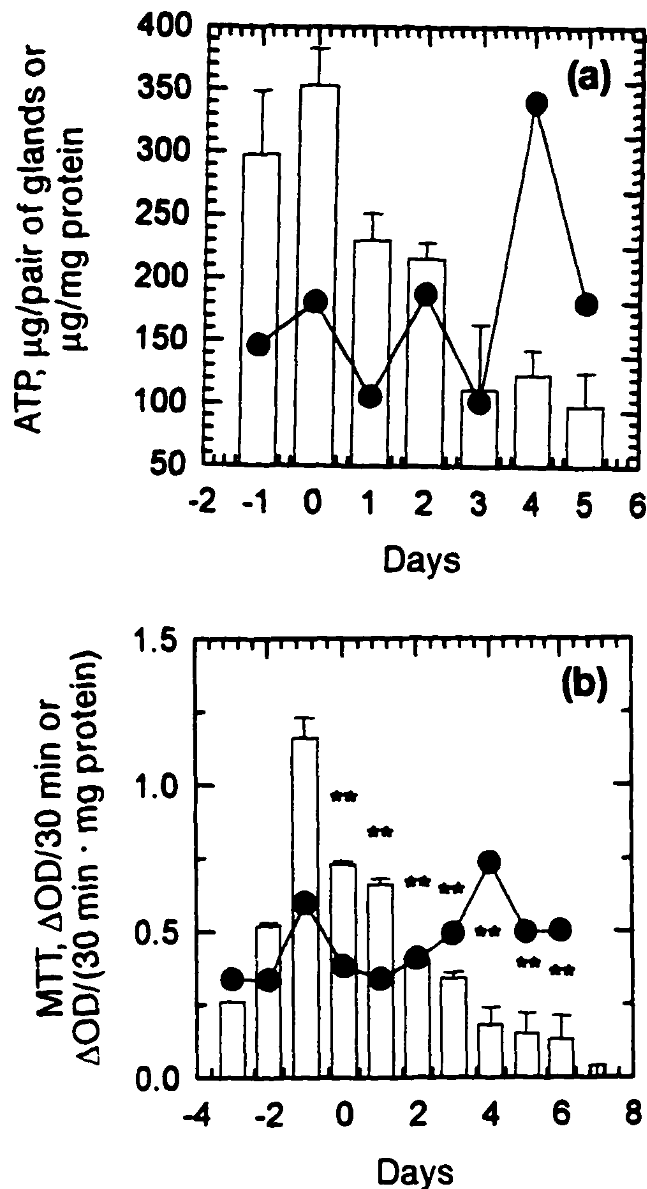

Figure 2. Energy Resources In Glands During Metamorphosis. (a) ATP levels. (b) Reduction of MTT. In both graphs, the bars represent total levels per gland and the lines represent total levels per milligram of protein. Double asterisks indicate values significantly different from day -1 at $p < 0.01$. Student's *t*-test was used to determine statistical significance. The values represent means of at least three experiments plus standard error of mean (SEM).

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Figure 3. MTT Staining Of Glands During Metamorphosis. Glands were stained with MTT as described in Materials and Methods of Chapter I. Ages of the animals at dissection are indicated in the upper left of each panel. Glands from earlier stages of metamorphosis, days -1 to 1, respire normally. Respiration begins to fail by day 3. The portion of the gland that is respiring normally in day 4 and 5 panels is the anterior duct (arrows). This portion of the gland does not die, rather it dedifferentiates into the adult labial gland. Magnification: 48X. Microscope: dissecting.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

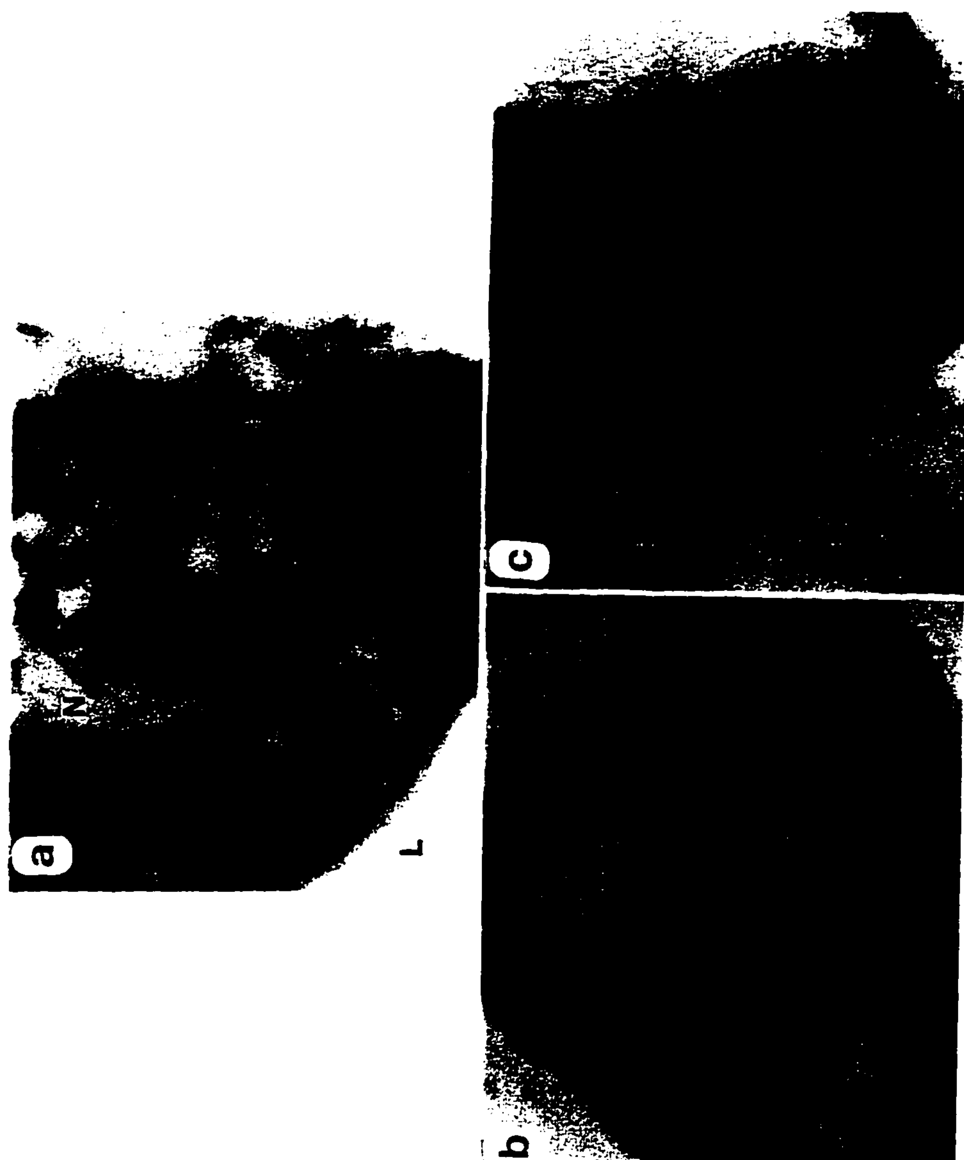

Figure 4. Histochemical Localization Of Acid Phosphatase In Labial Glands During Metamorphosis. (a) Day -1 gland. (b) Day 0 gland. (c) Day 3 gland. Lysosomes were localized using the histochemical acid phosphatase assay as described in Materials and Methods of Chapter I. Note that in the day -1 gland the lysosomes (arrows) are restricted to basal regions of the cell. In the day 3 gland lysosomes have increased in size and number and are located throughout the cytoplasm. In the day 0 gland lysosomes migrated from basal to lumenal regions (L). Nuclei (N) appear intact. Magnification: 1,000X. Microscope: compound.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

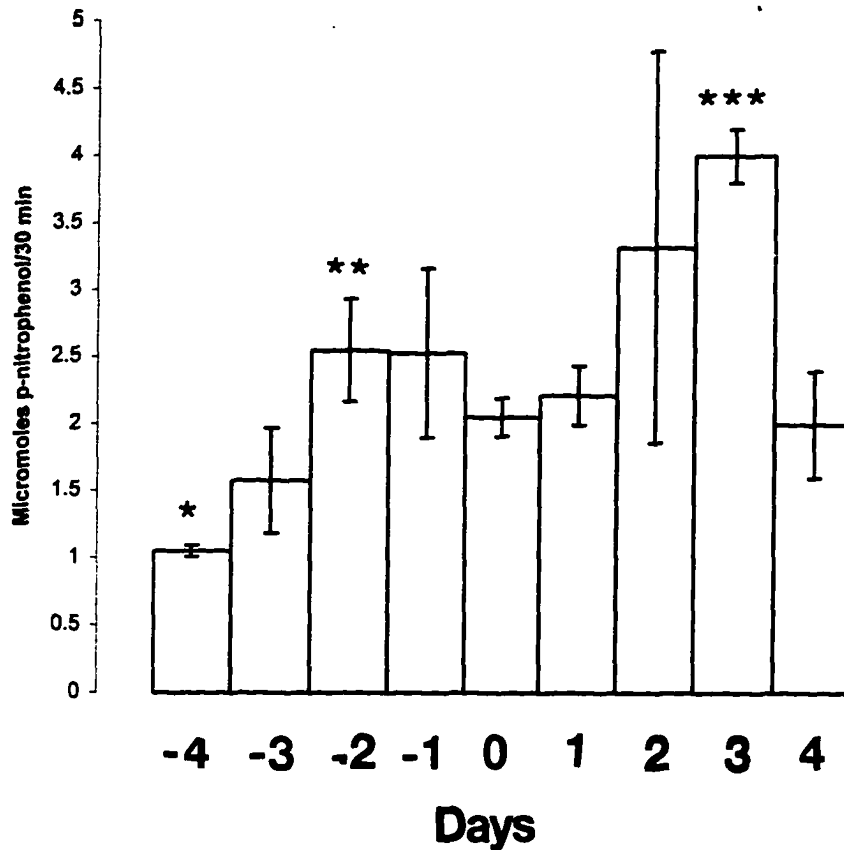

Figure 5. Acid Phosphatase Activity In Labial Glands During Metamorphosis. Labial glands were isolated at various stages of development and the biochemical AP assay was performed as described in Materials and Methods of Chapter I. The ages of the animals at dissection are indicated on the x-axis. The values represent means of at least three experiments ± SEM. Asterisks indicate values significantly different from day -3: *, $p < 0.02$; **, $p < 0.04$; and ***, $p < 0.004$. Student's *t*-test was used for determination of statistical significance. Total activity of AP is expressed as μmols p-nitrophenol released per 30 min.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

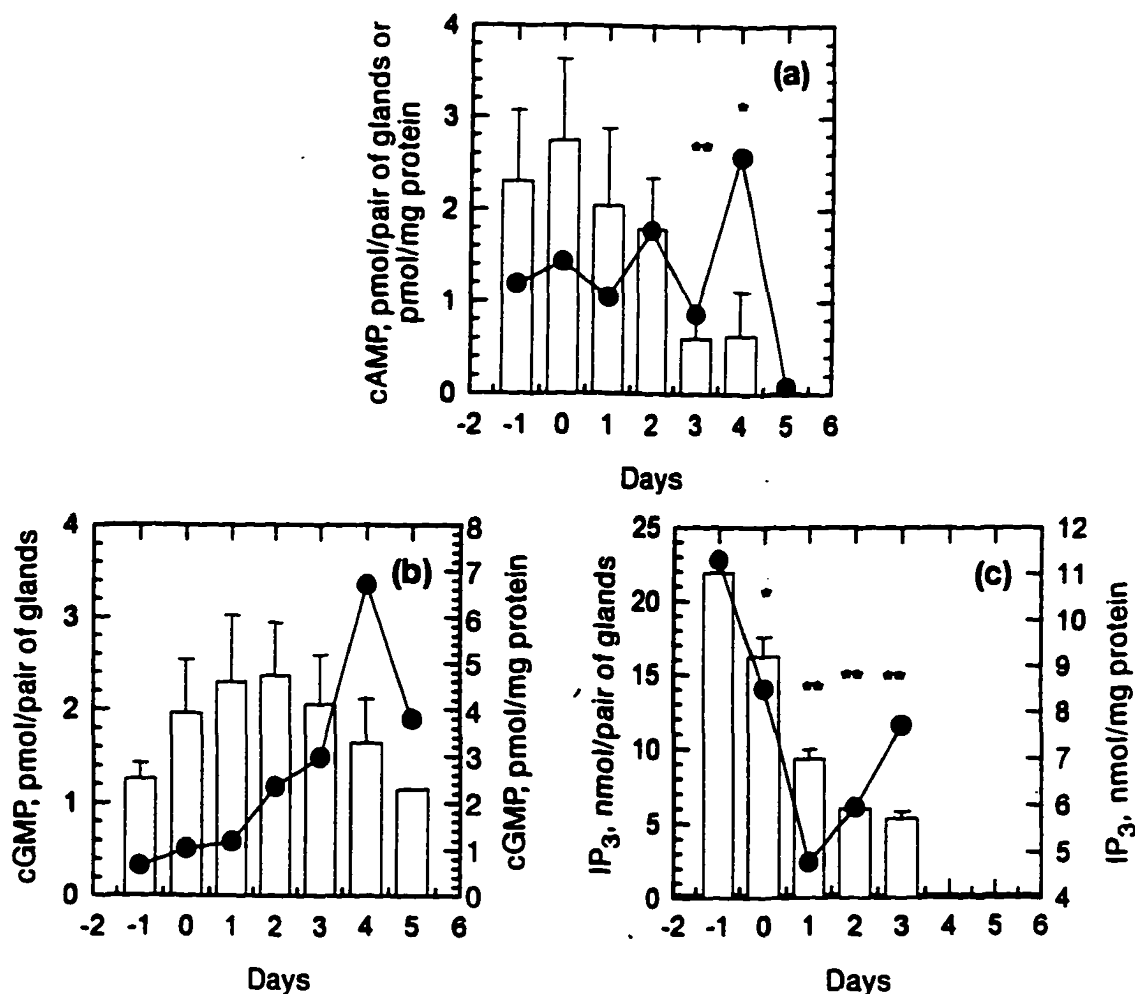

Figure 6. Levels Of Second Messengers In Glands During Metamorphosis. (a) cAMP levels. (b) cGMP levels. (c) IP_3 levels. The bars represent total levels per gland and the lines represent total levels per milligram of protein. Assays were performed according to Materials and Methods in Chapter I. Values represent mean plus SEM of at least three experiments. Single asterisks indicate statistically significant differences from day -1 at $p < 0.05$. Double asterisks indicate significantly different values from day -1 at $p < 0.01$. Student's *t*-test was used to determine statistical significance.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

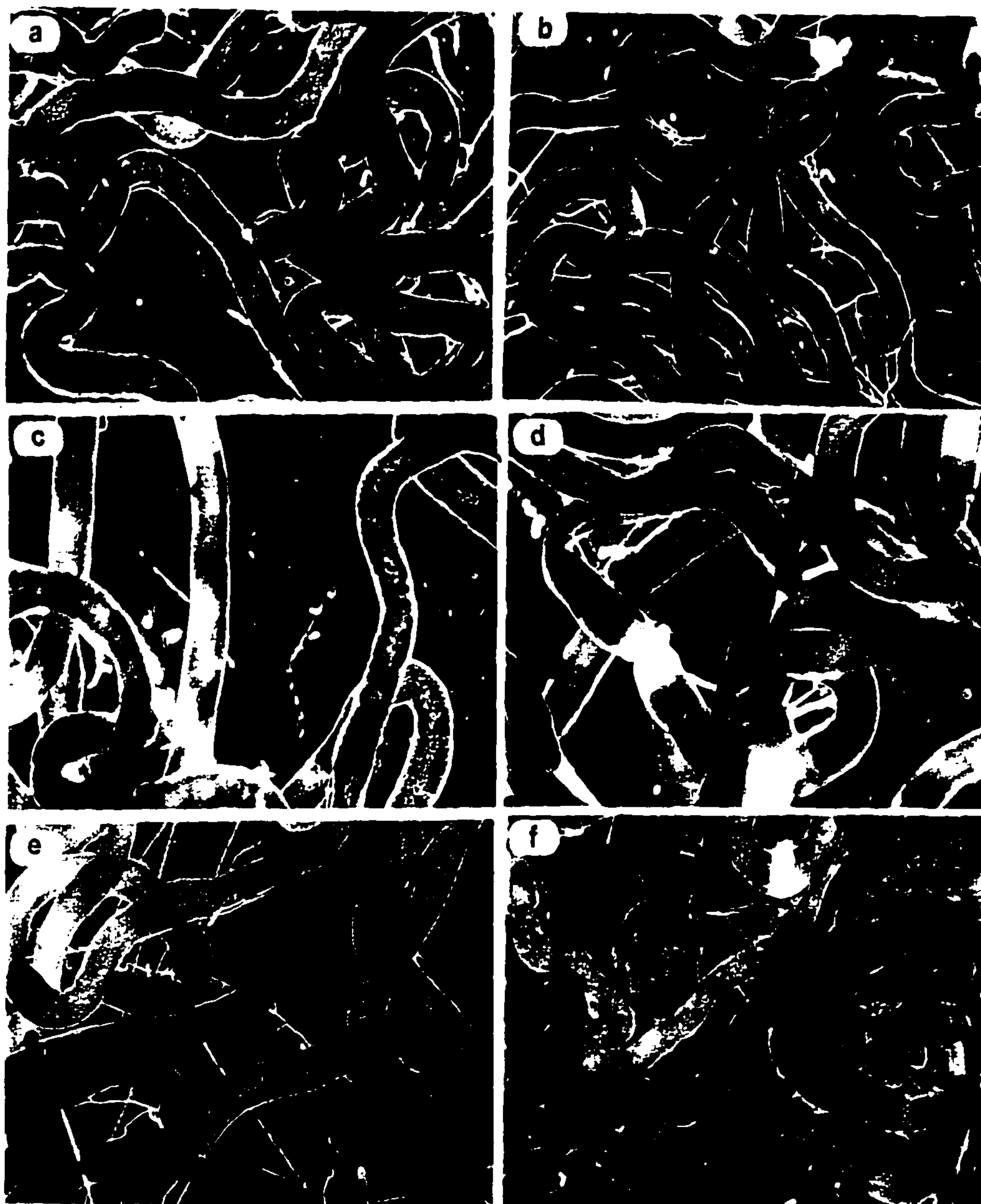

Figure 7. Histochemical Appearance Of Cultured Labial Glands Stained With MTT. Labial glands were freshly dissected (a,c,e) or cultured in Grace's medium (b,d,f) prior to staining with MTT according to Materials and Methods, Chapter I. (a) Day -3 freshly dissected gland. (b) Day -3 gland 18 hr in culture. (c) Day -1 freshly dissected gland. (d) Day -1 gland 24 hr in culture. (e) Day 1 freshly dissected gland. (f) Day 1 gland 6 hr in culture. Cultured labial glands appear to respire as well as glands that are undergoing spontaneous metamorphosis (as indicated by equivalent reddish purple staining). Magnification: a-d 30X; e and f 22.5X. Microscope: dissecting.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

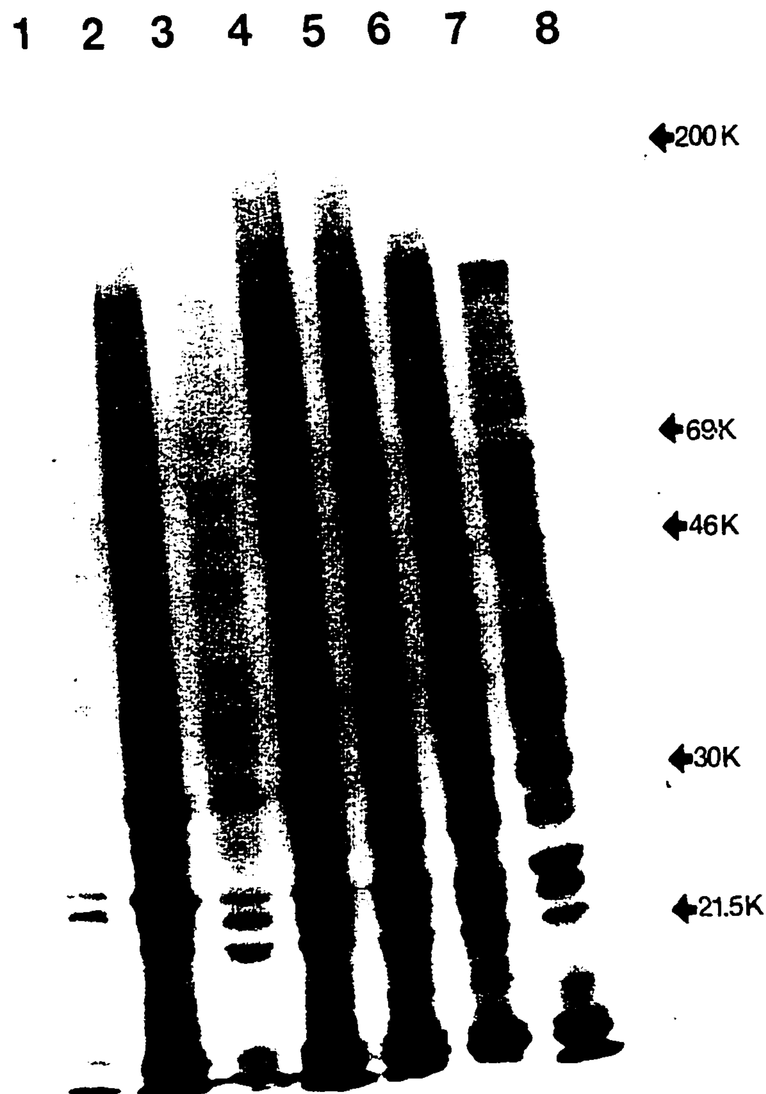

Figure 8. Effect Of 20-HE On Protein Synthesis. Glands were exposed to *in vitro* (lanes 5 and 6) or *in vivo (lanes 1-4, 7 and 8)* 20-HE as described in Chapters II or III, respectively. Isolated glands were then incubated *in vitro* with [^{35}S] methionine; proteins were separated by PAGE; and autoradiography was performed as described in Chapter II. The lanes are as follows: 1, day -4 control; 2, day -4 experimental; 3, day -3 control; 4, day -3 experimental; 5, day -3 cultured control; 6, day -3 cultured experimental; 7, day -2 control; and 8, day -2 experimental. Molecular size is indicated on the right of the gel in kDa. No significant changes in protein synthesis were detected at these stages. Protein degradation may have occurred in samples from lanes 1,3, and 8.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

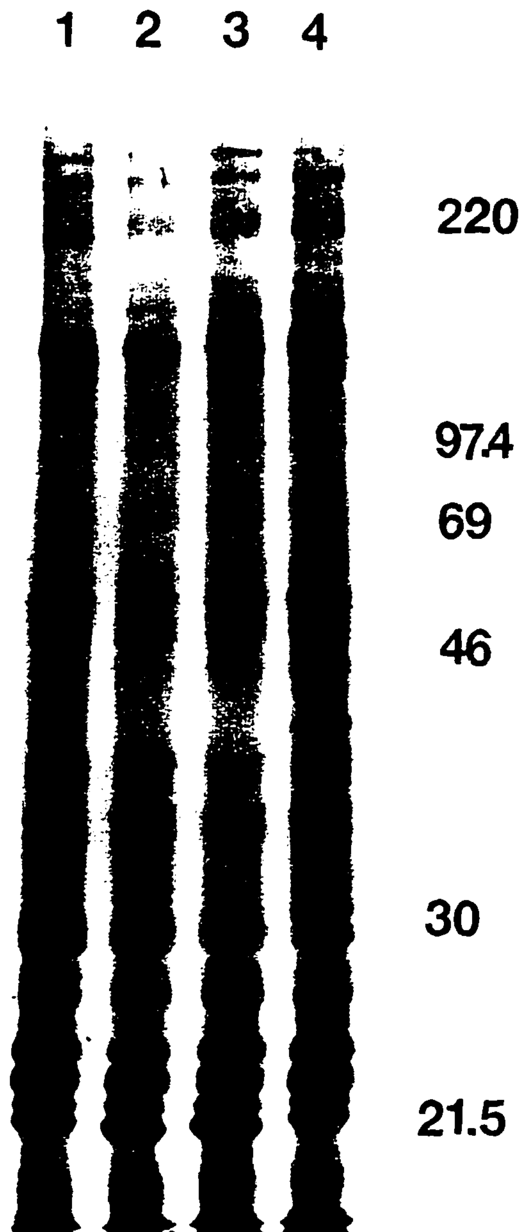

Figure 9. Effect Of Various 20-HE Doses On Gland Protein Synthesis. Day -3 glands were cultured in the presence or absence of either 5 X 10^{6} M or 5 X 10^{5} M 20-HE for 8 hr. Glands were then radiolabeled with [^{35}S] methionine and protein synthesis was analyzed by autoradiography as described in Materials and Methods in Chapter II. Lanes are as follows: 1, control, glands incubated in Grace's medium alone; 2, glands incubated in 5 X 10^{-6} M 20-HE; 3 control; and 4, glands incubated in 5 X 10^{-5} M 20-HE. Molecular size is indicated on the right of the gel in kDa. Protein synthesis was not affected by changes in 20-HE concentration.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

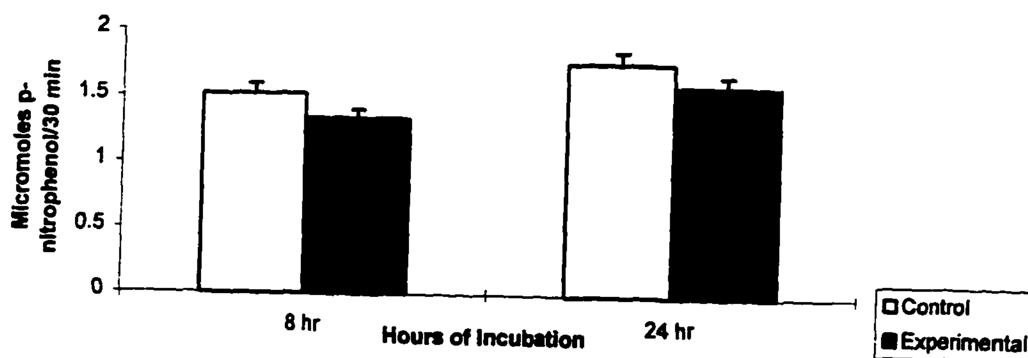

Figure 10. AP Activity In Day -2 Glands Cultured With 2 X 10 $^{-7}$ M 20-HE. Glands were cultured with or without 20-HE for 8 or 24 hr as described in Materials and Methods of Chapter II. The values represent means of at least three experiments. There were no significant differences between control and experimental glands at either incubation period.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

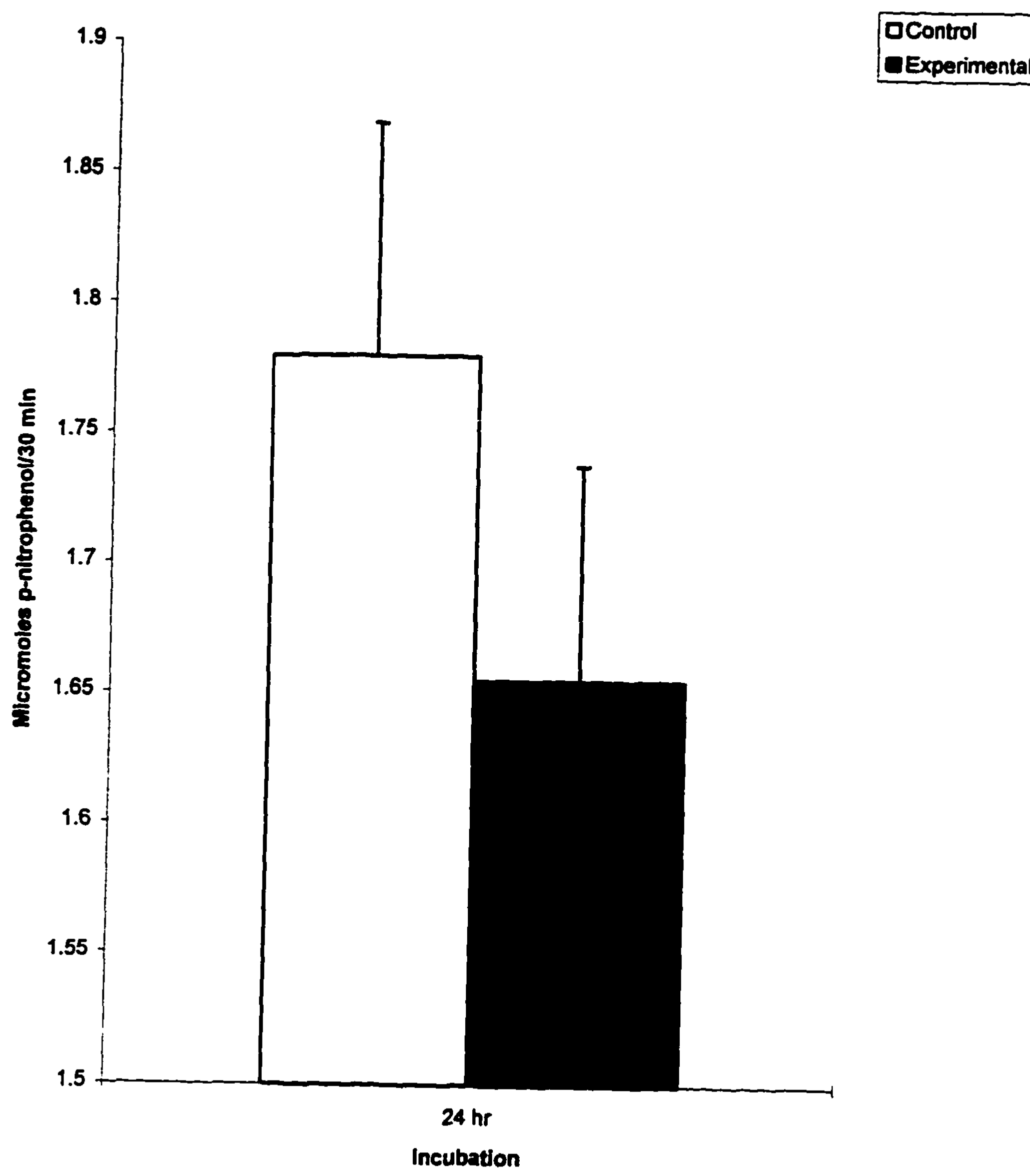

Figure 11. AP Activity In Day -2 Glands Cultured With 3 X 10^{-7} M 20-HE. Glands were exposed, *in vitro*, to a slightly higher than physiological concentration of 20-HE for 24 hr according to Materials and Methods of Chapter II. The values represent means of at least three experiments. There were no significant differences between control and experimental glands.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

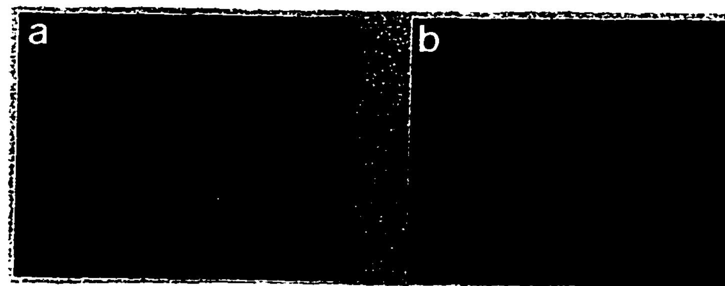

Figure 12. Localization Of Lysosomes In Cultured Metamorphic Glands.
Day 0 glands were maintained *in vitro* for 72 hr, in the absence (a) or presence (b) of 2.1 X 10^{-6} M 20-HE (physiological levels), and then stained with the acridine orange as described in Materials and Methods of Chapter II. Acridine orange stains acidic organelles, such as lysosomes, orange. *In vitro* exposure of glands to 20-HE does not appear to increase the number of lysosomes in the cytoplasm. Magnification: 400X. Microscope: compound.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

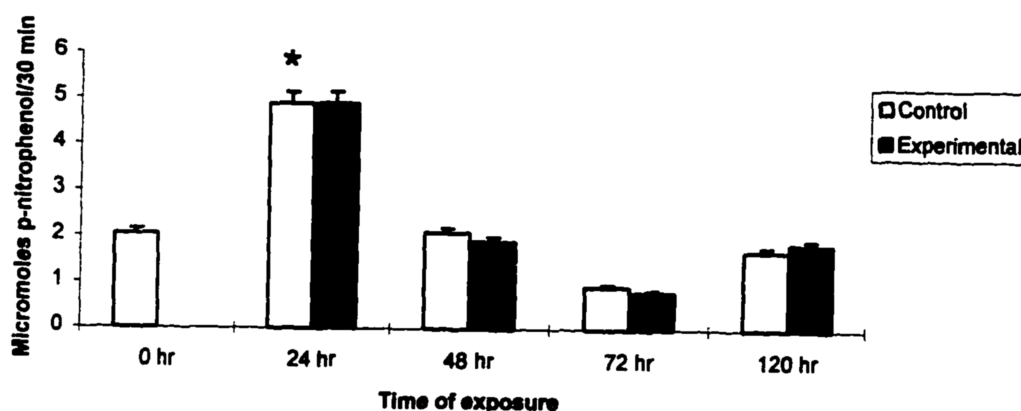

Figure 13. Effect Of *In Vitro* 20-HE On Metamorphic Glands. Glands were dissected from day 0 animals and cultured with or without 20-HE as described in Materials and Methods of Chapter II. After the glands were cultured for various exposure times, they were assessed by biochemical AP assay. The values represent means plus SEM of at least three experiments. The asterisk indicates statistically different value from 0 hr. This result suggests that the stress of culturing glands may reflect the increase in AP activity. Student's *t*-test was used to determine statistical significance.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

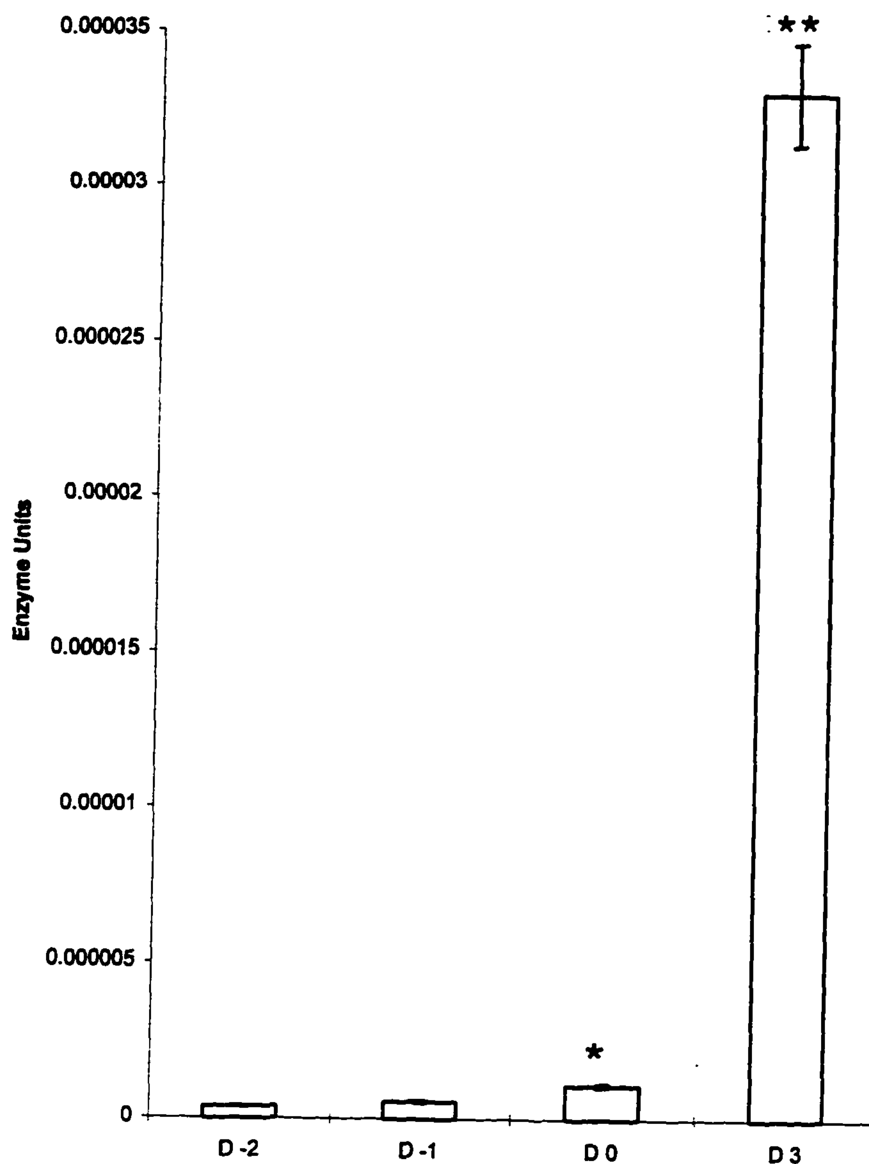

Figure 14. GalA Activity In Glands During Metamorphosis. Glands were isolated from spontaneously metamorphosing animals at various stages of development. The activity of α-galactosidase A was assessed as described in Materials and Methods of Chapter II. Values represent means ± SEM of at least three experiments. Asterisks indicate statistical differences from the previous value: *, $p < 0.01$; and **, $p < 0.0005$. Student's *t*-test was used to determine statistical significance. One enzyme unit is the amount of enzyme required to hydrolyze one nmol of substrate, 4-mehgylumbelliferyl-α-D-galactopyranoside (MUG), per hour at 37°C.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

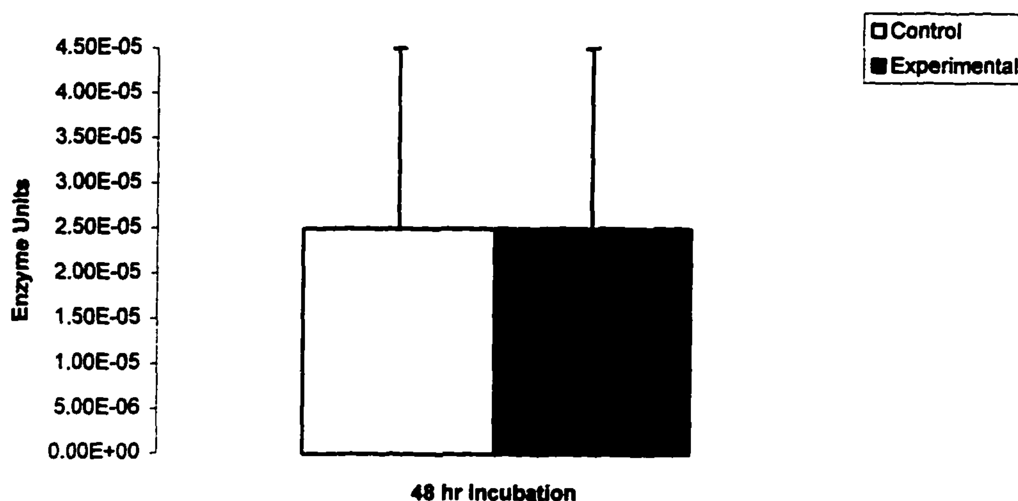

Figure 15. GalA Activity in Cultured Glands. α-Galactosidase A activity was assayed in day 2 glands that were cultured for 48 hr in the presence or absence of physiological levels of 20-HE, 2.1 X 10^{-6} M. The values represent means plus SEM of at least three experiments. The differences were not significant which may indicate that *in vitro* 20-HE may not directly affect lysosomal activity. One enzyme unit is the amount of enzyme required to hydrolyze one nmol substrate per hour at 37° C.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

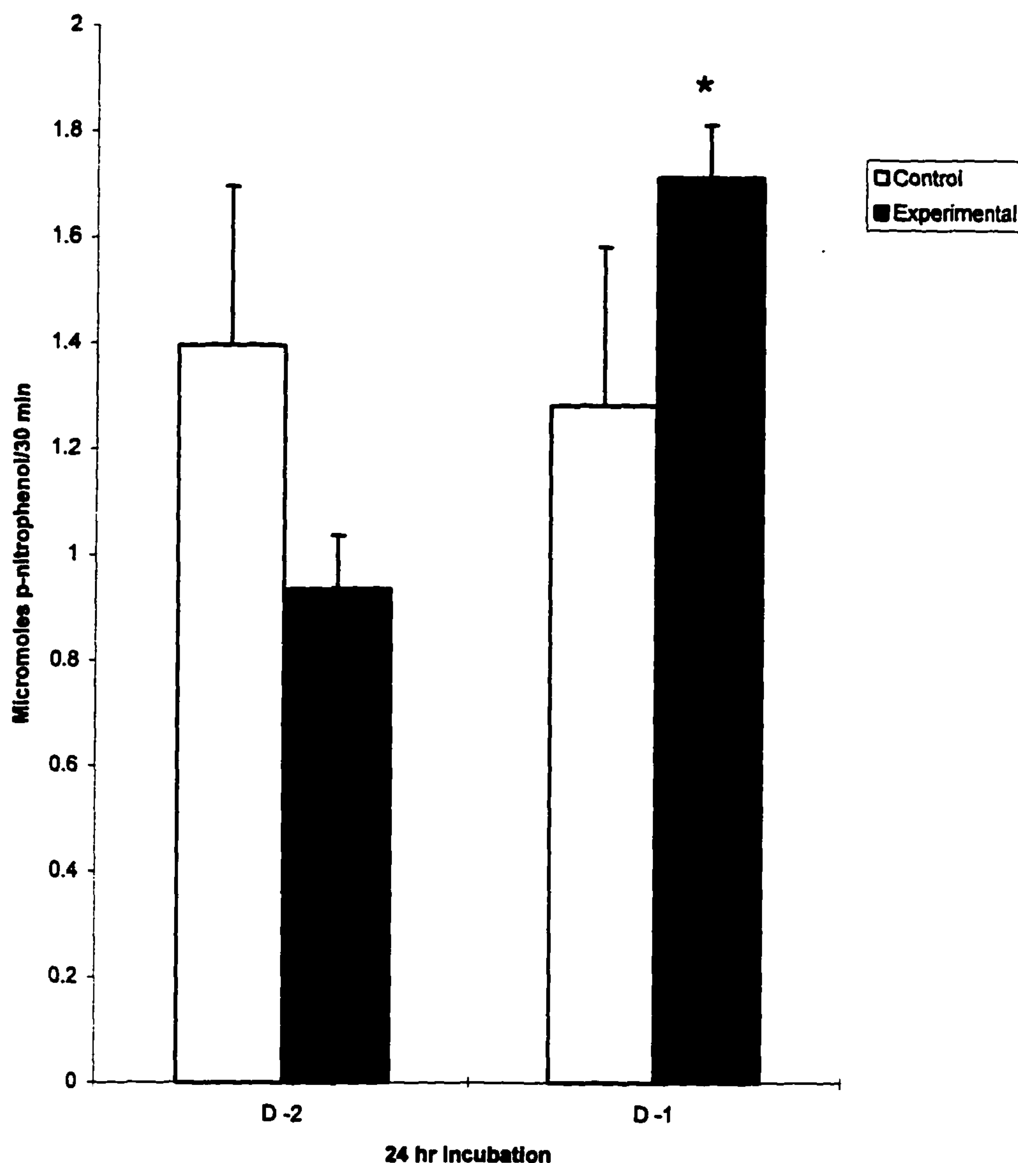

Figure 16. Effect Of *In Vivo* 20-HE On AP Activity In Premetamorphic Glands. Day -2 and day -1 animals were injected with vehicle or 20-HE and glands were dissected after 24 hr as described in Materials and Methods of Chapter III. The results of a biochemical AP assay are indicated as means plus SEM of at least three experiments. Asterisk indicates significant differences from control glands at $p < 0.004$. Student's *t*-test was used to determine statistical significance. This results suggest that 20-HE may prematurely induce cell death at this stage.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

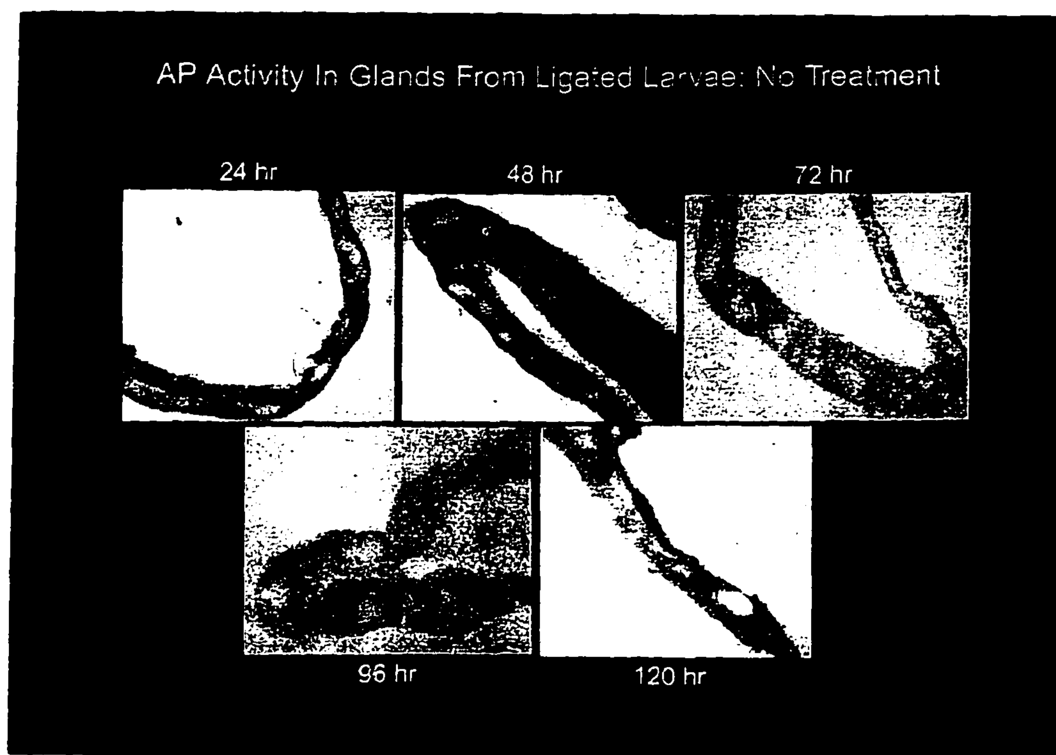

Figure 17. Histochemical Appearance Of AP In Untreated Glands From Isolated Abdomens. Animals were ligated at day 0 as described in Materials and Methods of Chapter 3. Isolated abdomens were left untreated (no injections) for 24-120 hr. Glands were then isolated and assessed by the histochemical acid phosphatase assay as described in Materials and Methods in Chapter I. Lysosomes were not appreciably detected until 96 hr. By 120 hr, the cytoplasm consisted mainly of remnant nuclei. This suggests that ligation may slow down the activation of lysosomes. Magnification: 100X. Microscope: compound.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

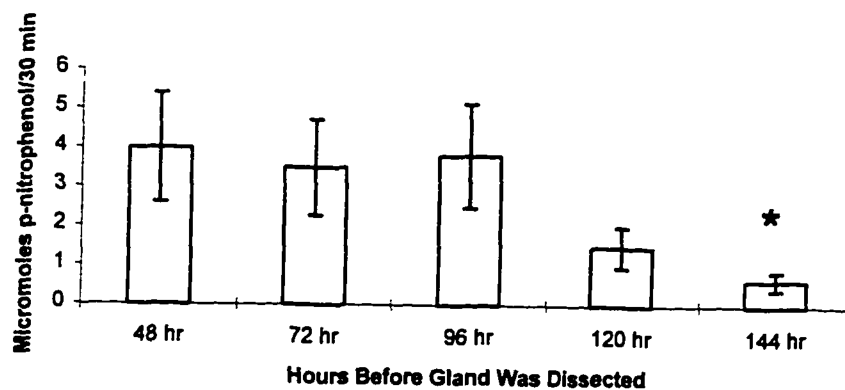

Figure 18. AP Activity In Untreated Glands From Isolated Abdomens. Day 0 animals were ligated and isolated abdomens were left untreated for various periods as described in Materials and Methods of Chapter III. The biochemical AP assay was performed at the end of the incubation periods. Values represent means ± SEM of at least three experiments. Asterisk indicates value significantly different from 120 hr, $p < 0.001$. Student's *t*-test was used to determine statistical significance.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

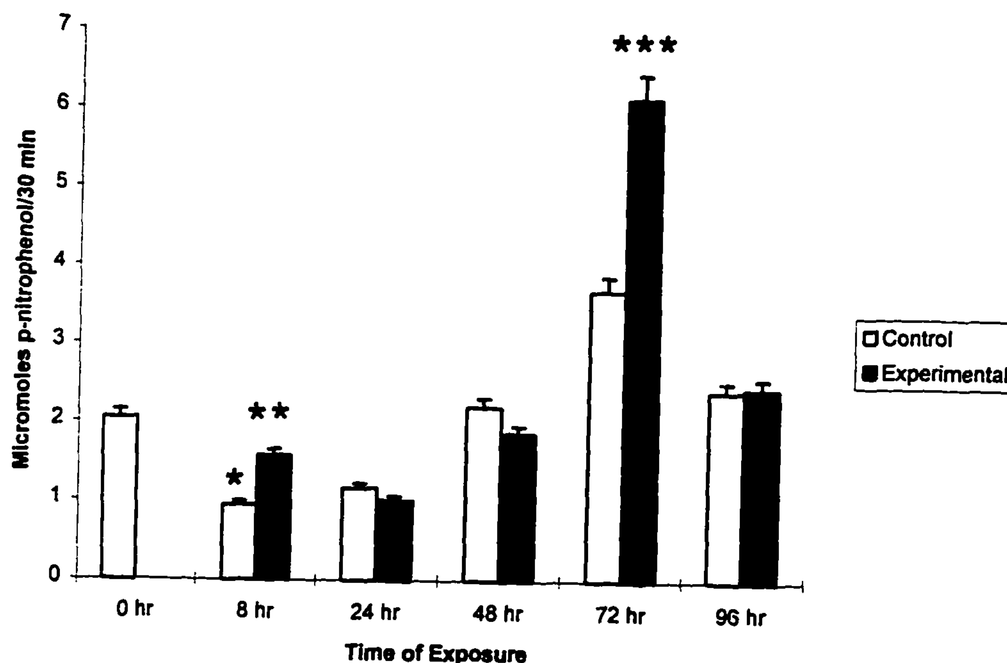

Figure 19. Effect Of 20-HE On AP Activity In Glands From Isolated Abdomens. Day 0 animals were ligated as described in Materials and Methods of Chapter III. Isolated abdomens were injected with vehicle or 20-HE. One injection was given for the 8 and 24 hr incubation periods, while one injection every 24 hr was given at the other times. Biochemical AP assay was performed on the glands. Values represent means plus SEM of at least three experiments. Single asterisk indicates significant differences from 0 hr, $p < 0.03$. Double and triple asterisks indicate significant differences between 20-HE treated and control (ethanol) groups: **, $p < 0.001$; and ***, $p < 0.004$. Student's *t*-test was used to determine statistical significance.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

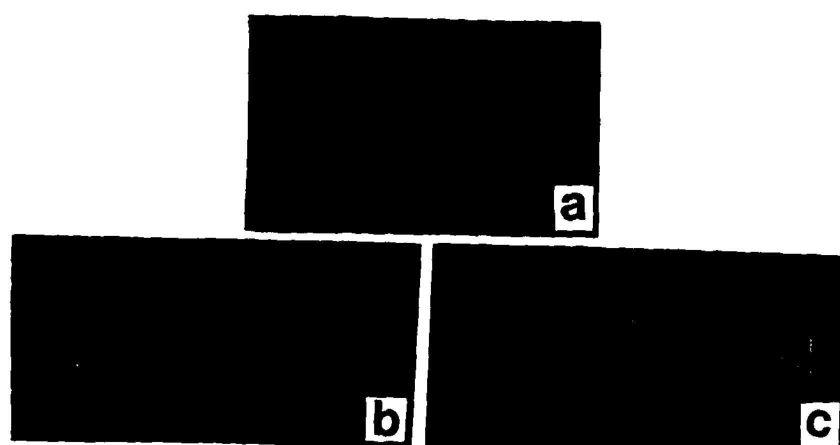

Figure 20. Localization Of Lysosomes In Glands That Were Treated *In Vivo* With Or Without 20-HE. (a) Day 3 labial gland during spontaneous metamorphosis. The following animals were ligated at day 0. (b) Gland from isolated abdomen that was injected with a single injection of vehicle. (c) Gland from isolated abdomen that was injected with a single injection of 20-HE. Glands were dissected after 72 hr and stained with acridine orange as described in Materials and Methods of Chapter III. Magnification: 400X. Microscope: compound.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

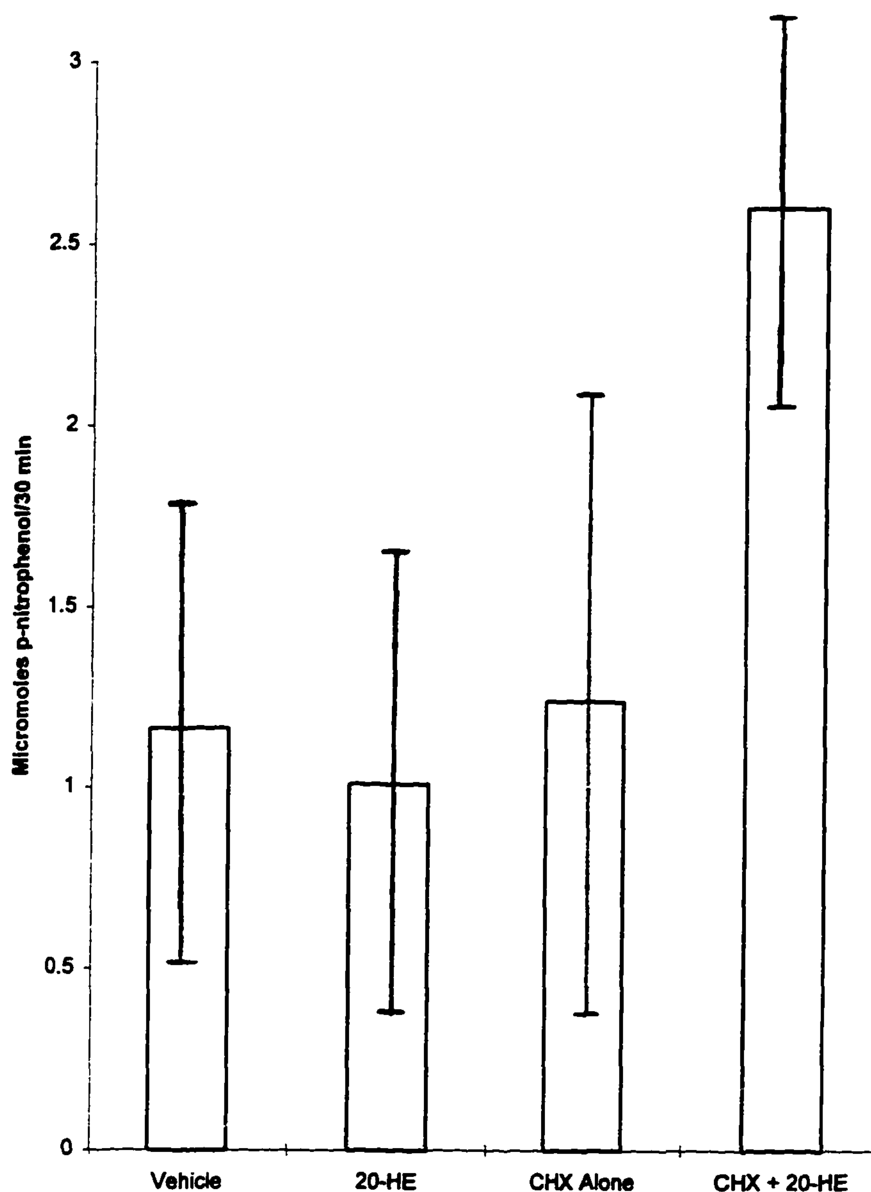

Figure 21. Effect Of *In Vivo* 20-HE & CHX On AP Activity. Isolated abdomens, from animals ligated at day 0, were injected with a single injection of vehicle, 20-HE, cycloheximide (CHX; inhibitor of protein synthesis), or 20-HE plus CHX. Glands were dissected after 24 hr and the biochemical AP assay was performed. Values represent means of at least three experiments. It appears that 20-HE plus CHX may have a synergistic effect of AP activity in the labial gland.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

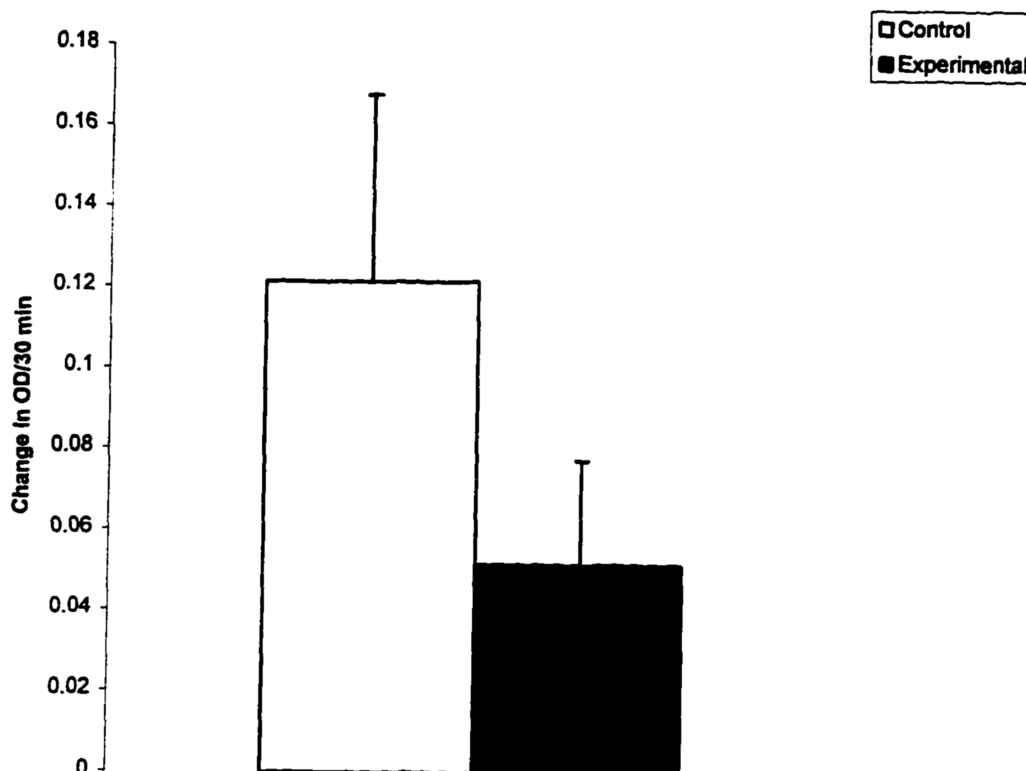

Figure 22. MTT Reduction In Glands From Isolated Abdomens. Animals were ligated at day 0. Isolated abdomens received a single injection of vehicle or 20-HE every 24 hr over a 72 hr period. Then glands were dissected and assessed for mitochondrial function by the biochemical MTT assay as described in Materials and Methods of Chapter I. Values represent means plus SEM of at least three experiments. 20-HE appears to have reduced mitochondrial function in half compared to control glands.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

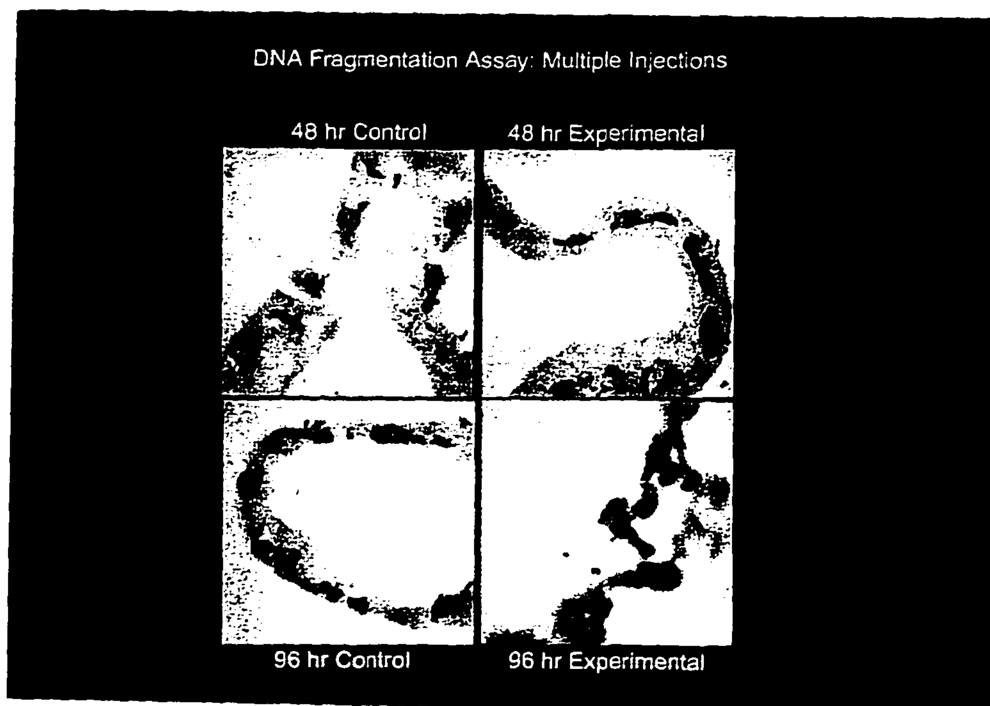

Figure 23. DNA Fragmentation: Multiple Injections. Animals were ligated at day 0. Isolated abdomens were injected with a single injection of vehicle or 20-HE every 24 hr. After various incubation periods, glands were assayed for *in situ* DNA fragmentation as described in Materials and Methods of Chapter III. 20-HE induced more nuclear degradation and morphological alterations (cytoplasmic shrinkage) compared to cells from control glands. Magnification: 100X. Microscope: compound.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

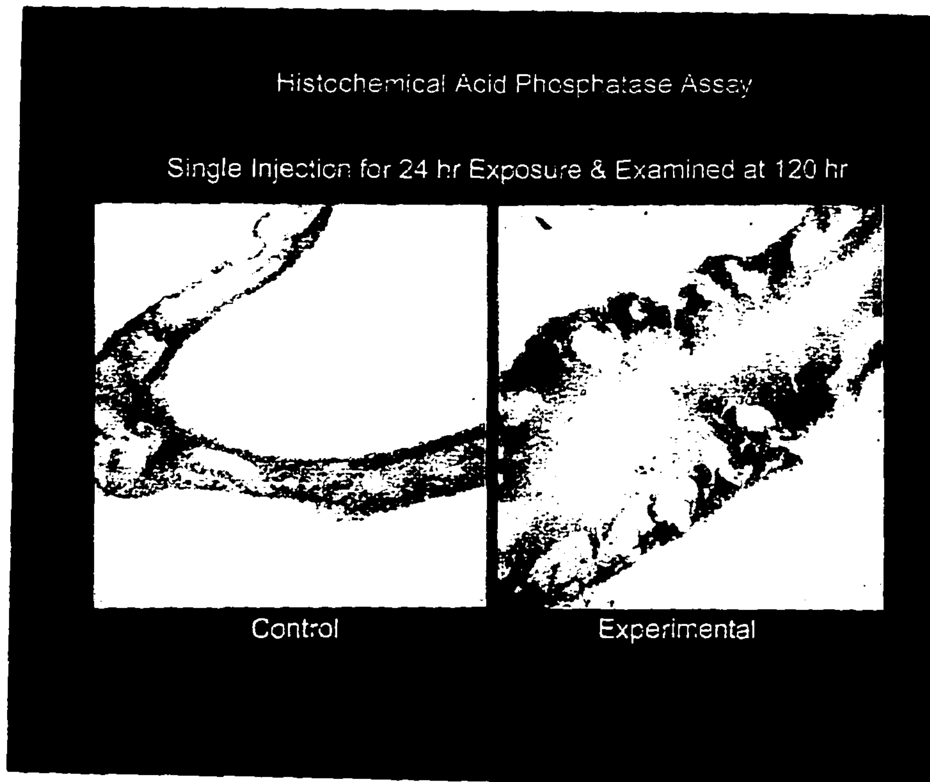

Figure 24. Effect Of A Single Pulse Of *In Vivo* 20-HE On Lysosomes. Animals were ligated at day 0. Isolated abdomens received a single injection of 20-HE or vehicle and glands were processed for the histochemical acid phosphatase assay, as described in Materials and Methods of Chapter I, after 120 hr. 20-HE induced movement and increased the number of lysosomes (right panel) compared to cell from control glands. This implies that a single pulse of 20-HE may be sufficient to trigger gland cell death. Magnification: 100X. Microscope: compound.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

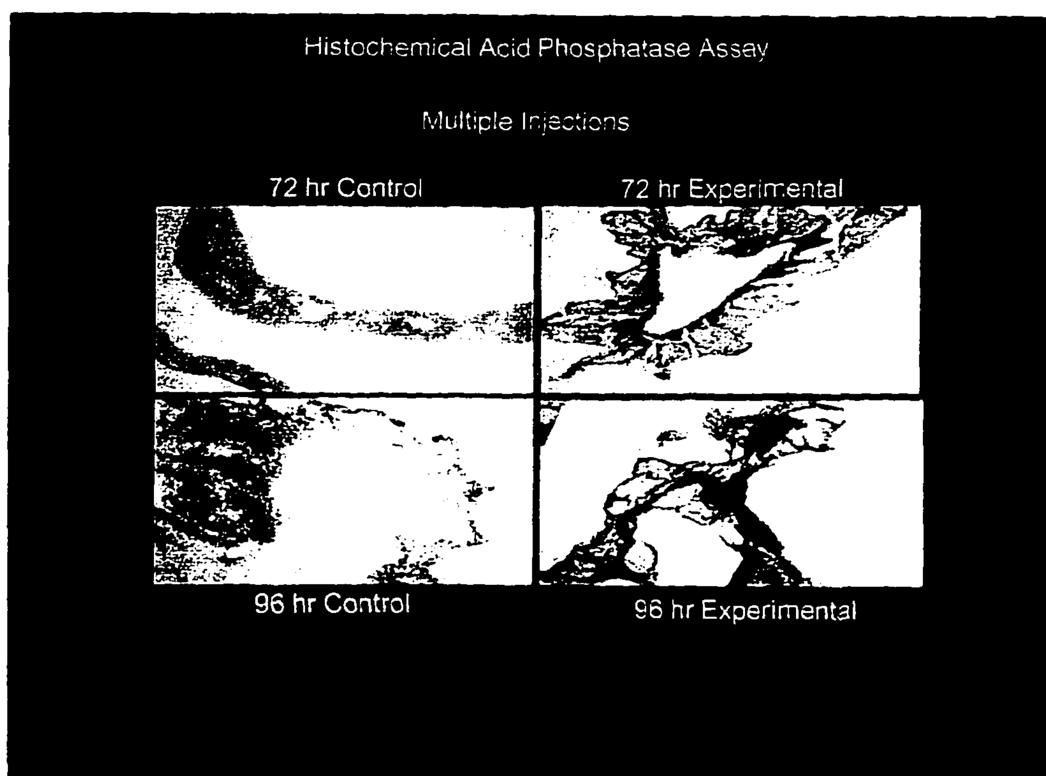

Figure 25. Effect Of Continuous *In Vivo* 20-HE On Lysosomes. Animals were ligated at day 0. Isolated abdomens received a single injection of vehicle or 20-HE every 24 hr and glands were dissected after the indicated exposure times. The glands were assessed with the histochemical acid phosphatase assay to localize lysosomes as described in Materials and Methods of Chapter I. The assay revealed that 20-HE induced lysosomal movement and cytoplasmic shrinkage of cells. Magnification: 100X. Microscope: compound.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

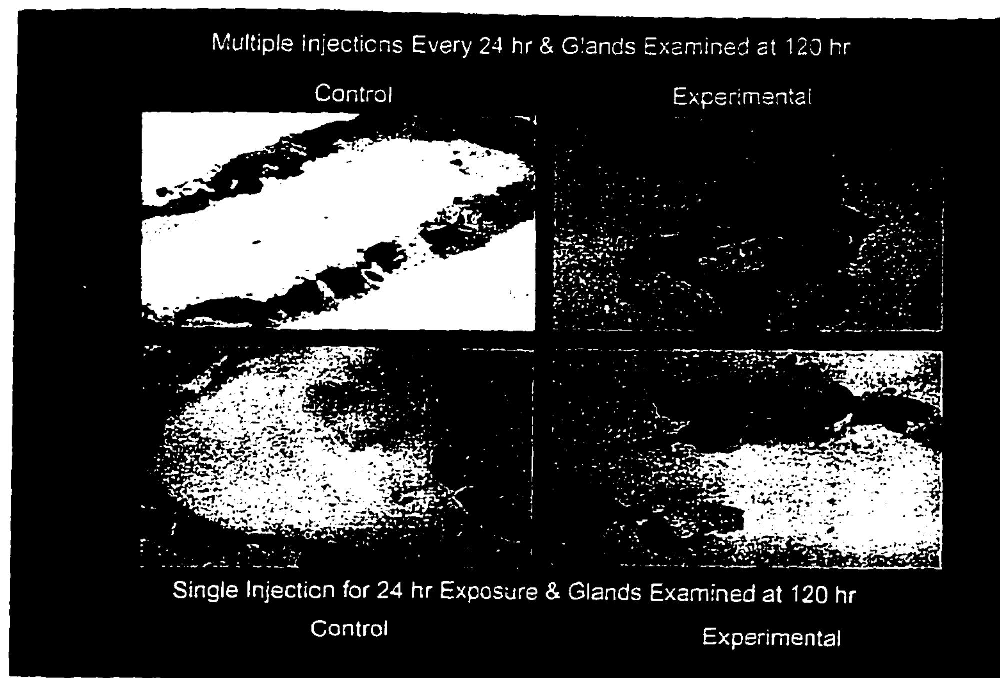

Figure 26. Evaluation Of Single & Multiple Injections Of 20-HE By DNA Fragmentation & Morphology. Isolated abdomens were exposed to *in vivo* 20-HE or vehicle. Some abdomens received one injection (lower panels) while others received a single injection at 24 hr intervals (upper panels). In either case glands were assayed at 120 hr for *in situ* DNA fragmentation as described in Materials and Methods of Chapter III. A single pulse of 20-HE (lower right panel) induced a level of DNA fragmentation that was comparable to glands exposed to multiple pulses of 20-HE (upper right panel). The morphological alterations induced by 20-HE are consistent with the appearance of a day 5 gland during spontaneous metamorphosis. Magnification: 100X. Microscope: compound.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

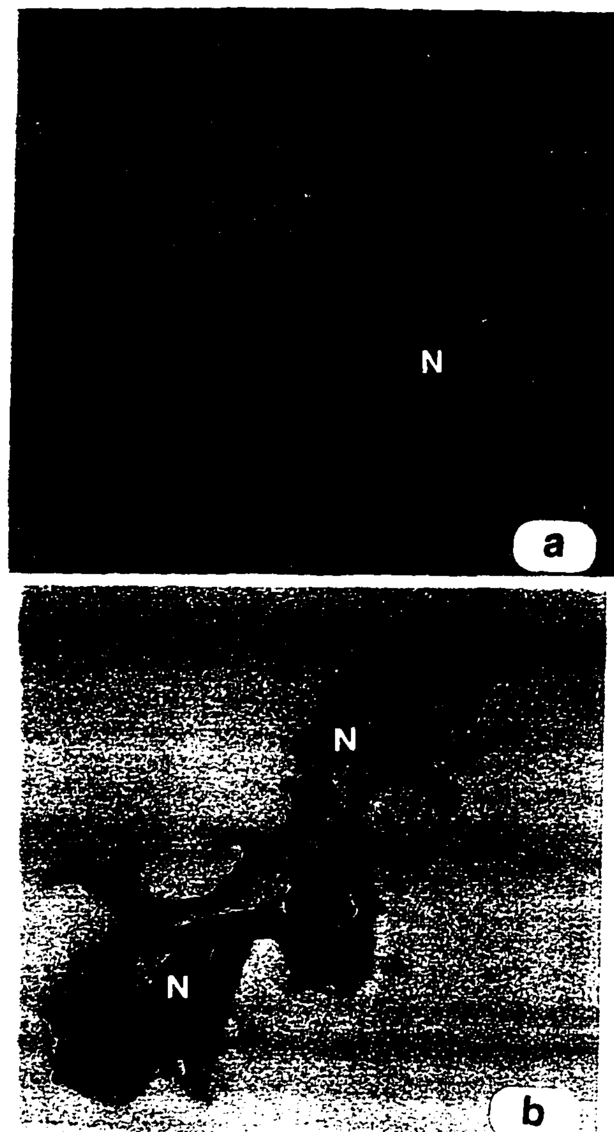

Figure 27. DNA Fragmentation During Metamorphosis. Glands were isolated from day 0 (a) and day 4 (b) spontaneously metamorphosing animals. DNA fragmentation was assessed by *in situ* staining as described in Materials and Methods of Chapter III. DNA fragmentation was detected only in the nucleus at day 0 (a). By day 4 (b) only remnant nuclei persist which displayed less intense staining compared to day 0. This may be due to the inability of terminal deoxynucleotidyl transferase (TdT) to access the 3'-OH ends of the DNA at later stages of cell death. Magnification: 100X. Microscope: compound.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

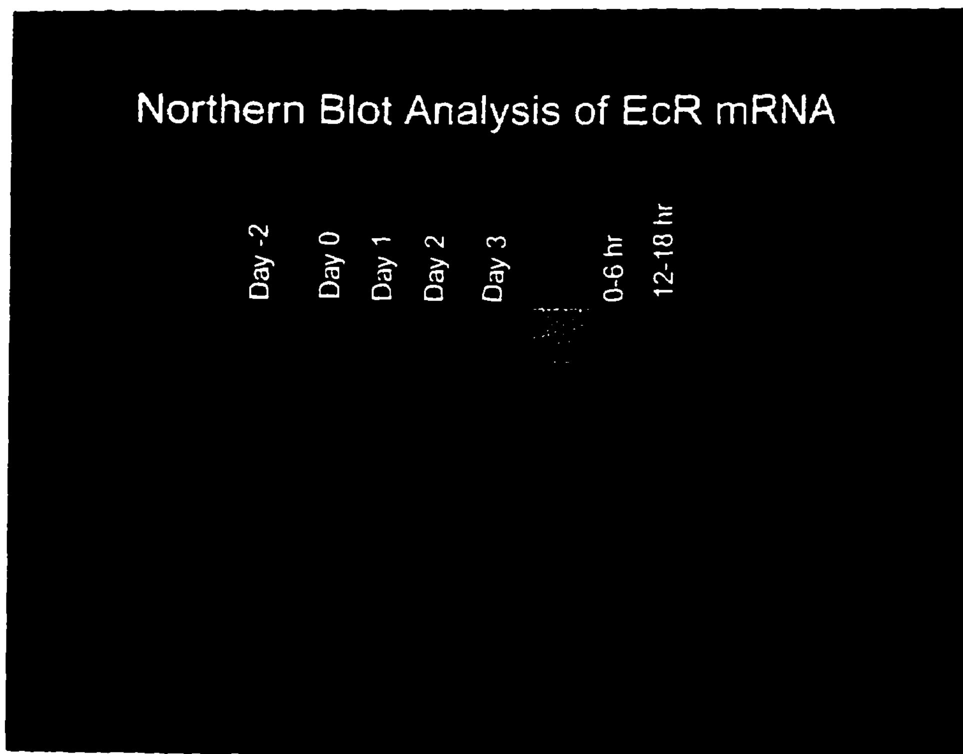

Figure 28. Northern Blot Analysis Of EcR mRNA During Metamorphosis. Total RNA was isolated from labial glands and intersegmental muscles (ISM) as described in Materials and Methods of Chapter IV. Thirty μg of total protein per lane was used. The expression of ecdysone receptor (EcR) mRNA is shown at various stages of gland development and times after adult emergence in ISM. Expression in labial glands was upregulated at day 0 and unchanged in metamorphic glands. The ISM are triggered to die by a decline in 20-HE levels. This correlated with downregulation of EcR mRNA at 0-6 hr and even more so at 12-18 hr.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

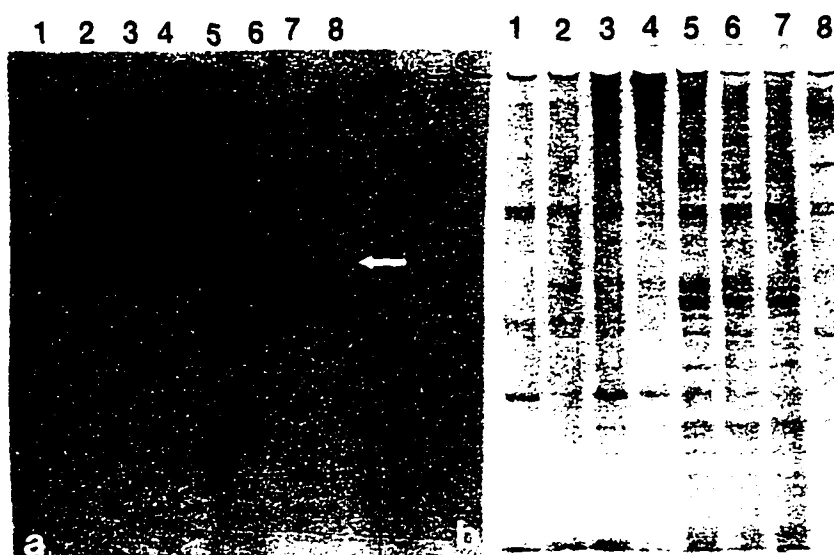

Figure 29. Western Blot Analysis Of EcR During Metamorphosis. (a) Western blot of EcR protein. (b) Coomassie blue-stained gel used in western blot. Labial glands and epidermis were dissected and protein was isolated as described in Materials and Methods of Chapter IV. Proteins were separated by PAGE and transferred to nitrocellulose membranes. The blot was reacted with an EcR antibody and the ECL kit was used for detection. Lanes are as follows: 1, day 4 gland; 2, day 3 gland; 3, day 2 gland; 4, day 1 gland; 5, day 0 gland; 6, day -1 gland; 7, day -2 gland; and 8, day -1 epidermis. No significant changes in the expression of EcR, at the level of protein, were detected. The arrow indicates EcR protein, between 75 and 80 kDa.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

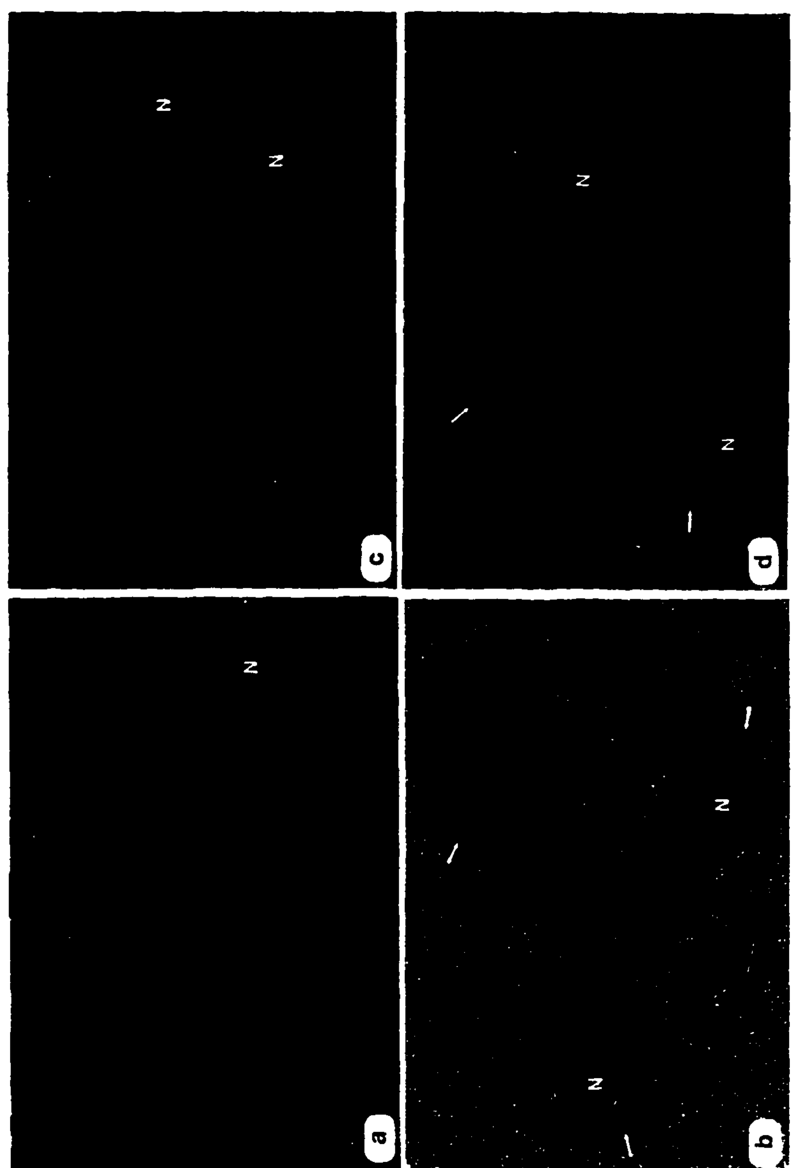

Figure 30. Immunocytochemistry Of EcR. (a) Day -1 gland. (b) Day 4 gland. (c) Gland from isolated abdomen injected with one injection of vehicle. (d) Gland from isolated abdomen injected with one injection of 20-HE. Glands, from treated abdomens, were dissected after 24 hr and were used along with spontaneously metamorphosing glands for immunocytochemistry as described in Materials and Methods of Chapter IV. EcR localization is mostly nuclear in premetamorphic (a) and control (c) glands. EcR protein, however, appears to translocate to the cytoplasm at later stages, day 4 (b), and in 20-HE treated glands (d). Nuclei are indicated as (N) and cytoplasmic labeling of EcR protein is indicated by arrows Magnification: 100X. Microscope: compound.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

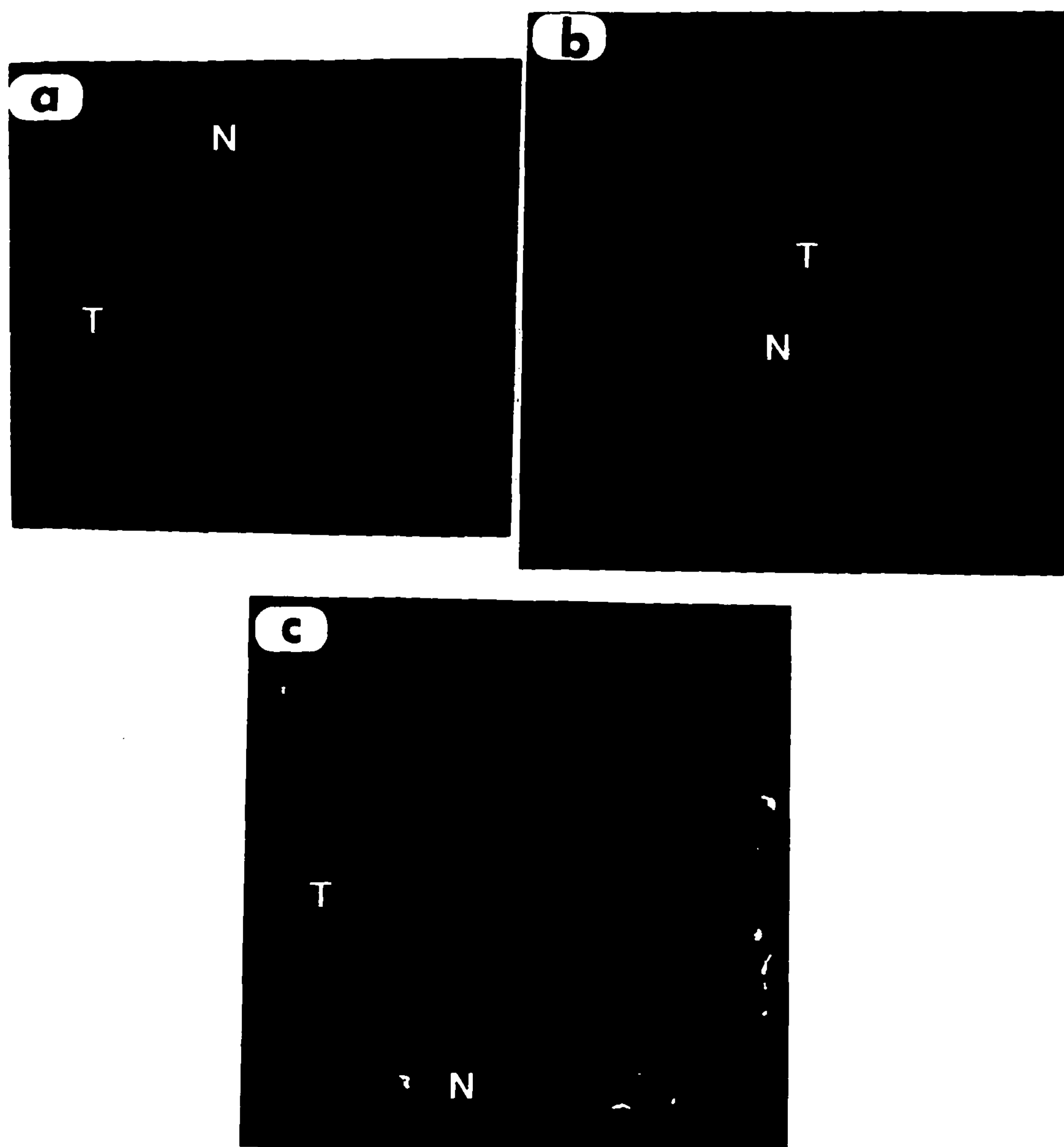

Figure 31. Examination Of Microtubules In Glands. (a) Day 1 gland. (b) Day 3 gland. (c) Day 5 gland. Labial glands were isolated from spontaneously metamorphosing animals and prepared for immunofluorescence of tubulin (T) and DAPI (stains DNA; seen as blue nuclear (N) stain) as described in Material and Methods of Chapter IV. Glands were observed by fluorescence and confocal microscopy using fluorescein and DAPI filters. The tubulin cytoskeleton appears to be reorganized as the glands undergo metamorphosis. Magnification: 100X. Microscope: compound.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

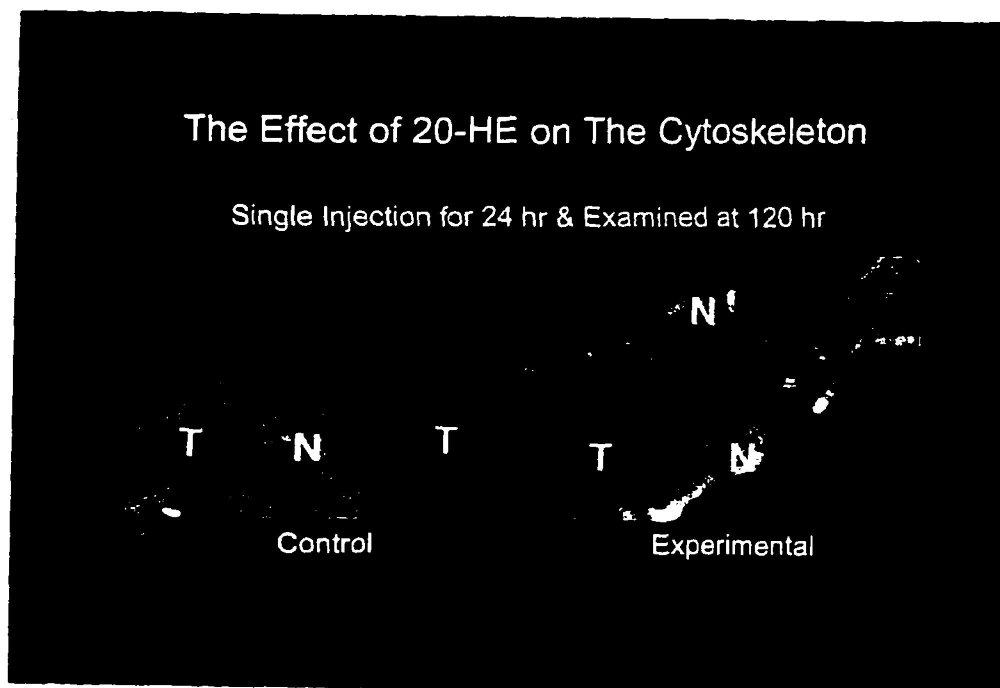

Figure 32. The Effect Of 20-HE On The Cytoskeleton. Animals were ligated at day 0. Isolated abdomens were injected with a single injection of vehicle or 20-HE as described in Materials and Methods of Chapter III. Glands were dissected at 120 hr and processed for tubulin (T) and DAPI (N; nuclear stain) immunofluorescence as described in Materials and Methods of Chapter IV. Experimental glands displayed more shrinkage and alterations in the tubulin cytoskeleton (T; pink stain) compared to controls. The cytoplasm of experimental glands was greatly reduced, resulting in more remnant nuclei (N; blue stain) compared to control glands. Magnification: 100X. Microscope: compound.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

PART IV. REFERENCES

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Aidells, B., Lockshin, R.A., and Cullin, A.M. 1971. Breakdown of the silk glands in *Galleria mellonella*. Acid phosphatase in involuting glands. J.Insect Physiol. 17:857-869.

Aizenzon, M.G., and Zhimulev, I.F. 1975. Hormonal control of lysis of the salivary gland in larvae of *Drosophila melanogaster*. Dokl.Akad.Nauk SSSR 221:1441-1443.

Akai, H. 1984. The ultrastructure and functions of the silk glands of *Bombyx mori*. In Insect Ultrastructure. (Eds. R.C. King and H. Akai). pp. 323-364. New York: Plenum Press.

Ameison, J.C., and Capron, A. 1991. Cell dysfunction and depletion in AIDS: the programmed cell death hypothesis. Immunol.Today 12:102-105.

Andres, A.J., and Thummel, C.S. 1992. Hormones, puffs and flies: the molecular control of metamorphosis by ecdysone. Trends Genet. 8:132-138.

Ankenbauer, W., Strahle, U., Schutz, G. 1988. Synergistic action of glucocorticoid and estradiol responsive elements. Proc.Natl.Acad.Sci. 85:7526-7530.

Antoniewski, C., Mugat, B., Delbac, F., and Lepesant, J.A. 1996 Direct repeats bind the EcR/USP receptor and mediate ecdysteroid responses in *Drosophila melanogaster*. Mol.Cell.Biol. 16:2977-2986.

Ashburner, M., Chihara, C., Meltzer, P., and Richards, G. 1974. Temporal control of puffing activity in polytene chromosomes. Cold Spring Harbor Symp.Quant.Biol. 23:655-662.

Ashburner, M. 1971. Induction of puffs in polytene chromosomes of in vitro cultured salivary glands of *Drosophila melanogaster* by ecdysone and ecdysone analogues. Nature 230:222-224.

Beal, M.F. 1992. Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? Ann.Neurol. 31: 119-130.

Beato, M. 1989. Gene regulation by steroid hormones. Cell 56:335-344.

Beaulaton, J., and Lockshin, R.A. 1977. Ultrastructural study of the normal degeneration of the intersegmental muscles of *Antheraea polyphemus* and *Manduca sexta* (Insecta, Lepidoptera) with particular reference to cellular autophagy. J.Morphol. 154:39-58.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Beckingham Smith, K., and Tata, J.R. 1976. The hormonal control of amphibian metamorphosis. In Developmental Biology of Plants and Animals. (eds. C. Graham and P.F. Wareing). pp. 232-245. Oxford: Blackwell.

Behl, C., Widmann, M., Trapp, T., and Holsboer, F. 1995. 17-beta estradiol protects neurons from oxidative stress-induced cell death *in vitro*. Biochem.Biophys.Res.Commun. 216:473-482.

Bell, R.A., and Joachim, F.G. 1976. Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. Ann.Entomol.Soc.Am 69:365-373.

Belyaeva, E.S., Aizenzon, M.G., Semeshin, V.F., Kiss, I., Koezka, K., Baritcheva, E.M., Gorelova, T.D., and Zhimulev, I.F. 1980. Cytogenetic analysis of the 2B3-4 to 2B11 region of the X-chromosome of *Drosophila melanogaster*. I. Cytology to the region and mutant complementation groups. Chromosoma 81:281-306.

Bennett, K.L., and Truman, J.W. 1985. Steroid-dependent survival of identifiable neurons in cultured ganglia of the moth *Manduca sexta*. Science 229:58-60.

Berger, E.M., Sloboda, R.D., and Ireland, R.C. 1980. Tubulin content and synthesis in differentiating *Drosophila* cells in culture. Cell Motil 1:113-129.

Bijovsky, A.T., and Abrahamsohn, P.A. 1992. Changes of the Golgi apparatus and lysosomes during decidualization in mice. Tissue Cell 24:635-642.

Billig, H., Furata, I., and Hsueh, A.J.W. 1993. Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. Endocrinology 133:2204-2212.

Birkenbeil, H., Eckert, M., and Gersch, M. 1979. Electron microscopical-immunocytochemical evidence of ecdysteroids in the prothoracic gland of *Galleria mellonella*. Cell Tissue Res. 200:285-290.

Bodenstein, D. 1943. Factors influencing growth and metamorphosis of the salivary gland in *Drosophila*. Biol.Bull. 84:13-33.

Bodwell, J.E., Orti, E., Coull, J.M., Pappin, D.J.C., Smith, L.I., and Swift, F 1991. Identification of phosphorylated sites in the mouse glucocorticoid receptor. J.Biol.Chem. 266:7549-7555.

Boise, L., Gonzalez-Garcia, M., Postema, C., Ding, L., Lindsten, T., Turka, L., Mao, X., Nunez, G., and Thompson, C. 1993. bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597-608.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Bollenbacher, W.E., Smith, S.L., Goodman, W., and Gilbert, L.I. 1981. Ecdysteroid titer during larval-pupal-adult development of the tobacco hornworm, *Manduca sexta*. Gen.Comp.Endocrinol. 44:302-306.

Bonyhadi, M.L., Rabin, L., Salimi, S., Brown, D.A., Kosek, J., McCune, J.M., and Kaneshima, H. 1993. HIV induces thymus depletion in vivo. Nature 363:728-732.

Brown, T.A., and DeLuca, H.F. 1990. Phosphorylation of the 1,25-dihydroxyvitamin D3 receptor. A primary event in 1,25-dihydroxyvitamin D3 action. J.Biol.Chem. 265:10025-10029.

Bruggenmeier, U., Kalff, M., Franke, S., Scheidereit, C., and Beato, M. 1991. Ubiquitous transcription factor OTF-1 mediates induction of the MMTV promoter through synergistic interaction with hormone receptors. Cell 64:565-572.

Burtis, K.C., Thummel, C.S., Jones, C.W., Karim, F.D., and Hogness, D. 1990. The *Drosophila* 74EF early puff contains E74, a complex ecdysone-inducible gene that encodes two ets-related proteins. Cell 61:85-99.

Bump, N.J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Ferenz, C., Franklin, S., Ghayur, T., Li, P., Licari, P., Mankovich, J., Shi, L., Greenberg, A.H., Miller, L.K., and Wong, W.W. 1995. Inhibition of ICE family proteases by baculovirus antiapoptotic protein p 35. Science 269:1885-1888.

Buttyan, R., Zakeri, Z., Lockshin, R., and Wolgemuth, D. 1988. Cascade induction of c-fos, c-myc, and heat shock 70K transcripts during regression of the rat ventral prostate gland. Mol.Endocrinol. 2:650-657.

Caglayan, S.H. 1990. Effect of 20-hydroxyecdysone on acid phosphatase activity in the larval fat body of *Manduca sexta*. Biochem.Int. 20:511-518.

Cathala, G., Savouret, J.F., Mendez, B., West, B.L., Karin, M., Martial, J.A., and Baxter, J.D. 1983. A method for isolation of intact, translationally active ribonucleic acid. DNA 2:329-335.

Chandler, V.L., Maler, B.A., and Yamamoto, K.R. 1983. DNA sequences bound specifically by glucocorticoid receptor in vitro render a heterologous promoter hormone responsive *in vivo*. Cell 33:489-499.

Chao, A.T., and Guild, G.M. 1986. Molecular analysis of the ecdysterone-inducible 2B5 early puff in *Drosophila melanogaster*. EMBO J. 5:143-150.

Cherbas, L., Lee, K., and Cherbas, P. 1991. Identification of ecdysone response

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

elements by analysis of the *Drosophila* Eip28/29 gene. Genes Dev. 5:120-131

Chinzei, Y. 1975. Induction of histolysis by ecdysterone in vitro: breakdown of anterior silk gland in silkworm, *Bombyx mori* (Lepidoptera:Bombycidae). Appl.Ent.Zool. 10:136-138.

Cho-Chung, Y.S. 1978. Interaction of cyclic AMP and estrogen in tumor growth control. In Endocrine Control of Neoplasia. (eds. R. Sharma and W.E. Cruss). pp. 335-346. New York: Raven Press.

Choi, M.K., and Fahrbach, S.E. 1995. Evidence for an endogenous neurocidin in the *Manduca sexta* ventral nerve cord. Arch.Insect Biochem.Physiol. 28:273-289.

Clever, U. 1964. Actinomycin and puromycin: effects on sequential gene activation by ecdysone. Science 146:794-795.

Clever, U., and Karlson, P. 1960. Induktion von puff-veranderungen in den speicheldrussen-chromosen von *Chironomous tentans* durch ecdyson. Exp.Cell Res. 20:623-626.

Collins, J.V. 1975. Soluble acid phosphatases of the hemolymph and fat body of *Calpodes ethlius* (Stoll) and the control of protein storage by fat body. Can.J.Zool. 53:480-489.

Compton, M.M., and Cidlowski, J.A. 1986. Rapid *in vivo* effects of glucocorticoids on the integrity of rat lymphocyte genomic deoxyribonucleic acid. Endocrinology 118:38-45.

Conneely, O.M., Sullivan, W.P., Toft, D.O., Birnbaumer, M., Cook, R., Maxwell, B., Zarucki-Schulz, T., Greene, G., Schrader, W., and O'Malley, B.W. 1986. Molecular cloning of the chicken progesterone receptor. Science 233:767-770.

Cowan, W.M., Fawcett, J.W., O'Leary, D.D.M., and Stanfield, B.B. 1984. Regressive events in neurogenesis. Science 225:1258-1265.

Cymborowski, B., Bogus, M., Beckage, N.E., Williams, C.M., and Riddirord, L.M. 1982. Juvenile hormone titres and metabolism during starvation-induced supernumerary larval moulting of the tobacco hornworm, *Manduca sexta*. J.Insect.Physiol. 28:129-135.

Dalman, F.C., Sturzenbecker, L.J., Levin, A.A., Lucas, D.A., Perdew, G.H., Petkovich, M., Chambon, P., Grippo, J.F., and Pratt, W.B. 1991. Retinoic acid receptor belongs to a subclass of nuclear receptors that do not form "docking'

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

complexes with hsp90. Biochemistry 30:5605-5608.

Dean, R.L. 1978. The induction of autophagy in isolated insect fat body by ß-ecdysone. J.Insect Physiol. 439-447.

DeMarzo, A.M., Beck, C.A., Onate, S.A., and Edwards, D.P. 1991. Dimerization of mammalian progesterone receptors occurs in the absence of DNA and is related to the release of the 90kD heat shock protein. Proc.Natl.Acad.Sci. 88:72-76.

Denner, L.A., Weigel, N.L., Maxwell, B.L., Schrader, W.T., and O'Malley, B.W. 1990. Regulation of progesterone receptor-mediated transcription by phosphorylation. Science 250:1740-1743.

De Priester, W., van Pelt-Verkuil, E., and de Leeuw, G. 1979. Demonstration of acid phosphatase activity induced by 20-hydroxyecdysone in the fat body of *Calliphora*. Cell Tissue Res. 200:435-442.

Desnick, R.J., and Bishop, D.F. 1989. Fabry disease: α-galactosidase A deficiency; Schindler disease: α-N-acetylgalactosaminidase deficiency. In The Metabolic Basis of Inherited Disease, II, 6 th ed. (eds. C.R. Scriver, A.L. Boudet, W.S. Sly, and D. Valle). pp. 1751-1796. New York: McGraw-Hill.

De The, I.L., Vivanco-Rulz, M., Trolluise, P., Stunnenborg, H., and Dejeun, A. 1990. Identification of a retinoic acid responsive element in the retinoic acid receptor beta gene. Nature 343:177-180.

DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W., and Guild, G.M. 1991 The *Drosophila* Broad-Complex encodes a family of related proteins containing zinc fingers. Genetics 129:385-397.

Dominick, O.S., and Truman, J.W. 1984. The physiology of wandering behaviour in *Manduca sexta*. I. Temporal organization and the influence of the internal and external environments. J.Exp.Biol. 110:35-51.

Dyer, C.A., and Benjamins, J.A. 1989. Organization of oligodendroglial membrane sheets: II. Galactocerebroside: antibody interactions signal changes in cytoskeleton and myelin basic protein. J.Neurosci.Res. 24:212-221.

Ellis, H.M., and Horvitz, H.R. 1986. Genetic control of programmed cell death in the nematode *C.elegans*. Cell 44:817-829.

Engelhardt, D., and Weber, M.M. 1994. Therapy of Cushing's syndrome with

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

steroid biosynthesis inhibitors. J.Steroid Biochem.Mol.Biol. 49:261-267.

Evans, R.M. 1988. The steroid and thyroid hormone receptor superfamily. Science 240:889-895.

Fahrbach, S.E, Choi, M.K., and Truman, J.W. 1994. Inhibitory effects of actinomycin D and cycloheximide on neuronal death in adult *Manduca sexta.* J.Neurobiol. 25:59-69.

Fahrbach, S.E. 1992. Developmental regulation of ecdysteroid receptors in the nervous system of *Manduca sexta.* J.Exp.Zool. 261:245-253.

Fahrbach, S.E., and Truman, J.W. 1987. Mechanisms for programmed cell death in the nervous system of a moth. Ciba Found.Symp. 126:65-81.

Farber, J.L., Kyle, M.E., and Coleman, J.B. 1990. Mechanisms of cell injury by activated oxygen species. Lab.Invest. 62: 670-679.

Finlayson, L.H. 1956. Normal and induced degeneration of abdominal muscles during metamorphosis in the lepidoptera. Q.J.Microsc.Sci. 97:215-234.

Fletcher, J.C., and Thummel, C.S. 1995. The *Drosophila* E74 gene is required for the proper stage-and tissue-specific transcription of ecdysone-regulated genes at the onset of metamorphosis. Development 121:1411-1421.

Forman, B.M., and Samuels, H.H. 1990. Dimerization among nuclear hormone receptors. New Biol. 2:587-594.

Fujiwara, H., Jindra, M., Newitt, R., Palli, S.R., Hiruma, K., and Riddiford, L.M. 1995. Cloning of an ecdysone receptor homolog from *Manduca sexta* and the developmental profile of its mRNA in wings. Insect Biochem.Mol.Biol. 25:845-856.

Gagliardini, V., Fernandez, P-A., Lee, R.K.K., Drexler, H.C.A., Rotello, R.J., Fishman, M.C., and Yuan, J. 1994. Prevention of vertebrate neuronal death by the crmA gene. Science 263:826-828.

Geiger, B., Rosen, D., and Berke, G. 1982. Spatial relationships of microtubule-organizing centers and the contact areas of cytotoxic T lymphocytes and target cells. J.Cell Biol. 95:137-143.

Glass, C.K., Lipkin, S.M., Devary, O.V., and Rosenfeld, M.G. 1989. Positive and negative regulation of gene transcription by a retinoic acid-thyroid hormone receptor heterodimer. Cell 59:697-708.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Godowski, P.J., Rusconi, S., Miesfeld, R., and Yamamoto, K.R. 1987. Glucocorticoid receptor mutants that are constitutive activators of transcriptional enhancement. Nature 325:365-368.

Golstein, P., Marguet, D., and Depraetere, V. 1995. Homology between reaper and the cell death domains of Fas and TNFR1. Cell 81:185-186

Gorski, J., Toft, D.O., Shyamala, G., Smith, D., and Notides, A. 1968. Hormone receptors: studies on the interaction of estrogens with the uterus. Recent Prog.Horm.Res. 24:45-80.

Grasl-Kraupp, B., Buttkay-Nedecky, B., Koudelka, H., Bukowska, K., Bursch, W., and Schulte-Hermann, R. 1995. In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 21:1465-1468.

Green, S., and Chambon, P. 1991. The oestorgen receptor: from perception to mechanism. In Nuclear Hormone Receptors. (ed. M.G. Parker). pp.15-38. New York: Academic Press.

Gregorio, C.C., Black, J.D., and Repasky, E.A. 1993. Dynamic aspects of cytoskeletal protein distribution in T lymphocytes: involvement of calcium in spectrin reorganization. Blood Cells 19:361-371.

Grether, M.E., Abrams, J.M., Agapite, J., White, K., and Steller, H. 1995. The head involution defective gene of *Drosophila melanogaster* functions in programmed cell death. Genes Dev. 9:1694-1708.

Guay, P.S., and Guild, G.M. 1991. The ecdysone-induced puffing cascade in *Drosophila* salivary glands: A Broad-Complex early gene regulates intermolt and late gene transcription. Genetics 129:169-175.

Guiochon-Mantel, A., Loosfelt, H., Lescop, P., Sar, S., Atger, M., Perrot-Applanat, M., and Milgrom, E. 1989. Mechanisms of nuclear localization of the progesterone receptor: evidence for interaction between monomers. Cell 57:1147-1154.

Haas, A.L., Baboshina, O., Williams, B., and Schwartz, L.M. 1995. Coordinated induction of the ubiquitin conjugation pathway accompanies the developmentally programmed death of insect skeletal muscle. J.Biol.Chem. 270:9407-9412.

Halaby, R., Zakeri, Z., and Lockshin, R.A. 1994. Metabolic events during programmed cell death in insect labial glands. Biochem.Cell Biol. 72:597-601.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Hammar, S.P., and Mottet, N.K. 1971. Tetrazolium salt and electronmicroscopic studies of cellular degeneration and necrosis in the interdigital areas of the developing chick limb. J.Cell Sci. 8:229-251.

Hann, S.K., Kim, H.I., Im, S., Park, Y.K., Cui, J., and Bystryn, J.C. 1993. The change of melanocyte cytotoxicity after systemic steroid treatment in vitiligo patients. J.Dermatol.Sci. 6:201-205.

Hard, T., Kellenbach, E., Boelens, R., Maler, B.A., Dahlman, K Freedman, L.PI, Carlstedt-Duke, J., Yamamoto, K.R., Gustafsson, J-A., and Kaptein, R. 1990. Solution structure of the glucocorticoid receptor DNA-binding domain Science 249:157-160.

Harmon, B., Bell, L., and Williams, L. 1984. An ultrastructural study on the "meconium corpuscles" in rat foetal intestinal epithelium with particular reference to apoptosis. Anat.Embryol. 169:119-124.

Hay, B.A., Wolff, T., and Rubin, G.M. 1994. Expression of baculovirus P35 prevents cell cell death in *Drosophila*. Development 120:2121-2129.

Helmberg, A., Auphan, N., Caelles, C., and Karin, M. 1995. Glucocorticoid-induced apoptosis of human leukemic cells is caused by the repressive function of the glucocorticoid receptor. EMBO J. 14:452-460.

Helminen, H.J., Ericsson, J.L.E, and Bengt, A. 1972. Differing patterns of acid phosphatase and cathepsin D activities in the rat ventral prostate gland during castration-induced prostatic involution. Acta Endrocrinol. 69:747-761

Hengartner, M.O., and Horvitz, H.R. 1994. *C.elegans* cell death gene ced-9 encodes a functional homolog of the mammalian proto-oncogene bcl-2. Cell 76:665-676.

Hengartner, M.O., Ellis, R.E., and Horvitz, H.R. 1992. *Caenorhabditis elegans* gene ced-9 protects cells from programmed cell death. Nature 356:494-499.

Hirschfeld, S., and Clearfield, H.R. 1995. Pharmacologic therapy for inflammatory bowel disease. Am.Fam.Physician 51:1971-1975.

Hollenberg, S.M., Weinberger, C., Ong, E.S., Carelli, G., Oro, A., Lebo, R., Thompson, E., and Evans, R.M. 1985. Primary structure and expression of a functional human glucocorticoid receptor. Nature 318:635-641.

Ishizuya-Oka, A., and Shimozawa, A. 1992. Programmed cell death and

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

heterolysis of larval epithelial cells by macrophage-like cells in the anuran small intestine *in vivo* and *in vitro*. J.Morphol. 213:185-195.

Jensen, E.V., Suzuki, T., Kawashima, T., Stumpf, W.E., Jungblut, P.W., and DeSombre, E.R. 1968. A two step mechanism for the interaction of estradiol with rat uterus. Proc.Natl.Acad.Sci. 59:632-638.

Jindra, M., Malone, F., Hiruma, K., and Riddirord, L.M. 1996. Developmental profiles and ecdysteroid regulation of the mRNAs for two ecdysone receptor isoforms in the epidermis and wings of the tobacco hornworm, *Manduca sexta*. Dev.Biol. 180:258-272.

Jochová, J., Zakeri, Z., and Lockshin, R.A. 1997. Rearrangement of the tubulin and actin cytoskeleton during programmed cell death in *Drosophila* salivary glands. Cell Death Differ. 4: 140-149.

Jones, H.E., and Bowen, I.D. 1993. Acid phosphatase activity in the larval salivary glands of developing *Drosophila melanogaster*. Cell Biol.Int. 17: 305-315.

Jurkowitz-Alexander, M.S., Altschuld, R.A., Hohl, C.M., Johnson, J.D., McDonald, J.S., Simmons, T.D., and Horrocks, L.A. 1992. Cell swelling, blebbing, and death are dependent on ATP depletion and independent of calcium during chemical hypoxia in a glial cell line (ROC-1). J.Neurochem. 59: 344-352.

Karim, F.D., and Thummel, C.S. 1991. Ecdysone coordinates the timing and amounts of E74A and E74B transcription in *Drosophila*. Genes Dev. 5:1067-1079.

Karin, M., Haslinger, A., Holtgreve, H., Richards, R.I., Westphal, H.M., and Beato, M. 1984. Characterization of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-IIA gene. Nature 308:513-519.

Karlson, P., and Bode, C. 1969. Die inaktivieung des ecdysons bei der schmeissfliege *Calliphora erythrocephala* Meigen. J.Insect Physiol. 15:111-118.

Kastner, P., Krust, A., Turcotte, B., Stropp, W., Tora, L., Gronemeyer, H., and Chambon, P. 1990. Two distinct estrogen-related promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J. 9:1603-1614.

Kerr, J.F.R., Wyllie, A.H., and Currie, A.R. 1972. Apoptosis: a basic biological phenomenon with wide range implications in tissue kinetics. Br.J.Cancer 26:239-257.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Khan, A.A., Soloski, M.J., Sharp, A.H., Schilling, G., Sabatini, D.M., Li, S.H., Ross, C.A., and Snyder, S.H. 1996. Lymphocyte apoptosis: mediation by increased type 3 inositol 1,4,5-trisphosphate receptor. Science 273:503-507.

Kiss, I., Beaton, A.H., Tardiff, J., Fristrom, D., and Fristrom, J.W. 1988. Interactions and developmental effects mutations in the Broad-Complex of *Drosophila melanogaster*. Genetics 118:247-259.

Kliewer, S.A., Umensono, K., Mangelsdorf, D.J., and Evans, R.M 1992. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone, and vitamin D3 signaling. Nature 355:446-449.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P.. and Hogness, D.S. 1991. The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell 67:59-77.

Kolber, M.S., Broschat, K.O., and Landa-Gonzalez. 1990. Cytochalasin B induced cellular DNA fragmentation. FASEB J. 4:3021-3027.

Kollros, J.J. 1981. Transitions in the nervous system during amphibian metamorphosis. In Metamorphosis. (eds. L.I. Gilbert and E. Frieden). pp. 445-459. New York: Plenum Press.

Koolman, J. 1982. Ecdysone metabolism. Insect Biochem. 12:225-250.

Kremer, M., Judd, J., Rifkin, B., Auszmann, J., and Oursler, M.J. 1995. Estrogen modulation of osteoclast lysosomal enzyme secretion. J.Cell Biochem. 57:271-279.

Kruman, I., Gukovskaya, A.S., Petrunyaka, V.V., Beletsky, I.P., and Trepakova, E.S. 1992. Apoptosis of murine VW 5147 thymoma cells induced by cold shock J.Cell Physiol. 315:112-117.

Kumar, V., and Chambon, P. 1988. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 55:145-156.

Kyprianou, N., and Isaacs, J.T. 1988. Activation of programmed cell death in the rat ventral prostate after castration. Endocrinology 122:552-562.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the heads of bacteriophage T4. Nature 227:680-685.

Landfield, P.W., Thibault, O., Mazzanti, M.L., Porter, N.M., and Kerr, D.S. 1992.. Mechanisms of neuronal death in brain aging and Alzheimer's disease; role of

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

endocrine-mediated calcium dyshomeostasis. J.Neurobiol. 23:1247-1260.

Lasnitzki, I., Dingle, J.T., and Adams, S. 1965. The effect of steroid hormones on lysosomal activity of rat ventral prostate gland in culture. Exp.Cell Res. 43:120-130.

Laufer, H. 1961. Forms of enzymes in insect development. Ann.N.Y.Acad.Sci. 94:825-835.

Lavie, G., Leib, Z., and Servadio, C. 1985. The mechanism of human NK cell-mediated cytotoxicity. Mode of action of surface-associated proteases in the early stages of the lytic reaction. J.Immunol. 135:1470-1476.

Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G., and Earnshaw, W.C. 1994. Cleavage of poly(ADP-ribose)polymerase by a proteinase with properties like ICE. Nature 371:346-347.

Levine, A.J., Momand, J., and Finlay, C.A. 1991. The p53 tumour suppressor gene. Nature 351:453-456.

Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., Salfeld, J., Towne, E., Tracey, D., Wardwell, S., Wei, F-Y., Wong, W., Kamen, R., and Seshardi, T. 1995. Mice deficient in IL-1β converting enzyme are defective in production of mature IL-1β and resistant to endotoxic shock. Cell 80:401-411.

Ling, Y.H., Waldemar, P., and Perez-Soler, R. 1993. Apoptosis induced by anthracycline in P388 parent and multidrug-resistant cells. Cancer Res. 53:1845-1852.

Linnik, M.D., Zobust, R.H., and Hatfield, M.D. 1993. Evidence supporting a role for programmed cell death in focal cerebral ischemia in rats. Stroke 24:2002-2009.

Lloyd, J.B., and Beck, F. 1969. Teratogenesis. In Lysosomes in Biology and Pathology Vol. I. (eds. J.T. Dingle and H.B. Fell). pp. 433-449. Amsterdam: North-Holland Publishing.

Lockshin, R.A., and Zakeri, Z. 1994. Programmed cell death: early changes in metamorphosing cells. Biochem.Cell Biol. 72:589-596.

Lockshin, R.A., and Beaulaton, J. 1974a. Programmed cell death. Cytochemical evidence for lysosomes during the normal breakdown of the intersegmental muscles. J.Ultrastructure Res. 46:43-62.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Lockshin, R.A., and Beaulaton, J. 1974b. Cytochemical appearance of lysosomes when the death of the intersegmental muscles is prevented. J.Ultrastructure Res. 46:63-78.

Lockshin, R.A. 1969. Lysosomes in insects. In Lysosomes in Biology and Pathology. (eds. J.T. Dingle and H.B. Fell). pp. 363-391. Amsterdam: North-Holland Publishing.

Lockshin, R.A., and Williams, C.M. 1965 a. Programmed cell death. I Cytology of degeneration in the intersegmental muscles of the *Pernyi* silkmoth. J.Insect Physiol. 11:123-133.

Lockshin, R.A., and Williams, C.M. 1965 b. Programmed cell death. V. Cytolytic enzymes in relation to the breakdown of the intersegmental muscles of silkmoths. J.Insect Physiol. 11:831-844.

Loosfelt, H., Logeat, F., Vu Hai, M.T., and Milgrom, E. 1984. The rabbit progesterone receptor. Evidence for a single steroid-binding subunit and characterization of receptor mRNA. J.Biol.Chem. 259:14196-14202.

Macchioni, P., Boiardi, L., Meliconi, R., Salvarani, C., Grazia Uguccioni, M., Rossi, F., Bulsatelli, L., and Facchini, A. 1994. Elevated soluble intercellular adhesion molecule 1 in the serum of patients with polymyalgia rheumatica: influence of steroid treatment. J.Rheumatol. 21:1860-1864.

MacManus, J.P., Buchan, A.M., Hill, I.E., Rasquinha, I., and Preston, E. 1993. Global ischemia can cause DNA fragmentation indicative of apoptosis in rat brain. Neurosci.Lett. 164:89-92.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R.M. 1995. The nuclear receptor superfamily: the second decade. Cell 83:835-839.

Mangelsdorf, D.J., Borgmeyer, U., Heyman, R.A., Yang Zhou, J., Ong, E.S., Oro, A.E., Kakizuka, A., and Evans, R.M. 1992. Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. Genes Dev. 6:329-344.

Maro, B., and Bornens, M. 1982. Reorganization of HeLa cell cytoskeleton induced by an uncoupler of oxidative phosphorylation. Nature 295:334-336.

Martin, D.P., Schmidt, R.E., DiStefano, P.S., Lowry, O.H., Carter, J.G., and Johnson, E.M. 1988. Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J.Cell Biol. 106:829-

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

844.

Matsura, S., Morimoto, T., Nagata, S., and Tashiro, Y. 1968. Studies on the posterior silk gland of the silkworm, *Bombyx mori*. II. Cytologic process in posterior silk gland cells during metamorphosis from larva to pupa. J.Cell Biol. 38:589-603.

Matteoni, R., and Kreis, T.E. 1987. Translocation and clustering of endosomes and lysosomes depends on microtubules. J.Cell Biol. 105:1253-1265.

Mattson, M.P., Cheng, B., Davis, D., Bryant, K., Leibenburg, I., and Rydel, R.E. 1992. Beta-amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. J.Neurosci. 12:376-389.

McConkey, D.J., Jondal, M., and Orrenius, S. 1992. Cellular signaling in thymocyte apoptosis. Semin.Immunol. 4:371-377.

Meltzer, Y.L. 1971. Hormonal and Attractant Pesticide Technology. Noyes Data Corp. Park Ridge: NJ.

Miller, A. 1950. The internal anatomy and histology of the imago of *Drosophila melanogaster*. In Biology of Drosophila. (ed. M. Demerec. pp. 420-534. New York: Wiley.

Miller, J., McLachlan, A.D., and Klug, A. 1985. Repetitive zinc-binding domains in the protein transcription factor IIIA from *Xenopus* oocytes. EMBO J. 4:1609-1614.

Mitrovic, B., Ignarro, L.J., Vinters, H.V., Akers, M.A., Schmid, I., Uittenbogaart, C., and Merrill, J.E. 1995. Nitric oxide induces necrotic but not apoptotic cell death in oligodendrocytes. Neuroscience 65:531-539.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J.Immunol.Methods 65:55-63.

Moudgil, V.K. 1990. Phosphorylation of steroid hormone receptors. Biochim.Biophys.Acta 1055:243-258.

Mountz, J.D., Wu, J., Cheng, J., and Zhou, T. 1994. Autoimmune disease. A problem of defective apoptosis. Arthritis Rheum. 37:1415-1420.

Muller, M., Baniahmad, C., Kaltschmidt, C., Schule, R., and Renkawitz, R. 1991. Cooperative transactivation of steroid receptors. In Nuclear Hormone Receptors. (ed. M.G. Parker) pp.155-174. New York: Academic Press.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Munck, A. 1971. Glucocorticoid inhibition of glucose uptake by peripheral tissues: old and new evidence, molecular mechanisms, and physiological significance. Persp.Biol.Med. 14:265-289.

Muro-Cacho, C.A., Pontaleo, G., and Fauci, A.S. 1995. Analysis of apoptosis in lymph nodes of HIV-infected persons. J.Immunol. 154:5555-5556.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., Munday, N.A., Raju, S.M., Smulson, M.E., Yamin, T-T., Yu, V.L., and Miller, D.K. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376:37-43.

Nijhout, H.F., and Williams, C.M. 1974. Control of moulting and metamorphosis in the tobacco hornworm, *Manduca sexta* (L.): Cessation of juvenile hormone secretion as a trigger for pupation. J.Exp.Biol. 61:493-501.

O'Malley, B.W., Tsai, S.Y., Bagchi, M., Weigel, N.L., Schrader, W.T., and Tsai, M-J. 1991. Molecular mechanism of action of a steroid hormone receptor. Recent Prog.Horm.Res. 47:1-24.

Oland, L.A., and Hayashi, J.H. 1993. Effects of the steroid hormone 20-hydroxyecdysone and prior sensory input on the survival and growth of moth central olfactory neurons *in vitro*. J.Neurobiol. 24:1170-1186.

Olson, L. 1993. NGF and the treatment of Alzheimer's disease. Exp.Neurol. 124:5-15.

Oppenheim, R.W., Maderdrut, J.L., and Wells, D.J. 1982. Cell death of motoneurons in the chick embryo spinal cord. VI. Reduction of naturally occurring cell death in the thoracolumbar column of Terni by nerve growth factor. J.Comp.Neurol. 210:174.

Oro, A.E., McKeown, M., and Evans, R.M. 1990. Relationship between the product of the *Drosophila* ultraspiracle locus and the vertebrate retinoid X receptor. Nature 347:298-301.

Penfold, P.L., and Provis, J.M. 1986. Cell death in the development of the human retina: phagocytosis of pyknotic and apoptotic bodies by retinal cells. Graefe's Arch.Clin.Exp.Opthalmol. 224:549-553.

Pfahl, M. 1982. Specific binding of the glucocorticoid-receptor complex to the mouse mammary tumor proviral promoter region. Cell 31:475-482.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Piacentini, M., Auruori, F., Dini, L., Ferrace, M.G., Ghibelli, L., Piredda, L., and Fesus L. 1991. Tissue transglutaminase is specifically expressed in neonatal rat liver cells undergoing apoptosis upon epidermal growth factor stimulation. Cell Tissue Res. 263:227.

Picard, D., and Yamamato, K.R. 1987. Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. EMBO J. 6:3333-3340.

Portera-Caillian, C., Hedreen, J.C., Price, D.L., and Koliatsos, V.E 1995. Evidence for apoptotic cell death in Huntington's disease and excitotoxic animal models. J.Neurosci. 15:3775-3787.

Price, J.M., Donahoe, P.K., Ito, Y., and Hendren, W.H. 3d. 1977. Programmed cell death in the Mullerian duct induced by Mullerian inhibiting substance. Am.J.Anat. 149:353-375.

Quarmby, V.E., Beckman, W.C., Wilson, E.M., and French, F.S. 1987. Androgen regulation of c-myc messenger ribonucleic acid levels in rat ventral prostate. Mol.Endocrinol. 1:865-874.

Rabizadeh, S., LaCount, D.J., Friesen, P.D., and Bredesen, D.E. 1993. Expression of the baculovirus p35 gene inhibits mammalian neural cell death. J.Neurochem. 61:2318-2321.

Radford, S.V., and Misch, D.M. 1971. The cytological effect of ecdysterone on the midgut cells of the flesh-fly *Sarcophaga bullata*. J.Cell Biol. 49:702-711

Richard, D.S., Amim, A.E., and Gilbert, L.I. 1993. A reappraisal of the hormonal regulation of larval fat body histolysis in female *Drosophila melanogaster*. Experientia 49:150-156.

Riddiford, L.M. 1982. Changes in translatable mRNAs during the larval-pupal transformation of the epidermis of the tobacco hornworm. Dev.Biol. 92:330-342.

Riddiford, L.M. 1978. Ecdysone-induced change in cellular commitment of the epidermis of the tobacco hornworm, *Manduca sexta*, at the initiation of metamorphosis. Gen.Comp.Endocrinol. 34:438-446.

Riddihough, G., and Pelham, H.R.B. 1987. An ecdysone response element in the *Drosophila* hsp27 promoter. EMBO J. 6:3729-3734.

Robinow, S., Talbot, W.S., Hogness, D.S., and Truman, J.W. 1993. Programmed cell death in the *Drosophila* CNS is ecdysone-regulated and coupled with a

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

specific ecdysone receptor isoform. Development 119:1251-1259.

Rotello, R.J., Lieberman, R.C., Lepoff, R.B., and Gerschenson, L.E. 1992. Characterization of uterine epithelium apoptotic cell death kinetics and regulation by progesterone and RU 486. Am.J.Path. 140:449-456.

Sanford, N.L., Searle, J.W., and Kerr, J.F.R. 1984. Successive waves of apoptosis in the rat prostate after repeated withdrawal of testosterone stimulation. Pathology 16:406-410.

Sass, M., and Kovacs, J. 1975. Ecdysterone and an analogue of juvenile hormone on the autophagy in the cells of fat body of *Mamestra brassicae.* Acta.Biol.Acad.Sci.Hung. 26:189-196.

Scheib, D. 1963. Properties and role of acid hydrolases of the Mullerian ducts during sexual differentiation in the male chick embryo. In Lysosomes Ciba Foundation Symposium. (eds. A.V.S. de Reuck and M.P. Cameron). pp. 264-277. Boston: Little Brown.

Schin, K.S., and Clever, U. 1965. Lysosomal and free acid phosphatase in salivary glands of *Chironomus tentans.* Science 150:1053-1055.

Schule, R., Muller, M., Kaltschmidt, C., and Renkawitz, R. 1988. Many transcription factors interact synergistically with steroid receptors. Science 242:1418-1420.

Schwabe, J.W.R., Neuhaus, D., and Rhodes, D. 1990. Solution structure of the DNA-binding domain of the oestrogen receptor. Nature 348:458-461.

Schwartz, L.M., Myer, A., Kosz, L., Engelstein, M., and Maier, C. 1990. Activation of polyubiquitin gene expression during developmentally programmed cell death. Neuron 5:411-419.

Schwartz, R.H. 1989. Acquisition of immunologic self-tolerance. Cell 57:1073-1081.

Schwartz, L.M., and Truman, J.W. 1984. Cyclic GMP may serve as a second messenger in peptide-induced muscle degeneration in an insect. Proc.Natl.Acad.Sci. 21: 6718-6722.

Schwartz, L.M., and Truman, J.W. 1983. Hormonal control of rates of metamorphic development in the tobacco hornworm, *Manduca sexta.* Dev.Biol. 99:103-114.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Schweichel, J.U., and Merker, H.J. 1973. The morphology of various types of cell death in prenatal tissues. Teratology 7:253-266.

Segraves, W.A., and Hogness, D.S. 1990. The E75 ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. Genes Dev. 4:204-219.

Sensibar, J.A., Liu, X.X., Patai, B., Alger, B., and Lee C. 1990. Characterization of castration-induced cell death in the rat prostrate by immunohistochemical localization of cathepsin D. Prostate 16: 263-276.

Sentman, C.L., Shutter, J.R., Hockenbery, D., Kamagawa, O., and Korsmeyer, S.J. 1991. Bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell 67:879-888.

Shapiro, D.J., Barton, M.C., McKearin, D.M., Chung, T.-C., Lew, D., Blurne, J., Nielsen, D.A., and Gould, L. 1989. Oestrogen regulation of gene transcription and mRNA stability. Recent Progr.Horm.Res. 45:29-58.

Smith, C.A., Williams, G.T., Kingston, R., Jenkinson, S.J., and Owen, J.J.T. 1989. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. Nature 337:181-184.

Smith, E., and Birt, L.M. 1972. Proteolytic activity during the metamorphosis of the blowfly, *Lucilia*. Insect Biochem. 2:218-225.

Smith, W.A., and Nijhout, H.F. 1983. *In vitro* stimulation of cell death in the moulting glands of *Oncopeltus fasciatus* by 20-hydroxyecdysone. J.Insect Physiol. 29:169-176.

Spelsberg, T.C., Rories, C., Rejman, J.J., Goldberger, A., Fink, K. Lau, C.K., Colvard, D.S., and Wiseman, G. 1989. Steroid action on gene expression: possible roles of regulatory genes and nuclear acceptor sites. Biol.Reprod. 40:54-69.

Sugimoto, A., Friesen, P.D., and Rothman, J. 1994. Baculovirus p35 prevents developmentally programmed cell death and rescues a ced-9 mutant in the nematode *Caenorhabditis elegans*. EMBO J. 13:2023-2028.

Sutherland, J.D., Kozlova, T., Tzertzinis, G., and Kafatos, F.C. 1995. *Drosophila* hormone receptor 38: a second partner for *Drosophila* USP suggests an unexpected role for nuclear hormone receptors of the nerve growth factor-induced protein B type. Proc.Natl.Acad.Sci. 92:7966-7970.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Szego, C.M., and Seeler, B.J. 1973. Hormone-induced activation of target-specific lysosomes: Acute translocation to the nucleus after administration of gonadal hormones *in vivo*. J.Endocrinol. 56:347-360.

Szego, C.M., Seeler, B.J., Steadman, R.A., Hill, D.F., Kimura, A.K., and Roberts, J.A. 1971. The lysosomal membrane complex. Focal point of primary steroid hormone action. Biochem.J. 123:523-538.

Talbot, W.S., Swyryd, E.A., and Hogness, D.S. 1993. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell 73:1323-1337.

Tartaglia, L.A., Ayres, T.M., Wong, G.H., and Goeddel, D.V. 1993. A novel domain within the 55 kd TNF receptor signals cell death. Cell 74:845-853.

Tata, J.R. 1966. Requirement for RNA and protein synthesis for induced regression of the tadpole tail in organ culture. Dev.Biol. 13:77-94.

Terai, C., Kornbluth, R.S., Pauza, C.D., Richman, D.D., and Carson, D.A. 1991. Apoptosis as a mechanism of cell death in cultured T lymphoblasts acutely infected with HIV-1. J.Clin.Invest. 87:1710-1714.

Teixeira, C., Reed, J.C., and Pratt, M.A. 1995. Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. Cancer Res. 55:3902-3907.

Thompson, C.C., Weinberger, C., Lebo, R., and Evans, R.M. 1987. Identification of a novel thyroid hormone receptor abundantly expressed in the mammalian central nervous system. Science 237:1610-1614.

Truman, J.W., Thorn, R.S., and Robinow, S. 1992. Programmed neuronal death in insect development. J.Neurobiol. 23: 1295-1311.

Truman, J.W., and Schwartz, L.M. 1984. Steroid regulation of neuronal death in the moth nervous system. J.Neurosci. 4:274-280.

Truman, J.W., Riddiford, L.M., and Safranek, L. 1974. Temporal patterns of response to ecdysone and juvenile hormone in the epidermis in the tobacco hornworm, *Manduca sexta*. Dev.Biol. 39:247-262.

Trump, B.F., and Berezesky, I.K. 1992. The role of cytosolic Ca^{2+} in cell injury, necrosis, and apoptosis. Curr.Opin.Cell Biol. 4:227-232.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Tsai, S.Y., Tsai, M-J., and O'Malley, B.W. 1989. Cooperative binding of steroid hormone receptors contributes to transcriptional synergism at target enhancer elements. Cell 57:443-448.

Van Pelt-Verkuil, E. 1979. Hormone mediated induction of acid phosphatase activity in the fat body of *Calliphora erythrocephala* prior to metamorphosis. J.Insect Physiol. 25:965-973.

Van Pelt-Verkuil, E, Van Rongen, E., De Priester, W. 1979. Normal and experimentally induced lysosomal activity in the larval fat body of *Calliphora erythrocephala* Meigen. Cell Tissue Res. 203:443-455.

Vaux, D.L., Haecker, G., and Strasser, A. 1994. An evolutionary perspective on apoptosis. Cell 76:777-779.

Vaux, D.L., Weissman, I.L., and Kim, S.K. 1992. Prevention of programmed cell death in *Caenorhabditis elegans* by human bcl-2. Science 258:1955-1957.

Walkinshaw, G., and Waters, C.M. 1995. Induction of apoptosis in catecholaminergic PC12 cells by L-DOPA. Implications for the treatment of Parkinson's disease. J.Clin.Invest. 95:2458-2464.

Walker, P.R., Kokileva, L., Leblanc, J., and Sikorska, M. 1993. Detection of the initial stages of DNA fragmentation in apoptosis. BioTechniques 15:1032-1040.

Weber, R. 1962. Induced metamorphosis in isolated tails of *Xenopus* larvae. Experientia 18:84-86.

Weeks, J.C., Davidson, S.K., and Debu, B.H. 1993. Effects of a protein synthesis inhibitor on the hormonally mediated regression and death of motoneurons in the tobacco hornworm, *Manduca sexta*. J.Neurobiol. 24:125-140.

Weeks, J.C., and Truman, J.W. 1985. Independent steroid control of the fates of motoneurons and their muscles during insect metamorphosis. J.Neurosci. 5:2290-2300.

Weller, M., Rodnight, R., and Carrera, D. 1972. Determination of adenosine 3'5'-cyclic monophosphate in cerebral tissues by saturation analysis. Biochem.J. 129: 113-121.

White, K., Grether, M.E., Abrams, J.M., Young, L., Farrell, K., and Steller, H. 1994. Genetic control of programmed cell death in *Drosophila*. Science 264:677-683.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Williams, G., and Smith, C. 1993. Molecular regulation of apoptosis: genetic controls on cell death. Cell 74:777-779.

Willman, T., and Beato, M. 1986. Steroid-free glucocorticoid receptor binds specifically to mouse mammary tumor virus DNA. Nature 324:688-691.

Woessner, J.F., Jr. 1969. The physiology of the uterus and mammary gland. In Lysosomes in Biology and Pathology. (eds. J.T. Dingle and H.B. Fell). pp. 299-329. Amsterdam: North-Holland Publishing.

Woo, D. 1995. Apoptosis and loss of renal tissue in polycystic kidney diseases. N.Engl.J.Med. 333:56-57.

Wyllie, A.H., Kerr, J.F.R., and Currie, A.R. 1980. Cell death: the significance of apoptosis. Int.Rev.Cytol. 68:251-306.

Wyllie, A.H., Kerr, J.F.R., Macaskill, I.A.M., and Currie, A.R. 1973. Adrenocortical cell deletion: the role of ACTH. J.Pathol. 111:85-94.

Xue, D., and Horvitz, H.R. 1995. Inhibition of the *Caenorhabditis elegans* cell-death protease ced-3 by a ced-3 cleavage site in baculovirus p35 protein. Nature 377:248-251.

Yanita, Y., Shi, Y.-B., and Brown, D.D. 1990. A correlation of thyroid hormone receptor gene expression with amphibian metamorphosis. Genes Dev. 4:1917-1924.

Yano, Y., Sakon, M., Kambayashi, J., Kawasaki, T., Senda, T., Tanaka, K., Yamada, F., and Shibata, N. 1995. Cytoskeletal reorganization of human platelets induced by the protein phosphatase 1/2 A inhibitors okadaic acid and calyculin A. Biochem.J. 307:439-449.

Yao, T., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.D., Mckeown, M., Cherbas, P., and Evans, R.M. 1993. Functional ecdysone receptor is the product of EcR and ultraspiracle genes. Nature 366:476-479.

Yao, T., Segraves, W.A., Oro, A.E., McKeown, M., and Evans, R.M. 1992. *Drosophila* ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell 71:63-72.

Young, R.G. 1979. Midgut lysosomal phosphatases and carbohydrases of the last larval instar of the southern armyworm, *Spodoptera eridania*. Ann.Entomol.Soc.Am. 72:193-196.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.

Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M., and Horvitz, H.R. 1993. The *C.elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1β converting enzyme. Cell 75:641-652.

Zacchary, D., and Hoffmann, J.A. 1980. Endocrine control of the metamorphosis of the larval muscles in *Calliphora erythrocephala* (Diptera): *In vitro* studies of the role of ecdysteroids. Dev.Biol. 80:235-247.

Zakeri, Z., Quaglino, D., Latham, T., Woo, K., and Lockshin, R.A. 1996. Programmed cell death in the tobacco hornworm, *Manduca sexta*: alterations in protein synthesis. Micr.Res.Techn. 34:192-201.

Zakeri, Z., and Lockshin, R.A. 1994. Physiological cell death during development and its relationship to aging. Annu.N.Y.Acad.Sci. 719:212-229.

Zakeri, Z.F., Quaglino, D., Latham, T., and Lockshin, R.A. 1993. Delayed internucleosomal DNA fragmentation in programmed cell death. FASEB J. 7:470-478.

Zelent, A., Mendelsohn, C., Kastner, P., Krust, A., Garnier, J-M., Ruffenach, F., Leroy, P., and Chambon, P. 1991. Differential expressed isoforms of the mouse retinoic acid receptor beta are generated by usage of two promoters and alternative splicing. EMBO J. 10:71-81.

Zelent, A., Krust, A., Petkovich, M., Kastner, P., and Chambon, P. 1989. Cloning of murine alpha and beta retinoic acid receptors and a novel receptor gamma predominantly expressed in skin. Nature 339:714-717.

Zelhof, A.C., Yao, T., Chen, J.D., Evans, R.M., and McKeown, M. 1995. Seven-up inhibits ultraspiracle-based signaling pathways *in vitro* and *in vivo*. Mol.Cell.Biol. 15:6736-6745.

Reproduced with permission of the copyright owner. Further reproduction prohibited without permission.