



Histochemical and electrophoretic analyses of hydrolytic enzymes during the embryogenesis of *Aulocara elliotti* (Thomas) (Orthoptera, Acrididae)
by Robert Edward Urban

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Zoology
Montana State University
© Copyright by Robert Edward Urban (1970)

Abstract:

The ontogeny of six hydrolytic enzymes was followed throughout the embryo genes is of the grasshopper *Aulocara elliotti* (Thomas). Nonspecific esterase, lipase, acid and alkaline phosphatase, and aminopeptidase activities were demonstrated using azo dye simultaneous coupling techniques. Protease activity was demonstrated using the substrate film methods. All enzymes except the proteases were separated and identified by polyacrylamide disc microgel electrophoresis.

The levels of enzymatic activity were recorded for the various organs and tissues in relation to the staging criteria of morphological development for this species (Van Horn, 1966a). A burst of acid phosphatase activity was observed in the presumptive prothorax region during stage 6. This site and time of enzyme activity correspond to the location of the physiological differentiation center reported for other species of insects. The presumptive region of subesophageal body cells exhibited a low level of aminopeptidase activity two stages prior to the morphological differentiation of these cells. Aminopeptidase activity was intense in these cells at the time of their differentiation, Neuropyle differentiation, but not neuroblast formation, was accompanied by high nonspecific esterase, lipase, acid phosphatase and aminopeptidase activities. The serosal membrane was found to be one of the most hydrolytically active tissues of the egg in contrast to the amniotic and provisional dorsal closure membranes which demonstrated negligible amounts of hydrolytic activity. The pleuropodia exhibited high levels of all of the hydrolytic enzymes studied at some stage in their development but dramatic shifts in enzyme activity were observed at different stages. These and other evidences indicate the possible function of these enzymes in nutritional metabolism. Shortly before the definitive stage the pleuropodia display intense hydrolytic activity and may be a major source of the hydrolytic enzyme activity found in the extra-embryonic fluids. The embryonic molting fluids and the serosal "cap" also may contribute hydrolytic enzymes to the extra-embryonic fluids. The abundant nonspecific hydrolytic activities observed in the degenerating pleuropodia and the serosal "cap" may account for the hydrolysis of the endocuticular layers without postulating a specific hatching enzyme. Intense aminopeptidase activity was found in the stomodeal- and proctodeal-midgut junctions in late post-diapause stages and within the presumptive cerci from stage 13 to hatching. The number of nonspecific esterase-active bands, as demonstrated with electrophoresis, was found to increase during development from 4 to 12 within the embryo and from 3 to 10 bands within the yolk. The zymogram patterns for the pleuropodia and the serosa were determined.

HISTOCHEMICAL AND ELECTROPHORETIC ANALYSES OF HYDROLYTIC
ENZYMES DURING THE EMBRYOGENESIS OF *AULOCARA ELLIOTTI*
(THOMAS) (ORTHOPTERA, ACRIDIDAE)

by

ROBERT EDWARD URBAN

A thesis submitted to the Graduate Faculty in partial
fulfillment of the requirements for the degree

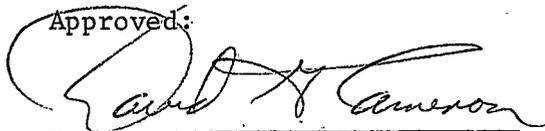
of

DOCTOR OF PHILOSOPHY

in

Zoology

Approved:



Head, Major Department



Chairman, Examining Committee



Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

June, 1970

ACKNOWLEDGMENT

I would like to offer my sincere gratitude to my acting major professor, Dr. Saralee Van Horn-Visscher, for her enthusiasm, encouragement, and direction throughout this research and for her constructive criticism of this manuscript. I would further like to extend acknowledgment to Dr. J. H. Pepper, my initial major professor, for his valuable counsel and advice during this study. I am indebted to Drs. P. D. Skaar and R. E. Moore for their critical reading of the manuscript. I would also like to thank Professor Ellsworth Hastings for collecting most of the experimental materials, and Dr. D. W. Blackmore for providing laboratory rats. To my wife, Kathlynn, and son, Ryan, I am extremely appreciative of their devotion to the cause and their needed support, patience and thoughtfulness throughout this endeavor.

This investigation was supported by the U. S. Department of Health, Education and Welfare through a Title IV Fellowship and from the Veterans Readjustment Benefits Act of 1966 (89-358).

TABLE OF CONTENTS

	Page
VITA	ii
ACKNOWLEDGMENT	iii
LIST OF TABLES	vi
LIST OF FIGURES	vii
ABSTRACT	xii
INTRODUCTION	1
MATERIALS AND METHODS	4
I. SOURCES OF BIOLOGICAL MATERIAL	4
II. PROCEDURES	4
III. METHODS FOR THE HISTOCHEMICAL DEMONSTRATIONS OF HYDROLYTIC ENZYMES	6
Nonspecific Esterases	6
Lipases	7
Acid Phosphatases	8
Alkaline Phosphatases	9
Aminopeptidases	10
Proteases	10
IV. PROCEDURES FOR DISCONTINUOUS POLYACRYLAMIDE GEL ELECTROPHORESIS	12
V. HISTOCHEMICAL MEDIA FOR THE ENZYME IDENTIFICATION IN GELS	15
Nonspecific Esterases	15
Lipases	16
Acid Phosphatases	17
Alkaline Phosphatases	18
Aminopeptidases	18

	Page
RESULTS	19
Introduction	19
PART I. HISTOCHEMICAL DEMONSTRATION OF HYDROLYTIC ENZY- MATIC ACTIVITY THROUGHOUT EMBRYOGENESIS	21
The Chorion	21
The Hydropyle	22
The Yolk and Vitellophages	25
The Extra-Serosal Fluid	28
The Serosal Membrane	29
The Amniotic and Provisional Dorsal Closure (PDC) Membranes	32
The Germ Anlage and Germ Band	33
The Central Nervous System	36
The Cerci	38
The Abdominal Walls	39
The Membrane Surrounding the Epineural Sinus	42
The Stomodeum and Gastric Caecae	43
The Proctodeum, Malpighian Tubules and Rectal Pads	44
The Midgut and Engulfed Yolk	48
The Pleuropodia	49
Hemocytes	57
PART II. DISCONTINUOUS POLYACRYLAMIDE GEL ELECTRO- PHORESIS	59
DISCUSSION AND CONCLUSIONS	97
Introduction	97
Hydrolytic Enzyme Activity in the Newly-laid Egg	98
Nonspecific Esterase and Lipase Activity in Early Pre- diapause Stages (1 to 12)	102
Indirect Evidence of a Differentiation Center (DC)	104
Subesophageal Body Cells (SEBC)	106
The Embryonic and Extra-embryonic Membranes	107
The Pleuropodia	111
Eclosion	112
Diapause Development	115
Polyacrylamide Disc Gel Electrophoresis	116
SUMMARY	119
LITERATURE CITED	124

LIST OF TABLES

Table	Page
I. NONSPECIFIC ESTERASE AND LIPASE ACTIVITY* IN EARLY PRE-DIAPAUSE STAGES OF <i>AULOCARA ELLIOTTI</i> (THOMAS)	63
II. NONSPECIFIC ESTERASE AND LIPASE ACTIVITY* IN LATE PRE-DIAPAUSE STAGES OF <i>AULOCARA ELLIOTTI</i> (THOMAS)	64
III. NONSPECIFIC ESTERASE AND LIPASE ACTIVITY* DURING DIAPAUSE DEVELOPMENT OF <i>AULOCARA ELLIOTTI</i> (THOMAS)	65
IV. NONSPECIFIC ESTERASE AND LIPASE ACTIVITY* IN POST-DIAPAUSE STAGES OF <i>AULOCARA ELLIOTTI</i> (THOMAS)	66
V. ACID PHOSPHATASE ACTIVITY* IN EARLY PRE-DIAPAUSE STAGES OF <i>AULOCARA ELLIOTTI</i> (THOMAS)	67
VI. ACID PHOSPHATASE ACTIVITY* IN LATE PRE-DIAPAUSE STAGES OF <i>AULOCARA ELLIOTTI</i> (THOMAS)	68
VII. ACID PHOSPHATASE ACTIVITY* DURING DIAPAUSE DEVELOPMENT OF <i>AULOCARA ELLIOTTI</i> (THOMAS)	69
VIII. ACID PHOSPHATASE ACTIVITY* IN POST-DIAPAUSE DEVELOPMENT OF <i>AULOCARA ELLIOTTI</i> (THOMAS)	70
IX. AMINOPEPTIDASE ACTIVITY* IN EARLY PRE-DIAPAUSE STAGES OF <i>AULOCARA ELLIOTTI</i> (THOMAS)	71
X. AMINOPEPTIDASE ACTIVITY* IN LATE PRE-DIAPAUSE STAGES OF <i>AULOCARA ELLIOTTI</i> (THOMAS)	72
XI. AMINOPEPTIDASE ACTIVITY* DURING DIAPAUSE DEVELOPMENT OF <i>AULOCARA ELLIOTTI</i> (THOMAS)	73
XII. AMINOPEPTIDASE ACTIVITY* IN POST-DIAPAUSE STAGES OF <i>AULOCARA ELLIOTTI</i> (THOMAS)	74
XIII. PROTEASE ACTIVITY* DURING EMBRYOGENESIS OF <i>AULOCARA ELLIOTTI</i> (THOMAS)	75
XIV. CONTROL SLIDES FOR HISTOCHEMICAL METHODS	76

LIST OF FIGURES

Figure	Page
1. Stage 25, inside of the hydropyle cap showing amino-peptidase activity	78
2. Stage 25, vitellophage nucleus showing nonspecific esterase activity	78
3. Stage NL (newly-laid), yolk showing lipase activity	78
4. Stage NL (newly-laid), yolk showing protease activity	78
5. Stage 3, yolk showing protease activity.	78
6. Stage 12, yolk showing protease activity	78
7. Stage 18, yolk control slide, 50% formalin fixation for 1 hour at 25°C. Protease activity	78
8. Stage 19 (4 months at 5°C), yolk showing lipase activity	78
9. Stage 25, yolk from midgut showing nonspecific esterase activity	80
10. Stage 25, extra-embryonic fluid showing nonspecific esterase activity	80
11. Stage 26, extra-embryonic fluid showing protease activity.	80
12. Stage 27 (late), extra-embryonic fluid showing protease activity	80
13. Stage 18, serosal membrane showing aminopeptidase activity in the cytoplasm	80
14. Stage 19 (at 5°C for 4 months), serosal membrane showing a mosaic pattern of nonspecific esterase activity	80
15. Stage 19 (at 5°C for 4 months), serosal membrane showing nonspecific esterase activity	80
16. Stage 19 (at 25°C for 4 months), serosal membrane showing acid phosphatase activity	80

17.	Stage 24, serosal membrane showing lipase activity	82
18.	Stage 20 (at 5°C for 4 months, then warmed to 25°C for at least 3 hours), serosal membrane showing acid phosphatase activity	82
19.	Stage 25, serosal membrane removed from the midgut yolk showing a mosaic pattern of aminopeptidase activity	82
20.	Stage 18, amniotic membrane showing acid phosphatase activity	82
21.	Stage 19 (at 5°C for 4 months), provisional dorsal closure membrane showing nonspecific esterase activity	82
22.	Stage 1, embryonic disc showing nonspecific esterase activity	82
23.	Stage 13, gnathal region of the embryonic head showing aminopeptidase activity	82
24.	Stage 13, gnathal region of the embryonic head showing aminopeptidase activity	82
25.	Stage 18, cerci showing aminopeptidase activity	84
26.	Stage 19 (at 25°C for 2 weeks), protocerebrum showing non-specific esterase activity	84
27.	Stage 19 (at 5°C for 4 months), subesophageal ganglion showing nonspecific esterase activity	84
28.	Stage 25, second and third thoracic ganglia showing non-specific esterase activity within the neuropyle	84
29.	Stage 20 (at 5°C for 4 months, then removed to 25°C for at least 3 hours). The pars intercerebralis region of the brain showing acid phosphatase activity	84
30.	Stage 20 (at 5°C for 4 months, then removed to 25°C for at least 3 hours), three thoracic ganglia showing acid phosphatase activity	84
31.	Stage 20 (at 5°C for 4 months, then removed to 25°C for at least 3 hours), abdominal wall containing dense crystalline deposits (uric acid)	84

32.	Stage 23, dorsal margin of abdominal wall showing amino-peptidase activity	84
33.	Stage 27, membrane surrounding the epineural sinus showing nonspecific esterase activity	86
34.	Stage 26, membrane surrounding the epineural sinus containing dense crystalline material (uric acid)	86
35.	Stage 26, gastric caecal buds extending from the stomodeum showing nonspecific esterase activity	86
36.	Stage 26, stomodeal-midgut junction showing amino-peptidase activity	86
37.	Stage 25, proctodeal-midgut junction showing nonspecific esterase activity	86
38.	Stage 27, proctodeal-midgut junction showing acid phosphatase activity	86
39.	Stage 25, one-half of proctodeum with three longitudinal bands of aminopeptidase activity	86
40.	Stage 26, proctodeal-midgut junction showing amino-peptidase activity	86
41.	Stage 26, Malpighian tubules showing lipase activity	88
42.	Stage 27, Malpighian tubules showing nonspecific esterase activity	88
43.	Stage 27, midgut showing nonspecific esterase activity	88
44.	Stage 25, midgut showing acid phosphatase activity	88
45.	Stage 19 (at 5°C for 4 months), pleuropodium showing nonspecific esterase activity	88
46.	Stage 19 (at 5°C for 4 months), pleuropodium control slide. Enzymatic activity destroyed with Altmann's fixative and 95% ethanol	88

47.	Stage 19 (at 5°C for 4 months), pleuropodium showing non-specific esterase activity	88
48.	Stage 25, pleuropodium showing aminopeptidase activity . .	88
49.	Stage 19 (at 5°C for 4 months), pleuropodium showing acid phosphatase activity	90
50.	Stage 20 (at 5°C for 4 months), then removed to 25°C for 3 hours), pleuropodium showing nonspecific esterase activity	90
51.	Stage 20 (as above), pleuropodium showing nonspecific esterase activity	90
52.	Stage 25 (5 days into post-diapause development), pleuropodium showing nonspecific esterase activity	90
53.	Stage 25, pleuropodium showing nonspecific esterase activity	90
54.	Stage 27 (late), pleuropodium found detached from body wall and floating free in the extra-embryonic fluid	90
55.	Stage 20 (at 5°C for 4 months, then removed to 25°C for 3 hours), pleuropodium showing acid phosphatase activity . .	90
56.	Stage 27, pleuropodium showing acid phosphatase activity . .	90
57.	Stage 23, pleuropodium showing aminopeptidase activity . .	92
58.	Stage 26, pleuropodium showing aminopeptidase activity . . .	92
59.	Stage 27 (late), pleuropodium found floating free in the extra-embryonic fluid showing protease activity	92
60.	Stage 25, an ellipsoid (20 x 27 μ) hemocyte showing non-specific esterase activity	92
61.	Stage 25, a round (20 μ) hemocyte showing nonspecific esterase activity	92
62.	Stage 25, three small round (6-10 μ) hemocytes showing nonspecific esterase activity	92

63.	Stage 25, hemocyte (12 x 14 μ) showing nonspecific esterase activity	92
64.	Stage 25, hemolymph, a plasma clot showing nonspecific esterase activity	92
65.	Disc gel electrophoretic (protein) pherograms in comparison with (enzyme) zymograms	93
66.	Direct tracings of nonspecific esterase zymograms from whole embryos at different stages in <i>Aulocara ellioti</i> (Thomas)	94
67.	Direct tracings of nonspecific esterase zymograms from yolk od embryos at different stages in <i>Aulocara ellioti</i> (Thomas)	95
68.	Direct tracings from nonspecific esterase zymograms from stage 19 (cold) embryonic tissues of <i>Aulocara ellioti</i> (Thomas)	96

ABSTRACT

The ontogeny of six hydrolytic enzymes was followed throughout the embryogenesis of the grasshopper *Aulocara ellioti* (Thomas). Non-specific esterase, lipase, acid and alkaline phosphatase, and aminopeptidase activities were demonstrated using azo dye simultaneous coupling techniques. Protease activity was demonstrated using the substrate film methods. All enzymes except the proteases were separated and identified by polyacrylamide disc microgel electrophoresis. The levels of enzymatic activity were recorded for the various organs and tissues in relation to the staging criteria of morphological development for this species (Van Horn, 1966a). A burst of acid phosphatase activity was observed in the presumptive prothorax region during stage 6. This site and time of enzyme activity correspond to the location of the physiological differentiation center reported for other species of insects. The presumptive region of subesophageal body cells exhibited a low level of aminopeptidase activity two stages prior to the morphological differentiation of these cells. Aminopeptidase activity was intense in these cells at the time of their differentiation, Neuro-pyle differentiation, but not neuroblast formation, was accompanied by high nonspecific esterase, lipase, acid phosphatase and aminopeptidase activities. The serosal membrane was found to be one of the most hydrolytically active tissues of the egg in contrast to the amniotic and provisional dorsal closure membranes which demonstrated negligible amounts of hydrolytic activity. The pleuropodia exhibited high levels of all of the hydrolytic enzymes studied at some stage in their development but dramatic shifts in enzyme activity were observed at different stages. These and other evidences indicate the possible function of these enzymes in nutritional metabolism. Shortly before the definitive stage the pleuropodia display intense hydrolytic activity and may be a major source of the hydrolytic enzyme activity found in the extra-embryonic fluids. The embryonic molting fluids and the serosal "cap" also may contribute hydrolytic enzymes to the extra-embryonic fluids. The abundant nonspecific hydrolytic activities observed in the degenerating pleuropodia and the serosal "cap" may account for the hydrolysis of the endocuticular layers without postulating a specific hatching enzyme. Intense aminopeptidase activity was found in the stomodeal- and proctodeal-midgut junctions in late post-diapause stages and within the presumptive cerci from stage 13 to hatching. The number of nonspecific esterase-active bands, as demonstrated with electrophoresis, was found to increase during development from 4 to 12 within the embryo and from 3 to 10 bands within the yolk. The zymogram patterns for the pleuropodia and the serosa were determined.

INTRODUCTION

"... enzymes are the quintessence of life." Florence Moog

In the past decade a series of coordinated investigations have been conducted at Montana State University to elucidate the factors underlying the enigmatic fluctuations of populations of *Aulocara ellioti* (Thomas). Van Horn (1963) described the embryonic morphology and histology. Hastings and Pepper (1964) found differences in the physiological responses to temperature and starvation stress in newly-hatched nymphs from different subpopulations. Other studies have been concerned with various aspects of the embryonic physiology. Roemhild (1965a & b) studied respiration and temperature effects, (1967) compartmentation of the eggs, and (1968) cationic effects on the eggs. Laine (1966) investigated patterns of respiration of the eggs. Van Horn (1966a & b) published on the embryonic morphogenesis, maternal age and developmental variability, and (1968) on gland volume changes in post-diapause development. Fluorescent antibody studies on muscle development were made by Horvath (1967). The vitellogenesis and post-embryonic ovarian development were reported by Leopold (1967). The biochemical aspects of embryonic development of *A. ellioti* were investigated by: Svoboda, Pepper, and Baker (1966) lipid metabolism; Bunde and Pepper (1968) biosynthesis and occurrences of free amino acids; Quickenden and Roemhild (1969) the effects of

maternal age and density upon carbohydrate metabolism; Quickenden (1970) carbohydrate changes during embryogenesis; and Robinson (in progress) patterns of protein synthesis during embryonic development. The results of these biochemical studies demonstrated qualitative and quantitative changes in substrates within eggs or whole embryos of *A. ellioti*, but they gave only limited information as to the specific embryonic tissues or organs in which these substrate changes occurred.

Although there have been many enzyme histochemical studies with vertebrate embryos (Moog, 1965), there is a paucity of such studies in insect embryogenesis, as noted by Krause and Sander (1962). As a part of the coordinated program of studies on the embryonic physiology of *A. ellioti*, the present investigations were undertaken, therefore, to identify the morphological sites of hydrolytic enzyme activities and to compare the relative levels of enzyme activities in different tissues throughout embryogenesis, including the period of diapause development.

Six hydrolytic enzymes were selected for study: nonspecific esterases, lipases, acid and alkaline phosphatases, aminopeptidases, and proteases. Several considerations were used in the selection of these enzymes. First, the hydrolytic enzymes are thought to be involved in embryonic nutrition (Boyer et al., 1960, 1961). Second, most of these enzymes have been shown in other insects to be composed of isozymes; that is, enzymes with similar substrate specificities

but having different electrophoretic mobilities. In other organisms each isozyme has been shown to develop at its own rate and according to its own unique pattern within a particular organ (Markert and Hunter, 1959). Third, populations of *Drosophila melanogaster* have been identified by their array of genetically determined esterase, alkaline phosphatase and aminopeptidase isozymes (Beckman and Johnson, 1964a, b & c). A knowledge of these isozyme patterns in *A. ellioti* could be used later as a basis for identification and comparison of populations. Fourth, Thomsen and Møller (1963) presented evidence that the neurosecretory cells and corpora allata glands appeared to control the intestinal protease activity in *Calliphora erythrocephala*. The possibility exists that protease activity in embryos also may be under endocrine control. The changes in activity levels could be related to variations in embryonic endocrine function in *A. ellioti* (Van Horn, 1968; Van Horn-Visscher, in progress).

The present investigations were divided into two phases: In the first, data were obtained using simultaneous coupling azo dye methods and, in the second, results were obtained using polyacrylamide disc microgel electrophoretic techniques.

MATERIALS AND METHODS

I. SOURCES OF BIOLOGICAL MATERIAL

Second, third, and fourth instar nymphs of *A. ellioti* were collected in the field and reared in cages and according to methods described by Anderson and Hastings (1966). The nymphs were obtained from the vicinity of Decker, Montana, in 1967, and Billings, Montana, in 1968 and 1969. Eggs were obtained from adults reared at a density of 3 pairs per cage. The egg pods were collected every two days by sifting the soil from the cage bottoms, and were incubated vertically in damp sand at room temperature (25°C). When the youngest embryos had reached the obligatory diapause stage (stage 19), the egg pods were placed in a cold room at 5°C. After 4 months of cold treatment, the egg pods were returned to room temperature (25°C) for post-diapause development.

II. PROCEDURES

Histochemical procedures for the demonstration of enzyme activity were performed on whole mounts of the various organs and tissues. A binocular dissecting microscope was used for all dissections. The dissections were performed under a modified Ringer's solution (Slifer, 1934) using a sharpened watch-maker's forceps and sharpened insect mounting pins. The material was rinsed in glass-distilled deionized water and transferred to a microslide or cover slip coated with diluted rubber

cement (Yos, 1961). Membranes were flattened and spread with a pin and the excess water was removed from the slide. The air-dried slides were held at 5°C until time of incubation.

The stage of development of the embryos was determined at the time of dissection according to the staging criteria established by Van Horn (1966a). To obtain a more genetically diverse sample of a particular developmental stage, embryos of the same stage were taken from different pods. A total of approximately 2,200 eggs (about 300 pods) were used for the histochemical portion of this study. An average of 9 eggs for each stage and each enzyme studied was used. A few stages had a minimum of 2 eggs at each stage for each enzyme, because embryos of a particular stage were unavailable. If, however, an irregularity of enzymatic activity was found in comparison to the activity of the preceding and following stages, the experiment was repeated. If it was impossible to repeat the experiment due to a lack of embryos of a particular stage, that stage was recorded as not tested (NT).

It was found necessary to photograph the results of the histochemical studies within at least 24 hours as most of the preparations were found to be unstable for longer periods. A 35 mm Carl Zeiss Camera, photometer, and microscope were used. The objectives used for this study were: a 6.3 X planapochromatic, a 10 X neofluar, a 25 X planapochromatic, a 40 X oil immersion apochromatic, and a 100 X oil immersion with an adjustable numerical aperture of 0.8 to 1.32 apochromatic. Kodak 35 mm Plus-X panchromatic B & W film (ASA 125) was

employed throughout this study. The film was developed in a daylight tank using the standard Kodak reagents and procedures. The enlarging and printing was performed using an Omega enlarger and Kodabromide F-3 and F-4 print paper. Colored slides were taken using 35 mm Eastman Kodak Ektachrome (ASA 160) daylight type film with a conversion filter. The color film was developed commercially.

III. METHODS FOR THE HISTOCHEMICAL DEMONSTRATION OF HYDROLYTIC ENZYMES

Nonspecific Esterases

Pre-incubation treatment. In order to detect the earliest appearance of enzymatic activity in pre-diapause embryos, no fixation prior to incubation was used. In diapause and post-diapause development a short pre-incubation fixation was employed to decrease diffusion artifacts in tissues with high activity. Calcium-formol (10% formalin with 1% CaCl_2) was used at 5°C for 15 minutes. The mounted tissues were washed in two 250 ml volumes of distilled water for 5 minutes each. The tissues were then placed in the incubation medium.

The preferred medium for nonspecific esterases.

1-Naphthol AS-D acetate	5.0 mg
Acetone	0.5 ml
0.2 M Tris [*] -maleate, pH 7.1	20.0 ml
Distilled deionized water	30.0 ml
Fast Blue RR salt (C.I. 37155)	20.0 mg
(from Barka and Anderson, 1965, p. 265)	

* Tris (hydroxymethyl) aminomethane; Trisma Base, Sigma Chemical Company, St. Louis, Missouri.

The freshly prepared medium was filtered and the final pH was adjusted with either NaOH or HCl on a Beckman Expandomatic pH meter. The standard incubation period was 30 minutes at room temperature (60 minutes was used for control slides and for tissues with little or no activity). All slides were mounted with glycerol-gelatin unless specified otherwise.

Two other nonspecific esterase media were tested for activity, (1) 1-naphthol AS-D chloroacetate (Maloney et al., 1960) which proved negative for embryonic material and (2) 1-Naphthyl acetate (Markert and Hunter, 1959) which showed excessive diffusion artifacts in unfixed whole mount preparations.

Lipases

Pre-incubation treatment. Without prior fixation, tissues were soaked in 0.05 M sodium taurocholate for 5 minutes. The slides were then transferred directly to the incubation medium without a distilled water rinse.

Standard incubation medium for lipase.

1-Naphthol AS-D acetate	5.0 mg
Acetone	0.5 ml
0.2 M Tris-maleate, pH 7.1	20.0 ml
Distilled deionized water	27.5 ml
1.0 M Sodium taurocholate	2.5 ml
Fast Blue RR salt (C.I. 37155)	20.0 mg

(modified from Barka and Anderson, 1965, p. 265, after consideration of Block, 1960) Incubation time was 30 minutes at 25°C.

Medium using 1-naphthol AS nonanoate, sodium taurocholate, and Fast Blue BB salt (C.I. 37175) was attempted (Abe et al., 1964). Results were all negative for yolk and embryo of various pre- and post-diapause stages. Fresh frozen rat pancreas sections showed a low level of lipase activity after 60 minutes of incubation which proved that the medium was functional.

The Tween methods for lipase activity also were tried. A 6% neutral formalin pre-incubation fixation for 30 minutes was employed. Tween 80* substrate was used with PbS for visualization (from Baraka and Anderson, 1965, p. 270). Controls, however, usually showed a positive reaction, particularly within the yolk. Bokdawala and George (1964) used a Versene pre-incubation soak to remove excess calcium ions. Tween 85 substrate (a trioleate) was employed with Alizarin Red S stain for visualization of the precipitated calcium soaps. The controls showed positive reactions. Other substrates used which also showed positive control reactions were Tween 65, a tristearate, 60, a monostearate, 40, a monopalmitate and 20, a monolaurate.

Acid Phosphatases (Phosphomonoesterase II)

Pre-incubation treatment. Embryos younger than stage 15 were not fixed prior to incubation. With embryos in later stages a weak fixation

* Tween 80, polyoxyethylene sorbitan monooleate, J. T. Baker Chemical Company, Phillipsburg, New Jersey.

using calcium-formol at 5°C for 30 minutes was employed to decrease diffusion artifacts. The tissues were washed in two 250 ml volumes of distilled water for 10 minutes each prior to incubation.

The preferred medium for acid phosphatase. The medium described by Barka and Anderson (1965, p. 245) employing 1-naphthol AS-TR phosphate, Michaelis veronal-acetate buffer, pH 5.0, and pararosanilin coupler (C.I. 42500) was used. The standard incubation time was 30 minutes at room temperature. The tissues were dehydrated, cleared, and mounted with Histoclad*. Sodium 1-naphthyl acid phosphate substrate also was tested using pararosanilin coupler. Acid phosphatase localization was superior with the substituted naphthol substrate, especially in unfixed tissues.

Alkaline Phosphatases (Phosphomonoesterase I)

Pre-incubation treatment. No pre-incubation fixation was used.

Two media for alkaline phosphatase activity.

1) 1-Naphthol AS-MX phosphate	5.0 mg
N-N Dimethylformamide	1.0 ml
0.05 M Tris-maleate buffer, pH 8.3	50.0 ml
MgCl ₂ (tested at final concentrations of 0.001, 0.002, 0.005, 0.01, and 0.05 M)	
Fast Blue RR salt	40.0 mg
(from Barka and Anderson, 1965, p. 234)	

* Clay Adams, Ind. N. Y.

The incubation period was extended to 60 minutes or more by replacing the spent medium with freshly prepared medium.

2) Sodium 1-naphthyl acid phosphate	50.0 mg
0.075 M Barbitol buffer, pH 9.2	50.0 ml
MgCl ₂ (final concentration 0.002 M)	
Fast Blue RR salt	50.0 mg
(from Barka and Anderson, 1965, p. 232)	

Incubation time was 60 minutes or more at room temperature. No alkaline phosphatase activity was detected with either method until stage 27.

Aminopeptidases

Pre-incubation treatment. Unfixed materials were used throughout development.

The preferred medium for aminopeptidase activity. The medium described in Barka and Anderson's (1965, p. 288) manual was used. The substrate employed was L-leucyl-4-methoxy-2-naphthyl amide. The coupler used was Fast Blue salt (C.I. 37235). No KCN activator was necessary. The substrate L-leucyl-2-naphthyl amide was also tried but excessive diffusion artifacts resulted.

Proteases

Pre-incubation treatment. Unfixed materials were used, except for controls. Extra-embryonic fluid was removed by simply pricking the anterior end of the egg with a pin and allowing the fluid to run out directly onto a prepared slide. The substrate film method adapted for

proteases by Adams and Tuqan (1961) was employed. Kodak projector slide plates, used for making transparencies, were exposed to the light for 2 minutes. The photographic plates were then developed in Microdol X print developer. The wash was extended for 4 hours to remove as much of the developing reagents as possible. The darkened photographic plates were dried in an oven at 40°C overnight. The plates were cut into slides 2" x 1". The freshly dissected tissues were transferred to the photographic plates and allowed 15 minutes to air dry.

The standard incubation media for proteases.

- 1) Tris-maleate buffer 0.05 M, pH 7.0 for embryonic tissues.
- 2) Acetate buffer 0.1 M, pH 6.5 for yolk and serosal membrane.

Two drops of buffer solution were placed over the air-dried tissues. The plates were then transferred to a water saturated atmospheric chamber in an oven at 37°C for 4 hours. Occasional additions of buffer solution were made to insure that the solutions did not dry. After the plates were removed from the chamber, they were dried in the oven. The plates were further dehydrated in ethanol, cleared in xylol, and mounted with Histo-clad. Areas with proteolytic activity appeared as bright holes in an otherwise darkened background. Degenerating eggs, which showed maximum proteolytic activity, were used for inactivation control tests. Also, plates were incubated without tissues to check for bacterial and fungal contamination. No activity was found using Tris-maleate buffer with overnight incubation. Incubation with acetate

buffer overnight did show a slight contamination.

Other buffer systems and pH attempted with limited success were: acetate buffer 0.1 M at pH 3.6, 4.0, 4.5, 5.0, 5.2, 5.4, and 5.6; bicarbonate buffer 0.1 M at pH 9.1, 9.5, 9.9, and 10.3; and Tris-HCl 0.2 M at pH 7.2, 7.5, 7.8, 8.2, and 8.5. Phosphate buffer 0.2 M at pH 7.1 was found to dissolve the silver salts of the photographic plates and consequently could not be used.

The Lagunoff and Benditt (1964) method was employed to test for chymotrypsin-like enzymatic activity using substrates 1-naphthol AS-beta-phenylpropionate and 1-naphthol AS-beta-chloropropionate and Fast Garnet GBC salt coupler. No enzymatic activity was demonstrated within the embryonic or extra-embryonic tissues or within the adult gut. Fresh frozen rat small intestine showed a positive reaction.

Controls for the histochemical methods. See Table XIV.

IV. PROCEDURES FOR DISCONTINUOUS POLYACRYLAMIDE GEL ELECTROPHORESIS

Preparation of gel columns. Polyacrylamide electrophoresis, as developed by Ornstein and Davis (1964) was employed in this study to further identify and characterize the hydrolytic enzymes previously demonstrated within the tissues. The solutions were identical to those of Davis's except that the reservoir Tris-glycine buffer working solution was diluted 1:1 (v/v) with distilled water rather than 1:10. This concentration of buffer solution gave a more discrete zymogram, particularly within microgels.

Modification of the microgel electrophoresis technique of Krause and Raunio (1967) was used in this study. Two sizes of tubes were employed: 2.26 mm ID X 60 mm "small" tubes and 0.92 mm ID X 50 mm microtubes. Five "small" tubes were cut from a single 1.0 ml glass pipette and four microtubes were cut from a single 0.1 ml pipette to insure uniformity of inside diameters.

All glass tubes were coated with Beckman's Descicote*, prior to filling with the gel solution. Gel columns could then be easily removed without distortion by hydrostatic pressure. The small tubes had a column of separation gel 45 mm tall, a stacking gel of 6 mm, a sample section of 6 mm, and a gel plug of 3 mm of additional stacking gel. The microtube gel dimensions were: separation gel 35 mm, stacking gel 6 mm, sample section 6 mm, and a plug gel of 3 mm. A Pasteur pipette was used to fill the tubes and a 1.0 ml injection syringe with a 22 gauge needle was used to adjust the final level of the gel solution and to layer distilled water over it. After the stacking gel was photopolymerized with a fluorescent light, the remaining portion of the tube was filled with distilled deionized water. A slurry of Sephadex G-25 was layered above the stacking gel to a height of 4-5 mm. The prepared tubes were placed in a cold room at 5°C pending application of the biological sample.

* Beckman Descicote number 18772, Beckman Instruments, Inc., Fullerton, California.

Preparation of biological samples. Tissues to be studied with electrophoresis were dissected in a cold room under cold modified Ringer's solution (Slifer, 1934).

The tissues were rinsed in distilled deionized water and pipetted with a medicine dropper to the top of the prepared tubes. A pin was used to push the tissues down to and into the Sephadex layer. The excess distilled water was removed with a syringe to just above the Sephadex-sampler layer. After four tubes were thus prepared, the tissues were homogenized individually at 1550 rpm with a slightly bent stainless steel needle attached to an electric stirrer. The homogenization proceeded for 15 minutes. The Sephadex G-25 serves two purposes: first, it aids in rupturing the swollen cells (due to the presence of distilled water) in a manner similar to a ball mill, and second, the Sephadex sample mixture is dense enough to allow the gel plug to layer over it. The gel plug was photopolymerized in the cold room. The samples were then ready for electrophoresis. The only deviation from this procedure was for acid phosphatase activity. Here the embryos were repeatedly frozen at -30°C and thawed in distilled water five times before being placed with its distilled water in the prepared tubes for homogenization.

Electrophoretic separation. The lower reservoir was entirely filled with Tris-glycine buffer pH 8.3, cooled to 5°C in order to protect the gel columns from ohmic heating. Four tubes were placed

equidistant from each other and from the central platinum electrode. A variable 500 volt DC power supply was used. A milliammeter was connected in series with the reservoir leads. A current of 0.5 mA per small tube (2.26 mm ID) was used. For the microtubes a current of 0.25 mA per tube was employed. The Bromphenol Blue marker migrated the length of the separation gel in the small tubes in about 2.5 hours and in the microtubes in about 4 hours.

Removal of the gel columns. After the electrophoretic separation was completed, the tubes were quickly removed from the reservoirs and placed in 5°C distilled water. A fine wire for cleaning a microsyringe was used to rim the gels. A large rubber bulb was filled with cold distilled water and fitted over the sample end of the tube. A steady pressure was applied to slide the gel column out of the glass tube. As soon as the gel had slid out of the tube, it was placed in a cold buffer solution appropriate for the enzymes to be demonstrated. While the gels were soaking in this buffer solution, the incubation medium was prepared.

V. HISTOCHEMICAL MEDIA FOR THE ENZYME IDENTIFICATION IN GELS

Nonspecific Esterases

0.02 M Tris-HCl buffer, pH 7.4	2.0 ml
Distilled deionized water	47.0 ml
1-Naphthyl acetate (1% in acetone)	1.0 ml
Fast Blue RR salt (C.I. 37155)	25 mg
(from Hunter and Maynard, 1962)	

The incubation was performed at 5°C in the dark for 2 hours (if longer incubation was necessary, new medium was prepared and used).

Another substrate used for esterase activity in polyacrylamide gels was 1-naphthol AS-D acetate, which was also the substrate used on the microslides. Because only two diffused bands of activity resulted after prolonged incubation, this method was discontinued.

Lipases

0.02 M Tris-HCl buffer, pH 7.4	2.0 ml
Distilled deionized water	45.5 ml
1.0 M Sodium taurocholate (final concentration 0.05 M)	2.5 ml
1-Naphthyl acetate (1% in acetone)	1.0 ml
Fast Blue RR salt (C.I. 37155) (modified from Hunter and Maynard, 1962)	25.0 mg

The gels were incubated at 5°C in the dark for 2 hours. For longer incubations, new medium was prepared and used.

Other methods tested for lipase activity were: Abe et al., (1964) method which used 1-naphthol AS nonanoate substrate and Fast Blue BB salt (C.I. 37175). The results were uniformly negative as they were on the microslides.

The Tween method was also attempted using Bokdawala and George (1964) method and modified after consideration of Bloch (1960). The medium tested was as follows. All solutions were made with distilled deionized water free of CO₂.

Distilled deionized water	27.5 ml
1.0 M Sodium taurocholate	2.5 ml
10% CaCl ₂	2.5 ml
0.5 M Tris-HCl buffer, pH 6.5	10.0 ml
2% Tween 85 (also used Tweens 80, 65, 60, 40, and 20)	7.5 ml

The 2% Tween solutions were treated with CaCl₂ overnight to precipitate the free fatty acids which are present in commercial preparations. The solution was decanted and filtered using Whatman #5 paper until the solution was clear. The gel columns were incubated at 37°C for 6 hours or more. After the incubation the gels were washed in four 500 ml volumes of distilled deionized water free of CO₂ at 5°C in order to remove free calcium ions. The washed gels were placed in 0.1% Alizarin Red S stain at pH 6.8 for 20 minutes at room temperature. The gels were rinsed and stored in 0.05 M Tris-HCl buffer, pH 6.8.

Although 2 to 3 faint diffused bands could be seen in some gels, the results were inconsistent.

Acid Phosphatases

Barka's (1961) method was employed using sodium 1-naphthyl acid phosphate with pararosanilin as coupler. Michaelis veronal acetate buffer at pH 5.0 was used. No Triton X 100 detergent was used, but freezing and thawing 5 times at -30°C was necessary to release a band of activity.

Alkaline Phosphatases

A medium of sodium 1-naphthyl acid phosphate in 0.075 M barbitol buffer pH 9.2 and using Fast Blue RR salt coupler was employed. $MgCl_2$ activator at final concentrations of 0.0001, 0.002, 0.005, 0.01, and 0.05 M was tried without success. All the gels tested for enzymatic activity were stored in methanol; water; acetic acid at 5:5:1 (v/v).

Aminopeptidases

The identical method described for demonstrating the enzymes in tissue whole mounts was used on the gel columns.

Controls for the zymogram methods. The tissues were boiled gently for 15 minutes, then placed in the prepared tubes and homogenized; tissues were placed in 1 N HCl for 30 minutes, the sample then was washed in distilled water for 15 minutes; $CuSO_4$ or $ZnCl_2$ at 0.005 M final concentration was used in incubation media as well as in a pre-incubation soak; and the gel columns were soaked prior to incubation in 1.0 N HCl for 30 minutes and washed in cold distilled water for another 20 minutes.

RESULTS

Introduction

The scoring of the enzyme activity was based on a 1 to 4 level of color intensity of the precipitated final reaction product within the individual cells of the organs or tissues examined. There are, however, a number of factors which could bias the score (Deuchar, 1966; Holt, 1956; Moog, 1965; Barka and Anderson, 1965, Chap. IX):

1) The relative size of the cells within a tissue - smaller cells appear to have a higher intensity (darker color) per cytoplasmic volume than larger cells.

2) Most tissues and organs change cytoplasmic volume during development.

3) Increase in number of cells increases the total relative enzymatic activity of the organ.

4) Some tissues are composed of cells of different sizes, for example, vitellophages, blood cells, fat body cells, coelomic sacs, contracting and stretching membranes, etc.

5) The phenomenon of heterochrony (precocious or retarded development between embryonic parts). All parts of the embryo do not always develop in a coordinated sequence, for example, embryos whose gnathal and thoracic appendages suggest a stage 8 embryo may have as yet an unsegmented abdomen.

6) Gormori (1955) states that differences in color intensity as much as $\pm 30\%$ can escape the human eye altogether (the human eye responds to light intensities on a logarithmic scale).

7) Duration and temperature of incubation must be kept constant. The end point of the incubation time was set at the time the tissues with known high enzymatic activity showed a maximum (++++) level of activity, i.e. no further incubation appreciably increased the color intensity within the individual cells.

8) Pre-incubation treatments such as dissection in modified Ringer's solution, fixation, distilled water rinses, air-drying of the tissues, and post-mortem degeneration definitely alter the enzymatic activity. These adverse effects on enzymatic activity are assumed to be rather constant for the particular enzyme studied.

9) Changes in enzymatic activity do not necessarily mean concurrent changes in enzyme concentration within a tissue. This phenomenon will be presented in the discussion section.

10) False localization of the colored precipitate due to differential solubilities between aqueous and lipoidal substances or differential absorption to structural elements, etc. must always be considered as well as false negative and/or positive reactions caused by inhibitory substances and spontaneous hydrolysis.

The results of tests for the activities of the nonspecific esterases and "lipases" were treated together for 2 reasons: the

histochemical localization of their activity consistently overlapped and the nonspecific esterase and "lipase" zymograms also showed an overlapping of 3 of the 4 "lipase" active bands. Sodium taurocholate at 0.05 M and at a pH of 7.1 inhibited 6 of the 10 nonspecific esterase active bands and appeared to induce only one "lipase" active band. So as not to convey the false impression that lipases per se were histochemically demonstrated, the results were combined under one heading, "nonspecific esterases and lipases".

The results are summarized in Table I through XIV. For reasons of brevity, the names of the various organs and tissues are listed by their definitive names although in the early embryonic stages they may be presumptive tissues or organs. Some terms also change during development due to morphogenetic movements. The extra-serosal fluid is extra-serosal until stage 25 at which time the yolk and its serosa are engulfed. The fluid then becomes an extra-embryonic fluid when no serosa is present. Fitzgerald, (1949), calls this fluid in *Melanoplus differentialis* extra-embryonic fluid even in pre-diapause stages.

PART I. HISTOCHEMICAL DEMONSTRATION OF HYDROLYTIC ENZYMATIC ACTIVITY THROUGHOUT EMBRYOGENESIS.

The Chorion

The chorion (egg shell) of *A. elliotti* is an unsculptured, semi-transparent, noncellular membrane surrounding the egg. This flexible membrane is secreted during oogenesis by the follicle cells of the

ovarioles shortly after vitellogenesis ends (Leopold, 1967).

The unfixed chorion was separated by removing the contents of the egg from it. The membrane was then rinsed in distilled water and mounted on a slide with its internal surface exposed to the incubation medium. In the early developmental stages the serosa was found to adhere, in part, to the inner surface of the chorion. The serosal membrane may be secreting the "secondary membrane" or serosal endocuticle at this time (Slifer, 1937, 1938). Enzymatic activity found on the inner surface of the chorion in the early stages, therefore, cannot be attributed to the chorion per se, but rather to the serosal membrane. The chorion itself showed no hydrolytic enzyme activity except at stages 26 and 27. The inner surface of the chorion during stage 27 shows a high level of protease activity. The source of these proteases is thought to be the extra-embryonic fluid which at this stage also showed a high protease activity. This will be further discussed in relation to the eclosion (hatching) process in the following section.

The Hydropyle

Pre-diapause stages. A low and sporadically occurring amount of acid phosphatase and nonspecific esterase and lipase activity was found within a thin proteinaceous layer lying beneath and usually adhering to the inner surface of the hydropyle cap at the posterior end of the newly-laid egg. This proteinaceous layer may have been a portion of the

plasmalemma (the periplasm of "yolk-poor" eggs) (Krause and Sander, 1962). In later embryonic stages, after the amniotic folds have joined ventrally and the serosal membrane completely surrounds the germ anlage and yolk, the serosal cells in the hydropyle region differentiate into large columnar cells which become attached to the inner surface of the hydropyle cap (Slifer, 1937; Matthée, 1951). In *A. ellioti* the hydropyle cells showed a low level of both acid phosphatase and nonspecific esterase and lipase activity throughout most of pre-diapause development. Aminopeptidase activity was not detected within the hydropyle cells in pre-diapause stages nor during diapause development. Because of the dark pigmentation and curved structure, the hydropyle region was not tested for protease activity.

Diapause development and post-diapause stages. Diapausing eggs (stage 19) which had been exposed to 5°C for 2 weeks, usually showed either a very slight or no nonspecific esterase and lipase activity within their hydropyle cells. Nonspecific esterase and lipase activity could not be detected in the hydropyle cells of diapausing eggs which were held for 4 months at 5°C. Eggs, which were exposed to cold for 4 months and then returned to room temperature for 3 hours, exhibited a very abrupt increase from no activity to a moderate level of nonspecific esterase and lipase activity within their hydropyle cells. This moderate level of activity was maintained in the hydropyle cells during blastokinesis (stages 20 to 24). During stage 24, this

enzymatic activity declined to a low level with the degeneration of the hydropyle cells. By stage 27 the inner surface of the hydropyle cap showed only a slight and dispersed amount of nonspecific esterase and lipase activity.

Diapausing eggs which were maintained at room temperature for 4 months continued to demonstrate a slight and dispersed nonspecific esterase and lipase activity within their hydropyle cells.

In contrast to the decrease of nonspecific esterase and lipase activity seen in the hydropyle cells of the diapausing eggs, acid phosphatase showed an increase in activity with or without exposure to cold temperature. After 4 months of cold exposure the acid phosphatase activity of the hydropyle cells further increased to a high level. Eggs which remained at room temperature for 4 months, however, indicated a decrease in activity to a low level. In post-diapause development the acid phosphatase activity in the hydropyle cells declined to a slight and sporadic occurrence.

Aminopeptidase activity was first observed within the hydropyle cells at stage 23. During stage 27 the activity increased to a high level. Figure 1 shows a stage 25 hydropyle with a moderate level of aminopeptidase activity.

The patterns of hydrolytic enzyme activity found in the hydropyle region must be considered as tentative in view of the wide variations in activity found between different embryos of the same stage. The

pigmentation of the hydropyle made it difficult to distinguish the colored precipitate in this region. Further investigations are needed before definite conclusions can be made.

The Yolk and Vitellophages

The yolk, with its vitellophages, was removed from the anterior end of the eggs and placed on a prepared slide. The yolk was spread carefully with a dissecting pin so as to leave yolk spherules intact but flattened. Vesicles of colored precipitate found within cytoplasmic islands and in the close vicinity to the large vitellophage nuclei were assumed to be enzymatic activity of the vitellophages. The vitellophage nuclei exhibited no activity of the hydrolytic enzymes studied throughout embryogenesis (Fig. 2).

Pre-diapause stages. A slight and widely dispersed amount of non-specific esterase and lipase activity was detected within the unfixed yolk of the newly-laid egg (Fig. 3). This enzymatic activity appeared to be confined within vesicles in the yolk's cytoplasmic reticulum.

While acid phosphatase and aminopeptidase activity was not detected, a low level of protease activity was observed in the yolk of the newly-laid egg (Fig. 4). Vesicles demonstrating nonspecific esterase and lipase activity were found to increase in number and size by stage 4, and by stage 13 there was a moderate level of activity throughout the yolk. This level of activity was maintained through the remainder of pre-diapause development. The vitellophages show a moderate

level of nonspecific esterase and lipase activity from stage 8 to stage 17. At stage 18, the activity was found at a high level.

Some lipid droplets within the yolk, but not all of them, showed coarse, blue, crystalline deposits. These deposits may indicate esterases and lipases present on or near the surface of these lipid droplets but they probably are diffusion artifacts caused by a greater solubility of the final reaction product in lipid substances.

A slight and sporadic occurrence of acid phosphatase activity was found within vesicles in the yolk at stage 2. This slight activity was maintained throughout pre-diapause development. The vitellophage cytoplasm showed a low to moderate level of acid phosphatase activity from stages 8 to 18. Aminopeptidase activity was not detected within the yolk or the vitellophages during pre-diapause stages. Protease activity appeared to be abundant in the yolk during pre-diapause development, and particularly so in the later stages (Fig. 5, 6 & 7).

Diapause development (stage 19). In diapausing embryos exposed to cold (5°C) for 2 weeks, there was an abrupt decrease in nonspecific esterase and lipase activity within the yolk and vitellophages. The greatest decrease occurred in the number of enzymatically active vesicles within the yolk. After 4 months of cold exposure, the vitellophages had accumulated an intense level of nonspecific esterase and lipase activity. The yolk, however, had only a moderate level of activity (Fig. 8).

Diapausing eggs, maintained at room temperature, did not show the decrease in nonspecific esterase and lipase activity within their yolk as did the eggs exposed to cold. After 4 months at room temperature the yolk exhibited a high level of activity whereas the vitellogophages had only a moderate level of nonspecific esterase and lipase activity. It appeared that there was no large accumulation of enzymatic activity within the vitellogophages which remained at room temperature during diapause.

Acid phosphatase activity within the yolk was found to increase rapidly during diapause with or without cold exposure. After 4 months at low temperature (5°C), the yolk showed a decline in activity; whereas the vitellogophages appeared to maintain their activity at a moderate level. Aminopeptidase activity was not detected within the yolk or vitellogophages during diapause in the cold or at room temperature. Protease activity decreased within the yolk with or without exposure to cold during diapause.

Post-diapause development (stage 20-27). The yolk demonstrated a sudden increase in nonspecific esterase and lipase activity when eggs were returned to room temperature after 4 months at 5°C. This intense activity declined to a moderate level by stage 23 and was maintained at this level even after yolk engulfment during stage 25 (Fig. 9). The vitellogophages displayed an intense amount of nonspecific esterase and lipase activity during yolk engulfment and during later stages of post-

diapause development.

Acid phosphatase activity in both the yolk and vitellophages increased to a high level by stage 23 and stayed at this level throughout the remainder of embryogenesis.

Aminopeptidase activity was not found within the yolk proper during post-diapause development. The first observation of aminopeptidase activity within the vitellophages occurred at stage 25, during yolk engulfment. In stage 26 and 27 the vitellophages showed an intense level of activity within the midgut cavity.

When eggs were returned to room temperature after 4 months of cold exposure, the proteolytic activity within the yolk exhibited a steep increase in activity from a low to a high level. Thereafter, the protease activity showed a gradual decline from stage 20 to 27.

The Extra-Serosal Fluid

The extra-serosal fluid was obtained by pricking the posterior end of the egg with a pin and allowing the fluid to escape directly onto a prepared slide. Care must be taken not to rupture the serosal membrane which would release the yolk into the extra-serosal fluid. The fluid was allowed to air dry before it was incubated in substrate medium.

The extra-serosal fluid was first observed in stage 7 at the time when the serosal membrane was detaching itself from the inner surface of the chorion and the yolk was contracting.

No nonspecific esterase and lipase activity could be demonstrated in the extra-serosal fluid until stage 25 (Fig. 10). Only a low quantity of activity was found in stages 26 and 27. Acid phosphatase activity was not found in the extra-serosal fluid throughout embryogenesis. Low alkaline phosphatase activity was found in the fluid during stage 27 and was associated with the degenerating pleuropodia, which also contained abundant alkaline phosphatase activity at that time. The first appearance of aminopeptidase activity was in stage 26 and by stage 27, activity was at a moderate level. Protease activity in the fluid reached a high level just prior to hatching during stage 27 (Fig. 11 & 12).

The Serosal Membrane

The serosal membrane was first observed attached to the inner surface of the chorion in eggs with embryos at stage 4. By stage 7, the serosa was located on the surface of the yolk with the extra-serosal fluid separating it from the chorion. The unfixed serosa was dissected from the yolk, flattened and stretched on a slide coated with rubber-adhesive, and allowed to air dry before incubating in substrate media.

Pre-diapause stages. A low level of nonspecific esterase and lipase activity was observed from stage 4 through stage 7. From stages 8 to 18 a moderate amount of nonspecific esterase and lipase activity was seen. With stage 18 and the beginning of diapause (stage 19) the

serosa showed a high level of activity.

The first evidence of acid phosphatase activity in the serosa was detected at stage 12; aminopeptidase activity was seen as early as stage 9. By stage 18 both aminopeptidase and protease activity was prominent (Fig. 13). The hydrolytic enzyme activity found within the serosal membrane consistently showed a mosaic pattern of distribution. Some areas exhibited a high amount of hydrolytic activity whereas adjacent areas showed only low or moderate levels of activity. The serosal membrane, as contrasted to the amniotic and provisional dorsal closure membranes, appeared to contain more hydrolytic enzyme activity than any other tissue during pre-diapause development.

Diapause development. The serosal membrane showed an abrupt decrease in nonspecific esterase and lipase activity after 2 weeks of cold (5°C) exposure. The enzymatic activity dropped from a high level to a low level. Eggs which were not exposed to cold only decreased in activity to a moderate level. The greatest difference in nonspecific esterase and lipase activity occurred between eggs which were in the cold for 4 months and eggs which were kept at room temperature for 4 months. Diapausing eggs kept at 5°C for 4 months showed a marked increase in activity to an intense level but the serosa still demonstrated the mosaic pattern (Fig. 14 & 15). Eggs, which remained at room temperature for 4 months, had only a very low and dispersed activity.

After 2 weeks of cold exposure, acid phosphatase activity observed within the serosal membrane showed no change from that observed at stage 18. After 4 months of cold exposure the activity was decreased to a low amount. This same decrease in activity was also seen in eggs which remained at room temperature for 4 months (Fig. 16).

In eggs exposed to 5°C for 2 weeks the serosal membrane exhibited a marked decline in aminopeptidase activity changing from a moderate quantity at stage 18 to an undetected level during diapause. Eggs, which were not exposed to cold also decreased in activity to an undetected level. When the eggs had been in the cold for 4 months, a moderate amount of activity was again restored. Aminopeptidase activity was not found in eggs that remained at room temperature for 4 months. This enzymatic pattern was similar to that of the nonspecific esterases and lipases.

Protease activity within the serosal membrane was found to suddenly increase with the onset of diapause. After 4 months of cold temperature, the protease activity was found at a high level.

Post-diapause stages. When diapause eggs were brought back to room temperature after 4 months at 5°C their serosal membranes continued to display intense amounts of nonspecific esterase and lipase activity. This activity persisted through stage 25, at which time the serosa, along with the yolk, was engulfed into the midgut by the process of dorsal closure (Fig. 17).

A burst of acid phosphatase activity was found in the serosal membrane within 3 hours after the eggs were removed from the cold. This intense activity remained in the serosa until stage 25. Most of this enzymatic activity was carried into the midgut when the serosa and the yolk were engulfed. Figure 18 shows a stage 20 serosa with intense acid phosphatase activity located exclusively within the cytoplasm and particularly around the outer surface of the nuclear membrane.

The serosal membrane exhibited high levels of aminopeptidase and protease activities throughout post-diapause development (Fig. 19). During the post-diapause development, the serosal membrane continued to exhibit more overall hydrolytic activity than most other tissues. Furthermore, it was noticed that usually a portion of the serosal membrane remained outside of the embryo after yolk engulfment. This small "serosal cap" was found near the posterior dorsal margin of the head. The serosal cap then appeared to degenerate, and it was thought to contribute its abundant hydrolytic enzymes to the extra-embryonic fluid. These enzymes may aid in the destruction of the inner serosal endocuticular layer of the chorion facilitating hatching. This will be treated more fully in the discussion section.

The Amniotic and Provisional Dorsal Closure (PDC) Membranes

The unfixed amniotic and PDC membranes were dissected from the embryos, flattened and spread on prepared slides. The amniotic membrane differed from the PDC membrane in its adhesiveness, adhering to

itself as well as to the dissecting pin. Consequently, the amnion proved difficult to handle and was not tested in many stages.

The amniotic membrane was first observed on the ventral surface of the germ anlage at stage 4. Nonspecific esterase and lipase activity could not be demonstrated in the amnion at any stage tested. Trace amounts of acid phosphatase activity were found in the amnion after stage 13 (Fig. 20). Only a slight amount of aminopeptidase activity was detected at stage 23. All the other stages were negative for this activity. The amnion was not tested for protease activity.

The provisional dorsal closure membrane was first observed in stage 16, although it may have been present earlier. None of the hydrolytic enzymes studied could be detected in this membrane (Fig. 21). The PDC membrane separated the yolk from the inner dorsal surface of the embryo and enclosed the epineural sinus. By stage 16, and probably earlier, the embryo was completely enclosed within the amnion and the PDC membrane. It is thought that these membranes must be permeable to the hydrolyzed nutrients of the yolk, but the membranes themselves appeared not to be the site nor source of those hydrolytic enzymes.

The Germ Anlage and Germ Band

About 7 days after oviposition at room temperature, a small round disc of cells develops on the surface of the yolk below the hydropyle at the posterior end of the egg. This disc of cells is the germ anlage, and is designated as stage 1 (Van Horn, 1966a). The unfixed embryonic

tissue of the early stages was dissected from the egg and mounted whole on a slide. The germ anlage was not considered a germ band until after gastrulation had occurred with the formation of the germ layers, the ectoderm and the mesoderm (Krause and Sander, 1962).

Only a background level of nonspecific esterase and lipase activity was seen within the blastomeres of the germ anlage and germ band from stages 1 through 11 (Fig. 22). Acid phosphatase and aminopeptidase activity was not detected in the germ band until stages 6 and 7, respectively.

Acid phosphatase activity was first observed in three distant locations within the germ band during stage 6. A plaque of high activity was seen first in the gnathal region. A moderate level of activity was secondarily found at the posterior tip of the abdomen, and at about the same time, a slight activity was located within the protocephalon. The burst of high acid phosphatase activity seen in the gnathal region will be treated in relation to a proposed physiological differentiation center in the discussion section.

Aminopeptidase activity first was seen at stage 7 in the gnathal region slightly anterior to the location of acid phosphatase activity. By stage 9, the gnathal region showed a sharp increase in aminopeptidase activity. This intense enzymatic activity first appeared in a group of cells lying across and slightly anterior to the stomodeum. Later this group of cells became elongated dorsolaterally forming a

"handlebar" configuration (Fig. 23 & 24). Since the subesophageal body cells are morphologically differentiated by stage 9 and are seen in the same location as the aminopeptidase activity (Van Horn, 1963), it was thought that this enzymatic activity was indicative of the chemodifferentiation of subesophageal body cells. A low level of aminopeptidase activity also was observed at the posterior tip of the abdomen during stage 7. This activity increased to a high level by stage 9 at the time of the proctodeal invagination. The aminopeptidase activity then was seen to migrate laterally during stages 13 and 14 and finally to lie in the region of the rudimentary cerci (Fig. 25). These intriguing patterns of acid phosphatases and aminopeptidases will be discussed later in the paper.

The first observation of nonspecific esterase and lipase activity above background levels of activity occurred in the germ band during stage 12 within the protocerebrum. This increase in enzymatic activity was concurrent with the onset of neuropyle differentiation (Van Horn, 1963). In stage 13 the optic lobes, deutocerebrum, tritocerebrum, and the subesophageal ganglion all show a similar increase in nonspecific esterase and lipase activity. This activity increased to a moderate level by stage 15. Between stages 14 and 18, a posterior progression of activity was seen first within the thoracic ganglia and then into the abdominal ganglia paralleling the posterior differentiation of the neuropyle. By stage 18, prior to diapause, the neuropyle regions of

the protocerebrum and the subesophageal ganglion exhibited the highest level of nonspecific esterase and lipase activity of the entire central nervous system.

The Central Nervous System (CNS)

The CNS was not dissected from the embryos at early stages, but was observed in situ through stage 16. In the later stages the CNS or portions of it were removed and placed whole and unfixed upon a prepared slide for incubation in the various media.

The early patterns of nonspecific esterase and lipase activity were described above. When stage 19 (diapause) eggs were exposed to 5°C for 2 weeks the nonspecific esterase and lipase activity was seen to greatly diminish from a high level in stage 18 in the protocerebrum and subesophageal ganglion to only a low level. However, in eggs which remained at room temperature the nonspecific esterase and lipase activity did not show this steep decline in activity. Even after 4 months at room temperature the activity remained at approximately the same level as in early diapause (Fig. 26). Eggs which were exposed to 4 months of cold showed an intense level of nonspecific esterase and lipase activity within the subesophageal ganglion and high level of activity in the protocerebrum (Fig. 27). This activity was primarily within the central neuropyle areas of the brain and ventral ganglia. During post-diapause development the CNS continued to demonstrate high to intense levels of activity (Fig. 28). After the initial high activity

at stage 6, acid phosphatase activity declines to a moderate level from stage 9 to 12. In stage 12 the entire embryonic brain and optic lobes exhibit an intense level of activity. By stage 15, activity has declined to a moderate level; by stages 17 and 18 the acid phosphatase activity was at a high level within the brain, optic lobes, and ventral ganglia.

Acid phosphatase activity remained high in the CNS after 2 weeks of apparent diapause whether exposed to cold or not. After 4 months at either 5°C or 25°C temperature the activity had decreased to a moderate level. When diapause eggs which were kept for 4 months at 5°C were again exposed to warm temperature for at least 3 hours the acid phosphatase of the CNS demonstrated a striking increase in activity to an intense level (Fig. 29 & 30). This intense level of activity was then maintained throughout the remainder of post-diapause development.

The protocerebrum, optic lobes, and subesophageal ganglion first showed aminopeptidase activity at stage 13. This activity was of a moderate level. By stage 18 the protocerebrum had a high level of aminopeptidase. The other regions of the brain and the subesophageal ganglion showed a moderate level of activity. At stage 18 the thoracic and abdominal ganglia demonstrated a low amount of activity within their neuropyle.

With the apparent onset of diapause whether exposed to cold or not the aminopeptidase activity within the central nervous system decreased, particularly within the subesophageal ganglion where no aminopeptidase activity could be detected by this method. Even after 4 months of cold exposure and subsequent return to room temperature, there was only a low level of aminopeptidase within most of CNS. The subesophageal ganglion still gave a negative result for aminopeptidase activity. However, the subesophageal ganglion of embryos which were not exposed to cold temperatures showed a moderate level of activity even after 4 months in warm temperature. It appears that cold treatment during diapause repressed, inhibited, or degraded aminopeptidases within the subesophageal ganglion. Aminopeptidase activity in stages 20 and 21 was very low or undetectable within the brain and subesophageal ganglion. The remaining ventral ganglia showed a low to moderate level of activity. At stage 23, however, the brain and subesophageal ganglion suddenly showed a high level of activity which they maintained through stage 27. In stage 27 the protocerebrum and the subesophageal ganglion had intense levels of aminopeptidase activity whereas the ventral ganglia continued to show a moderate level of activity. Protease activity within the central nervous system was not tested.

The Cerci

The rudimentary cerci were observed in situ extending from the 10th abdominal segment. Their acid phosphatase and nonspecific esterase

