



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

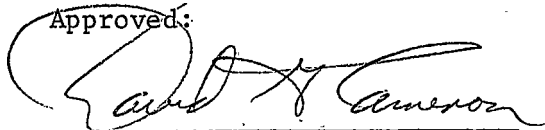
of

DOCTOR OF PHILOSOPHY

in

Zoology

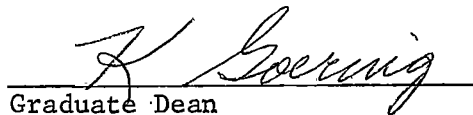
Approved:



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 Vitellogophages	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, vitellogophore 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 of 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. elliotti* 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 Histoclad. 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 Desicote 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)	25.0 mg
(modified from Hunter and Maynard, 1962)	

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 CaCl_2 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 CO_2 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, vitellogophages, 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. ellioti* 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 vitellograph 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 vitellographs 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 vitellographs. The greatest decrease occurred in the number of enzymatically active vesicles within the yolk. After 4 months of cold exposure, the vitellographs 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. Amino-peptidase 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

and lipase activity was not conspicuous as compared to the surrounding abdominal tissues throughout embryogenesis. Aminopeptidase activity was found at the tip of the abdomen at stage 27 and at stage 9 a high level of activity was exhibited. During stages 13 and 14 this aminopeptidase activity appeared to migrate laterally from the tip of the abdomen, and at stage 15 an intense activity was located in the presumptive cerci (as previously stated under the germ band results). This intense reaction continued through stage 18 (Fig. 25). A decrease from the intense level to a high level of activity resulted after 2 weeks of cold (5°C) exposure during apparent diapause (stage 19). This level of activity persisted after 4 months at 5°C . Eggs (at stage 19) kept at room temperature for 4 months showed a decline in aminopeptidase activity to a moderate level. There was no alteration in the high level of activity within the cerci with the termination of diapause at stage 20. In stages 23 to 27 the activity fell to a moderate level, but at stage 27 the cerci again increased to a high level of activity prior to eclosion (hatching).

The Abdominal Walls

Lateral segmental cell clusters. A lateral segmental cell cluster, exhibiting a higher acid phosphatase activity than the surrounding abdominal wall, was first detected at stage 10. By stage 12 a moderate level of activity was found, and by stage 13 the activity abruptly

increased to an intense level. Stages 14 to 19 displayed a conspicuously high amount of acid phosphatase activity. Consideration of the embryonic histology of *A. ellioti* (Van Horn, 1963) led to the belief that the enzymatic activities observed in the cell clusters were indicative of the chemodifferentiation of the oenocytes.

These lateral segmental cell clusters maintain their high level of acid phosphatase activity after 4 months of incubation at 5°C. There is a decrease in activity seen with the termination of cold treatment (stage 20). Embryos, thought to be in diapause, were held at 25°C for 4 months. These embryos displayed only a low level of acid phosphatase activity within these cell clusters. In post-diapause development these cells were no longer distinguished by contrasting levels of acid phosphatase activity (Fig. 31).

Nonspecific esterase and lipase activity was not seen in these cell clusters until stage 17 and then only at a low level. With the nonspecific esterase and lipase methods, discrete cell clusters were not as evident as with the acid phosphatase method. Therefore, it is tentatively suggested that nonspecific esterases and lipases were probably associated with the fat body cells which make their first morphological appearance concurrent with onset of this enzymatic activity at stage 17 (Van Horn, 1963). After 4 months of incubation at 5°C an increase in activity to a moderate level was observed. In post-diapause development lateral segmental cell clusters showed a high level of nonspecific esterase and lipase activity during stages 25 and 26.

In stage 27 this activity declined to an inconspicuous moderate level.

A low level of aminopeptidase activity first was seen within lateral cell clusters along the thoracic walls of embryos at stage 13. Not until stage 17 did the abdominal walls show aminopeptidase activity. In stage 18 both the thoracic and abdominal walls demonstrated a moderate level of activity. The amount of aminopeptidase activity in these structures during diapause decreased to a low level. In post-diapause development the activity was obscured because of an overall increase in aminopeptidase activity within the thoracic and abdominal walls. Methods of demonstrating protease activity did not reveal any outstanding areas of high activity in the abdominal wall.

The dorso-lateral margins of the abdominal walls. A dorso-longitudinal region extending the length of the abdominal wall near or at the junction of the provisional dorsal closure membrane showed conspicuous acid phosphatase and aminopeptidase activity at stages 13 and 14, respectively. By stage 18 the acid phosphatase activity was found at a high level along this dorsal margin. The activity continued at this level during diapause incubation at 5°C for 4 months and also throughout post-diapause development. At stage 18 aminopeptidase activity, showing a moderate reaction, was less pronounced than the acid phosphatase activity. This moderate level of aminopeptidase activity was maintained throughout diapause whether the embryos were exposed to cold temperature or not. In post-diapause development the activity remained at a moderate level through stage 23 (Fig. 32). In stages 25,

26, and 27 the aminopeptidase activity in this region was seen at a high level. No protease and nonspecific esterases or lipases were distinguishable along the dorsal margin of the abdominal wall throughout embryogenesis.

Because the embryos were not studied with cytochemical methods, it could not be determined whether the above enzymatic activity was present in the pericardial cells, the gonads, or the fat body cells. It is likely, however, that the enzymatic activity observed along the dorsal margins of the abdominal walls is only associated with the high mitotic rate of this rapidly proliferating region.

The Membrane Surrounding the Epineural Sinus

A membrane surrounding the epineural sinus first was dissected from a stage 17 embryo, after the provisional dorsal closure membrane had been formed. This membrane appeared to be attached to the internal dorsal edge of the abdominal wall and lie ventral to the provisional dorsal closure membrane and dorso-lateral to the ventral ganglia. This cellular membrane was easily removed as it does not adhere to other mesodermal elements of the abdomen. In late post-diapause stages large white crystalline deposits were found within this membrane, which are thought to be uric acid crystals (Polivanova, 1965). After yolk engulfment (stage 25) this membrane was found to lie ventro-lateral to the midgut (mesenteron) and could still be easily removed.

No nonspecific esterase and lipase activity could be detected within this membrane throughout embryogenesis (Fig. 33). A slight amount of acid phosphatase activity was first observed in the membrane during stage 25. In stages 26 and 27 the membrane demonstrated a moderate level of activity, but this activity was not found adjacent to the white crystalline deposits (Fig. 34). A slight and dispersed amount of aminopeptidase activity was also found in this membrane during stage 25. This meager level of activity was maintained through stage 27.

The Stomodeum and Gastric Caecae

The stomodeal invagination was first observed during stage 6. After stage 17, the entire stomodeum was dissected from the embryo and placed separately on a slide for incubation in substrate media.

Nonspecific esterase and lipase activity was not detected within the stomodeum above background levels until stage 26. A moderate level of activity was found in both stages 26 and 27 (Fig. 35). The gastric caecae were first observed as small buds extending from the posterior end of the stomodeum during stage 24. The buds themselves showed only a background of nonspecific esterase and lipase activity. By stage 25, however, a sudden burst of enzymatic activity was seen. The gastric caecae demonstrated an intense reaction in stages 25, 26, and 27 (Fig. 35).

The stomodeum demonstrated no acid phosphatase until stage 25, and then only a slight amount. In stages 26 and 27 this activity increased to a moderate level. The gastric caecae showed a slight amount of acid phosphatase activity in stage 25 and a sudden high level of activity in stages 26 and 27.

No aminopeptidase activity could be detected in the stomodeum until stage 26. Here, however, the aminopeptidase activity appeared as a ring of activity at the junction of the stomodeum with the midgut (Fig. 36). By stage 27 this stomodeal-midgut junction demonstrated a high level of activity. The gastric caecae showed a low amount of aminopeptidase activity from their first appearance as caecal buds at stage 24. Not until stage 27, however, did the gastric caecae show a high level of aminopeptidase activity. The stomodeum did not demonstrate any protease activity at any time during embryogenesis.

The Proctodeum, Malpighian Tubules and Rectal Pads

The proctodeal invagination was first observed by stage 9. It was observed in situ during stages 9 to 16 and thereafter dissected and mounted separately. The proctodeum only showed a background level of nonspecific esterase and lipase activity from stages 9 through 23. In stage 24 a low level of activity of these enzymes was found in most of the cells. By stage 25, an abrupt increase in activity to a high level was noted (Fig. 37). This level of activity persisted through

stage 27. Six longitudinal rectal pads were distinguished by their intense nonspecific esterase and lipase activity in stages 26 and 27.

The proctodeum first demonstrated a low amount of acid phosphatase activity by stage 17. There was a slight decrease in activity after incubation for 4 months at 5°C in diapause embryos. During blastokinesis (stages 20 to 24) acid phosphatase activity could not be detected within the proctodeum. By stage 25 a low level of activity was seen, and at stage 26 a sudden burst of activity occurred throughout the proctodeum. The rectal pads were conspicuous from their first observation at stage 25 with a moderate level of activity. During stage 27 the acid phosphatase activity of the rectal pads increased to an intense level (Fig. 38). Furthermore, the margin around the anus also showed an intense reaction for acid phosphatase. The acid phosphatase activity of the anal margin was studied from stage 6 at which time the tip of the abdomen showed a moderate activity with a subsequent intense level of activity at stage 13. By stage 15 this activity had declined to a moderate level which was maintained through stage 18. Acid phosphatase activity in the anal margin of the rectum during diapause incubation demonstrated an increase in activity to a high level whether exposed to 5°C for 2 weeks or not. After 4 months of incubation at 5°C the anal margin showed a moderate activity whereas the embryos which remained at 25°C showed only a low level of activity. There was no change in the level of acid phosphatase activity within the anal margin with

the termination of diapause. A moderate level of activity was found to remain constant throughout blastokinesis. At stage 25 a high level of activity was seen, and in stages 26 and 27, as was previously mentioned, an intense level of acid phosphatase was observed within the anal margin of the rectum.

No aminopeptidase activity could be demonstrated in the proctodeum until stage 25. At stage 25 a moderate level of aminopeptidase activity could be seen at the proctodeal-midgut junction. The proctodeum itself, however, contained only a slight and dispersed level of activity. The rectal pads were conspicuous during stage 25 with a high level of aminopeptidase activity (Fig. 39). By stage 26 the proctodeal-midgut junction was seen as a ring of intense activity which persisted through stage 27 (Fig. 40). The rectal pads could not be distinguished from the rectum proper during stage 27 due to a large increase in aminopeptidase activity of the rectum itself.

A low level of protease activity was found for the first time in the proctodeum during stage 26; and by stage 27 the activity was scored at a moderate level. The rectal pads showed the same level of protease activity as the proctodeum.

The Malpighian tubules were first observed as 6 small evaginations from the anterior end of the proctodeum during stage 16. The Malpighian tubules showed no nonspecific esterase and lipase activity until stage 25 at which time a slight amount of activity was found in some but not

all of the tubules (Fig. 37). The enzymatic activity was first observed at the base of the tubules and it appeared to extend outward with development. In stage 26 most of the tubules demonstrated a moderate level of nonspecific esterase and lipase activity, some of the tubules showed activity all the way to their bulbous tips (Fig. 41). During stage 27, most of the Malpighian tubules demonstrated intense levels of nonspecific esterase and lipase activity throughout their entire length (Fig. 42).

A slight amount of acid phosphatase activity was found in Malpighian tubule buds at stage 17. A low level of activity was observed in the bulbous tips by stage 18. With exposure to 5°C for 2 weeks during stage 19 (diapause) a small decrease in activity was found, and after 4 months at 5°C no acid phosphatase activity could be detected within the Malpighian tubules. In eggs which were kept at room temperature during diapause, a low level of acid phosphatase activity continued in the presumptive Malpighian tubules. The tubules showed a moderate activity at their tips in stages 26 and 27 (Fig. 38). It appeared that the acid phosphatase was most evident at the tip of the Malpighian tubules.

No aminopeptidase activity could be detected in the Malpighian tubules until stage 26 at which time a slight amount was found within their bulbous apical tips (Fig. 40). During stage 27 this activity increased to a moderate level. The Malpighian tubules also demonstrated

a low level protease activity during stage 27 along their entire length.

The Midgut and Engulfed Yolk

The midgut was first observed at stage 24 surrounding the newly ingested yolk. A dorsal longitudinal incision was made through the provisional dorsal closure membrane to expose the midgut. The midgut was dissected from the embryo and the yolk contents were removed. The midgut wall was rinsed in modified Ringer's, then in distilled water, and was flattened and spread onto a prepared slide with its internal surface exposed. The midgut lining at stage 24 appeared to be only one cell layer thick. In later stages (26 and 27) the midgut was found to be very irregular in cellular thickness, perhaps due to the migration of vitellogophages to the periphery of the ingested yolk where they appear to become incorporated into the midgut epithelium (Van Horn, 1963).

The midgut at stage 24 was found to have only a background level of nonspecific esterase and lipase activity. The midgut by stage 27 showed only a moderate level of activity (Fig. 43). The vitellogophages and yolk at this time, however, demonstrated an intense amount of non-specific esterase and lipase activity and were found to adhere to the midgut membrane making it difficult to assess the activity of the midgut lining itself.

A slight and sporadic occurrence of acid phosphatase activity was also found in the midgut at stage 24. In stage 25 there was a moderate activity (Fig. 44) and in stages 26 and 27 a high acid phosphatase

activity. The engulfed yolk and vitellophages demonstrated a high acid phosphatase activity from stage 23 through stage 27.

The midgut showed no aminopeptidase activity until stage 26 and then only a slight and dispersed amount. In stage 27 a moderate level of aminopeptidase activity was found. The engulfed yolk exhibited no aminopeptidase itself, but the vitellophages for the first time during embryogenesis showed aminopeptidase activity at the time of yolk engulfment. Stage 25 vitellophages exhibited a moderate level of activity, and stages 26 and 27 showed intense aminopeptidase activity.

A remnant of the ingested serosal membrane could be found within the yolk at stage 25. This engulfed serosal membrane showed a high to intense reaction for nonspecific esterases and lipases, acid phosphatases, and aminopeptidases (protease activity was not tested).

The Pleuropodia

The pleuropodia originate from the first abdominal appendages which appear at stage 8 in this species. Not until stage 14, however, does the hypoderm of the first abdominal appendage show a specialization into cells with enlarged nuclei (Van Horn, 1963). These appendages subsequently develop into paired reniform structures made up of a single layer of glandular cells surrounding a lumen. For histochemical studies, the pleuropodia were plucked from the abdominal walls, rinsed in distilled water, and placed separately on slides.

Pre-diapause stages. The pleuropodia had only a background level of nonspecific esterase and lipase activity from its first appearance, as the first abdominal appendage, at stage 8 through stage 13. From stage 14 to 18, the activity was found to gradually increase from a low to a high level of nonspecific esterase and lipase. The nonspecific esterase and lipase activity was located uniformly throughout the cytoplasm of the pleuropodial cells during pre-diapause.

Acid phosphatase activity was not detected in the pleuropodial cells until stage 15 and at stage 18 was found to be at a moderate level.

Aminopeptidase activity was first seen in the pleuropodia at stage 17 and by stage 18 there was still only a low activity present. The pleuropodia also contained a low level of protease activity in stage 18. Early stages were tested for protease activity with the gland attached to the abdomen. The pleuropodia showed little, if any, protease activity in earlier pre-diapause stages.

Diapause (stage 19). The pleuropodia, in contrast to the serosal membrane, yolk, and central nervous system, did not decrease in nonspecific esterase and lipase activity with the onset of diapause and cold exposure (5°C). The high level of activity in pleuropodia at stage 18 continued after 2 weeks of cold temperature. After 4 months of 5°C the pleuropodia consistently showed an increase in nonspecific esterase and lipase activity to an intense level (Fig. 45 & 46). The gland at this time also was found to increase in cytoplasmic volume (Van Horn,

1963). The highest enzymatic activity appeared to be located on the internal lumen side of the cells (Fig. 47). This distribution was at first thought to be a diffusion artifact or perhaps a consequence of the glandular morphology. Pleuropodia were, therefore, allowed to soak in distilled water for 30 minutes prior to incubation to determine whether the "border effect" would be further enhanced. The same pattern of distribution resulted. Cold (5°C) calcium-formal (10%) fixation was employed for periods up to 6 hours but the border effect remained, although overall activity was greatly diminished. The incubation time then was shortened to 10 minutes and the pleuropodia were placed directly into the medium without being dissected under a modified Ringer's solution or rinsed in distilled water. The greatest activity remained toward the inner margin of the gland and near the nuclei. If this asymmetrical distribution of esterase and lipase activity was due to the morphology of the gland, then a similar pattern would be expected with the other enzymes tested. This, however, was not the case. Both acid phosphatase and aminopeptidase activity were found to be high or intense at stage 25 but their distribution was uniform throughout the cells (Fig. 48). The accumulation of nonspecific esterase and lipase activity on the internal lumen side of the pleuropodia could still be an artifact caused by an extreme solubility of the esterases and lipases within the substrate incubation medium. However, the possibility also exists that these glands secrete nonspecific esterases and lipases into

the lumen of the gland which is continuous with the embryonic hemocoel.

Eggs, which were not exposed to cold during diapause, showed a decrease in nonspecific esterase and lipase activity in their pleuropodia from a high level at stage 18 to a low level after 2 weeks in apparent diapause. After 4 months incubation at room temperature the pleuropodia of (stage 19) diapause embryos continued to demonstrate only a moderate level of activity in sharp contrast to the intense activity in the pleuropodia which had been exposed to 5°C during diapause.

The pattern of acid phosphatase activity during diapause is the opposite of the nonspecific esterases and lipases. After 2 weeks at 5°C the pleuropodia showed a decrease in activity and after 4 months of cold duration no acid phosphatase activity could be detected within these organs (Fig. 49). Pleuropodia from embryos which remained at room temperature during diapause, on the other hand, exhibited a high level of acid phosphatase after 2 weeks. After 4 months at room temperature the acid phosphatase activity declined to a low level.

The level of aminopeptidase within the pleuropodia during diapause exhibited a wide variation between different embryos having the same temperature treatment. Of the six embryos (12 pleuropodia) tested for aminopeptidase activity after 28 days at 5°C, 4 pairs of pleuropodia demonstrated a high level of aminopeptidase activity uniformly

distributed throughout the cytoplasm of the cells. One embryo showed a negative reaction and the remaining embryo displayed a moderate level of activity within the lumen of the gland and particularly at the junction of the pleuropodia with the abdominal wall.

The pleuropodial cells themselves showed only a low level of activity in this particular embryo. Van Horn (1963) noted that occasionally a few fat body cells and hemocytes were found near or within the lumen of the pleuropodia. It is possible that these cells are responsible for part of this activity within the lumen of the gland, though this is not likely because of the low number of cells found.

Embryos, which were kept at room temperature during diapause, also showed variation in activity between the embryo's pleuropodia. Most of the embryos displayed a moderate level of aminopeptidase activity in their pleuropodia after 2 weeks at room temperature during diapause. After 1 month, however, most pleuropodia showed only a low amount of activity. After 4 months at room temperature the majority of the pleuropodia again exhibited a moderate level of activity within the pleuropodial cells.

Protease activity within the pleuropodia after 2 weeks of cold (5°C) exposure at stage 19 showed a low level of activity, the same as it did in stage 18. After 4 months of cold exposure the pleuropodia exhibited a higher level of activity reaching a moderate level. Embryos, which were not exposed to cold temperature during diapause did

not increase in activity above the pre-diapause low level after 4 months of warm temperature.

Post-diapause stages (20 to 27). The pleuropodia of embryos exposed to 5°C for 4 months at stage 19 showed an intense level of non-specific esterase and lipase activity as stated above (Fig. 45 & 47). The glands increased in size to approximately twice the pre-diapause volume during this period. When the eggs were brought back to room temperature and allowed to warm up for just 3 hours, the intense esterase and lipase activity of the pleuropodia was rapidly reduced to only a moderate and uniform level (Fig. 50 & 51). The gland's volumes, likewise, were decreased to approximately the pre-diapause size. This abrupt decrease in nonspecific esterase and lipase activity occurred in all 20 embryos tested.

A moderate level of nonspecific esterase and lipase activity was maintained during blastokinesis. At stage 24 the pleuropodia showed an increase in activity to a high level, and by stage 25 the pleuropodia were again swollen and exhibited an intense level of activity (Fig. 52 & 53). The intense level of nonspecific esterase and lipase activity continued during stages 26 and 27; however, the gland was found to decrease in volume after stage 25. In late stage 27 the pleuropodia usually were found detached and floating free in the extra-embryonic fluid. The glands appeared to be degenerating, becoming sticky to the touch of a pin and continually decreasing in size (Fig. 54).

Acid phosphatase activity, which could not be detected in the pleuropodia after 4 months of cold, was found at a moderate level after 3 hours of exposure to room temperature with the beginning of post-diapause development (Fig. 55). The acid phosphatase activity appeared to decrease gradually during blastokinesis from a moderate level at stage 20 to a slight amount at stage 24 and stage 25. In stage 26, a burst of high activity was found in the pleuropodia in most of the embryos studied. After the glands were found detached from the abdominal wall during stage 27, they showed only a low level of acid phosphatase activity (Fig. 56).

The only organs to demonstrate alkaline phosphatase activity during embryogenesis were the pleuropodia, which had a moderate level of activity during stage 27. A wide range of magnesium ion concentrations were tested with embryos of younger stages with negative results. Alkaline phosphatase activity was found in 3 abnormal embryos at stage 19. These abnormal embryos had received 4 months of cold (5°C) exposure and were returned to room temperature for post-diapause development. After 6 days at 25°C the serosal attachments of these embryos were still intact, whereas normal embryos would have proceeded through blastokinesis and been at stage 25. These embryos were found to have their stomodeums and proctodeums everted into the yolk masses, and their definitive dorsal closures had formed over the ventral surface of the embryos and enclosed their thoracic appendages (Ando, 1955).

These 3 abnormal embryos demonstrated a moderate to a high alkaline phosphatase activity laterally along their abdominal walls. This activity may have been present in the oenocytes cells which differentiate from the hypodermis surrounding the invaginated (here evaginated) spiracles. Embryos at stage 27 which had failed to undergo blastokinesis, also showed a low level of alkaline phosphatase activity along the lateral abdominal wall.

Only a trace of aminopeptidase activity could be detected in the pleuropodia of embryos which were returned to room temperature after 4 months of cold. Stage 21 embryos showed no aminopeptidase activity whatsoever. At stage 23 (Fig. 57) there was a moderate level of activity and by stage 25 an intense level of aminopeptidase activity (Fig. 48). This intense activity decreases to a moderate amount in stage 26 (Fig. 58) and in late stage 27 the aminopeptidase activity was again at a high level. The activity was found to be distributed quite uniformly throughout the gland unlike the nonspecific esterase and lipase activity.

Protease activity was found to be intense in late stage 27 pleuropodia (Fig. 59). Such proteolytic activity was thought to be one of the consequences of cellular degeneration, for dead eggs which were used for control tests also showed intense protease activity.

No distinct pH optimum could be found for protease activity. High protease activity was found in the pleuropodia between pH 6.4 and 7.8

using 0.05 M Tris-maleate buffer. The standard pH of 7 was therefore chosen for all protease analyses in the embryonic tissues.

Hemocytes

Van Horn (1963) found loose, round mesodermal cells lying below the stomodeum within the thoracic segments of stage 13 embryos. These cells were thought to be the first evidence of embryonic hemocytes.

The hemolymph was removed from the epineural sinus by making an opening along the dorso-medial line. The slightly green tinted hemolymph was allowed to flow out directly onto a prepared slide. The droplet was then smeared with a cover slip and allowed to air-dry with no fixation prior to incubation in the substrate media. No attempt was made to classify the hemocytes, only their general outline, dimensions, and their enzymatic activity were recorded.

Four different cell sizes and/or shapes were found in late post-diapause stages (25, 26, and 27). No vermiform cells were observed. The largest but least numerous type was an ellipsoidal cell with diameters of about 20 and 27 μ . It appeared to have relatively little cytoplasm (Fig. 60). This hemocyte showed a high nonspecific esterase and lipase activity, a moderate acid phosphatase activity and a low aminopeptidase activity within its nucleus.

A medium size (about 20 μ in diameter) round cell type with moderate amount of cytoplasm was found to have a moderate level of

nonspecific esterase and lipase activity (Fig. 61). These cells appeared to be quite numerous. They showed a low level of acid phosphatase and aminopeptidase activity, although some cells of this size showed no activity whatsoever.

The smallest and most numerous cells had diameters from 6 to 10 μ and were found to contain small nuclei and abundant cytoplasm (Fig. 62). These small round cells exhibited a slight amount of nonspecific esterase and lipase activity, within their cytoplasm. No acid phosphatase or aminopeptidase activity could be detected within these cells.

The fourth and the most rare cell type found measured 12 x 14 μ . Its cytoplasm showed numerous vesicles (spherules) and it only demonstrated a low level of nonspecific esterase and lipase activity (Fig. 63).

The hemolymph was found to "clot" into noncellular proteinaceous masses although occasionally cells were present within them. These plasma clots showed a moderate to high nonspecific esterase and lipase activity as well as a moderate level of acid phosphatase activity (Fig. 64). Hemolymph from embryos at stage 19 also showed a moderate level of nonspecific esterase activity.

PART II. DISCONTINUOUS POLYACRYLAMIDE GEL ELECTROPHORESIS

Whole embryos at stage 19 (held at 5°C for 4 months) were used for comparing pherogram patterns with the zymogram patterns (Fig. 65). When 2 homogenized embryos were assayed per tube (2.26 mm ID) a total of 28 protein staining bands were seen. Using only one embryo per tube, 26 protein staining bands were observed; but, there were 4 protein bands which did not correlate between them (tracing #2, bands 13, 17, and 20, numbered consecutively from the origin (0) to the anode (+), were not found in tracing #3. Tracing #3 also contained an extra band (#26) which tracing #1 did not demonstrate). The number of protein bands found in a whole diapausing embryo using the 0.92 mm ID microtubes was 27. One whole embryo proved to be an excessive amount of protein for separation in the microtubes.

The zymogram patterns for nonspecific esterases, lipases, aminopeptidases and acid phosphatases also are shown in Figure 65. Ten bands showing nonspecific esterase activity were found in embryos at stage 19. Two artifact bands which stained purple were seen. This purple stain was also found within the tissues when the histochemical methods were employed.

Sodium taurocholate (0.05 M) at pH 7.4 inhibited esterase-active bands number 1, 4, 5, 6, 7, 8, and 10, whereas lipase-active bands 1, 2, and 3 corresponded to esterase-active bands 2, 3, and 8. Lipase-active band number 4 appeared to be a new band of activity not

demonstrated in the nonspecific esterase zymogram. The 2 purple artifact bands were also present in the lipase zymograms.

Aminopeptidase zymograms demonstrated 2 slowly migrating bands of activity as well as a yellow artifact band. A pale yellow background stain was observed in the tissues using the same histochemical method.

Acid phosphatase zymograms demonstrated only a single band of activity after the embryo had been frozen (-30°C) and thawed repeatedly (5 times). Another acid phosphatase active band usually was found within the stacking gel. A relatively fast migrating yellow artifact band also was present.

The anodal zymograms patterns do not correlate well with the anodal pherogram patterns. Some of the enzymatically active bands coincided with the positions of certain protein bands, while others did not have a stainable protein moiety. Hunter and Maynard (1962) and others similarly found no correlation between esterase zymograms and pherograms in their work.

The ontogeny of nonspecific esterases was followed from pre-diapause to post-diapause stages (Fig. 66). Eleven whole embryos were used per tube with an average stage of 9.2 (stages 8 through 11). Four nonspecific esterase bands were found plus a fast migrating purple artifact band. A slowly migrating greenish-yellow pigment was seen in all of the gels prior to the enzymatic incubation of the gels. Six embryos per tube were homogenized from stages 12 through 14 (tracing #2).

Six bands of nonspecific esterase activity were found using these stages (average stage of 12.9). In older embryos (average stage of 15.2) 8 nonspecific esterase bands were seen (tracing #4). As previously mentioned, embryos of late stage 19 showed 10 active bands. Embryos at stage 24 decreased to 9 active bands and in the late stage 26 embryo, 12 nonspecific esterase bands were observed.

The yolk of the newly-laid egg had 3 bands of nonspecific esterase activity seen within a broad, slowly migrating yellowish-green pigment (Fig. 67). This confirmed the presence of nonspecific esterases within the yolk of the newly-laid egg since the histochemical method for nonspecific esterases showed only a very slight and dispersed enzymatic activity at this stage. The yolk from a stage 10 embryo continued to show 3 active bands (tracing #2). Yolk from eggs at stage 14 showed 5 active bands; stage 17, 7 active bands; stage 18, 8 active bands; stage 19 (incubated at 5°C for 4 months), 9 active bands. The pherogram for yolk of stage 19 showed 10 protein bands with a pattern not directly corresponding with the esterase zymogram (Fig. 67, tracing #7). Yolk not yet engulfed from a stage 24 embryo, demonstrated 9 esterase active bands (Fig. 67, tracing #8). Engulfed yolk from the same egg showed only 6 bands of nonspecific esterase activity in tracing #9.

Various organs from embryos at stage 19 (incubated at 5°C for 4 months) were electrophoresed within 0.92 mm ID microtubes. No protein staining could be detected using 10 pairs of pleuropodia per microtube.

A maximum of 8 nonspecific esterase bands was found using 10 pairs of pleuropodia per microtube (Fig. 68, tracing #3). Four bands of nonspecific esterase were found using a single pair of pleuropodia per microtube (tracing #1). A rapidly migrating purple artifact band was always present.

The serosal membranes from 10 eggs (stage 19) were homogenized for analysis. While yolk was carefully dissected from the membrane, some contamination was inevitable. The zymograms revealed 8 nonspecific esterase bands when the 2.26 mm ID tubes were used (Fig. 68, tracing #4). The serosal membranes from 2 embryos of stage 19 were used with the 0.92 mm ID microtubes, and a total of 10 nonspecific esterase active bands could be seen (tracing #5). A homogenate of the whole proctodeum, the Malpighian tubules and remnants of the PDC membrane from 2 embryos (stage 19) demonstrated 3 slowly migrating nonspecific esterase bands using the microtubes for separation (tracing #6). These tissues showed only a trace of nonspecific esterase and lipase activity using the histochemical methods.

TABLE I. NONSPECIFIC ESTERASE AND LIPASE ACTIVITY* IN EARLY PRE-DIAPAUSE STAGES OF *AULOCARA ELLIOTTI* (THOMAS).

Tissues	Stages									
	NL**	1	2	3	4	5	6	7	8	9
Inside of chorion	-	-	-	-	-	-	NT	-	-	-
Hydropyle cap	+-	+-	+-	+-	+-	+-	NT	+	+	+
Vesicles in yolk	+-	+	+	+	+	+	+	+	+	+
Vitellophages	FO;+-	+-	+-	+	+	+	+	+	++	++
Serosal membrane					FO;+	+	NT	+	++	++
Extra-serosal fluid								FO;NT	-	-
Amniotic membrane					FO;NT	-	NT	NT	-	-
Germ anlage & band	FO;BG	BG	BG	BG	BG	BG	BG	BG	BG	BG
Stomodeum							FO;BG	BG	BG	BG
Proctodeum										FO;BG
First abdominal appendages (pleuropodia)									FO;BG	BG

* Nonspecific esterase activity was demonstrated by using 1-naphthol AS-D acetate with Fast Blue RR salt coupler (from Barka and Anderson, 1965; p, 265). Lipase activity was demonstrated by using the above medium with 0.05 M sodium taurocholate.

** Stage NL designates a newly-laid egg less than 24 hours old.

RELATIVE ENZYMATIC ACTIVITY DESIGNATIONS FOR TABLES I THROUGH XII

- (-) No activity at 1250 X.
- (BG) Background activity at 1250 X.
- (+-) Slight activity, either dispersed or sporadic in occurrence at 500 X.
- (+) Low activity at 500 X.
- (++) Moderate activity at 312.5 X or less.
- (+++)
- (++++)
- (NT) Not tested.
- (FO) First morphological observation of tissue.

TABLE II. NONSPECIFIC ESTERASE AND LIPASE ACTIVITY* IN LATE PRE-DIAPAUSE STAGES OF *AULOCARA ELLIOTTI* (THOMAS).

Tissues	Stages								
	10	11	12	13	14	15	16	17	18
Inside of chorion	-	NT	-	-	-	NT	NT	-	-
Hydropyle cap	+	NT	+	+	+	NT	NT	+	+
Vesicles in yolk	+	+	+	++	++	++	++	++	++
Vitellophages	++	++	++	++	++	++	++	++	+++
Serosal membrane	++	++	++	++	++	++	++	++	+++
Extra-serosal fluid	-	-	-	-	-	-	NT	-	-
Amniotic membrane	NT	-	NT	-	NT	NT	NT	-	-
Provisional dorsal closure membrane							FO;NT	-	-
Protocerebrum	BG	BG	+	++	++	++	++	++	+++
Optic lobes	BG	BG	BG	+	+	+	+	+	+
Deutocerebrum	BG	BG	BG	+	+	++	++	++	++
Tritocerebrum	BG	BG	BG	+	+	++	++	++	++
Subesophageal ganglion	BG	BG	BG	+	+	++	++	++	+++
Thoracic ganglia	BG	BG	BG	BG	+	++	++	++	++
Abdominal ganglia	BG	BG	BG	BG	BG	BG	+	+	+
Gnathal region	BG	BG	BG	+	+	++	++	++	++
Stomodeum	BG	BG	BG	BG	BG	BG	BG	BG	BG
Proctodeum	BG	BG	BG	BG	BG	BG	BG	BG	BG
Malpighian tubules							FO;-	-	-
Pleuropodia	BG	BG	BG	BG	+	+	++	++	+++
Membrane surrounding the epineural sinus								-	-
Abdominal cell clusters	BG	BG	BG	BG	BG	BG	BG	+	+

* See Table I for methods used.

See Table I for relative enzymatic activity designations.

TABLE III. NONSPECIFIC ESTERASE AND LIPASE ACTIVITY* DURING DIAPAUSE DEVELOPMENT OF *AULOCARA ELLIOTTI* (THOMAS).

Tissues	Stages					
	18	19a	19b	20	19c	19d
Inside of chorion	-	-	-	-	-	-
Hydropyle cap	+	-	-	++	+-	+-
Vesicles in yolk	++	+-	++	++++	++	+++
Vitellophages	+++	++	++++	++++	+++	++
Serosal membrane	+++	+	++++	++++	++	+-
Extra-serosal fluid	-	-	-	-	NT	-
Amniotic membrane	-	NT	-	-	NT	-
Provisional dorsal closure membrane	-	-	-	-	-	-
Proctocerebrum	+++	+	+++	+++	++	+++
Optic lobes	+	+	+	+	+	+
Deutocerebrum	++	+	++	++	++	++
Tritocerebrum	++	+	++	++	++	++
Subesophageal ganglion	+++	+	++++	+++	+++	+++
Thoracic ganglia	++	+	++	+++	++	+-
Abdominal ganglia	+	+	+	++	+	+
Gnathal region	++	++	++	++	++	+-
Stomodaeum	BG	BG	BG	BG	BG	BG
Proctodaeum	BG	BG	BG	BG	BG	BG
Malpighian tubules	-	-	-	-	-	-
Pleuropodia	+++	+++	++++	++	++	++
Membrane surrounding the epineural sinus	-	-	-	-	-	-
Abdominal cell clusters	+	+	++	++	+++	+

* See Table I for methods used.

See Table I for relative enzymatic activity designations.

Stage 19a, diapause, exposed to 5°C temperature for 15 days.

Stage 19b, diapause, exposed to 5°C temperature for 4 months.

Stage 20, diapause termination, eggs were exposed to 5°C for 4 months then removed to room temperature for at least 3 hours.

Stage 19c, diapause, remained at room temperature for 2 weeks.

Stage 19d, diapause, remained at room temperature for 4 months.

TABLE IV. NONSPECIFIC ESTERASE AND LIPASE ACTIVITY* IN POST-DIAPAUSE STAGES OF *AULOCARA ELLIOTTI* (THOMAS).

Tissues	Stages							
	20	21	22	23	24	25	26	27
Inside of chorion	-	-	NT	-	-	-	-	-
Hydropyle cap	++	++	NT	++	+	+	+	+-
Vesicles in yolk	++++	+++	NT	++	NT	+++*	+++*	+++*
Vitellophages	++++	++++	NT	++++	NT	+++*	+++*	+++*
Serosal membrane	++++	NT	NT	++++	++++	++++**	++++**	++++**
Extra-serosal fluid	-	NT	NT	-	NT	+-	+	+
Amniotic membrane	-	NT	NT	-	-	-	Not found	
Provisional dorsal closure membrane	-	-	NT	-	-	Closure completed		
Membrane surrounding the epineural sinus	-	NT	NT	-	NT	-	-	-
Protocerebrum	+++	+++	NT	+++	+++	++++	+++	++++
Optic lobes	+	+	NT	++	++	++	++	++
Deutocerebrum	++	++	NT	++	++	++	+++	+++
Tritocerebrum	++	++	NT	++	++	++	+++	+++
Subesophageal ganglion	+++	+++	NT	+++	+++	+++	+++	+++
Thoracic ganglia	+++	+++	NT	+++	+++	+++	+++	+++
Abdominal ganglia	++	++	NT	NT	++	++	+++	+++
Stomodaeum	BG	BG	BG	BG	BG	BG	++	++
Gastric caeca					FO;BG	++++	++++	++++
Midgut				FO;BG	+	++	++	++
Proctodeum	BG	BG	NT	BG	+	+++	+++	+++
Malpighian tubules	-	-	NT	-	-	+-	++	++++
Rectal pads	BG	BG	NT	BG	+	++	++++	++++
Pleuropodia	++	++	NT	++	+++	++++	++++	++++
Abdominal cell clusters	++	++	NT	++	++	+++	+++	++
Hypodermis	BG	BG	BG	BG	BG	BG	BG	BG
Exuviae							FO;-	-
Hemocytes	NT	NT	NT	NT	NT	++	+++	+++
Tracheae							-	-

* See Table I for methods used.

** Yolk was removed from the midgut.

See Table I for relative enzymatic activity designations.

TABLE V. ACID PHOSPHATASE ACTIVITY* IN EARLY PRE-DIAPAUSE STAGES OF *AULOCARA ELLIOTTI* (THOMAS).

Tissues	Stages									
	NL	1	2	3	4	5	6	7	8	9
Inside of chorion	-	-	NT	NT	-	-	NT	NT	-	-
Hydropyle cap	+	+	NT	NT	+	+	NT	NT	+	+
Vesicles in yolk	-	-	+-	NT	+-	+-	NT	+-	+-	+-
Vitellophages		FO;-	-	NT	-	-	NT	-	+-	+
Serosal membrane					FO;-	-	NT	-	-	-
Extra-serosal fluid								FO;-	-	-
Amniotic membrane					FO;NT	NT	NT	NT	NT	-
Germ band, cephalad			FO;-	NT	-	-	+-	+	++	++
Germ band, caudad			FO;-	NT	-	-	-	-	-	-
Stomodeum							FO;-	-	-	-
Gnathal region					-	-	+++	+++	+++	++
Tip of abdomen					-	-	++	+++	+	+
Proctodeum										FO;-
First abdominal appendages (Pleuropodia)									FO;-	-

* Acid phosphatase activity was demonstrated by using substrate 1-naphthol AS-TR phosphate with pararosanilin coupler (Barka and Anderson, 1965, p, 245).

See Table I for relative enzymatic activity designations.

TABLE VI. ACID PHOSPHATASE ACTIVITY* IN LATE PRE-DIAPAUSE STAGES OF *AULOCARA ELLIOTTI* (THOMAS).

Tissues	Stages								
	10	11	12	13	14	15	16	17	18
Inside of chorion	-	NT	NT	-	-	NT	NT	-	-
Hydropyle cap	+	NT	NT	+	+	NT	NT	+	+
Vesicles in yolk	+-	+-	+-	+-	NT	+-	NT	+-	+-
Vitellophages	+	+	+	++	NT	++	NT	++	++
Serosal membrane	NT	-	+-	+	NT	++	NT	++	++
Extra-serosal fluid	NT	-	-	-	NT	-	NT	NT	-
Amniotic membrane	NT	NT	-	-	+-	NT	NT	+-	+-
Provisional dorsal closure membrane							FO;NT	-	-
Membrane surrounding the epineural sinus	NT	NT	NT	NT	NT	-	NT	-	-
Brain & optic lobes	++	++	++++	++++	+++	++	NT	+++	+++
Ventral ganglia	+	++	++	++	++	++	NT	+++	+++
Abdominal cell clusters	+	+	++	++++	+++	+++	NT	+++	+++
Dorsal margin of abdominal wall	-	-	-	+	+	+	NT	++	+++
Stomodeum	-	-	-	-	-	-	NT	-	-
Gnathal region	++	+++	++++	++++	++	++	NT	++	++
Tip of abdomen	+	+	++	++++	+++	++	NT	++	++
Ingluvial ganglia							FO;NT	+-	+
Proctodeum	-	-	-	-	-	-	NT	+	+
Malpighian tubules							FO;NT	+-	+
Pleuropodia	-	NT	-	-	-	+-	NT	+	++

* See Table V for method used.

See Table I for relative enzymatic activity designations.

TABLE VII. ACID PHOSPHATASE ACTIVITY* DURING DIAPAUSE DEVELOPMENT OF *AULOCARA ELLIOTTI* (THOMAS).

Tissues	Stages					
	18	19a	19b	20	19c	19d
Inside of chorion	-	-	-	-	-	-
Hydropyle cap	+	++	+++	++	++	+
Vesicles in yolk	+-	++	+	+	++	+
Vitellophages	++	++	++	++	++	+
Extra-serosal fluid	-	+	-	-	-	-
Serosal membrane	++	++	+-	++++	++	+
Provisional dorsal closure membrane	-	-	-	-	-	-
Amniotic membrane	+-	-	-	+	-	-
Membrane surrounding the epineural sinus	-	-	-	-	-	-
Brain & optic lobes	+++	+++	++	++++	+++	++
Ventral ganglia	+++	+++	++	+++	+++	++
Abdominal cell clusters	+++	+++	+++	++	+++	+
Dorsal margin of abdominal wall	+++	+++	+++	++	+++	+
Stomodeum	-	-	-	-	-	-
Gnathal region	++	++	+	+++	++	+
Ingluvial ganglia	+	+	+	++	+	+
Proctodeum	+	+	+-	+-	+	-
Anal margin of rectum	++	+++	++	++	+++	+
Malpighian tubules	+	+-	-	+	+	+-
Pleuropodia	++	+	-	++	+++	+

* See Table V for method used.

See Table I for relative enzymatic activity designations.

Stage 19a, diapause, exposed to 5°C temperature for 2 weeks.

Stage 19b, diapause, exposed to 5°C temperature for 4 months.

Stage 20, diapause termination, eggs were exposed to 5°C for 4 months then removed to room temperature for at least 3 hours.

Stage 19c, diapause, pods remained at room temperature for 2 weeks.

Stage 19d, diapause, pods remained at room temperature for 4 months.

TABLE VIII. ACID PHOSPHATASE ACTIVITY* IN POST-DIAPAUSE DEVELOPMENT OF *AULOCARA ELLIOTTI* (THOMAS).

Tissues	Stages							
	20	21	22	23	24	25	26	27
Inside of chorion	-	NT	NT	-	NT	-	-	-
Hydropyle cap	++	NT	NT	+-	NT	+-	+-	+-
Vesicles in yolk	+	NT	NT	+++	+++	+++**	+++**	+++**
Vitellophages	++	NT	NT	+++	+++	+++**	+++**	+++**
Extra-serosal fluid	-	NT	NT	-	NT	-	-	+
Serosal membrane	++++	NT	NT	++++	++++	++++	no longer present	
Provisional dorsal closure membrane	-	NT	NT	-	-	Closure completed		
Amniotic membrane	+	NT	NT	NT	-	Not found		
Membrane surrounding the epineural sinus	-	NT	NT	NT	NT	+-	++	++
Brain & optic lobes	++++	NT	NT	++++	++++	++++	++++	++++
Ventral ganglia	+++	NT	NT	+++	+++	+++	+++	+++
Abdominal cell clusters	++	NT	NT	++	++	++	++	++
Dorsal margin of abdominal wall	++	NT	NT	+++	+++	+++	+++	+++
Stomodeum	-	NT	NT	-	-	+	++	++
Gastric caeca					FO;-	+-	+++	+++
Ingluvial ganglia	++	NT	NT	++	++	++	++	++
Gnathal region	+++	NT	NT	+++	+++	+++	+++	+++
Midgut					FO:+-	++	+++	+++
Proctodeum	+-	NT	NT	-	-	+	+++	+++
Rectal pads						FO:++	+++	++++
Anal margin of rectum	++	NT	NT	++	++	+++	++++	++++
Malpighian tubules	+	NT	NT	+	+	+	++	++
Pleuropodia	++	NT	NT	+	+	+	+++	+
Hemocytes	NT	NT	NT	NT	NT	NT	++	++

* See Table V for method used.

** Yolk removed from midgut.

See Table I for relative enzymatic activity designations.

TABLE IX. AMINOPEPTIDASE ACTIVITY* IN EARLY PRE-DIAPAUSE STAGES OF *AULOCARA ELLIOTTI* (THOMAS).

Tissues	Stages									
	NL	1	2	3	4	5	6	7	8	9
Inside of chorion	-	-	NT	-	NT	-	-	-	-	-
Hydropyle cap	-	-	NT	-	NT	-	-	-	-	-
Yolk	-	-	NT	-	-	-	-	-	-	-
Vitellogophages		FO;-	NT	-	-	-	-	-	-	-
Extra-serosal fluid								FO;-	-	-
Serosal membrane					FO;NT	-	-	-	-	-
Amniotic membrane					FO;NT	NT	NT	NT	-	-
Germ anlage & band		FO;-	NT	-	-	-	-	-	-	-
Brain region					-	-	-	-	-	-
Ventral ganglia region					-	-	-	-	-	-
Gnathal region (subesophageal body cells)								FO;+-	+	++++
Tip of abdomen					-	-	-	+-	+	+++
Stomodeum							FO;-	-	-	-
Proctodeum										FO;-
First abdominal appendages (Pleuropodia)									FO;-	-

* Aminopeptidase activity was demonstrated by using L-leucyl-4-methoxy-2-naphthylamide with Fast Blue B salt coupler (from Barka and Anderson, 1965, p, 288).

See Table I for relative enzymatic activity designations.

TABLE X. AMINOPEPTIDASE ACTIVITY* IN LATE PRE-DIAPAUSE STAGES OF *AULOCARA ELLIOTTI* (THOMAS).

Tissues	Stages								
	10	11	12	13	14	15	16	17	18
Inside of chorion	-	NT	NT	-	-	-	-	-	-
Hydropyle cap	-	NT	NT	-	-	-	-	-	-
Yolk	-	NT	NT	-	-	-	-	-	-
Vitellophages	-	NT	NT	-	-	-	-	-	-
Extra-serosal fluid	-	NT	NT	-	-	-	NT	NT	-
Serosal membrane	+	NT	NT	NT	+	+	++	NT	++
Amniotic membrane	NT	NT	NT	NT	-	-	NT	NT	-
Provisional dorsal closure membrane							FO;-	-	-
Protocerebrum	-	NT	NT	++	+	++	++	++	+++
Optic lobes	-	NT	NT	++	+	+	+	+	++
Subesophageal ganglion	NT	NT	NT	++	NT	+	NT	NT	++
Gnathal region (subesophageal body cells)	++++	NT	NT	++++	++++	++++	++++	++++	++++
Ventral ganglia	-	NT	NT	-	-	-	-	-	+
Thoracic cell clusters	-	NT	NT	+	+	+	+	+	++
Abdominal cell clusters	-	NT	NT	-	-	-	-	+	++
Tip of abdomen	+++	NT	NT	+	+	+	+	+	+
Cerci	-	NT	NT	++	++	++++	++++	++++	++++
Dorsal margin of abdominal wall	-	NT	NT	-	+	+	+	+	++
Pleuropodia	-	NT	NT	-	-	-	-	+-	+
Stomodeum	-	NT	NT	-	-	-	-	-	-
Proctodeum	-	NT	NT	-	-	-	-	-	-
Malpighian tubules							FO;-	-	-

* See Table IX for method used.

See Table I for relative enzymatic activity designations.

TABLE XI. AMINOPEPTIDASE ACTIVITY* DURING DIAPAUSE DEVELOPMENT OF *AULOCARA ELLIOTTI* (THOMAS).

Tissues	Stages						
	18	19a	19b	20	19c	19d	19e
Inside of chorion	-	-	-	-	-	-	-
Hydropyle cap	-	-	-	-	-	-	-
Yolk	-	-	-	-	-	-	-
Vitellophages	-	-	-	-	-	-	-
Extra-serosal fluid	-	-	-	-	-	-	-
Serosal membrane	++	-	++	+++	+	-	-
Amniotic membrane	-	-	-	-	-	-	-
Provisional dorsal closure membrane	-	-	-	-	-	-	-
Protocerebrum	+++	++	+	+	+++	+	++
Optic lobes	++	++	+	+	++	+	-
Subesophageal ganglion	++	-	-	-	++	++	++
Gnathal region (subesophageal body cells)	++++	+++	+++	+++	+++	++	++
Ventral ganglia	+	+	+	+	+	+	+
Thoracic cell clusters	++	++	+	+	+	++	++
Abdominal cell clusters	++	+	+	+	++	+	+
Tip of abdomen	+	+	+	+	+	+	+
Cerci	++++	+++	+++	+++	++++	+++	++
Dorsal margin of abdominal wall	++	++	++	++	++	++	++
Pleuropodia**	+	+++	++	+	++	+	++
Stomodeum	-	-	-	-	-	-	-
Proctodeum	-	-	-	-	-	-	-
Malpighian tubules	-	-	-	-	-	-	-

* See Table IX for method used.

** Wide variation in activity was found, see result section for a more complete description.

Stage 19a, diapause, exposed to 5°C temperature for 28 days.

Stage 19b, diapause, exposed to 5°C temperature for 4 months.

Stage 20, diapause termination, eggs were exposed to 5°C for 4 months then removed to room temperature for at least 3 hours.

Stages 19c, 19d, and 19e; diapause, remained at room temperature for 2 weeks, 1 month, and 4 months, respectively.

TABLE XII. AMINOPEPTIDASE ACTIVITY* IN POST-DIAPAUSE STAGES OF
AULOCARA ELLIOTTI (THOMAS).

Tissues	Stages							
	20	21	22	23	24	25	26	27
Inside of chorion	-	-	NT	-	NT	-	-	-
Hydropyle cap	-	-	NT	+	NT	++	++	+++
Yolk	-	-	NT	-	NT	***	***	***
Vitellophages	-	-	NT	-	NT	+++*	++++*	++++*
Extra-serosal fluid	-	-	NT	-	NT	NT	+	++
Serosal membrane	+++	+++	NT	+++	NT	+++	No longer present	
Amniotic membrane	-	NT	NT	+	NT	Not found		
Provisional dorsal closure membrane	-	-	NT	-	NT	Closure completed		
Protocerebrum	+	-	NT	+++	NT	+++	++++	++++
Optic lobes	+	-	NT	+++	NT	+++	+++	+++
Subesophageal ganglion	-	-	NT	+++	NT	+++	+++	+++
Gnathal region (subesophageal body cells)	+++	+++	NT	+++	NT	+++	+++	++++
Ventral ganglia	+	++	NT	++	NT	++	++	++
Thoracic cell clusters	+	+	NT	+	NT	+	+	+
Abdominal cell clusters	+	-	NT	-	NT	-	+	+
Tip of abdomen	+	+	NT	+	NT	+	+	+
Cerci	+++	+++	NT	++	NT	++	++	+++
Dorsal margin of abdominal wall	++	++	NT	++	NT	+++	+++	+++
Pleuropodia	+-	-	NT	++	NT	++++	++	+++
Membrane surrounding the epineural sinus	NT	NT	NT	-	NT	+-	+	+-
Stomodaeal-midgut junction	-	-	NT	-	NT	-	++	+++
Gastric caeca					FO;+	+	+	+++
Midgut					FO;NT	-	+-	++
Proctodeal-midgut junction	-	-	NT	-	NT	++	++++	++++
Malpighian tubules	-	-	NT	-	NT	-	+-	++
Rectum	-	-	NT	-	NT	+-	+	+++
Rectal pads	-	-	NT	-	NT	+++	+++	+++
Hemocytes	NT	NT	NT	NT	NT	+	+	+

* See Table IX for method used.

** Yolk removed from midgut.

See Table I for relative enzymatic activity designations.

TABLE XIII. PROTEASE ACTIVITY* DURING THE EMBRYOGENESIS OF
AULOCARA ELLIOTTI (THOMAS).

Tissues	Stages											
	NL	1	2	3	7	8	9	12	13	14	16	17
Yolk	+	++	++	++	++	++	++	++	++	++	++	+++
Embryo		-	-	-	+-	-	-	+	+	-	+	+

Tissues	Stages											
	18	19a	19b	20	19c	19d	21	25	26	27		
Inside of chorion	-	-	-	-	-	-	-	-	-	+	+++	
Extra-serosal fluid		+-	-	-	-	+-	+	+	++	++	+++	
Yolk		+++	++	+	+++	+	+	++	+++	+++	+++	
Serosal membrane		+	++	+++	+++	+	+	++	+++	No longer present		
Provisional dorsal closure membrane		-	-	-	-	-	-	-	Closure completed			
Stomodeum		-	-	-	-	-	-	-	-	-	-	
Proctodeum		-	-	-	-	-	-	-	-	+	++	
Malpighian tubules		-	-	-	-	-	-	-	-	+-	+	
Abdominal wall		+	+	+	+	+	+	+	+	+	+	
Hypodermis		NT	NT	NT	NT	NT	NT	NT	+-	-	-	
Exuviae									FO;NT	++++	++++	
Hemolymph		NT	NT	NT	NT	NT	NT	NT	NT	++	++	
Pleuropodia		+	+	++	++	+	+	+	+	++	++++	

* Protease activity was demonstrated by the substrate film digestion method of Adams and Tuqan (1961).

** Yolk removed from midgut.

Stage 19a, diapause, at 5°C for 2 weeks.

Stage 19b, diapause, at 5°C for 4 months.

Stage 20 eggs were in the cold at 5°C for 4 months then removed to room temperature for at least 3 hours.

Stage 19c, diapause, pods remained at room temperature for 2 weeks.

Stage 19d, diapause, pods remained at room temperature for 4 months.

RELATIVE PROTEASE ACTIVITY DESIGNATIONS

- (-) No activity at 200 X.
- (+-) Slight activity, present in some tissues but not in others at 200 X.
- (+) Slight activity at 200 X.
- (++) Moderate activity at 200 X.
- (+++)
- (++++)

TABLE XIV. CONTROL SLIDES FOR HISTOCHEMICAL METHODS.

Tissues from stages demonstrating high enzymatic activity were used for inactivation and inhibition control tests e.g., stages 19, 26 and 27.

PART 1. Duration of pre-incubation treatments which completely inactivated the following enzymes as demonstrated by the standard histochemical methods used in this study. The inactivated tissues were incubated together with active tissues.

TREATMENTS	ENZYMES			
	ESTERASES- LIPASES	ACID PHOSPHATASES	AMINO- PEPTIDASES	PROTEASES
Altmann's II fixative* at 25°C	2 hours	15 min.	15 min.	Used 50% formalin in place of buffer
95% ethanol or 100% methanol, 25°C	2 min.	30 sec.	30 sec.	1 min.
100% acetone at 5°C	30 sec.	15 min.	10 min.	NT
Boiled in dist. water	15 min.	10 min.	10 min.	10 min.
Dry heat at 120°C	1 hour	30 min.	30 min.	1 hour

PART 2. Final concentration of salts in incubation media which gave complete inhibition of enzymatic activity or nearly so. A five minute pre-incubation soak in the salt solutions of the same respective concentrations was used. No rinse followed this soaking.

SALTS TESTED	ENZYMES			
	ESTERASES- LIPASES	ACID PHOSPHATASES	AMINO- PEPTIDASES	PROTEASES
NaF	0.01 M**	0.01 M**	NT	NT
CuSO ₄	0.005 M	0.003 M	0.002 M	0.005 M
ZnCl ₂	0.005 M	0.003 M	0.002 M	0.005 M
HgCl ₂	0.005 M	0.003 M	0.002 M	0.005 M
KCN	NT	NT	0.03 M	0.03 M

NT Not tested.

* Gretchen L. Humason, Animal Tissue Techniques; 1967; p. 17.
W. H. Freeman and Co., San Francisco.

** Slight activity was still found in the most active tissues.

PLATE I-VI

- Figure 1. Stage 25, inside of the hydropyle cap showing a moderate (++) amount of aminopeptidase activity (LMNA with FB-B). Not all dark areas denote activity. 240 X.
- Figure 2. Stage 25, vitelloghage nucleus showing a negative (-) reaction of nonspecific esterase activity (NAS-DA with FB-RR). 1480 X.
- Figure 3. Stage NL (newly-laid), yolk showing a slight (+-) level of lipase activity (NAS-DA with FB-RR and Na T). 150 X.
- Figure 4. Stage NL (newly-laid), yolk showing a low (+) level of protease activity (Ag-G). 240 X.
- Figure 5. Stage 3, yolk showing a high (+++) level of protease activity (Ag-G). 95 X.
- Figure 6. Stage 12, yolk showing a high (+++) level of protease activity (Ag-G). 95 X.
- Figure 7. Stage 18, yolk control slide, 50% formalin fixation for 1 hour at 25°C. Protease activity is negative (-) (Ag-G). 240 X.
- Figure 8. Stage 19 (4 months at 5°C), yolk showing a moderate (++) level of lipase activity (NAS-DA with FB-RR and Na T). 150 X.

Symbol designations for Plates I through VIII.

Nonspecific esterases:

NAS-DA, 1-naphthol AS-D acetate
FB-RR, Fast Blue RR salt

Aminopeptidases:

LMNA, L-leucyl-4-methoxy-2-naphthyl amide
FB-B, Fast Blue B salt

Lipases:

NAS-DA, 1-naphthol AS-D acetate
FB-RR, Fast Blue RR salt
Na T, Sodium taurocholate 0.05 M

Proteases:

Ag-G, Silver - gelatin substrate film

Acid phosphatases:

NAS-TRP, 1-naphthol AS-TR phosphate
p-R, Pararosanilin dye

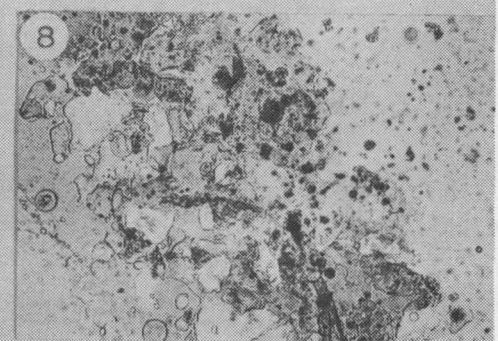
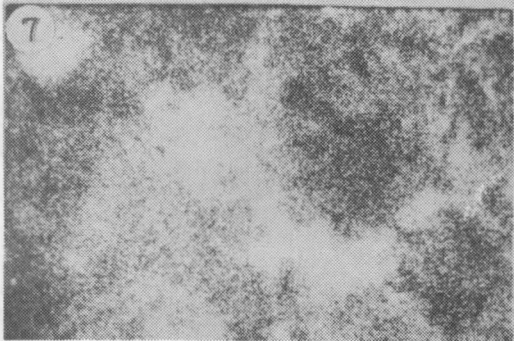
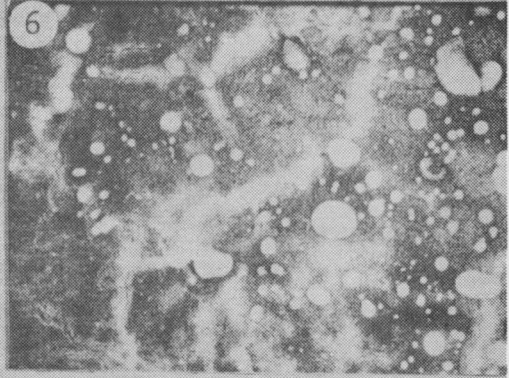
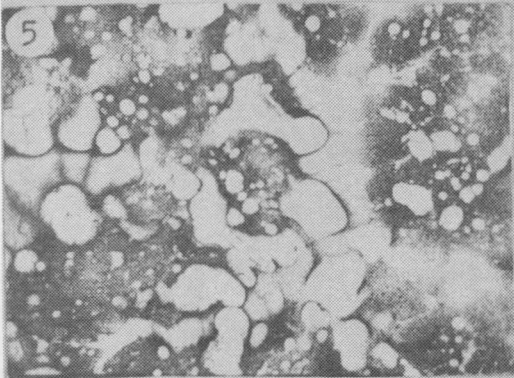
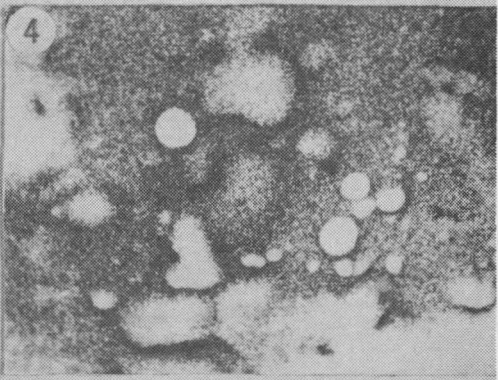
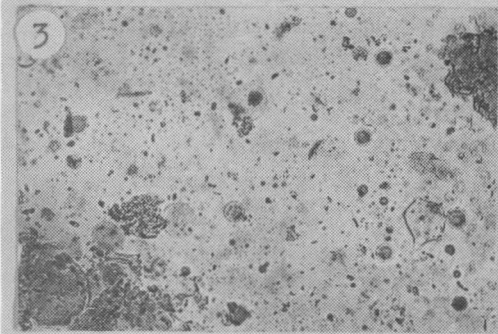
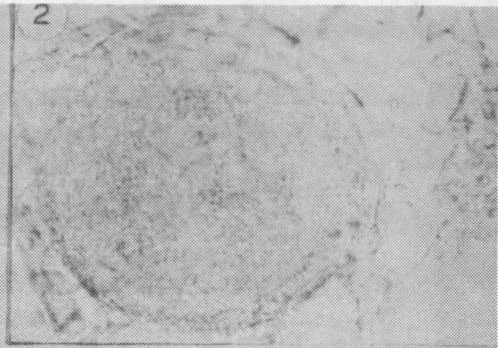
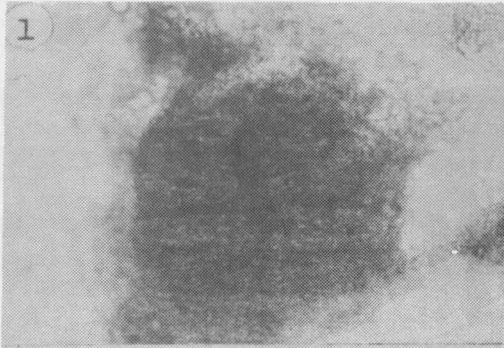


PLATE II

- Figure 9. Stage 25, yolk from midgut showing a moderate (++) amount of nonspecific esterase activity (NAS-DA with FB-RR). 240 X. See arrow.
- Figure 10. Stage 25, extra-embryonic fluid showing a slight (+-) amount of nonspecific esterase activity (NAS-DA with FB-RR). 590 X. See arrow.
- Figure 11. Stage 26, extra-embryonic fluid showing a moderate (++) level of protease activity (Ag-G). 150 X.
- Figure 12. Stage 27 (late), extra-embryonic fluid showing a high (+++) level of protease activity (Ag-G). 150 X.
- Figure 13. Stage 18, serosal membrane showing a moderate (++) level of aminopeptidase activity in the cytoplasm (LMNA with FB-B). 1480 X.
- Figure 14. Stage 19 (at 5°C for 4 months), serosal membrane showing a mosaic pattern of nonspecific esterase activity from an intense (+++++) reaction to a moderate (++) reaction (NAS-DA with FB-RR). 240 X.
- Figure 15. Stage 19 (at 5°C for 4 months), serosal membrane showing a moderate (++) level of nonspecific esterase activity (NAS-DA with FB-RR). 590 X. Note that most of the activity is located near the nuclear margin.
- Figure 16. Stage 19 (at 25°C for 4 months), serosal membrane showing a slight (+-) amount of acid phosphatase activity (NAS-TRP with p-R). 240 X.

See Plate I for symbol designations.

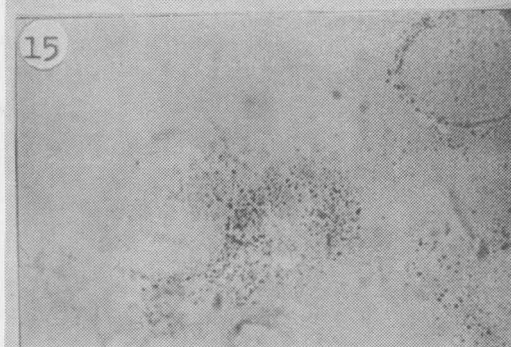
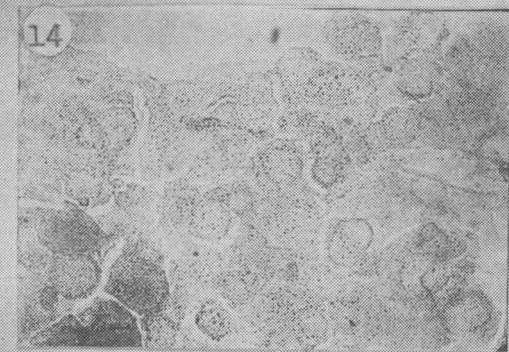
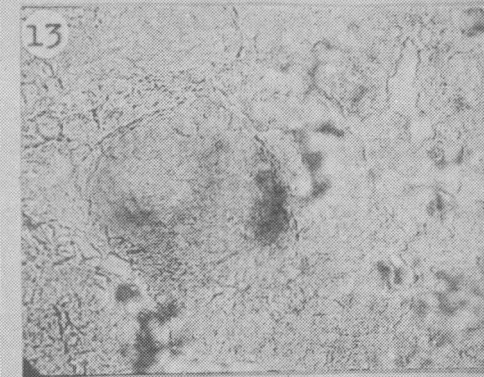
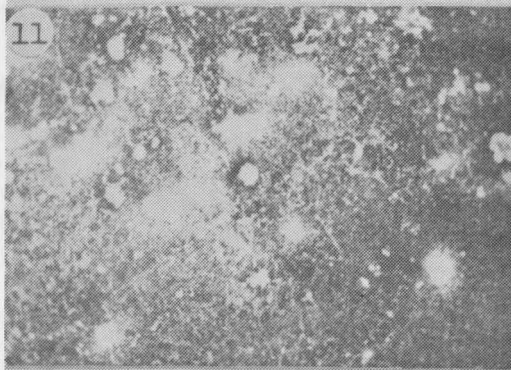
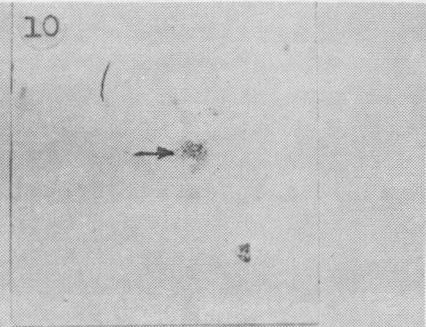
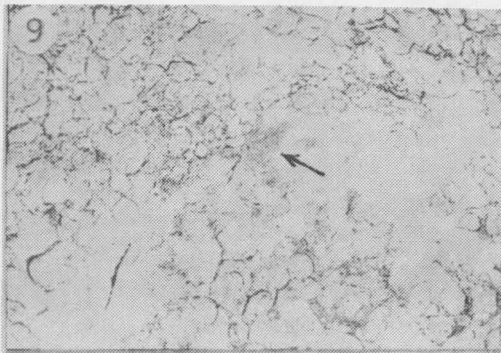


PLATE IIB⁸-

- Figure 17. Stage 24, serosal membrane showing a moderate (++) level of lipase activity (NAS-DA with FB-RR and Na T). 590 X. Note that most of the activity is located near the nuclear margin.
- Figure 18. Stage 20 (at 5°C for 4 months, then warmed to 25°C for at least 3 hours), serosal membrane showing an intense (++++) reaction for acid phosphatase activity (NAS-TRP with p-R). 240 X. Note that nuclei do not show acid phosphatase activity.
- Figure 19. Stage 25, serosal membrane removed from the midgut yolk showing a mosaic pattern of a high (+++) level of aminopeptidase activity (LMNA with FB-B). 370 X. See arrow.
- Figure 20. Stage 18, amniotic membrane showing a slight (+-) level of acid phosphatase activity (NAS-TRP with p-R). 590 X.
- Figure 21. Stage 19 (at 5°C for 4 months), provisional dorsal closure membrane showing a negative (-) reaction for nonspecific esterase activity (NAS-DA with FB-RR). 590 X.
- Figure 22. Stage 1, embryonic disc showing a background (BG) level of nonspecific esterase activity (NAS-DA with FB-RR). 1480 X. See arrows.
- Figure 23. Stage 13, embryonic head region showing an intense (++++) level of aminopeptidase activity in gnathal region (LMNA with FB-B). 95 X.
- Figure 24. Stage 13, embryonic head showing an intense (++++) level of aminopeptidase activity in the gnathal region (LMNA with FB-B). 240 X. Same as Figure 23 but with higher magnification.

See Plate I for symbol designations.

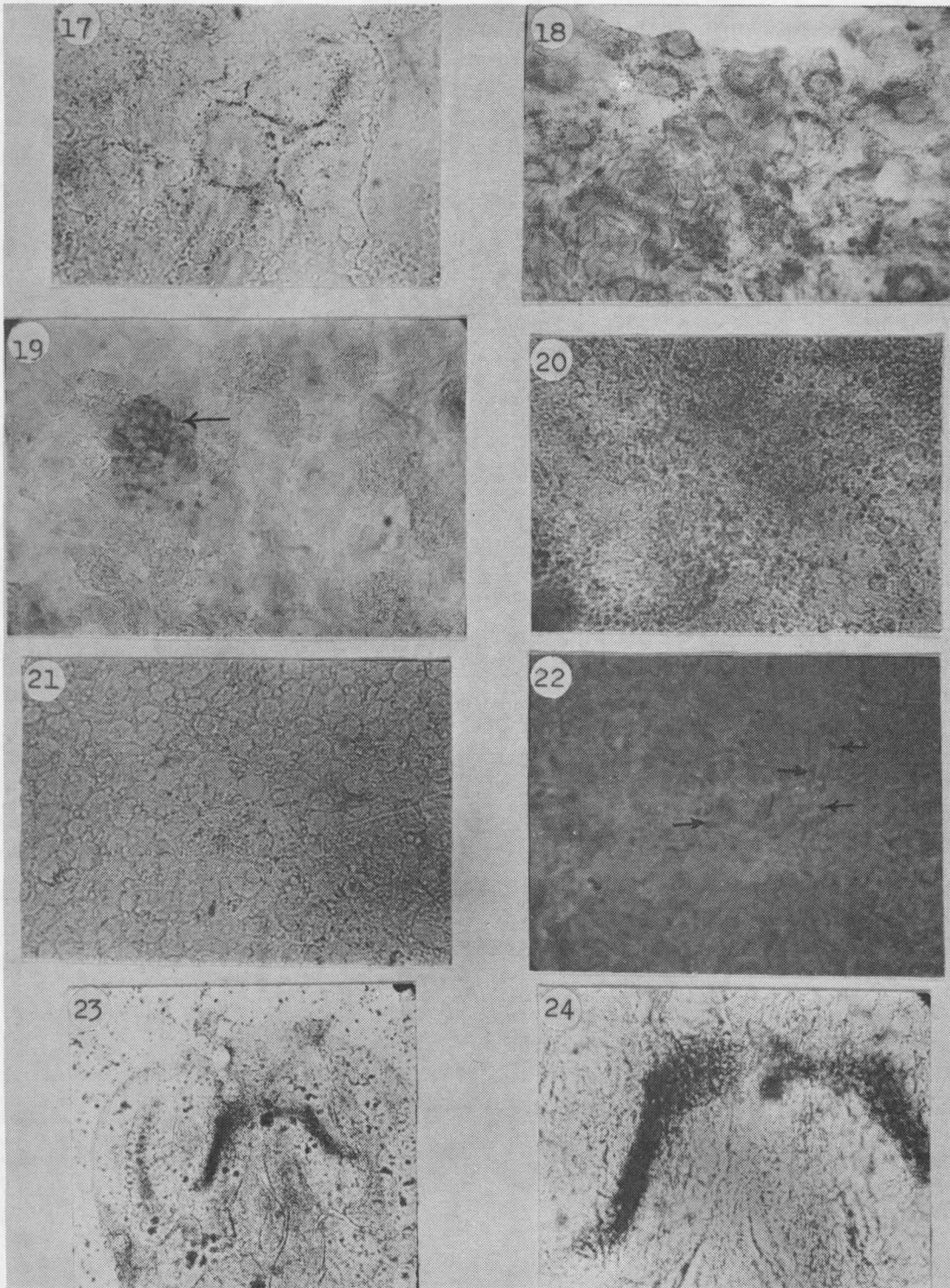


PLATE IV

- Figure 25. Stage 18, cerci showing an intense (++++ level of aminopeptidase activity (LMNA with FB-B). 240 X.
- Figure 26. Stage 19 (at 25°C for 2 weeks), protocerebrum showing a moderate (++) level of nonspecific esterase activity, particularly within the median lobes (pars intercerebralis) (NAS-DA with FB-RR). 95 X. See arrows.
- Figure 27. Stage 19 (at 5°C for 4 months), subesophageal ganglion showing an intense (++++ reaction for nonspecific esterase activity (NAS-DA with FB-RR). 150 X. See arrows.
- Figure 28. Stage 25, second and third thoracic ganglia showing a high (+++) level of nonspecific esterase activity within the neuropyle (NAS-DA with FB-RR). 240 X.
- Figure 29. Stage 20 (at 5°C for 4 months, then removed to 25°C for at least 3 hours), brain region showing an intense (++++ amount of acid phosphatase activity within the pars intercerebralis. The optic lobes show a high (+++) level of activity (NAS-TRP with p-R). 95 X.
- Figure 30. Stage 20 (at 5°C for 4 months, then removed to 25°C for at least 3 hours), three thoracic ganglia showing a high (+++) level of acid phosphatase activity (NAS-TRP with p-R). 150 X.
- Figure 31. Stage 20 (as above), abdominal wall containing dense crystalline deposits, probably uric acid. There is no association of acid phosphatase activity with these deposits. Most mesodermal cells show a moderate (++) acid phosphatase activity (NAS-TRP with p-R). 95 X.
- Figure 32. Stage 23, dorsal margin of abdominal wall showing a moderate (++) level of aminopeptidase activity (LMNA with FB-B). 240 X. See arrow. (A) is an artifact.

See Plate I for symbol designations.

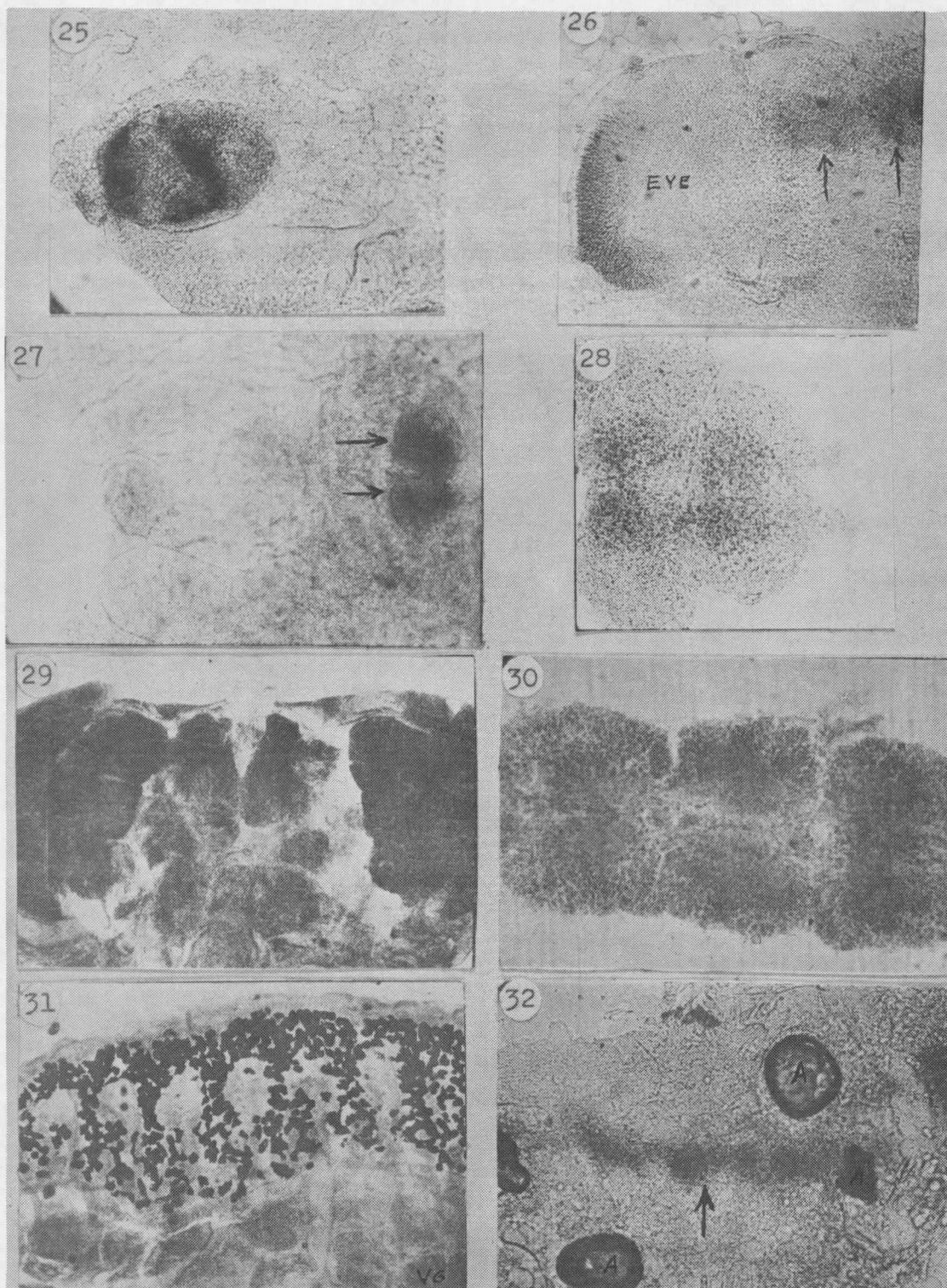


PLATE V. ca-

- Figure 33. Stage 27, membrane surrounding the epineural sinus showing a negative (-) reaction for nonspecific esterase activity (NAS-DA with FB-RR). 150 X.
- Figure 34. Stage 26, membrane surrounding the epineural sinus containing dense crystalline material, probably uric acid. The membrane shows a moderate (++) level of acid phosphatase activity (NAS-TRP with p-R). 370 X. See arrows.
- Figure 35. Stage 26, gastric caecal buds (GC) extending from the stomodeum (S) showing an intense (++++) level of nonspecific esterase activity (NAS-DA with FB-RR). 240 X.
- Figure 36. Stage 26, stomodeal (S) -midgut (M) junction showing a ring of moderate (++) aminopeptidase activity (LMNA with FB-B). 240 X.
- Figure 37. Stage 25, proctodeal-midgut junction showing a high (+++) level of nonspecific esterase activity within the proctodeum (P) and a moderate (++) level of activity in the midgut (M). The Malpighian tubules (MT) show only a slight (+) level of activity (NAS-DA with FB-RR). 150 X.
- Figure 38. Stage 27, proctodeal (P) -midgut (M) junction showing an intense (++++) level of acid phosphatase activity at the origins of the Malpighian tubule evaginations. Also note the ribbons of intense (++++) acid phosphatase activity lying longitudinally along the proctodeum. The Malpighian tubules on the right side of the photomicrograph show a moderate (++) amount of acid phosphatase activity (NAS-TRP with p-R). 150 X.
- Figure 39. Stage 25, one-half of proctodeum with three longitudinal bands of high (+++) amounts of aminopeptidase activity. The Malpighian tubules show no (-) aminopeptidase activity (LMNA with FB-B). 150 X.
- Figure 40. Stage 26, proctodeal (P) -midgut (M) junction showing a ring of intense (++++) aminopeptidase activity. Malpighian tubules (MT) show a negative (-) reaction for aminopeptidase activity (LMNA with FB-B). 150 X.

See Plate I for symbol designations.

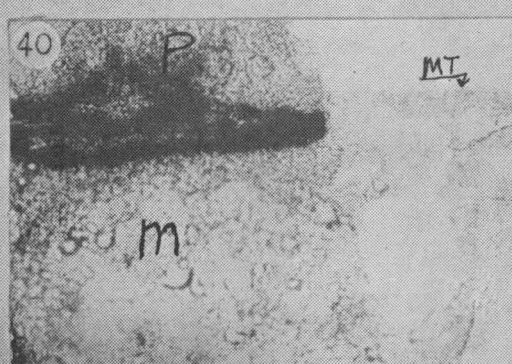
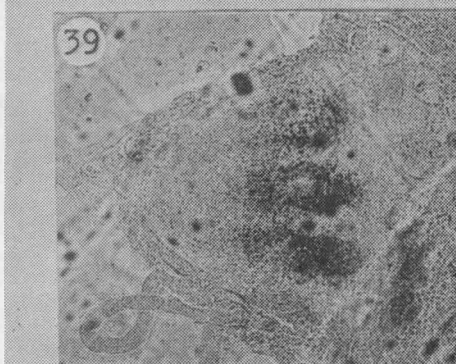
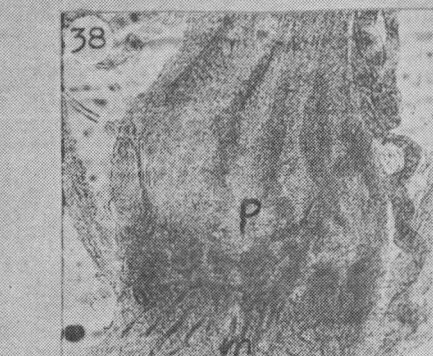
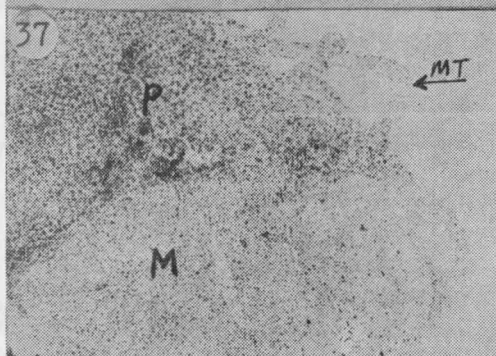
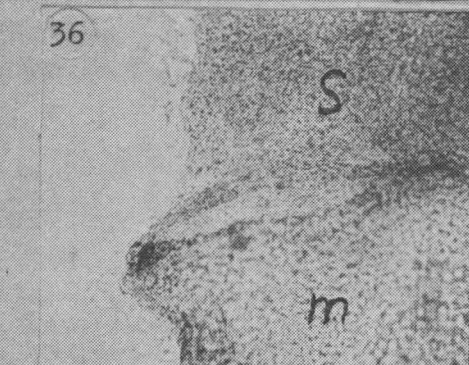
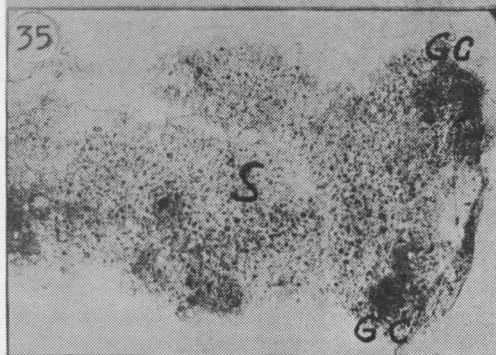
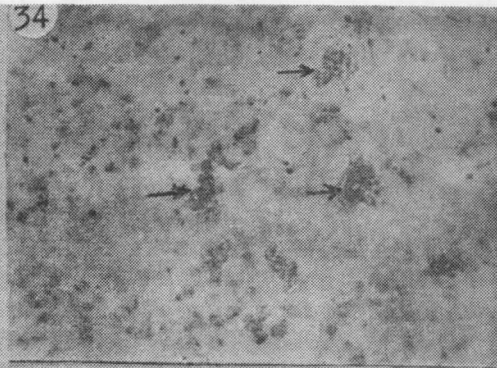
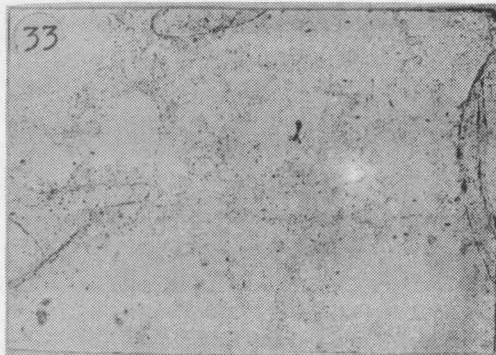


PLATE VI 78-

- Figure 41. Stage 26, Malpighian tubules showing a moderate (++) level of lipase activity (NAS-DA with FB-RR and Na T). 240 X.
- Figure 42. Stage 27, Malpighian tubules showing an intense (++++ level of nonspecific esterase activity (NAS-DA with FB-RR). 240 X.
- Figure 43. Stage 27, midgut showing a moderate (++) level of non-specific esterase activity (NAS-DA with FB-RR). 150 X. Some adhering yolk is present also.
- Figure 44. Stage 25, midgut showing a moderate (++) level of acid phosphatase activity (NAS-TRP with p-R). 150 X.
- Figure 45. Stage 19 (at 5°C for 4 months), pleuropodium showing an intense (++++ nonspecific esterase activity. Note the abundance of activity toward the lumen of the gland (NAS-DA with FB-RR). 240 X.
- Figure 46. Stage 19 (at 5°C for 4 months), pleuropodium control slide. Enzymatic activity destroyed with Altmann's fixative and 95% ethanol. A negative (-) reaction for nonspecific esterase activity (NAS-DA with FB-RR). 590 X.
- Figure 47. Stage 19 (at 5°C for 4 months), pleuropodium showing an intense (++++ nonspecific esterase activity. Note the demarcation in enzymatic activity (NAS-DA with FB-RR). 720 X.
- Figure 48. Stage 25, pleuropodium showing an intense (++++ amount of aminopeptidase activity. Note the uniformity of activity (LMNA with FB-B). 240 X.

See Plate I for symbol designations.

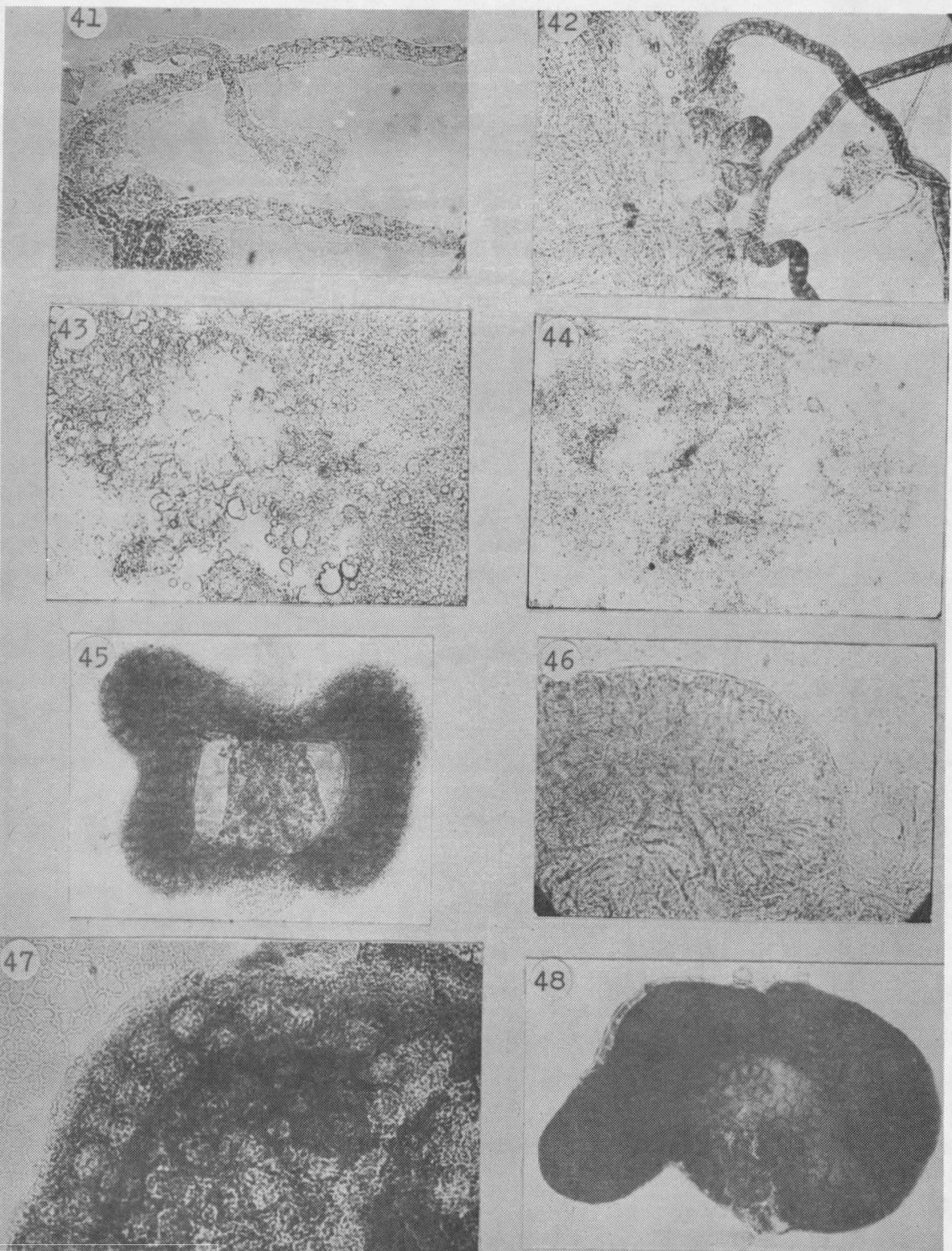


PLATE VII~~98~~

- Figure 49. Stage 19 (at 5°C for 4 months), pleuropodium showing a negative (-) reaction for acid phosphatase activity (NAS-TRP with p-R). 240 X.
- Figure 50. Stage 20 (at 5°C for 4 months, then removed to 25°C for 3 hours), pleuropodium showing a moderate (++) level of nonspecific esterase activity. Note the uniformity of the reaction at this level of activity (NAS-DA with FB-RR). 240 X. Compare with Figure 45.
- Figure 51. Stage 20 (as above), pleuropodium showing a moderate (++) level of nonspecific esterase activity. A higher magnification of Figure 50. (NAS-DA with FB-RR). 590 X.
- Figure 52. Stage 25 (5 days into post-diapause development), pleuropodium showing an intense (++++ level of nonspecific esterase activity (NAS-DA with FB-RR). 150 X.
- Figure 53. Stage 25, pleuropodium showing an intense (++++ level of nonspecific esterase activity (NAS-DA with FB-RR). 590 X. Higher magnification than Figure 52.
- Figure 54. Stage 27 (late), pleuropodium found detached from body wall and floating free in the extra-embryonic fluid. An intense (++++ nonspecific esterase activity. The gland has decreased by about 1/2 in volume from that of stage 25, Figure 52. (NAS-DA with FB-RR). 150 X.
- Figure 55. Stage 20 (at 5°C for 4 months, then removed to 25°C for 3 hours), pleuropodium showing a moderate (++) level of acid phosphatase activity (NAS-TRP with p-R). 240 X.
- Figure 56. Stage 27, pleuropodium showing a low (+) level of acid phosphatase activity, particularly at the stalk of the gland. (NAS-TRP with p-R). 150 X.

See Plate I for symbol designations.

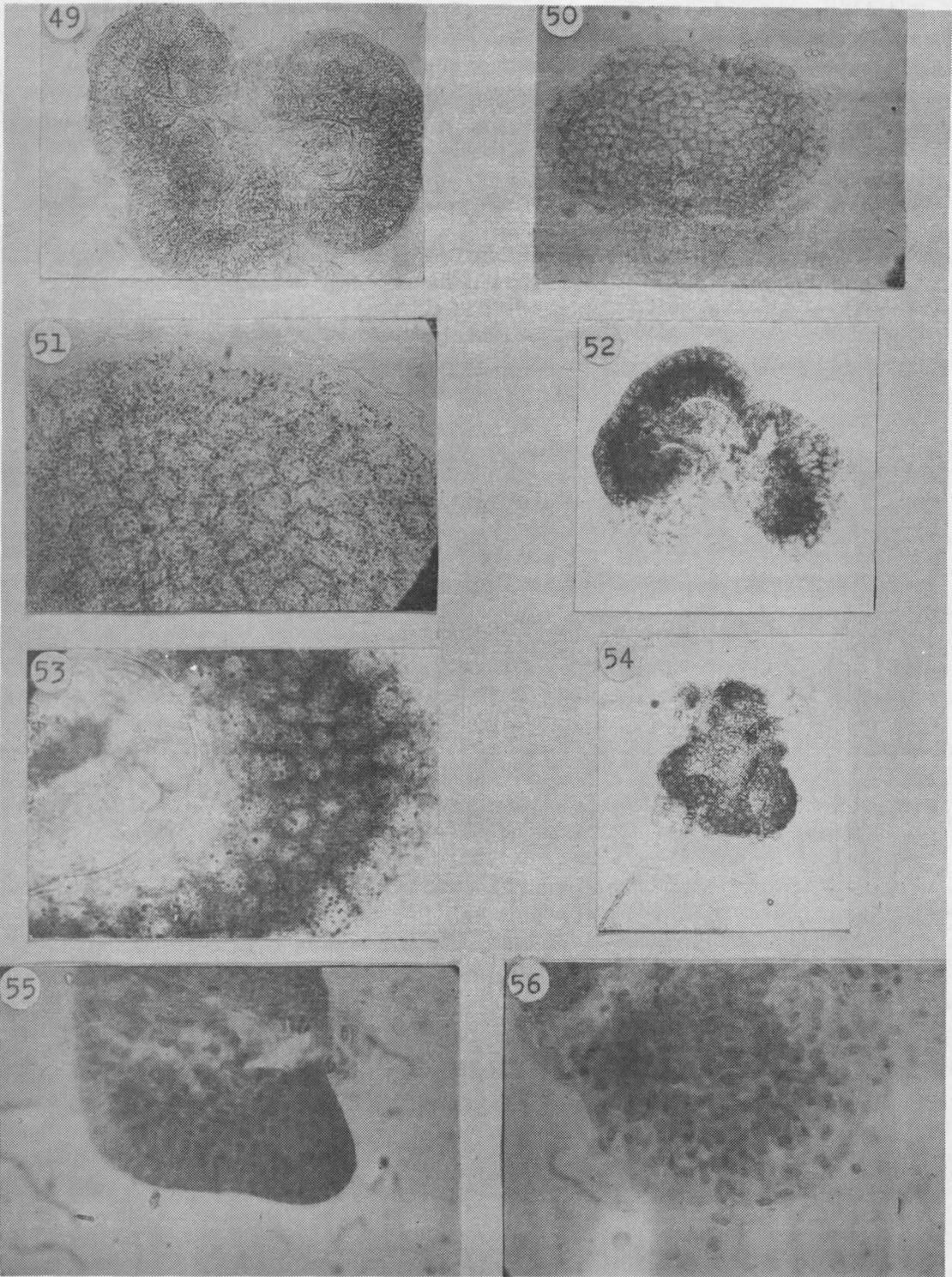
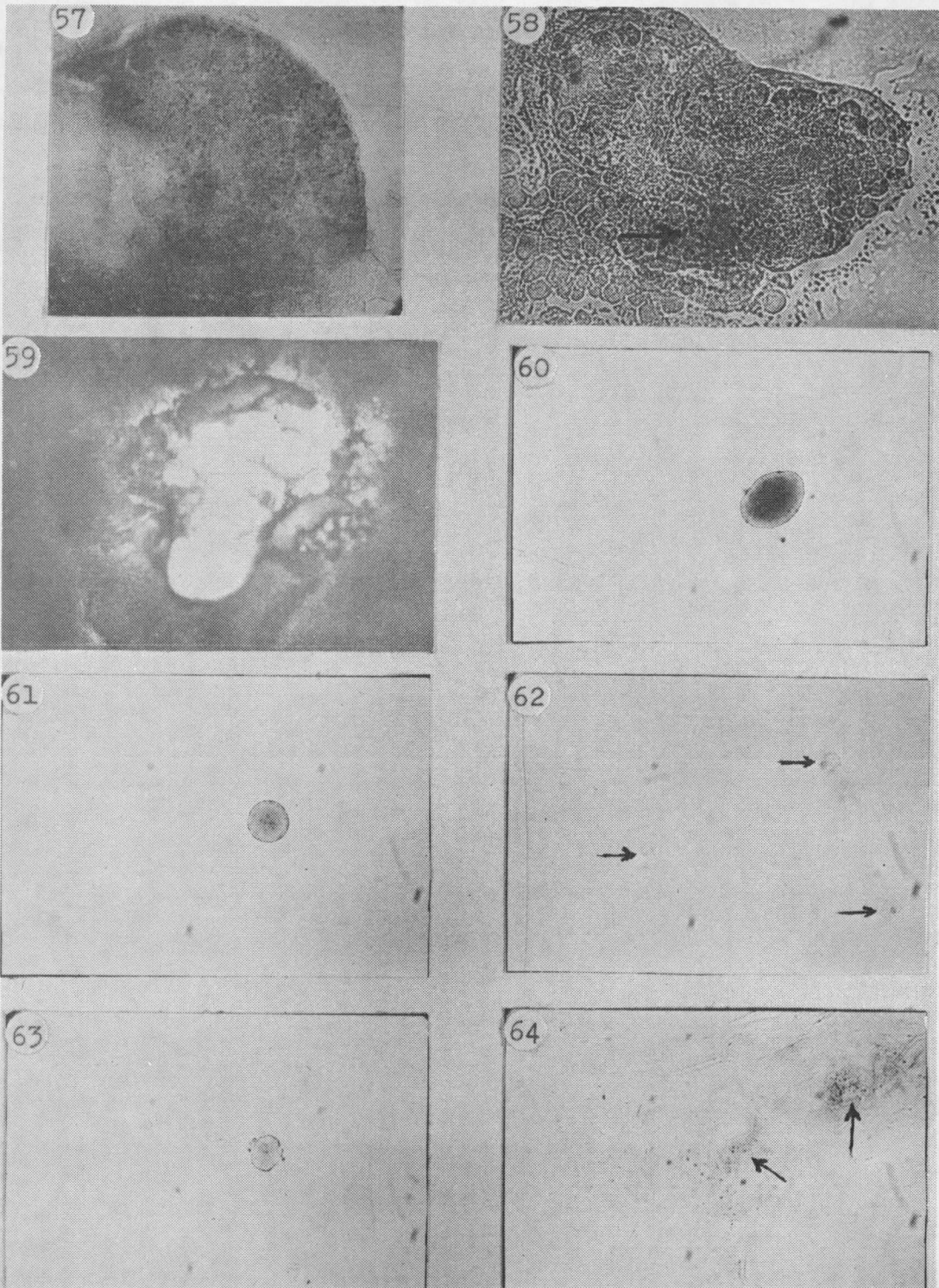


PLATE VIII

- Figure 57. Stage 23, pleuropodium showing a moderate (++) level of aminopeptidase activity (LMNA with FB-B). 240 X.
- Figure 58. Stage 26, pleuropodium showing a moderate (++) level of aminopeptidase activity. Most of this activity is located at the junction of the gland to the abdominal wall. (LMNA with FB-B). 370 X. See arrow.
- Figure 59. Stage 27 (late), pleuropodium found floating free in the extra-embryonic fluid showing an intense (++++ level of protease activity (Ag-G). 150 X.
- Figure 60. Stage 25, an ellipsoid (20 x 27 μ) hemocyte showing a high (+++) level of nonspecific esterase activity (NAS-DA with FB-RR). 590 X.
- Figure 61. Stage 25, a round (20 μ) hemocyte showing a low (+) level of nonspecific esterase activity (NAS-DA with FB-RR). 590 X.
- Figure 62. Stage 25, three small round (6-10 μ) hemocytes showing a slight (+) level of nonspecific esterase activity (NAS-DA with FB-RR). 590 X.
- Figure 63. Stage 25, hemocyte (12 x 14 μ) showing a low (+) reaction for nonspecific esterase activity (NAS-DA with FB-RR). 590 X.
- Figure 64. Stage 25, hemolymph, a plasma clot showing a moderate (++) level of nonspecific esterase activity (NAS-DA with FB-RR). 590 X. See arrows.

See Plate I for symbol designations.

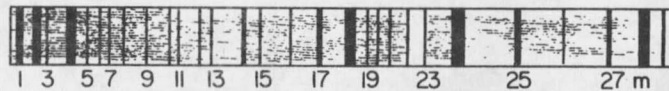


STAGE 19 DIAPAUSING EMBRYOS

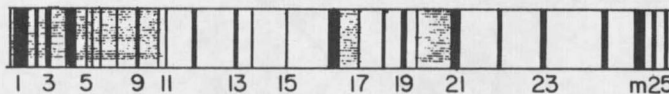
(1) Protein stained,
0.92mm ID tube,
27 bands



(2) Protein stained,
2 embryos/2.26mm ID
tube, F&T 3X, 28 bands



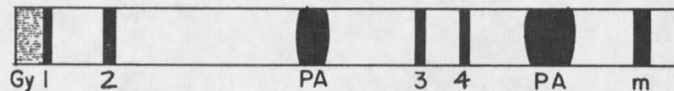
(3) Protein stained,
2.26mm ID tube,
26 bands



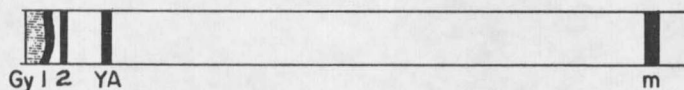
(4) Nonspecific
esterase 2.26mm ID
tube, 10 bands of
activity



(5) Lipases, 2.26mm
ID tube, 4 bands of
activity



(6) Aminopeptidases
2.26mm ID tube,
2 bands of activity



(7) Acid phosphatase,
2.26mm ID tube, F&T
5X, 1 band of activity

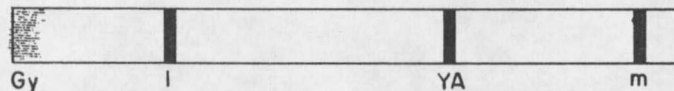
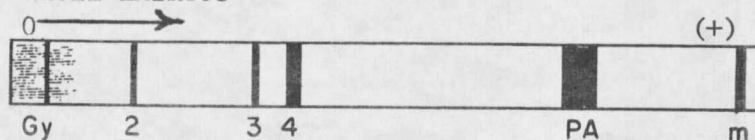


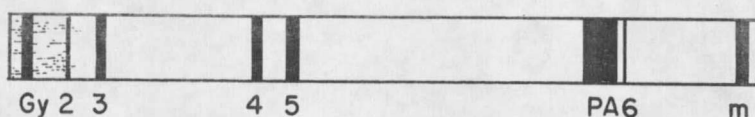
Fig. 65. Disc gel electrophoretic (protein) phereograms in register with (enzyme) zymograms. The method of Ornstein and Davis (1964) was used. All samples, except for tracing (2) consisted of a single stage 19 embryo per tube. Stage 19 embryos were held at 5°C for 4 months and at no time were they exposed to warm temperature. The direct tracings from the gels were enlarged with an Omega photographic enlarger. Protein staining was accomplished with Amido-Black dye. The media employed for the zymograms were as follows: (4) 1-naphthyl acetate with Fast Blue RR salt; (5) 1-naphthyl acetate with 0.05 M sodium taurocholate and Fast Blue RR salt; (6) L-leucyl-4-methoxy-2-naphthyl amide with Fast Blue B salt; (7) Sodium 1-naphthyl acid phosphate with pararosanilin dye. F&T, Frozen and Thawed; PA, Purple artifact; YA, Yellow artifact; GY, Greenish-yellow pigment; M, Marker, Bromphenol Blue; ID, Inside diameter.

NONSPECIFIC ESTERASES IN WHOLE EMBRYOS

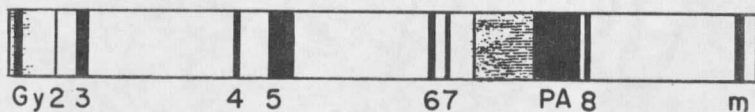
(1) Stages 8-11,
average stage 9.2, 11
embryos/tube; 4 bands
of activity



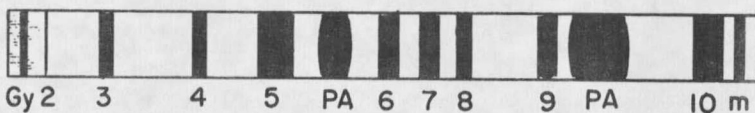
(2) Stages 12-14,
average stage 12.9, 6
embryos/tube; 6 bands
of activity



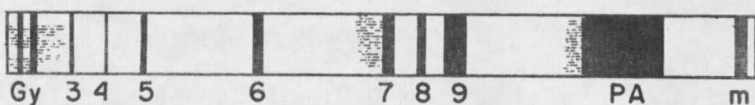
(3) Stages 14-16,
average stage 15.2, 5
embryos/tube; 8 bands
of activity



(4) Stage 19 (cold),
1 embryo/tube; 9 bands
of activity



(5) Stage 24,
1 embryo/tube; 9 bands
of activity



(6) Stage 26 (late),
1 embryo/tube; 12
bands of activity

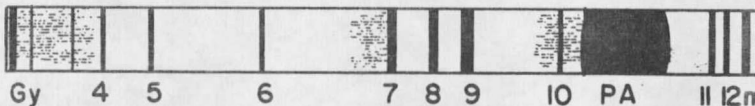


Fig. 66. Direct tracings of nonspecific esterase zymograms from various embryonic stages of *Aulocara elliotti* (Thomas). The tracings were enlarged with an Omega photographic enlarger. Nonspecific esterase was demonstrated with 1-naphthyl acetate using Fast Blue RR salt. Tube size for the zymograms was 2.26mm ID X 60mm. The Ornstein and Davis (1964) method was employed. The whole embryo was homogenized in distilled water within the prepared gel tube at 5°C. The samples were electrophoresed at 5°C with a current of 0.50mA/tube. GY, Greenish-yellow pigment; PA, Purple artifact; M, Marker, Bromphenol Blue.

NONSPECIFIC ESTERASES IN YOLK

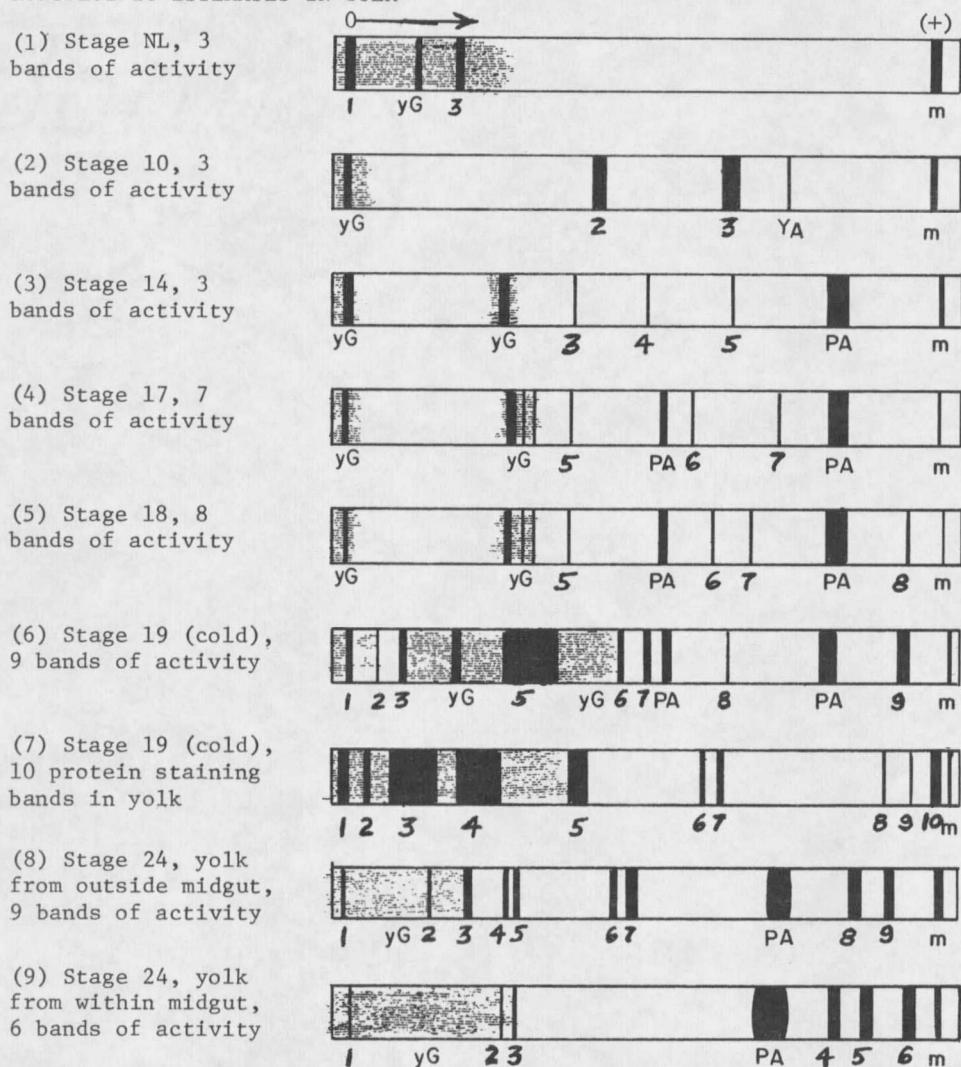
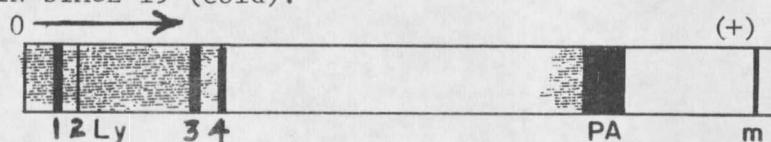


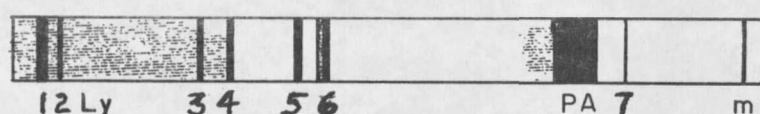
Fig. 67. Direct tracings of nonspecific esterase zymograms from various embryonic stages of *Aulocara elliotti* (Thomas). The tracings were enlarged with an Omega photographic enlarger. Each gel tube contains yolk from a single egg. Tube size for the above zymograms was 2.26mm ID X 60mm. The Ornstein and Davis (1964) method was used. Nonspecific esterase activity was demonstrated with 1-naphthyl acetate with Fast Blue RR salt. YG, Yellowish-green pigment; YA, Yellow artifact; PA, Purple artifact; M, Marker, Bromphenol Blue.

NONSPECIFIC ESTERASES IN STAGE 19 (cold).

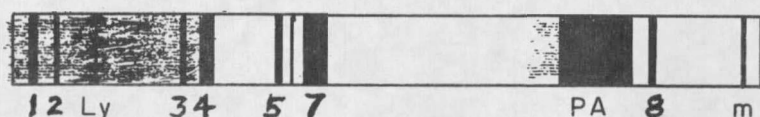
(1) Pleuropodia,
1 pair/microtube; 4
bands of activity



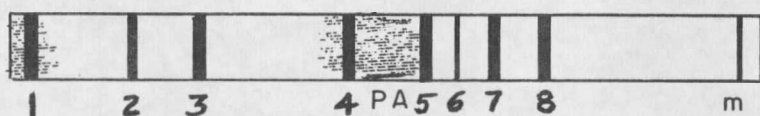
(2) Pleuropodia,
2 pair/microtube; 7
bands of activity



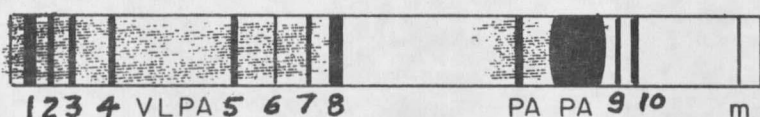
(3) Pleuropodia,
10 pair/microtube; 8
bands of activity



(4) Serosal membrane
from 10 eggs/2.26mm ID
tube; 8 bands of activ-
ity



(5) Serosal membrane
from 2 eggs/microtube;
10 bands of activity



(6) Proctodeum with
PDC and Malpighian
tubules from 2 eggs/
microtube; 3 bands of
activity

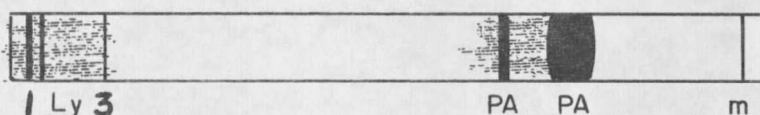


Fig. 68. Direct tracings from nonspecific esterase zymograms from stage 19 (cold) embryonic tissues of *Aulocara elliotti* (Thomas). The tracings were enlarged on Omega photographic enlarger. All the zymograms except (4) were electrophoresed in 0.92mm ID X 50mm gel tubes at 5°C with a current of 0.25 mA/tube. The tissues were homogenized in distilled water within the prepared gel tubes at 5°C. The method of Ornstein and Davis (1964) was employed. Nonspecific esterase was demonstrated with 1-naphthyl acetate using Fast Blue RR salt coupler. LY, Light yellow artifact; PA, Purple artifact; VLP, Very light purple artifact; M, Marker, Bromphenol Blue; PDC, Provisional dorsal closure membrane.

DISCUSSION AND CONCLUSIONS

Introduction

It is usually tacitly assumed that changes in enzymatic activity as demonstrated by histochemical methods reflect commensurate changes in the enzyme concentration within a tissue. This, however, may not always be the case. There may be gains or losses in dissociable activators or inhibitors within the tissues, affecting the steric configurations of the enzymes and/or altering their polymeric states. Furthermore, the activities observed by the amount of end product precipitated seldom, if ever, reflect the natural activities of these enzymes, which may be inhibited by lack of natural substrate and/or other regulatory mechanisms. Caution must be exercised, therefore, in correlating enzymatic activity levels with biochemical substrate changes (Pearse, 1968). The physiological significances of these hydrolytic enzymes in embryos are not well understood at the present time. The difficulty of assigning a particular function to these enzymes is due, in part, to their multiple molecular forms and overlapping substrate specificities (Laufer, 1960).

In studies where precise cellular localization of enzymatic activity is important, considerable amounts of enzymatic activity are sacrificed in the various processes necessary to section the material. Fixation, dehydration, embedding and storage of tissue blocks prior to

sectioning and incubation must be kept strictly uniform or the resulting activity variations will nullify the results of relative enzymatic activity between different tissues. The present study was concerned with describing the relative enzymatic activity between embryonic tissues and stages rather than with the precise cellular localization of enzymatic activity. Using unfixed whole organs has the advantage of not introducing fixation and embedding artifacts resulting from displacement of materials by solvent fronts migrating through the cells and tissues (Burstone, 1962). Pearse (1968) strongly recommends that fresh unfixed tissues (frozen sections) be used when comparing parallel biochemical and histochemical data. The use of whole mounts for studying insect tissues has become more prevalent (Raychaudhuri, 1968), especially since the advent of substituted naphthols (1-naphthol AS-TR phosphate, etc.) which form a more insoluble end product than the unsubstituted naphthyls such as 1-naphthyl acetate (Burstone, 1962). Diffusion artifacts occurring in fresh whole mounts are more easily recognized than the artifacts found in fixed and embedded tissues.

Hydrolytic Enzyme Activity in the Newly-laid Egg

Nonspecific esterase and lipase activity was demonstrated in the yolk of the newly-laid egg by both histochemical methods and electrophoresis. These enzymatic activities were observed with histochemical

methods within small (0.6-1.0 μ) vesicles thinly distributed throughout the yolk system. The yolk system of an insect egg is composed of particulate yolk bodies which are enmeshed within a cytoplasmic network referred to as an endoplasmic reticulum by Krause and Sander (1962) or called a protoplasmic or cytoplasmic reticulum by Johannsen and Butt (1941). The cytoplasmic reticulum of yolk rich eggs, such as *A. ellioti*, is sparse and was not directly observed by the methods used in these studies. Since the particulate yolk materials were found not to show enzymatic activity, it is concluded that the activities observed probably were associated with the cytoplasmic reticulum and apparently were derived from the maternal parent.

Carlson (1942) found esterases which hydrolyzed methyl butyrate and tributyrin within glycerol extracts of newly-laid eggs of *Melanoplus differentialis*. He was unable, however, to demonstrate lipase activity using an olive oil method. Fodor (1947, 1948) using olive oil substrate, found lipase activity within homogenates of newly-laid eggs of *Schistocera gregaria* and *Doclostaurus maroccanus*. In characterizing this lipase activity he found that sodium taurocholate inhibited lipase activity. Sodium taurocholate (a bile salt) is reported in histochemical literature to activate lipases and to inhibit esterases although not exclusively or completely (Nachlas and Seligman, 1949; Gormori, 1952). In a review of the subject, Bloch (1961) stated that sodium taurocholate (and other bile salts) either activates or inhibits

pancreatic lipases, depending upon the concentration of the salt and the pH of the medium. The activation of lipase activity was thought to result from removal of the reaction products (free fatty acids and mono- and diglycerides) from the reaction site by rearranging them into micellar form rather than emulsifying the substrate or acting directly upon the enzyme's active cleft.

Yolk of the newly-laid egg of *A. elliotti* also demonstrated a slight hydrolysis of the photographic emulsion (gelatin) in acetate buffer (0.1 M, pH 6.5). A sharp pH optimum was not observed over a wide range of pH values tested (3.6 to 10.3). This suggests that a number of proteolytic enzymes may be present with overlapping pH optima. Other investigations have indicated multiple forms of proteolytic enzymes in insects. Patel and Richards (1960) using paper electrophoresis found 3 separate gelatin-hydrolyzing enzymes, each with a separate pH optimum, in the midgut of houseflies. None of these 3 protease-active bands corresponded to known proteases: papain, pepsin, or trypsin. Kuk-Meiri et al., (1966) found a cathepsin-type of proteolytic activity in homogenates of 3-day-old eggs of *Locusta migratoria* with a pH optimum between 3.5 and 4.1. Shulov et al. (1957) reported no hydrolysis of casein by homogenates of newly-laid eggs of *L. migratoria* and indicated little if any proteolytic activity at that stage. Cleavage of casein was found in later stages of development (polypod and early oligopod stages) with various pH optima 5.6, 6.5

and 7.8.

The yolk system is known to act as a significant morphogenetic agent during formation of the germ anlage in insects. The localized retraction of the yolk system below the region of presumptive germ anlage is thought to be produced by liquifaction or contraction of the yolk (Counce, 1961). The yolk of the newly-laid egg of *A. elliotti* was found electrophoretically to contain at least 3 nonspecific esterase-active bands in addition to the lipases and proteases demonstrated histochemically. It is suggested that these enzymes may not be concerned only with the nutrition of the embryo, but may as well contribute to the early morphogenetic patterns in the yolk system, such as migration of the cleavage nuclei and yolk cleavage. A precise localization of these enzymes, in the newly-laid egg, however, will have to await a technique for sectioning the yolk in which enzymes are not destroyed.

A low activity of acid phosphatase was detected within a cytoplasmic layer adhering to the internal surface of the hydropyle cap of the newly-laid egg. In some eggs nonspecific esterase and lipase activity also was noted in this area. This region apparently has morphogenetic significance in the Acrididae (short germ egg) for it is the site at which the germ anlage is differentiated from the remaining blastoderm. The relationships between enzyme activities and morphogenetic induction in the early embryology are not clear and await

experimental demonstration.

Nonspecific Esterase and Lipase Activity in
Early Pre-diapause Stages (1 to 12).

Svoboda, Pepper, and Baker (1966) investigated the lipids in eggs of *A. elliotti* and found that the triglycerides composed about 90% of the total lipids and that the unsaturated fatty acids predominated over saturated fatty acids. Allais et al. (1964) found in the egg of *L. migratoria* that the lipids constitute 26% of the total dry weight of the eggs. There was, however, a decrease of 31% in the total lipids from laying to hatching. The triglycerides were far the major components of this decrease. Slifer (1930) found that the fatty acids of *M. differentialis* decreased by about 50% during embryogenesis. Carbohydrates and proteins were not utilized to any great extent during the embryogenesis of *M. differentialis* (Hill, 1945, and Bodine, 1946). Clearly, lipids play a major role in embryonic metabolism of the Acrididae and other insects as well (Williams, 1967).

Clements (1967) studied the lepidopteran esterases using starch gel electrophoresis and 1-naphthyl acetate as the substrate. He showed that the majority of the esterases were aliesterases and not proteases as was hypothesized by Cook and Forgash (1965). Clements also found that some of the esterase bands could hydrolyze C¹⁴ -tripalmitate and consequently, could be classified as lipase bands. The nonspecific esterase and lipase activity demonstrated within the tissues of *A. elliotti*, at least in part, therefore, reflects the morphological

sites of high lipid metabolism.

The importance of lipid substrates in the early embryology of *A. ellioti* is indicated by the presence of background levels of non-specific esterase and lipase activities in nearly all embryonic tissues. In stages 1 to 12 these enzymes showed even higher levels of activities in extra-embryonic structures: the serosal membrane, the vitellophages, the vesicles within the yolk, and the hydropyle cap.

At stage 12 increased levels of nonspecific esterase and lipase activities were observed within the protocerebrum, coincident with the onset of neuropyle differentiation (Van Horn, 1963). Tahmisian (1943), using biochemical methods, studied the enzymogenesis of cholinesterase in the eggs of *M. differentialis*. He suggested that this enzyme appeared at the time of neuroblast cell formation. He also found, using the leech muscle contraction technique, that acetylcholine (the substrate) was not formed until the 17th day of post-diapause development. The enzyme then appeared to have preceded the substrate by 31 developmental days (excluding diapause). Enzymatic activity, therefore, is not always a criterion for function. Salkeld (1961) characterized the nonspecific esterases within the developing embryos and nymphs of the milkweed bug *Oncopectus fasciatus*. She found no acetylcholinesterase activity in the 3-day-old embryo in which neuroblast differentiation had begun. In *A. ellioti* neuroblast differentiation begins

at stage 7 (Van Horn, 1963) and is not accompanied by increased non-specific esterase activity levels. Salkeld did find cholinesterase activity in the 4-day-old embryo in which neuropyle formation in the brain and ventral ganglia was obvious. This appeared to be the case in *A. elliotti*. Salkeld was unable to find any nonspecific esterase activity within the central nervous system after inhibition with eserine and paraxon, indicating that this activity was specifically acetylcholinesterase.

Indirect Evidence of a Differentiation Center (DC)

Since Seidel's demonstration of an activation and differentiation center in *Platynemís* (Bodenstein, 1955), it is generally agreed that the organization of the insect embryo involves the operation of these centers. Although the activation center has been demonstrated in only four species, the differentiation center has been found in most species in which it has been sought (Kuwana and Takami, 1958). Yao (1950) found a burst of alkaline phosphatase in the presumptive thorax of *Drosophila melanogaster* during or shortly after germ band contraction. This enzyme activity then was found to spread to other parts of the embryo. The center of origin of alkaline phosphatase activity was considered by Yao to be the differentiation center for the *Drosophila* embryo. Yao also found high alkaline phosphatase activity near the anal opening of the proctodeum. He proposed an antero-posterior

gradient of alkaline phosphatase activity with the posterior predominating.

A burst of acid phosphatase was found in *A. ellioti* in the gnathal region, near the presumptive prothorax. Almost simultaneously, another site of acid phosphatase activity was seen near the posterior tip of the abdomen. There has been no experimental evidence in any insect to establish whether the histochemically demonstrated enzymes do, in fact, delineate, in space and time, the differentiation center (DC).

Counce (1961) has summarized the German literature on this subject. She states that the activity of the DC in different insects may become apparent at the time of cleavage, or at the time of nuclei migration, or as late as external segmentation. In *A. ellioti* the site of acid phosphatase activity was found to originate during external segmentation at stage 6 (Van Horn, 1963). In some insects the DC is differentiated morphologically or it shows biochemical differentiation such as the acid phosphatase activity in *A. ellioti* would indicate. Mesoderm formation as well as segmentation was said to begin in the region of the DC region (Counce, 1961), and in *A. ellioti* the region of acid phosphatase activity corresponds to the region where segmentation begins (Van Horn, 1963). The DC may shift in location in some species toward a more anterior position in later phases of development. The site of acid phosphatase activity in *A.*

elliotti also was observed to shift anteriorly. In *Tachycines* the DC is said to establish the major body regions-procephalon, gnathocephalon, thorax and abdomen (Counce, 1961), but its presence or action in *A. elliotti* has not been studied. A cause-effect relationship between the site of acid phosphatase activity and the duration of action of a differentiation center, therefore, has not been established, nor has this relationship been excluded.

Subesophageal Body Cells (SEBC)

The first embryonic cells to differentiate as a distinct cell type in *A. elliotti* are the subesophageal body cells (Van Horn, 1963). These cells are binucleate with large pale nuclei. The subesophageal body is regarded as an embryonic structure although the cells have been reported in nymphs of *A. elliotti* (Van Horn-Visscher, personal communication, 1970). They were found also in nymphs of *Gryllus*, in Blattidae, and in adult termites (Johannsen and Butt, 1941). Early investigators considered the cells to be excretory in function, similar to the green glands of Crustaceans. Kessel (1961) studied the cytochemistry of these cells and could find no evidence of uric acid or urates, melanin, or glycogen. He also was unable to detect alkaline and acid phosphatase although a trace of lipase was thought to exist. Kessel used the Gormori lead sulfide method for visualization. In the present study a moderate level of acid phosphatase and nonspecific esterase and lipase activity was observed in the

subesophageal body region, but it was not particularly different in activity in comparison to the surrounding mesoderm. Aminopeptidase was first seen in the presumptive area of the subesophageal body cells two stages prior to their morphological differentiation. By stage 9, the aminopeptidase activity of these cells was maximal. Kessel (1961) concluded that the subesophageal body cells played a role in protein and lipoprotein metabolism and in excretion. The intense aminopeptidase activity of these cells in *A. ellioti* also suggests their involvement in a specialized protein metabolism. Aminopeptidase activity was found to be at a high level throughout diapause at 5°C and during post-diapause development.

The Embryonic and Extra-embryonic Membranes

Salkeld (1961) reported that the serosal membrane of the 3-day-old embryo of the milkweed bug (*Oncopeltus fasciatus*) contained non-specific esterase activity. Slifer (1937) and Matthée (1951) described the serosal membrane and the secretion of yellow and white cuticles on the inner surface of the chorion in the early stages of acridid development. After secreting these cuticles, the serosal membrane is said to draw away from the chorion and an extra-serosal fluid was found between the new cuticles and the serosa. In *A. ellioti* the serosal membrane was observed attached to the chorion in stage 4 embryos and was found to have a low nonspecific esterase and lipase activity. This activity increased after the retraction of serosa from the chorion.

By stage 7 the extra-serosal fluid was first observed. Polivanova (1965) in studying the embryonic membranes of the stink bug *Eurygaster intergriceps*, found weak acid phosphatase activity in the serosal membrane (serous) at the time of cuticular production. Acid phosphatase activity in *A. ellioti* was not observed in unfixed serosal membrane until stage 12, considerably after separation from the chorion. By stage 18, prior to diapause, the serosa exhibited moderate to high levels of nonspecific esterase and lipase, acid phosphatase, aminopeptidase and protease activity in a mosaic pattern throughout the membrane. A recent report by Bregman (1968) on the serosa in the male embryos of the mealy bug, *Pseudococcus obscurus*, showed that the serosa demonstrated a mosaic pattern of euchromatic and heterochromatic nuclei. That this may account for the mosaic pattern in enzymatic activity seen in *A. ellioti* is doubtful since all the embryos examined, both males and females demonstrated the mosaic pattern. It is not known whether the same groups of cells showing high nonspecific esterase and lipase activity also show high acid phosphatase or other enzyme activities. It may be that groups of cells in the serosa specialize for a particular product or that a cyclic change in products and/or function results in this mosaic pattern. Because the histochemical methods for demonstration of nonspecific esterases and aminopeptidases both have similar pH requirements, these enzymes could be serially tested on the same piece of

serosa to observe whether their activities overlap or are distinct. All of the hydrolytic enzymes studied, except alkaline phosphatase, showed high to intense activity in the serosal membrane during post-diapause development. Since the serosa, or most of it, was engulfed with the yolk during blastokinesis, this high hydrolytic activity was contributed to that of the rudimentary midgut which shows little or no hydrolytic activity at this time.

At stage 16 the amniotic and provisional dorsal closure (PDC) membrane completely encloses the embryo. The hydrolytic enzyme activities of these membranes were negligible, as demonstrated by these methods. Roemhild (1967) examined the various compartments in the eggs of *A. ellioti* and found each of them to have different "physical, biochemical and biotic characteristics". He proposed that the membranes separating the different egg compartments may be responsible for maintaining diapause. Because the embryo is isolated from the yolk within its amniotic and PDC membranes by stage 16, and since these membranes contain little if any hydrolytic enzyme activity, these membranes must either be permeable to the hydrolyzed nutrients of the yolk or the embryo itself has stored sufficient nutrients to sustain itself through the last few stages of pre-diapause and diapause development. Other authors, however, have reported evidence for the transfer of substrates to the embryo during diapause. Svoboda (1964) reported in *A. ellioti* that the weight of the total crude lipid extracts from isolated embryos

increased from 12.9 mg to 19.6 mg per 200 embryos after 2 months of diapause development. The triglyceride fraction increased from 4.4 mg to 8.0 mg per 200 embryos. After 5 months of diapause the total crude lipid of 200 embryos was 19.3 mg and the triglyceride fraction was 9.2 mg. Svoboda, however, did not conclude that the lipid content or triglyceride content of the embryo proper increased during diapause development. He noted that his values for the remaining yolk were variable due to losses of yolk during dissection. It appears to this author, however, that a trend was evident. Van Horn (1963) noted large increases in lipoidal material within mesodermal elements of *A. ellioti* during extended diapause. Kaocharern (1958), studying embryos of *M. differentialis*, found the deposition of lipid droplets within the mesoderm to increase in late pre-diapause and during diapause. Bunde and Pepper (1968) and Quickenden (1970), respectively, found the highest levels of free amino acids and trehalose were reached in *A. ellioti* whole eggs during prolonged diapause. It would appear the hydrolytic enzymes are active during extended diapause, particularly within the yolk, serosal membrane and pleuropodia. The PDC and the amniotic membranes may be permeable to these hydrolyzed nutrients although they apparently are neither the site nor source of abundant hydrolytic enzyme activity.

The Pleuropodia

The hydrolytic enzyme activity changes in the pleuropodia of *A. ellioti* have been reported earlier in some detail. All six of the enzymes studied were found in these organs at some stage. Salkeld (1961) also found the pleuropodia of the milkweed bug, *O. fasciatus*, to contain nonspecific esterase activity. Hagan (1931, 1939), studying the embryology of two viviparous species, noted their unusually large pleuropodia and proposed the name "pseudoplacenta" for what he considered to be important nutritive organs. Kaucharern (1958) found that the pleuropodia in *M. differentialis* increased in lipid droplets during diapause. Van Horn (1963) reported an increase in the diameter of pleuropodial cells from 10 μ to 80 μ during extended diapause development. Laine, however, (1966) measured the pleuropodia cell diameters in *A. ellioti* during diapause and found them to range from 20 to 42 μ with no particular pattern to this variation. The hydrolytic enzyme changes found in pleuropodia in this study make it appear likely that they are involved in nutritional metabolism prior to their involvement in the hatching process. With the termination of diapause these glands lose much of their intense nonspecific esterase and lipase activity and decrease about one-half in total gland volume. This decrease in activity seems to represent a release of these enzymes into the embryonic hemocoel. Nonspecific esterase activity was observed in the embryonic hemolymph (epineural

sinus fluid) at stage 19. The pleuropodia are known not to secrete an embryonic cuticle although the hypodermal cells from which they are derived, do. The physiological significance of this in *A. ellioti* is not clear.

Eclosion

The pleuropodia were seen to increase in hydrolytic activity again after diapause, and by stage 25 they demonstrated intense levels of activity. Slifer (1937, 1938) proposed that the pleuropodia in *M. differentialis* were the source of a hatching enzyme which digested the inner white serosal cuticle, (originally a secretion product of the serosa). Slifer measured the thickness of white cuticle during post-diapause development and found that it decreased abruptly a few days before hatching. From numerous cytological tests, she reported that the pleuropodia began to show secretory granules near the time when blastokinesis ends, which corresponds to stage 23 in *A. ellioti*. In *M. differentialis* these granules were said to reach a maximum concentration 4 or 5 days before hatching and, subsequently, to disappear completely. The disappearance of these granules was closely correlated in time with the disintegration of the white cuticle. Just prior to hatching the pleuropodia became shrivelled and obviously were degenerating. The pleuropodia of *A. ellioti* undergo similar degeneration at stage 27. In the present study nonspecific

esterases and lipases were seen for the first time in the extra-embryonic (serosal) fluid at stage 25. A trace of acid phosphatase activity was seen at stage 27. The first observation of alkaline phosphatase activity was found in the extra-embryonic fluid and in the pleuropodia at stage 27. Fitzgerald (1949), using biochemical methods, found alkaline phosphatase activity only within the extra-embryonic fluid of *M. differentialis*. It reached a maximum level at the time of hatching. In *A. ellioti* the extra-embryonic fluid also demonstrated moderate protease activity at stages 25 and 26 and a high level of activity at stage 27. The inner surface of the chorion at stage 26 showed a low level of protease activity, and by stage 27 the chorion demonstrated high protease activity. The exuviae (remnants of the embryonic cuticles) which were found within the extra-embryonic fluid during stages 26 and 27 also exhibited intense protease activity. Although the pleuropodia showed a low level of protease activity during blastokinesis, by stage 27 an intense level was seen. The protease activity found in the extra-embryonic fluid may be from three sources: 1) the serosal "cap", which is left outside of the embryo after yolk engulfment, degenerates and may contribute its hydrolytic enzymes to the extra-embryonic fluid; 2) the embryonic molt which occurs during stage 25 (Van Horn, 1968) may release some of the molting fluid with its enzyme complement to the extra-embryonic fluid; and 3) the pleuropodia, which are degenerating during stage 27, may release protease activity into the extra-embryonic fluid. The

protease activity seen on the chorion may have been a contamination from the extra-embryonic fluid although a thorough distilled water rinse did not appreciably remove these proteases. Nonspecific esterases and lipases and alkaline phosphatases also demonstrated intense activity levels during stages 25 to 27. The abundant hydrolytic enzymes associated with the degeneration of the pleuropodial glands, as well as that of the serosal cap, could easily account for hydrolysis of endocuticular layers of the chorion in *A. elliotti*. There was no evidence to support the production of a specific "hatching enzyme" by the pleuropodia in *A. elliotti*, either with histochemical or electrophoretic methods.

Roonwal (1937) described the development of the midgut in *L. migratoria* and noted that shortly after blastokinesis, definite groups of cells proliferate on the blind ends of both the proctodeal and stomodeal invaginations and appear to form the midgut. In *A. elliotti* although the stomodeum and proctodeum had invaginated by stage 9 in pre-diapause development, it was not until shortly before hatching that they began to show moderate to high levels of hydrolytic enzyme activity. In stage 26 and 27 the stomodeal-midgut junction and the proctodeal-midgut junction each showed a ring of intense aminopeptidase activity. These rings of enzyme activity may be related to cavitation of the blind ends of these organs or to the further elaboration of a midgut lining. Van Horn (1963) found the midgut lining at stage 26 to

be composed primarily of a single cell layer.

Diapause Development

In Tables III, XI, and XIII data were tabulated concerning relative hydrolytic enzyme activity within the various organs and tissues during diapause. Columns 18, 19a, 19b, and 20 represented enzymatic activities in the normal sequence of development. Columns 19c and 19d show the enzymatic activity of eggs which were retained at room temperature during apparent diapause. *A. ellioti* has an obligatory diapause. Most eggs developing at room temperature will progress to stage 19 but will develop no further until exposed to cold temperature (5°C) for about 50 days. Although morphogenesis has halted, other biochemical processes continue, albeit, at a lower rate. After extended exposure to cold temperature many of the embryonic organs and tissues of *A. ellioti* show hydrolytic enzyme activities as high or higher than the pre-diapause levels. Bunde and Pepper (1968) found in eggs of *A. ellioti* their highest levels of free amino acids during prolonged diapause (stage 19). Quickenden (1970) reported the highest trehalose levels in eggs at this stage. Robinson (personal communication, 1970) also has found evidence that at least a low rate of protein synthesis is evident at 8°C in diapausing eggs of *A. ellioti*. It would appear from these biochemical studies that transformation of substrates occurs during diapause. The hydrolytic enzyme activity profiles of the various organs and

tissues observed in the present study suggest that after an initial minimum cold duration (probably about 50 days at 5°C) certain biochemical changes occur if cold exposure continues.

Polyacrylamide Disc Gel Electrophoresis

The enzymes seen within the tissues may not be the same enzyme as demonstrated by electrophoresis. Hannibal and Nachlas (1959) suggested that only the desmo (insoluble) enzymes are primarily demonstrated using histochemical methods. The soluble or lyo-enzymes may be investigated by homogenizing tissues in distilled water and separating them by electrophoresis. This distinction, however, is more physical than qualitative for the esterases. Hunter et al. (1964) eluted the origin of the acrylamide gel which contained the "desmo-esterases" that had not migrated and, using the same techniques as in the first separation, they found the two patterns to be essentially the same, although the intensity was lower for the second separation. The lyo-esterase, therefore, appear to be identical to the desmo-esterases. Allen and Gockerman (1964) found that freezing and thawing or the use of detergents, such as Triton X-100, would release two different fractions of acid phosphatase from liver lysosomes. Here the distinction between lyo- and desmo- forms appears to have a molecular basis.

Since the acid phosphatase, aminopeptidase and lipase zymogram patterns in *A. ellioti* were found to be composed of only a few bands, while 10 nonspecific esterase-active bands were seen in embryos at stage 19, the latter were chosen for further investigation. The number of nonspecific esterase-active bands were found to increase with developmental stage. The maximum number of anodal migrating esterases from whole embryos was found to be 12. Salkeld (1965) found, using starch gel electrophoresis, a total of 11 anodal nonspecific esterase-active bands in the newly-hatched milkweed bug, *O. fasciatus*. She also found 7 cathodal migrating esterases. The number of esterase-active bands in embryos of *A. ellioti* at stage 19 were found to increase from that observed in pre-diapause stages, although in the histochemical demonstration of nonspecific esterase and lipase activities, these enzymes were found to decrease in activity. A rapidly-migrating band seen in diapause embryos was not found in post-diapause embryos. The zymogram patterns of pre- and post-diapause embryos differ in the mobility of a number of other bands, indicating that new or altered esterases are distinctive to each stage of development.

The yolk of *A. ellioti* was seen to increase in the number of esterase-active bands during development. In addition, a yellow pigment was found in all zymograms prior to staining. Salkeld (1965) also found a yellow non-protein staining pigment using the starch gel technique. Ten protein-staining bands were found in *A. ellioti* in the yolk from diapause eggs. Koch (1965) found a maximum of 11

protein bands in the yolk of *Carausius morosus* in his study of the differentiation of the endoplasmic reaction system in relation to embryonic development.

A technique using microgels was developed whereby the isolated organs of the embryo were electrophoresed and their esterase zymograms compared. Each organ was shown to have its own characteristic profile of esterase-active bands. The ontogeny of the enzyme-active bands within the various organs will have to await future study. This microgel technique should prove useful in characterizing the embryos at stage 6 for new enzyme and/or protein products in relation to the activity of the proposed differentiation center. Krause and Raunio (1967) found that the 0.9 mm inside diameter tube gave the maximum sensitivity without requiring the use of a micromanipulator. These authors established the sensitivity of this method using diluted blood serum and found that a total protein sample of 10 μ g per a 20 μ l sample was required to visualize the protein bands. The method used in the present study employed the zymogram technique to these microgels amplified the sensitivity many fold.

SUMMARY

The ontogeneses of six hydrolytic enzymes were followed throughout the embryogenesis of the grasshopper *Aulocara elliotti* (Thomas) using both enzyme histochemical and electrophoretic methods. Non-specific esterase, lipase, acid and alkaline phosphatase, and aminopeptidase activities were demonstrated histochemically using the azo dye simultaneous coupling techniques. Protease activity was detected using the substrate-film method. The above enzymes, with the exception of the proteases, were separated and identified by polyacrylamide disc microgel electrophoresis. The principal findings and conclusions are listed below:

- 1) The yolk of the newly-laid egg was endowed with nonspecific esterases, lipases and proteases which it probably derived from the maternal parent.

- 2) Nearly all cells throughout embryogenesis demonstrated at least a background level of nonspecific esterase and lipase activity within their cytoplasm. This supports the biochemical evidence that lipids are the major source of energy during acridid embryogenesis.

- 3) A burst of acid phosphatase activity was observed within the gnathal region near the presumptive prothorax during stage 6 of embryonic development (Van Horn, 1966a). This activity is thought to be indicative of a physiological differentiation center at this site.

4) A low level of aminopeptidase activity was observed in the presumptive area of subesophageal body cells at stage 7, two stages before they were morphologically differentiated. By stage 9 the aminopeptidase activity was found to be maximal at this site.

5) A high level of acid phosphatase and aminopeptidase activity was found at the posterior tip of the abdomen in stages 7 and 9, respectively. This enzymatic activity appeared concurrently with the high activity in the gnathal region.

6) Although the stomodeum and proctodeum had invaginated by stage 9 in pre-diapause development, it was not until shortly before hatching that they began to show high levels of hydrolytic enzyme activity.

7) High nonspecific esterase, lipase, and acid phosphatase activity appeared with or slightly before neuropyle differentiation (stage 12) within the protocerebrum. This enzymatic activity followed the posterior progression of neuropyle formation along the ventral nerve cord. By stage 13 almost all of the cells of the brain demonstrated high acid phosphatase activity.

8) The serosal membrane was found to be one of the most hydrolytically active tissues of the egg. The serosa showed a mosaic pattern of high and low enzymatic activity throughout its existence.

9) The amniotic membrane showed trace amounts of acid phosphatase activity after stage 13. Both the provisional dorsal closure

membrane and the amnion were found to be almost completely lacking in hydrolytic enzyme activity. It would appear that these two membranes serve as partitional structures, whereas the serosa, in addition, appears to be a major source of hydrolytic enzymes.

10) Nonspecific esterase and lipase activity within the pleuropodia of diapause embryos showed a large increase after 4 months of incubation at 5°C. This intense level was found to decrease sharply with the termination of diapause and the initiation of blastokinesis. The gland appeared to secrete these enzymes into the embryonic hemocoel rather than externally. By the end of blastokinesis the pleuropodia again demonstrated an intense level of nonspecific esterase and lipase activity. Acid phosphatase and aminopeptidase activity were found to change independently of each other and of nonspecific esterase and lipase activity.

11) Alkaline phosphatase activity was observed only in the pleuropodia and the extra-embryonic fluid at stage 27.

12) Prior to hatching (stage 27) the pleuropodia, the extra-embryonic fluid, and the inner surface of the chorion demonstrated high protease activity. The hydrolytic enzyme activities found at stages 26 and 27 in the extra-embryonic fluid are thought to be associated with the degeneration of the pleuropodial glands, rather than with the production of a specific hatching enzyme as reported by Slifer (1938) in *Melanoplus differentialis* (Thomas).

13) In stages 26 and 27 the stomodeal-midgut junction and the proctodeal-midgut junction both demonstrated rings of intense aminopeptidase activity. This enzymatic activity may be related to the cavitation of the blind ends of the fore and hind gut and the elaborations of the midgut lining.

14) The presumptive cerci by stage 15 demonstrated an intense aminopeptidase activity which appeared to have migrated to them from the tip of the abdomen.

15) Diapause embryos incubated at 5°C for 2 weeks showed an overall level of hydrolytic enzyme activity equal to or less than that observed during pre-diapause development. After 4 months of cold exposure many organs and tissues demonstrated an increase in hydrolytic enzyme activity from their pre-diapause levels. Since biochemical studies have shown that the highest peak of substrate concentration (trehalose and free amino acids) has occurred in embryos maintained for extended periods in diapause, it would appear that the hydrolytic enzymes are functional during diapause and that diapause is not a quiescent state with respect to hydrolytic enzyme activity.

16) Nonspecific esterase activity appeared in all of the embryonic hemocytes observed but it differed widely between cell types.

17) No chymotrypsin-like enzyme activity could be demonstrated.

18) The nonspecific esterase zymograms obtained from homogenates of whole embryos showed an increase from 4 enzyme-active bands at

stages 8-11 to 12 enzyme-active bands at stage 26.

19) Yolk of the newly-laid egg contained 3 nonspecific esterase-active bands. Yolk from a stage 10 egg also showed 3 active bands but these had different electrophoretic mobilities from the yolk of the newly-laid egg. The zymogram patterns of yolk from eggs with embryos at stages 14, 17, 18, 19, and 24 were obtained.

20) The pleuropodial zymogram at stage 19 showed a total of 8 nonspecific esterase bands but no protein staining bands could be observed.

21) The proctodeum, the Malpighian tubules and the provisional dorsal closure membrane was found to have 3 nonspecific esterase bands at stage 19. These tissues showed only a trace of nonspecific esterase activity using the histochemical methods.

22) Sodium taurocholate was found to inhibit 6 out of a total of 10 esterase-active bands. Three "lipase" bands had nonspecific esterase counterparts, but one rapidly-migrating band appeared to be a new lipase band. The nonspecific esterases could not be distinguished from lipases using these histochemical methods.

LITERATURE CITED

- Abe, M., S. P. Kramer and A. M. Seligman. 1964. The histochemical demonstration of pancreatic-like lipase and comparison with the distribution of esterase. *J. Histochem. Cytochem.* 12:364-383.
- Adams, C. W. M. and N. A. Tuqan. 1961. The histochemical demonstration of protease by a gelatin-silver film method. *J. Histochem. Cytochem.* 9:469-472.
- Allais, J. P., J. Bergerard, J. Etienne and J. Polonovski. 1964. The nature and evolution of lipids during embryogenesis of *Locusta migratoria*. *J. Insect Physiol.* 10:753-772.
- Allen, J. M. and J. Gockerman. 1964. Electrophoretic separation of multiple forms of particle associated acid phosphatase. *Ann. N. Y. Acad. Sci.* 121:616-633.
- Anderson, N. L. and E. Hastings. 1966. Some notes on rearing *Aulocara elliotti* (Orthoptera:Acrididae). *Ann. Entomol. Soc. Am.* 59: 718-719.
- Ando, H. 1955. Everted embryos of dragonflies produced by ligation. *Sci. Rep. Tokyo Bunrika Daigaku, Sec. B*, 8(119):65-74. In: *Invertebrate Embryology*. Edited by M. Kumé and K. Dan(1958). pp. 452-453. Translated from Japanese and reprinted for the National Science Foundation (Grant G-8663) in 1968 by NOLIT, Publ. House, Belgrade, Yugoslavia.
- Barka, T. J. 1961. Electrophoretic separation of acid phosphatase in rat liver on polyacrylamide gels. *J. Histochem. Cytochem.* 9:542-547.
- Barka, T. and P. J. Anderson. 1965. *Histochemistry: Theory, Practice and Bibliography*. 631 pp. Hoeber Medical Division. Harper & Row, Publ. Inc., New York.
- Beckman, L. and F. M. Johnson. 1964a. Variations in larval alkaline phosphatase controlled by Aph alleles in *Drosophila melanogaster*. *Genetics* 49:829-841.

Beckman, L. and F. M. Johnson. 1964b. Esterase variations in *Drosophila melanogaster*. Hereditas 51:212-220.

. 1964c. Genetic control of aminopeptidase in *Drosophila melanogaster*. Hereditas 51:221-230.

Bloch, K. E. 1960. (Editor) Lipide Metabolism. 411 pp. John Wiley and Sons, Inc., New York.

Bodenstein, D. 1955. In: Analysis of Development. Edited by B. H. Willier, P. A. Weiss and U. Hamberger. W. B. Saunders Co., Philadelphia. Chap. 4, pp. 337-345.

Bodine, J. H. 1946. Uric acid formation in the developing egg of the grasshopper, *Melanoplus differentialis*. Physiol. Zool. 19: 54-57.

Bokdawala, F. D. and J. C. George. 1964. Histochemical demonstration of the muscle lipase. J. Histochem. Cytochem. 12:768-771.

Boyer, P. D., H. Lardy and K. Myrback. 1960-61. (Editors) The Enzymes, Vol. 4 and 5, Academic Press, New York.

Bregman, A. A. 1968. The development of the serosa in male embryos of the mealy bug *Pseudococcus obscurus* Essig. J. Exp. Zool. 169:173-196.

Bunde, D. E. and J. H. Pepper. 1968. Biosynthesis and occurrence of free amino acids in eggs of the grasshopper, *Aulocara ellioti*. J. Insect Physiol. 14:1635-1649.

Burstone, M. S. 1962. Enzyme Histochemistry and its Application in the Study of Neoplasms. 621 pp. Academic Press, New York.

Carlson, L. D. 1942. Esterases of the grasshopper egg. Biol. Bull. 81:375-384.

Clements, A. N. 1967. A study of soluble esterases in *Pieris brassica* (Lepidoptera). J. Insect Physiol. 13:1021-1030.

Cook, B. J. and A. J. Forgash. 1965. The identification and distribution of carboxylic esterases in the American cockroach, *Periplaneta americana* (L.). J. Insect Physiol. 11:237-250.

- Counce, S. J. 1961. The analysis of insect embryogenesis. *Ann. Rev. Entomol.* 6:295-312.
- Deuchar, E. M. 1966. *Biochemical Aspects of Amphibian Development.* 206 pp. John Wiley and Sons, Inc., New York.
- Fitzgerald, L. R. 1949. The alkaline phosphatase of the developing grasshopper egg. *J. Exp. Zool.* 110:461-487.
- Fodor, P. J. 1947. Studies on the nature of insect lipases and esterases. *Enzymologia* 12:343-351.
- _____. 1948. The specificity of action of insect lipases. *Enzymologia* 13:57-61.
- Gomori, G. 1952. The histochemistry of esterases. *Internat. Rev. Cytol.* 1:323-335.
- _____. 1955. Histochemistry of human esterases. *J. Histochem. Cytochem.* 3(6):479-484.
- Hannibal, M. J. and M. M. Nachlas. 1959. Further studies on the lyo- and desmo- components of several hydrolytic enzymes and their histochemical significance. *J. Biophys. Biochem. Cytol.* 5(2):279-288.
- Hastings, E. and J. H. Pepper. 1964. Population studies on the big-headed grasshopper, *Aulocara elliotti*. *Ann. Entomol. Soc. Amer.* 57(2):216-220.
- Hill, D. L. 1945. Carbohydrate metabolism during development of *Melanoplus differentialis*. *J. Comp. Cell. Physiol.* 25:205-216.
- Hagan, H. R. 1931. The embryogeny of the Polychaetid, *Hesperoetes fumarius* Westwood, with reference to viviposity in insects. *J. Morph.* 51:3-118.
- _____. 1939. *Diploptera dylliscoides*, a viviparous roach with elongated pleuropodia. *Ann. Entomol. Soc. Amer.* 32:264-266.
- Holt, S. J. 1956. The value of fundamental studies of staining reactions in enzyme histochemistry with reference to the indoxyl methods for esterases. *J. Histochem. Cytochem.* 4(6):541-544.

- Horvath, B. Z. 1967. Studies on the development of muscle in embryos of *Aulocara ellioti* (Thomas) (Orthoptera, Acrididae) using the fluorescent antibody technique. Ph.D. Thesis, Montana State University, Bozeman. 69 pp.
- Hunter, R. L. and E. A. Maynard. 1962. Esterase in the trigeminal and superior ganglion of the rabbit. J. Histochem. Cytochem. 10(6):677.
- _____, J. T. Rocha, A. R. Pfrender and D. C. De Jong. 1964. The impact of gel electrophoresis upon our understanding of the esterases. Ann. N. Y. Acad. Sci. 121(2):532-543.
- Johannsen, O. A. and F. H. Butt. 1941. Embryology of Insects and Myriapods. 462 pp. McGraw-Hill Book Co. Inc., New York.
- Kaocharern, P. 1958. Cytological changes associated with diapause in embryos of the grasshopper, *Melanoplus differentialis* (Thomas). Ph.D. Thesis, University of Wisconsin, Madison. 82 pp.
- Kessel, R. G. 1961. Cytological studies of the subesophageal body cells and pericardial cells in embryos of the grasshopper, *Melanoplus differentialis*. J. Morph. 109(3):189-322.
- Koch, P. 1965. Veränderungen im Dotterproteinmuster der Stabheuschrecke *Carausius morosus* im Verlauf der Embryonalentwicklung. Verh. Dtsch. Zool. Ges. Jena (1965) Zool. Ans. Suppl. 29:251-255 (1966).
- Krause, G. and K. Sander. 1962. In: Advances in Morphogenesis. Edited by M. Abercrombie and J. Brachet. 2:259-303. Academic Press, New York.
- Krause, U. and V. Raunio. 1967. Micro gel electrophoresis. Clin. Chim. Acta. 17:251-254.
- Kuk-Meiri, S., N. Lichtenstein, A. Shulov and M. P. Pener. 1966. Cathepsin-type proteolytic activity in the developing eggs of the African migratory locust (*Locusta migratoria migratorioides* R. & F.). Comp. Biochem. Physiol. 18:783-795.
- Kuwana, J. and T. Takami. 1957. In: Invertebrate Embryology. Edited by M. Kume and K. Dan (1958). pp. 418-420. Translated from Japanese and reprinted for the National Science Foundation (Grant G-8663) in 1968 by NOLIT, Publ. House, Belgrade, Yugoslavia.

- Lagunoff, D. and E. P. Benditt. 1964. Benzosalicylanilide ester substrates of proteolytic enzymes. Kinetics and histochemical studies. I. Chymotrypsin. *Biochemistry* 3(10):1427-1431.
- Laine, I. I. 1966. Temperature effects on the oxygen consumption during development of *Aulocara elliotti* (Thomas). Ph.D. Thesis, Montana State University, Bozeman. 74 pp.
- Laufer, H. 1960. Blood proteins in insect development. *Ann. N. Y. Acad. Sci.* 89:490-515.
- Leopold, R. A. 1967. A study on the postembryonic ovarian development and vitellogenesis of *Aulocara elliotti* (Thomas) (Orthoptera, Acrididae). Ph.D. Thesis, Montana State University, Bozeman, 96 pp.
- Moloney, W. C., K. McPherson and L. Fliegelman. 1960. Esterase activity in leukocytes demonstrated by the use of naphthol AS-D chloroacetate substrate. *J. Histochem. Cytochem.* 8:200-207.
- Markert, C. L. and R. L. Hunter. 1959. The distribution of esterases in mouse tissue. *J. Histochem. Cytochem.* 7:42-49.
- Matthée, J. J. 1951. The structure and physiology of the egg of *Locustana pardalina* (Walk). Union of South Africa, Dept. of Agri. Science Bull. 316. 83 pp.
- Moog, F. 1965. In: *The Biochemistry of Animal Development*. Edited by R. Weber. Vol. 1, Chap. 6. pp. 307-365. Academic Press, New York.
- Nachlas, M. M. and A. M. Seligman. 1949. Evidence for the specificity of esterase and lipase by the use of three chromogenic substrates. *J. Biol. Chem.* 181:343-355.
- Ornstein, L. and B. J. Davis. 1964. Disc electrophoresis I and II. *Ann. N. Y. Acad. Sci.* 121(2):321 and 404.
- Patel, N. G. and A. G. Richards. 1960. Proteolytic enzymes from the midgut of the house fly. *J. Insect Physiol.* 4:146-153.
- Pearse, A. G. E. 1968. *Histochemistry: Theoretical and Applied*. 759 pp. Little, Brown and Co., Boston.

- Polivanova, E. N. 1965. The functional significance of the embryonic membranes of insects. Translated from Doklady Akademii Nauk SSSR. 160(1):243-245.
- Quickenden, K. L. 1970. Carbohydrates in eggs of the grasshopper, *Aulocara ellioti*, during development. J. Insect Physiol. 16:171-183.
- _____. and G. R. Roemhild. 1969. Maternal age and density effects on carbohydrates partitioned to eggs of the grasshopper, *Aulocara ellioti*. J. Insect Physiol. 15:1215-1223.
- Raychaudhuri, A. 1968. Staining whole mounts of an insect *Tribolium confusium* reproductive tracts for succinate dehydrogenase and nonspecific esterases. Stain Tech. 43(1):9-14.
- Roemhild, G. R. 1965a. Respiration of the eggs and parts of eggs of *Aulocara ellioti* (Thomas). Physiol. Zool. 38(3):213-218.
- _____. 1965b. Cyclical temperature effects on diapause termination of eggs of *Aulocara ellioti*. J. Insect Physiol. 11:1633-1639.
- _____. 1967. Compartmentation in the eggs of *Aulocara ellioti* (Thomas). Physiol. Zool. 40:182-188.
- _____. 1968. Cationic effects and levels in developing eggs of the grasshopper, *Aulocara ellioti*. J. Insect Physiol. 14:1035-1043.
- Roonwal, M. L. 1937. Studies on the embryology of the African migratory locust, *Locusta migratoria migratorioides* R. & F. (Orthoptera, Acrididae). II. Organogeny. Philos. Trans. Roy. Soc. (London). B, 227:175-244.
- Salkeld, E. H. 1961. The distribution and identification of esterases in the developing embryo and young nymph of the milkweed bug, *Oncopeltus fasciatus* (Dallas). Can. J. Zool. 39:589-595.
- _____. 1965. Electrophoretic separation and identification of esterases in eggs and young nymphs of the large milkweed bug, *Oncopeltus fasciatus* (Dallas). Can. J. Zool. 43:593-602.

- Shulov, A., M. P. Pener, S. Kuk-Meiri and N. Lichtenstein. 1957. Proteolytic enzymes in various embryonic stages of the eggs of *Locusta migratoria migratorioides* (R. & F.). J. Insect Physiol. 1:279-285.
- Slifer, E. H. 1930. Insect development I. Fatty acids in the grasshopper egg. Physiol. Zool. 3:503-518.
- _____. 1934. Insect development VI. The behavior of grasshopper embryos in anisotonic, balanced salt solutions. J. Exp. Zool. 67:137-157.
- _____. 1937. The origin and fate of the membranes surrounding the grasshopper egg; together with some experiments on the source of the hatching enzyme. Quart. J. Micro. Sci. 19:493-512.
- _____. 1938. A cytological study of *Melanoplus differentialis* (Orthoptera, Acrididae), which furnishes new evidence that they produce the hatching enzyme. J. Morph. 63(1):181-196.
- Svoboda, J. A. 1964. Studies on some of the lipids of the egg of the grasshopper species *Aulocara elliotti* (Thomas). Ph.D. Thesis, Montana State College, Bozeman. 71 pp.
- _____, J. H. Pepper and G. L. Baker. 1966. On the lipids of the egg of the grasshopper, *Aulocara elliotti*. J. Insect Physiol. 12:1540-1565.
- Tahmisian, T. N. 1943. Enzymes in Ontogenesis: Choline-esterase in development of *Melanoplus differentialis* eggs. J. Exp. Zool. 92(2):199-213.
- Thomsen, E. and I. Møller. 1963. Influence of neurosecretory cells and of corpus allatum on intestinal protease activity in the adult *Calliphora erythrocephala* Meig. J. Exp. Biol. 40(2): 301-321.
- Van Horn, S. N. 1963. The embryogenesis and embryonic variability of *Aulocara elliotti* (Thomas) (Acrididae, Orthoptera). Ph.D. Thesis, Montana State College, Bozeman. 172 pp.
- _____. 1966a. Studies on the embryogenesis of *Aulocara elliotti* (Thomas) (Orthoptera, Acrididae). I. External Morphogenesis. J. Morph. 120(1):83-114.

- Van Horn, S. N. 1966b. Studies on the embryogenesis of *Aulocara ellioti* (Thomas) (Orthoptera, Acrididae). II. Developmental variability and the effects of maternal age and environment. J. Morph. 120(2):115-134.
- _____. 1968. Changes in gland volume during the post-diapause development in embryos of *Aulocara ellioti* (Thomas) (Orthoptera, Acrididae). Proc. Mont. Acad. Sci. 28:72-75.
- Williams, J. 1967. In: The Biochemistry of Animal Development. Edited by R. Weber. 2:375-376.
- Yao, T. 1950. Cytochemical studies on the embryonic development of *Drosophila melanogaster*. II. Alkaline and acid phosphatases. Quart. J. Micro. Sci. 91:79-90.
- Yos, D. A. 1961. The mounting of carbowax sections with lighter-fluid and rubber cement. Stain Tech. 36(1):163-167.



3 1762 10011643 1

D378

Ur15

cop.2

Urban, Robert E.

Histochemical and electrophoretic analyses of hydrolytic enzymes...

[illegible]

D378
Ur. 15
cap. 2