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
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Abstract:
The ontogeny of six hydrolytic enzymes was followed throughout the embryo genes is of the grasshopper AuloaaTa 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 contract 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)

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ROBERT EDWARD URBAN

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

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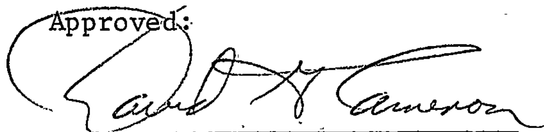

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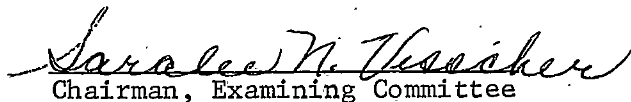

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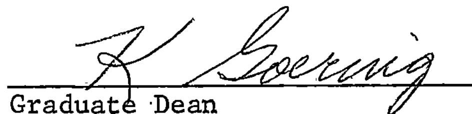

Graduate Dean

MONTANA STATE UNIVERSITY
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ABSTRACT

The ontogeny of six hydrolytic enzymes was followed throughout the embryogenesis 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.

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 elliotti* (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. elliotti* 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. elliotti*, 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. elliotti*, 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. elliotti* 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. elliotti* (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 aperature 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 Omaga 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 Barka 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-HCI 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 CO_2.

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 commerical 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, vitellophages, blood cells, fat body cells, coelomic sacs, contracting and stretching membranes, etc.

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

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

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

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

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

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

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

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

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

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

The Chorion

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

ovarioles shortly after vitellogenesis ends (Leopold, 1967).

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

The Hydropyle

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

plasmalemma (the periplasm of "yolk-poor" eggs) (Krause and Sander, 1962). In later embryonic stages, after the amniotic folds have joined ventrally and the serosal membrane completely surrounds the germ anlage and yolk, the serosal cells in the hydropyle region differentiate into large columnar cells which become attached to the inner surface of the hysropyle cap (Slifer, 1937; Matthée, 1951). In *A. elliotti* 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 nonspecific esterase and lipase activity was detected within the unfixed yolk of the newly-laid egg (Fig. 3). This enzymatic activity appeared to be confined within vesicles in the yolk's cytoplasmic reticulum.

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

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

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

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

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

Diapausing eggs, maintained at room temperature, did not show the decreae 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 vitellophages had only a moderate level of nonspecific esterase and lipase activity. It appeared that there was no large accumulation of enzymatic activity within the vitellophages 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 vitellophages appeared to maintain their activity at a moderate level. Aminopeptidase activity was not detected within the yolk or vitellophages during diapause in the cold or at room temperature. Protease activity decreased within the yolk with or without exposure to cold during diapause.

<u>Post-diapause development</u> <u>(stage 20-27)</u>. 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 vitellophages 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 prominant (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 hysrolytic 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. elliotti* (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.

<u>The dorso-lateral margins of the abdominal walls</u>. 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 vitellophages 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 vitellophages and yolk at this time, however, demonstrated an intense amount of nonspecific esterase and lipase activity and were found to adhere to the midgut membrane making it difficult to assess the activity of the midgut lining iteslf.

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 hemocoele.

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 nonspecific 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 acitivity, 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 prediapause 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).

	Stages									
Tissues	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
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 L 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.
(+++)	High activity at 200 X or less.
(++++)	Intense activity at 125 X or less (overdeveloped reaction).
(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).

	Stages								
Tissues	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).

	Stages					
	18	19a	19b	20	19c	19d
Tissues						
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	++	++	++	++	++	+-
Stomodeum	BG	BG	BG	BG	BG	BG
Proctodeum	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).

	Stages							
Tissues	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	++	++	+++	+++
Stomodeum	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).

	Stages								
Tissues	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	-	-	-	-	-	-	-
Vitellophages		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).

	Stages								
Tissues	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	+-	+-	+-
Stomodeal-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.
- (+++) High activity at 125 X.
- (++++) Intense activity at 125 X or less.

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.

	ENZYMES			
TREATMENTS	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.

	ENZYMES			
SALTS TESTED	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

Figure 1. Stage 25, inside of the hydropyle cap showing a moderate (++) amount of aminopeptidase activity (LMNA withFB-B). Not all dark areas denote activity. 240 X.

Figure 2. Stage 25, vitellophage 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.

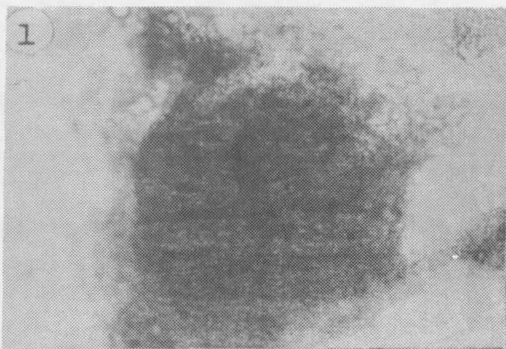
Nonspecific esterases:
- NAS-DA, 1-naphthol AS-D acetate
- FB-RR, Fast Blue RR salt

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

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

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

Proteases:
- Ag-G, Silver - gelatin substrate film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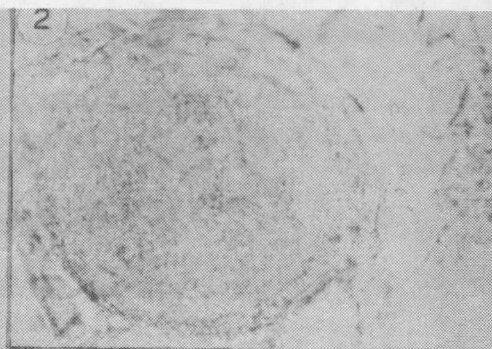
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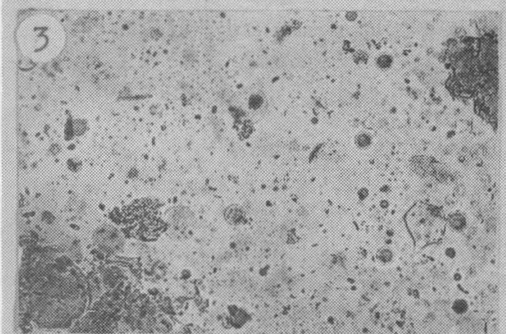
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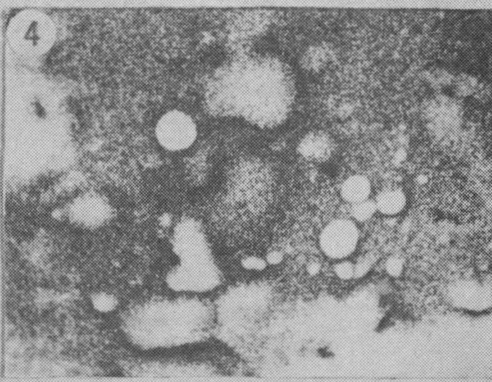
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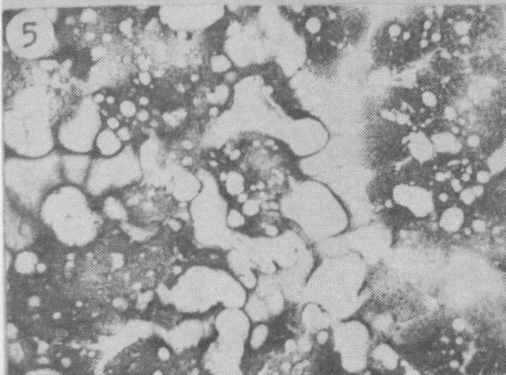
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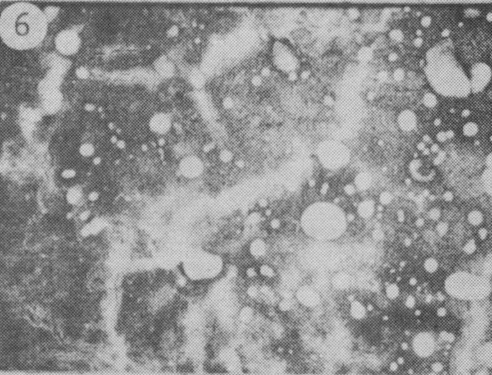
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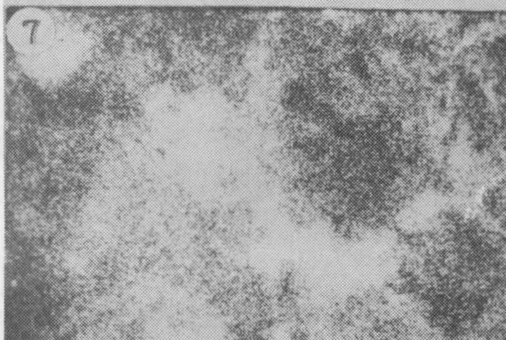
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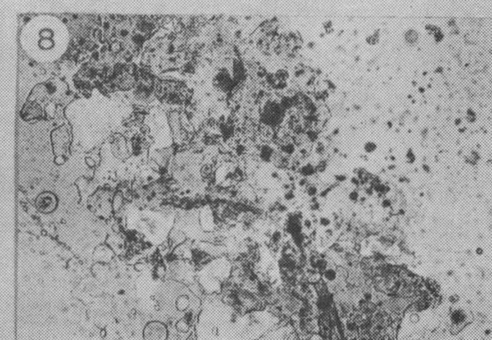
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PLATE II

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.

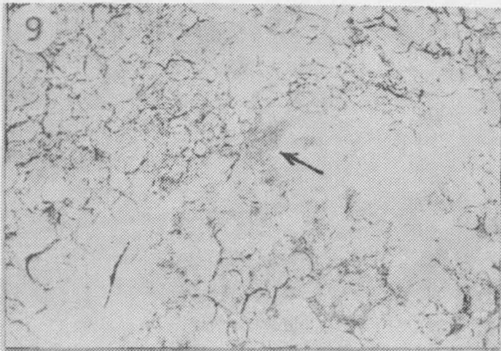
Figure 13. Stage 18, serosal membrane showing a moderate (++) level of aminopeptidase activity in the cytoplasm (LMNA with FB-B). 1480 X.

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.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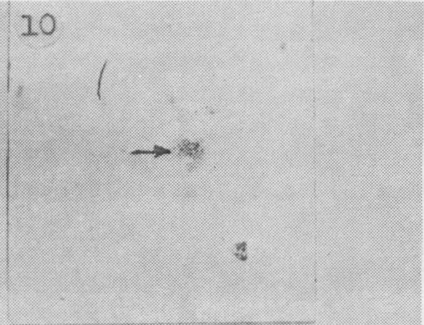
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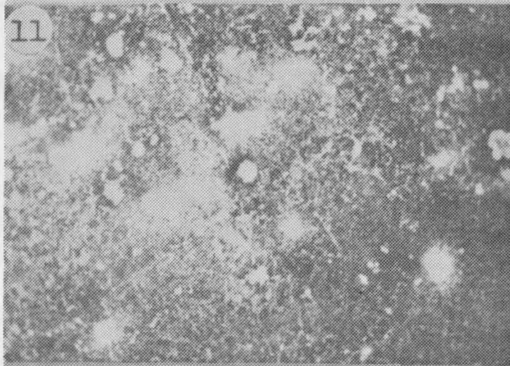
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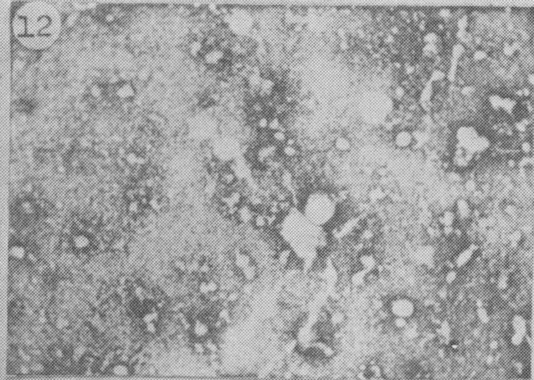
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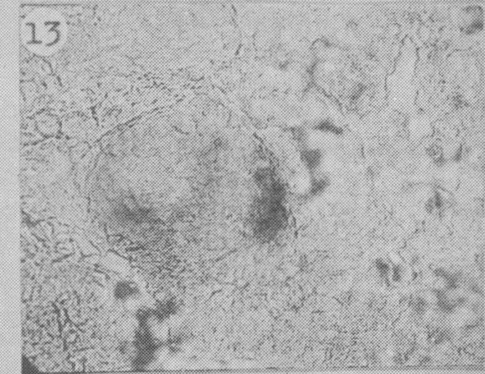
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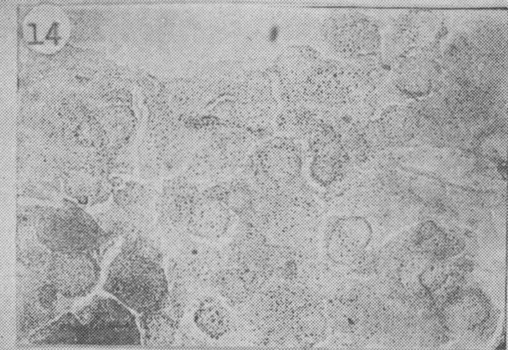
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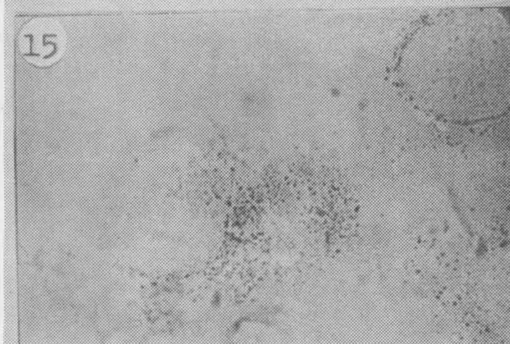
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PLATE III

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.