The distribution, rate of synthesis and characterization of protein from the eggs of Aulocara elliotti (Thomas) (Orthoptera, Acrididae) during development

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A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Zoology
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Abstract:
Dry weight, protein content and rate of protein synthesis of whole eggs and separate egg fractions (embryo, yolk and shell) were followed throughout the embryogenesis of Aulocara elliotti (Thos.). Protein content was determined by the biuret assay. The protein of whole eggs and the yolk fraction decreased markedly during the transition from prediapause to diapause development. The embryonic protein increased during the first 50 days of diapause development and gradually decreased with longer times in the diapause state. Increases in embryonic protein paralleled morphogenetic changes during post-diapause development.

There was an increase in the ratio of non-protein to protein material in the embryo during late pre-diapause which reached a high level that was maintained throughout diapause and decreased during post-diapause development. Uniformly-labeled valine-C14 was introduced by a desiccation-absorption technique to allow a 4-day period of in vivo incorporation into protein. The embryo fraction showed a high rate of protein synthesis at 36 days of pre-diapause development. The rate of protein synthesis was low in embryos and relatively high in yolk during diapause. The observations indicate that the increase in the ratio of non-protein to protein material in the diapausing embryo is due to continued hydrolysis and transfer of yolk reserves to the embryo while a low embryonic metabolic rate allows the accumulation of the products.

Embryonic proteins and yolk proteins that were soluble in 40 % sucrose were separated electrophoretically on polyacrylamide disc gels.

Yolk protein not soluble in 40 % sucrose (over 50 %) was dissolved in 0.6 N KC1 and separated. The number of protein bands was determined by-staining with Amido-Schwarz. Proteins conjugated with carbohydrates or lipoid material were indicated by use of the PAS reaction and Sudan Black B staining. The general pattern of protein bands was determined in embryos and yolk during pre-diapause, diapause and post-diapause development. Protein from embryos and yolk contained the largest number of bands during diapause. Three lipid-positive bands were found in diapausing embryos while only one was found during pre-diapause and post-diapause development. Four protein bands in diapausing embryos correspond with bands in the yolk pattern during diapause. This may mean that intact proteins are transferred from the yolk to the embryo. Two, five and six lipoprotein bands, respectively, appeared in 40 % sucrose-soluble yolk during pre-diapause, diapause and post-diapause development. Yolk soluble in 0.6 N KC1 contained two major protein bands which were positive for both carbohydrate and lipid.

THE DISTRIBUTION, RATE OF SYNTHESIS AND CHARACTERIZATION OF PROTEIN FROM THE EGGS OF *AULOCARA ELLIOTTI* (THOMAS) (ORTHOPTERA, ACRIDIDAE) DURING DEVELOPMENT

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of

DOCTOR OF PHILOSOPHY

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Zoology

Approved:

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MONTANA STATE UNIVERSITY
Bozeman, Montana

June, 1970

ACKNOWLEDGMENT

I am grateful to my major professor, Dr. George Roemhild, for suggestions made during the course of this study and valuable constructive criticisms of the manuscript. I wish to thank Drs. Harold Picton and Saralee Visscher for their excellent critical reading of the manuscript. Drs. A. M. El-Negoumy, Gordon Julian and Gary Strobel contributed valuable technical advice during the course of this study that was much appreciated. I wish to thank Professor Ellsworth Hastings for providing grasshoppers and rearing facilities. My wife, Marie, deserves special acknowledgment for her patience, understanding, encouragement and help during this study.

I appreciate the financial support for this study which was provided by the U. S. Department of Health, Education and Welfare through a Title IV NDEA Fellowship and the Zoology and Entomology Department at Montana State University through a teaching assistantship.

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ABSTRACT

Dry weight, protein content and rate of protein synthesis of whole eggs and separate egg fractions (embryo, yolk and shell) were followed throughout the embryogenesis of *Aulocara elliotti* (Thos.). Protein content was determined by the biuret assay. The protein of whole eggs and the yolk fraction decreased markedly during the transition from pre-diapause to diapause development. The embryonic protein increased during the first 50 days of diapause development and gradually decreased with longer times in the diapause state. Increases in embryonic protein paralleled morphogenetic changes during post-diapause development. There was an increase in the ratio of non-protein to protein material in the embryo during late pre-diapause which reached a high level that was maintained throughout diapause and decreased during post-diapause development. Uniformly-labeled valine-C^{14} was introduced by a desiccation-absorption technique to allow a 4-day period of *in vivo* incorporation into protein. The embryo fraction showed a high rate of protein synthesis at 36 days of pre-diapause development. The rate of protein synthesis was low in embryos and relatively high in yolk during diapause. The observations indicate that the increase in the ratio of non-protein to protein material in the diapausing embryo is due to continued hydrolysis and transfer of yolk reserves to the embryo while a low embryonic metabolic rate allows the accumulation of the products.

Embryonic proteins and yolk proteins that were soluble in 40 % sucrose were separated electrophoretically on polyacrylamide disc gels. Yolk protein not soluble in 40 % sucrose (over 50 %) was dissolved in 0.6 N KCl and separated. The number of protein bands was determined by staining with Amido-Schwarz. Proteins conjugated with carbohydrates or lipoid material were indicated by use of the PAS reaction and Sudan Black B staining. The general pattern of protein bands was determined in embryos and yolk during pre-diapause, diapause and post-diapause development. Protein from embryos and yolk contained the largest number of bands during diapause. Three lipid-positive bands were found in diapausing embryos while only one was found during pre-diapause and post-diapause development. Four protein bands in diapausing embryos correspond with bands in the yolk pattern during diapause. This may mean that intact proteins are transferred from the yolk to the embryo. Two, five and six lipoprotein bands, respectively, appeared in 40 % sucrose-soluble yolk during pre-diapause, diapause and post-diapause development. Yolk soluble in 0.6 N KCl contained two major protein bands which were positive for both carbohydrate and lipid.

INTRODUCTION

The grasshopper species *Aulocara elliotti* (Thomas) is indigenous to the Great Plains area of the United States and has been of considerable interest from an economic standpoint for many years. Although control of this insect is possible by chemical means, an understanding of the factors which cause population changes and subsequent economic damage would be of value when planning any sort of control program. Consequently, during the past decade a number of biochemical studies have been undertaken on the egg stage of this species with the idea that an intimate knowledge of the physiology might result in some understanding of the reasons for population change.

Several classes of compounds have been investigated. Svoboda (1964) studied the lipid fraction of developing eggs and found that there was a marked increase of lipids within the embryo during early diapause development. The lipid content and especially the triglyceride fraction of whole eggs decreased considerably during the post-diapause period suggesting that these materials were being utilized for energy or as a carbon source. Boell (1935) reported that 73 % of the oxygen consumed during the development of *Melanoplus differentialis* can be accounted for on the basis of oxidation of fats. Thus it appears that the pattern of utilization in *A. elliotti* is similar to that found in *M. differentialis*.

Quickenden and Roemhild (1969) and Quickenden (1970) determined the carbohydrate levels in eggs throughout embryogenesis. They noted increases in mannitol and trehalose during diapause. Since glycogen levels did not decline and the quantity of free mannose was insufficient to serve as the only precursor for mannitol synthesis, it was postulated that the hydrolysis of protein-carbohydrate complexes could provide precursors for mannitol and/or trehalose synthesis.

Bunde (1965) and Bunde and Pepper (1968) studied the free amino acids present at various stages in the development of *A. elliotti* eggs. They found that the total free amino acid level was much higher during the diapause period than during other developmental periods. They suggested that the increase during diapause might be due to a decrease in the utilization of amino acids for protein synthesis while the hydrolysis of yolk proteins continued.

Fluctuations in free amino acid levels may be reflected in the protein metabolism of the egg. Changes in the distribution of protein and the rate of protein synthesis should provide information relating to growth and physiological activity during embryogenesis. Such findings would be valuable in the integration of the physiological picture of this organism.

Numerous studies on protein metabolism in insects have been reviewed recently by Gilmour (1965), Agrell (1964), Chefurka (1965) and

Chen (1966). It is generally accepted that the total nitrogen of the insect egg remains constant throughout embryogenesis. The distribution of nitrogen has been followed in the egg of *M. differentialis* by Trowbridge and Bodine (1940). They found an increase in the nitrogen of the embryo and a concomitant decrease in the nitrogen of the yolk, while shell nitrogen increased at the beginning of embryogenesis and decreased at the end. No change was found during diapause.

Agrell (1964) states that uric acid should be the end product of nitrogen metabolism in insect eggs, however, findings are controversial. Shaw (1955) has found high levels of ethanolamine phosphoric acid in grasshopper eggs. This compound could be important in nitrogen elimination (Agrell, 1964). Bodine (1946) has reported that the uric acid content could be equivalent to no more than 6.6 % of the nitrogen of the egg protein of *M. differentialis*. Ludwig and Rothstein (1952) indicated that protein was not utilized as an energy source in *Popillia japonica* since total nitrogen remained constant and there was no evidence for the accumulation of waste nitrogen.

Metabolic changes are difficult to demonstrate in insect eggs due to their small size and the large numbers needed for biochemical analyses. An understanding of the basic structural changes and temperature requirements of acridid eggs during embryogenesis is essential to design a meaningful study.

No studies of the *A. elliotti* egg shell have been performed, however, the general features of acridid egg shells appear to be the same. Hartley (1961) performed light microscope studies of the shell of newly-laid eggs in seven acridid species. He calls the shell of the newly-laid egg the chorion. It is of maternal origin and is divided into; a thick inner layer consisting of an interlocking, tangled system of fine struts; a thin but more dense layer or area adjacent to it; and a thin granular outer layer. He also noted that the complex inner layer was much thicker at the posterior end of the egg and in some cases had pits passing into it from the outside. Such an arrangement would appear to be conducive to water absorption and might be responsible for trapping certain molecules. The chorion remains on the egg throughout embryogenesis although it may become cracked and discontinuous in older eggs. This may be of little importance since the serosal cuticles, which are of embryonic origin, become the principle layers of the shell soon after laying. Slifer (1937) and Mathée (1951) have studied the cuticular layers in acridid egg shells and have found that there is an outer layer (yellow cuticle) and an inner, thicker layer (white cuticle).

The growth and morphogenesis of embryos of *A. elliotti* have been described in terms of 27 discrete morphological stages (Van Horn 1963, 1966a). An obligatory diapause usually begins at stage 19. Fifty days of exposure to temperatures of 8°C or lower are required to break

the diapause state (Roemhild, 1965a).

Roemhild (1967) has shown that when eggs are held at 25°C for about 25 days after laying certain membranes in the egg allow the formation of distinct compartments. The embryo becomes separated from the yolk by the provisional dorsal closure forming the epineural sinus. The serosa then detaches from the cuticle allowing the formation of the hydropylar, yolk and extra-serosal compartments. Roemhild (1967, 1968) has found that the physical and chemical characteristics of these compartments differ and suggested that compartmentation yields conditions unfavorable for further morphological development. The membranes are ruptured during blastokinesis which marks the end of diapause. The compartments are obliterated at this time and morphogenetic activity resumes.

During compartmentation the embryo was in an environment of pH 7.4 and high sodium ion concentration. After blastokinesis the embryo comes in contact with fluids of pH 6.6 and low sodium concentration. Roemhild (1968) has noted that incorporation of valine-C^{14} and acetate-C^{14} into embryo protein *in vitro* is higher at lower pH values in the range of 6.8-7.0. Agrell (1952) has found that histolysis is associated with high pH values while periods of histogenesis occur when pH values are low. The low sodium ion concentration *in vivo* after blastokinesis could also effect an increase in the rate of protein synthesis. Stevenson and Wyatt (1962) and Price (1967) have found that sodium

levels are critical to the rate of protein synthesis. Apparently sodium is inhibitory to the activation system as it is in mammals (Sachs, 1957).

Since proteins are intimately involved in growth and function, the rate of protein synthesis should reflect the growth rate and other physiological activities during embryogenesis. Most studies of protein synthesis in insects have involved the *in vitro* incorporation of labeled amino acids into tissues or tissue minces or *in vivo* incorporation after the injection of labeled amino acids. One of the requirements for protein synthesis is the functioning of oxidative metabolism (Chefurka, 1965). The diapause period appears to be unfavorable for protein synthesis in some insects because the O_2 consumption is low.

The diapause period in insects has been characterized as one of lowered metabolism (Keister and Buck, 1964). Decreases in the respiratory rate of *M. differentialis* during diapause have been noted by Bodine (1929), and Burkholder (1934) and Boell (1935). Similar findings have been reported for *Bombyx* by Ashbel (1930). Quite different observations have been obtained for diapausing *A. elliotti* eggs. The respiratory rate, as measured by O_2 consumption, is maintained at the relatively high late pre-diapause level throughout diapause (Roemhild, 1965b; Laine, 1966). Harvey (1962) has enumerated three processes responsible for O_2 consumption; morphogenesis, maintenance and function.

In species with low 0_2 consumption during diapause, one would expect only maintenance since this requires less 0_2 than biosynthesis (Lees, 1955). Bunde (1965) found that acetate-C^{14} was incorporated into 8 amino acids during diapause in *A. elliotti*; thus, at least some biosynthesis occurs. Since the respiratory rate is maintained at the relatively high pre-diapause level during diapause in *A. elliotti* (Roemhild, 1965b; Laine, 1966), the biochemical transformations during this period may be considerable (Van Horn, 1963; Svoboda, 1964; Quickenden, 1969; Urban, 1970).

Changes in protein synthetic activity might be indicated by the separation and partial characterization of the proteins in the egg. Comparisons of hemolymph and yolk proteins were made electrophoretically in *Periplaneta americana* by Neilsen and Mills (1968) and in *Leucophaea maderae* by Sheurer (1969). Duke (1966) used this method to study the hemolymph proteins in *Locusta migratoria migratorioides*. These workers have indicated that some hemolymph proteins are deposited directly into the yolk during vitellogenesis. Since the yolk is the source of energy and metabolic materials for embryogenesis, it could be informative to separate and compare the embryonic proteins and yolk proteins throughout this period.

Partial characterization of the protein bands can be accomplished using histochemical techniques. Wang and Patton (1968) found positive reactions for five types of conjugated protein in the larval hemolymph

of *Galleria mellonella, Acheta domesticus* and *Tenebrio molitor*. Dejmal and Brookes (1968) have identified the major component of *Leucophaea maderae* yolk as a lipoprotein by a histochemical technique and Vinson and Lewis (1969) have detected 4 glycoproteins and 2 lipoproteins in sub-adults and adults of three species of *Heliothis*. Since carbohydrates and lipids are probably the most important energy sources during insect embryogenesis (Rothstein, 1952; Ludwig and Ramozzotto, 1965), the detection of these materials conjugated with protein was undertaken.

In the present study, whole eggs, embryos, yolk and shells were analyzed separately to determine protein content and the rate of protein synthesis throughout embryogenesis. Embryonic protein and yolk protein were separated on polyacrylamide gels. Additional gels were run to test for protein-carbohydrate and lipoprotein complexes.

MATERIALS AND METHODS

Experimental Design

Egg samples of known-age representing various stages in embryogenesis were collected in 1967. These eggs were allowed to absorb valine-C^{14} as a tracer to determine the rate of protein synthesis. Radioactive valine uptake, protein content and sample dry weights were determined. The eggs were divided into two groups, hereafter called Series I and Series II. The first series was composed of whole egg samples while the second series was made up of separate egg fractions; that is, the embryo, yolk and shell. A whole egg sample held at 25°C for 140 days in the diapause state to determine the effect of no cold exposure is marked (*) below. Sample age, condition and temperature regime are represented schematically as:

	Pre-diapause State Eggs at 25°C						Diapause State Eggs at 8°C					Post-diapause State Eggs at 25°C				
Series I Days of development in state when sample was taken:	8	15	26	36	42	52	26	50	76	140	140*	1	3	5	10	15
Morphological stage (Van Horn, 1966):	1	5	10	13	18	19	19	19	19	19	19	20-24		25		26
Series II Days of development	8	15	20	28	35	41	26	45	51	110		1	3	5	10	15
Morphological stage:	1	5	7	10	13	18	19	19	19	19		20-24		25		26

Further information about the egg proteins was obtained through an electrophoretic study of the proteins in the embryo fraction and yolk fraction of Series II. For this study two additional egg fraction samples, one in diapause and the other in the post-diapause state, were prepared from eggs which had not been exposed to valine-C^{14}.

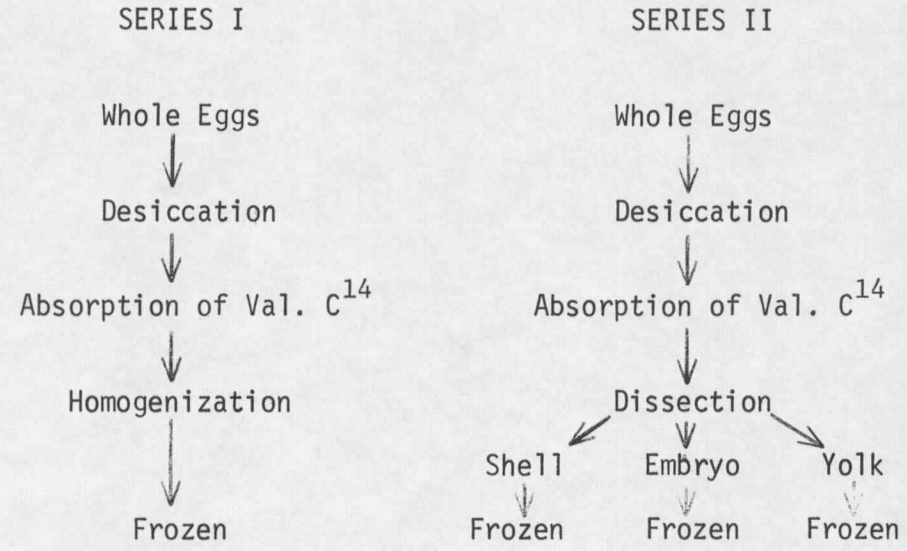
Biological Samples

The reproductive adults of *A. elliotti* were reared from a population near Decker, Montana, in the summer of 1967. The grasshoppers were reared under greenhouse conditions in cylindrical lucite cages, 20.5 cm in diameter and 27.5 cm high, set on dirt-filled pans according to the procedures described by Anderson and Hastings (1966). They were maintained in 20 cages, each with a density of three pairs per cage, and were fed fresh western wheatgrass, *Agropyron smithii*, one of the preferred food-plants of *A. elliotti* (Pfadt, 1949; Anderson, 1964).

Egg pods were collected on alternate days by sifting the dirt in the pans. The pods were then stored in plaster of Paris blocks at 25°C for development (Van Horn, 1966b). After a period of 52 days, egg pods selected for diapause or post-diapause samples were placed in an 8°C refrigerator. After about 4 months of cold exposure egg pods to be used for post-diapause samples were returned to 25°C. At the appropriate time eggs for each of the pre-diapause, diapause and post-diapause samples were removed and treated using methods described later To prevent desiccation, egg pods maintained at 25°C were watered lightly

twice a week and those kept at 8°C only once a week.

The scheme by which eggs were processed is shown on the following page. When the eggs had reached the desired age they were removed from the pods, washed, air-dried and weighed. Isotope introduction was accomplished by a desiccation-absorption technique (Bunde, 1965; Quickenden, 1969). The eggs were desiccated for a period of four days over anhydrous calcium sulfate. Early pre-diapause eggs were desiccated for shorter periods due to rapid weight loss. Weight loss in all samples was limited to less than one-tenth of the starting weight. The eggs were then immersed in a radioactive solution consisting of 25 μc of uniformly labeled L-valine-C^{14} (specific activity = 190 mc/mM) per ml of distilled water. This amino acid was chosen because Bunde (1965) found that the concentration of free valine was quite constant and that it was not synthesized from acetate-C^{14} during the embryogenesis of *A. elliotti*. During absorption the lids of the incubation vials were lifted periodically to prevent oxygen depletion. After absorption for four days the eggs were washed thoroughly in distilled water, air-dried and weighed. The age in days reported for all samples includes the 4 days of desiccation followed by 4 days of absorption except for early pre-diapause samples in which desiccation times were shorter. The age in days of each sample is coincident with the end of the absorption period.



From this point on, whole eggs and egg fraction samples were separately subjected to the following steps:

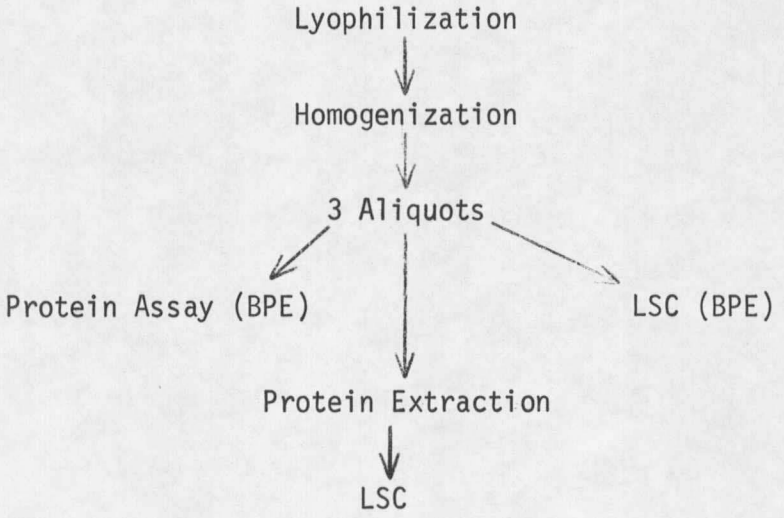


Figure 1. Major steps in the procedure for determining the protein content and valine-C^{14} activity of extracted protein in *A. elliotti* whole eggs and egg fractions. Abbreviations: Val. C^{14}-uniformly labeled L-valine-C^{14}, LSC-liquid scintillation counting, BPE-before protein extraction.

In series I, whole egg samples used varied from 13 to 34 eggs. Embryos in the diapause and post-diapause periods were categorized using staging criteria for this species established by Van Horn (1966a). After the absorption period and staging were completed, the eggs were homogenized for three minutes in distilled water at 4°C and then frozen at -20°C.

In Series II, samples contained 41 to 94 eggs. When the absorption period was completed, the eggs were placed in distilled water for dissecting. Embryos and egg shells were removed separately from the dissecting medium with as little water as possible. They were rinsed briefly in distilled water and placed in separate containers. The remaining material (dissecting medium, yolk and rinse water) formed the yolk fraction. In the first three pre-diapause samples the embryos were left in the yolk fraction because of their small size; the yolk and shells of only 20 eggs were separated in these samples. In late post-diapause eggs the yolk mass had been engulfed by the embryos but was removed to maintain separate fractions.

Sixteen whole egg samples for Series I and 45 egg fraction samples for Series II were prepared. Some samples were stored at -20°C for as long as 2 years. For easier handling and to obtain the dry weights, all samples were lyophilized prior to analysis. Very little difference was noted in the electrophoretic patterns of protein from fresh and stored material.

Two additional post-diapause whole egg samples were analyzed to check the possibility that microorganisms might contaminate the incubation medium and affect the results. Two mgs each of Penicillin G, Mycostatin and streptomycin sulfate were added to the incubation medium of one sample. These bacteriostatic and fungicidal agents eliminated the possibility of protein synthesis by microorganisms in the medium. In the other sample, 2 mgs of chloramphenicol, a broad spectrum bactericide and protein synthesis inhibitor, were added to the medium. Incorporation of valine-C^{14} into protein by both samples indicated that microorganisms were not responsible for this protein synthesis and that chloramphenicol in the incubation medium did not stop protein synthesis. The antibiotics probably did not penetrate the shells of these eggs.

Biuret Assay

Colorimetric quantitative analysis of the protein concentration of each lyophilized sample was made. One large aliquot was divided into three parts for the determination of protein concentration, uptake of valine-C^{14} and incorporation of valine-C^{14} into protein. A biuret standard curve was devised using bovine serum albumin. Linearity in accordance with Beer's Law was established in the range of 0.1 to 7.0 mgs of protein by reading the absorbance at 540 mμ on a Bausch and Lomb Spectronic 20 Colorimeter. The biuret reagent used was modified from that described by Gornall et al. (1949) by the addition of ethyl ether as used by Karnavar and Nair (1969). The protein standards were treated in the same manner as the samples.

A large aliquot of lyophilized material from each sample was homogenized in distilled water in a Ten Broeck ground glass tissue grinder. Final volumes of homogenates were from 5 to 10 ml. Biuret assay aliquots of 1 to 3 ml were taken from the homogenates. Whenever the amount of sample material was sufficient, 3 ml of the homogenate were taken and divided into 3 aliquots for triplicate analysis. A 1:4:1 mixture of homogenate-biuret reagent-ethyl ether was used. After adding ether for lipid extraction the mixture was shaken for 15 seconds and centrifuged for 10 minutes at 4,000 rpm. Without the ether extraction the biuret medium becomes cloudy. With the lipids in the ether layer, the aqueous layer was transferred to a cuvette for a colorimeter reading 30 minutes after the initial mixing.

Protein Extraction

Protein was extracted from the remaining homogenate by a procedure similar to that of Wannemacher et al. (1965). An equal volume of cold 20 % trichloroacetic acid was added to the homogenate. The precipitate formed was separated from the supernatant fluid by centrifugation in an International HN centrifuge at 4,000 rpm at 4°C. Two more extractions with 8 ml of cold 10 % trichloroacetic acid followed. To remove residual acid, as well as some of the lipid, the precipitate was extracted with 8 ml of a 95 % ethanol solution saturated with sodium acetate (Wannemacher, 1965 and Lowry et al., 1951). Additional lipid extractions were performed with 8 ml of 3:1 ethanol-ethyl ether and 8 ml

of anhydrous ethyl ether. Excess ether was removed by drying in a convection hood. The precipitate was then placed over anhydrous calcium sulfate in a desiccator for 24 to 48 hours and weighed.

Liquid Scintillation Counting

Radioactivity in the samples was monitored with a model 6804 Nuclear Chicago liquid scintillation counter. Blanks were counted to determine background activity.

The dry protein precipitates were very insoluble. Consequently, they were ground to a fine consistency in a Ten Broeck gound glass tissue grinder with ethyl ether as the solvent. The resultant slurry was transferred to a scintillation vial containing a Cab-O-Sil gel scintillation medium. This medium contained 34.35 g of Cab-O-Sil, 4 g of PPO and 0.250 g of POPOP per liter of toluene (Ott et al., 1959 and Jackson, personal communication). The ether was evaporated in a convection hood and the thixotropic gel medium was shaken to evenly suspend the protein for counting. Samples were counted for times of 1 to 10 minutes. An error of less than five percent was obtained for all samples according to a standard chart composed by Aronoff (1956) depicting counting time in relation to percentage error. A Cab-O-Sil quenching curve was prepared after Wang and Willis (1965). This allowed the calculation of the specific activity (disintegrations per minute per mg) of the valine-C^{14} labeled protein.

Since all samples did not absorb the same amount of tracer per egg, it was necessary to measure the uptake of valine-C^{14}. For the first measurement, a 10 µl aliquot of the incubation medium in which whole eggs were immersed for 4 days, was taken before and after absorption and counted in the liquid scintillation counter. The difference in counts represented the amount absorbed by whole eggs. In the second set of measurements, the uptake of valine-C^{14} was determined for the different egg fractions as well as the whole egg samples. Aliquots of the crude lyophilized material were taken from each sample and homogenized in 0.5 ml of distilled water. The homogenates were counted in a p-dioxane scintillation medium containing 6 g of PPO, 0.568 g of POPOP and 120 g of naphthalene per liter of p-dioxane (Bush and Hansen, 1965). Another quenching curve was prepared for this scintillation medium. The specific activity of the extracted protein then was divided by the specific activity of the unextracted material to obtain percentage incorporation. This calculation allows samples to be compared even though they absorbed different levels of valine-C^{14}.

Disc Electrophoresis

Protein from the separate embryo fractions and yolk fractions was subjected to disc electrophoresis on polyacrylamide gels using a method modified from Davis (1964). The name of this technique was derived from its dependence on *disc*ontinuities in the electrophoretic matrix

and, coincidentally, from the discoid shape of the bands of separated ions (Ornstein, 1964). The polyacrylamide gel columns were formed in cylindrical glass tubes 65 mm long with an inner diameter of 5.5 mm. The gel was composed of three layers: 1) uppermost: a large pore (3.75 % acrylamide) sample gel 7 mm in length containing the protein ions; 2) middle: a large pore (3.75 % acrylamide) spacer gel 7 mm in length; and 3) bottom: a small pore (7 % acrylamide) separation gel 45 mm in length. The separation gel was chemically polymerized with ammonium persulfate as the catalyst and the large pore gels were photopolymerized. The large pore spacer gel was eliminated in most gels since it offered no improvement in the resolution of protein bands.

An eight-position plexiglass electrophoresis cell (Thurston, 1966) was built by the Instrument Service Shop at Montana State University. The cell consisted of separate upper and lower buffer reservoirs, each containing a platinum electrode. The gels were placed vertically (sample gel up) in double O-rings equidistant from the platinum electrode of the upper reservoir. When the buffer (Tris-glycine, pH 8.3) was added the upper end of the gel was in contact with the buffer in the upper reservoir and the lower end of the gel was in contact with the buffer in the lower reservoir. Current was applied at 3-4 milliamperes per tube at 250 to 350 V. The polarity was set so that the positive pole was the electrode in the bottom

reservoir. Therefore, all protein ions which were negatively charged at pH 8.3 migrated downward in the direction of the separation gel. Five drops of 0.001 % aqueous Bromphenol Blue were added to the upper reservoir as a tracking dye at the start of electrophoresis. After about 75 minutes of electrophoresis the tracking dye band had migrated to a point 1 cm from the lower end of the separation gel and electrophoresis was stopped. The gels were then removed from the tubes for staining. A separate cell, that accommodated larger tubes which were constricted at the bottom, was used for destaining. The protein, lipoprotein and polysaccaride-containing protein patterns of embryos and yolk in the pre-diapause, diapause and post-diapause periods of development were studied. Individual patterns within any one period displayed only minor differences and were not studied. It should be noted that several embryos or yolks from eggs in different stages of development were pooled. Several gels from at least two separate electrophoretic runs were studied to determine patterns for each of the three major developmental periods.

For each gel 3 to 6 mg of lyophilized embryo or yolk fractions were processed. The material was homogenized in 40 % sucrose in a Ten Broeck ground glass tissue grinder after Nielsen and Mills (1968). It was then centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatant solution was carefully removed and mixed with 2 volumes of the sample gel minus the sucrose solution of Davis (1964).

Over fifty percent of the yolk material was insoluble in 40 % sucrose. This insoluble material was also analyzed electrophoretically. It was dissolved in 0.6 M KCl with the aid of a cyclo-mixer and centrifuged at 12,000 rpm for 10 minutes at 4°C. A residue of about 5 % remained after this centrifugation. Sucrose was added to the supernatant solution to a concentration of about 40 % to facilitate polymerization. Two volumes of sample gel were added to the supernatant solution as described above and the material was divided among 3 gel tubes for electrophoresis. One gel was used for general protein staining and the others were stained for lipoprotein and polysaccharide-containing protein.

Protein Characterization

General protein staining.

Protein bands were discerned by staining gels for 1 hour in a solution of 1 % Amido Schwarz in 7 % acetic acid. Gels were destained by leaching or electrophoresis in 7 % acetic acid.

Periodic acid-Schiff reaction.

This reaction is well known for the detection of polysaccharides. PAS-positive protein bands were demonstrated by the method of Hotchkiss as described by Gurr (1958). The following steps were performed after electrophoretic separation: (1) the gels were oxidized in 0.8 % aqueous periodic acid for five minutes and washed in 70 % ethanol; (2) the gels were reduced for five minutes in an acidic solution (1 g of KI and 1 g of sodium thiosulfate dissolved in

20 ml of distilled water, 30 ml of absolute ethanol and 0.5 ml of 2 N HCl) and washed in 70 % ethanol; (3) the gels were placed in Schiff's reagent (Davenport, 1960) for ten minutes; and (4) the gels were washed in a solution of 0.2 g of potassium metabisulfite in 45.5 ml of distilled water and 0.5 ml of concentrated HCl. PAS-positive material stained a rose to purple color in Schiff's reagent. Bands became more apparent with prolonged sulfite washes and storage in 7 % acetic acid.

Since sucrose is PAS-positive and was included in the sample gel it was necessary to determine its effect. Control gels were subjected to electrophoresis: (1) without protein in the sample gel; (2) without sucrose in the sample gel; and (3) without protein or sucrose in the sample gel. PAS tests on these controls showed that sucrose in the sample gel does not interfere by entering the separation gel.

Lipoprotein staining.

Lipoid bands were visualized in the gels by the Sudan Black method after Wang and Patton (1968). A 0.5 % solution of Sudan Black B in a 3:3:4 mixture of ethanol-ethylene glycol-water was used. Brown to dark brown bands appeared after 5 hours of staining.

RESULTS AND DISCUSSION

Isotope Introduction and Egg Weight Changes

In most insect biochemical tracer studies the isotope is included in the diet, injected into the animal or put into an *in vitro* medium (Winteringham, 1962; Lockshin, 1966; Chefurka, 1965). Obviously dietary introduction cannot be used with the cleidoic eggs of *A. elliotti* and an *in vitro* study connotes disruption of egg structure which might interfere with normal embryogenesis. Likewise, injection of the eggs could result in injury and resultant wound repair might mask normal events (Harvey and Williams, 1961; Stevenson and Wyatt, 1962). The desiccation-absorption technique of isotope introduction circumvents these difficulties.

The average weight loss per day per egg during desiccation and the average weight gain per egg during four days of absorption are shown in Table I. Pre-diapause eggs less than 20 days old have fragile shells and lose weight readily upon desiccation. Shell fragility and weight loss upon desiccation decreases in eggs over 20 days old. These changes are thought to be due to serosal cuticle proliferation (Slifer, 1937; McFarlane, 1960). All of the egg samples exhibited gains in weight during the four day absorption period (Table I). Considerable variation in both loss and gain of weight was noted. Since uptake of valine-C^{14} was not always proportional to the weight gain during absorption, it was necessary to gain information regarding the

Table I. Changes in the weight of eggs from *A. elliotti* during desiccation and absorption.

Average weight loss per day per egg[a]		Days in state at the end of the absorption period		Average weight gain per egg after four days of absorption	
Series I	Series II	Series I	Series II	Series I	Series II
		Pre-diapause eggs at 25°C			
0.82 mg	0.54 mg	8 days	8 days	0.85 mg	0.50 mg
.95	.70	15	15	1.10	1.04
1.08	.76	15	16	1.46	.89
.31	.60	26	20	1.05	.68
.20	.09	36	28	1.27	1.68
.18	.12	42	35	1.33	.93
.16	.15	42	41	2.45	1.56
.17		42		2.32	
.13		52		2.42	
		Diapause eggs at 8°C			
.03	.02	26	26	1.79	1.18
.12	.04	50	45	1.71	1.85
.08	.13	76	51	1.89	1.66
.09	.08	140	110	1.11	2.21
.08		140[b]		1.88	
		Post-diapause eggs at 25°C			
.04	.04	1	1	2.14	1.08
.08	.04	3	3	2.52	.99
.11	.06	5	5	2.14	2.48
.23	.21	10	10	1.96	2.04
0.21	0.24	15	15	0.89	0.88

[a] Eggs were desiccated until one-tenth of their original weight was lost or for no more than four days.

[b] This sample was kept at 25°C, whereas all other diapause samples were stored at 8°C.

concentration of valine-C^{14} available for incorporation into protein (Figure 2, Table II). The use of this information is described in the following paragraphs.

Radioactive Valine Uptake Measurement

Two measurements of the concentration of valine-C^{14} in the samples were described earlier. The first measurement determined the amount of valine-C^{14} absorbed from the incubation medium by older eggs. This was accomplished by monitoring valine-C^{14} radioactivity in the incubation medium before and after absorption. The difference was assumed to be the amount absorbed. The equation $\frac{\text{valine-}C^{14}\text{ absorbed per egg}}{\text{valine-}C^{14}\text{ available per egg}} \times 100$ (see Figure 2) was used to calculate the percentage absorption of the isotope. Pre-diapause eggs were highly variable with respect to their absorption of the isotope, an observation noted previously by Bunde (1965). Structural changes in the shell could cause variable absorption of valine-C^{14}. Eggs became more resistant to desiccation as pre-diapause development progressed, while shell dry weight increased and shell fragility decreased. This may have been due to the processes of phenolic tanning and serosal cuticle deposition. These processes have been studied in other orthopterans by Slifer (1937), Mathée (1951) and McFarlane (1960).

Diapause and post-diapause samples were less variable with respect to the absorption of valine-C^{14} than were the pre-diapause samples. The percentage of absorption was high and appeared to increase in older eggs. The structure of the shell of some acridid eggs

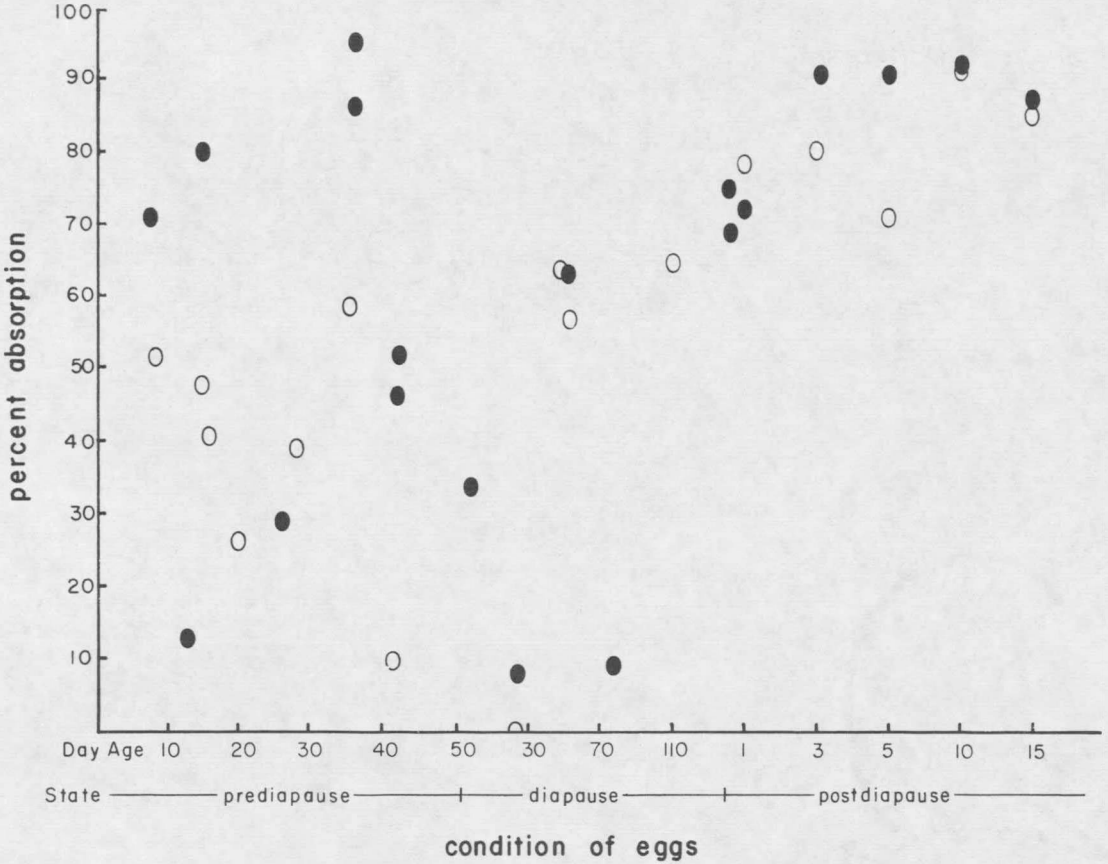


Figure 2. Percent absorption of valine-C^{14} by *A. elliotti* eggs during development. Series I whole egg samples—●. Series II egg samples (before fractioning)—O. Percent absorption was calculated from $\frac{\text{valine-}C^{14}\text{ absorption per egg}}{\text{valine-}C^{14}\text{ available per egg}} \times 100$.

Table II. Valine-C^{14} uptake measurements expressed in disintegrations per minute per *A. elliotti* egg or egg fraction.

Whole egg samples		Egg fraction samples			
Age of sample	Average dpm/egg	Age of sample	Average dpm/embryo	Average dpm/yolk	Average dpm/shell
		Pre-diapause eggs at 25°C			
8 days	12,568	8 days		20,493	2,326
15	22,458	15		25,358	3,274
15	29,800	16		9,887	2,110
26	24,598	20		16,454	8,230
36	60,406	28	437	3,414	618
36	76,537	35	1,001	2,180	1,499
42	32,049	41	1,339	12,950	10,150
42	39,675				
52	88,476				
		Diapause eggs at 8°C			
26	17,033	26	36	716	810
50	1,544	45	199	1,540	1,991
76	3,060	51	476	1,616	1,471
140	90,895	110	640	5,043	3,924
140[a]	37,905				
		Post-diapause eggs at 25°C			
1	45,341	1	1,238	17,113	22,847
3	73,170	3	3,922	11,620	16,761
5	99,013	5	1,728	32,209	19,365
10	81,967	10	8,537	13,362	24,242
15	39,639	15	5,578	10,185	18,327

[a] This sample was kept at 25°C, whereas all other diapause samples were stored at 8°C.

such as *M. differentialis*, has been reported to be nearly complete at the time the egg enters diapause (Slifer, 1937). If the development of the egg shell is similar in *A. elliotti* the differential absorption rate observed during pre-diapause could be due to changes in the shell at this time. Absorption of acetate also was found to be more constant during diapause and post-diapause in eggs of *A. elliotti* (Bunde, 1965). The measurements described above determined the uptake of valine-C^{14} by whole eggs. Data derived from these measurements were used to show percentage absorption of valine-C^{14} but were not used to correct samples for variable isotope concentration.

Embryos, yolk and shells did not take up valine-C^{14} in proportion to their weights. Since it was necessary to know the amount of valine-C^{14} available for incorporation into protein in each egg fraction to determine meaningful incorporation values, another set of concentration measurements was obtained. An aliquot of the crude lyophilized material from each whole egg sample and each egg fraction sample was homogenized and counted as described earlier. These counts were used to calculate the specific activity of valine-C^{14} for all samples (Table II). This information was used to correct for variable isotope uptake and will be elaborated on in a later section.

Dry Weights of Whole Eggs and Egg Fraction Samples

The average dry weight per whole egg or egg fraction was determined for all samples after lyophilization (Table III, Figure 3). The

Table III. Percent dry weights[a] of whole eggs and egg fractions from *A. elliotti*.

Age of samples:	Whole egg samples:	Age of samples:	Whole egg[b] samples:	Egg fraction samples:		
Series I	Series I	Series II	Series II	Series II Embryo	Series II Yolk	Series II Shell
		Pre-diapause eggs at 25°C				
8 days	46.17 %	8 days	44.68 %		43.69 %	0.99 %
15	45.32	15	45.73		44.77	.96
15	39.35	16	41.91		41.42	.49
26	36.38	20	46.13		44.81	1.32
36	37.80	28	38.94	1.01 %	36.72	1.21
36	34.40	35	36.39	2.09	33.15	1.15
42	34.92	41	31.22	2.70	27.20	1.32
42	37.13					
52	30.02					
		Diapause eggs at 8°C				
26	29.83	26	30.22	3.80	25.09	1.33
50	29.73	45	27.86	3.88	22.86	1.12
76	27.19	51	32.98	5.62	26.10	1.16
140	27.81	110	26.69	3.71	21.65	1.33
140[c]	25.93					
		Post-diapause eggs at 25°C				
1	26.34	1	28.75	4.14	23.45	1.16
3	26.83	3	27.45	4.06	22.20	1.19
5	29.20	5	27.33	3.86	22.29	1.18
10	26.69	10	27.20	6.95	19.05	1.20
15	31.35	15	28.96	8.85	19.12	0.99

[a] $\frac{\text{Average lyophilized weight per egg}}{\text{Average wet weight per egg after absorption}} \times 100.$

[b] The dry weights of the egg fractions were summed to calculate the percent dry weight of Series II whole eggs.

[c] This sample was kept at 25°C, whereas, all other diapause samples were stored at 8°C.

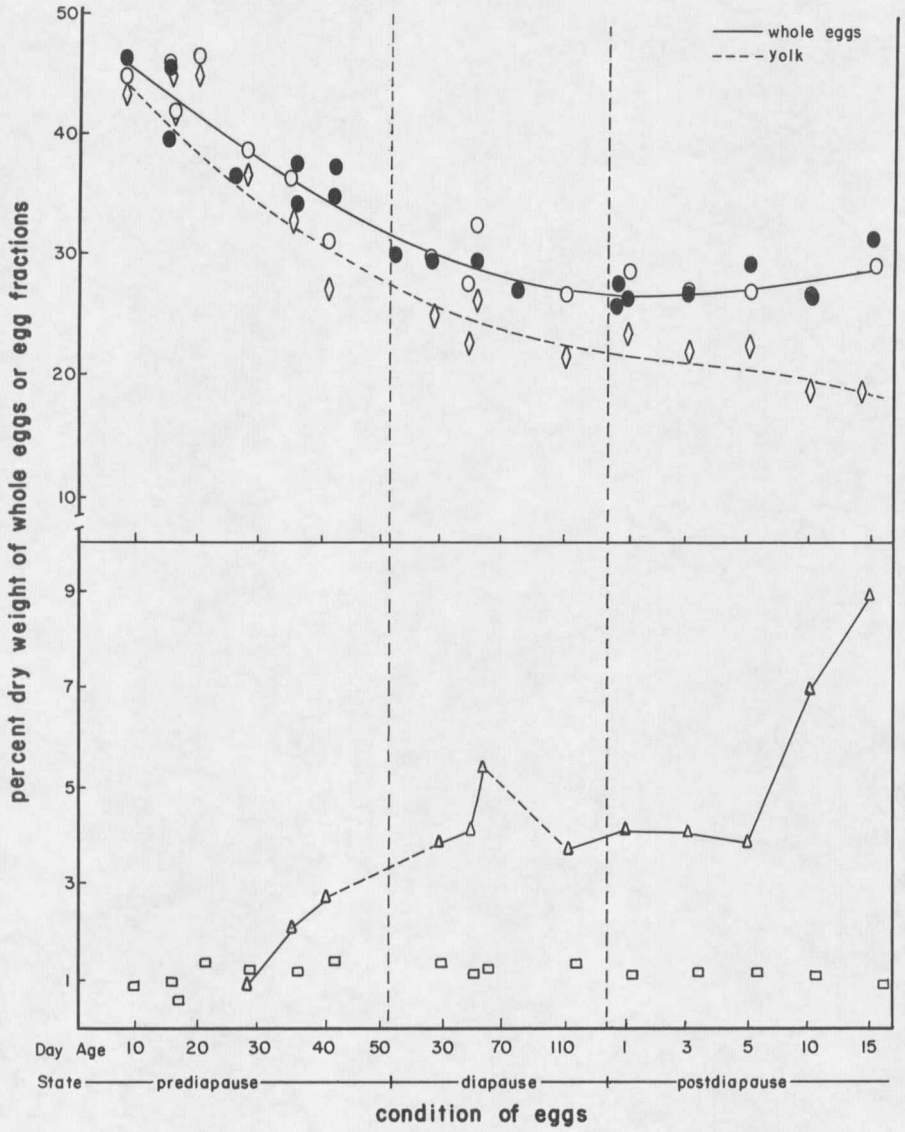


Figure 3. Percent dry weight of *A. elliotti* whole eggs and egg fractions. Series I whole eggs—●. Series II eggs (sum of egg fractions) —○. Series II yolk fraction—◇. Series II embryo fraction—△. Series II shell fraction—□. Percent dry weight is the product of $\frac{\text{average lyophilized weight per whole egg or egg fraction}}{\text{average wet weight per egg after absorption}} \times 100$.
Broken lines in the lower graph are used to denote time periods between samples that are too great to establish a trend.

dry weights of embryo, yolk and shell fractions of Series II were summed to obtain the whole egg values. Percentage dry weights for the whole egg samples and egg fraction samples were calculated as follows: $\frac{\text{average lyophilized weight per egg or egg fraction}}{\text{average wet weight per egg after absorption}} \times 100$. Percentage values are free of apparent changes due to size variations in the eggs. Quickenden (1969) determined percentage dry weight in 1 to 7-day-old pre-diapause *A. elliotti* eggs to be about 45 %. This is in agreement with the percentage dry weight values of 45 and 46 % found in two 8-day-old pre-diapause samples in the present study. The percentage dry weight of whole eggs showed that egg hydration increases during pre-diapause and remains high throughout the remainder of embryogenesis.

Embryos exhibited a gradual increase in dry weight throughout the pre-diapause period and for 50 days of diapause development. Embryonic dry weight shows a decrease to the early pre-diapause level after 110 days of exposure to 8°C during diapause and rises again during post-diapause development at 25°C. The material accumulated by the embryo during the transition from pre-diapause to diapause is apparently utilized during prolonged cold exposure while in the diapause state. The yolk displayed a gradual decline in percentage dry weight throughout development which is consistent with its use as a source of energy and anobolic materials. The dry weight of the egg shell showed a marked increase during pre-diapause which leveled off during diapause

and dropped in post-diapause just prior to hatching (Figure 4). The increase during pre-diapause was probably due to deposition of serosal cuticle as was mentioned earlier in relation to the decrease in shell fragility and resistance to desiccation. The decrease in the dry weight of the shell prior to hatching (post-diapause sample, 15 days old: Figure 3) can be attributed to the breakdown of the shell. Slifer (1937) noted a decrease in the thickness of the inner layer of the serosal cuticle during post-diapause in *M. differentialis* and Trowbridge and Bodine (1940) found a decrease in the total nitrogen of the shell in this species prior to hatching. Slifer (1937) attributed the decrease in cuticle thickness to the action of a hatching enzyme produced by the pleuropodia. Urban (1970) found that the pleuropodia of *A. elliotti* exhibited high protease activity in the embryonic exuviae, extra-embryonic fluid and on the inside of the shell. It appears that the decrease in the dry weight of the shell observed in the present study may have been due to enzymatic digestion of part of the shell.

Protein Content of Whole Eggs and Egg Fractions

The protein content of Series I and Series II samples was determined throughout embryogenesis. Protein was assayed by a modified biuret method. Most of the values are averages of several analyses: 2-6 analyses were performed on Series I whole egg samples and Series II yolk fraction samples were analyzed in triplicate; the values obtained for diapause embryos are the average of 2-3 analyses, except

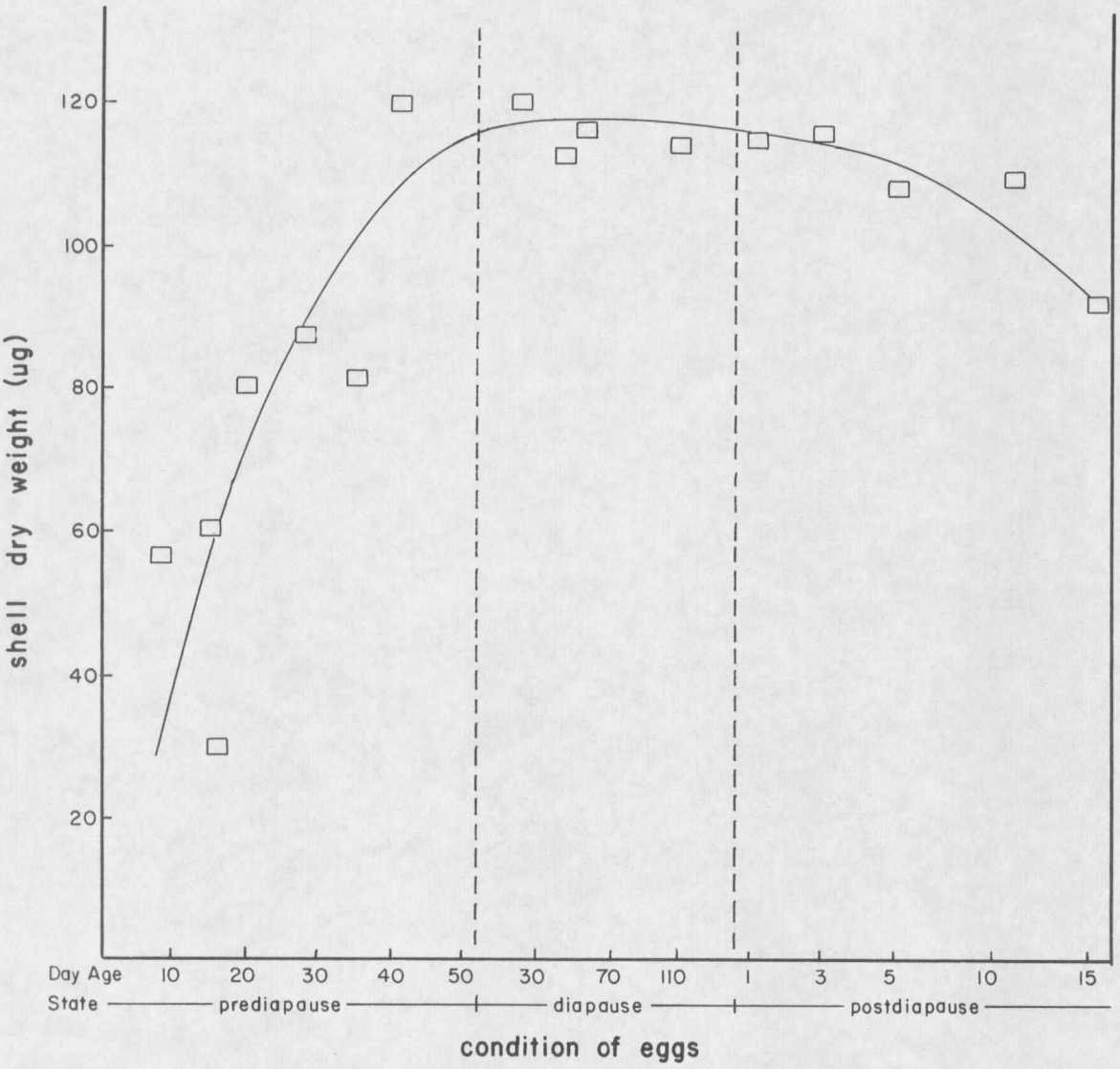


Figure 4. Shell dry weight of *A. elliotti* eggs.

for two samples with single analyses: single analyses were used for Series II shell fractions. The Q test (Skoog and West, 1963) was used to evaluate the validity of biuret readings from Series I whole egg samples. Three readings were rejected by this criterion.

Biuret determinations of the protein content of Series I whole eggs are presented in Table IV and Figure 5. The protein contents of the embryo, yolk and shell from Series II egg fraction samples were summed to obtain whole egg values (Table V, Figure 6). The most consistent observations in Figures 5 and 6 are that the protein content of whole eggs decreased during the first 26 days of diapause at 8°C, increased during the next 25 days and decreased after more than two months of cold exposure. The Series I whole egg samples maintained at 25°C for 140 days in the diapause state showed a slightly higher percentage of protein than the comparable sample maintained at 8°C (Table IV). The wide range of values and the lack of a sufficient number of samples make it difficult to interpret these results. The protein content of embryo fractions and yolk fractions is reported below as corroborative information.

In oviparous animals the yolk is consumed during embryogenesis as a source of energy and anabolic materials. It seems apparent that the protein content of the embryo should increase while that of the yolk decreases during *A. elliotti* embryogenesis. Changes which occur in protein levels, especially during the diapause period, however,

Table IV. Protein content of Series I whole eggs throughout the embryogenesis of *A. elliotti*.

Description of samples				Protein content	
Day age	Stages	Mean stage	No. of eggs	Average mg/egg	% protein[a]
		Pre-diapause eggs at 25°C			
8			18	1.78	59.14
15			13	2.03	79.61
26			22	1.75	59.52
36			25	1.40	46.35
36			25	1.42	52.59
42			24	1.68	57.93
42			18	1.58	54.43
52	19	19	34	1.46	52.31
		Diapause eggs at 8°C			
26	19	19	34	1.23	47.69
50	19	19	24	1.63	57.60
76	19	19	25	1.52	53.90
140	19-23	20.0	25	1.33	54.73
140[b]	19-23	19.6	27	1.38	62.16
		Post-diapause eggs at 25°C			
1	21-23	21.8	25	1.52	54.09
3	no data		25	1.57	57.93
5	20-25	22.6	23	1.77	62.11
10	25-26	25.5	24	1.56	57.14
15	25-27	26.2	25	1.48	52.86

[a] Percent protein was calculated from $\frac{\text{mg protein per egg}}{\text{mg dry weight per egg}} \times 100$.

[b] This sample was maintained at 25°C.

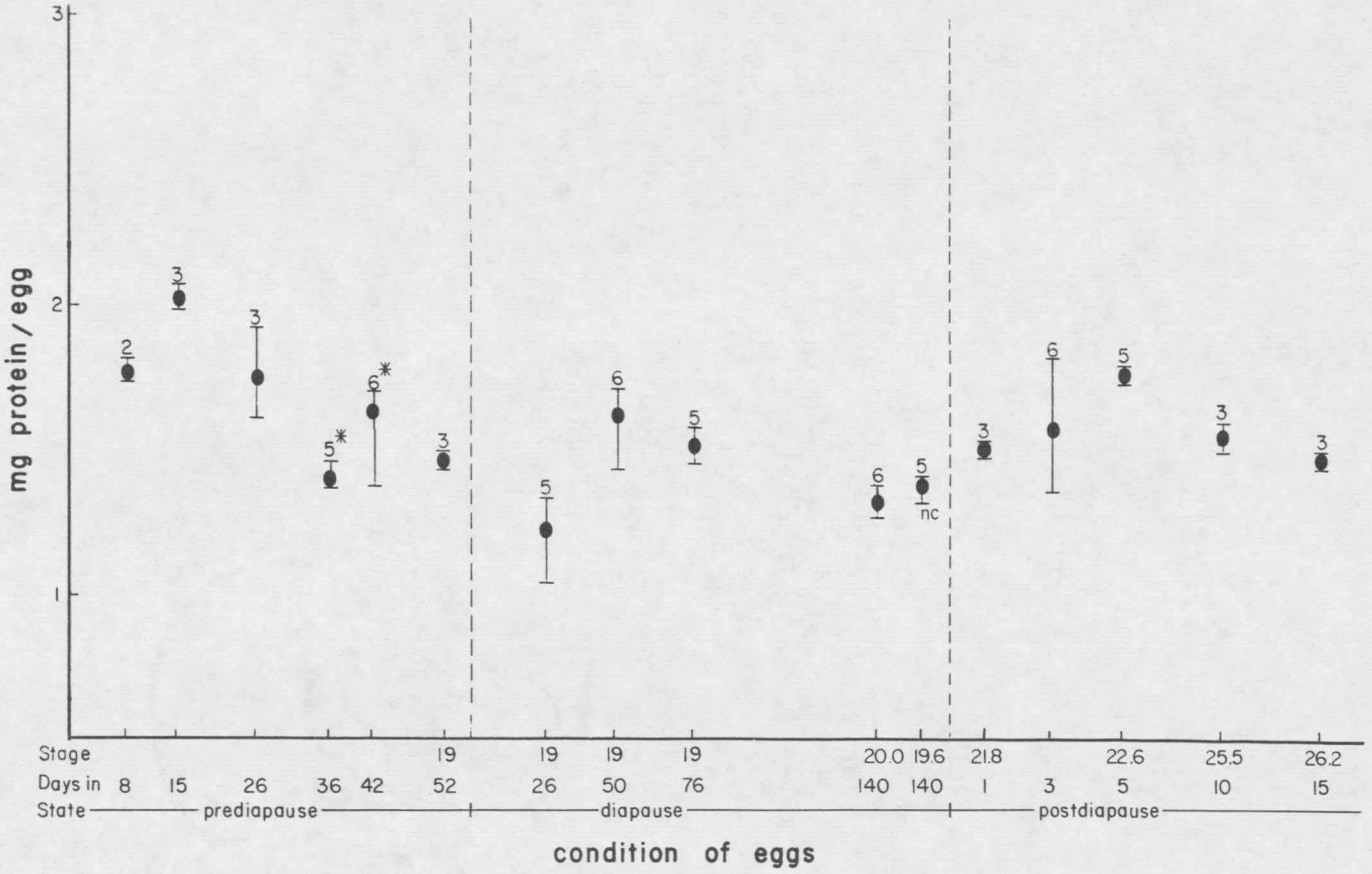


Figure 5. Protein content of Series I whole eggs throughout the embryogenesis of *A. elliotti*. Numerals above the symbols represent the number of analyses on that sample. *—denotes two samples. nc—denotes no cold exposure.

Table V. Protein content of Series II egg fraction samples from *A. elliotti*.

Description of samples				Average protein content per whole egg or egg fraction							
				Embryo		Yolk		Shell		Whole eggs[a]	
Day age	Stages	Mean stage	No. of eggs	mg	percent[b]	mg	percent[b]	mg	percent[b]	mg	percent[b]
				Pre-diapause eggs at 25°C							
8			20			1.71	63.56	23	41.03	1.73	64.31
15			20			1.49	54.78	17	56.63	1.51	54.32
16			20			1.50	53.38	17	28.57	1.52	54.09
20			46			1.43	55.86	12	13.34	1.42	53.79
28	9-12	10.4	86	0.040	57.97	1.37	53.94	31	35.84	1.44	53.33
35	13-16	14.6	48	.090	58.06	1.40	56.91	27	32.94	1.52	56.30
41	18-19	18.9	53	.114	47.93	1.44	59.02	51	42.32	1.61	57.50
				Diapause eggs at 8°C							
26	19	19	51	.125	37.54	1.19	54.19	30	25.49	1.34	50.94
45	19	19	47	.177	46.58	1.25	55.86	27	23.58	1.45	52.73
51	19	19	43	.239	45.70	1.36	56.11	29	25.75	1.63	53.10
110	19	19	47	.139	42.90	1.08	56.89	35	30.45	1.25	53.65
				Post-diapause eggs at 25°C							
1	19-24	20.5	50	.182	44.83	1.27	55.22	52	45.53	1.50	54.35
3	19-24	20.6	48	.164	41.08	1.25	56.87	34	26.64	1.45	53.31
5	19-24	21.1	41	.212	51.39	1.18	56.69	43	40.15	1.41	55.29
10	24-26	24.7	49	.371	57.84	1.08	60.00	33	29.67	1.39	54.09
15	25-27	26.0	50	.461	58.97	1.07	63.31	31	33.82	1.56	60.94

[a] The protein contents of embryo, yolk and shell fractions were summed to obtain whole egg values.

[b] Percent protein = $\frac{\text{average mg protein per whole egg or egg fraction}}{\text{average mg dry weight per whole egg or egg fraction}} \times 100$.

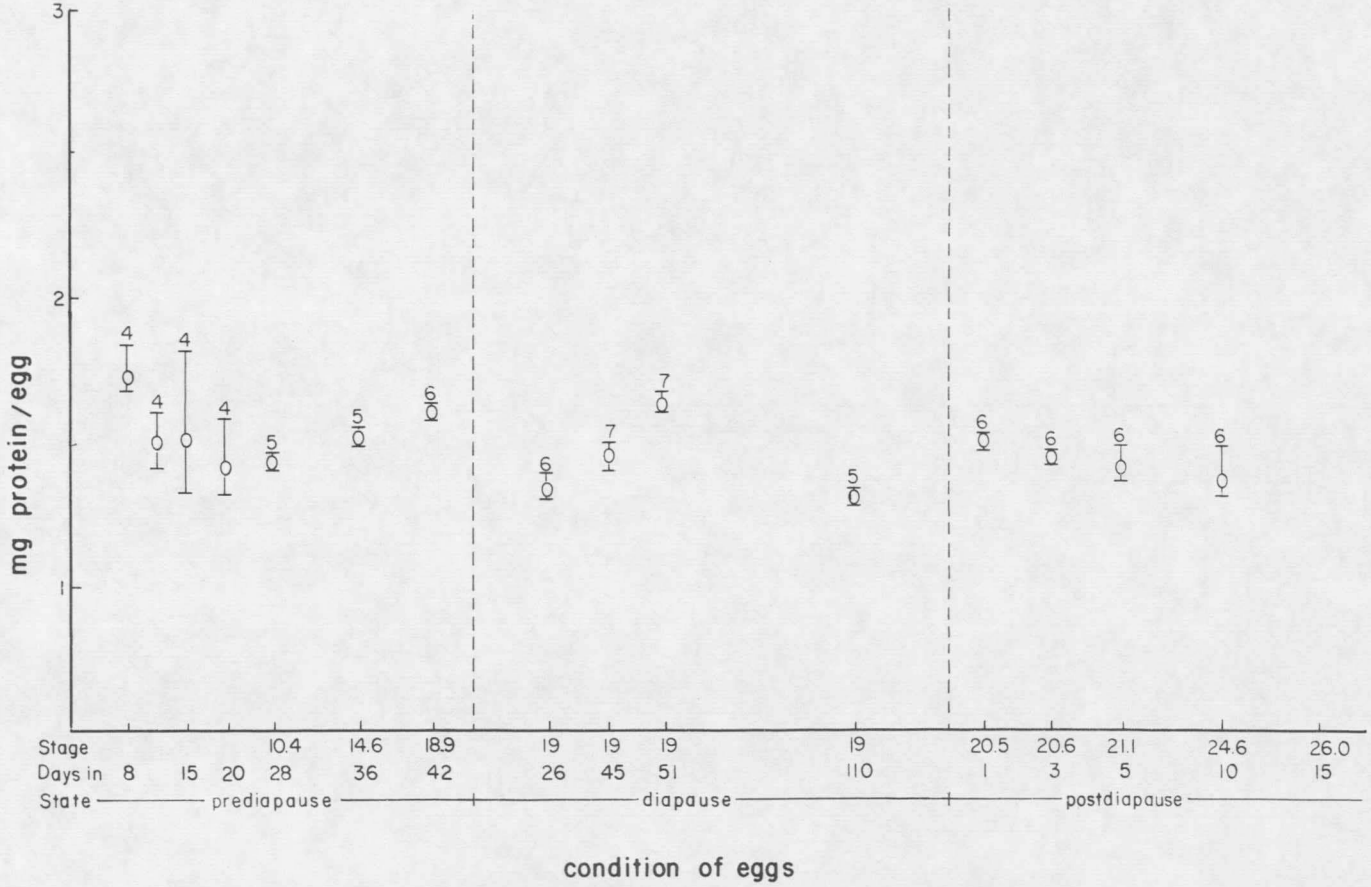


Figure 6. Protein content of Series II egg fraction samples throughout the embryogenesis of *A. elliotti*. Embryo, yolk and shell protein were summed to obtain the whole egg protein content. Numerals above the symbols represent the number of analyses on the sample.

may be of special interest.

The protein content of the embryo fractions exhibited an increase during pre-diapause development as shown in Table V and Figure 7. A slight increase in embryonic protein appeared during the transition from the pre-diapause to the diapause state. Two diapause samples exposed to the cold for 45 and 51 days respectively, showed increases in protein content which corroborate the increase seen in the egg as a whole (Figures 5 and 6) at about 50 days of diapause development. After 110 days of cold exposure the protein content had decreased almost to the early diapause level. Thus, an increase in protein during the first 50 days of diapause, followed by a decrease during prolonged cold exposure, may be typical of diapausing embryos. The first three post-diapause samples displayed little increase in protein which is probably related to the observation that many of the embryos in these samples were retarded as evidenced by their low average morphological stage (Van Horn, 1966b). Post-diapause embryo fractions from the eggs of two samples that had been removed from the cold for 10 and 15 days respectively, had high levels of protein.

Variation in the size of embryos may bring about differences in protein content between one sample and another. The use of percentage values will eliminate variation due to differences in weight and show the amount of protein in relation to the rest of the dry material of the embryo. The percentage of protein in embryos was calculated from

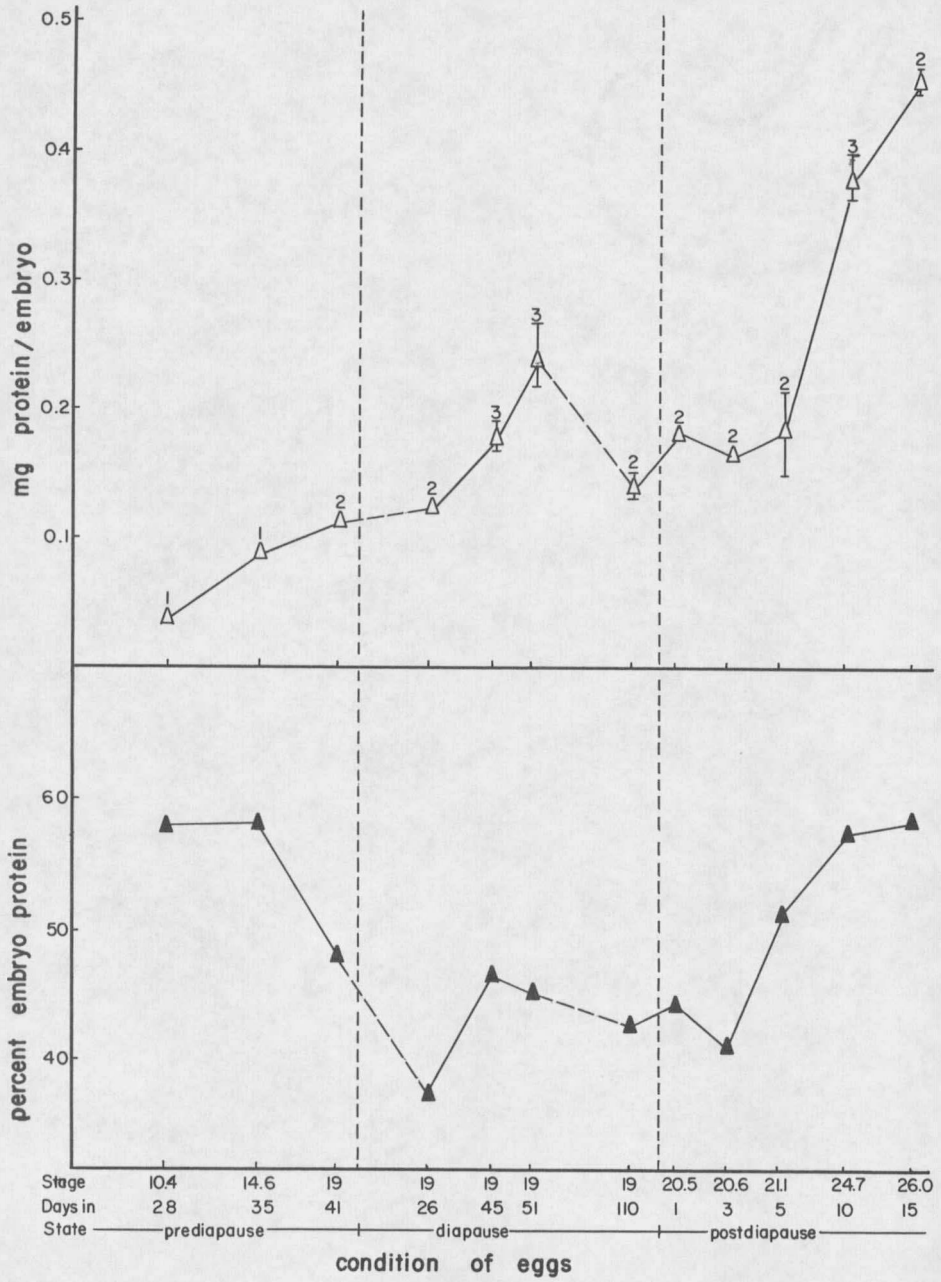


Figure 7. Protein content and percent protein in *A. elliotti* embryos throughout embryogenesis. Average protein per embryo in mg—Δ, percent embryo protein (calculated from $\frac{\text{average mg protein per embryo}}{\text{average mg dry weight per embryo}}$ X 100)—▲. Numerals above symbols-number of analyses. Broken lines-time period too great to establish a trend.

$\frac{\text{average mg protein per embryo}}{\text{average mg dry weight per embryo}}$ X 100. These calculations show that the percentage of protein in embryos drops about 20 % from the 35-day-old pre-diapause sample to the 26-day-old diapause sample (Figure 7). It has been stated, however, that the absolute protein content actually increased during this time. This means that there must be a substantial increase in the non-protein material of the embryo which masks the increase in protein content.

The protein content of the yolk displayed a gradual decline throughout embryogenesis (Table IV, Figure 8). The percentage of protein in yolk (calculated from $\frac{\text{average mg yolk protein}}{\text{average mg yolk dry weight}}$ X 100) was high in samples at the beginning and at the end of embryogenesis. A sharp increase was noted in the percentage of protein in yolk of 35 and 41-day-old pre-diapause samples. This could mean that there was a loss of non-protein material from the yolk to the embryo or that yolk protein was being formed. Data to be presented later shows that the former is probably most important while the latter may be partly responsible. The marked decrease in the percentage of protein in the embryo seen in the transition from late pre-diapause to diapause (Figure 7) and the increase in percentage yolk protein observed simultaneously (Figure 8) indicates that non-protein material was transferred from the yolk to the embryo at this time. Figure 9 is an idealized representation of the shift in the amounts of protein and non-protein material in the embryo throughout development. The sharp drop in percentage yolk

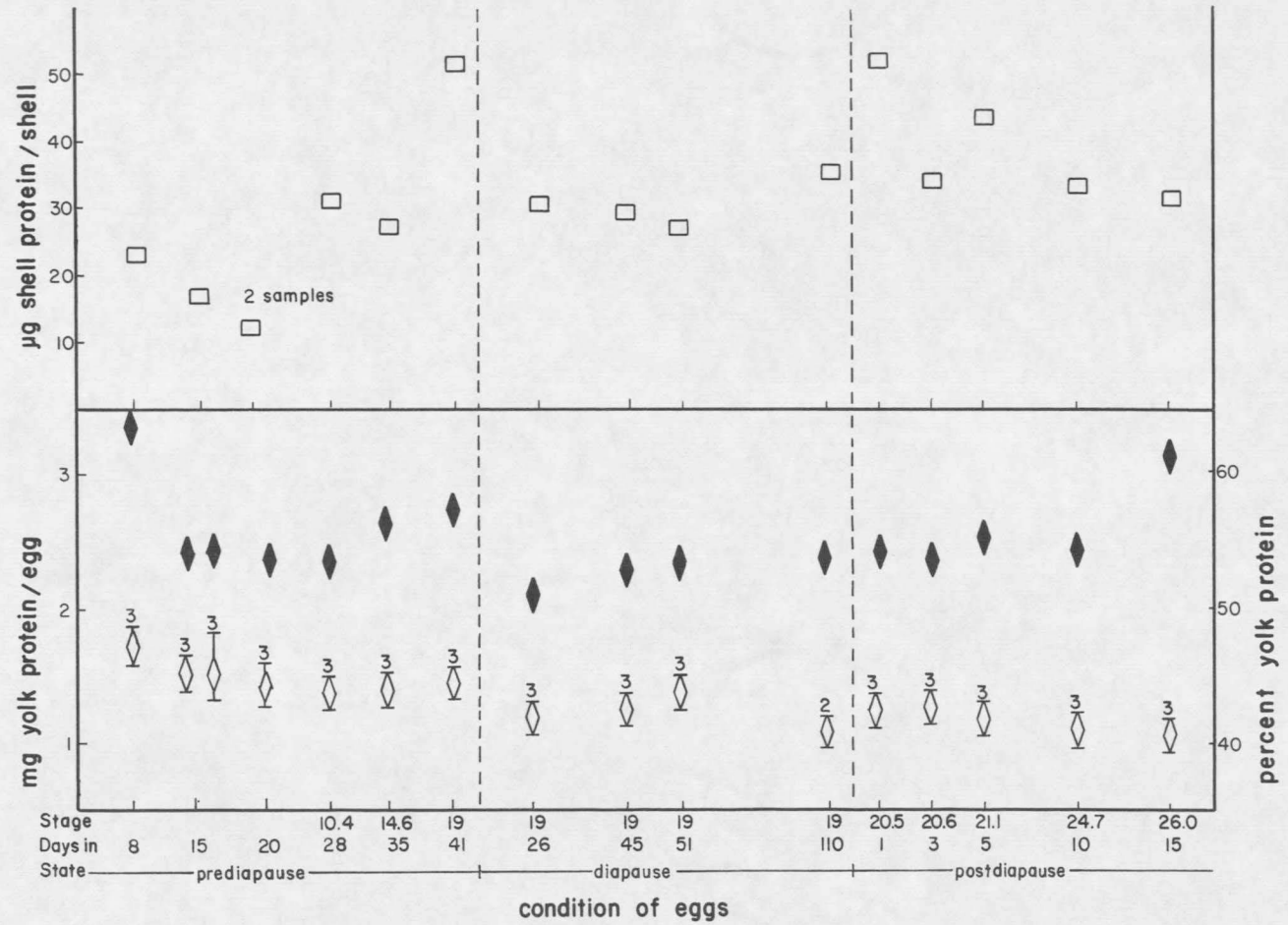


Figure 8. Protein content of the yolk fraction and shell fraction throughout the embryogenesis of *A. elliotti*. Average mg protein per yolk—◊, average µg protein per shell—□, percent yolk protein (calculated from $\frac{\text{average mg yolk protein per egg}}{\text{average mg yolk dry weight per egg}} \times 100$)—◆. Numerals above symbols represent the number of analyses on that sample.

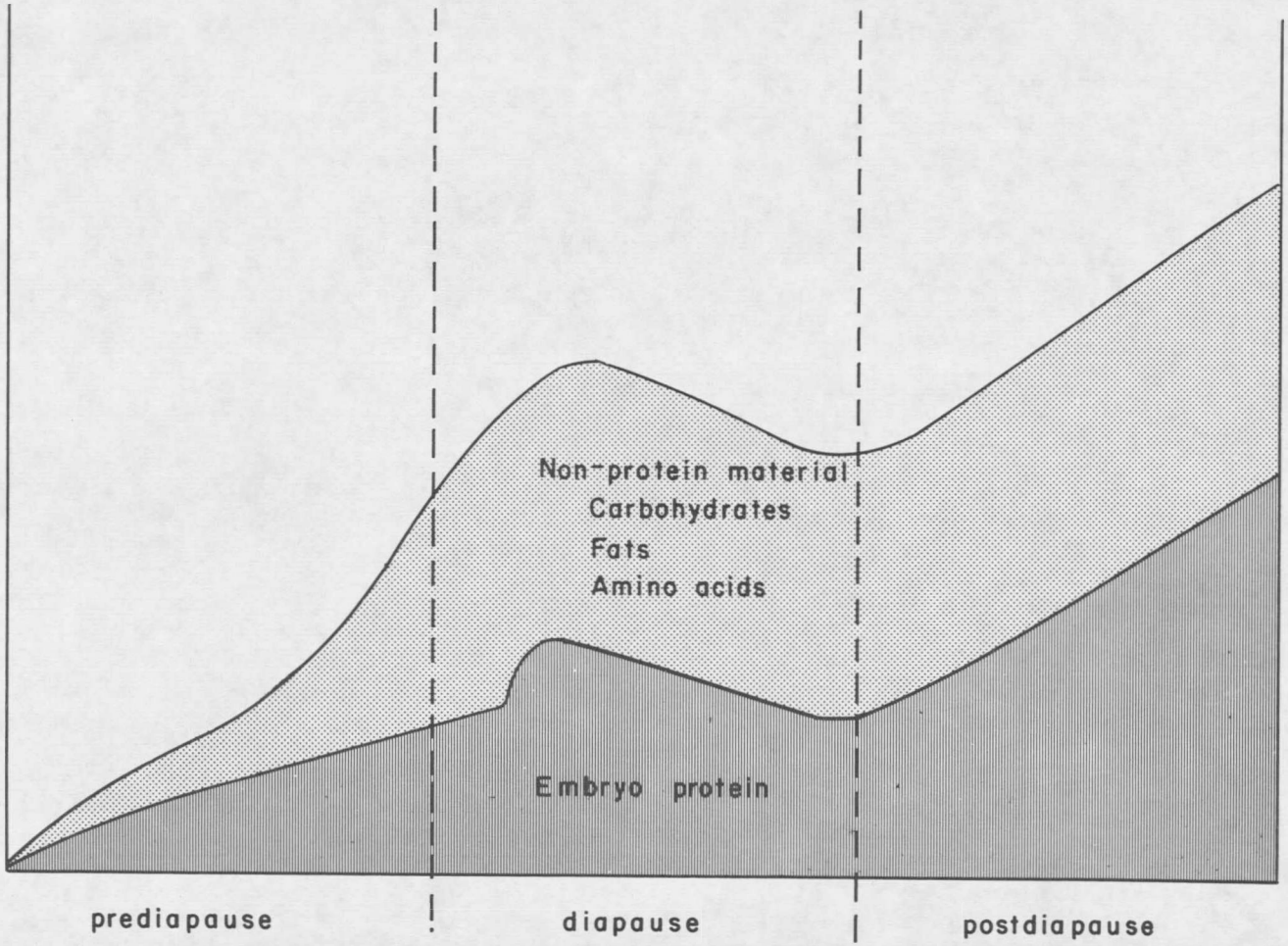


Figure 9. An idealized representation of the amount of protein and non-protein material in *A. elliotti* embryos throughout embryogenesis.

protein observed in the transition from pre-diapause to the diapause state was followed by little fluctuation until 15 days of post-diapause development. The percentage of yolk protein increased at that time.

Shell protein content appeared to be quite variable (Table IV, Figure 8). It should be noted that the highly insoluble nature of the shell protein was not conducive to accurate protein assay by the biuret method and since the shell fraction yielded a relatively small amount of material only single analyses were possible. A possible trend toward an increase in protein content was noted in late pre-diapause samples. This suggestion is supported by the observation that shell dry weights increased during pre-diapause (Table III, Figure 3).

As previously mentioned, it is generally accepted that total nitrogen remains constant throughout embryogenesis in the insect egg. Total nitrogen in whole eggs was determined in the present study by a micro-Kjeldahl technique (Kabat and Mayer, 1961). With the number of determinations in parentheses the average values for pre-diapause, diapause and post-diapause whole eggs respectively, are (4) 8.38, (2) 8.66 and (2) 8.48 % nitrogen. This indicates that total nitrogen was probably constant at about 8.5 % of the whole egg weight throughout development.

It has been reported that protein is not utilized to a great extent for energy in *Bombyx mori* or *M. differentialis* (Williams, 1967). As

mentioned earlier, Ludwig and Rothstein (1952) could find no evidence that protein was an energy source in *P. japonica* and uric acid production during the embryogenesis of *M. differentialis* corresponded to only 6.6 % of the egg protein (Bodine, 1946). The present study also indicates that protein is not utilized to a great extent as an energy source but is transferred for anabolic purposes. The total increase in embryonic protein shown in Figure 7 is very close to the amount of protein lost by the yolk during the same period (Figure 8). Several studies have demonstrated the transfer of yolk material to the embryo. Trowbridge and Bodine (1940) found that the transfer of nitrogen from the yolk to the embryo was quantitative and closely paralleled the growth curve of *M. differentialis*. The transfer of Cu and Fe followed the same pattern (Bodine and Wolkin, 1934; Bodine and Fitzgerald, 1948). These workers found pronounced increases in the transfer of these materials during pre-diapause and especially post-diapause development in *M. differentialis* while transfer during diapause was negligible.

The increase in yolk protein in 45 and 51-day-old diapause samples and, as reported earlier, the increase in embryo protein at the same time indicates that the embryo and/or yolk was synthesizing a significant amount of protein at this stage of development. Evidence to be presented later will show that most of the protein synthesis at this time occurred in the yolk (Figure 11).

Reports of synthesis during insect diapause have been criticized because of the possible injury response with the injection techniques used for isotope introduction. Harvey and Williams (1961) and Stevenson and Wyatt (1962) found increases in protein biosynthesis and O_2 consumption due to such wounding in the diapausing pupae of *Hyalophora cecropia*. The desiccation-absorption technique of isotope introduction used in the present study also might cause some injury to the eggs resulting in stimulated metabolic activity which could mask normal events. The eggs were handled carefully, however, and desiccation was carefully controlled. The data, therefore, are thought not to be influenced by injury response phenomena.

Roemhild (1961) noted that a small percentage (exact value not known) of *A. elliotti* eggs lose the connection between the serosa and the hydropyle during diapause. He found that this occurred in both chilled and non-chilled eggs and resulted in a 50 % increase in respiration. In the present study one sample of twenty-five stage-19 eggs incubated 140 days at 8°C had 24 eggs without the hydropylar connection and one that had completed blastokinesis. In another 25-egg sample of the same age, which was maintained at 25°C, 22 eggs had lost the connection and 3 had completed blastokinesis. No embryos in other diapause samples completed blastokinesis but a few (number undetermined) had lost their hydropylar connection. These embryos were still in stage 19 and no morphological change was evident. It will be remembered that all eggs were immersed in an aqueous incubation medium for 4 days prior to

sampling. The above observations suggest that the longer eggs are in the diapause state the more susceptible they are to losing their hydropylar connection and that this may be related to water absorption.

Roemhild (1967) reported that the fluid in the extra-serosal compartment of *A. elliotti*, which surrounds the hydropylar connection, has a higher osmotic pressure than the fluid in the epineural sinus. He found an opposite relationship between the total solids of these compartments. The epineural sinus contained high levels of protein while the extra-serosal compartment contained more free amino acids. The marked decrease in yolk protein noted from pre-diapause to diapause in the present study could be due to hydrolysis and transfer of some of the products to the extra-serosal compartment. If such a process continued during diapause and external contact water was available, the accumulation of small molecules in the extra-serosal compartment could result in hydration due to osmotic phenomena. Hydration occurred in the present study when eggs were immersed for 4 days in water and valine-C^{14} during isotope introduction. Eggs might be subjected to similar conditions of immersion during rainy weather in the field. The effects of such environmental conditions on the embryonic physiology should not be disregarded. For example, late in the autumn many eggs in the field have been at stage 19 for at least several weeks. If heavy rains occurred at this time excessive hydration of the egg could result in the premature rupture of the hydropylar

connection and bring about an increase in respiratory rate, yolk reserves then might be depleted before the warm temperatures necessary for post-diapause morphogenesis arrived, and result in limited survival.

Under natural conditions the obligatory diapause period of *A. elliotti* is temporally associated with cold environment. Certain biochemical transformations could increase cold-hardiness in diapausing eggs. Since freezing point depression is a colligative property, hydrolysis of a large molecule into its constituent subunits would depress the freezing point in proportion to the number of residues it contained. Several workers have reported increases in the concentration of small molecules in the diapausing eggs of *A. elliotti*.

Bunde (1965) found that the free amino acid content in whole eggs of *A. elliotti* was highest during the diapause state. High levels of trehalose and mannitol were found to be associated with the yolk in diapausing eggs of this species by Quickenden (1969, 1970). He also reported high levels of trehalose in the embryo fraction of diapausing eggs. A large increase in the size of the fat body of the embryo during diapause was reported by Van Horn (1963). The fat body is known to serve as a storage reservoir for proteinaceous and lipoid materials. Results of Svoboda (1964) showed increases in lipid in embryos of *A. elliotti* during diapause. His data showed that the weight of the lipid extract in samples containing 200 embryos increased

from 12.9 to 19.6 mg during the first two months of diapause development. In the same samples the weights of the triglycerides increased from 4.4 to 8.0 mg, although he explained that this could be due to variation in egg size or residual solvent. He also noted that after thin-layer chromatography both embryo and yolk showed spots corresponding to free fatty acids.

The Rate of Protein Synthesis in Whole Eggs and Egg Fractions

In this study it was found that a 4-day absorption period was necessary to introduce enough valine-C^{14} into the egg to show measurable incorporation into protein. The concentration of valine-C^{14} in each sample displayed considerable variation (Table II).

Higher concentrations of labeled amino acids in free amino acid pools should result in higher incorporation of them, up to a certain saturation limit. In order to quantitatively compare the values of valine-C^{14}, it was necessary to correct for variations in concentration due to variable uptake.

It has been found that the relationship between the concentration of a labeled amino acid within a sample and the specific activity of the extracted protein in the samples is linear (Hyden and Lange, 1968). Assuming that this linear relationship exists in the eggs of *A. elliotti*, it can be stated that the specific activity of the protein in one sample can be compared with that of other samples by dividing the specific activity of the protein by the concentration of the labeled amino acid

present.

The concentration of valine-C^{14} in the unextracted sample material is proportional to the specific activity of this material. Concentration measurements were obtained as described earlier. These data and the specific activity of the extracted protein as expressed in the following relationship:

$\frac{\text{specific activity of the extracted protein}}{\text{specific activity of unextracted material}} \times 100$ give the percentage incorporation of the isotope. This allows all samples to be compared even though they showed variable uptake of valine-C^{14}. The percentage incorporation values should be proportional to the rate of protein synthesis because of the linear relationship described above.

Protein was extracted from all samples by a technique modified from Wannemacher et al. (1965) to determine the incorporation of valine-C^{14}. The amount of protein extracted per egg or egg fraction is presented in Table VI. These values were not used to show the protein content of eggs or egg fractions because biuret determinations were deemed more efficient.

In cases where the amount of protein extracted was less than that indicated by biuret testing the protein extraction was assumed to be incomplete. In this instance the amount of protein per egg or egg fraction detected by biuret testing was divided into the weight of the extracted protein to determine the equivalent number of eggs, embryos, yolks or shells in the extracted protein. This enabled the calculation

Table VI. The amount of protein extracted from whole egg and egg fraction samples of *A. elliotti*.

Series I whole egg samples		Series II egg fraction samples			
	Extracted protein		Extracted protein		
Day age	Average mg/whole egg	Day age	Average mg/embryo	Average mg/yolk	Average µg/shell
	Pre-diapause eggs at 25°C				
8	0.67[b]	8		1.91	23
15	1.38[b]	15		2.00	17
26	.60[b]	16		2.00	12
36	1.39[b]	20		1.94	78
36	1.76	28	0.288	1.95	31
42	2.05	35	.106	2.04	66
42	0.82[b]	41	.175	2.00	108
52	2.03				
	Diapause eggs at 8°C				
26	1.54	26	.190	1.47	103
50	2.01	45	.230	1.43	101
76	1.95	51	.309	1.64	99
140	1.64	110	.226	1.26	91
140[a]	1.65				
	Post-diapause eggs at 25°C				
1	1.65	1	.249	1.10	94
3	1.69	3	.245	1.50	101
5	1.80	5	.189	1.59	89
10	1.80	10	.362	1.46	94
15	1.83	15	.454	1.34	78

[a] This sample was maintained at 25°C, whereas all other diapause samples were kept at 8°C.

[b] The original homogenate in this sample was made 0.3 N in KOH which caused a loss of protein in the first trichloroacetic acid extraction but did not effect biuret values since a standard curve corrected for KOH was used.

of dpm per egg or egg fraction since the egg equivalent of the protein in the counting vial was known. In cases where the weight of the extracted protein was more than could be accounted for by the biuret value it was assumed that protein extraction was complete but that the protein was contaminated with some non-protein materials. Here the activity of the extracted material was divided by the egg or egg fraction equivalent of the material before extraction to obtain specific activity in per egg or egg fraction units.

The data used to calculate percentage incorporation in the whole egg samples of Series I are presented in Table VII. The egg shells are thought to absorb or otherwise non-specifically bind valine-C^{14} and therefore, the incorporation values of whole eggs cannot be considered accurate. The apparent rate of protein synthesis in samples of whole eggs during pre-diapause reached a peak at 36 days of development and descended to the lowest level observed during the next 16 days at 25°C (Figure 10). All of the eggs in this 52-day-old sample were in stage 19, the morphological stage most characteristic of the diapause state. No staging data was obtained on the first seven pre-diapause samples of whole eggs or on the second post-diapause sample. The rate of protein synthesis in whole egg samples incubated at 8°C appeared to increase gradually during diapause development. It will be noted that the percentage incorporation is highest in two samples that were in diapause for 140 days. Of these two samples, the one with

Table VII. The apparent rate of protein synthesis[a] in Series I whole egg samples from *A. elliotti* measured as percent incorporation of valine-C^{14}.

Description of eggs				Whole egg samples		
Day age	Stages	Mean stage	No. of eggs	Average $\frac{dpm}{egg}$ EP[b]	÷	Average $\frac{dpm}{egg}$ UM[c] X 100 % inc.
			Pre-diapause eggs at 25°C			
8			18	838		12,568 6.67
15			13	2,271		29,800 7.62
26			22	6,591		24,598 28.26
36			25	29,816		60,406 49.36
36			25	32,778		76,537 42.83
42			24	6,659		32,049 20.78
42			18	6,024		39,675 15.18
52	19	19	34	2,416		88,476 2.73
			Diapause eggs at 8°C			
26	19	19	34	2,125		17,033 12.48
50	19	19	24	295		1,544 19.11
76	19	19	25	1,084		3,060 35.43
140	19-23	20.0	25	42,662		90,895 46.54
140[d]	19-23	19.6	27	19,419		37,905 51.23
			Post-diapause eggs at 25°C			
1	21-23	21.8	25	27,299		45,341 60.21
3	no data		25	41,208		73,170 56.32
5	20-25	22.6	23	51,679		94,013 52.19
10	25-26	25.5	24	47,537		81,967 58.00
15	25-27	26.2	25	37,020		39,639 93.39

[a] Because of non-specific binding of valine-C^{14} by egg shells the values cannot be considered representative of the real rate of protein synthesis.

[b] EP-extracted protein

[c] UM-unextracted material

[d] This sample was maintained at 25°C, whereas all other diapause samples were at 8°C.

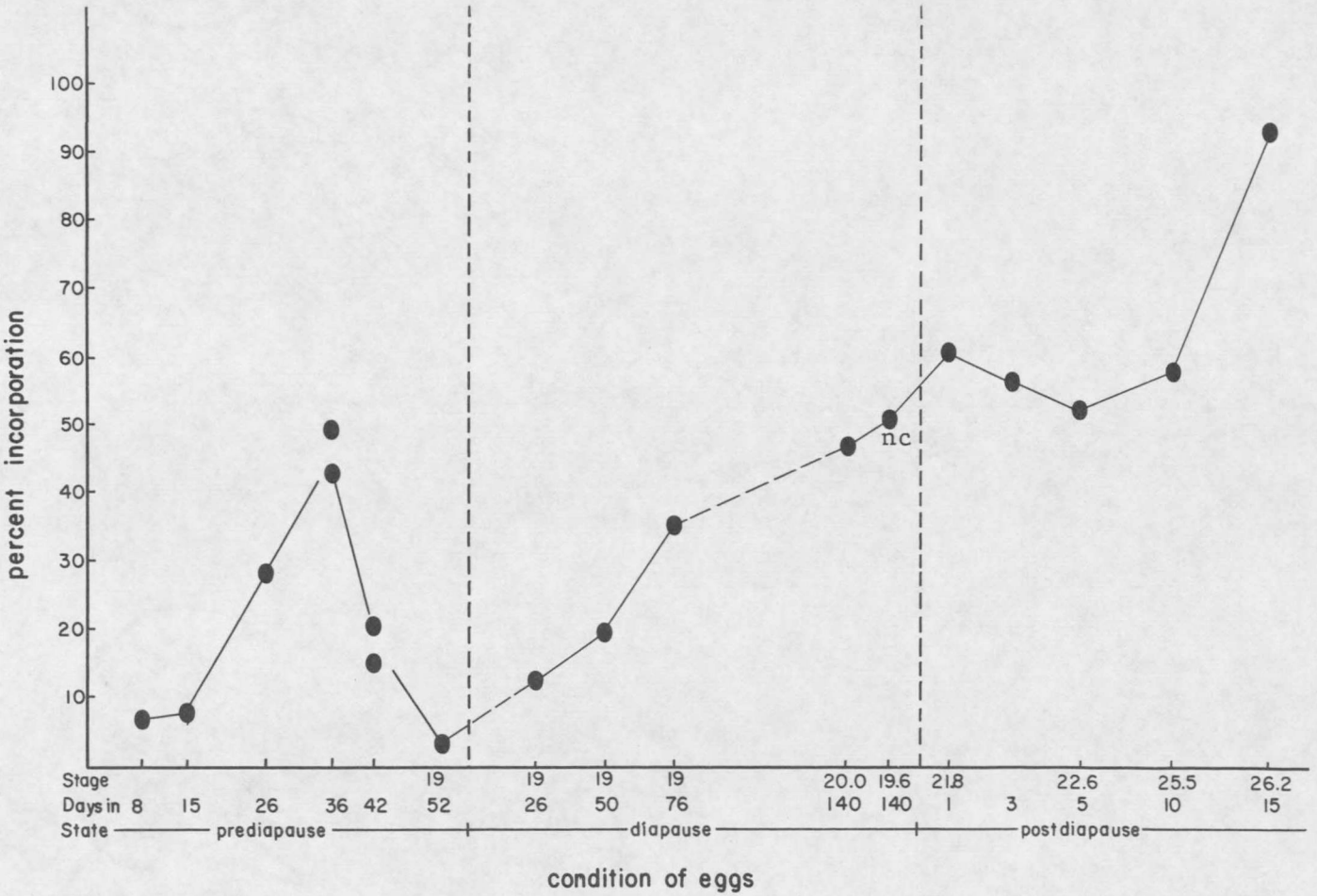


Figure 10. Percent incorporation of valine-C^{14} into protein in Series I whole egg samples throughout the embryogenesis of *A. elliotti*. Broken lines are used where the time period between samples is too great to establish a trend. nc-denotes no cold exposure.

a slightly higher percentage of incorporation was maintained at 25°C rather than at 8°C. Post-diapause samples that had been returned to 25°C for 1, 3, 5 and 10 days maintained a high level of protein synthesis with no apparent increase until 15 days at 25°C had elapsed. A sharp increase is noted at this time.

The rate of protein synthesis in egg fraction samples from Series II was similar to data obtained from the samples of whole eggs. Percentage incorporation in the embryonic fraction exhibited a peak during pre-diapause after 35 days of development at 25°C (Table VIII, Figure 11). At 41 days of development a marked decrease in the rate of embryonic protein synthesis appeared. A slight increase occurred after 26 days in diapause at 8°C; however, the uptake of valine-C^{14} and the specific activity of the extracted protein were so low that it is difficult to say that this increase is valid (Table VIII). The remaining fractions of diapause embryos showed very low levels of protein synthesis. The growth rate of the embryo, as evidenced by the average morphological stages in Figure 11, reflects the rate of protein synthesis of the embryo during post-diapause development at 25°C.

The rate of protein synthesis detected in yolk fractions from Series II peaked at a rather low level after 35 days of pre-diapause development at 25°C (Figure 11). After 41 days at 25°C the average morphological stage was 18.9 indicating that most of the eggs had reached stage 19 which is characteristic of diapausing eggs. The

Table VIII. The rate of protein synthesis in Series II egg fraction samples from *A. elliotti* measured as percent incorporation.

Description of samples				Embryo fraction			Yolk fraction			Shell fraction		
Day age	Stages	Mean stage	No. of eggs	$\frac{dpm}{emb.}EP^a \div \frac{dpm}{emb.}UM^b \times 100 = \%$ inc.			$\frac{dpm}{yolk}EP \div \frac{dpm}{yolk}UM \times 100 = \%$ inc.			$\frac{dpm}{shell}EP \div \frac{dpm}{shell}UM \times 100 = \%$ inc.		
Pre-diapause eggs at 25°C												
8			20				1,319	20,493	6.44	594	2,326	25.94
15			20				975	25,358	3.84	487	3,274	14.87
16			20				491	9,887	4.97	709	2,110	33.60
20			46				443	16,454	2.69	5,081	8,230	61.74
28	9-12	10.4	86	62	437	14.19	262	3,414	7.67	95	618	15.37
35	13-16	14.6	48	281	1,001	28.07	235	2,180	10.78	721	1,499	48.10
41	18-19	18.9	53	55	1,339	4.11	439	12,950	3.39	3,418	10,150	33.60
Diapause eggs at 8°C												
26	19	19	51	2	36	5.56	114	716	15.92	356	810	43.95
45	19	19	47	6	476	1.26	576	1,616	35.64	1,068	1,471	72.60
51	19	19	43	2	199	1.01	85	1,540	5.52	546	1,991	27.42
110	19	19	47	6	640	.94	1,137	5,043	22.55	1,977	3,924	50.38
Post-diapause eggs at 25°C												
1	19-24	20.5	50	93	1,238	7.51	7,396	17,113	43.22	11,484	22,847	50.26
3	19-24	20.6	48	713	3,922	18.18	4,147	11,620	35.69	10,020	16,761	59.78
5	19-24	21.1	41	278	1,728	16.09	12,985	32,209	40.31	11,284	19,365	58.27
10	24-26	24.7	49	2,195	8,537	25.71	6,084	13,862	43.89	15,469	24,242	63.81
15	25-27	26.0	50	3,176	5,578	56.94	5,918	10,185	58.11	9,535	18,327	52.03

[a] EP-extracted protein

[b] UM-unextracted material

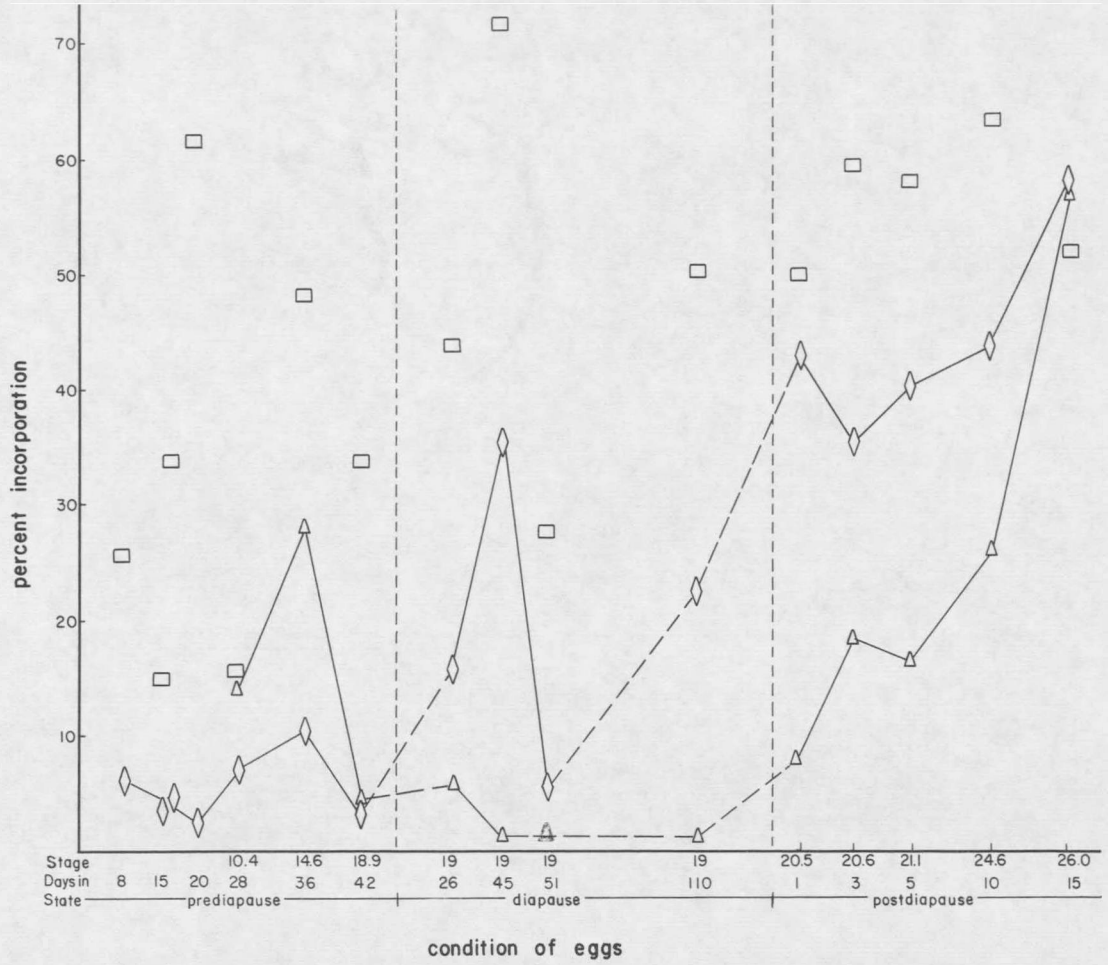


Figure 11. Percent incorporation of valine-C^{14} into protein in Series II egg fraction samples throughout the embryogenesis of *A. elliotti*. Embryo-Δ, yolk-◊, shell-□. Broken lines indicate that the time period between samples is too great to establish a trend.

percentage of incorporation decreased in the yolk fraction at this time, although not as sharply as it did in the embryo fraction. During the first 26 days of diapause development at 8°C the yolk fraction exhibited a higher rate of protein synthetic activity than that noted during pre-diapause, and after 45 days at 8°C, this rate had more than doubled. After 51 days at 8°C, the percentage of incorporation in the yolk fraction showed an abrupt decrease. The yolk in the 110-day-old diapause sample had an incorporation value of 25 %. The yolk fraction displayed a high rate of protein synthesis during post-diapause development at 25°C.

The percentage of incorporation values obtained for the shell fraction exhibited wide variation during pre-diapause and diapause development (Figure 11). The oldest diapause samples and all of the post-diapause samples showed more consistent and high incorporation values. It will be recalled that the shell was thought to bind the isotope non-specifically. In the following discussion of information obtained on the rate of protein synthesis in whole eggs and egg fractions, attempts to determine the nature of the association of valine-C^{14} with the shell will be described.

Measurements of the rate of protein synthesis are complicated by the simultaneous breakdown of protein and reutilization of breakdown products. The four-day absorption period for isotope introduction used in the present study gives ample time for such processes to reincorporate the isotope. However, the continuous supply of isotope to

the egg during this time should counteract the effect of protein breakdown somewhat and since "total" protein was extracted the recycling of the isotope from one protein molecule to another would not result in completely fallacious values.

Ambiguous results also may be obtained with the use of C^{14}- or H^{3}- labeled amino acids because of non-specific binding of the amino acids to proteins and other cellular constituents (Haurowitz, 1963). Detection techniques used in monitoring the rate of protein synthesis are subject to this phenomenon. Liquid scintillation counting is a sensitive technique and allows the detection of very small amounts of radioactivity. For example, the specific activity of diapause embryos was only 2-6 dpm per embryo (Table VII). Whether such a small amount of activity represents protein biosynthesis or just non-specific binding of valine-C^{14} to a protein is unknown, although it seems that necessary maintenance functions could account for at least that much incorporation of valine-C^{14}.

Relatively high specific activity values (dpm/shell) were found in shell fractions (Table VII). These high values seem more significant when one considers that each shell comprises less than 5 % of the whole egg dry weight (Table III). Acridid egg shells are reported to be non-cellular while protein synthesis has always been found to be associated with cellular structures. Although an intensive study of *A. elliotti* egg shells has not been performed, general information about the egg

shells of other acridids has been presented by Slifer (1937), Mathée (1951) and Hartley (1961).

It appears that the only cells that could remain associated with the *A. elliotti* egg shell after fractioning would be the hydropylar cells and possibly fragments of the cellular serosa. As mentioned earlier, increases in dry weight and protein during pre-diapause (Figures 3 and 7) probably indicate the deposition of the serosal cuticles during this period. The high specific activity in the protein fraction of the shell during pre-diapause could be attributed to protein synthesis in serosal cells. During diapause and post-diapause no changes are noted in the dry weight or protein content of the shell other than a decrease in dry weight just before hatching. Although it was observed that the serosa no longer has contact with most of the shell as it did during pre-diapause, the specific activity of the material in the shell extract was still very high.

Hartley (1961) has shown that the chorions of newly-laid eggs in seven acridid species are constructed of a complicated meshwork as described in the introduction to the present study. Soon the serosal cuticles are laid down and these layers become the principle wall of the acridid egg (Slifer, 1937; Edney, 1957; and Hartley, 1961). Slifer (1946) has shown that water absorption occurs through a specialized region of the posterior end called the hydropyle in the egg of *M. differentialis*. Mathée (1951) states that this structure is secreted

by the specialized hydropylar cells of the serosa in *Locustana pardalina*. He finds that cytoplasmic filaments from these cells follow small pore canals in the white cuticle which lead directly to larger pore canals in the outer or yellow cuticle. He also depicts pore canals in the serosal cuticles in the remainder of the egg shell. If such an arrangement exists in *A. elliotti* then valine-C^{14} could be trapped in these canals. Mathée (1951) did not find a waxy layer between the white and yellow portions of the serosal cuticle in the hydropyle region indicating that this area is specialized for water absorption. It is interesting to note that Slifer (1946) proposed that a wax layer was secreted over the hydropyle region of *M. differentialis* eggs to prevent desiccation during diapause. Mathée (1951) found that a proteinaceous layer was secreted by the hydropyle cells of *L. pardalina* and that it behaved like a keratin membrane in preventing desiccation at low humidities. At 25°C this protein was secreted after 16 to 18 days of development. It would appear that such a phenomenon may be operating in eggs of *A. elliotti* since resistance to desiccation develops after a similar period (Table II). Mathée (1951) also noted that the protein dissolves or disappears before or simultaneously with water absorption. In the present study increases in weight were noted in all samples during the absorption period, indicating that there was no barrier to water uptake. Since the hydropylar cells are few in number in *A. elliotti* (Roemhild, 1961) and the amount of proteinaceous material

secreted would be very small; it seems unlikely that such a phenomenon could be responsible for more than a small portion of the radioactivity noted in the extracted protein from the shell. The formation of tanned lipoprotein in the yellow cuticle and protein-carbohydrate complexes in the white cuticle could account for considerable incorporation of valine-C^{14} during the formation of these structures in early pre-diapause development. However, high percentage incorporation values were observed throughout embryogenesis (Table VII, Figure 9). The best hypothesis, based on what is known about acridid egg shells, suggests that molecules may be trapped or bound within these structures.

To determine the state of the association of the valine-C^{14} in the shell, egg shells were removed from 50 post-diapause eggs and placed in an incubation medium containing valine-C^{14} for 4 days. Protein was extracted using the methods reported earlier and was monitored for radioactivity. The specific activity was almost identical to that of the shell fractions in which whole eggs were incubated *in vivo* This was s strong indication that the isotope was non-specifically bound or trapped by the shell. In another experiment the shells were removed from a group of post-diapause eggs and boiled for three minutes. Then they were immersed for two days in a solution of valine-C^{14} containing antibiotics to prevent protein synthesis by microorganisms. Protein extracted from these shells showed that less than 0.6 % of the isotope originally present in the incubation medium was associated with

the extracted protein. This low activity may mean that the boiling disrupted certain structural alignments of the shell which would normally trap valine. The same procedure was used to extract protein from samples of embryos and yolk. Similar low activity values were obtained indicating that the extraction technique was good and that only insignificant non-specific binding of valine occurred in these materials.

Since the values for the shell fraction probably do not represent a true rate of protein synthesis, it seems clear that the whole egg values would be affected. It can be seen, however, that high rates of protein synthesis coincident with major morphogenetic change are not masked by this binding phenomenon. For example, a sharp increase in the rate of protein synthesis was noted in whole eggs at 36 days of pre-diapause development in Figure 10 and was also present in the embryo fraction and to a lesser degree in the yolk fraction (Figure 11). These fractions would not be affected by the non-specific binding of valine-C^{14} by egg shells. The morphological stages of the embryos in these egg fraction samples ranged from stage 13 to 16 and the average stage was 14.6. From stages 13 to 16 many organs, tissues and cells are differentiating in *A. elliotti*. Van Horn (1963) observed differentiation of the endoskeletal elements at this time, including the anterior and posterior arms of the tentorium, the metathoracic apodemes of the femur, the coxal apodemes and the mandibular apodemes. She also noted the differentiation of the corpora allata, corpora cardiaca, ventral head glands and salivary glands at this time. Tracheal

diverticula, oenocytes, nerve ganglia, sense organs, Malpighian tubules, rectal pads and splanchnic musculature also show differentiation during stages 13 to 16.

When the embryo reaches stage 19 morphogenetic activity appears to cease. Most embryos do not reach this stage of development until after 40 days in the pre-diapause state (Van Horn, 1966a). Figures 10 and 11 show a marked decline in percentage incorporation from 36 to 42 days of development in pre-diapause for the whole egg, embryo fraction and yolk fraction samples. A whole egg sample that had been at 25°C for 52 days showed a very low rate of protein synthesis. All of the embryos in the sample were stage 19 and apparently were in diapause. It would be of interest to know how long this low level of protein synthesis would persist in eggs not exposed to the cold. All samples except one in the present study were placed at 8°C for diapause development. After 140 days of diapause development at 25°C this whole egg sample showed a high rate of protein synthesis similar to a comparable sample with cold exposure. This may be because all of the eggs in these samples had lost their hydropylar connection and it is presumed had elevated respiratory rates. The increase in respiratory rate reported to be associated with this phenomenon (Roemhild, 1961) may be related to the high rate of protein synthesis seen in whole eggs and, more specifically, the yolk fraction.

Disc Electrophoresis

Changes in the morphology and physiology in embryos of *A. elliotti* should be reflected in the composition of embryonic protein and yolk protein. Such changes have been detected in this study by electrophoretic means. The number and pattern of conjugated protein bands were studied to indicate whether protein was complexed with other metabolites. Increases in the number or staining intensity of such bands may indicate the importance of these metabolites during development.

Electrophoresis was performed as described earlier to determine the pattern of protein bands characteristic of 40 % sucrose-soluble embryonic protein, 40 % sucrose-soluble yolk protein and 0.6 N KCl-soluble yolk protein during each major developmental period. Less than half of the yolk was soluble in 40 % sucrose. The remainder was soluble in 0.6 N KCl. The number of gels used to determine the patterns characteristic of pre-diapause, diapause and post-diapause is shown above each gel diagram in Figures 12, 13 and 14. Separate gels were used for: (1) general protein staining with Amido-Schwarz; (2) "polysaccharide" detection with the periodic acid-Schiff reaction; and (3) lipoprotein staining with Sudan Black B. Only strongly positive PAS bands will be discussed since the specificity of this reaction is questionable. A strong reaction is given by compounds with 1,2-glycol groups such as alpha-glycerophosphate, mannitol and muscle glycogen, while serum albumin and casein also react (Gurr, 1958).

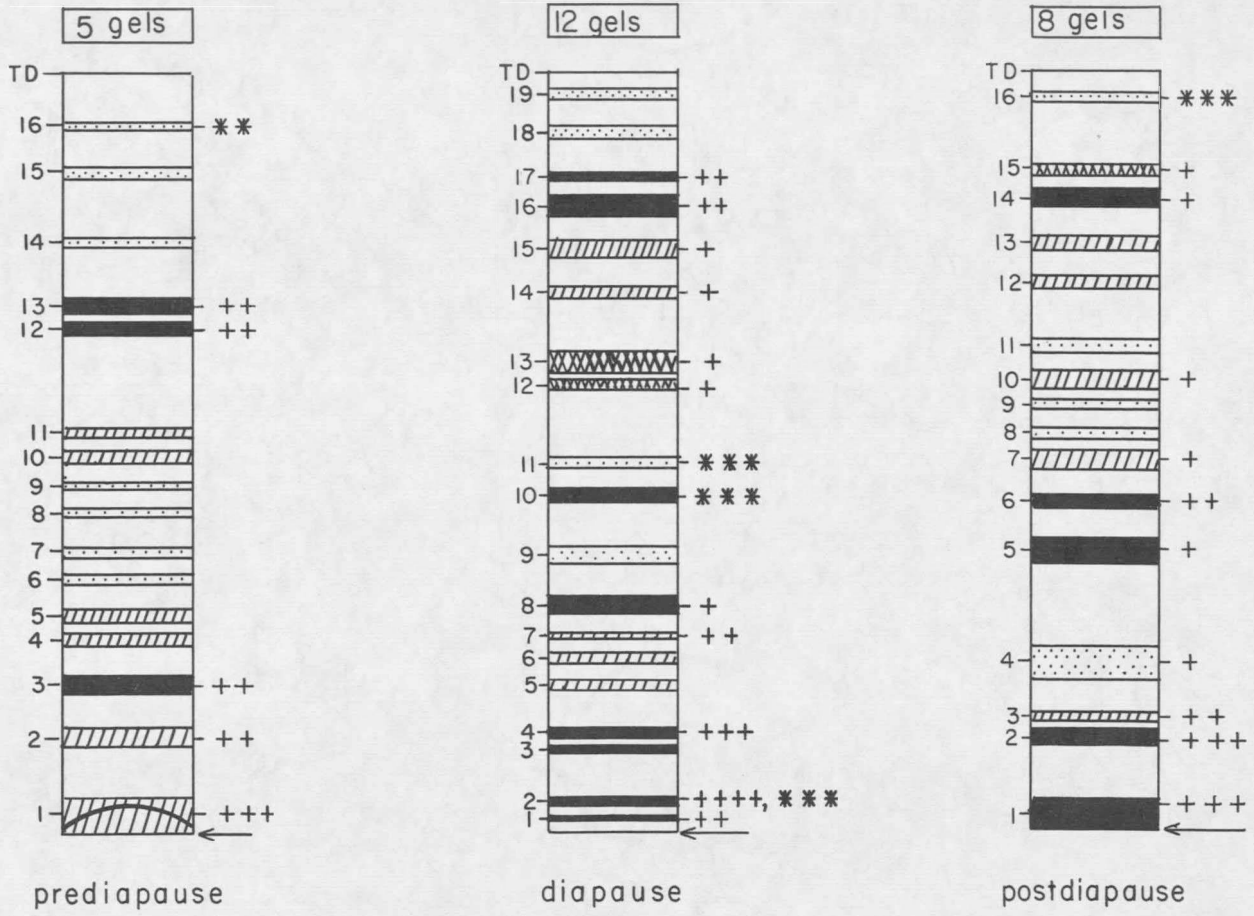


Figure 12. The electrophoretic patterns of 40 % sucrose-soluble embryo protein during the embryogenesis of *A. elliotti* and an indication that some of the protein bands are conjugated with carbohydrate and/or lipoid material. Origin ←, periodic acid-Schiff-positive —(+), Sudan Black B-positive—(*), tracking dye—TD. The number of (+) or (*) symbols indicate staining intensity. The number of gels used to determine each pattern is enclosed above each diagram.

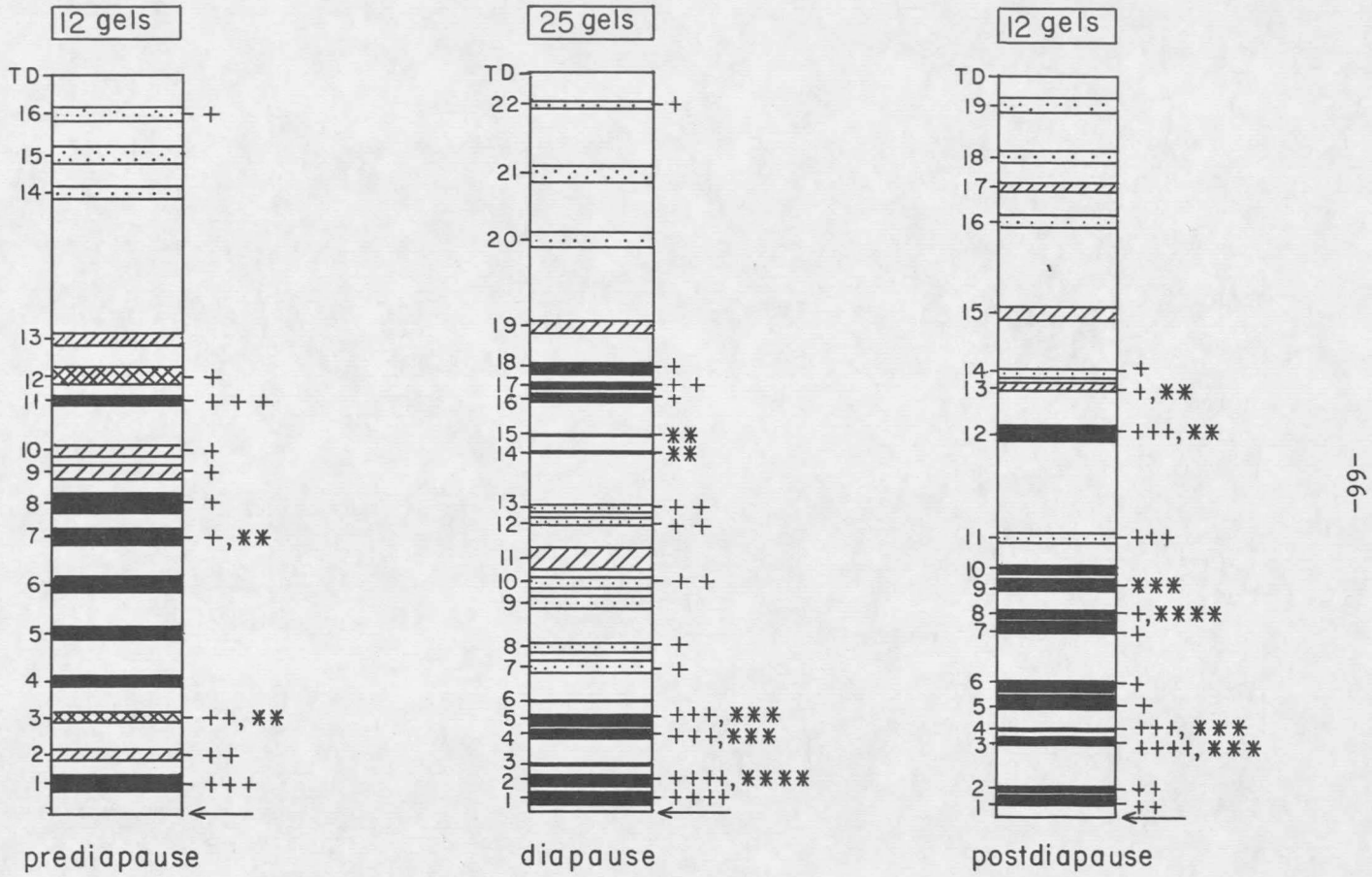


Figure 13. The electrophoretic patterns of 40 % sucrose-soluble yolk protein during the embryogenesis of *A. elliotti* and an indication that some of the protein bands are conjugated with carbohydrate and/or lipoid material. Origin ⟵, periodic acid-Schiff-positive—(+), Sudan Black B-positive—(*), tracking dye—TD. The number of (+) or (*) symbols indicate staining intensity. The number of gels used to determine each pattern is enclosed above each diagram.

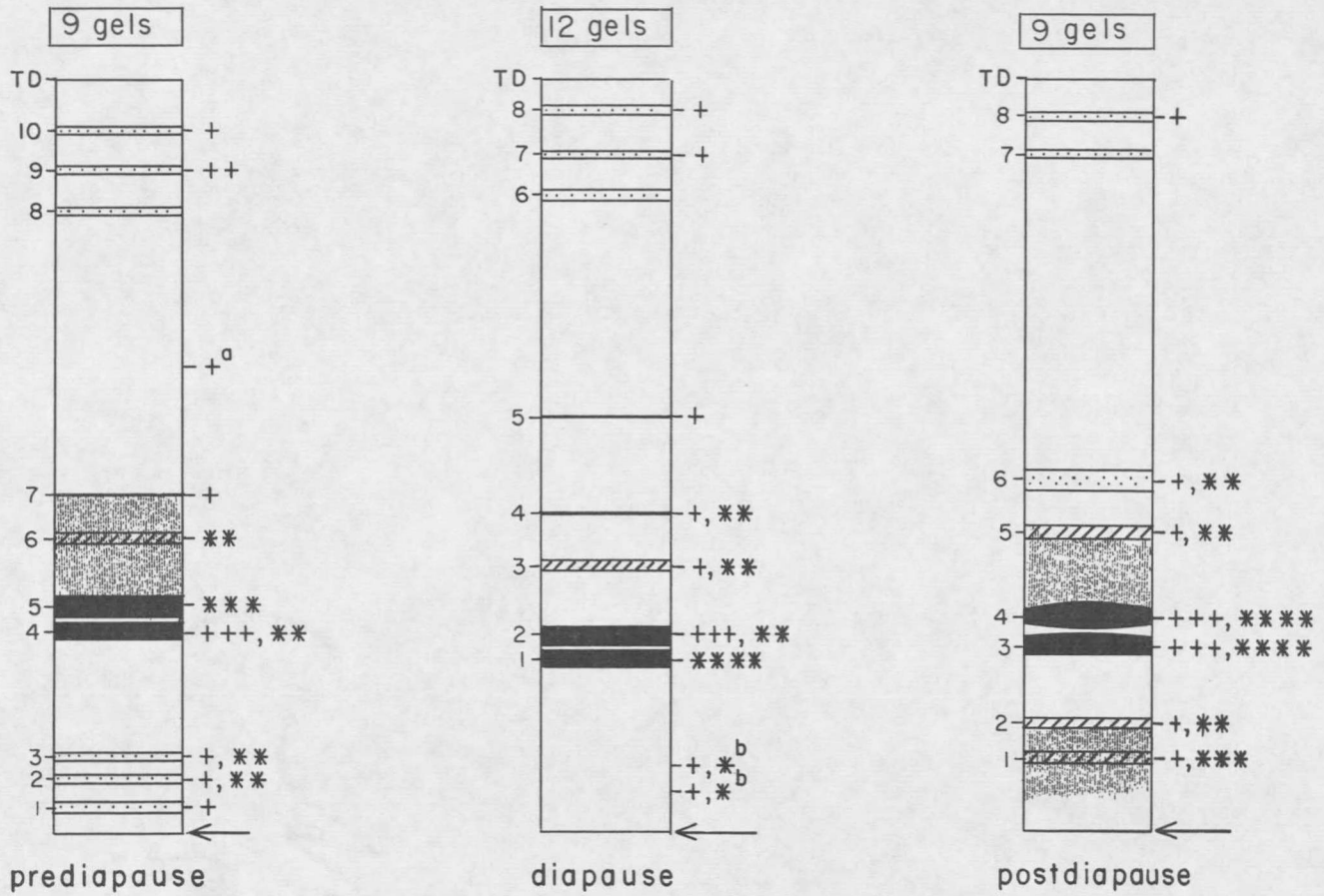


Figure 14. The electrophoretic patterns of 0.6 N KCl-soluble yolk protein during the embryogenesis of *A. elliotti* and an indication that some of the protein bands are conjugated with carbohydrate and/or lipoid material. Origin ⟵ , periodic acid-Schiff-positive —(+), Sudan Black B-positive —(*), tracking dye—TD. The number of (+) or (*) symbols indicate the staining intensity. The symbols with (a) or (b) superscripts represent carbohydrate and/or lipoid positive areas in which no protein was detected.

Some tests and observations relating to the specificity of this reaction will be discussed later.

Diapausing embryos displayed 3 more protein bands than did pre-diapause or post-diapause embryos (Figure 10). The number of intensely PAS-positive bands in the embryo increased by one from pre-diapause to diapause. Only one lipid-positive band of high mobility was present in pre-diapause and post-diapause embryos. Three new lipid-positive bands of low mobility were observed in diapause, none of which correlated with the fast band noted above.

Twenty-two protein bands were detected in 40 % sucrose-soluble yolk during diapause. Only 16 were found in pre-diapause yolk and 19 in post-diapause yolk (Figure 13). The three fastest bands in pre-diapause and diapause yolk are thought to be identical with bands 14, 15 and 16 shown in the pre-diapause embryo gel diagram of Figure 12. There were two strong PAS-positive bands in 40 % sucrose-soluble yolk during pre-diapause, four during diapause and three in post-diapause. Figure 13 shows that the number of Sudan Black B-positive bands increased from 2 to 5 during the transition from pre-diapause to diapause while post-diapause yolk had 6 bands.

The protein of the yolk that was soluble in 0.6 N KCl exhibited 10 protein bands in pre-diapause and 8 in diapause and post-diapause yolk (Figure 14). It appears that up to 3 bands in both the areas of low and high mobility in 0.6 N KCl-soluble yolk also are present in 40 %

sucrose-soluble yolk. The problem of determining whether the bands of intermediate mobility from 0.6 N KCl-soluble yolk protein correspond with certain areas in gel patterns derived from 40 % sucrose-soluble yolk is complicated by the fact that these bands apparently represent more than one protein. Wang and Patton (1968) found that some protein bands in the larval hemolymph of *G. mellonella*, *A. domesticus* and *T. molitor* were positive for as many as five different conjugated proteins. Some bands were both PAS- and lipid-positive in the present study. This is shown by the presence of both (+) and (*) symbols beside these bands in Figures 12, 13 and 14. Two heavily stained bands were found in the 0.6 N KCl-soluble yolk throughout development (Figure 14). They apparently represent the major proteins of the yolk and are lipoprotein in nature, although each band also gave a positive PAS reaction. The symbols ($+^a$) and ($*^b$) in Figure 14 represent areas of weak PAS and lipid staining in which no protein band was detected by Amido-Schwarz staining. This may mean that there was no protein present or that the amount was too low to be detected. The latter appears to be the case since protein bands in these areas did stain in gels from other developmental periods. The area marked ($+^a$) in the gel diagram for pre-diapause yolk corresponds with band 5 of the diapause gel. The gel diagram for diapause yolk has two lipid positive areas marked ($*^b$) in which no protein was detected. These areas correspond with bands 2 and 3 in the gel diagram for pre-diapause yolk and bands 1 and 2 of the gel diagram for post-diapause yolk.

Lipid-positive areas were indicated by the affinity of Sudan Black B dye for lipid material. Humason (1967) has noted that Sudan Black B is the most sensitive of the lipid dyes. The PAS reaction also is sensitive but does not appear to be very specific. The PAS technique used in this study was the same as that used by Wang and Patton (1968). They found large numbers of PAS-positive protein bands in insect hemolymph following electrophoresis. Large numbers of PAS-positive bands also were found in the present study, and some gels were found to give a positive reaction for every protein band. Haurowitz (1963) has stated that most, if not all, proteins contain small amounts of carbohydrate. Whether this is true or not, there are several structures other than those common to carbohydrates that can elicit a positive PAS reaction (Barka and Anderson, 1965; Gormori, 1952). The structures of interest in the present study are those associated with proteins. Barka and Anderson (1965) have theorized that certain amino acids in proteins could give a positive PAS reaction. To test this, the following proteins were subjected to electrophoresis individually in the present study; ribonuclease, D-amino acid oxidase, pepsin and bovine serum albumin. Each gave a positive PAS reaction although the bands were not intensely colored and appeared slowly. It also was noted that certain PAS-positive bands in the embryonic protein and yolk protein appeared rapidly and were intensely colored. These bands have been designated by three or four (+) symbols and are assumed to be

conjugated proteins containing a sizeable "polysaccharide" moiety. Weak PAS-positive bands that appeared slowly are designated with one or two (+) symbols. These areas are not considered to be representative of proteins conjugated with "polysaccharides" in the following discussion, since it has been shown that proteins not thought to be conjugated with a sizeable prosthetic group can give this type of a reaction. It is recognized that small quantities of a protein containing carbohydrates may appear in this group.

The gels showing embryonic protein and yolk protein had 4 corresponding bands during diapause. They are yolk bands 1, 2, 4 and 5 and embryonic bands 1, 2, 3 and 5. This is consistent with the idea that proteins are being synthesized in the yolk and transferred to the embryo during diapause. Feldherr (1962) studied the utilization of yolk protein in *H. cecropia* throughout embryogenesis and suggested that some proteins released from the yolk were incorporated unmodified in the embryo. Barth and Barth (1954) have suggested that the storage protein of yolk from frog eggs probably is not broken down and that some prefabricated structural proteins of the yolk may be incorporated unchanged into the embryo. In *A. elliotti* the transfer of intact proteins from the yolk to the embryo could account for some of the increase in the embryonic protein content observed during the first 50 days of diapause development (Figure 6). Two lines of evidence suggest that peptides or preformed protein may be transferred from the

yolk to the embryo. First, the rate at which valine-C^{14} was incorporated indicates that protein synthesis in the embryo was low during diapause (Figure 11), although there was a marked increase in embryonic protein content (Figure 6) during the first 50 days of diapause development. Second, a greater number of protein bands appeared in both embryo and yolk fractions during diapause and some of the new yolk bands appeared to correspond with new embryo bands (Figures 12 and 13). The present study indicates that unlabeled yolk protein is transferred to the embryo during the first 50 days of diapause while new labeled protein is being synthesized in the yolk. This is supported by the following observations: (1) the high rate of protein synthesis in the yolk during the first 45 days of diapause development and the low rate of protein synthesis in the embryo at this time (Figure 11); (2) the increase in protein content of whole eggs during the first 50 days of diapause development (Figures 5 and 6); (3) the increase in embryonic protein content during the same period (Figure 7); and (4) the appearance of new correlating protein bands in both embryo and yolk during diapause (Figures 12 and 13). The large amount of protein noted in the fluid of the epineural sinus in diapausing *A. elliotti* embryos by Roemhild (1967) is consonant with this idea.

Further comparisons of protein bands in the gels from different developmental periods are difficult and interpretations uncertain, since great differences exist in the general patterns for pre-diapause,

diapause and post-diapause embryos and yolk. Not enough is known about the character or function of the Amido-Schwarz stained proteins to relate them to any specific event. However, some conjugated proteins have been partially characterized with regard to their lipid or "polysaccharide" content, and therefore, the number and intensity of these bands may indicate changes in the levels of these materials in the egg.

Protein-carbohydrate complexes were suggested to comprise a major portion of the yolk in oocytes of *A. elliotti* on the basis of histochemical tests (Leopold, 1967). PAS staining of electrophoretically separated yolk protein in the present study also suggested the presence of a considerable amount of this material; however, lipoproteins may comprise a greater portion of the yolk. The latter suggestion is based on the predominance of lipoprotein-positive areas in that portion of the yolk which was not soluble in 40 % sucrose but was soluble in 0.6 N KCl. It will be remembered that this portion represents over 50 % of the yolk fraction. Dejmal and Brookes (1968) found that about 80 % of the yolk protein of *Leucophaea maderae* was soluble in saline at an ionic strength of 0.4 or greater. These proteins migrated as 5 bands during electrophoresis, although most of the protein appeared in one band which was identified as lipoprotein.

The hydrolysis of protein-carbohydrate complexes in the diapausing egg of *A. elliotti* has been suggested by Bunde (1965) as a mechanism

for the increase in free amino content and by Quickenden (1969) as an explanation for the increase in trehalose. Urban (1970) has noted protease activity in the serosa and yolk from eggs of *A. elliotti* during diapause that could affect these changes. In the present study "polysaccharide-positive" protein bands appeared to increase in number and quantity in the 40 % sucrose-soluble yolk fraction from pre-diapause to diapause (Figure 11). Partial hydrolysis of these complexes could be responsible for the increase in the number of yolk protein bands. Nothing definite can be ascertained in this regard except that such molecules and the enzymes to degrade them are present.

It has been suggested that carbohydrates, proteins and fats are utilized in that order during embryogenesis (Needham, 1931). The importance of carbohydrates or proteins as an energy source during embryogenesis is rather obscure for *A. elliotti*. Quickenden (1969, 1970) found slight early and late utilization of glycogen in *A. elliotti* but could not conclude that this moiety was an important energy source for embryogenesis. In the present study an increase in the protein content of the embryo during the first 50 days of diapause was followed by decreasing levels of protein. This may mean that some protein is utilized by the embryo during diapause, however, the actual utilization of protein as an energy source cannot be shown.

The importance of fats during insect embryogenesis has been noted by several workers. The amount of lipid decreases by more than one-

half during embryogenesis in *Bombyx* (Williams, 1967) and in *M. differentialis* (Slifer, 1930). Slifer also noted a gradual decrease in fatty acids during post-diapause. Considerable evidence has accrued for the accumulation of fats in the embryo during diapause in *A. elliotti*. The observations of Van Horn (1963) and the data of Svoboda (1964) have been discussed in this respect. Kaocharern (1958) has noted the accumulation of fat in the embryo of *M. differentialis* during diapause. Results presented earlier in the present study (Figure 12) showed that 3 new lipoprotein bands appeared in diapause embryos. Pre-diapause and post-diapause embryonic protein had only one lipoprotein band (Figure 12). Three new lipoprotein bands appeared in diapause yolk and one additional band was found in post-diapause yolk. These findings could mean that lipids accumulate in the embryo during diapause and were utilized during post-diapause development. Svoboda (1964) found that considerable lipid material was utilized during post-diapause in eggs of *A. elliotti*. Results of the present study and of others (Svoboda, 1964; Bunde, 1965; Quickenden, 1969) concerning possible embryonic metabolites in *A. elliotti* establish no definite sequence of utilization for carbohydrates, proteins or fats, but it appears that lipids are the most important energy source, especially during post-diapause.

SUMMARY

Proteins in the egg of *A. elliotti* were studied throughout embryogenesis. Quantitative analyses were made on whole eggs and egg fractions (embryo, yolk and shell). A qualitative study of embryonic protein and yolk protein during embryogenesis involved the electrophoretic separation of these proteins and a partial characterization of those conjugated with carbohydrate or lipoid material. The major findings and conclusions are enumerated below.

1. The protein content of the embryo increased gradually during pre-diapause development and the percentage of protein in the embryo was high until a decline was noted after 41 days of pre-diapause development at 25°C.

2. An increase in the protein content of whole eggs and embryos was found during the first 50 days of diapause development at 8°C.

3. A marked decrease in the percentage of protein in embryos and a concurrent increase in protein content was observed during the transition from pre-diapause to diapause development. This indicated that an increase in the amount of non-protein material in the embryos occurred at that time. A decrease in the protein content of whole eggs and the yolk fraction observed at this time suggests the hydrolysis of protein-carbohydrate complexes and the transfer of the products to the embryo. The transfer of intact proteins from the yolk to the embryo was indicated by the observance of 4 bands in the electrophoretically

separated protein of both embryos and yolk that had identical mobilites.

4. The protein content of whole eggs and the embryo fraction decreased gradually after 50 days of diapause development. This may mean that some protein is utilized as an energy source at this time. The protein content of embryos increased sharply after 10 days of post-diapause development at 25°C. Most of these embryos had completed blastokinesis and therefore, may have resumed mitotic activity at this time as noted previously by Roemhild (unpublished research data).

5. The rate of protein synthesis during pre-diapause was highest in whole eggs and embryos at about 35 days of development. Van Horn (1963) has found that many tissues and organs are differentiating at this time.

6. Embryos exhibited a low rate of protein synthesis during diapause development while the yolk fraction yielded high values. Whole egg samples displayed a gradual, but marked increase during this period. It was suggested that the loss of the hydropylar connection in diapausing eggs may cause an increase in the rate of protein synthesis.

7. A high rate of protein synthesis during post-diapause development was observed in whole eggs, embryos and yolk and a definite increase occurred after blastokinesis.

8. The shell fraction is thought to non-specifically bind or trap valine-C^{14}.

9. The electrophoretic separation of embryonic protein and yolk protein showed that the greatest number of protein bands were resolved

in diapause material. Partial hydrolysis of yolk protein reserves with the subsequent transfer of some of the products to the embryo could produce this phenomenon.

10. The presence of protein-carbohydrate and lipid-protein complexes was indicated by the presence of PAS-positive and Sudan Black B-positive bands in electrophoretically separated embryonic protein and yolk protein.

11. New lipoprotein bands appeared in embryonic protein and yolk protein during diapause development. One additional new band positive for lipoprotein appeared in post-diapause yolk soluble in 40 % sucrose.

12. Over 50 % of the yolk was insoluble in 40 % sucrose but was dissolved in 0.6 N KCl. This material contained two major bands thought to be lipoprotein.

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