



Studies on the development of muscle in embryos of *Aulocara elliotti* (Thomas) (Orthoptera, Acrididae) using the fluorescent antibody technique
by Bela Zoltan Horvath

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree
DOCTOR OF PHILOSOPHY in Zoology
Montana State University
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Abstract:

Muscle differentiation and the development of contractility was investigated in embryos of *Anlocara elliotti* (Thomas). Antibodies, prepared against adult femoral myosin and labelled with fluorescein isothiocyanate were used to analyze muscle development during the various embryonic stages. The purity of antigen was established through viscometry, spectrophotometry and enzyme tests. Through the use of various control procedures, it was found that the antimyosin was bound by the A band in the cross-striated myofibrils. The first structures that bound the antibodies in the developing embryo were observed at the beginning of diapause. These structures were uniformly fluorescent filaments or fibrils, within the myoblasts from the ventral abdominal region of the embryo. At the end of diapause as blastokinesis began, cross-striated fibrils and multinucleated myotubes appeared. As development proceeded the number of cross-striated myofibrils increased. By stage 24 definite muscle bands were present in the embryo. The contractility of embryonic muscle was tested by ATP. It was found that only those cells which contained cross-striated fibrils contracted when exposed to ATP. The differentiation of myoblasts and of cross-striated myofibrils is presumed to be attributable to a direct effect of hormonal control.

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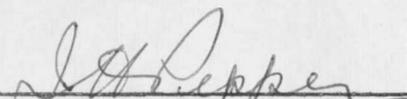
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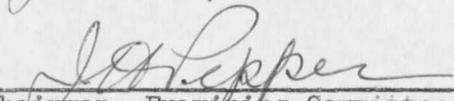
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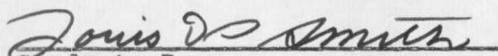
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ABSTRACT

Muscle differentiation and the development of contractility was investigated in embryos of Aulocara ellioti (Thomas). Antibodies, prepared against adult femoral myosin and labelled with fluorescein isothiocyanate were used to analyze muscle development during the various embryonic stages. The purity of antigen was established through viscometry, spectrophotometry and enzyme tests. Through the use of various control procedures, it was found that the antimyosin was bound by the A band in the cross-striated myofibrils. The first structures that bound the antibodies in the developing embryo were observed at the beginning of diapause. These structures were uniformly fluorescent filaments or fibrils, within the myoblasts from the ventral abdominal region of the embryo. At the end of diapause as blastokinesis began, cross-striated fibrils and multinucleated myotubes appeared. As development proceeded the number of cross-striated myofibrils increased. By stage 24 definite muscle bands were present in the embryo. The contractility of embryonic muscle was tested by ATP. It was found that only those cells which contained cross-striated fibrils contracted when exposed to ATP. The differentiation of myoblasts and of cross-striated myofibrils is presumed to be attributable to a direct effect of hormonal control.

INTRODUCTION

The movement of the embryo within the egg is a characteristic phenomenon of development in many insects. Those movements which lead to a change in the orientation of the embryo in the egg are generally classed under the term blastokinesis (Torre-Bueno, 1950). In the different insect groups, however, blastokinesis may vary greatly (Snodgrass, 1935). In the grasshopper species A. ellioti blastokinesis consists of a revolution of the embryo on its transverse axis by which it reverses its orientation from the posterior toward the anterior end of the egg (Van Horn, 1963).

Although blastokinesis is a well known event, the mechanisms associated with it have not been satisfactorily explained. The rupture and contraction of embryonic membranes was thought to be the mechanical force that initiated these movements (Slifer, 1932; Le Berre, 1952, as reported by Roemhild, 1967). Nelsen (1931) noted that, although the rupture of serosa may cause some passive movements, the revolution of the embryo is accompanied by active movements of the embryo itself. In some cases Slifer (1932) found that blastokinesis was nearly completed without the serosa being ruptured indicating that the contraction of the embryonic membranes is not the primary cause of revolution.

Microscopic examination by Slifer (1934) revealed that, before the onset of blastokinesis, unicellular spindle-shaped nonstriated fibers are present and that they occupy the position of the future abdominal muscle. She suggested that these presumptive muscle cells are responsible for the movements of the embryo. This implies then since she did not detect striated muscle until about nine days after the first movements, that these

undifferentiated muscle cells were capable of contraction long before cross-striation was present. There are other references in the literature to this effect, suggesting that contraction can take place before the appearance of the cross-striated condition. Neusch (1963) investigated the development of muscle function in Antheraea polyphemus and found that myofibrils of the developing flight muscles contracted two days prior to the appearance of cross-striation. Similarly, Carlson and Meek (1908) reported that, in embryos of Limulus, heart beats could be observed before cross-striation could be distinguished. Such reports are not unique to Arthropods. Contraction of cardiac myoblasts and skeletal muscle fibrils of vertebrates, before the appearance of cross-striation, was reported by several investigators (Lewis and Lewis, 1917; Copenhaver, 1939; Olivio, et al., 1946).

Since cross-striation of myofibrils is due to a very precise localization of certain proteins (Hanson and Huxley, 1953), it is doubtful whether myofibrils lacking this particular arrangement of contractile components can contract. Although cross-striation per se is not a prerequisite for contraction, nevertheless, there must be a requirement for these proteins to be present in a highly organized condition (Holtzer, et al., 1959; Holtzer, 1961).

Studies by Holtzer, et al. (1957) indicated that failure to detect cross-striation in contracting myofibrils is not necessarily due to the absence of striation, but may be due to the limited sensitivity of the techniques used. Their investigations disclosed that striation in mono or multinucleated myoblasts could be detected at a much earlier stage by

immunological means, using fluorescent antibodies against myosin, than with conventional histological techniques. Experiments with labeled antimyosin also demonstrated that neither skeletal nor cardiac myofibrils could contract until after cross-striation was present (Holtzer and Abbott, 1958; Holtzer, et al., 1959).

Although considerable work has been done on the development and morphology of the various insect muscles (Edwards, et al., 1954; Philpott and Szent-Györgyi, 1955; Tiegs, 1955; Hodge, 1956; Smith, 1962; Pringle, 1965; and others), studies to correlate morphological differentiation with the development of function have been limited (Slifer, 1934; Wigglesworth, 1956; Neusch, 1963). The present study was initiated to obtain basic information on muscle development and to correlate morphological differentiation of myofibrils with the appearance of first embryonic movements in Aulocara ellioti. To accomplish the purposes of this investigation, the fluorescent antibody techniques of Coons and Kaplan (1950) and Coons (1956) were used.

The use of fluorescent antibody technique as an analytical tool requires that the antisera contain antibodies only against the specific protein, in this case insect myosin. It is important, therefore, that the antigen used for the production of antibodies has a high degree of purity. To establish the homogeneity of myosin preparations, several tests were performed. Data obtained from viscosity measurements, spectrophotometry, ultracentrifugal analyses, and enzyme studies were used as criteria of purity. Preliminary studies were conducted using chick muscle and some of

the results were used for comparative purposes with insect muscle.

MATERIALS AND METHODS

Insect Material

The grasshoppers which were used as a source of myosin were collected as adults from various localities in Montana. Because A. ellioti is available only during the summer months, several thousand specimens were procured during these summer periods in order to ensure an adequate supply of material.

The insects, brought in from the field, were inactivated by placing them in a 4° C cold room, then the femora of their hind legs were removed and placed in a solution of 50 percent glycerol and water at 4° C. When approximately 50 femora were obtained they were ground in a mortar to break up the exoskeleton in order to allow for a better penetration of glycerol. The suspension was held for 24 hours at 0° C, after which it was transferred to a fresh 50 percent glycerol-water solution and placed at -20° to -25° C until used (Szent-Györgyi, 1949).

A. ellioti eggs also were collected from wild populations. The eggs were removed from the pods and the embryos dissected free from the yolk and other extraneous material. These embryos were then placed in a mixture of 50 percent glycerol-water and held at 0° C for 24 hours after which they were transferred into a fresh mixture of 50 percent glycerol-water solution and kept at -20° C to -25° C until used.

The embryos were staged according to the criteria established by Van Horn (1963, 1966a). She showed that, stages 1 to 18 represent pre-diapause development, and at stage 19, which is reached approximately 40 days at 25° C after the eggs are laid, the embryos of A. ellioti enter an

obligatory diapause. Stage 20 represents the beginning of the post-diapause development, and at this stage the embryo begins to revolve around the posterior pole of the egg. Blastokinesis is completed at stage 23, and stage 27 represents the definitive embryo.

Preparation of Proteins

Myosin from insect muscle, unlike that of rabbit muscle, cannot be obtained by direct extraction because even a short extraction period yields a typical actomyosin (Gilmour and Calaby, 1953). Pure myosin can be prepared, however, from actomyosin by differential centrifugation in the presence of ATP (Adenosine-5'-triphosphate) and magnesium ions (Weber, 1956; Maruyama, 1958). Accordingly, myosin was prepared essentially as described by Maruyama (1958; Kominz, et al., 1962).

The glycerol-suspended insect muscle was washed twice in a solution containing 0.04 M KCl and 0.01 M Tris (Tris [hydroxymethyl]-aminomethane, SIGMA Chemical Co., St. Louis, Mo., U.S.A.) buffer, pH 7.0. It was then homogenized in a blender for 30 seconds at a high speed in a solution consisting of 0.6 M KCl and 0.04 M KHCO_3 . The extraction was then carried out for two to three hours at 4^o C with occasional agitation. The above suspension was then diluted with one volume of 0.6 M KCl, filtered through several layers of gauze, and then centrifuged at 12,000 RPM for 30 minutes. The supernatant was filtered again through gauze to remove suspended fat particles. The precipitation of actomyosin was effected by the addition of ten volumes of cold water, and adjusting the pH to 6.5 with dilute acetic acid. The precipitated actomyosin was then removed by centrifugation at 6,000 RPM for 30 minutes, after which it was dissolved in 2 M KCl and

enough water added to bring the final KCl concentration to 0.6 M. The pH was adjusted to 7.2 with 0.15 M imidazole - HCl buffer. The dissolved actomyosin was then reprecipitated twice with cold water, and finally clarified by centrifugation at 12,000 RPM for 30 minutes. The actomyosin solution obtained by the above procedure was adjusted to about 0.3 percent concentration and centrifuged for three hours at 40,000 RPM in the presence of 0.6 M KCl, 2 mM MgCl₂, 5 mM ATP, and 0.02 M Tris buffer, pH 7.2. The upper three-fourths of the supernatant which contained the myosin was carefully removed and dialyzed against a solution containing 0.6 M KCl, 0.02 M Tris, pH 7.2. A few mM of beta-mercaptoethanol was added to the dialyzing solution to avoid denaturation of the protein (Maruyana, 1961). The myosin solution, thus freed from ATP and Mg ions, was then diluted 15 fold with cold water to precipitate the protein. The myosin was collected by centrifugation at 6,000 RPM for 30 minutes. It was then dissolved in 0.6 M KCl, and 0.02 M Tris, pH 7.2, and was further purified by subsequent reprecipitations with cold water. Finally, the precipitated myosin was dissolved in 0.6 M KCl containing the desired buffer.

Methods for Characterization of Actomyosin and Myosin

Viscometry.

An Ostwald type viscometer with a flow time of 25 seconds (0.6 M KCl) was used. The volume of protein solution introduced was 5 ml, and when applicable ATP and MgCl₂ was added in quantities of 0.1 ml each.

Ultracentrifugal analysis.

A Beckman Analytical Ultracentrifuge Model E equipped with

schlieren optics and an AN-D rotor at a top speed of 59,780 RPM was employed.

Spectrophotometry.

A Beckman DU spectrophotometer was used. The absorption spectra between 250 m micra. and 320 m micra. were investigated.

Protein concentration.

The protein concentration was determined by the method of Lowry et al., (1951). For each determination six different concentrations containing between 6 to 100 micro g of protein were used. To a 1 ml volume of protein 5 ml copper - alkali and 0.5 ml of Folin - Ciocalteu reagent (Hartman-Ledden Co., Philadelphia, Pa., U.S.A.) was added, and the color read at 750 m micra using either a Beckman DU spectrophotometer or a Spectronic 20 with a red filter and red sensitive phototube. Standards were based on micro Kjeldahl nitrogen determinations. The digestion mixture consisted of three parts of CuSO_4 , one part of K_2SO_4 , and 0.005 parts of selenite, and 2 ml of concentrated reagent grade sulfuric acid. A protein solution of 0.5 ml volume was introduced. A mixed indicator containing boric acid and methyl red - bromocresol green was used. The conversion factor, to obtain the quantity of actomyosin and myosin, was 6.0 (Maruyama, 1957).

Myosin ATP-ase test.

Myosin was present in a solution containing 0.6 M KCl, and in addition either 0.02 M Tris or 0.05 M imidazole buffers. The reaction mixture consisted of 0.2 to 0.6 M KCl, 0.02 M Tris or 0.05 M imidazole

buffer, 3 mM CaCl_2 , and 2 to 3 mM ATP. Incubation was at 23° C at pH 7.0 for various lengths of time depending upon the objective of study (Maruyama, 1958).

The ATP-ase activity for the insect myosin was determined by measuring the free inorganic phosphate liberated from ATP. The determination was carried out using a modification of the method of Fiske and Subbarow (1925).

The enzyme reaction was started with the addition of 1 ml of protein to 2 ml of a solution containing the appropriate salts, buffer, and ATP as described previously. The reaction was stopped by the addition of 1 ml of ten percent TCA (Trichloroacetic acid) solution. The precipitate formed was removed by centrifugation and 2 ml of the supernatant was used for phosphate determination. To the 2 ml sample was added 6 ml of water, 1 ml Photol (Eastman Kodak Co., Rochester, N. Y., U. S. A.) mixture (0.2 percent Photol in 30 percent NaHSO_3) and 1 ml of 5 percent ammonium molybdate in 5 N H_2SO_4 . The solution was mixed and allowed to stand for ten minutes, and the optical density was read in a colorimeter at 640 m μ .

Preparation of Antibodies

Rabbits were immunized with insect myosin as described by Laki, Horváth, Katzo (1958). About ten mg doses of the myosin dissolved in 0.5 N NaCl were injected intravenously via the marginal ear vein every other day for a two-week period. Six days after the last injection, 15 ml of blood were drawn and the titres determined. In some cases alum-precipitated

protein was also used (Stevens, et al., 1957). With this latter technique the formation of nonspecific antibodies may have been reduced because much less protein had to be injected to produce a high titre serum.

Preparation of Gamma Globulin

The gamma globulin from the sera was isolated by precipitation with ammonium sulfate at 50 percent saturation (Coons, 1958). The purification of antibodies was achieved by two additional precipitations with ammonium sulfate at a final concentration of one-third saturation. The final precipitate was dissolved in buffered saline and dialyzed against the same solution to eliminate ammonium ions.

The protein concentration was determined colorimetrically by the method of Lowry et al., (1951). Standards were based on micro Kjeldahl nitrogen determinations using a conversion factor of 6.25 (Campbell, et al., 1964) to obtain the quantity of protein in the sample.

Conjugation of Antibodies with Fluorescein Isothiocyanate

The conjugation procedure as described by Rinderknecht (1963) and George, et al., (1961) was used with some modifications.

To 2 ml of gamma globulin solution, diluted with an equal part of 0.05 M sodium carbonate - bicarbonate buffer pH 8.5, 0.5 mg of fluorescein isothiocyanate (10 percent on celite) (Nutritional Biochemical Co., Cleveland, Ohio, U.S.A.) per mg of protein was added. This fluorescein isothiocyanate powder was carefully deposited on the surface of the protein solution to prevent agglomeration of the particles. Wetting of the powder was facilitated then by centrifugation. The powder, thus sedimented, was suspended throughout the liquid by gentle stirring. The conjugation was allowed to proceed at least one hour at room temperature, or at 4° C for

24 hours with continuous slow mechanical stirring. The unbound dye was removed by dialysis or by gel filtration as described below.

Dialysis was carried out at 4° C against large volumes of buffered saline. To eliminate the green fluorescence from the dialysate, a period of three to five days was required even though the dialysing solution was changed frequently.

A much more rapid separation of unbound dye was achieved by gel filtration using Sephadex G-25 Fine Gel (Pharmacia Fine Chemicals, Inc., Sweden). The column was prepared by pouring the water-suspended gel into a container and washing the column with several column-volumes of 0.02 M sodium phosphate buffer pH 6.5. From 2 to 5 ml aliquots of fluorescein isothiocyanate labelled protein solution were applied to the top of the gel, and the column developed with 0.02 M sodium phosphate buffer pH 6.5. With this method an excellent separation of labelled protein and unbound dye was obtained.

Preparation of Myofibrils

Femoral muscles of adults of A. ellioti were dissected out and placed in a mixture of equal parts of glycerol and water. The suspension was left overnight at 0° C after which the muscles were resuspended in a fresh glycerol-water mixture and stored at -20° to -25° C until used.

The muscles of three femora were used for each preparation. After storage in the glycerol-water solution, the muscles were washed in a solution containing 0.04 M KCl and 0.01 M Tris, pH 7.0. The muscles were homogenized at a high speed in a Virtis micro-homogenizer for one minute

in a solution of 0.04 M KCl and 0.01 M Tris, pH 7.0 or in a solution of 25 percent glycerol containing 0.016 M phosphate buffer, pH 7.5 (Szent-Györgyi and Holtzer, 1960). The myofibrils were sedimented by centrifugation, and suspended in a medium of 25 percent glycerol and 0.016 M phosphate buffer, pH 7.5. When the myofibrils were stored for an extended period of time, the suspending solution used consisted of 50 percent glycerol and phosphate buffer.

Techniques for Studying Myofibrils and Embryos

Conventionally stained material.

Grasshopper embryos and muscle were fixed in Bouin's solution and embedded in paraffin, and sectioned serially at 5 micra. The stain used was Heidenhain's iron-hematoxylin (Humason, 1962).

Phase contrast microscopy.

Muscles and embryos, used for the phase contrast observations, were unstained-glycerol-extracted preparations. Specimens were mounted on a slide in 25 percent glycerol containing 0.016 M phosphate buffer, pH 7.5.

Fluorescent antimyosin stained material.

Insect myofibrils and insect embryos were extracted in glycerol as already described. The staining of myofibrils and embryos was performed by using either of the following two procedures. In one case the myofibrils or squashed embryos were placed on a microscope slide in a medium consisting of 25 percent glycerol and 0.016 M phosphate buffer, pH 7.5. A few drops of fluorescein-labelled antimyosin solution was drawn under

the coverslip, and the reaction allowed to proceed for ten minutes. Then the preparation was washed with the glycerol-phosphate solution to remove unbound antibodies.

In the other case myofibrils and embryos were transferred into either the fluorescein-labelled antimyosin or the fluorescein-labelled normal globulin solution in buffered glycerol. After 24 hours the specimens were removed and washed repeatedly through centrifugation in the glycerol-phosphate solution at 0° to 4° C.

Contraction of glycerol-extracted muscle by ATP.

The contractility of myofibrils and embryonic muscle was tested by exposing them to a solution of ATP (0.04 M ATP in 0.6 M KCl, pH 7.0). A drop of ATP solution, applied to the edge of the cover slip, was drawn under the cover by the aid of blotter paper placed on the opposite side of the cover slip. The contraction of muscle preparations could thus be followed under the phase contrast microscope.

Tests for the Specificity of Antimyosin Sera

To demonstrate that the antimyosin sera contained, at least to a high degree, only antibodies against myosin and that the antimyosin reacted only with myosin in the myofibrils, the following experiments were performed.

a) After the removal of A band material, myofibrils were treated with fluorescein-labelled antimyosin. For extraction of A band material from insect muscle a solution consisting of 1.0 M KCl, 0.01 M sodium pyrophosphate, 0.001 M MgCl₂, and 0.01 M Tris, pH 7.0 was used (Gilmoure,

and Robinson, 1964). After the extraction the fibrils were washed in a solution of 0.04 M KCl, 0.016 M phosphate buffer, pH 7.0.

b) Myofibrils, extracted with 0.6 M KI solution, were treated with fluorescein-labelled antimyosin. The KI solution from insect muscle is known to extract both myosin and actin (Szent-Györgyi, 1951; Gilmour and Robinson, 1964).

c) Fluorescent antibody-stained preparations were washed in either the KI or Mg-pyrophosphate solution.

d) The muscle and embryonic preparations were treated with fluorescein-labelled normal globulin solution, and subsequently were washed in either the KI or Mg-pyrophosphate solution.

Microscopy

A Zeiss Photomicroscope equipped with phase contrast and fluorescence accessories was used. Observations with the fluorescence microscope were made by using either a dark-field or a bright/darkfield phase contrast fluorescence condenser (Zeiss), and Achromatic, Plan/Opochromatic, and Neofluar objectives (Zeiss). Excitation was accomplished by using HBO 200 mercury lamp with UG and BG Zeiss filters. Photomicrographs were taken on Kodak Tri-X or Panatomic-X film and developed in Diafine.

RESULTS

Viscometry.

The viscosity of insect actomyosin and myosin was measured both in the absence and in the presence of ATP. Figure 1 represents the relative viscosity of actomyosin before and after the addition of ATP. As ATP was introduced, the viscosity dropped to a low level (Fig. 2). The hydrolytic breakdown of ATP by the actomyosin began immediately as indicated by a gradual increase of viscosity, and in about 35 minutes when most or all of the ATP was hydrolyzed, the original viscosity was almost completely recovered (Fig. 2).

On the other hand the viscosity of myosin remained the same after ATP was added (Fig. 3).

The viscosity of the lower portion of the supernatant, (actin plus undissociated actomyosin) obtained by centrifugation of actomyosin in the presence of ATP and $MgCl_2$, was also investigated. A comparison of the relative viscosities of actomyosin, myosin and the lower portion of the supernatant (Table I) shows that the viscosity of actomyosin in the presence of ATP changed the most, while that of the lower supernatant changed only slightly, and the myosin did not alter its viscosity.

Ultracentrifugal analysis.

Ultracentrifugal analysis of insect actomyosin showed one main peak and two smaller components (Plate I). The main peak which had a sedimentation constant of 40S is thought to be actomyosin. One of the slow peaks with a constant of 5 S is probably myosin, while the nature of the other slow peak having a constant of 2 S has not been determined (Maruyama, 1957).

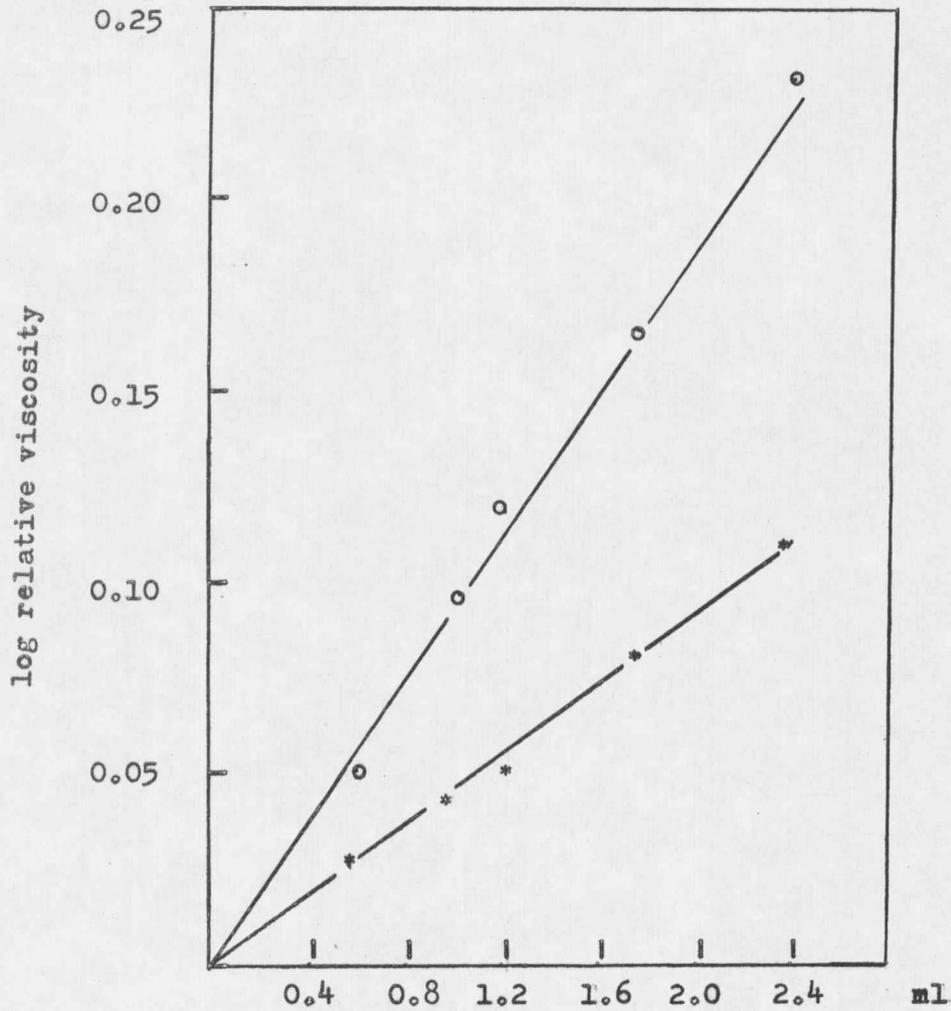


Fig. 1. Viscosity of A. ellioti femoral actomyosin at different concentrations in the absence and presence of ATP. Conditions: 0.6 M KCl, 0.02 M Tris, 1 mM ATP, pH 7.2, 23° C; o - without ATP; * - with ATP.

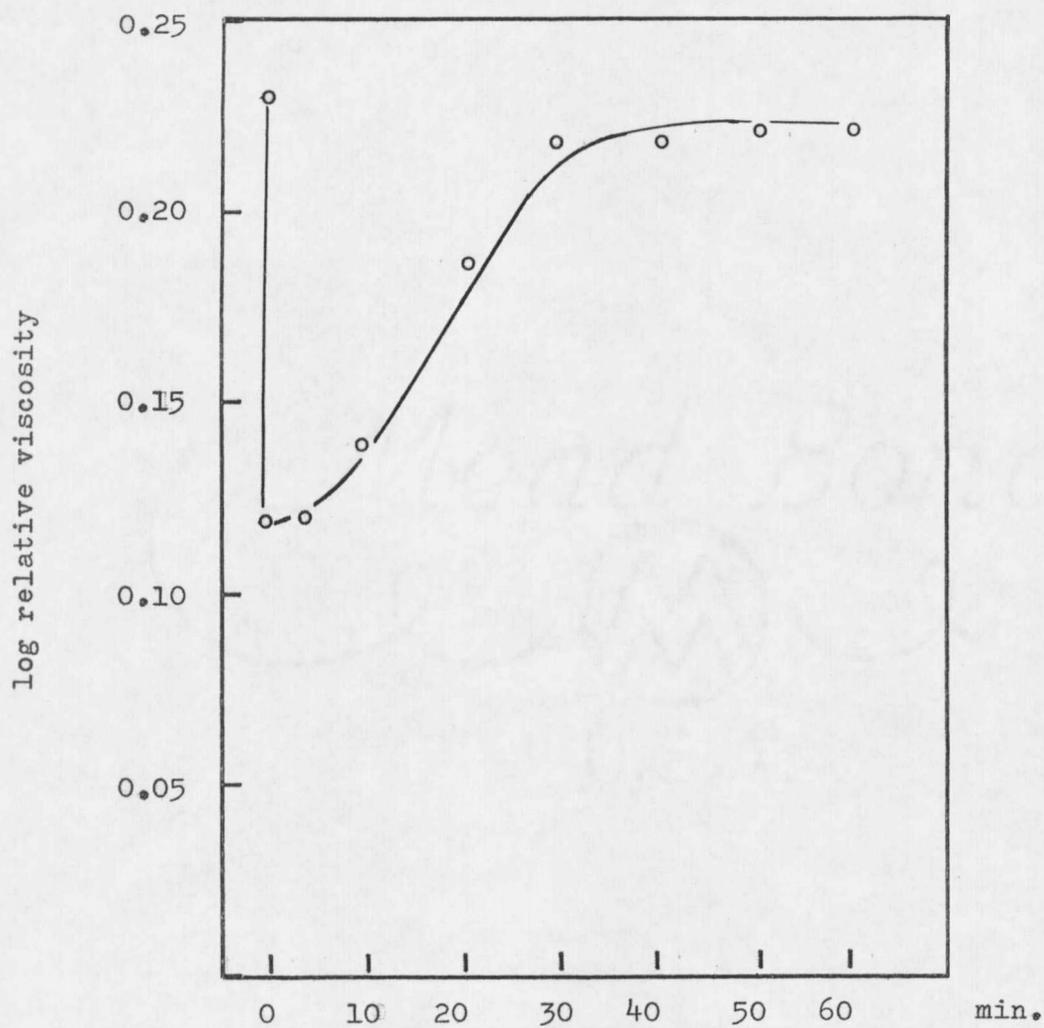


Fig. 2. Viscosity of actomyosin from femoral muscle of A. ellioti in the presence of ATP. Conditions: 0.6 M KCl, 0.02 M Tris, 1 mM ATP, pH 7.2, 23° C.

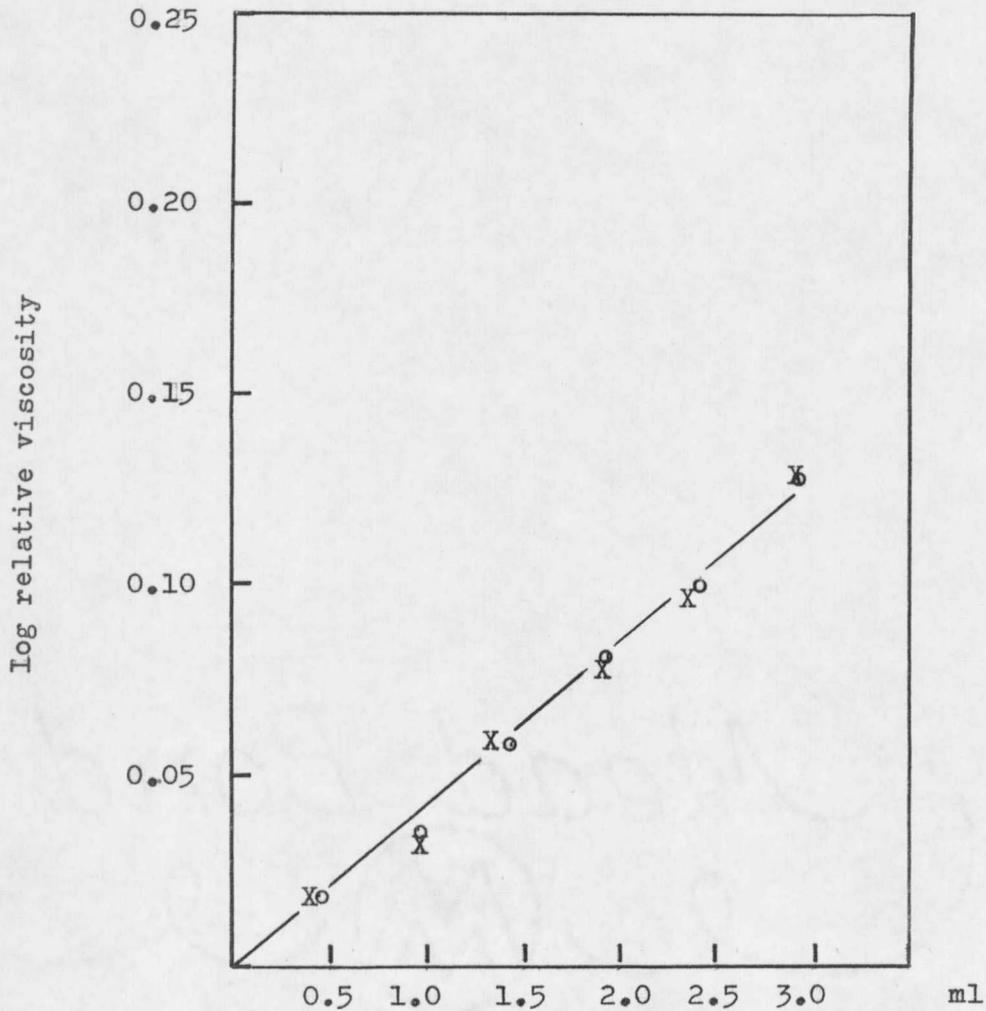


Fig. 3. Viscosity of A. ellioti femoral myosin at different concentrations in the absence and presence of ATP. Conditions: 0.6 M KCl, 0.02 M Tris, 1 mM ATP, pH 7.2, 23° C; o - in the absence of ATP; X - in the presence of ATP.

TABLE I

Comparison of relative viscosities, both in the absence and presence of ATP, of actomyosin, upper-half of supernatant, and lower-half of supernatant obtained by centrifuging insect actomyosin in the presence of ATP and Mg ions.

	Relative Viscosity	
	without ATP	with ATP
Actomyosin	1.298	1.148
Upper-half of supernatant	1.025	1.025
Lower-half of supernatant	1.039	1.032

In the presence of 3.85 mM ATP and 4 mM $MgCl_2$, the sedimentation pattern changed significantly as a result of the dissociation of actomyosin (Plate II). The sedimentation constant for the main peak was approximately 5 S which corresponds to the 5 S peak in the absence of ATP and which is assumed, therefore, to be myosin (Maruyama, 1957). Two small peaks also were noticeable in addition to one main one but no S value was computed for these.

Spectrophotometry.

The absorption spectra between 250 and 320 m micra were investigated (Fig. 4). Maximum absorption was observed at 280 m micra.

ATP-ase activity.

The ATP-ase activity of insect myosin was measured at different protein concentrations (Fig. 5). The activity level of several preparations

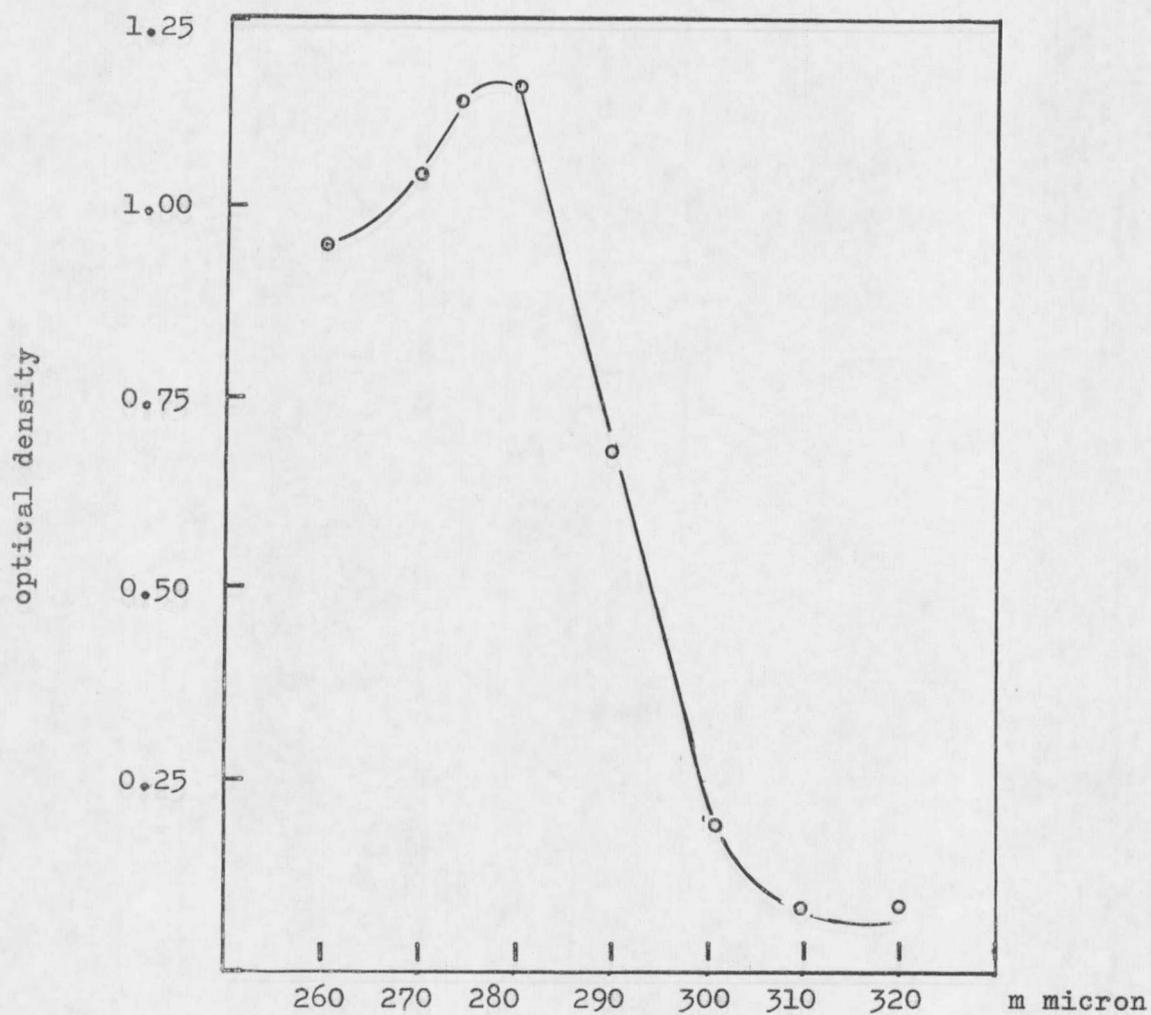


Fig. 4. Absorption spectra of *A. eliotti* myosin. Conditions: 0.6 M KCl, pH 7.0, 23° C.

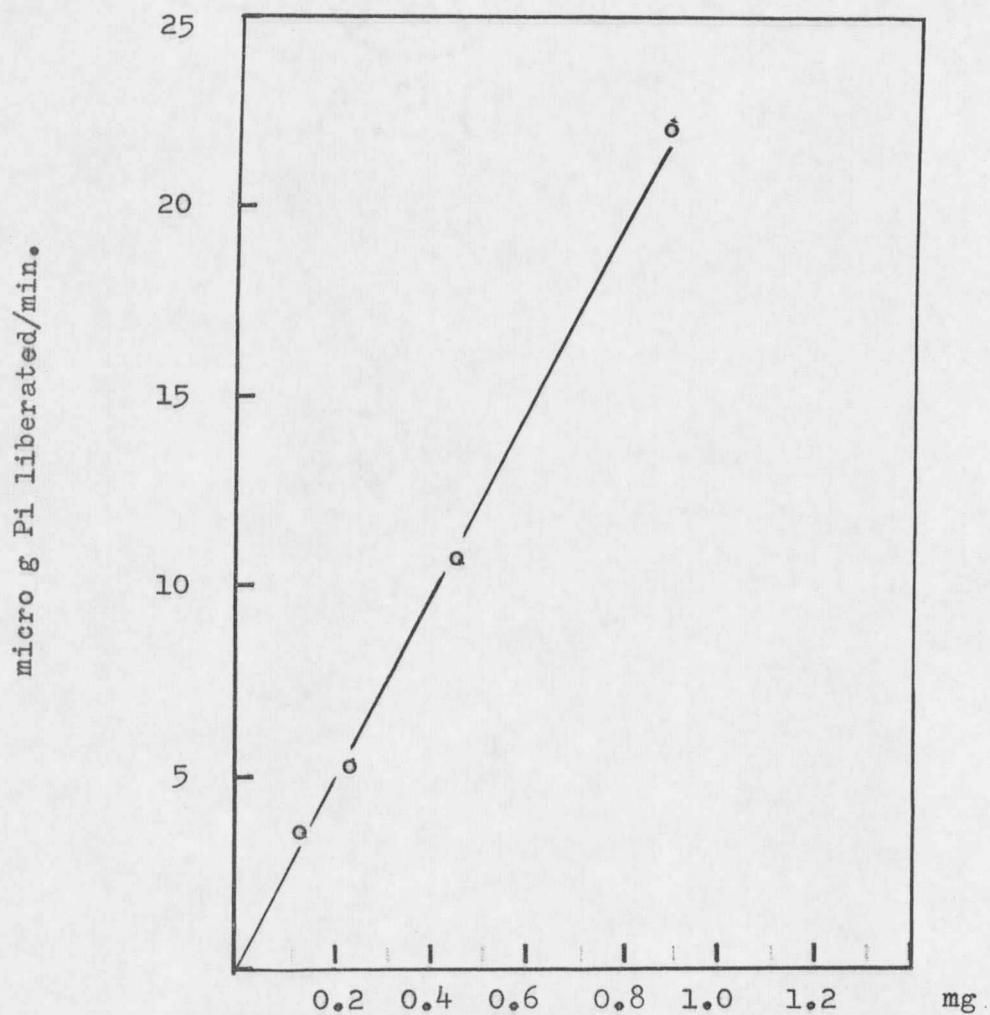


Fig. 5. ATP-ase activity of *A. eliotti* femoral myosin at different protein concentrations. Conditions: 0.2 M KCl, 3 mM CaCl₂, 0.02 M Tris, 3 mM ATP, pH 7.0, 23° C.

was determined and the average amount of phosphate liberated was 0.85 micro M Pi/min/mg protein. The Q_p , microliters of hypothetical P gas liberated in one hour at 37° C per milligram of protein, was about 3700.

To check the purity of myosin preparations, the time activity of myosin ATP-ase was also determined. Figure 6 shows the time activity of insect myosin ATP-ase in the presence of 5 micro M ATP. As the data show, the maximum amount of phosphate liberated is 156.3 micro g, the amount being approximately the same as that of terminal labile phosphate introduced with ATP (154.8 micro g). A slight discrepancy between the terminal P introduced with ATP and the Pi liberated was observed in all enzyme tests which is either due to some impurity of myosin solution or to the sensitivity of the quantitative techniques used. Because myosin was precipitated several times before the ATP-ase activity was determined, it is assumed that the slight difference is due to the sensitivity of the methods.

Appearance of adult myofibrils; phase contrast observations.

Although no precautions were taken in preparing rest-length myofibrils, the majority of fibrils were in a relaxed state when examined. In relaxed fibrils sarcomeres ranged in length from 7 to 15 micra with a peak at about 9 micra. The A bands of these fibrils varied according to the size of the sarcomere, the majority being about 5 micra in length. In these relaxed fibrils A and I bands and the Z lines were clearly distinguished, and an H zone was present in the middle of the A band, but no M line could be detected (Fig. 7).

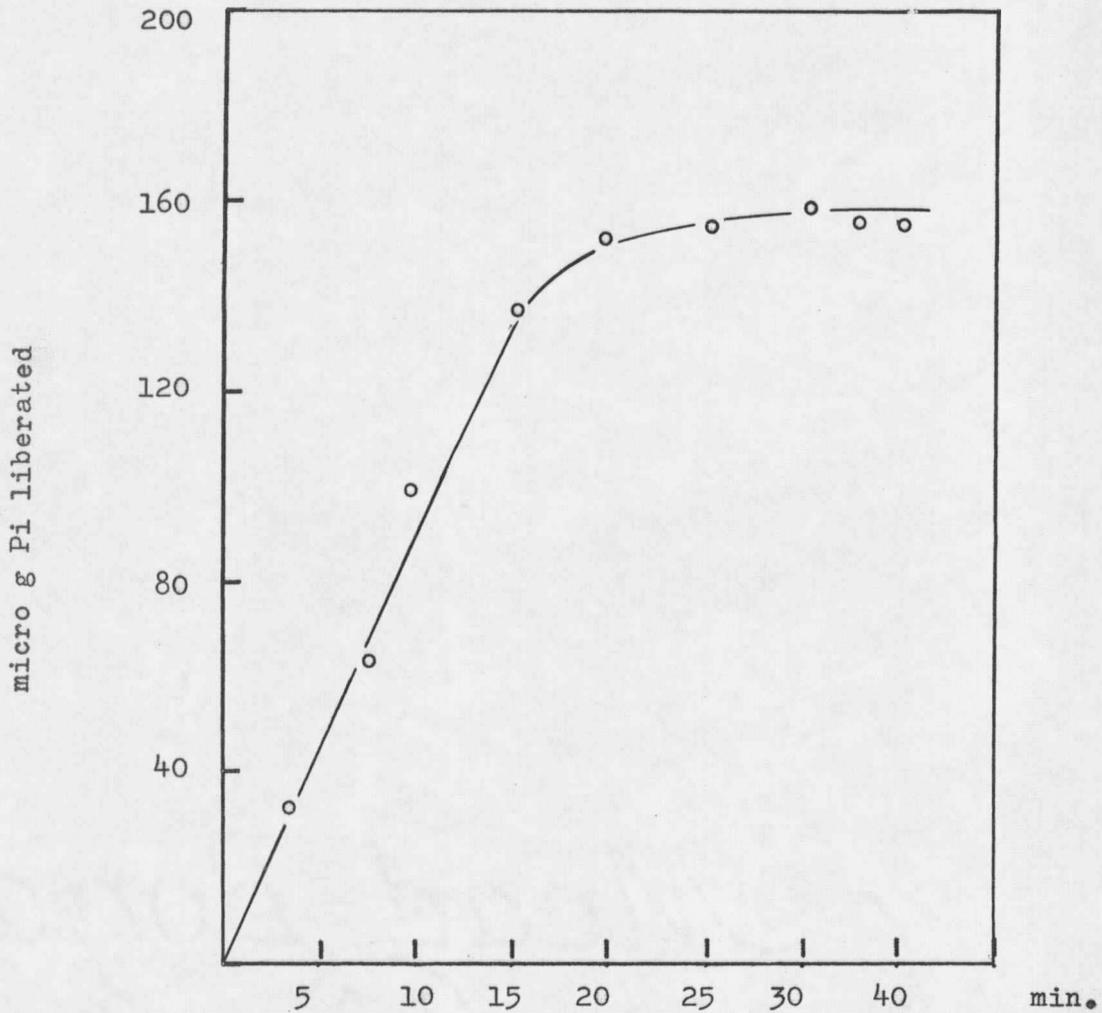


Fig. 6. Time activity of myosin ATP-ase from A. elliotti femoral muscle.
Conditions: 0.2 M KCl, 3 mM CaCl₂, 0.02 M Tris, 5 micro M ATP, pH 7.0, 23^oC, 0.45 mg protein.

The addition of Mg-pyrophosphate solution to the suspended fibrils changed their appearance drastically (Fig. 8). This solution extracted most or all of the A band material leaving a light background material and the dark Z lines. The H zone of the formed A bands showed very low density. A simultaneous swelling and shortening of myofibrils also took place. A subsequent washing of fibrils in a mixture consisting of 0.04 M KCl and 0.01 M Tris (neutral), and the addition of ATP did not alter the appearance of these extracted myofibrils.

The exposure of myofibrils to the KI solution resulted in the removal of both the A and I bands including the lighter background material (Fig. 9). In addition to this change in the band pattern, a swelling and shortening of fibrils also took place which was more prominent than that caused by the Mg-pyrophosphate solution alone.

Staining with antimyosin.

Treating the normal myofibrils with the antimyosin solution resulted in an increased optical density of their A bands (Figure 10). This increase in optical density seemed to be more pronounced at the lateral edges of the A band than at any other region.

Washing these antimyosin treated myofibrils in either the KI or Mg-pyrophosphate solution did not cause any visible change in the appearance of their A bands; however, after exposing them to the KI solution, the I bands became invisible and the Z lines seemed to disappear. It appeared, therefore, that antimyosin fixed the A band material in such a way as to prevent its removal by the extracting solutions.

Myofibrils, treated with normal globulin solution, exhibited no visible change in the optical density of the regions of the sarcomeres.

Upon the addition of Mg-pyrophosphate solution, the A band substance was removed, and the general appearance of fibrils was similar to the extracted but untreated preparations. The KI solution affected the whole sarcomere by extracting both the A and I band material, and caused the disappearance of the Z lines. It seemed, therefore, that the normal globulin did not react with any of the material localized in the sarcomere, and thus did not prevent the extraction of these substances.

Myofibrils, pretreated with either the Mg-pyrophosphate or KI solution, did not seem to bind antimyosin.

Fluorescence microscopy of adult myofibrils.

Relaxed myofibrils, treated with the fluorescein-labelled antimyosin solution, showed a green fluorescence at the A band region (Fig. 11) however, there was considerable variation in the intensity of fluorescence. The I bands remained unstained but the Z lines occasionally showed a very faint green fluorescence which, after repeated washings, usually disappeared. The H zone of the fibrils seemed to be free of fluorescence.

Treatment of myofibrils with fluorescein-labelled normal globulin solution did not cause any specific region of the sarcomere to fluoresce. In most cases an extremely faint staining of the outer limits of the fibrils was observed which was probably due to the presence of free dye in the solution.

When myofibrils were pretreated with either the Mg-pyrophosphate or KI solution and then exposed to the labelled antimyosin, fluorescence was completely absent. On the other hand, myofibrils, previously stained with

the labelled antimyosin and then washed with the Mg-pyrophosphate solution, preserved their fluorescing A bands. Treatment with the KI solution gave similar results.

Muscle development.

In embryos younger than stage 16 no elongated cells could be found in the ventral abdominal region where future muscle bands later occur in embryo.

In iron-hematoxylin stained sections of embryos of stages 16 and 17 oval-shaped cells, occasionally with mitotic figures, were observed (Fig. 12). These cells were in close association with each other and formed an almost continuous band between the lateral abdominal walls of the embryo. When squashed glycerol-extracted material from this abdominal region of stage 17 embryos was viewed under the phase contrast microscope, oval-shaped and somewhat elongated cells were found; however, spindle-shaped cells were absent.

Iron-hematoxylin stained sections of stages 17 (possibly late) and 18 embryos revealed the presence of an increased number of elongated cells in the position of the future abdominal muscle bands. In glycerol-extracted and squashed material of the same ages these elongated cells were very distinct under phase contrast observation. In these cells no filamentous material could be detected under phase contrast. When such preparations were exposed to fluorescein-labelled antimyosin, and subsequently washed to remove unbound antibodies, no fluorescence could be detected with the fluorescence microscope. The failure of these cells to bind the antibody indicated that in stage 18 or in younger embryos no

protein with the immunological specificity of myosin was present. In view of these observations, these elongated cells which did not bind the antimyosin were termed presumptive myoblasts. It may be significant that neither presumptive myoblasts nor any other cell outside the muscle forming regions fixed antimyosin at any stage of development.

A large number of the elongated cells became spindle-shaped by early stage 19. In embryos which were fixed and stained with iron-hematoxylin these spindle-shaped cells did not reveal the presence of any filaments. In glycerol-extracted preparations, after treatment with the labelled antimyosin, fibrillar structures could be detected under the phase contrast microscope (Fig. 13). When this latter preparation was viewed under the fluorescence microscope, uniformly fluorescent fibrils could be observed which were distributed at the periphery of cells in close association with the sarcolemma (Fig. 14). These filaments were found to be the first structures that bound the antimyosin in the developing embryo (Fig. 15).

Antibody was bound frequently by the sarcoplasm or by cytoplasmic inclusions and this resulted in a diffused fluorescence throughout the cell (Fig. 16). Intensely fluorescing spots or particles within the sarcoplasm, however, may have indicated the presence of some definitive elements that fixed the antimyosin to a higher degree than did other components of the cell. Such fluorescing particles may have resulted from the breaking of long myosin-containing filaments during the squashing procedures. When such injured cells are treated with the fluorescein-labelled antimyosin, myosin or myosin-containing particles dispersed in the sarcoplasm will fix

the antibodies in such a way that a diffused fluorescence will be observed in the cell (Holtzer, et al., 1959). This seems to be a plausible explanation for the presence of fluorescence in the sarcoplasm since presumptive myoblasts having no filamentous structures did not fix the antimyosin in their sarcoplasm to detectable extent.

When glycerol-extracted and squashed embryos of late stage 19 were treated with the labelled-antimyosin, fluorescence could be observed in the abdominal region of the embryos (Fig. 17). As can be seen, the fluorescing structures are organized into distinct bands which are uniformly fluorescent without the evidence of cross-striation.

The most conspicuous change from the state of mononucleated myoblasts is the appearance of multinucleated myotubes and cross-striated myofibrils at the end of diapause in stage 20. In myotubes of this age the nuclei are densely packed together, while during late stage 20 and in latter stages, the nuclei usually lie apart in the sarcoplasm (Figs. 18 and 19). The once round nuclei, by the end of stage 20 in older myotubes, become much elongated and occupy a position with their longitudinal axes being in the direction of the longitudinal axis of the myotubes (Fig. 20).

When inspected with the phase contrast microscope, glycerol extracted preparations or iron-hematoxylin stained sections, do not reveal the presence of cross-striated fibrils in stage 20. However, when stage 20 material was treated with fluorescein-labelled antimyosin, the cross-striated condition of fibrils became evident under the fluorescence microscope (Fig. 21). Although most of the fibrils in late stage 20

appeared to be cross-striated, there were fibrils which were still uniformly fluorescent.

During stage 21 most, if not all, of the fibrils appeared to be cross-striated when treated with antimyosin and observed under the fluorescence microscope. In phase contrast, however, many of the fibrils appeared to be non-striated before antibody treatment. It may be mentioned that the failure to detect cross-striation in some of the fibrils with the fluorescence method may have resulted from the limitations of the techniques used. Squashed preparations may be several hundred microns in thickness. As a consequence not all of the fibrils are in the same plane of focus and thus a diffused fluorescence will be observed.

Except for an increase in the number of multinucleated myotubes, embryos in stages 22 and 23 did not reveal any additional detail over the stage 21 material.

Beginning with stage 24 or possibly late stage 23, the formation of muscle bands becomes conspicuous. The once individual myotubes are now in an intimate association with each other, and they can no longer be distinguished as individual units in squashed preparations. Glycerol-extracted and squashed muscle of this age, when exposed to the labelled-antimyosin and observed under the fluorescence microscope, is almost indistinguishable from matured muscle treated in a similar manner (Figs. 22 and 23).

Contraction of embryonic muscle by ATP.

In order to correlate morphological differentiation with the

development of contractility, observations were made on the effect of ATP on the myofibrils from embryos of various ages. During the course of these tests it was found that only those cells containing cross-striated myofibrils were able to contract when exposed to ATP. Cells without cross-striated myofibrils were not observed to contract during any stage of embryonic development. The pattern of cross-striation in the contracted embryonic fibrils was found to be similar to that in contracted adult muscle (Fig. 24).

As in mature muscle, a previous treatment of cross-striated embryonic myofibrils with antimyosin prevented them from contracting when they were exposed to ATP.

PLATE I

Sedimentation pattern of actomyosin from A. ellioti.

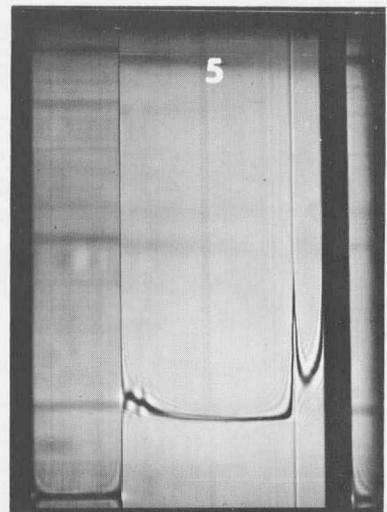
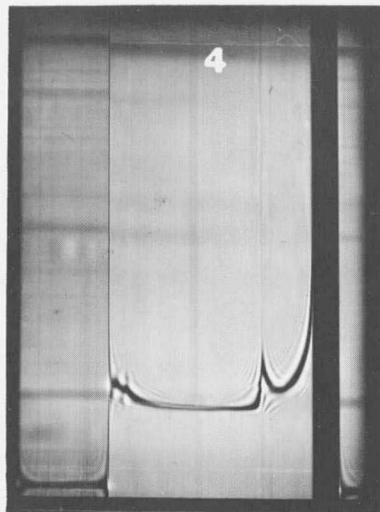
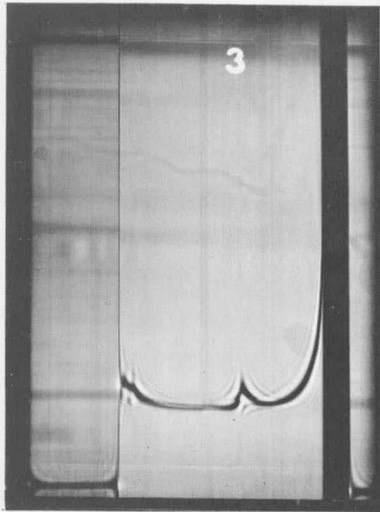
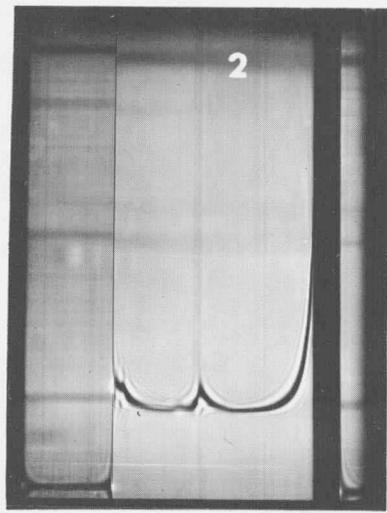
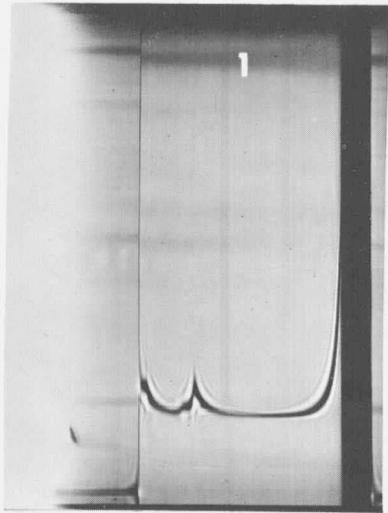


PLATE II

Sedimentation pattern of actomyosin from A. ellioti in the presence of ATP.

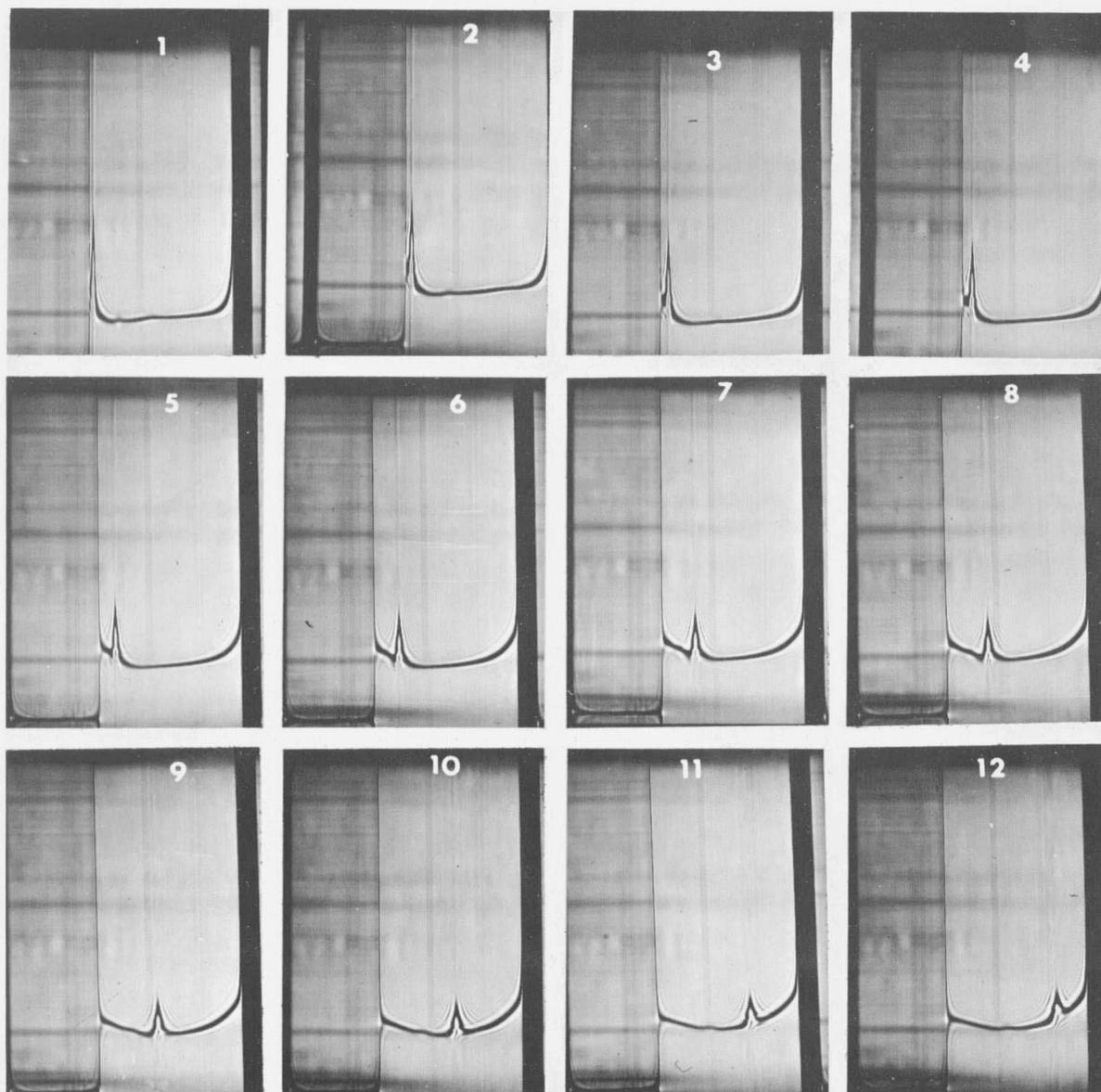


PLATE III
(Figures 7-12)

- Fig. 7. Phase contrast photomicrograph of myofibrils from an adult of A. elliotti. Glycerol-extracted, 1720X.
- Fig. 8. Myofibrils extracted with Mg-pyrophosphate solution. Phase contrast, 1720X.
- Fig. 9. Myofibrils extracted with KI solution. Phase contrast, 1720X.
- Fig.10. Phase contrast photomicrograph of an antimyosin-treated myofibril, 1720X.
- Fig.11. Fluorescence photomicrograph of an antimyosin-treated myofibril. 1800X.
- Fig.12. Presumptive myoblasts in the ventral abdominal region of a stage 17 embryo. Iron-hematoxylin stained. Bright field, 630X.

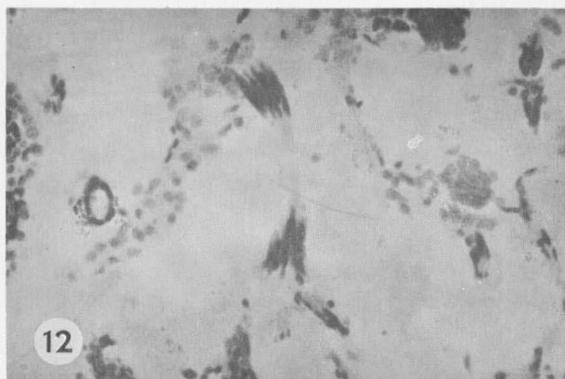
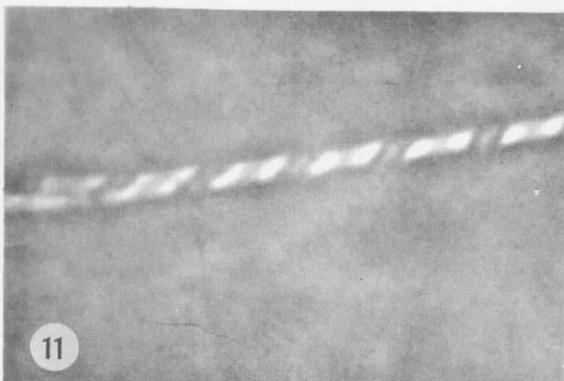
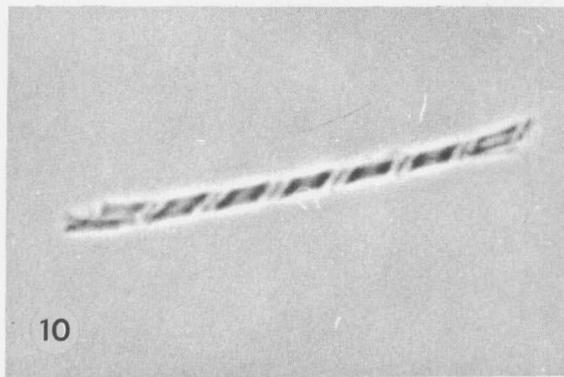
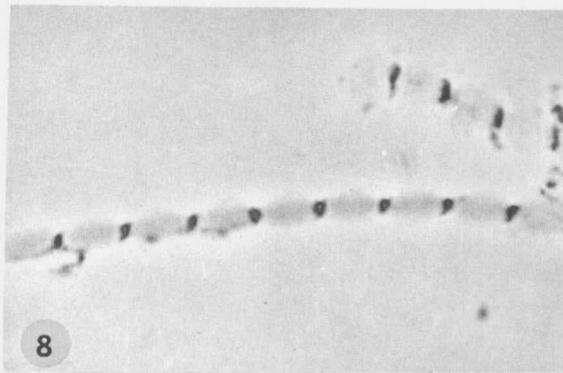
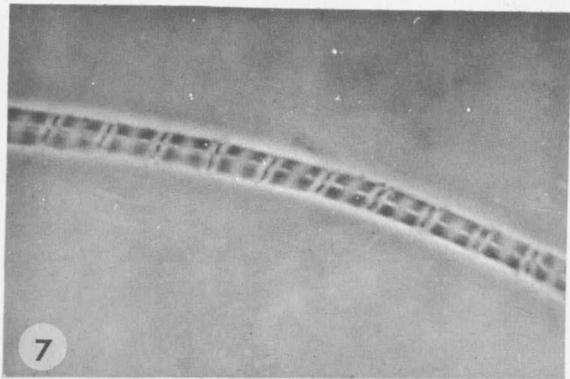


PLATE IV
(Figures 13-18)

- Fig. 13. Spindle-shaped myoblasts from a stage 19 embryo. Glycerol-extracted, squashed, and antimyosin-treated. Phase contrast, 1920X.
- Fig. 14. Myoblasts from a stage 19 embryo. Glycerol-extracted, squashed, and antimyosin treated. Phase contrast-fluorescence, 1800X.
- Fig. 15. Myofilaments from a stage 19 embryo. Glycerol-extracted, squashed and fluorescent-antimyosin treated. Fluorescence microscopy, 3600X.
- Fig. 16. Fluorescent-antimyosin stained myoblasts. Glycerol-extracted and squashed. Fluorescence microscopy, 1800X.
- Fig. 17. Low-power phase contrast-fluorescence photomicrograph of the abdominal region of a stage 19 embryo. Glycerol-extracted, squashed, and fluorescein-labelled antimyosin-stained, 300X.
- Fig. 18. Myotubes from an embryo of early stage 20. Glycerol-extracted and squashed. Phase contrast, 1600X.

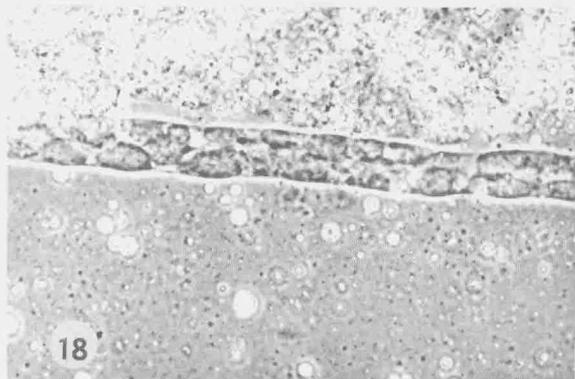
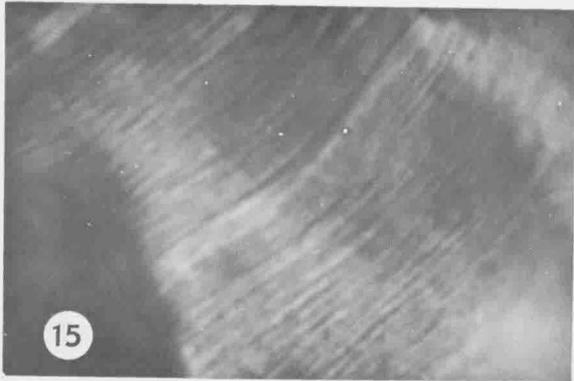
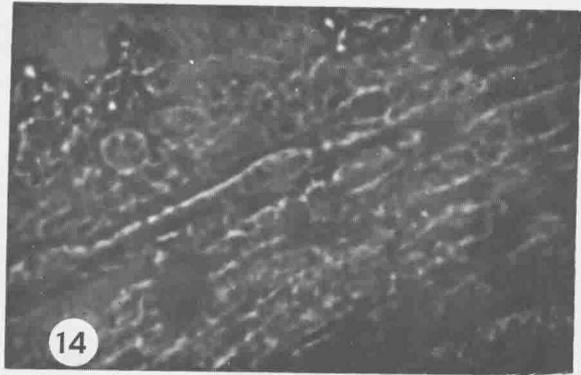
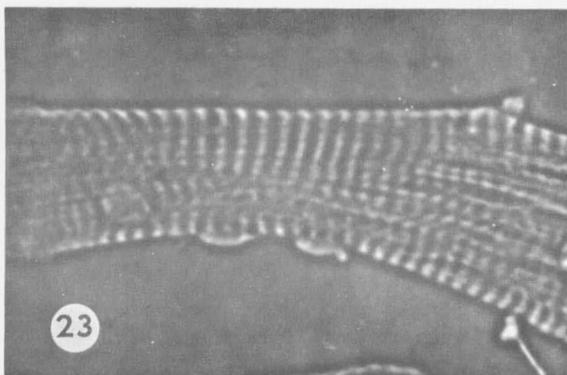
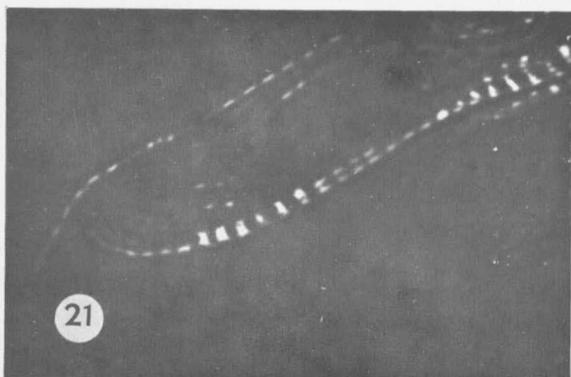
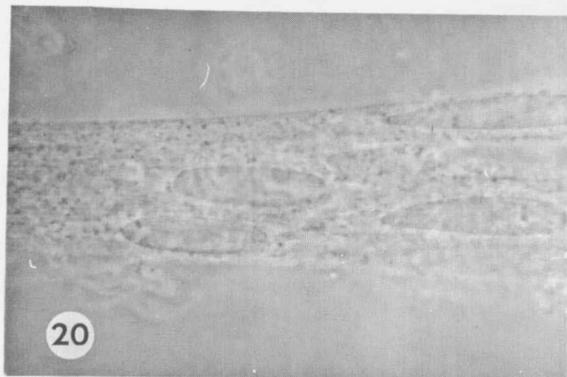


PLATE V
(Figures 19-24)

- Fig. 19. Phase contrast photomicrograph of myotubes from a late stage 20 embryo. Glycerol-extracted and squashed. 1800X.
- Fig. 20. Elongated nuclei within the myotubes of a stage 21 embryo. Glycerol-extracted and squashed. Phase contrast. 1920X.
- Fig. 21. Fluorescence photomicrograph of fluorescent-antimyosin treated myotubes from a stage 20 embryo. Glycerol-extracted and squashed. 800X.
- Fig. 22. Phase contrast photomicrograph of glycerol-extracted muscle from a stage 24 embryo. 800X.
- Fig. 23. Fluorescent-antimyosin stained muscle from a stage 24 embryo. The light bands are A bands and the dark ones are I bands. Phase contrast-fluorescence, 1200X.
- Fig. 24. Contracted myofibrils from a stage 24 embryo. Glycerol-extracted, fluorescent-antimyosin treated, and squashed. Fluorescence microscopy, 1600X.



DISCUSSION AND CONCLUSIONS

Viscosity.

The viscosity of insect actomyosin, dissolved in about 0.6 M KCl solution, decreased significantly upon the addition of ATP. This drop in viscosity is thought to be due to the dissociation of the actomyosin complex into its components actin and myosin (Szent-Györgyi, 1947; Gergely, 1956). The viscosity of myosin however, changed only slightly in the presence of ATP. This decrease in viscosity which was occasionally observed is most likely caused by contamination with actin. When myosin was repeatedly reprecipitated, no noticeable change in its viscosity was observed upon the addition of ATP. Myosin preparations were considered, therefore, to be highly homogenous when their viscosity remained unaltered after ATP was introduced.

Ultracentrifugal analysis.

The primary purpose of ultracentrifugal analysis was to determine if the conditions used for the preparations of myosin from actomyosin were appropriate. As the results show in the presence of ATP actomyosin dissociated into myosin and is presumed that the other component is actin. Furthermore, the results indicate that the protein preparations were homogenous to a high degree. The sedimentation constant of both myosin and actomyosin approximates those found by Maruyama (1957) for these insect proteins, i.e. $s=30S$ and $s=4-5 S$ respectively. The discrepancy between the data of Maruyama and that of the author may be due to several things, such as the concentration of proteins, the purity of the preparations, and the fact that different insects were used.

ATP-ase Tests.

Purified insect myosin catalyzes the hydrolytic split only of the terminal phosphate group of ATP (Maruyama, 1958). If other enzymes such as adenylate kinase and apyrase are present, ATP is broken down to AMP and 2 Pi. By measuring the amount of Pi liberated from a known amount of ATP, therefore, the homogeneity of myosin preparations can be determined.

The amount of Pi set free by myosin in all tests was approximately the same as the quantity of terminal labile P introduced with ATP. It can be assumed therefore, that the myosin preparations, after several precipitations, did not contain any significant quantity of other ATP-ases. The absence of these enzymes becomes important in considering the specificity of antibody preparations. The presence of these enzymes in the myosin solution used as the antigen would have stimulated the production of nonspecific antibodies and subsequently caused nonspecific reactions when the specificity of fluorescent antibodies was tested.

Spectrophotometry.

In order to determine the presence of nucleotides in the myosin preparations the UV absorption of myosin solutions was determined. A maximum absorption was observed at 280 m microns which is caused by the aromatic amino acid residues. The ratio of the optical density at 280 and 260 m microns did not exceed 1.12 thus indicating the relative absence of nucleotides (Haurowitz, 1963).

Observations of myofibrils.

The appearance of adult A. ellioti femoral myofibrils, under the

phase contrast microscope, was similar to that of other orthopteroid insect muscle (Gilmour and Robinson, 1964). As in other insect or rabbit muscle, glycerol treatment did not seem to influence the general morphology of myofibrils from A. ellioti.

The treatment of myofibrils with the Mg-pyrophosphate and KI solutions was probably the most important experimental criterion in establishing the specificity of antimyosin preparations. The Mg-pyrophosphate solution in rabbit muscle is assumed to selectively remove myosin (Hanson and Huxley, 1957) and to remove actomyosin from insect muscle (Gilmour and Robinson, 1964). The KI solution, in addition to myosin, also extracts the I band material from the myofibrils (Szent-Györgyi, 1951). That myosin was removed from the fibrils by both the Mg-pyrophosphate, and KI solution, was indicated by the failure of myofibrils to respond to ATP. In untreated relaxed fibrils under similar conditions, ATP caused contraction which was easily distinguished under the phase contrast microscope.

The failure of binding the antimyosin by the extracted fibrils was also indicated after the application of antibody by the unchanged density of the former A band regions. These results suggest that myosin alone was responsible for the fixation of antibodies which resulted in an increased optical density of the A band.

That the normal globulin solution did not contain a component that reacted with myosin in the A band, was illustrated by the failure of this solution to enhance the density of A bands. While pretreatment of myofibrils with antimyosin prevented the removal of A band material by

either the Mg-pyrophosphate or KI solution, a pretreatment with normal globulin did not affect the extractability of A band substance.

Before the cause of the localization of antibodies in the myofibrils can be postulated, it is important to establish that the antimyosin molecules combined selectively with myosin. The experiments here demonstrated that the antimyosin solution combined and fixed some antigen in the A band. Since myosin is known to be localized only in the A band of sarcomere (Hanson and Huxley, 1953; Hanson and Huxley, 1957), the presence or absence of the A band should distinctly change the staining pattern of myofibrils when they are treated with the antimyosin solution.

The experiments showed that, when myosin was extracted with the Mg-pyrophosphate solution, the former A bands did not fix the antibodies. A treatment of myofibrils with the labelled antisera, however, protected the A band from the Mg-pyrophosphate solution which is known to extract myosin selectively. At the same time, the cytological detail was lost in myofibrils pretreated with labelled normal globulin and then extracted with either Mg-pyrophosphate or KI solutions. These results indicate, therefore, that the antisera combined and selectively fixed the Mg-pyrophosphate extractable myosin in glycerinated myofibrils.

Most of the studies on the fixation of antimyosin by myofibrils have been carried out on chick and rabbit skeletal muscle (Finck, et al., 1956; Holtzer, et al., 1957; Szent-Györgyi and Holtzer, 1963; Peppe and Huxley, 1964). These and similar other investigations indicate that antimyosin fixed and protected myosin in the A band from extraction by KI or

Mg-pyrophosphate solution while normal globulin failed to do the same. These workers also found that when the viscosity of extracts from chick myosin, pretreated with normal globulin, was examined, there was a sharp drop of viscosity in the presence of ATP, a characteristic reaction of a solution containing myosin and actin. However, it was found that ATP when added to extracts of myofibrils, pretreated with antimyosin, failed to produce a change in viscosity (Holtzer, 1959). These results were explained by assuming that antimyosin forms a complex with myosin in the A band thus selectively fixing the antigen in the myofibrils by rendering it insoluble (Szent-Györgyi and Holtzer, 1960).

Results obtained using the double gel diffusion technique also indicated that most of the KI extractable myosin was combined and fixed by the antimyosin molecules in the myofibrils (Holtzer, 1961).

Szent-Györgyi and Holtzer (1963) made a quantitative study to determine the amount of myosin that reacted in striated myofibrils with antibodies prepared against myosin. When an excess of antibodies was added to myofibrils, they found that the antigenic sites in the myofibrils became saturated. On the basis of this they calculated the amount of anti-myosin bound by the myofibrils and found that one mole of myosin in the myofibril reacted with 16 moles of antibody. This result concurs with that of Samuels (1961) who found a similar ratio when antimyosin reacted with purified myosin.

On the basis of these studies it seems likely that the material which reacts with the antimyosin molecules in the myofibrils is myosin. It is

possible, however, that antibodies to other unknown antigens are also present in the antisera and that these may contribute to the staining pattern of myofibrils (Holtzer, et al., 1957). While the unknown antibodies may influence the staining pattern of myofibrils, it is nevertheless true that the lack of staining, in conditions when myosin is fixed, indicates a lack of myosin in those regions of the sarcomere which did not stain (Szent-Gy^{org}yi and Holtzer, 1960). While these studies deal with antibody staining of chick and rabbit myofibrils, many of their results are similar to those obtained in the present investigation on insect muscle. On the basis of these results obtained in this study, it may be assumed that at least one major component of the fluorescein labelled antimyosin complexes with myosin in the myofibrils of A. ellioti.

Muscle development.

This research was focused primarily on muscle differentiation during prediapause, diapause and the beginning of post-diapause development in the embryos of A. ellioti. The observations were concentrated on those embryonic stages during which cross-striated myofibrils were differentiated and contractility developed.

The results obtained in this study concerning muscle development depend largely upon two factors: one, the sensitivity of the fluorescence technique; the other, the immunological specificity of the reaction between the antimyosin containing serum and the myosin containing structures.

The results of this study indicate that, while in iron-hematoxylin stained material or material prepared for phase contrast observation,

cross-striated myofibrils could not be detected in embryos of stage 20, after treatment with fluorescein-labelled antimyosin cross-striated structures could readily be seen under the fluorescence microscope. According to the calculations of Holtzer et al., (1957) a locus of 0.1 micron in diameter which contains less than a thousand myosin molecules could be detected with the fluorescence technique. The great sensitivity of the fluorescence method is further supported by the observations of Okazaki and Holtzer (1966) who could not detect myosin filaments with the electron microscope before they could be detected with fluorescein-labelled anti-myosin. The limit of the sensitivity of the antibody technique cannot be stated with certainty, but there is little doubt that its resolution exceeds that of phase contrast microscopy or that of conventionally stained material when viewed under the bright field microscope.

The evidence supporting the view that the myosin solution used for the injection of animals was of high purity, and that the antisera reacted only with the A band material in the sarcomere can be summarized as follows:

1. Ultracentrifugal analyses indicated that the conditions used for the preparation of myosin were appropriate.
2. The presence of ATP did not cause a change in the viscosity of myosin solutions.
3. The amount of Pi liberated by myosin corresponded to the amount of terminal P in introduced ATP.
4. When the A band material was removed from the myofibrils, both

adult and embryonic, the fibrils failed to bind the antibody.

5. Myofibrils extracted with the KI solution did not fix the antibodies.

6. The washing of antimyosin stained fibrils in either KI or in Mg-pyrophosphate solution did not change the morphology of the fibrils.

7. When myofibrils, adult or embryonic, were treated with labelled normal globulin and subsequently washed in either the KI or Mg-pyrophosphate solution, no fluorescence was detected along the fibrils.

8. A previous treatment of myofibrils with antimyosin prevented them from contracting when exposed to ATP.

9. Antimyosin was not bound by tissues other than muscle either in the embryo or in the adult insect.

Although the evidence cited here indicates that the myosin antisera were highly specific for the A band material which is known to contain myosin, the possibility of there being non-specific antibodies present in the sera and that these may have influenced the staining pattern to some degree cannot be excluded.

The results of this investigation, when compared with those of earlier studies (Slifer, 1934; Neusch, 1963), reveal that the use of fluorescence antibody technique permitted a more detailed cytological observation of insect muscle development than conventionally stained material or unstained material prepared for phase contrast observation. As a consequence of the sensitivity of the technique used, some of the results of this study on the differentiation of insect muscle differ in certain specific detail from those found in the literature (Slifer, 1934; Neusch,

1963). Contrary to the observations of Slifer and Neusch, the present author found that myoblasts were able to contract only after they had differentiated to a state where they contained cross-striated filaments or myofibrils. Furthermore, the present investigation on A. ellioti also revealed that, at the time when blastokinesis begins, cross-striated myofibrils are already present in the ventral abdominal region of the embryo. These results differ from those of Slifer (1934) who could only detect spindle-shaped cells without the evidence of cross-striated fibrils during blastokinesis in grasshopper embryos. Her observation that these spindle-shaped cells, without the presence of cross-striated myofibrils, can contract is not supported by the results of the present investigation. The results of Neusch's study (1963) on the development of muscle function in Antheraea polyphemus also differ from those of the present study. He states that myofibrils within multinuclear strings of myoblasts were contracting two days prior to the appearance of cross-striation. Since it was established in the present study as well as in studies on various types of chick muscle (Holtzer et al., 1957; Holtzer and Abbott, 1958; Holtzer, et al., 1959) that only those myoblasts can contract which contain cross-striated myofibrils, the observations of both Slifer and Neusch may have been limited by the sensitivity of their techniques. As the results of the various investigations indicate, it is significant that by antibody staining and fluorescence microscopy more precise morphological observations are possible than can be achieved with other less sensitive methods.

The course of muscle development in A. ellioti, as found by using the

fluorescence method, is described as follows: Prior to and during stage 17 there is mitotic activity in the muscle forming loci of the ventral abdominal region of the embryo. Some of the cells became elongated and clustered into more or less definitive bands. At these band forming regions general mitotic activity subsided and no mitotic figures were present in the elongated cells. In stage 19 a number of these cells became spindle-shaped and differentiated to a state where they contained non-striated filaments or fibrils. At the end of diapause which corresponds to stage 20 cross-striated fibrils appear, and at the same time, the formation of multinucleated myotubes begins. Myoblasts or myotubes containing the cross-striated fibrils contracted when exposed to ATP.

The observation that mitotic activity decreased among the cells forming the future muscle bands and that mitotic figures were absent from myoblasts is in good agreement with observations made on the development of chick muscle. Stockdale and Holtzer (1961), studying the relationship between myogenesis and DNA synthesis, found that mitotic figures were never present in elongated mononucleated myoblasts which bind the antibodies. By exposing cultures of presumptive myoblasts and myoblasts to tritiated thymidine, the above authors could not detect radioactivity in the DNA of those cells which were committed to myogenesis, thus indicating that DNA synthesis ceased in these cells. The observations of Konigsberg (1963) also corroborate these results. His experiments demonstrated that myofibril formation was restricted to only those cells in which mitotic

activity had ceased. In agreement with these results are those of Marchok and Herrmann (1967) who found that the proliferation of cells continued outside the muscle forming regions, but not within the myoblasts.

The observation made in the present study that presumptive myoblasts in the A. ellioti embryos did not bind antimyosin is in accord with the findings of Holtzer et al., (1957), Stockdale and Holtzer (1961), and Okazaki and Holtzer (1966), on chick myogenesis. Furthermore, as the results of this study show, antimyosin was always bound by definite structures and not by the sarcoplasm itself in the myoblasts or in the myotubes. As already mentioned, even when fluorescence was diffused throughout the cell, there were particles present within the sarcoplasm that exhibited a stronger fluorescence than the rest of the cell. According to Holtzer (1959; 1961) such diffused fluorescence may result from injuring the cell, whereby myosin containing fibrils are broken up and dispersed in the sarcoplasm.

While these results indicate that only muscle forming cells react with antimyosin, there are observations (Ebert, et al., 1966; Ebert, 1959; Ranzi, 1962) which are not in agreement with these results. For example Ranzi and his co-workers (1962) found that precipitation occurred between antimyosin and some extract from the gastrula of the toad. They found likewise, that some fraction of the total body extract precipitated supposed myosin in the blastula of the toad. It should be noted, however, that the antisera which these investigators used was prepared against total body extracts and therefore, the serum may have contained not only

antimyosin, but other antibodies as well. It is doubtful then that the precipitation reaction in these systems was due to myosin and antimyosin alone.

It is of some interest that in both chick myoblasts (Holtzer, et al., 1957; Holtzer, 1961) and in A. ellioti the first fibrils within the myoblasts are found to be in close association with the sarcolemma. According to Holtzer (1961) such intimate association of myofibrils with the sarcolemma may indicate that the cell surface serves as a site for the synthesis of cell structures.

It has been noted previously that the first structures which bind the antimyosin are uniformly fluorescent, and that cross-striated fibrils appear only during a later stage in development. That this highly organized state is preceded by one in which only non-striated fibrils could be found, is in accordance with observation made during development of both insect and chick muscle. Wigglesworth (1956) found that in Rhodnius prolixus the first filaments that appeared were uniformly birefringent when viewed with the polarizing microscope, and that the isotropic bands appeared later in development. The observations of Slifer (1934) and Neusch (1963) that non-striated fibrils can be detected in insects before cross-striated ones also corroborate the results of the present investigation. Using fluorescein-labelled antimyosin Holtzer (1961) and Holtzer et al., (1957) also found that in myotomes of chick embryos the myofibrils which first appeared were uniformly fluorescent.

These observations, however, are not in agreement with those of

Shafiq (1963), who reported that in the developing flight muscles of Drosophila melanogaster non-striated fibrils were absent at any stage of development. His observations with the electron microscope revealed that even the first fibrils which could be detected were already periodically divided into sarcomeres. Shafiq's results, while they differ from those mentioned above, may indicate that in different groups of insects and in different muscle types, the course of development may follow different paths (Snodgrass, 1935; Johannsen and Butt, 1941; Tiegs, 1955; Pringle, 1965).

The first cross-striated myofibrils appeared to bind the antimyosin the same way as did mature muscle. That the antibodies were localized in the A band was demonstrated by the failure of myofibrils to bind the antimyosin when the A band material was previously extracted. Similar results have been obtained in chick muscle (Holtzer et al., 1957; Holtzer, et al., 1959; Holtzer, 1961) where the staining pattern of embryonic myofibrils corresponded to that of mature muscle.

The longitudinal dimension of the fluorescing A band in A. ellioti embryos was in the same range (4-7 micron) as was found for adult muscle. Likewise, the longitudinal dimension of the entire sarcomere in the embryonic fibrils corresponded to that found for the adult muscle (7-15 microns). Measurements made by Koshira and Maruyama (1958) on the dimensions of the sarcomere in the developing thoracic muscles of honeybee also indicated that the length of the sarcomere remains constant during the development of insects. Observations made on chick muscle (Holtzer,

1961) are similar to those described above.

The increase in the number of cross-striated myofibrils as development proceeds is conspicuous. The number of fibrils within the myotubes increased until they seemed to form a thin sheet over the entire myotube. The way by which new fibrils were formed was not investigated in detail during this study, but in view of the present investigation and those of others (Tiegs, 1955; Wigglesworth, 1956; Koshira and Maruyama, 1958), certain comments can be made. The absence of fluorescence in the sarcoplasm and in the nuclei of both the myoblasts and of myotubes may be regarded as an indication that new fibrils are not synthesized in the nuclei or in the sarcoplasm but may be derived from already existing fibrils or may be synthesized in situ along the pre-existing fibrils (Holtzer, 1961). According to Wigglesworth (1956) in Rhodnius prolixus new myofibrils are the result of a longitudinal splitting of pre-existing filaments. Tiegs (1955) also observed in certain types of insect muscle that the growth of fibrils is achieved by a cleavage of the older fibrils. The possibility therefore, that in A. elliotti the multiplication of myofibrils is achieved by a longitudinal splitting of pre-existing fibrils cannot be excluded.

The use of antimyosin, prepared against the myosin of the adult insect, to analyze muscle development in the embryo gives rise to an important question. Is there any structural or immunological difference between embryonic and mature form of a protein species? Some immunologists have claimed (Ebert, 1955, 1959) that proteins in embryonic cells

are different from the homologous proteins in mature cells. One example of certain immunological dissimilarities between embryonic and mature forms of a protein was demonstrated by Stein et al., (1957) with these two forms of hemoglobin.

A certain degree of difference in the immunological specificity between young and adult rabbit myosin was found by Varga et al., (1962). Through a series of precipitation tests, these investigators demonstrated that a very highly concentrated adult immune serum was necessary to precipitate myosin derived from a young rabbit. In accord with their results, they suggested that during the course of embryonic development, the change in muscular activity is accompanied by a change in the structure of myosin or by a change in its immunological specificity.

Biochemical changes in the nature of the protein components of the myofibrils during embryonic development in insects was studied by Maruyama and his associates (Maruyama, 1954; Maruyama and Moriwaki, 1958; Ohshima, Maruyama, and Noda, 1965). Their results, like those of Varga et al., (1962), indicated that there exists a certain relationship between the quantity and quality of some of the contractile proteins and the activity of an insect. For example, it was found by Maruyama (1954) that in the honeybee the actomyosin content decreased at pupation but increased again during the emergence of the adult. In addition, not only the amount of actomyosin was at a low level during the pupal period but its ATP-ase activity was also low when compared to that of the adult.

Ohshima, Maruyama, and Noda (1965) found definite changes in the

characteristics of contractile proteins during embryonic development of chick. Viscosity measurements indicated that the ATP sensitivity of actomyosin increased more than ten fold upon hatching as compared to the sensitivity of 11 day old embryos. In addition, the ATP-ase activity of actomyosin also increased at hatching. Furthermore, they found that the size of the actomyosin molecule increased during development, but they did not find such a change in the size of the myosin.

Although the above observations suggest that some of the proteins of the myofibril may undergo change as development proceeds, in the present study there was no indication that the myosin, first detected in the cross-striated filaments, is different from that in mature muscle. The fluorescence pattern exhibited by the embryonic fibrils when stained with antimyosin which was derived from adult muscle was similar to that in adult myofibrils. The similarity between adult and embryonic myofibrils was also indicated by the response to ATP of the cross-striated embryonic fibrils. As in adult muscle, antimyosin-treated embryonic fibrils were resistant to ATP and did not contract. The observation that antimyosin made the A band in embryonic fibrils resistant to extracting solutions, as it did in adult fibrils, also suggests that embryonic fibrils bound the antimyosin the same way as did adult ones. These results are in agreement with those of Finck, et al., (1956), Holtzer and Abbott (1958), and Holtzer (1961) who studied chick muscle. Holtzer et al., (1957) found that antimyosin, prepared against adult chick myosin, localized in the same way in both embryonic and adult muscle. Furthermore, it was found

by Finck, et al., (1956) that myoblasts which contained cross-striated myofibrils contracted upon exposure to ATP in the same way as did mature muscle, and that the pattern of cross-striation which appeared during the contraction of embryonic fibrils was the same as during the contraction of adult muscle. These observations and those of the present study indicate similarities between mature and embryonic myofibrils. It is possible that differences exist between embryonic and mature myofibrils of A. eliotti, but they could not be detected using the techniques of this investigation.

It has been suggested that the mechanism of blastokinesis involves active movements of the embryo, and thus muscular activity is required (Nelsen, 1931; Slifer, 1932, 1934). Slifer (1934) stated that spindle-shaped cells without cross-striated myofibrils in the abdominal region of the grasshopper embryo were responsible for embryonic movements. According to the results of the present study, only those cells contracted which contained cross-striated myofibrils. It is possible that Slifer's techniques were not sensitive enough to detect cross-striation in contracting cells even though cross-striated myofibrils may have been present. The results of the present study support Slifer's proposal regarding the possible mechanism of blastokinesis. Although there was no attempt made in the present investigation to determine whether the presence of functional muscle is a prerequisite for blastokinesis, there is a possibility that muscular activity contributes to these movements along with other factors as proposed by Le Berre (1952) (as reported by Roemhild, 1967). It is of interest that cross-striated muscle appears in A. eliotti embryos at the

time when blastokinetic movements are initiated.

Diapause in insect embryos is a period of arrested development independent of environmental factors (Van Horn, 1966b). While the differentiation of new structures may cease during this period, there are indications that biosynthetic activity and certain morphological changes in existing structures may take place (Kaocharern, 1958; Van Horn, 1963, 1966b). In studying embryonic development of A. elliotti Van Horn (1963, 1966b), found the ventral head gland, an endocrine structure, to be present by stage 14. Between stages 14 and 19, which includes diapause, she observed a very conspicuous increase and morphogenetic change within this structure, however, she could not detect any functional activity in this gland during this period. As diapause is terminated, the ventral head glands increase in size, undergoing a cycle of activity, then gradually decrease in size in the last stages of embryonic development (Van Horn, 1967).

Much consideration has been given to the possibility that hormonal regulation is involved in insect diapause (Harvey, 1962). For example, Williams (1946) found in Platysamia pupae that the brain released some factor which was responsible for the termination of diapause and for the initiation of further development. The diapause of silkworm embryos has been shown by Fukuda (1951) (as reported by Jones, 1956) to be regulated by a diapause factor originating in the suboesophageal ganglion of the maternal parent. Jones (1953) has suggested that during diapause in embryos of Locustana pardalina the brain liberates some factor or factors

at the time when diapause is terminated. He concluded that, "The initiation of growth and differentiation on the termination of diapause in the egg of pardalina takes place before the ventral head glands are formed." On the other hand, observations by Van Horn (1963, 1966b) revealed that the embryonic neuroendocrine system of A. elliotti had differentiated prior to the time of diapause (40 days at 25° C). She proposed (1967) that as diapause development proceeds, the accumulation of brain hormone may be responsible for the simultaneous activation of the corpora allata and ventral head gland which leads to the termination of diapause. These glands, through their secretions, provide a suitable environment for the further growth and differentiation of the developing embryo.

That the presence of some factors, possibly hormones, is required for cell differentiation is also born out by the experiments of Nöthiger and Oberlander (1967). They found that male genital disc cells may remain embryonic for extended periods of time when cultured in an adult environment. When these cells are placed in an embryonic environment, they begin to differentiate. This indicates that the capacity of the cell to differentiate can only be realized in the embryonic milieu in which the necessary factors, possibly hormones, are present. These observations further suggest that the development of insects may be arrested either because some inhibitor is present or because of the lack of a factor which is necessary for further development.

Further evidence for the requirement of hormone-like substances to be

present in tissue culture for myogenesis to proceed was given by de la Haba et al., (1966). They found that, if insulin and somatotropin was added to the culture media, the formation of myotubes was enhanced. Insulin seemed to exert its influence by stimulating the fusion of myoblasts to form myotubes. They proposed that insulin or an insulin-like substance is present in the yolk of the chick egg, and that it provides an environment in which myogenesis can take place. That some agents present in the microenvironment of the cell stimulate myosin synthesis and the fusion of myoblasts, was also suggested by Okazaki and Holtzer (1965).

In light of the observations and suggestions discussed above one may speculate concerning the course of muscle development in A. ellioti. It is possible that, prior to diapause, some developmental factor may be present in the embryo which stimulates presumptive myoblasts to synthesize myosin. At the time of diapause this factor is either exhausted, chemically bound and thus unavailable, or is inhibited. The brain hormone may accumulate, however, so that at the termination of diapause the corpora allata and ventral head gland are stimulated to secrete the juvenile hormone and ecdysone. These substances may provide a new environment for the cell populations within the embryo in which further synthesis and differentiation will be favored. Such hormonal agents may activate molecules on the surfaces of myoblasts and thus facilitate fusion to form myotubes as well as to activate other species of molecules within the cytoplasm to resume myosin synthesis. To confirm these hypotheses further experimental evidences are necessary.

SUMMARY

Muscle differentiation in A. ellioti embryos was studied by immunological means. Antibodies, against myosin of adult grasshopper, were labelled with fluorescein isothiocyanate and used to investigate the development of muscle in the prediapause, diapause and early postdiapause periods of development.

The embryos were subjected to certain of the following treatments:

- 1) Specimens were fixed, sectioned, stained with iron-hematoxylin and examined by bright field microscopy.
- 2) Embryos were extracted in glycerol, squashed and observed under the phase contrast microscope.
- 3) Glycerol extracted embryos or squashed preparations were treated with fluorescein-labelled antimyosin and observed under the phase contrast and fluorescence microscope.

In specimens stained with fluorescent antimyosin, uniformly fluorescent non-striated myofibrils were detected in the ventral abdominal region of the embryo by the beginning of stage 19. Cross-striated fibrils appeared first as diapause was terminated at which time blastokinesis movements began. These cross-striated fibrils appeared to bind the antimyosin in the same way as did mature muscle. It was found that only those fibrils which were cross-striated contracted when ATP was added.

Data obtained from viscometry, ultracentrifugal analysis, spectrophotometry and ATP-ase test were used as criteria of purity for the myosin preparations. To establish the distribution of antibodies, both in the adult and embryonic myofibrils, various procedures were used. Treatment of specimens with labelled normal globulin, Mg-pyrophosphate, and KI

solutions indicated that the antimyosin was bound by the A band in the sarcomere.

It is suggested that muscular activity is involved in the blastokinetic movements of the embryo, and that muscle development is under hormonal control.

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