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

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.

STUDIES ON THE DEVELOPMENT OF MUSCLE IN EMBRYOS OF AULOCARA ELLIOTTI (THOMAS) (ORTHOPTERA, ACRIDIDAE) USING THE FLUORESCENT ANTIBODY TECHNIQUE

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ABSTRACT

Muscle differentiation and the development of contractility was investigated in embryos of Aulocara 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.

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. elliotti 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-stration, 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 elliotti. 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 homogeniety 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. elliotti 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. elliotti 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 prediapause development, and at stage 19, which is reached approximately 40 days at 25° C after the eggs are laid, the embryos of A. elliotti 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° 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 $MgCl_2$, 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 $MgCl_2$ 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 micra.

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 sodiumcarbonate - 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. elliotti 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