Muscle proteins in the chick myotome examined by the immunofluorescent method

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INTRODUCTION

In previous studies on the development of the somite (Langman & Nelson, 1968), the majority of the cells of the myotome appeared to arise from the dermatome and not, as previously suggested, from the cells of the so-called dorso-medial somite lip (Williams, 1910; Hamilton, 1952; Boyd, 1960). Once the myotome cells, characterized by a pale, round to irregular, nucleus and darkly stained nucleolus, are formed, they fail to synthesize DNA. During subsequent development, the myotome appears to extend in ventro-lateral direction by the addition of new cells originating primarily from the dermatome. After the dermatome has lost its epithelial structure, the myotome, in transverse section, consists of a tissue band with an abundance of cytoplasm and a large number of round to spindle-shaped nuclei. Though DNA synthesis was rarely observed among the cells of the extended myotome, considerable proliferative activity was observed in the adjacent tissues.

To determine at which stage of development the cells of the myotome begin to synthesize muscle proteins, Holtzer, Marshall & Finck (1957) used the fluorescent antibody technique on glycerol-extracted, squashed, and teased samples of myotome cells obtained from Hamburger & Hamilton (1951) stage 14–29 embryos. According to the authors, two types of cells can be distinguished in muscle differentiation, one type consisting of elongated, spindle-shaped cells and the other of round to irregular cells similar to mesenchyme cells. The elongated cells, referred to as ‘myoblasts’, begin to synthesize myosin at stages 15–16; they have then lost their ability to synthesize DNA. The mesenchyme-like cells, referred to as ‘presumptive myoblasts’, will differentiate into myoblasts, but cannot histochemically be differentiated from surrounding mesenchyme cells. Unlike the myoblasts they are capable of synthesizing DNA (Stockdale & Holtzer, 1961).

In contrast to the work of Holtzer et al. (1957), who stated that myosin is the first specific muscle protein to appear during development, Ogawa (1962) found

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by means of precipitin tests that actin appears in the muscle fibers at the 72 h stage and myosin approximately 24 h later.

When the data obtained with the immunofluorescent and precipitin techniques are compared with information from electron-microscopic studies, considerable agreement about the stage of development at which the muscle proteins appear is noted. Przybylski & Blumberg (1966) state that at stages 15–16 the cells of the myotome, dermatome and adjacent mesenchyme are ultrastructurally similar to each other. By stages 16–17, however, some of the myotome cells assume an elongated shape and the first myofilaments, 50–70 Å in diameter, appear. Slightly later dense filaments 100–120 Å in diameter become visible. During subsequent stages the 100 Å filaments are separated by smaller 50 Å filaments, suggesting an early A and I band configuration. Allen & Pepe (1965), Dessouky & Hibbs (1965), and Obinata, Yamamoto & Maruyama (1966), who likewise studied muscle differentiation in the chick, come to only slightly different conclusions and it is now generally accepted that the thin filaments, believed to represent actin, coincide with or precede slightly the appearance of the thick filaments thought to be myosin (Huxley, 1957).

Since the fluorescent studies have been carried out mainly with squashed or teased cells, thereby disturbing the interrelationship of the presumptive myoblasts, myoblasts and myotubes, and the ultrastructural investigations are handicapped by their limited field of vision, the precise interrelationship of the cells in the developing myotome remains unknown. Hence, this work was undertaken to examine by the immunofluorescent antibody technique at which stage and at what place in the myotome plate the first specific muscle proteins arise. In addition, by using transverse sections we hoped to obtain a better insight into the spatial interrelationship of presumptive myoblasts, myoblasts and myotubes.

MATERIALS AND METHODS

Preparation of myosin antiserum. Chicken myosin was prepared according to the Szent-Györgyi method (1951) with some minor modifications as previously described (Ikeda, Abbott & Langman, 1968). One gram of myosin was dissolved in 1 ml 0·6 M Tris/sodium EDTA/boric acid (TEB) buffer (pH 8·4) and to this solution was added an equal volume of Freund's complete adjuvant (Difco). Two ml of the emulsion was injected subcutaneously into each of a number of albino rabbits. The injections were repeated with 7-day intervals for 2–3 months. Serum was collected repeatedly throughout the course of immunization and tested for its specificity by the agar-gel immuno-electrophoretic technique (Ikeda & Zwaan, 1967). If satisfactory results were obtained, the serum was stored in small aliquots at −20 °C. In order to examine the specificity of the myosin antiserum, it was tested against total muscle extract, actin, myosin, adenylic acid deaminase, chicken serum and liver extract. Actin and adenylic acid deaminase were prepared as previously described (Ikeda et al. 1968).
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Tissue preparation. Fertilized White Leghorn eggs were incubated at 38 °C and the embryos removed 52 h to 5 days after the beginning of incubation. The embryos were then staged according to Hamburger & Hamilton (1951) and fixed in either Carnoy's solution, acetone or 95 % ethyl alcohol. Since the latter two fixatives damage the histological structure of the myotome area, Carnoy's solution was finally selected as the best fixative for our experiments. It gave a minimum of autofluorescence and a maximum specific reaction. After the tissue was fixed for 2 h in Carnoy's solution at 3 °C, it was dehydrated and embedded in paraffin in the usual way. Sections were cut at 3–5 μ and paraffin removed in two changes of xylene for 15 min, followed by three changes of 95 % ethanol for 15 min. Finally, they were rinsed twice for 10 min in buffered saline. To determine whether myosin was denatured by the heat of the embedding process myosin extract was heated at 60 °C for 15 min (Locker, 1956). In subsequent immunoelectrophoretic tests the heated myosin reacted similarly to the unheated control.

Immunofluorescent method. The indirect fluorescent method ('sandwich technique') was selected for our experiments because of its great sensitivity (Coons, 1956). Goat gamma-globulin solution, prepared against rabbit gamma-globulin and conjugated with fluoresceinisothiocyanate, was obtained commercially (Difco). To reduce the non-specific staining, the solutions were absorbed with mouse tissue powder (Difco).

The tissue sections were exposed to chicken myosin antiserum for 20 min in a moisture chamber at 20 °C. They were then washed twice for 10 min with buffered saline and covered with fluorescent goat globulin for 20 min. After rinsing twice in buffered saline, the sections were mounted in a buffered aqueous solution of polyvinyl alcohol (Elvanol, grade 5105; Dupont Electrochemicals Department, Wilmington, Delaware) (Rodriguez & Deinhardt, 1960). Control experiments were performed as described previously (Ikeda & Zwaan, 1967).

The slides were examined under a Reichert Zetopan fluorescence microscope equipped with a high-pressure mercury vapor lamp HBO-200 with primary filters (Schott) UG 1 and BG 12 and barrier filter GG 9. Photographs were made on 35 mm Tri-X Pan film (Kodak), which was developed with Rodinol (Agfa) 1:100 for 13 min at 20 °C.

RESULTS

Immunoelectrophoretic experiments. When myosin extract is tested against total muscle antiserum, one distinct and one small precipitin band appear (Text-fig. 1A). When, however, total muscle extract is tested against myosin antiserum, a minimum of 3–4 precipitin bands appear, indicating that the myosin antiserum to be used in our further experiments contains antibodies not only against myosin but also against several other fractions (Text-fig. 1 B). Since one of these bands has a position corresponding to that formed by adenylic acid deaminase when tested against total muscle antiserum (Text-fig. 1C), and
another band corresponds to that formed by actin (Text-fig. 1D), it is evident that the myosin antiserum contains antibodies against adenylic acid deaminase, actin and possibly some other antigens. It does not contain as many antibodies as total muscle antiserum, as is apparent from the precipitin pattern formed by testing total muscle extract against its own antiserum (Text-fig. 1E).

**Immunofluorescent experiments.** The first fluorescent reaction over the cells

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**Text-fig. 1.** Drawing of the immunoelectrophoretic precipitin pattern formed by testing the following. A, Myosin against total muscle antiserum: note the dense precipitin band and the small band close to the antigen well. B, Total muscle extract against myosin antiserum: in comparison with A it is evident that the myosin antiserum contains antibodies against several proteins. C, Adenylic acid deaminase against total muscle antiserum: compare the position of this band with those found in B. D, Actin against total muscle antiserum: compare the position of the band with those found in B. E, Total muscle extract against total muscle antiserum.

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**PLATE 1**

Figs. A–D. Semi-schematic drawings of transverse sections through the brachial myotomes of chick embryos at various stages of development. The outline of the dermatome and myotome is indicated by white dotted lines. The myotome cells giving a positive reaction for the presence of muscle proteins are indicated with white ink. L, Lateral; M, medial; d, dermatome. Note the gradual expansion of the positive myotome cells and the disappearance of the dermatome.

Fig. E. Transverse section through the brachial myotome of a chick embryo (stage 20). Note that the fluorescence of the cells on the lateral side of the myotome is stronger than that of the cells on the medial side. The dorsal portion of the myotome does not contain any positive cells.

Fig. F. Similar section as in fig. E, but passing through two successive myotomes (stage 22). Note the bud extending from the medial side of the ventral portion of the myotome (arrow).

Fig. G. Similar section as in fig. E. Note the bud extending from the ventral extremity of the myotome (arrow).
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of the brachial myotomes is found in embryos of stages 17–18 (Plate 1, fig. A). At this stage of development the myotome is extended in the ventro-lateral direction and reaches the coelomic cavity. The dermatome has lost its epithelial structure except for the most dorsal portion. The few cells showing a positive fluorescent reaction, indicating the presence of muscle proteins, are located on the lateral side of the myotome plate, with the exception of an occasional area where they are found throughout the thickness of the myotome (Plate 1, fig. A). The most dorsal portion of the myotome, which is still in contact with the dermatome, does not show any fluorescence. Hence, the synthesis of muscle proteins starts cell by cell in a random fashion, and occurs initially on the lateral side of the myotome.

In subsequent stages, the fluorescent reaction not only spreads to other cells on the lateral side of the myotome (Plate 1, fig. B) but also increases in intensity (Plate 2, fig. A). At stages 19–20 (3½ days) the fluorescent reaction gradually spreads medially, but the dorsal end of the myotome plate and the extreme medial side remain negative (Plate 1, figs. C, E; Plate 2, fig. B). Simultaneously with the spreading of the fluorescent reaction in medial direction, the myotome increases in thickness. Many negative cells are seen on the medial side of the myotome among the positive ones (Plate 2, fig. C). During stages 21–24 the epithelial structure of the remaining portion of the dermatome gradually disappears and the fluorescent reaction then spreads further in dorsal direction (Plate 1, fig. D; Plate 2, fig. B).

At stages 24–26 (4½–5 days) the cells on the lateral side of the myotome begin to form myotubes (Plate 2, figs. C, D) which under higher magnification show cross striations characteristic for the myofibrils. In the central area of the myotome the cells are spindle-shaped and arranged into parallel bundles; on the

Plate 2

Fig. A. Transverse section through a brachial myotome of a stage-19 chick embryo. The dotted line indicates the medial border of the myotome plate. Note the heavily fluorescent cells on the lateral side of the myotome and the negative and lightly fluorescent cells on the medial side. L, Lateral; M, medial.

Fig. B. Transverse section through a stage-22 embryo. The dorsal portion of the myotome still contains negative cells. In ventral direction the fluorescent reaction becomes gradually more intense. The dermatome cells have almost completely lost their epithelial structure. × 440.

Fig. C. Transverse section through the brachial myotome of a chick embryo, stage 24. Throughout the thickness of the myotome and particularly on the medial side (arrow) some fluorescent negative cells are found interspersed between the strongly fluorescent cells. Note the negative nuclei surrounded by a fluorescent circle. × 440.

Fig. D. Sagittal section through the myotome of a chick embryo, stage 26. On the lateral side are found many long fluorescent bundles representing the myofibrils of the myotubes. In the more central area are found many strongly fluorescent spindle-shaped cells; on the medial side some weakly fluorescent cells are present. Unlabeled cells are found throughout the myotome, but particularly on the medial side. Note the fluorescent granules over the myotube region. × 440.
medial side some cells are weakly fluorescent while others are negative. Fluorescent granules appear in the region of the myotubes, and are seen only after myotube formation has started (Plate 2, fig. D).

**DISCUSSION**

One of the difficulties of the fluorescent antibody technique is that the precise localization of a protein can only be undertaken with success after the specificity of the antibody has been thoroughly tested. Our immunoelectrophoretic studies on the purity of the myosin antiserum, indeed, demonstrated the presence of a number of contaminating antibodies. Among these were antibodies against actin and adenylic acid deaminase and possibly some other components. Holtzer *et al.* (1957) likewise reported that their myosin antiserum contained one major antibody and one or two trace components. Samuels (1961) and Finck (1965) suggested that the myosin fractions were contaminated with adenylic acid deaminase, actomyosin or nucleoprotein. Despite repeated fractionation of the chemically prepared myosin with ammonium sulfate, the contaminating components could not be completely removed (Holtzer *et al.* 1957; Finck, 1965). Since our fluorescent antibody experiments were performed with impure myosin antiserum, we have refrained from stating that the positive reaction is caused by myosin, but rather by the presence of muscle proteins.

In our work the first fluorescent cells appeared at Hamburger & Hamilton stage-17 embryos. This is slightly later than indicated by the work of Holtzer *et al.* (1957), who found the first reaction at stages 15–16. Initially the cause of the minor discrepancy was thought to be a difference in the specificity and strength of our antiserum in comparison with that of Holtzer. Considering, however, that our antiserum demonstrated the presence of muscle proteins in widely separated, individual cells and that immunoelectrophoretically it not only reacted with myosin but also with actin, it seems more likely that the difference is caused by our experimental technique. Holtzer *et al.* (1957) used glycerol extracted, squashed and teased tissues, while we used \( \frac{3}{4} \) transverse sections. The amount of muscle proteins capable of reacting with the antiserum was undoubtedly, in our work, considerably less than in the case of whole cells. Whether the first reaction found in our work is caused by actin or myosin or possibly by both remains to be studied with more highly purified antibodies. Allen & Pepe (1965) found that thin filaments (50–70 Å in diameter), in all likelihood representing actin molecules, appear at stage 16 and are initially randomly dispersed through the cytoplasm. This observation was confirmed by Obinata *et al.* (1966) and Przybylski & Blumberg (1966). Dessouky & Hibbs (1965), on the contrary, found that the thin and the thick filaments, representing myosin, appear approximately at the same time. Hence, it is impossible at present to state which of the two proteins is synthesized first, or whether both appear at the same time.
In previous work (Langman & Nelson, 1968) the cells of the myotome were found to appear first in the dorsal region and from this position to extend in ventro-lateral direction by the addition of new cells formed by the overlying dermatome. Hence we expected that the muscle proteins would appear first in the dorsal region of the myotome. On the contrary the first fluorescent cells, indicating synthesis of muscle proteins, appear in the more central regions of the myotome. Muscle protein synthesis in the most dorsal portion of the myotome does not start until the adjacent dermatome cells have lost their epithelial structure. Only then does the fluorescent reaction gradually extend in dorsal direction. Two possible explanations of this observation must be considered: (1) the cells of the dermatome inhibit the synthesis of muscle proteins in the adjacent myotome cells; (2) the first formed myotome cells migrate from their initial position in a ventro-lateral direction and are continuously replaced dorsally by new cells formed by the dermatome. Since the latter possibility seems more probable to us, this would mean that the ventro-lateral extension of the myotome is partially caused by migration of cells from dorsal to ventral and partially by the addition of new cells.

Holtzer et al. (1957) and Stockdale & Holtzer (1961) called the cells which synthesized muscle proteins ‘myoblasts’, while the cells destined to become myoblasts, but not yet synthesizing muscle proteins, are referred to as ‘presumptive myoblasts’. Though the myoblasts do not synthesize DNA, the latter cells still maintain this ability. Our observations indicate that differentiation from presumptive myoblast to myoblast and from myoblast to myotome occurs first on the lateral side of the myotome and subsequently in the central and medial areas. Initially the myotome cells formed by the dermatome synthesize neither muscle proteins nor DNA. With the disappearance of the dermatome the first muscle proteins become evident in the cells along the lateral border of the myotome. These cells we will call myoblasts. The cells on the medial side of myotome plate still synthesize neither muscle protein nor DNA. Since they form an intermediate stage between the myoblast and presumptive myoblast, we will refer to them as ‘primitive myoblasts’. In preliminary experiments in which the fluorescent technique was combined with the radioautographic technique, it was noted that adjacent to the medial side of the original myotome many cells were synthesizing DNA. Since these cells, according to our observations, will later start to synthesize muscle proteins, we will call them presumptive myoblasts. The progressive differentiation from presumptive myoblasts to myotubes becomes particularly evident by stage 24. At this stage myotubes with distinct cross striations are visible on the lateral side of the myotome. In the central area (or medial side of the original myotome) are found spindle-shaped myoblasts, sometimes arranged in parallel bundles, sometimes as individual cells. On the medial side some cells have just started to synthesize muscle proteins, while others are still negative.

Simultaneously with the formation of the myotubes, we noted the appearance
of fine granular fluorescent droplets. These droplets, localized over the myotube area and immediately adjacent to it, were not seen over the spindle-shaped myoblasts or on the medial side of the myotome. Though the granules may be artefacts, Przybylski & Blumberg (1966) reported that during the fusion of myoblasts into myotubes, the cell membranes break down and some cytoplasmic material and ribosomes appear outside the cells. It can be argued that these particles are much too small to show a fluorescent reaction, but keeping in mind that in our experiments the sensitive indirect 'sandwich' method was used, it seems possible that some of the fluorescent granules contain muscle proteins which leaked out of the myoblasts during myotube formation.

Considering the progressive differentiation from presumptive myoblasts to myotube as a process which begins initially on the lateral side of the myotome, which factor or factors initiate the synthesis of muscle proteins? When Holtzer & Detwiler (1954) grafted tissue strips of spinal cord and somites obtained from salamanders into similarly aged hosts, the somite cells formed muscle and cartilage. When they did not include spinal cord in the graft, the somites failed to differentiate. In similar experiments performed in the chick embryo, the spinal cord was likewise found to be of primary importance for the differentiation and growth of muscle cells (Lash, Holtzer & Holtzer, 1957). According to Holtzer and co-workers, the influence of the spinal cord appears to be mediated by a factor which is transmissible through mesenchyme. Whether this factor is transmissible through such a thick layer of mesenchyme, as is present in stage 17–20 embryos, remains, in our opinion, questionable.

SUMMARY

This work was undertaken to examine by the immunofluorescent antibody technique at which stage of development of the chick embryo the first muscle proteins are formed. In addition the spatial interrelationship of presumptive myoblasts, myoblasts and myotubes was studied. Chick embryos ranging in age from 52 h to 5 days were fixed in Carnoy and transverse sections cut at 3–4 μ. The indirect fluorescent method (sandwich technique) was applied using fluorescent goat gamma-globulin and myosin antiserum.

1. When the specificity of the myosin antiserum was tested by the immunoelectrophoretic technique, antibodies against myosin as well as against actin, adenylic acid deaminase and some other fractions were detected. Consequently, the fluorescent reactions found in the embryo do not specifically indicate the presence of myosin, but rather that of muscle proteins.

2. The first fluorescent reaction over the myotome was found at stages 17–18, and was restricted to a few cells on the lateral side of the central myotome region. From here the reaction spread rapidly to other cells on the lateral side in a ventral and dorsal direction. Simultaneously with the increase in thickness of the myotome, the fluorescent reaction spread in a medial direction. The cells
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of the myotome, particularly those in the most dorsal portion, did not begin to show fluorescence until the adjacent dermatome cells had lost their epithelial structure.

3. At stages 24–26 the lateral side of the myotome is formed by myotubes with fluorescent myofibrils. In a medial direction are found subsequently heavily fluorescent spindle cells, either in bundles or as individual cells, which in turn are followed by irregular cells either slightly or not at all fluorescent. The latter cells sometimes showed DNA synthesis. Hence, the differentiation from presumptive myoblast to myoblast and from myoblast to myotube proceeds from lateral to medial.

RESUME

Examens des protéines musculaires du myotome de poulet par la méthode d’immunofluorescence

Ce travail a été entrepris pour examiner, à l'aide de la technique des anticorps immunofluorescents, à quel stade du développement de l'embryon de poulet sont formées les premières protéines musculaires. On a étudié en outre les relations spatiales mutuelles entre myoblastes présomptifs, myoblastes et myotubes. Des embryons de poulet d'un âge compris entre 52 h et 5 jours ont été fixés au Carnoy et débités en coupes transversales de 3 à 4 μ. On a appliqué la méthode de fluorescence indirecte (technique du 'sandwich') en utilisant de la gamma-globuline fluorescente de chèvre et un sérum anti-myosine.

1. Quand la spécificité du sérum anti-myosine a été éprouvée par la technique d'immunoélectrophorèse, on a décelé des anticorps anti-myosine ainsi que anti-actine, anti-adénylacidodésaminase et quelques autres fractions. Par conséquent, les réactions fluorescentes trouvées chez l'embryon n'indiquent pas spécifiquement la présence de myosine mais plutôt celle de protéines musculaires.

2. La première réaction fluorescent sur le myotome a été trouvée aux stades 17–18; elle est restreinte à quelques cellules sur la face latérale de la région centrale du myotome. A partir de là, la réaction s'étend rapidement aux autres cellules de la face latérale, dans les directions ventrale et dorsale. En même temps que s'accroît l'épaisseur du myotome, la réaction fluorescente s'étend en direction médiane. Les cellules du myotome, en particulier celles de la partie la plus dorsale, n'ont pas commencé à présenter de fluorescence avant que les cellules adjacentes du dermatome aient perdu leur structure épithéliale.

3. Aux stades 24–26, la face latérale du myotome est formée de myotubes avec des myofibrilles fortement fluorescentes. Dans la direction médiane, on ensuite des cellules fusiformes fortement fluorescentes, soit en faisceaux, soit isolées, qui sont à leur tour suivies par des cellules irrégulières, soit légèrement fluorescentes, soit pas du tout. Ces dernières cellules ont parfois montré une synthèse d'ADN. De là, la différenciation des myoblastes présomptifs en myoblastes et des myoblastes en myotubes a lieu depuis la région latérale vers la région médiane.
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