Ultrastructural changes during transition of larval to adult intersegmental muscle at metamorphosis in the blowfly Calliphora erythrocephala

II. The formation of adult muscle

By A. C. CROSSLEY

From the School of Biological Sciences, University of Sydney, Sydney 2006, Australia

SUMMARY

A residual myofibre resulting from autolysis of larval muscle, and from myoblast fusion, has been described in an accompanying paper. This report traces the reorganization of such a myofibre during development of contractile adult muscle at metamorphosis. Microtubules remain in the residual myofibre, but they are not oriented with respect to the fibre axis. Organization of microtubules into an array precedes myofilament formation, and this array is oriented with respect to the fibre long axis. The distribution of microtubules is ordered but not precise, and statistical evidence is presented to show that there is a preferred separation distance of 800 Å between adjacent microtubules. Possible mechanisms for control of this distribution are discussed. It is suggested that long-range electrostatic forces may be involved, rather than structural cross-bridges.

Coated vesicles occur on the plasma membrane, but are not obviously associated with sarcomere organization. There is no morphological evidence that larval sarcomere organization persists in residual myofibres, and the first indication of adult sarcomeres is the development of periodic electron dense deposits at the periphery of the myofibre. The electron dense deposits develop into Z-bodies and define the adult sarcomeres. Finely filamentous material is associated with the Z-bodies, but the nature of this material is obscure. The filaments have been termed 'initial filaments' by other authors working on developing muscles, but an anatomical similarity with tertiary 'ultrathin', 'residual', or 'C' filaments, described in contractile muscle, is pointed out. The first-formed thick primary myofilaments are of reduced diameter, as in certain other insect and rat muscles. The orientation of developing myofilaments is related to the pre-existing microtubule array, which appears to serve the function of 'scaffolding'. The ratio of myofilaments to microtubules slowly increases, but microtubules remain in adult muscle. The presence of a microtubule disturbs the precision of the myofilament paracrystalline array. Infoldings of the plasma membrane extend into the T-system, and develop an association with small cisternae (that have already formed) between microtubules. The association becomes a diad, and the cisternae become sarcoplasmic reticulum. Large nuclei derived from larval muscle bear many nuclear pores and a well-developed fibrous lamina, and are believed to be highly polyploid. Small nuclei derived from myoblasts by cell fusion bear few nuclear pores and an indistinct fibrous lamina, and are believed to be diploid. Changes in both types of nuclei during muscle development were followed by staining methods and autoradiography. Cessation of RNA synthesis by large nuclei was accompanied by separation of chromosomes from the nuclear membrane and eventual pycnosis, but not by
detectable changes in the number of nuclear pores. In muscle preparations maintained *in vitro*, RNA synthesis declined in the large nuclei during the period of burgeoning of the myofilaments, but continued in small nuclei derived from myoblasts. It is concluded that control of the syncytial cytoplasm by both types of nucleus ceases in favour of small nucleus autonomy before the adult muscle becomes functional.

In the adult muscle an average of ten, and a maximum of twelve, thin secondary myofilaments surround each thick primary myofilament. In strongly contracting adult muscle both classes of myofilament pass through holes in the Z-discs, and the sarcomere becomes shorter than the length of a thick filament. This 'supercontraction' has not been described in adult insects, although it is well known in larval dipteran muscles.

**INTRODUCTION**

The transition of larval muscle to adult muscle in *Calliphora* begins with autolysis, during which the organized larval contractile apparatus is destroyed. The residual myofibre is a tube of cytoplasm containing a variety of membranous elements together with a number of large polyploid nuclei, but no myofilaments or Z-discs. Fusion of small bipolar myoblasts with the residual myofibre precedes formation of adult myofibrils (Crossley, 1972). This report traces the pattern of reorganization of the myofibre leading to a contractile adult muscle.

It will be shown that analogies can be drawn between the *Calliphora* transitional muscles and the muscles of *Rhodnius* that are subject to an involution cycle (Wigglesworth, 1956). These *Rhodnius* muscles have now been extensively investigated with the aid of the electron microscope (Toselli & Pepe, 1965, 1968a, b; Warren & Porter, 1966, 1969; Auber-Thomay, 1967).

However, since periodic deposits of Z-line material and hence an outlined sarcomere configuration are retained in resting *Rhodnius* muscle, *de novo* formation of myofibril three-dimensional architecture is presumably not involved, as it must be in the *Calliphora* muscle investigated here.

Apart from the work on *Rhodnius*, ultrastructural studies of insect muscle differentiation have been confined to the indirect flight muscles of the thorax (Koshihara & Maruyama, 1958; Auber, 1961, 1962, 1964, 1965a, b, 1967a, b, c, 1969; Shafiq, 1963; Brosemer, Vogell & Bücher, 1963), and the tergotrochanteral muscle of the thorax (Auber, 1969). Insect intersegmental muscles are markedly different in fine structure from indirect flight muscles (Smith, 1966; Osborne, 1967; Crossley, 1968), and the first object of the present work has been to discover whether these differences extend to development. Are the departures from the established pattern of development of vertebrate muscle that are reported in thoracic muscles (Auber, 1969) common to other types of insect muscle?

A second important point that needs clarification concerns the role of microtubules in myofilament differentiation. Microtubules about 200 Å in diameter were reported in insect flight muscle by Auber some years ago, when they were said to be associated with developing myofilaments and possibly at the origin of primary myofilaments (Auber, 1962). It appeared, subsequently, more likely that primary myofilaments had their origin in tubular filaments 80 Å in diameter,
i.e. much smaller than microtubules. Furthermore, in these flight muscles microtubules 200 Å in diameter became scarce before the time of most active myofilament formation, and it was suggested that a direct role in myofilament formation could be excluded (Auber, 1965a). In a recent paper Auber (1969) suggests that, in insect flight muscle, microtubules lend rigidity during the early formative stages. Fischman (1967) reports that microtubules do not have a structural relationship to developing myofilaments in embryonic chick skeletal muscle (cf. Przybylski & Blumberg, 1966), nor does there seem to be a strict relationship between the plane of the lattice formed by myofilaments and adjacent microtubules (cf. Firket, 1967). However, in the intersegmental muscles of Calliphora, a superficial examination indicated that an irregular disposition of microtubules, like that reported for flight muscle (Auber, 1962), was unlikely, and the quantitative study reported here shows that microtubules are not disposed independently of each other. The nature of the factors bringing about orientation of microtubules in developing muscle, and their significance for myofilament differentiation, is discussed in terms of recent work on intermolecular forces and integrated into our knowledge of other oriented microtubule systems.

The two types of nuclei present in the developing muscle syncytium are, first, a group of large polyploid nuclei derived from larval muscle and, secondly, a group of small nuclei, probably diploid, present as the result of fusion of myoblasts derived from imaginal embryonic cells. The third main objective of the present work has been to determine the role of each type of nucleus in the formation of the adult myofibre. This has been furthered by a fine-structural analysis of nuclear changes in relation to a study of RNA synthesis by means of autoradiography.

**MATERIALS AND METHODS**

*Calliphora erythrocephala* (Meig.) (= *vicina*, R.-D.) larvae were reared at 25 °C on excess quantities of liver and allowed to form puparia in sand. Puparia were maintained in an incubator at 25 °C and 60 % relative humidity. Under these conditions the stages within the puparium occupied 210–220 h. The transitional abdominal intersegmental muscles studied were those numbered 1–4 in an earlier anatomical study (Crossley, 1965). Preliminary dissection of the muscles under fixative was followed by osmium post-fixation, which made the extremely thin (about 100 μm diameter) muscles visible and facilitated final dissection in alcohol during dehydration.

The fixative used was a 2·5 % glutaraldehyde solution, buffered at pH 7·2 with HCl-cacodylate or phosphate, containing 0·15 M-sucrose. Initial fixation was at 20 °C for 10 min, followed by reduction to 4 °C for a further period of 90 min. After thorough washing in buffered sucrose over a period of 18 h at 4 °C, tissues were post-fixed in veronal-buffered 1 % osmium tetroxide and dehydrated in a graded ethanol series. Some specimens were treated with 5 % uranyl acetate in
90% ethanol during dehydration. Dehydrated muscle was embedded in Araldite and thin sections were cut on a Porter-Blum MT-2 or a Reichert OM-U2 microtome equipped with diamond knives. Contrast in electron micrographs was enhanced by treatment of sections with 5% uranyl acetate in 20% methanol, followed by lead citrate, the latter according to Reynolds (1963). Some sections were also treated with 2% phosphotungstic acid in 95% methanol (Fischman, 1967). Sections were examined in a Siemens Elmiskop I operated at 80 kV, or in a Hitachi HU 11E operated at 75 kV. In both instruments 30 μm self-heating apertures were used and the magnifications were calibrated using a germanium shadowed carbon replica of 463 nm diffraction grating.

In order to analyse nuclear RNA synthesis by autoradiography, the dorsal integument of the abdomen was dissected from the pupa or pharate adult immersed in maintenance medium. The muscles were exposed by dissection using fine jets of medium from a pipette, and adhering fat body was removed during the process. The integument with attached muscles was then transferred to medium containing 0.007 mg/ml uridine T (G) specific activity 13.6 mCi/mg (Radiochemical Centre, Amersham, England) and incubated for 90 min. The maintenance medium used in these experiments is designed to adhere to known parameters of Calliphora haemolymph, particularly with regard to osmotic pressure, pH, ionic ratios, and amino acid composition. It is adequate to permit rhythmic contraction in isolated fly hearts for up to 14 days, and allows some tissue growth. It has the following composition (in mg/100 ml): KCl 63.7, CaCl₂ 22.2, MgCl₂.6H₂O 264.3, K₃PO₄ 94, NaHCO₃ 88.6, sodium succinate 20, sodium citrate 55, sodium malate 200, α-ketoglutaric acid 40, glucose 200, fructose 200, sucrose 4800, penicillin 3, streptomycin 10, lactalbumin hydrolysate 700, L-aspartic acid 33, L-asparagine 55, β-alanine 100, L-glutamic acid 72, L-glutamine 132, L-lysine HCl 30, L-proline 103, L-tyrosine HCl 115. This is brought to pH 7.2 by addition of 30 ml of 1.65 N-NaOH, then 3% Antheraea serum and vitamins are added, as in the tissue culture medium of Grace (1962). Radioactive tissues were embedded and sectioned as outlined above, then the sections were coated with Ilford L-4 emulsion by the technique of Caro & van Tubbergen (1962). After exposure for 3 months, the grids were developed in ID-19 developer, stained with lead citrate, and examined.

RESULTS

(1) The organization of microtubules

Microtubules are present in larval abdominal intersegmental muscle, and are retained during metamorphosis in transitional muscles, although their orientation with respect to the fibre axis changes. Thirty hours after puparium formation, shortly before autolysis of the larval contractile apparatus begins, 240 Å diameter microtubules are numerous in the peripheral sarcoplasm of the myofibre (Fig. 1). The orientation of a particular microtubule is related to its
neighbours so that they run parallel, but clusters are not regularly disposed with respect to the axis of the myofilaments of the contractile apparatus.

Destruction of the larval myofilaments by autolysis is completed by 50 h after puparium formation, but microtubules remain in the residual myofibre. The distribution of the remaining microtubules is apparently random (Fig. 2). Indeed the cytoplasm of the 50 h myofibre is conspicuous in its complete lack of overt organization, either axial or sarcomeric. It appears to be a long (> 1.8 mm), narrow (< 100 μm) tube of plasma membrane, enclosing nuclei and randomly distributed inclusions, which are embedded in a rather dense flocculent matrix (Fig. 2). The breakdown of the larval contractile apparatus, the nature of the residual inclusions, and the fusion of myoblasts with the residual myofibre are described in an accompanying paper (Crossley, 1972). In that paper it was shown that elongated bipolar myoblasts come to lie alongside a residual myofibre, so that myoblast microtubules assume the orientation of the myofibre. Fusion of myoblast and myofibre follows. It can now be shown that, concurrently, a striking reorganization of the microtubules of the myofibre begins. This reorganization brings existing microtubules into the general axial alignment of the myofibre-myoblast complex. At first, alignment is only precise at the periphery of the myofibres (Figs. 3–4), but the ordering influence gradually extends towards the core. Precise axial alignment of microtubules is not achieved throughout the myofibre, because the core is occupied by nucleic and other inclusions which apparently interfere with microtubule disposition (Fig. 6). Myofibres with clear axial alignment of microtubules always show close association or fusion with myoblasts, indicating at least a temporal relationship between alignment and fusion.

A qualitative examination of micrographs of 70–100 h myofibres in transverse section (such as Figs. 3 and 4) suggests that the distribution of microtubules is ordered but not precise. The distribution has been analysed quantitatively to test for the existence of a factor controlling microtubule separation. Skellam (1952) has derived an expression to describe the distribution of the distance between an individual and its nearest neighbour, which allows an observed distribution to be compared with that which could be expected on an assumption of randomness. Thus if λ is the number of individuals in a circle of unit radius, and x is the nearest neighbour distance, then, for random distribution,

\[ dF(x) = e^{-\lambda x^2} 2 \lambda x \, dx, \]

where \( F(x) \) is the probability integral of \( x \). This expression gives the curve plotted in Fig. 5. Superimposed on the curve is a histogram of the observed nearest neighbour distribution of microtubules in a peripheral area of a 96 h myofibre. It is apparent that the distribution of nearest neighbour distances differs from that which could be expected on an assumption of randomness, and indicates that influences tending to regularize microtubule separation exist. Although the nature of such influences is conjectural (see Discussion), it can be
stated that they result in a preferred separation distance of about 800 Å between adjacent microtubules.

An analysis of angular relationships between adjacent microtubules failed to reveal any bias, the average value for the angle between a microtubule and its two nearest neighbours was 45°.

Each microtubule is surrounded by a zone of low electron density 75 Å or more wide, from which other organelles are excluded. Thin strands of material are frequently observed traversing this zone, particularly in sections treated with 2% phosphotungstic acid in 95% methanol to enhance contrast (Fig. 4). However, the strands of material did not span the entire gap between adjacent microtubules.

(2) The development of membranous elements and sarcomere organization

The formation of microtubule arrays is accompanied by the development of pronounced longitudinal folds in the plasma membrane of the myofibre. Some folds become isolated as subsidiary fibres, particularly near the insertions of the muscle (Fig. 3). About 90 h after puparium formation, membranous vesicles appear between the microtubules (Figs. 3, 4). Subsequent developments reveal that these vesicles are rudiments that give rise to the endoplasmic reticulum.

Coated vesicles occur on the plasmalemma of muscles during the period 50–100 h after puparium formation (Fig. 4). It has been suggested that coated vesicles on the *Calliphora* residual myofibre are associated with uptake of macromolecules from the haemolymph prior to the reassembly of the contractile apparatus (Crossley, 1972).

There is no morphological evidence that larval sarcomere organization persists in residual myofibres, and Z-bodies are first detected as diffuse electron dense deposits near the periphery of the fibre 120 h after puparium formation (Figs. 7, 8). At this time, longitudinal orientation and regular spacing of microtubules extends throughout the myofibre, except in the vicinity of nuclei. Early

---

**Note.** The number encircled in the bottom right-hand corner of each figure is the time interval in hours, from formation of the puparium to the fixation of the tissue.

**Figures 1, 2**

Fig. 1. Longitudinal section of supercontracted transitional intersegmental muscle 30 h after puparium formation, shortly before autolysis of the larval contractile apparatus begins. Many microtubules are present (mt), but their axis of alignment does not follow the myofilaments. Attachment of the basement membrane at hemidesmosomes (hd) is seen, as is an indication of connexion between hemidesmosome and adjacent Z-disc (arrow). Both the latter are composed of similar electron dense material. Supercontraction leads to heavy folding of the peripheral sarcoplasm (fo), and the passage of both thick and thin filaments through the Z-discs (Z). (×27000.)

Fig. 2. Residual myofibre 50 h after puparium formation. Myofilaments have now disappeared, and microtubule orientation is random (arrows). A heterogeneous collection of vacuoles is also present. (×29000.)
Adult muscle formation in the blowfly

Z-discs are elongated in the same axial alignment as adjacent microtubules. The distance separating Z-discs along the myofibre is variable, but averages 4 µm (Fig. 7). Thus the length of the sarcomere when it is first defined is within the normal operating range of contractile muscle. This range is 10.5–1.75 µm in the supercontracting larval intersegmental muscle of Calliphora (Crossley, 1968). Before contraction is initiated, sarcomeres have an average length of 3.5 µm. Z-bodies have not been detected in myofibres before filaments are present in the vicinity, and thin myofilaments have invariably been associated with recognizable Z-band material.

Mitochondria are present throughout the transition of the myofibre. At 120 h after puparium formation each mitochondrion takes the form of an elongated tube about 0.2 µm by 3.5 µm (Fig. 9), arranged with its long axis corresponding to that of the myofibre. The mitochondria gradually increase in size and develop a more extensive series of cristae (cf. Figs. 9, 10). In the adult muscle the mitochondria are about 0.4 µm by 3.5 µm, significantly broader than those of the residual myofibre (Figs. 16–18).

Infoldings of the plasma membrane extend into the sarcoplasm forming the T-tubule system, and each infolding becomes associated with one or more of the small cisternae already formed between the microtubules (Fig. 9). This association subsequently develops into a diad, but no elaboration of the surface of the sarcoplasmic reticulum is detected at this early stage.

(3) Formation of myofilaments

Early Z-bodies, at 120 h after puparium formation, each bear a brush-like group of thin filaments (Fig. 7). In transverse section the Z-body appears as an electron dense deposit associated with thin filaments (Fig. 8). Most of the thin filaments attached to the Z-body appear to be less than 50 Å in diameter after staining with uranyl and lead salts, but a few are as large as 80 Å in diameter. Since the thin secondary myofilaments of the fully developed muscle are 70–80 Å in diameter, it appears that the majority of first formed thin filaments at the

Figs. 3, 4. Both show longitudinally folded residual myofibre areas 70 h after puparium formation, cut in transverse section. In Fig. 3 a fold has become partially isolated as a tubular peninsula. Axial alignment of 240 Å diameter microtubules is obvious at the periphery of each myofibre. A quantitative analysis of distribution of microtubules at this time reveals that the distribution is non-random, and that there is a preferred separation distance of about 800 Å between adjacent microtubules. Each microtubule is surrounded by a zone of low electron density 75 Å or more wide. In Fig. 4 the section has been stained with 2 % phosphotungstic acid in 95 % methanol in addition to lead and uranyl salts, revealing thin strands of material connected to microtubules (large arrows). Coated vesicles (cv) are present on the plasma membrane, which now almost entirely lacks a basement membrane. Membranous vesicles appear between microtubules (small arrows). Vesicular associations with the plasma membrane may be precursors of diads. (Fig. 3, x 56000; Fig. 4, x 94000.)
Z-disc are either not secondary myofilaments, or are incomplete secondary myofilaments (see Discussion).

Thicker filaments, 110–190 Å in diameter also appear in the myofibre at 120 h after puparium formation, and myofibres containing only one species of filament were not encountered in this study. Thick and thin myofilaments rapidly assume the interdigitation characteristic of primary and secondary myofilaments. In transverse sections the distance separating thick myofilaments from their nearest neighbours is between 325 and 350 Å, and hexagonal packing is apparent as soon as seven or more thick filaments form in a particular area (Fig. 9).

Fig. 5. Observed microtubule nearest neighbour distance (histogram) is here compared with the distribution that could be expected on an assumption of random distribution (curve). A preferred separation distance of ca. 800 Å rather than the expected value of 400 Å is revealed. The data is from a 70 h myofibre.

Figures 6, 7

Fig. 6. A low magnification survey micrograph of a longitudinal section of a 140 h myofibre. In the core of the myofibre, examples of large (l) and small (s) nuclei are seen, derived from larval muscle and imaginal histoblasts respectively. Heterogeneous inclusions, including a large electron lucent vacuole (on right), are also present in the core region, whilst longitudinally aligned filaments, microtubules, and mitochondria occupy the peripheral sarcoplasm. (x 5000.)

Fig. 7. By 120 h after puparium formation the first traces of sarcomere organization can be detected in longitudinal sections of the myofibre. Diffuse electron dense Z-bodies (Z) appear, spaced about 4 µm apart along the axis of the myofibre. Filaments attached to the Z-bodies are apparently mostly less than 50 Å in diameter, but a few are as large as 80 Å. Microtubules (mt) and ribosomes (r) are prominent. (x 37500.)
The thick filaments formed at the periphery of a bundle have a diameter close to 120 Å. This is a strikingly lower value than that obtained for thick primary myofilaments in the interior of the bundle at the same time (120 h), which have an average diameter of 190 Å. The lower peripheral value is also significantly different from that obtained for primary myofilaments of adult muscle. The thick myofilaments appear to be made up of components of differing electron density, giving rise to a tubular appearance (Fig. 10). This phenomenon is affected by fixation procedures, but is never as marked here as in insect flight muscle (Auber, 1962).

The spatial relationship between myofilaments and microtubules is not precise, and myofilaments are found as far as 2000 Å from the nearest microtubule. Nevertheless, major differences in orientation between microtubules and adjacent myofilaments are rare (Figs. 9, 10). In the core of the myofibre, divergent microtubules are often accompanied by similarly divergent myofilaments. Regular structural cross-linkages between myofilaments and microtubules were not observed.

By 140 h after puparium formation the ratio of thick myofilaments to microtubules is 1:1 in peripheral regions (Fig. 9). By 190 h the ratio is 10:1 (Fig. 10), and in contractile adult muscle at emergence (220 h) is 50:1 (Fig. 16). Microtubules are frequently incorporated into the myofilament array. It appears from a morphological standpoint that the microtubule is unable to substitute directly for a myofilament in the array, since introduction of a microtubule disturbs the regular paracrystalline array of myofilaments (Fig. 10). A region of low electron density still surrounds microtubules when they are incorporated into the myofilament array (Fig. 10).

(4) Coexistence of larval muscle nuclei and myoblast nuclei within the myofibre

Nuclei derived from larval muscle and nuclei derived from myoblasts are present together in the myofibre following myoblast fusion (Crossley, 1972).

Figures 8, 9

Fig. 8. In transverse sections of the 120 h myofibre Z-bodies are seen in the peripheral cytoplasm. Filamentous (arrows) and amorphous material appears in the vicinity of the Z-bodies, but ribosomes (r) are not particularly numerous. Mitochondria and small smooth-walled cisternae are also present between the transversely-cut microtubules. (×110 000.)

Fig. 9. A transverse section through part of a 140 h myofibre, cut at the level of the newly formed A-band. Note the interdigitation of thick and thin myofilaments, with hexagonal packing when seven or more myofilaments form in a particular area. The thick myofilaments formed at the periphery of the bundle (arrows) are about 120 Å in diameter, whereas those at the centre have a diameter close to 190 Å. A short T-tubule (T) is seen to be associated with two smooth-walled cisternae. It is suggested that this may represent an early diad. Note the large number of microtubules cut in cross-section. (×105 000.)
Adult muscle formation in the blowfly
There is great disparity in size, and in several other features, between the two kinds of nuclei (Figs. 6, 11). An attempt has been made to obtain quantitative data to form a basis for comparison and to illustrate differences between nuclei.

The nuclei derived from larval muscle are ellipsoids with a long axis close to 15 μm, and a short axis close to 8 μm. The nuclear membrane is studded with pores (Fig. 12). An approximate estimate of pore density has been obtained by measurement from sections. The average value for pore separation of 0.27 μm can be used for such a calculation, assuming the nucleus to be a perfect ellipsoid. Each pore is calculated to have an area of influence of 0.07–0.09 μm², depending on whether square or hexagonal packing is assumed. It follows that an approximate value for pore density in these large nuclei is 18 per μm², or 5000 per nucleus. As we shall see, this is more than twenty times the density found in small nuclei.

No changes in the numbers of nuclear pores were detected as the activity of the nucleus changed, as evidenced by pycnosis, discussed below. The inner membrane of the nuclear envelope is coated with a fibrous lamina about 300 Å wide (Fig. 13). Finely granular chromatin is dispersed throughout the nucleoplasm of these nuclei during myofibril formation (Fig. 12), but the chromatin becomes condensed towards the centre of most nuclei before adult emergence (Fig. 11). In the light microscope, similar changes in distribution of DNA were followed using Feulgen or methyl green staining methods, and the nuclei can be described as pycnotic at the time of adult emergence. Chromosome examination using aceto-orcein squash preparations reveals that the large nuclei derived from larval muscle are highly polyploid, but not polytene. In addition to chromosomes, the nuclei contained specialized zones of perichromatin, interchromatin and a nucleolus (Fig. 12).

The nuclei derived from myoblasts by fusion are small spheroids averaging 4 μm in diameter (Figs. 6, 11). Although these nuclei appear closely apposed in pairs or chains, there is no evidence of mitosis once fusion with the myofibre has taken place (Crossley, 1965). Nuclear pores are infrequent, with an average separation of 1.2 μm. By calculation, the area of influence of each pore is 1.3 μm² and the pore density is approximately 0.8 per μm², or 20 per nucleus. No significant difference was detected in the size of nuclear pores from different types of nuclei, or the same type of nuclei at different developmental stages, in measurements taken from sections tangential to the nuclear envelope. However, measurements of pore frequencies and sizes from sectioned material lack precision (see Figure 10).

Transverse section of a myofibre 190 h after puparium formation. There are now ten thick myofilaments for every microtubule. Notice that microtubules are frequently incorporated into the myofilament packing lattice. A region of low electron density surrounds microtubules. The thick myofilaments sometimes have a tubular profile (T, T-system; f, myofilaments; m, mitochondrion). (× 86000.)
Fig. 11. Large polyploid nuclei derived from larval muscle (n) are present together with small nuclei derived from myoblasts (my.n) in the developing adult muscle. Numerous pores stud the nuclear envelope of the large nuclei, but pores are infrequent on the small nuclei. Condensation of chromatinic material is already beginning in the nucleus derived from larval muscle (n) 150 h after puparium formation. No fibrillar organization is apparent, although myofilaments are numerous (f). (hl = haemolymph.) (×12000.)
Adult muscle formation in the blowfly

A fibrous lamina was not detected in the small nuclei derived from myoblasts, but chromatin, interchromatin, perichromatin, and a nucleolus are present. The dispersed distribution of chromatin in electron micrographs, and of DNA in Feulgen preparations, is not subject to redistribution during myofibre development, and these nuclei do not become pycnotic. They appear to be diploid, like most nuclei derived from imaginal discs in diptera. It thus appears from the morphological evidence that, at the time of emergence of the adult fly, only the small nuclei are active. What further evidence can be obtained to clarify the roles of the two types of nuclei? It should be possible to determine when RNA synthesis is in progress in each class of nucleus by staining procedures, and by autoradiography. Pyronin-Y positive material was present in all nuclei examined.

(5) RNA synthesis in larval and myoblast nuclei

Tracer experiments on the intact developing fly are technically difficult, since the pharate adult must be exposed by opening the puparium in order to administer the isotope. The fragility of the developing adult cuticle, the sluggish circulation of the pupa, and the fatty tissues surrounding the developing muscles, all mediate against successful labelling. After a number of inconclusive experiments, an in vitro method was substituted (see Materials and Methods). Muscles were examined by autoradiography at 98, 140 and 190 h after puparium formation. Incorporation of label was detected in small nuclei derived from myoblasts at all three intervals. In the large nuclei derived from larval muscle incorporation was detected at 98 and 140 h after puparium formation (Fig. 14), but no incorporation was detected at 190 h after puparium formation. The interpretation placed on these observations is that both large and small myoblast nuclei actively synthesize RNA up to and during the period of myofilament formation. Synthesis of RNA by large nuclei derived from larval muscle ceases well before the adult emerges, whereas it continues in the small nuclei until the end of the pharate period. These results are in accord with those obtained by study of nucleus ultrastructural anatomy, as reported above. It seems probable that in the adult muscle, control of the syncytium passes to the small nuclei derived from imaginal discs.

(6) The structure of adult muscle

Fully formed adult intersegmental muscles take the form of flattened straps 1-2-1-6 mm long and about 0-15 mm wide. Separation into bundles of fibrils occurs near the insertions of the muscle. The sarcolemma bears a thin basement membrane and occasional hemidesmosomes (Figs. 16-18). T-system clefts 150-200 Å wide extend into the contractile apparatus, and contain occasional electron dense deposits. The sarcoplasmic reticulum is fenestrated (Fig. 17), but is not particularly extensive (Figs. 15-18). Mitochondria, fused membrane
Adult muscle formation in the blowfly

aggregates, and latticed cisternae derived from larval sarcoplasmic reticulum, are present (Fig. 15). The latticed cisternae are less abundant than in pupal myofibres, but their presence in actively contractile muscle is interesting in the light of the suggestion made earlier that they might arise under the influence of abnormal afferent nervous stimuli. Mitochondria are elongated (< 5 μm) in the long axis of the myofibre (Fig. 17), but become buckled and disoriented during supercontraction (Fig. 18).

In the adult myofilament lattice, an average of ten, and a maximum of twelve, thin secondary myofilaments surround each primary myofilament, giving a ratio of 4:1 in transverse sections of the A-band. A paracrystalline array is present in relaxed muscle. The Z-bands have a perforated appearance in sections of relaxed muscle (Fig. 17). In muscle fixed in contraction, both thick and thin myofilaments pass through the perforations of the Z-disc, a characteristic of supercontracted fibrils. At the Z-discs in such muscles the regular myofilament array is disturbed, and some thick filaments appear to be bent (Fig. 18).

There is a superficial subdivision into fibrils by the interpolation of mitochondria, sarcoplasmic reticulum, glycogen deposits, and microtubules between groups of filaments, but these groupings are not maintained beyond the length of a few sarcomeres. The phenomenon has been discussed by Hoyle (1967).

In all parameters, with the exception of the presence of latticed cisternae, the adult Calliphora intersegmental muscle resembles its larval counterpart described earlier (Crossley, 1968).

DISCUSSION

The organization of microtubules

Microtubules present in Calliphora intersegmental muscles assume orientation parallel to the long axis of the myofibre before formation of myofilaments begins, as has been reported in Calliphora indirect flight muscle (Auber, 1962, 1965a) and chick muscle (Firket, 1967; Fischman, 1967). In Rhodnius intersegmental muscle microtubules and myofilaments are said to appear and increase in

Figures 12–14

Fig. 12. Nucleus in 140 h myofibre showing chromatin (c), perichromatin (p), interchromatin (i) and a granular nucleolus (no). This nucleus shows no signs of pycnosis. Nuclear pores are separated by an average distance of 0.27 μm. (x 28000.)

Fig. 13. Enlarged portion of the nuclear envelope from a nucleus such as that shown in Fig. 12. The unit membrane bordering on the nucleoplasm bears a fibrous lamina (fl) which is electron dense. This coat is absent from the membrane facing the cytoplasm, which bears ribosomes (n, nucleoplasm; c, cytoplasm). (x 120000.)

Fig. 14. Autoradiograph of a nucleus in a muscle fixed 140 h after puparium formation, after 2 h incubation in a maintenance medium containing tritiated uridine. Silver grains indicative of RNA synthesis are distributed throughout the nucleus, but are particularly numerous in the vicinity of the nucleolus (no). Folds (fo) in the nuclear envelope are also seen in control nuclei at this time. (x 16000.)
Adult muscle formation in the blowfly

numbers concomitantly, and large numbers of microtubules are only present when myofilament differentiation is well advanced (Toselli & Pepe, 1968). This is the reverse of the situation reported in Calliphora flight muscle where increase in myofilament numbers is accompanied by a decrease in microtubule numbers (Auber, 1965a). In Calliphora intersegmental muscle, microtubules are at first randomly oriented and few in number, but become oriented parallel to the long axis of the myofibre, and more numerous, before myofilament formation. In Rhodnius intersegmental muscles the parallel disposition of microtubules persists through involution (Toselli & Pepe, 1968).

In the present quantitative study of microtubule distribution, a preferred separation of 700–800 Å has been demonstrated. Such a separation would not be anticipated on a basis of random distribution, and implies that influences tending to maintain separation of microtubules exist. The nature of such influences is a matter for speculation. Two general types of theory might be invoked. One would seek to explain microtubule separation on the basis of structural bridges. Such bridges have been reported in Hela cells, and plant endosperm cells by Hepler, McIntosh & Cleland (1970), and are postulated for the microtubule array in the filopodia of Echinopsphaerium (Tilney & Byers, 1969). However, as Harris (1970) has pointed out, the evidence for macromolecular links connecting microtubules is not strong. A second type of theory would seek to explain microtubule distribution on a basis of long-range electrostatic forces. A theory of this type has already been developed to account for the distribution of myofilaments and has been extended recently into a theory of muscular contraction (Elliot, Rome & Spencer, 1970). These workers suggest that long-range electrostatic repulsive forces are involved in the maintenance of the filament lattice in vertebrate striated muscles. In the resting condition, it is suggested that filament separation is determined by the balance of van der Waals, electrical double layer, and hydration effects. Long-range repulsion has recently been demonstrated experimentally between actin filaments in vitro (Spencer, 1969). The separation distance of microtubules in the 90 h myofibre, about 700–800 Å, is within the range of influence of electrostatic forces, which may extend up to 1000 Å from the surface of proteins (Bernal, 1959). The existence

---

**Figures 15, 16**

Fig. 15. A transverse section of the core region of a muscle from a newly emerged adult fly. Microtubules are still present (arrows). Notice also latticed cisternae (l.c.) and membrane aggregates (ma) which are absent in larval muscle and arise during transition. (A) = Adult muscle. (× 60000.)

Fig. 16. Muscle from a newly emerged adult fly. This transverse section shows, A-band (A), I-band (I), and Z-band (Z) regions. A T-system cleft 200 Å wide, containing a dense deposit (d), extends into the contractile apparatus, where it is associated with a flattened cisterna containing electron-dense material (arrow). The basement membrane is much thinner than that derived from larval muscle (cf. Fig. 1), but hemidesmosomes are well developed. m, mitochondrion. (A) = Adult muscle. (× 70000.)
of such forces around microtubules has yet to be experimentally demonstrated; nevertheless, a theory of microtubule distribution based on long-range electrostatic forces is attractive for two reasons. First, it could account well for the statistical but not paracrystalline precision of the microtubule array in some developing muscles. A more regular arrangement might be expected if crossbridges were involved, as in the completed myofibre. Furthermore, the intertubular distances involved are greater than those probable for physical links. Secondly, an electrostatic physical basis could also be invoked for the distribution of myofilaments between microtubules. Thus when myofilaments appear between predisposed microtubules, they share the same orientation, although, as in chick muscle (Fischman, 1967), the relationship between the plane of the lattice formed by thick myofilaments and the plane of adjacent microtubules is not precise. Parallel orientation would, on the basis of an electrostatic repulsion theory, represent the state of minimum energy.

The observation of Toselli & Pepe (1968) for Rhodnius muscle, that microtubules were almost completely absent from the cytoplasm at the time of active myofilament formation, runs contrary to a ‘scaffolding’ theory, unless in this muscle pre-existing sarcomere organization negates the need for microtubule ‘scaffolding’. Many earlier reports of muscle differentiation in the absence of microtubules may also be cited in opposition to such a theory, but in view of the labile nature of the type of microtubule involved, the preservation of structure may be questionable.

The work of Bischoff & Holtzer (1968) shows that $10^{-8}$ M-colchicine prevents elongation of chick myoblasts to form myotubes, and leads to the development of myofibrils in rounded sacs. This result argues against a direct involvement of microtubules in the assembly of myofilament protein, but supports a ‘scaffolding’ analogy.

The plasma membrane may play a part in the orientation of microtubules, since longitudinal orientation develops first, and is most precise, at the periphery of the myofibre. The present work, and many earlier reports, indicate that myofilament differentiation is initiated at the periphery of the myofibre (Shafiq, 1963; Auber, 1964; Dessouky & Hibbs, 1965; Fischman, 1967; Auber-Thomay, 1967; Toselli & Pepe, 1968a, b; Warren & Porter, 1969).

Fig. 17. Longitudinal section of slightly contracted muscle from a pharate adult fly, fixed about 10 h before the adult should have emerged from the puparium. Note the perforated Z-discs (Z), part of the fenestrated sarcoplasmic reticulum (arrow), and the axially aligned mitochondria. Sarcomere length in this area is 5.8 μm. (× 23 000.)

Fig. 18. Longitudinal section of supercontracted muscle. Age, preparation, and magnification identical to Fig. 17. Notice that the thick myofilaments now penetrate the Z-discs, and additional contraction has led to folding of the sarcolemma, and buckling of the mitochondria between myofibrils. Sarcomere length in this area is 3.5 μm. (× 23 000.)
It has been proposed by Bernal (1965) that water can generally be considered to exist in a semi-crystalline form up to 10-20 Å from protein surfaces, and there may be a certain restriction of water and ion movement up to 100 Å from such surfaces. It is possible that organized water could account for the 75 Å wide zone of low electron density that surrounds microtubules in the myofibre and in other systems (Porter, 1966).

**The deposition of myofilaments**

The suggestion of Auber (1964), later retracted (Auber, 1965a), that microtubules may be converted into thick myofilaments, is not supported by the present study. No filaments intermediate between microtubules 240 Å in diameter and myofilaments 190 Å in diameter have been detected in *Calliphora* intersegmental muscles. Furthermore, the incorporation of microtubules into the myofilament array, without reference to the paracrystalline arrangement of the thick myofilaments, suggests that the two classes of structure are quite distinct.

Synthesis of thin myofilaments before thick myofilaments has been reported in chick muscle by Allen & Pepe (1965), in rat muscle by Price, Howes & Blumberg (1964), and in *Calliphora* flight muscle (Auber, 1969). In *Calliphora* intersegmental muscle at transition, deposition of thin and thick myofilaments occurs simultaneously or in rapid sequence, as has been shown in salamander muscle (Hay, 1963), and chick muscle (Przybylski & Blumberg, 1966; Fischman, 1967).

Sarcomere spacing, in *Calliphora* intersegmental muscle, appears to be regulated by prior spatial distribution of Z-band material on to which filaments are inserted, a tentative conclusion also reached by Fischman (1967) for chick muscle. In the present muscles, formation of thick myofilaments apparently begins with the deposition of a group of myosin molecules to form a fibril about 120 Å in diameter, which subsequently expands to form a definitive primary myofilament 190 Å in diameter. This type of expansion was reported for insect flight muscle by Auber (1965a), and for rat muscle by Heuson-Stiennon (1965). However, in chick muscle primary myofilaments are of definitive maximal diameter (160-170 Å) at the time they are first formed (Fischman, 1967). The nature of the change in insect and rat primary myofilament diameter is unknown. It could involve addition of further myosin molecules, or rearrangement of existing ones. In the latter context it can be noted that the core of insect primary myofilaments is composed of material of low electron density and, since the core is reduced or absent in newly formed myofilaments, rearrangement of myosin molecules about a core of another material may occur. The materials of the ‘C’ filament of Auber & Couteaux (1963), or of the ‘residual filament’ of dos Remedios (1969) are obvious candidates for such a role, and the establishment of a method for their characterization in preparation for the electron microscope is urgently needed.

There is uncertainty in the literature about the nature of the thin filaments
Adult muscle formation in the blowfly

(50 Å diameter) that are associated with Z-discs in developing insect muscle. These were first described by Auber (1965a) and he later termed them ‘initial filaments’ (Auber, 1969). Filaments of similar nature have also been described by Warren & Porter (1966), Auber-Thomay (1967), Toselli & Pepe (1968b), and are seen in the present material. We can conclude with Auber (1969) that it is not clear whether secondary myofilaments form between the 50 Å filaments, or from them in their place. Recent work suggests that there may be a third type of filament in contractile striated muscle, the ‘ultrathin’ filament of McNeill & Hoyle (1967), or the ‘resistant’ filament of Guba, Harsanyi & Kajda (1964), or the ‘residual’ filament of dos Remedios (1969). These filaments are 50 Å or less in diameter, and are rather difficult to stain, and hence resolve, in electron microscope preparations. There is no agreement as to whether such ultrathin filaments run between or within thick primary myofilaments, at the level of the A-band. It is clear, nevertheless, that a filament running the length of the sarcomere could play a crucial part in the development of the myofilament array and sarcomere organization. Indeed it is possible that ‘initial’ filaments and ultrathin or residual filaments may be the same structures. It is hoped that experimental application of negative staining techniques to developing muscle may illuminate this area.

This paper is believed to be the first report of supercontraction in the muscle of an adult insect, although supercontraction is well established for dipteran larvae (Osborne, 1967; Crossley, 1968).

The role of the nuclei

The involvement of two distinct populations of nuclei within a single body of cytoplasm is believed to be a novel phenomenon for metazoan development. The development of myofilaments could involve transcription of DNA from either or both classes of nucleus. The change from a disperse finely granular DNA distribution towards the end of myofilament formation suggests that these nuclei become less active at this time. Indeed the anatomical evidence suggests that RNA synthesis may be limited to the small class of nuclei in adult muscle. This proposal is supported by the preliminary results of labelling experiments with tritiated thymidine. These suggest that RNA synthesis ceases in the large nuclei after the period of myofilament formation, by 190 h after puparium formation.

There is no measurable change in the distribution of nuclear pores during the development of the muscle, and this cannot be used as a criterion of nuclear activity.

REFERENCES


Adult muscle formation in the blowfly


(Manuscript received 16 April 1971)