Changing muscle patterns in a segmental epidermal field

By G. J. A. WILLIAMS and S. CAVENEY

From The Cell Science Laboratories, Department of Zoology,
University of Western Ontario, London, Ontario, Canada

SUMMARY

The spatial rearrangements that take place during metamorphosis in the abdominal sternites and associated retractor muscles of the beetle Tenebrio molitor are described. This paper provides the descriptive background needed to consider whether a morphogenetic gradient is involved in specifying the position at which adult muscle attachments develop. (Experimental work in support of this gradient hypothesis is published in a companion paper.)

The ventral abdominal retractor muscles are extensively remodelled at metamorphosis so that the adult muscles differ considerably in appearance from the larval muscles from which they are derived. In particular, there is a change in both the absolute and relative positions of the sites of muscle attachment. Rearrangement of muscles takes place during both the pre-pupal period and the pupal stage. It is achieved by means of two separate and temporally distinct mechanisms. Epidermal remodelling in the pre-pupal period results in the movement of attached retractor muscles (epidermokinetic muscle movement). In the pupal stage, however, the muscles move over the basal surface of the epidermis (myokinetic muscle movement). Myokinetic movements may be brought about by extension of myoblast processes from the metamorphosing muscles. These findings are considered in terms of Poyarkoff’s theory that the pupa serves as an integumental mould, approximating the shape of the adult, within which certain adult muscles develop.

INTRODUCTION

Many morphogenetic problems are posed by the dramatic changes in body form that occur at metamorphosis in holometabolous insects. These changes involve remodelling of the integument (composed of the single-layered epidermis, and its secretory product, the cuticle) as well as internal components, such as the muscles. Much has been written about the histological changes which occur in insect muscles during metamorphosis, and the control mechanisms involved in the degeneration and regeneration of muscles at this time (reviewed by Finlayson, 1975). Attention has also focused upon the mechanisms involved in generating integumental patterns in insects. However, problems associated with the generation of new muscle patterns have hardly been considered.

1 Author’s address: Department of Zoology, University of Western Ontario, London, Ontario, N6A 5B7, Canada.
The morphogenesis of muscles and integument are obligatorily interrelated since muscles provide the basis for functional interactions between integumental plates. Also, as has been shown ultrastructurally by Lai-Fook (1967) and Caveney (1969), the integument is modified at muscle attachment sites. For these reasons we became interested in the possibility that the segmentally repeating epidermal gradient of positional information, known to govern cuticular patterns in insects (see review by Lawrence, 1973), might also dictate the sites at which new muscle attachments are formed at metamorphosis.

To investigate this idea we chose to study remodelling of retractor muscles in the abdomen of the beetle, Tenebrio molitor. This paper describes the changes that occur during metamorphosis in these muscles, and in the associated abdominal sternites. It also provides the necessary background for experimental work demonstrating that an epidermal gradient specifies the position at which adult muscle attachments form (Williams & Caveney, 1980).

Each retractor muscle has its origin lying within one segment, and its insertion at the anterior margin of the next posterior segment (Snodgrass, 1935). In Tenebrio, the four most superficial pairs of ventral abdominal retractors, unlike the inner intersegmental muscles which persist unchanged into the adult stage, undergo a transformation at metamorphosis. Remodelling of these retractors begins at the pupal apolysis (at the beginning of the pre-pupal period), and continues throughout most of the pupal stage so that the adult retractors are fully developed at the time of adult emergence. The adult retractors differ considerably in appearance from the larval muscles from which they are derived. In particular, both the relative and absolute positions of the sites of muscle attachments are changed. Repositioning of the origins of these muscles takes place entirely within the segmental field which is bounded at anterior and posterior by the intersegmental membranes. This repositioning can therefore be analysed in terms of what is already known about the segmental gradient which governs cuticular patterning.

Translocation of retractor muscles occurs by means of two separate and temporally distinct mechanisms. In the pre-pupa, the muscles are carried to new positions by rearrangements of the epidermal cells to which they are attached – the epidermokinetic mechanism of muscle movement. In contrast, in the pupa, the anterior ends of the muscles move relative to the basal surface of the overlying epidermis – the myokinetic mechanism of muscle movement.

**MATERIALS AND METHODS**

*Beetle culture*

Animals were taken from a culture reared on a wholewheat flour–bran–yeast mixture maintained at 27 °C with approximately 70 % relative humidity. Last-instar larvae were removed when they stopped feeding and came to the surface of the food, and staged, after apolysis, by the method of Stellwaag-Kittler (1954).
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(We have found that, by a number of histological criteria, the eyestaging technique of Stellwaag-Kittler is a reliable indicator of the temporal sequence of changes taking place in the abdominal sternite epidermis during the prepupal period.) Pupae were staged as time from pupation; adults as time from emergence.

At 26 °C the times to pupation for the difference eyestages are approximately as follows: eyestage 3 (155 h), 4 (145 h), 5 (130 h), 6 (120 h), 7 (115 h), 8 (100 h), 9 (95 h), 10 (80 h), 11 (70 h), 12 (65 h). The pupal stage lasts 189 ± 3 h (mean ± S.D.).

Dissections

Gross morphological changes in the retractor muscles were studied by dissecting animals of known age. All animals were dissected from the dorsal surface, immersed in 70 % ethanol; or sequentially, in 70 % and 100 % ethanol. The ethanol, acting as a precipitative fixative, renders the muscles easily visible and permits resolution of individual fibres in the adult muscles during dissection.

Histological techniques

(I) Integumental features were studied using wholemounts of sternite integument. Sternites were fixed in Zenker’s fixative, and washed in distilled water before hydrolysis in HCl for 10 min at 56–80 °C. They were then briefly rinsed in cold 1N HCl, and distilled water, and stained with Schiff’s leucobasic fuchsin (Feulgen reagent). After treating with bisulphite, the sternites were dehydrated and mounted in Permount.

Staining with Feulgen reveals the presence of both mitotic and dying cells in the epidermis. The dying cells can be recognized by the presence of chromatic droplets within their cytoplasm (Wigglesworth, 1972).

(II) Muscle histology was studied using unstained wholemounts of sternite integument and associated retractor muscles examined under polarized light; or wholemounts stained with toluidine blue or Feulgen reagent. For more detailed study, muscles were removed from the integument and mounted individually, after Feulgen staining.

Preparations to be stained with toluidine blue were fixed in Galigher’s formalin–acetic acid–alcohol mixture (Galigher & Kozloff, 1971) and stained with 1 % toluidine blue in 1 % sodium borate. After staining, specimens were rinsed in tap and distilled water, dehydrated in alcohols, cleared in a 1:3 terpineol–toluene mixture, and mounted in Canada balsam in terpineol. This procedure was evolved to eliminate the use of xylene which makes the cuticle brittle so that preparations tend to crack on mounting.

Tissue culture

Last-instar larvae were allowed to pupate on clean filter paper. Under sterile conditions sternites were removed from pupae 24 h, or less, after pupation,
using a mounted chip of broken razor blade. (Two lateral incisions were made along the sides of the sternite to be removed, a third in the posterior intersegmental region, and a fourth slightly posterior to the anterior intersegmental region.) Each sternite preparation was then transferred to sterile culture medium (Caveney, 1976). Adhering fat body and any remaining intersegmental muscles were carefully removed using watchmaker’s forceps. The resulting preparations, which consist of sternite integument and the stumps of the retractor muscles (severed near their intersegmental insertions), were cultured at 28 °C in 2% CO₂ in air for periods of up to 1 month.

Study of the epidermal basal lamina

Sternites were removed from pre-pupae and pupae of known age and transferred to culture medium, as described above. Preparations were then clamped individually in 35 mm petri dishes containing medium and probed with a microneedle (tip diameter 1 μm) attached to a micromanipulator. Preparations were observed with a Reichert inverted microscope. The basal lamina was said to be ‘loose’ if it could be pulled away, as an intact sheet, from the epidermis without killing the cells. (Cells were presumed to be living and intact when they maintained normal resting potentials after removal of the basal lamina.)

Water anaesthetization

Animals were immobilized for grafting operations, and for pricking through marker bristles, by immersion in distilled water for a period of about 6 h at room temperature. Water anaesthetization of last-instar larvae delays pupation by as much as 30 h whether followed by an operation or not, the effect being more pronounced in younger larvae. Subsequent development of these animals is, however, apparently normal. Immersion of newly moulted pupae (less than 24 h old) in distilled water does not affect the timing of their development to any detectable extent.

Grafting operations

All grafts were performed on abdominal sternites 4 or 6 of last instar larvae between eyestages 3 and 8. The results were then analysed by dissection of adults 24–30 h after eclosion. The graft site and operational techniques were the same as those used by Caveney (1973). Each graft square was transplanted into an identical site on a second animal. As the morphology of the sternal pit glands of adult male and female Tenebrio differs (see Caveney, 1973, fig. 1), the fate of grafted integument could be established, in adults, when host and donor insects were of opposite sex. Retractor muscle patterns are identical in males and females at the stages used for these operations.

Grafts were not deemed acceptable if (a) host and donor animals did not develop at the same rate; or if (b) the level of wounding in the graft area was excessive. Three indicators of excessive wounding were used: (1) extensive
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melanization of the larval exuvium at the site of the operation; (2) untanned patches of cuticle on the adult sternite; and (3) abnormalities in the adult retractor muscle patterns in the operated sternite. As there are no external features which differentiate the sexes of larvae, only an average of 50% of 'acceptable' grafts were useful. Using these criteria, 11 'acceptable' intersex grafts were performed. Thirty-nine control operations, in which a square of integument was removed and replaced in the same site, produced no abnormalities in the adult sternites. (There are a disproportionate number of controls for the intersex grafting experiments as these controls were also used for grafting experiments described in a companion paper (Williams & Caveney, 1980).)

RESULTS

Histological changes in the muscles

Extensive changes occur in the retractor muscles R1, R2, R3 and R4 (Fig. 1) during the course of metamorphosis. These changes result in a transformation of the rather compact tubular-shaped larval muscles into the more dispersed ribbon-like adult muscles. The adult muscles consist of many distinct fibres which can be resolved easily using a dissecting microscope, whereas the larval muscles consist of a smaller number of closely packed fibres.

The retractors all go through the same sequence of histological changes during their remodelling. However, each pair of muscles undergoes remodelling at a characteristic time. The muscles R4, R3 and R2 show degenerative changes during the pre-pupal period: R4 muscles first, then R3 and R2 (Fig. 2). In contrast, the R1 muscles are probably fully functional at the pupal ecdysis and do not show any perceptible histological changes until immediately after this ecdysis.

The first recognizable histological change is the appearance of numerous mitotic figures in the muscle fibres. An extended period (several days) of mitotic activity ensues and results in a dramatic increase in nuclear density. Concurrently, many mononucleated cells (presumably myoblasts) become associated with the muscle surfaces. There is a negligible amount of nuclear degeneration within the muscles at any stage during their remodelling.

As the nuclear density increases, transverse striations become less distinct, reflecting a loss of alignment of the Z bands; and, after the pupal ecdysis, there is a reduction of whole muscle birefringence. This decrease in birefringence immediately precedes myokinetic muscle movements (Fig. 2). As myokinetic movements occur, individual muscle fibres containing many nuclei, but with poorly defined transverse striations and weak birefringence, separate from one another. The muscles fan out and divide into separate fibres, first at their intersegmental insertions and then at their origins (Fig. 3). The ends of the muscles therefore assume the dispersed adult morphology before the central regions.
Fig. 1. Polarized light micrographs of wholemounts of abdominal sternites 4 and 6 with retractor muscles R1, R2, R3 and R4 from the newly moulted larva, pupa and adult of *Tenebrio*. (A) Sternite 4 of the larva. (B) Sternite 4 of the pupa. (C) Sternite 4 of the adult. (D) Sternite 6 of the larva. (E) Sternite 6 of the pupa. (F) Sternite 6 of the adult. Note that the R3 muscle, which becomes a dorso-ventral muscle in the adult, was removed from the adult sternites. The R4 muscle is poorly developed or absent in sternite 4 of the adult. Ant, anterior; Post, posterior; M, mid-line of the sternite; or, R1 muscle origin; in, R1 muscle insertion; O, marker bristle; •, position of the anterior marker bristle-forming epidermal cells. (For simplicity the position of the posterior marker bristle-forming cells has been omitted. These cells are incorporated into the intersegmental region in the adult). *, Position of epidermal cells adjacent to the origin of the R1 muscle in the newly moulted pupa. Scale bars are 1 mm.

Metamorphosis of the retractor muscles is complete when distinct transverse striations and an increase in birefringence appear in the dispersed fibres of the adult muscles, about 2 days before eclosion (Fig. 2). The muscles are functional at eclosion, and contraction of these muscles facilitates the emergence of the adult beetle from its pupal skin.
Spatial rearrangement of the muscles

The retractor muscles undergo a series of changes in position during the pre-pupal period and pupal stage (Figs. 3 and 4). These rearrangements will be described in the order in which they occur.

Epidermokinetic movement of the R4 muscle during the pre-pupal period

In the pre-pupa, the R4 muscles are displaced laterally (Fig. 4). They undergo nuclear multiplication, and there is a loss of alignment of the Z bands prior to movement. However, the motive force for this movement is not generated by components of the muscles themselves, but rather by epidermal remodelling.

The epidermal cells of Zone II (a region of flexible integument between the abdominal segments) move in an anterior direction in the centre of the sternite, between the R4 muscles, during the pre-pupal period (Figs. 2, 4). This movement can be seen in Feulgen-stained sternite wholemounts due to the greater density of the nuclei in epidermis from Zone II than Zone I regions. The movement is a visible indicator of epidermal rearrangements taking place in the posterior part of the sternite which result in lateral displacement of the R4 muscles. A high level of mitotic activity in the epidermis is accompanied by an increase in epidermal cell density along the mid-line of the sternite at the time of R4
Fig. 2. Diagram showing events in the epidermis and retractor muscles during the pre-pupal period and pupal stage. Developmental time (in days), as well as pre-pupal eyestages are indicated on the abscissa. The timing of certain epidermal events (*) is taken from Delbecque, Hirn, Delachambre & De Reggi (1978). Their data were derived from electron microscopy of abdominal sternites 1-4 of Tenebrio, and are therefore directly comparable with our own observations. However, as Delbecque et al. report observations for a 9-day pupal stage we have reduced their measurements by 8/9 to bring them into line with our observations. d, Period of differentiative epidermal mitoses. p, Period of proliferative epidermal mitoses. Zone II, a region of flexible integument between the abdominal segments. Arrows indicate the beginning of a period of reduced whole muscle birefringence in each retractor muscle during metamorphosis.
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Fig. 3. Diagrams showing the gross morphology of the retractor muscles R1, R2, R3 and R4 in abdominal sternite 4 at selected stages during metamorphosis. Note that as the R3 muscle becomes a dorso-ventral muscle the position of its insertion in the adult is omitted. M, Mid-line of the sternite; ZI, Zone-I cuticle; ZII, pliable Zone-II cuticle. Scale bar is 1 mm.

movement (Fig. 4c). Therefore epidermal growth may also contribute to movement of the R4 muscles in the pre-pupa.

Translocation of the R4 muscles occurs after detachment of the epidermis from the overlying cuticle (apolysis) and after separation of the epidermal cells from their basal lamina, but before pupal cuticle deposition begins (Fig. 2). Thus, the epidermal cells are detached both from the cuticle above and the basal lamina beneath during this period of epidermal growth and rearrangement.

Epidermokinetic movement of all retractors at the pupal ecdysis

Retractor muscle movements at the pupal ecdysis are due to expansion of the folded (or 'pre-expanded') pupal integument coupled with detachment of the muscles from the larval cuticle. Changes in the position of the muscles, although they are not apparent until the ecdysis, are a direct result of the epidermal
remodelling which brings about the transformation of larval sternites into pupal ones.

This point is illustrated by comparing the arrangement of the R1 muscles in abdominal sternites 4 and 6 (Fig. 1). It appears that lateral movement of the R1 muscles is a secondary effect of the overall sternite shape changes that occur at
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pupation. In the larva, the R1 muscles lie at the same distance from the mid-line in sternites 4 and 6 (Fig. 1a, d); and the shapes of the two sternites are similar, although sternite 6 tapers more than sternite 4. However, in the pupal and adult stages (Fig. 1b, e and 1c, f), the origins and insertions of the R1 muscles are closer to the mid-line in sternite 6 than they are in sternite 4. This observation can be correlated with a more pronounced increase in epidermal nuclear density along the mid-line of sternite 4, than sternite 6, in the late prepupa; and also relatively greater broadening of the central region of sternite 4 at the pupal ecdysis. (The positions of four sets of marker bristles provide reference points for comparison of the shape changes in the central regions of the sternite 4 and 6 during metamorphosis. These bristles are produced by the same epidermal cells in both larval and pupal stages, since pricking through the marker bristles of last-instar larvae with a fine mounted dissecting pin results in the absence of marker bristles in the pupa. The adult beetle lacks these bristles. However, the position of the epidermal cells in the adult that gave rise to the bristles in the pupa can be defined by pricking through the pupal bristles with a fine dissecting pin dipped in the juvenile hormone analogue, farnesol. Animals treated in this way emerge as adults with white spots of pupal cuticle above the epidermal cells affected by the prick (Wigglesworth, 1958).)

Lateral movements of the R2, R3 and R4 muscles at pupation, like those of the R1 muscles, result from overall broadening of the sternites. Repositioning of the insertion of the R4 muscles can be attributed to remodelling of the lateral sternite boundaries which occur at pupation (Fig. 3). Some flaring of the ends of the retractor muscles becomes apparent during the late pre-pupal period, and immediately after the pupal ecdysis (Fig. 1b, e). This is thought to be the result of localized growth of the epidermis at the muscle attachment sites leading to spacing of the muscle fibres.

Myokinetic movements of the retractor muscles in the pupa

Changes in the position of retractor muscles during the pupal stage are brought about by movements of the muscles relative to the epidermis.

This has been demonstrated experimentally for the R1 muscles by means of homografts between larvae of opposite sex (Fig. 5 and Materials and Methods). In the larval sternite (Fig. 5a) the origin of the R1 muscle lies outside the graft area. However, in a newly emerged adult carrying an intersex homograft (Fig. 5b) the origin of the R1 muscle lies well within the boundary of the grafted tissue. Therefore the R1 muscle must have migrated across the host epidermis, and across the graft boundary, to insert upon donor integument.

The interpretation of the results of the homograft experiment in the case of the R2 muscle is more complex. The origin of the R2 muscle apparently moves from donor integument (Fig. 5a), to a position in which some fibres attach to host and others to donor integument (Fig. 5b). However, the R2 muscle, unlike the R1 muscle, is severed during grafting. Therefore both host and donor R2
Fig. 5. Diagram showing a homograft experiment in which integument from sternite 4 of a female larva was grafted onto a identical site on sternite 4 of a male larva. (A) The larval sternite showing the position of the graft area, and retractor muscles R1 and R2. Note that the origin of R1 lies outside the graft area, whereas the origin of R2 lies inside the graft area at this stage. (B) The adult sternite showing the position of the graft boundary and of muscles R1 and R2 after metamorphosis. The origin of R1 lies inside the graft area. Fibres of the R2 muscle attach to both host and graft integument. ♂, Male (host) integument; ♀, female (donor) integument. Scale bars are 1 mm.

Muscle components could contribute to the development of the adult R2 muscle. However, dissections reveal that the R2 muscle does undergo a change in position during the pupal stage (Fig. 3), and since the sequence of changes in the R2 muscles at this time are so similar to those of the R1 muscles, we feel that both move by a common (i.e. myokinetic) mechanism.

A diagram of selected stages in the myokinetic movements of the R1 and R2 muscles is provided in Fig. 6. Myokinetic movement of the R1 muscle commences on day 3 of the pupal stage (Fig. 6b). The anterior end of the muscle moves in a posterior direction until it reaches its final position in the antero-posterior axis of the sternite at day 5 (Fig. 6d). Trailing cytoplasmic processes mark the path of this migration (Fig. 6b, c). As the anterior end of R1 begins to move, the insertion of the muscle, at the intersegmental region expands in a medio-lateral direction due to fanning out of the muscle fibres (Fig. 6b, c). In contrast, the majority of medio-lateral movements at the muscle anterior do not occur until after movement in a posterior direction is almost complete. However, from day 6 onwards (Fig. 6c, d) the entire muscle begins to spread out as new attachments are established at its anterior end. Individual fibres separate, initially near their ends, then along their entire lengths (Fig. 6d). The adult R1 muscles are completely developed at day 6 of the pupal stage. Posterior migration of the R2 muscle (Fig. 6g, h) commences at 1-5 days and is complete at day 3 of the pupal stage. It therefore precedes R1 migration, and in this way the R2 muscle moves out of the path of the R1 muscle before R1 begins to migrate. As in the case of
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Fig. 6. Diagrams showing selected stages in the myokinetic migration of the R1 and R2 muscles in the pupal sternite. The inner margin of each diagram lies at the mid-line of the sternite. (A–E) R1 migration. Successive positions of the R1 fibres during days 5 and 6 of the pupal stage are indicated by dotted lines. (F–J) R2 migration. Arrows indicate the direction of movement at each stage. cp, cytoplasmic processes. Scale bar is 1 mm.
Fig. 7. Myoblast migration from severed retractor muscles in vitro. Sternite integument with adhering muscle stumps was excised from a pupa 12 h old and maintained in vitro for 8 days. During this period, myoblast processes extend in a medio-lateral direction across the epidermis from the stumps of muscles R2, R3 and R4. Camera-lucida drawing of sternite wholemount in phase contrast. M, Mid-line of sternite. Scale bar is 1 mm.

Fig. 8. Micrograph of region between the stumps of muscles R2 and R3 in the upper right of Fig. 7. In polarized light, the strong birefringence and transverse striations of the myofibres are evident. Spontaneous contraction of myofibres occurs within 4 days in vitro. Arrows indicate the direction of migration of myoblasts which form the myofibres. Scale bar is 0.1 mm.

the R1 muscle, trailing cytoplasmic processes extend between the old and new sites of the R2 muscle attachment (Fig. 6g, h) and mark both the path of posterior migration and the original position of the muscle. As the anterior end of R2 approaches its final position in the antero-posterior axis of the segment, medio-lateral movements begin. At first these movements have a strong net lateral
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component and the origin of the muscle is shifted laterally (Fig. 6g). The anterior end of the muscle fans out as new anterior attachments are formed (Fig. 6g, h, i). The muscle fibres then separate along their entire lengths to give rise to the disperse adult muscle morphology (Fig. 6j).

In vitro investigation of myokinetic migration

We have been able to mimic part of the process of myokinetic migration in vitro. When sternites are removed from newly moulted pupae and cultured for several days, myoblasts emerge from the stumps of muscles R2, R3 and R4. These myoblasts extend processes in a highly directed, medio-lateral fashion across the sternite epidermis (Figs. 7, 8). Myofibres derived from these myoblasts develop striations and become spontaneously contractile.

Myoblasts were not obtained from the R1 muscles in vitro. This is probably due to the fact that tissue was not taken from animals at an appropriate stage in development. The R1 muscles undergo metamorphic changes somewhat later than the other muscles (Fig. 2).

Differential rearrangement of homologous muscles

Myokinetic migration of muscles is involved in generating differences in the arrangement of homologous retractor muscles in different abdominal sternites in the late pupal and adult stages.

This was demonstrated by comparing the R1 muscles in sternites 4 and 6 (Fig. 1). In the adult beetle the origins of the R1 muscles in sternite 6 (Fig. 1f) are more anterior in position than those of the homologous muscles in sternite 4 (Fig. 1c), whereas in the larva (Fig. 1a, d) and newly ecdysed pupa (Fig. 1b, e) they lie at equal distances from the anterior sternite boundaries. The position of cells adjacent to the R1 muscle origins was marked in both sternite 4 and 6 of newly ecdysed pupae by pricking the integument with a fine dissecting pin (Fig. 1b, c). This results in slight distortions of the adult cuticle above the cells affected by the prick but does not produce any detectable changes in the R1 muscles. In the adult, the marked integument is the same distance from the anterior margin of both sternite 4 and 6 (Fig. 1c, f), but different distances from the origins of the R1 muscles. As intersex homograft experiments have revealed that there is myokinetic migration of R1 muscles in both sternites 4 and 6, these results indicate that the R1 muscles migrate further, in a posterior direction, with respect to the integument in sternite 4 than they do in sternite 6.

DISCUSSION

In insects with a complete metamorphosis the external forms of the larval and adult stages are markedly different. In order to accommodate for these changes in body form there is extensive modification of internal tissues, including skeletal muscle, during the pupal stage.
Consideration of the relationship between metamorphosis of the exoskeleton and internal tissues led to the suggestion (Poyarkoff, 1914) that the prime function of the pupal stage in holometabolous insects was to permit adult muscles to differentiate within an integumental mould that approximated the final adult form. He suggested that the pupal stage was interposed between those of the larva and adult because two molts were required to complete metamorphosis of the skeletal muscles. The first (pupal) molt was required for the generation of a new external form similar to that of the adult, the second (adult) molt for the development of adult muscle attachments.

While we feel that Poyarkoff over-stated his case by trying to establish the dominance of one aspect of development during the pupal stage, our observations of muscle metamorphosis in *Tenebrio* show that the image of the pupa as an integumental mould still bears consideration. In *Tenebrio* the pre-pupal period is primarily a time of epidermal remodelling resulting in formation of an adult integumental mould, with changes in the positions of retractor muscles due to epidermokinetic muscle movements. In contrast, the pupal stage provides a time for precise repatterning of muscles with respect to the new exoskeletal mould, by myokinetic movements, before the adult cuticle is secreted. Using current terminology (Wolpert, 1969), muscle repatterning at metamorphosis can be described as a two-stage process in which (a) new positional information is established and expressed in the epidermis, and (b) this positional information is used by migrating muscles as they seek their adult attachment sites.

This view provides a conceptual framework for discussion of changes in the abdominal sternites which are related to retractor muscle movements and also of the mechanisms underlying these movements. Specific points will be considered in the following three sections: Epidermal remodelling; epidermokinetic muscle movements; myokinetic muscle movements.

**Epidermal remodelling**

Epidermal remodelling occurs in a relatively brief period prior to ecdysis in insects. The epidermal cells undergo divisions which, in association with increases in cell size, cell differentiation, death and rearrangement, generate changes in body form.

At, or shortly after, the onset of mitosis the epidermis undergoes apolysis. This event, because it frees the apical surfaces of the cells, probably facilitates epidermal rearrangements. In *Tenebrio* the epidermis is freed of a further constraint to cell rearrangement at certain times during metamorphosis because the basal lamina becomes loose. In the context of the Poyarkoff theory, this is interesting because the epidermal cells are detached from both cuticle and basal lamina for a longer time in the pre-pupal period than in the pupal stage (Fig. 2). This can be related to the requirement for more extensive epidermal remodelling at the larval–pupal, than at the pupal–adult transition. In fact, Caveney (1973), using colchicine, showed that all the epidermal divisions that occur during the
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pupal stage are differentiative divisions associated with the development of the adult pit glands, so that these divisions result in rather minor modifications of what is already, essentially, the adult form. Loosening of the basal lamina in the pupa does not occur concurrently with detachment of the apical surface of the cells from the cuticle (Fig. 2). It is probably associated with changing contours of the basal surface of the epidermis during the development of the pit glands. In contrast, loosening of the basal lamina in the pre-pupal period, which occurs soon after apolysis, may be necessary to permit the extensive epidermal re-arrangements (movement of Zone-II cells, for example), as well as the epidermokinetic muscle movements, that occur at this time.

Loosening of the epidermal basal lamina is probably a general, if over-looked structural modification facilitating extensive epidermal remodelling (either epidermal differentiation or rearrangement) in insects. It has been reported to occur in both hemimetabolous and holometabolous insect species. For example, the basal lamina can be removed from the abdominal epidermis of Oncopeltus (Hemiptera) during periods of proliferative and differentiative cell divisions during the metamorphic moult (Lawrence, 1968). Ultrastructural studies of development in the lepidopteran Hyalophora reveal that the basal lamina is temporarily lost from both wing epidermis (Greenstein, 1972), and ventral abdominal epidermis (Sedlak & Gilbert, 1976) in the pupa when the apical surfaces of these cells are also detached from the cuticle. In the wing epidermis this occurs at a time when the products of epidermal differentiation (bristle and scale cells) can first be distinguished from other epidermal cells. The basal lamina is also detached from epidermal cells in the wing of the lepidopteran Manduca, at the adult apolysis (Nardi & Kafatos, 1976). Berridge, Gupta, Oschman & Wall (1976) have reported that in Calliphora (Diptera) epidermal cells of the adult salivary glands lack both basal lamina and cuticle when the adult glands are expanding. Reinhardt (pers. comm.) has observed that in the pupa of Drosophila (Diptera) the basal lamina is disrupted or absent from polytene epidermal cells during their replacement by spreading of (imaginal) histoblasts. Moreover the histoblasts are also detached from the cuticle at this time. He suggests that detachment from both basal lamina and cuticle facilitates histoblast spreading.

Epidermokinetic muscle movements

The generally accepted view of insect muscle repatterning at metamorphosis is that it results from growth of the integument to which the muscles are attached. Amongst different orders of holometabolous insects the epidermokinetic type of muscle movement has been described in Simulium (Diptera) (Hinton, 1961); in Apis (Hymenoptera) (Daly, 1964); the thoracic muscles of Tenebrio (Coleoptera) (Lee, 1964); and in Galleria (Lepidoptera) (Millen, 1975). (These are only a few references to what appears to be a commonly observed phenomenon.)

The exact mechanisms which underlie epidermokinetic muscle movements have not been fully investigated by these authors, although the movements have been related to concurrent epidermal remodelling. For example, Hinton (1961) states that changes in the position and orientation of the indirect flight muscles of Simulium are brought about by ‘differences in the rate of growth of the epidermis of different parts of the mesothorax’. In Tenebrio, the lateral displacement of the origins of muscles R1, R2 and R3 at pupation are readily explained by such a general mechanism, since there is a general broadening of the sternite at this time. However, the R4 muscle is unusual in that its displace-
ment from the sternal mid-line, in the pre-pupa, is relatively greater than can be accounted for by overall sternite shape changes. We have attempted to determine what specific epidermal changes are involved in producing this localized epidermokinetic movement.

Changes in the distribution of epidermal cells may be due to (a) rearrangement of existing cells or (b) cell division (Fristrom, 1976). Therefore both epidermal divisions and cell rearrangement could contribute to lateral displacement of the R4 muscles. Many proliferative epidermal divisions do take place at the time of R4 movement (Fig. 2). However, it was not possible to relate specific areas of epidermal mitosis, or epidermal cell death, to the muscle movements. Furthermore the majority of the epidermal divisions that take place in the immediate vicinity of the R4 muscle origins around the time of epidermokinetic movement are differentiative divisions. These are unlikely to result in displacement of the muscles as the daughter cells leave the plane of the epidermis. Therefore the exact role of epidermal growth in generating R4 movement remains to be determined. It seems clear, however, that epidermal rearrangements are involved, as R4 movement is accompanied by a forward movement of the Zone-II cells (Fig. 4). This epidermal movement presumably imparts some lateral force of displacement upon neighbouring Zone-I cells, resulting, in turn, in the lateral displacement of the R4 muscle origins. These findings suggest that, in general, it may be necessary to question the assumption that epidermokinetic muscle movements are specifically the product of epidermal growth; epidermal rearrangements must also be considered.

Myokinetic muscle movements

From the time that pupal cuticle starts to be deposited, until apolysis in the pupal stage, epidermal cells are subject to constraints upon their movement. (They are attached to the pupal cuticle at their apical surfaces.) The translocation of retractor muscles that occurs during the early pupal stage is accomplished, therefore, by movements of the muscles relative to the epidermis. Or, more positively: rearrangement of the muscles at this time is brought about by myokinetic migrations within a fixed integumental mould. These migrations cease when appropriate adult muscle attachment sites are encountered on the epidermal surface, and the muscle attachments are elaborated as the adult cuticle is secreted.

Myokinetic movement of the R1 and R2 muscles of *Tenebrio* takes place in two stages. First there is a net shortening of the muscles that results from posterior migration of their anterior ends; and then there is an overall broadening of the muscles as individual fibres separate from one another (Fig. 6). The mechanisms underlying these two processes are incompletely understood. For example, the origin and function of the cytoplasmic processes (Fig. 6) that are observed during posterior migration are not known. It seems clear, however, in view of the fact that all myokinetic movements are preceded by a marked reduction
of whole muscle birefringence (Fig. 2), that muscular contraction is not involved.

Posterior myokinetic movement of the retractor muscles may require that the muscles are intact and attached to the integument at their posterior ends, as it does not occur in vitro when the muscles are severed. However, medio-lateral migration of myoblasts does occur in vitro (Figs. 7 and 8), and is thought to be analogous to the medio-lateral spreading of the retractor muscles which occurs in vivo. The rather long range migrations that are observed in vitro probably result from the fact that myoblasts are completely released from the cut surfaces of the muscles, and perhaps also released from events that would normally terminate migration in vivo. We regard the in vitro migrations as an uncontrolled, or exaggerated, version of the in vivo process.

The only previous description of myokinetic muscle movement is in the lepidopteran *Galleria mellonella* (Millen, 1975). In this moth, the anterior end of the dorsal scutellar longitudinal muscle migrates over the epidermis during metamorphosis. Millen suggests that the motive forces behind this migration are generated by ruffled membranes, or pseudopodial activity, at the anterior end of the muscle. It is possible that retractor muscles migrate using a similar mechanism in *Tenebrio*. However, we favour the idea that, in *Tenebrio* at least, the motive forces for net muscle movements are generated by myoblast processes which are extended from the metamorphosing muscles. The larval muscles are therefore seen to serve two functions. They are a source of myoblasts; and also act as a scaffolding, or substratum, upon which the adult muscles are built. This arrangement facilitates controlled 'sampling' of the epidermal surface by processes extended from myoblasts within the muscles. At the onset of myokinetic muscle movement myoblast processes form transient attachments with the epidermis in the immediate vicinity of the muscle. Net posterior movement of the muscle then occurs as the body of the muscle moves from old to newly formed attachment sites. Then, as the anterior end of the migrating muscle approaches its final position in the antero-posterior axis of the sternite, myoblasts start to extend processes in a medio-lateral direction. These processes form the final (adult) muscle attachments. Meanwhile individual myoblasts fuse together and also fuse with the remnants of the larval muscles to form the adult muscle fibres. These events produce the fanned-out muscle fibres that are observed in later stages of myokinetic migration (Fig. 6). The adult muscle fibres then separate along their lengths as they take up slack between their points of attachment to the integument.

The control of processes underlying myokinetic muscle movements must be complex. For example, what factors control the timing at which movements are initiated, the direction of net movement, and the position at which the adult muscle attachments are finally elaborated? The observation that there is a different degree of myokinetic migration of homologous muscles in different abdominal sternites indicates that the extent of migration is differentially con-
trolled in each sternite. This observation adds another tier of complexity to the morphogenetic problems posed by our observations of muscle metamorphosis within individual sternites. We have attempted to solve some of these problems by using grafting experiments to demonstrate that an epidermal morphogenetic gradient specifies the position at which adult muscle attachments are formed. This experimental work is presented in a companion paper (Williams & Caveney, 1980).

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REFERENCES


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