ASPECTS OF THE STRUCTURE AND FUNCTION OF SOME GASTROPOD COLUMELLAR MUSCLES (MOLLUSCA)

by

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THESIS

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ERRATA

1. Page 10, line 7 should read .... were made accessible by ....

2. Page 14, lines 4 and 5 of legend should read ....B: detail of A showing transverse section....

3. Page 15, line 2 of legend should read ....horizontal antero-posterior section....

4. Page 16, lines 6 and 11 should read ....columellar muscle....

5. Page 50, line 12 should read ....over distances several times the length of the shell (Fretter....

6. Page 158, line 22 should read ....ultrastructure of the pedal musculature of patellid....

To Fabio

Madame, you are a woman,

and that is altogether too much.

Justification offered to the Austrian Physicist Marietta Blau
for rejecting her from a post at the University of Vienna.

Contents

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Abstract

The columellar muscle of both limpets and coiled shell gastropods is of the paramyosin smooth type. Collagen forms an integral part of the musculature constituting about 35 % of the tissue. In limpets, muscle organisation is typical of a muscular hydrostat. Tightly packed blocks of muscle, dense arrays of cross-linked collagen, large muscle cells (9 μm diameter) and thick filaments (70 nm diameter, 30 μm long) produce a tough, relatively rigid but powerful muscle. In coiled shell gastropods, muscle organisation is intermediate between a muscular and a fluid hydrostat. Finer muscle cells (6 μm diameter), thick filaments (60 nm diameter) and a loose intercellular network of collagen interspersed with fluid vesicles are features of a more pliable and extensible muscle. In addition, ultrastructural differences, such as larger numbers of mitochondria and sarcolemmal invaginations distinguish the tarsal from the columellar muscle in both limpets and coiled shell gastropods.

About 25 % of muscle cells in most species examined, contain a novel arrangement of thin filaments with periodic electron-dense regions. These are similar in appearance to intrafusal cells and stress-fibres of non-muscle cells.

Structural analysis of isolated filaments, optical diffraction and SDS gel electrophoresis confirm the large dimensions and the paramyosin nature of the thick filaments. Microdensitometry of the gel proteins confirms the high proportion of collagen present.

No significant differences in muscle ultrastructure were found between limpets from different tidal heights. Muscle attachment areas are shown to be species-specific and positively correlated to tenacity and wave exposure. The muscle attachment mechanism is similar to that described for other molluscs. It consists of a special epithelial layer and a mucous-like material at the muscle-shell interface that possibly has an adhesive function.

Although the ultrastructure of *Patella* is very similar to that of the anterior byssus retractor of *Mytilus*, its mechanical behaviour is not. The muscle has a narrow working range where maximum tensions and "catch-like" contractions develop. This narrow length range is co-incident with the *in situ* length at which clamping occurs. It is suggested that the large component of collagen has an important influence over the mechanical behaviour of the muscle during clamping, by cross-linking in a manner similar to that described for some echinoderm connective tissues.

Chapter 1

GENERAL INTRODUCTION

The muscular foot is one of the characteristic features of the phylum Mollusca. The particular form of the foot amongst different molluscan classes is closely correlated with habitat. In Placophora, for example, and some Gastropoda, the foot is characterised by a broad sole for both adhesion to the substratum and for locomotion by means of cilia, or muscular pedal waves (Trueman, 1983; Trueman & Clarke, 1988). In the Bivalvia, Scaphopoda and some Gastropoda, the foot is adapted for burrowing both in shape and by the presence of a haemocoelic cavity for pedal protraction (Trueman & Brown, 1976; Trueman, 1983). In each case, the form of the foot is adapted to a specific life-style.

Another major feature of the phylum Mollusca is the calcareous shell, which also, in general, exhibits adaptations to habitat. The bivalved shell with its elastic ligaments affords a locomotory device as well as protection, while the shell becomes a buoyancy aid in cephalopods. In gastropods, two forms of shell exist which are thought to have evolved from a common cuticular or spiculose shell (Trueman & Clarke, 1988). One form is a simple shallow cone, as in limpets, the other is a helical coil (Hughes, 1986). Both forms afford protection from harsh environmental factors and predation (Branch, 1985).

The foot is connected to the shell by means of the columellar muscle. The particular form of this muscle is largely determined by the shell and foot form, and thus, in turn, by adaptation to habitat and life-style. In limpets, the columellar muscle runs dorso-ventrally and attaches to the inner surface of the shell in a horseshoe-shaped region (Fig. 1.1A). In coiled shell gastropods the muscle is attached to a coiled pillar of shell, the columella (Fig. 1.1B). The archaeogastropod *Haliotis* illustrates an intermediate case, where the columellar muscle has no columella shell origin, even though the

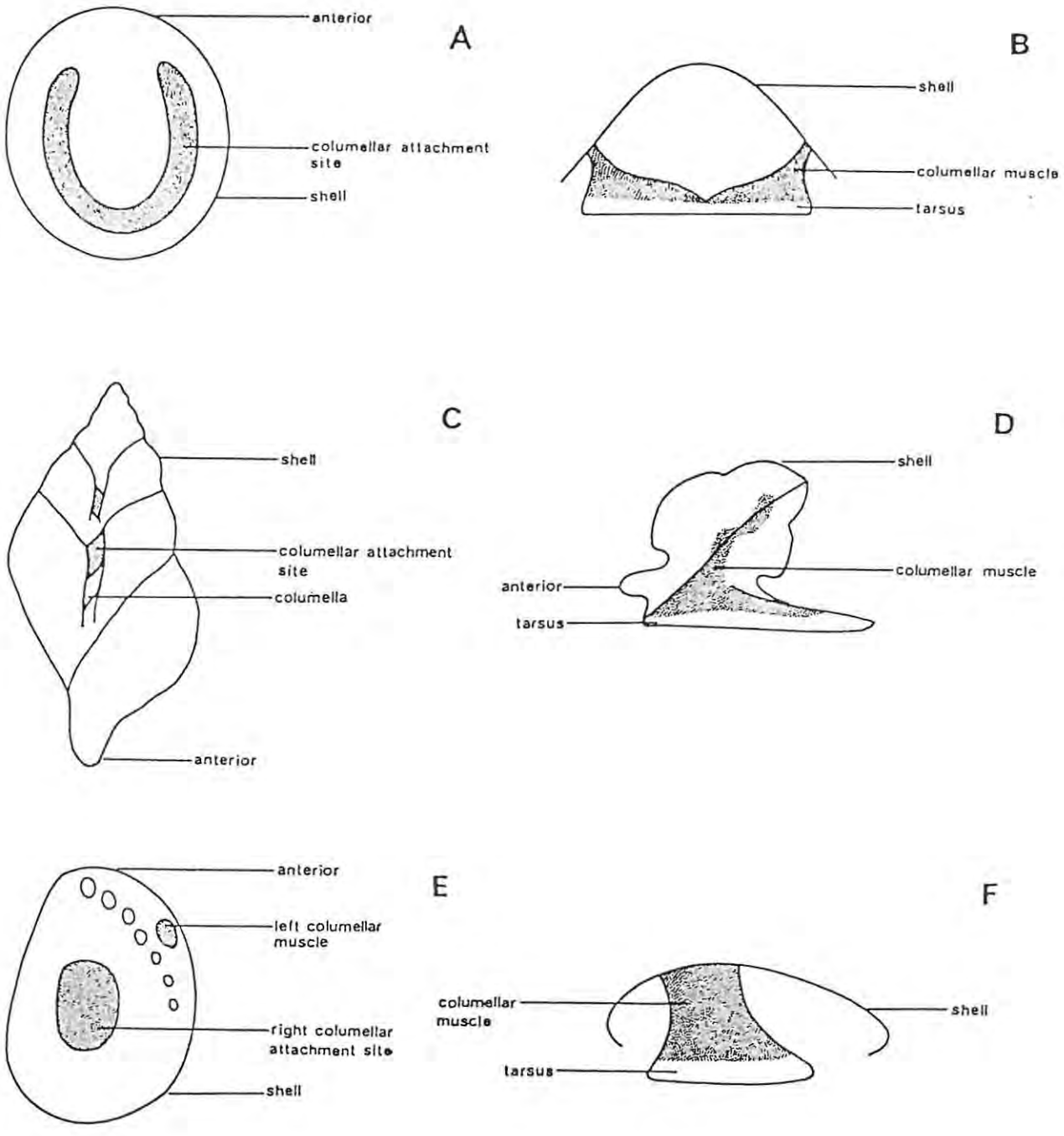

Figure 1.1. Schematic diagrams to show the attachment site on the shell interior and the distribution of the columellar muscle of three forms of gastropod. A, limpet ventral view. B, limpet antero-posterior section. C, coiled shell gastropod longitudinal section. D, coiled shell gastropod sagittal section. E, haliotid ventral view. F, haliotid sagittal section.

coiled shell form persists (Fig.1.1C). In the earliest known prosobranchs Bellerophontacea, which were contemporary to the Cambrian period (Fretter & Graham, 1962), two bilaterally symmetrical muscles inserted onto the columella (Knight, 1947). It is thought that by gradual extension backwards the two muscles joined to form the characteristic horseshoe columellar muscle of present day limpets (Fretter & Graham, 1962). In the coiled shell gastropods, the left columellar muscle was lost. In the intermediate form of *Haliotis* it remains, but is much reduced.

Common to both coiled shell gastropods and limpets is the use of the flat sole of the foot to "adhere" to and "creep" over the substratum. Yet in no other gastropods but the patellid limpets are adhesive forces so large (Branch & Marsh, 1978), that they may approach values as high as 7×10^5 Pa (Branch, 1981) in some species. Clamping can be seen as an adaptation of limpets that enables them to inhabit regions of the intertidal rocky shore where other gastropods would not survive (Fretter & Graham, 1962). The desiccating effects of wind and sun and dislodgement by strong wave action and predation are substantially reduced by clamping (Branch, 1985). When threatened by a predator clamping can be used by limpets both defensively and aggressively. Adult species of *Patella oculus* are known to raise their shells aggressively then pull them down firmly onto a predator (Branch, 1985).

A distinction can be made between adhesion *per se*, which is a function of the layer of mucus secreted at the sole of the foot, (Branch & Marsh, 1978; Grenon & Walker, 1981) and clamping, which is a function of the columellar muscle that holds the shell closely apposed to the substratum. These two functions, though distinct, must operate simultaneously if clamping is to be effective. Adhesion or muscle tension alone will not produce clamping. An essentially isometric contraction of the columellar muscle pulls the shell against the substratum. Although a stiffened muscle has been shown to be necessary if clamping is to persist (Grenon & Walker, 1981) maintaining the clamped state by persistent muscular contraction for any length of time

would be energetically uneconomical. The mechanism of maintaining muscle rigidity has not been investigated.

A closer look at the phenomenon of limpet clamping shows that three principal elements are involved: the shell, the mucus layer at the sole of the foot and the columellar muscle. Several authors have dealt at length with the adhesive aspect of tenacity in limpets. Properties of adhesive mucus have been investigated (Grenon & Walker, 1980, 1981; Denny, 1983), measurements of tenacity have been made and mechanisms of adhesion proposed (Aubin, 1892; Abé, 1951; Miller, 1974; Branch & Marsh, 1978; Grenon & Walker, 1981; Davenport, 1988). Shell shape and texture has also been studied in relation to tenacity (Branch & Marsh, 1978). However, in all these studies, no consideration was given to how the structure and performance characteristics of the columellar muscle might relate to clamping.

Although contraction of the columellar muscle is fundamentally the same in coiled shell gastropods and limpets, in the one case it is used principally to withdraw the foot into the shell, while in the other, it pulls the shell down onto the substratum (Fretter & Graham, 1962; Trueman & Brown, 1976). Another role of the muscle in coiled shell gastropods may be to support the shell (Frescura & Hodgson, 1989). The different role of the columellar muscle in coiled shell gastropods and limpets prompted the question of whether or not specialised structural adaptations have been acquired in limpets to enable them to clamp and thus successfully colonise the intertidal zone. To answer this question a comparative study of gastropod columellar muscles is necessary.

Studies of the organisation of columellar muscle at the light microscope level have been undertaken recently (Voltzow, 1985, 1988, 1990; Kier, 1988; Trueman & Hodgson, 1990). However, very few ultrastructural studies have been done on gastropod columellar muscles except for work on *Lymnaea* (Plesch, 1977) and *Nassarius* (Trueman & Hodgson, 1990) and none on limpets. This study deals with both structural and functional aspects of gastropod columellar muscles. The first part compares the structure at

both the light and electron microscope level between several marine prosobranch gastropods including other archeogastropods, caenogastropods and one pulmonate genus chosen from a range of different habits (e.g. territorial, generalist grazers) and intertidal zones (Table 1.1). In addition, the muscle-shell attachment site of limpets is examined. The second part investigates some of the performance characteristics of limpet columellar muscle comparing results with those of other molluscan smooth muscles.

The columellar muscle of limpets, or shell muscle as it is sometimes called, is defined in this work to be the entire pedal musculature except a region immediately above the sole of the foot (Fig. 1.1). This latter region which differs morphologically (Voltzow, 1988,1990) and ultrastructurally (Chapter 3) from the rest of the muscle is hereafter called the tarsus following Voltzow's (1990) suggested terminology. In coiled shell gastropods, the extent of the tarsus may vary, but the region is still differentiated from the columellar proper. The term columellar muscle is used consistently for all gastropods examined in this study emphasising the common derivation of the muscle.

The work commences in Chapter 2 with a histological study of the columellar muscles of several species of prosobranch limpet and one species of pulmonate limpet as well as selected representatives of other gastropod genera with coiled shells. Chapters 3 and 4 deal with the ultrastructure of limpet columellar muscle, and for comparison, that of a range of other gastropod genera. Chapter 5 takes a closer look at the fine structure of muscle filaments using a variety of techniques including, isolation of filaments, optical diffraction, gel electrophoresis, microdensitometry and immunocytochemistry. Chapter 6 is a study of the external morphology and size of the columellar muscle attachment scar of limpet shells. Histology, scanning electron microscopy and freeze-fracture methods are used to examine the muscle-shell interface, and muscle attachment areas are correlated to the zonal habitat of each species. Chapter 7 is an investigation of the effects of some neurotransmitters on whole and isolated bundles of limpet columel-

Species	Intertidal Zone and Habits
Order: Archaeogastropoda	Herbivorous grazers
Patella granularis	high shore, migratory
Patella oculus	high to mid shore, roams while feeding
Patella longicosta	mid to low shore, vagrant when older
Patella miniata	low tide pools and gulleys, roams while feeding, aggressive
Patella cochlear	subtidal rocks exposed to wave action, non-vagrant
Patella tabularis	subtidal, moves short distances, territorial, aggressive
Patella vulgata	mid shore, avoids the most sheltered places
Patella aspera	ditto
Patella intermedia	ditto but may extend to bare rocks
Helcion pectunculus	upper mid shore rocks, pools and gulleys
Helcion pruinosus	upper mid shore undersides of exposed rocks
Haliotis spadicea	upper region of subtidal, restricted zone, shelters under rocks
Oxystele tigrina	mid shore under rocks
Oxystele sinensis	mid shore often where surf is rough, a little lower than *O. tigrina*
Turbo sarmaticus	mid to low shore under submerged rocks
Order: Caenogastropoda	Carnivorous scavengers
Thais capensis	mid shore down to brisk wave action, under rocks and in pools
Burnupena catarrhacta	mid shore shallow pools, rock crevices
Burnupena cincta	mid to low shore, rock pools
Bullia rhodostoma	surf of open sandy beaches
Bullia digitalis	wave-swept sandy beaches, lower than *B. rhodostoma*
Subclass: Pulmonata	Herbivorous grazers
Siphonaria concinna	high to mid shore, exposed rocks
Siphonaria aspera	high to mid shore, more exposed rocks, more vagrant than *S. concinna*

Table 1.1. Habits and habitats of intertidal gastropods examined in this study. Reference sources: Fretter & Graham, 1962; Kilburn & Rippey, 1982; Branch & Branch, 1981.

lar muscle. Specific neurotransmitters are used to determine some of the mechanical properties of the muscle and to test the hypothesis that limpet columellar is a "catch" muscle. Chapter 8 investigates the length-tension relationships of patellid columellar muscle to determine its working range. A synthesis of all aspects of the work is made in the final Chapter and a model is proposed for the mechanism of limpet clamping. Problems that remain unsolved are highlighted and possible methods for their solution given.

Chapter 2

HISTOLOGICAL STRUCTURE OF COLUMELLAR AND TARSAL MUSCLE : LIMPETS AND COILED SHELL GASTROPODS

2.1 INTRODUCTION

The gastropod foot is a muscular organ capable of a large range of different functions, *inter alia* locomotion, adhesion to the substratum, egg capsule formation, mating and prey capture (Voltzow, 1990). In order to understand how the musculature accomplishes these different roles an appreciation of the organisation of the muscle is necessary. There have been several descriptions of the anatomical organisation of gastropod muscle (for reviews see Fretter & Graham, 1962; Hyman, 1967; Voltzow, 1990). Few studies have described internal organisation in relation to function. More recently, descriptions have been given of the general arrangement of the pedal musculature of the terrestrial slug (*Agriolimax reticulatus*: Jones, 1973), the freshwater snail (*Lymnaea stagnalis*: Plesch, Janse & Boer, 1975) and several marine gastropods (*Patella vulgata*: Jones & Trueman, 1970; *Neritina reclivata* and *Thais rustica*: Gainey, 1976; *Bullia digitalis* and *Haliotis midae*: Trueman & Brown, 1976, 1985, 1987; *Busycon contrarium* and *Haliotis kamtschatkana*: Voltzow, 1990; *Nassarius kraussianus*: Trueman & Hodgson, 1990). From these studies it appears that most gastropod muscles are based on a muscular hydrostat system as described by Kier (1988). An area of poor consideration is the connective tissue component of gastropod muscles. Only a few studies have been concerned with the connective tissue component of pedal muscle (Frescura & Hodgson, 1989; Voltzow, 1990; Trueman & Hodgson, 1990) which is here proposed as an essential element of gastropod columellar and tarsal muscles and the muscular hydrostat in general.

The pedal musculature however, can be considered to be composed of two distinct regions, the columellar and the tarsal muscle (Chapter 1). There is evidence from this study that these two regions are distinguished both functionally and structurally. Voltzow (1990) has come to the same conclusion independently from her studies on *Busycon contrarium* and *Haliotis kamtschatkana*. Since this distinction has not been made clear before, much of what appears in the literature on pedal muscle structure includes both these regions and needs careful examination to determine precisely which region of muscle is under scrutiny.

This Chapter deals with a histological examination of the columellar and tarsal muscle of a range of gastropods with different habits and habitats. These include several species of patellid limpet, coiled shell gastropods and the pulmonate limpet *Siphonaria*. A generalised model of the gastropod pedal musculature is given.

2.2 MATERIALS AND METHODS

Animals were collected from the east coast of South Africa. Species of patellid limpet were chosen to cover a full range of habitats across the different shore zones (Table 1.1) from very sheltered niches (e.g. *Helcion pectunculus*) to regions exposed to brisk wave activity (e.g. *Patella cochlear*). Similarly, the choice of coiled shell gastropods covered a range of habitats and a variety of life-styles from scavengers (e.g. *Burnupena*) to more sedentary grazers (e.g. *Turbo*). *Haliotis* was selected as a gastropod morphologically intermediate between the coiled shell gastropods and limpets (Chapter 1). The pulmonate *Siphonaria* lives a sedentary existence clinging to rocks like the patellid limpets but lacking their powers of tenacity. Finally, a representative from the genus *Bullia* was chosen in complete contrast to limpets having an extremely active life-style and a sandy beach habitat.

The columellar muscle of seven species of patellid limpet: *Patella oculus* (Born, 1778), *P. miniata* (Born, 1778), *P. granularis* (Linné, 1758), *P. cochlear* (Born, 1778), *P. longicosta* (Lamarck, 1819), *Helcion pectuncu-*

lus (Gmelin, 1791) and *H. pruinosus* (Krauss, 1848), the haliotid *Haliotis spadicea* (Donovan, 1808), the pulmonate limpet *Siphonaria concinna* (Sowerby, 1824), and five species of coiled shell gastropods: *Oxystele sinensis* (Gmelin, 1791), *Turbo sarmaticus* (Linné, 1758), *Thais capensis* (Petit, 1852), *Burnupena cincta* (Roding, 1798) and *Bullia digitalis* (Dillwyn, 1817) were examined.

Coiled shell gastropod columellar muscles were accesssed by crushing shells in a vice. The muscle from both limpets and coiled gastropods was either pulled gently away or cut from the shell. Some complete specimens of the columellar muscle were made including the tarsal region. Mostly, tissue from the mid to upper regions of the columellar muscle was used. Tissue was fixed either in 10 % formalin or Bouin's aqueous fixative. After being dehydrated through a series of alcohols and cleared in xylene, tissue was embedded in Paraplast, sectioned in three planes (Fig. 2.1) at thicknesses varying from 5 to 8 μm and stained by one of two methods which differentiate between collagenous connective tissue and muscle: Milligan's trichrome (staining of blood cells with Orange G omitted, Humason, 1967) and Mallory's trichrome, and a third method, haematotoxylin counterstained with eosin (Humason, 1967).

2.3 RESULTS

Comparison of the columellar muscle of the pulmonate *Siphonaria* with that of patellid limpets showed no differences in organisation. Coiled shell gastropods showed some obvious differences from patellid limpets and *Haliotis* showed a combination of both patellid and coiled shell gastropod features.

2.3.1 Columellar Muscle - Limpets

The limpet columellar musculature has a complex arrangement of muscle fibres (Figs. 2.1 & 2.2A). The outer layer of muscle is made up of circumferential bundles of fibres that form a thin sphincter around the entire foot

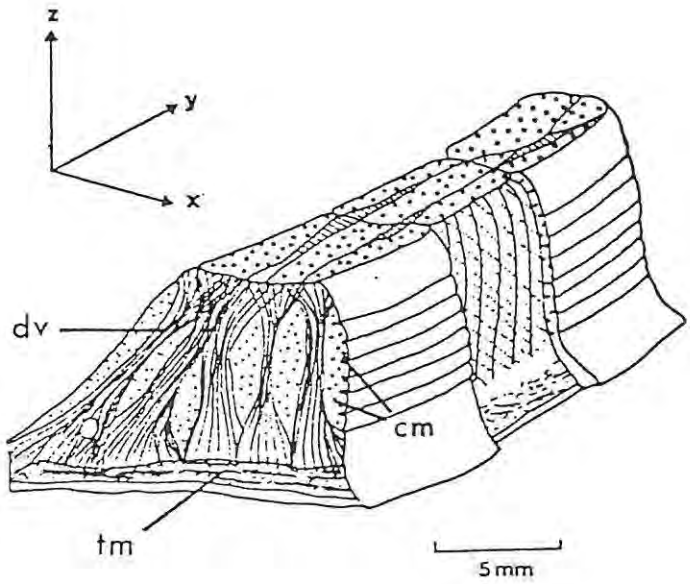

Figure 2.1. 3-dimensional drawing of a section of columellar muscle from a patellid limpet. Note the large dorso-ventral muscle bundles (dv) which ramify at the sole and sides of the foot. cm: circumferential muscle bundles, tm: transverse muscle bundles, x-z plane: cross section, x-y plane: antero- posterior section, y-z plane: sagittal section.

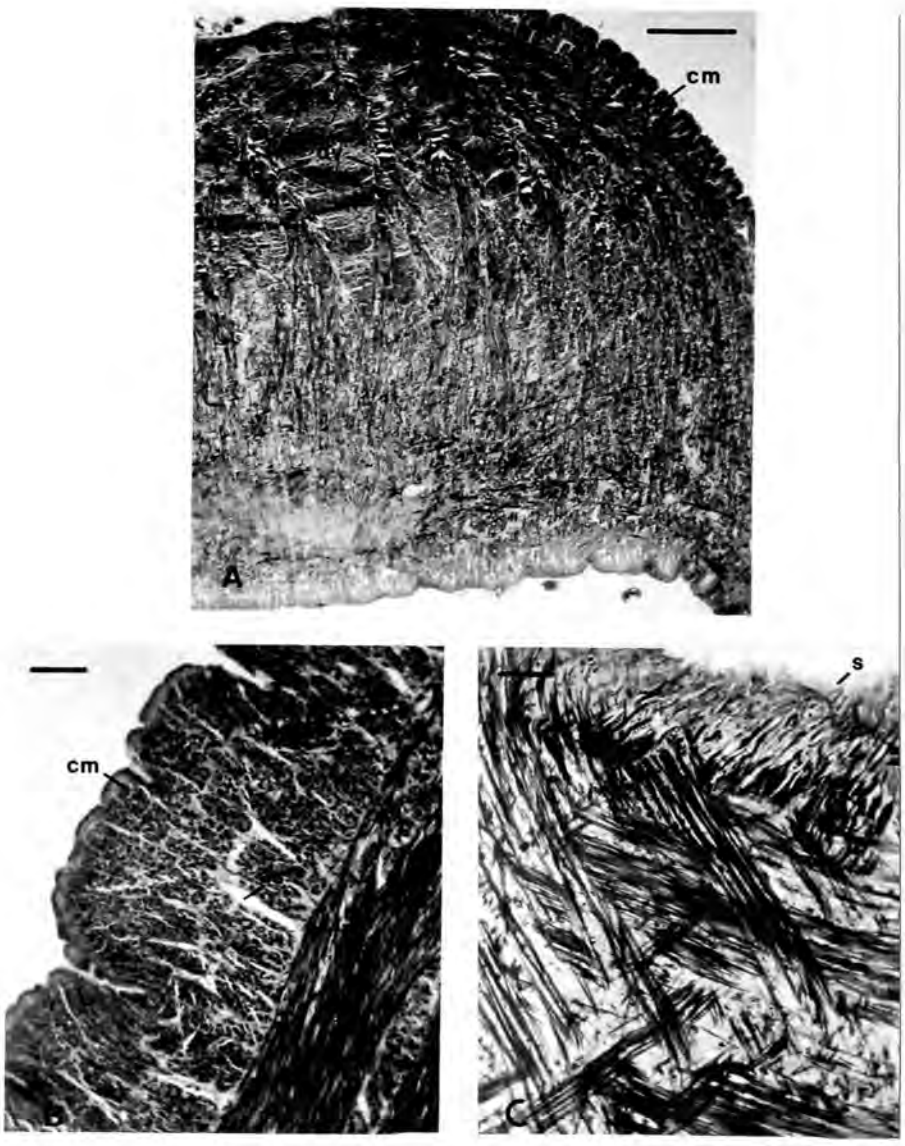

Figure 2.2. Photomicrographs of sections of the foot muscle from *P. oculus*. Tissue differentiated with Mallory's trichrome stain, muscle stains red, collagen stains blue. A: transverse section of whole foot muscle showing dorso-ventral (dv), circumferential (cm) and transverse (tm) muscle bundles. Scale bar = 500 μm. B: detail of circumferential muscle bundles (cm) in transverse section. Scale bar = 50 μm. C: detail of sagittal section to the inside of the circumferential muscle. Scale bar = 50 μm. s: shell attachment region, as: artifactual space from tissue processing.

(Fig. 2.1 & 2.2B). The tissue to the inside of the sphincter is composed of fibres running cross-diagonally from shell to pedal muscle (Fig. 2.1 & 2.2C). The majority of muscle fibres within the foot are found in large dorso-ventral bundles (z-direction) of mean diameter 180 $\pm$ 41 μm (SD, N=18) (Figs. 2.1 & 2.2A) while other muscle bundles run circumferentially (y-direction) (Figs. 2.1 & 2.2A & B) and a few run transversely (x-direction) near the sole of the foot (Figs. 2.1 & 2.2A). Between the dorso-ventral bundles distinct blocks of muscle are evident (Fig. 2.3B). Dorso-ventral fibres ramify at the sole and sides of the foot of the limpets forming a network of very fine muscle fibres (Fig. 2.1, 2.2A & 2.3A).

Histological observations also show that collagenous connective tissue permeates the entire musculature and constitutes 30 % or more of the pedal musculature (as estimated from relative areas of differentially stained collagen and muscle). Further estimations of the collagen to muscle ratio are given in Chapter 5. Collagen is particularly conspicuous towards the shell attachment site, the sole and sides of the foot (Figs. 2.3A & C). There are no blood sinuses in the pedal musculature but mucus storage sites are sometimes evident in the sole of the foot. These are particularly obvious in *P. oculus* (Fig. 2.3C).

2.3.2 Columellar Muscle - Coiled Shell Gastropods and *Haliotis*

A similar complex organisation of interwoven muscle fibres and collagen is seen in the other gastropods examined. Bundles of muscle are again seen to branch into finer fibres towards the tarsus. To avoid repetition, only the most significant features of organisation pertaining to each genera are described below.

The columellar muscle of *Haliotis spadicea* is the most homogeneous in appearance of all the gastropods with fine muscle fibres interweaving throughout (Fig. 2.4A & B). The muscle fibres of *Oxystele sinensis* appear to be fine, lying predominantly in the longitudinal direction (Fig. 2.4C & D).

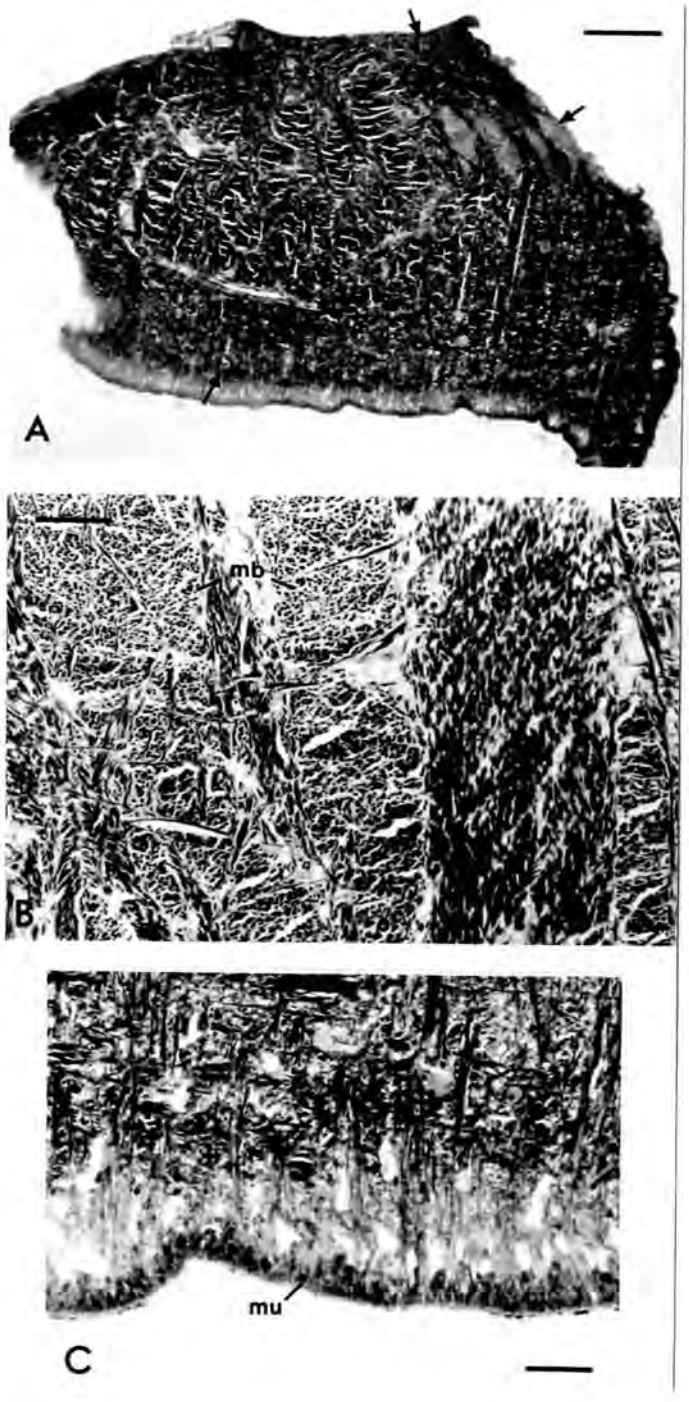

Figure 2.3. Photomicrographs of sections of the foot muscle of *P. oculus*. Tissue differentiated with Milligan's stain (Orange G omitted), muscle stains red, collagen stains green. A: transverse section of foot. Note the distribution of collagenous connective tissue at the shell attachment region, sole and sides of foot (arrows). Scale bar = 1000 μm. B: detail of cross section between dorso-ventral muscle bundles (dv) showing muscle blocks (mb). Scale bar = 100 μm. C: detail of tarsus showing mucocytes (mu). Scale bar = 150 μm.

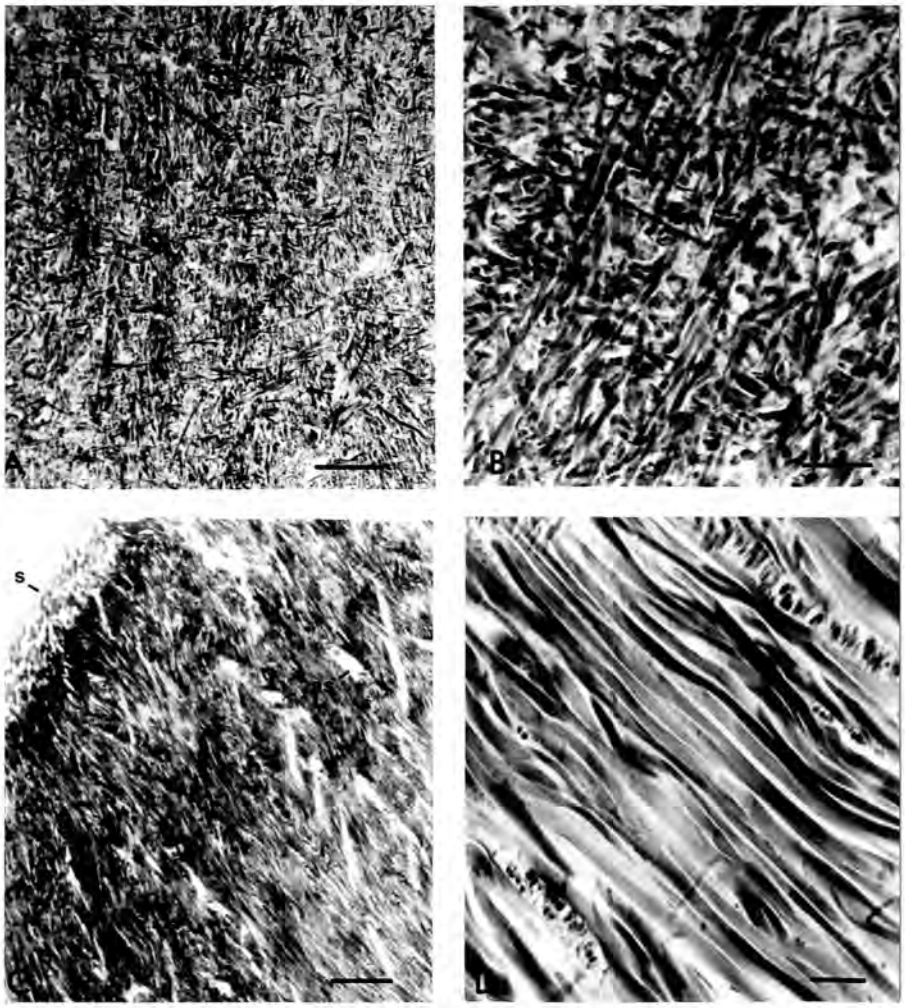

Figure 2.4. Photomicrographs of representative sections of gastropod columellar muscle. Tissue differentiated with Mallory's trichrome stain. A: antero-posterior section of columellar muscle of *Haliotis spadicea*. Note the lack of defined muscle blocks. Scale bar = 250 μm. B: detail of section in A. Scale bar = 50 μm. C: sagittal section of columellar muscle of *Oxystele sinensis*. Scale bar = 150 μm. D: detail of section in C. Scale bar = 25 μm. as: artifactual space from tissue processing, s: shell attachment region.

Turbo sarmaticus has distinct layers of muscle running in given directions (Fig. 2.5A). Large muscle bundles of mean diameter 112 ± 24 μm (SD, N=11) run dorso-ventrally (Fig. 2.5B). *Thais capensis* has a basket-weave appearance with muscle fibres crossing obliquely between the dorso-ventral fibres (Fig. 2.5C). Fluid spaces are much in evidence in the tarsal region of *Burnupena*, below the columellar, but are also seen to extend into the columellar region where they lie between the bundles of longitudinal muscle (Fig. 2.6A). *Bullia digitalis* has no distinct layering of muscle bundles (Fig. 2.6B).

Collagen is abundant in all species examined and is more prominent in some species in the lower region of the columellar towards the sole of the foot (e.g. *Thais*, Fig. 2.5C).

2.3.3 Comparative Results in Summary

All species examined have an interwoven arrangement of muscle fibres and collagen. The arrangement of fibres varies from distinct layers of muscle bundles running in specific directions (*Turbo* and limpets) to a more homogeneous mix of muscle fibres in many directions (*Haliotis*). The principal muscle bundles run longitudinal to the long axis of the columellar muscle. The tarsus typically contains finer, branched muscle bundles and a larger proportion of collagenous connective tissue than the columellar muscle (Figs. 2.2A, 2.3A & 2.5C). Coiled shell gastropod muscle bundles are less obvious than those in limpets (*Turbo* excepted) being finer and obscured by muscle fibres interweaving in other directions (measurement of bundle diameters for these species was not possible). Fluid spaces were seen in the gastropods *Burnupena* and *Thais* where they were present mostly in the tarsus (Fig. 2.6A). Figures 2.7 & 2.8 summarise from the findings of this study, the principal features that distinguish a typical limpet columellar muscle from a typical coiled gastropod columellar muscle as seen at the light microscope level. The columellar muscle of *Haliotis* falls between these two generalisations.

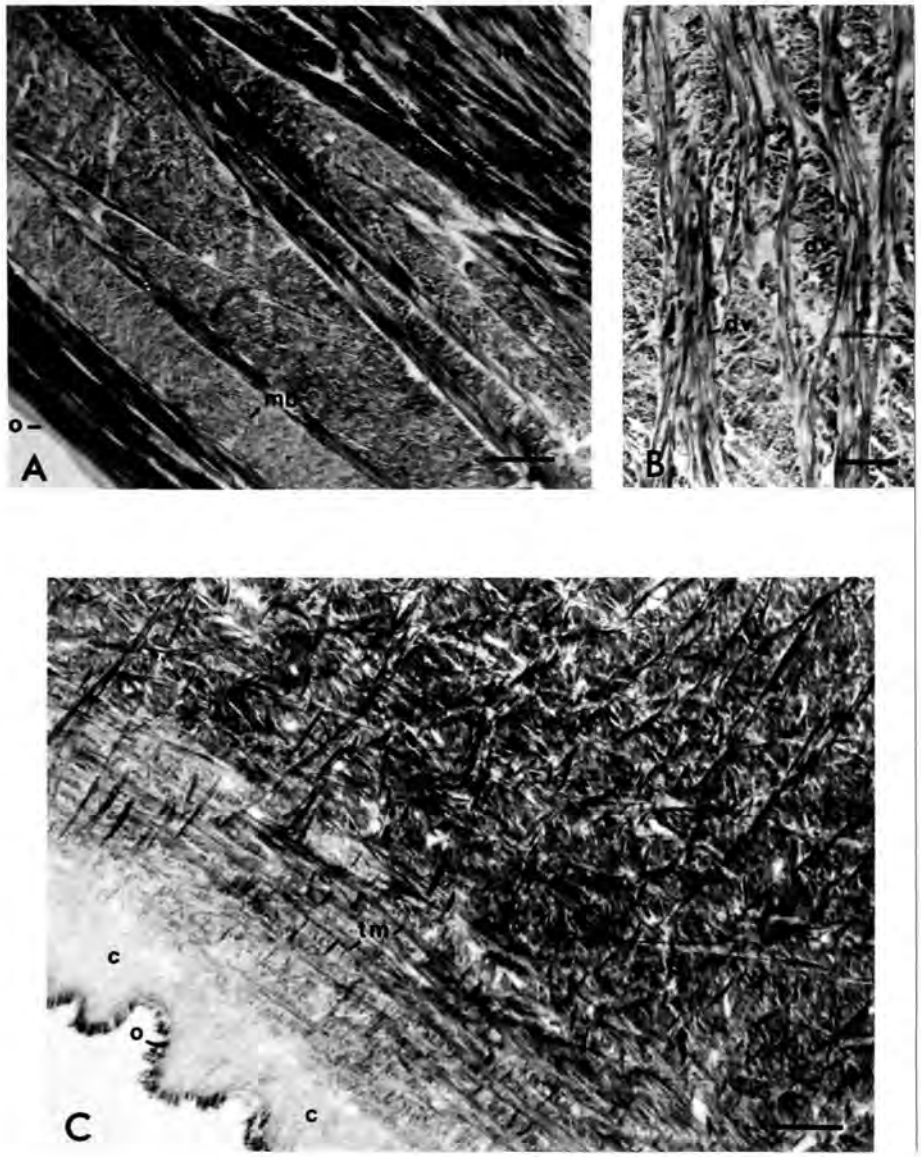

Figure 2.5. A: sagittal section of columellar muscle of *Turbo sarmaticus* showing distinct muscle blocks (mb) between longitudinal muscle bundles (lm) of columellar muscle. Scale bar = 150 μm. B: detail of dorso-ventral muscle bundles (dv) from a region of columellar muscle close to the shell. Scale bar = 50 μm. C: sagittal section of foot of *Thais capensis* which includes part of columellar (top right hand portion of picture) and tarsal muscle (lower left hand portion). Scale bar = 50 μm. o: outer edge of muscle, c: collagenous connective tissue, tm: transverse muscle bundles.

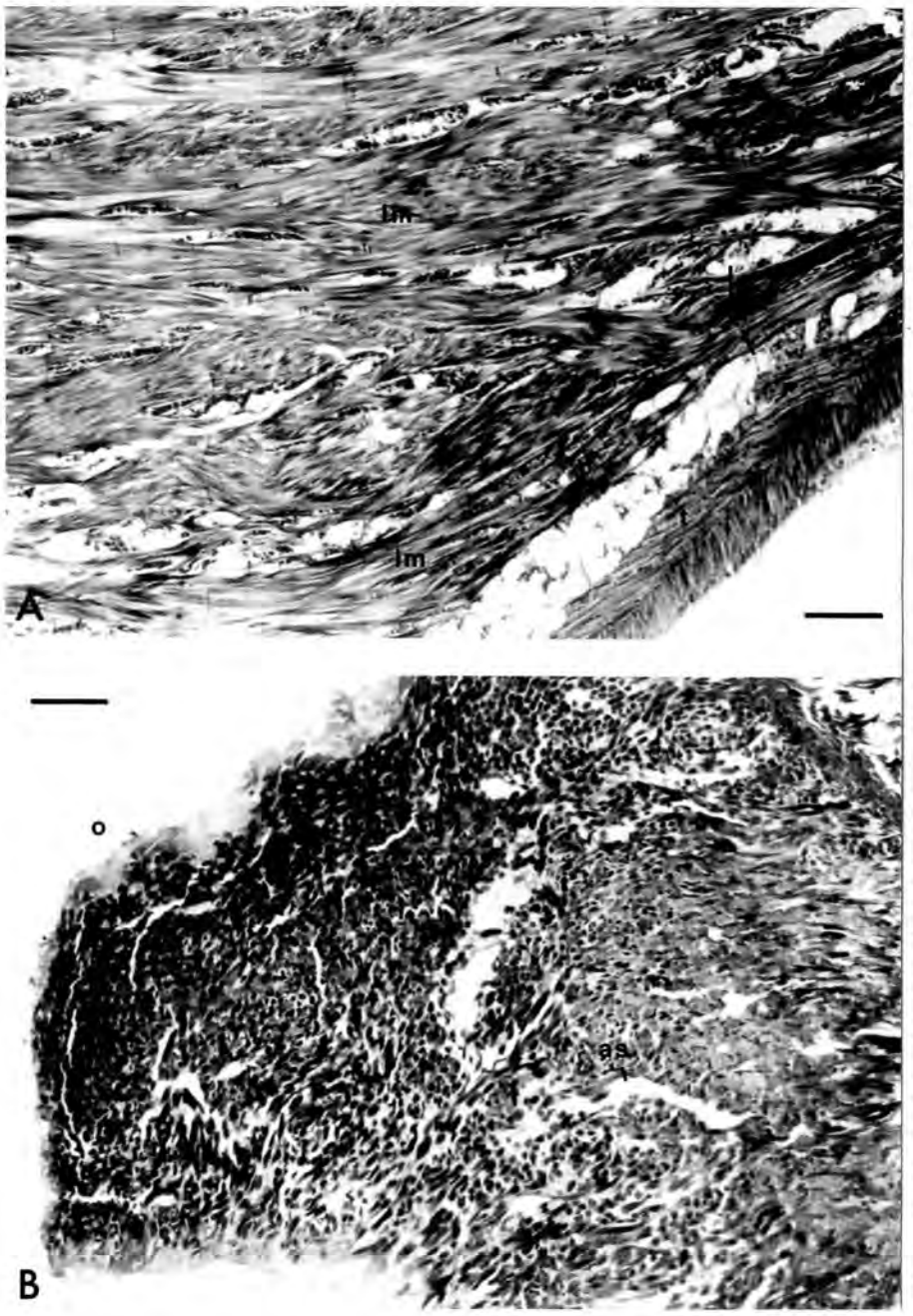

Figure 2.6. Photomicrographs of gastropod columellar muscle. Tissue differentiated with Mallory's trichrome stain. A: sagittal section of columellar and tarsal muscle from *Burnupena cincta*. Note the prevalence of fluid vesicles (fv) especially conspicuous in the tarsal region (t) below the longitudinal columellar muscle bundles (lm). Scale bar = 150 μm. B: transverse section through longitudinal columellar muscle bundles of *Bullia digitalis*. Scale bar = 150 μm. as: artifactual spaces from tissue processing, o: outer edge of muscle.

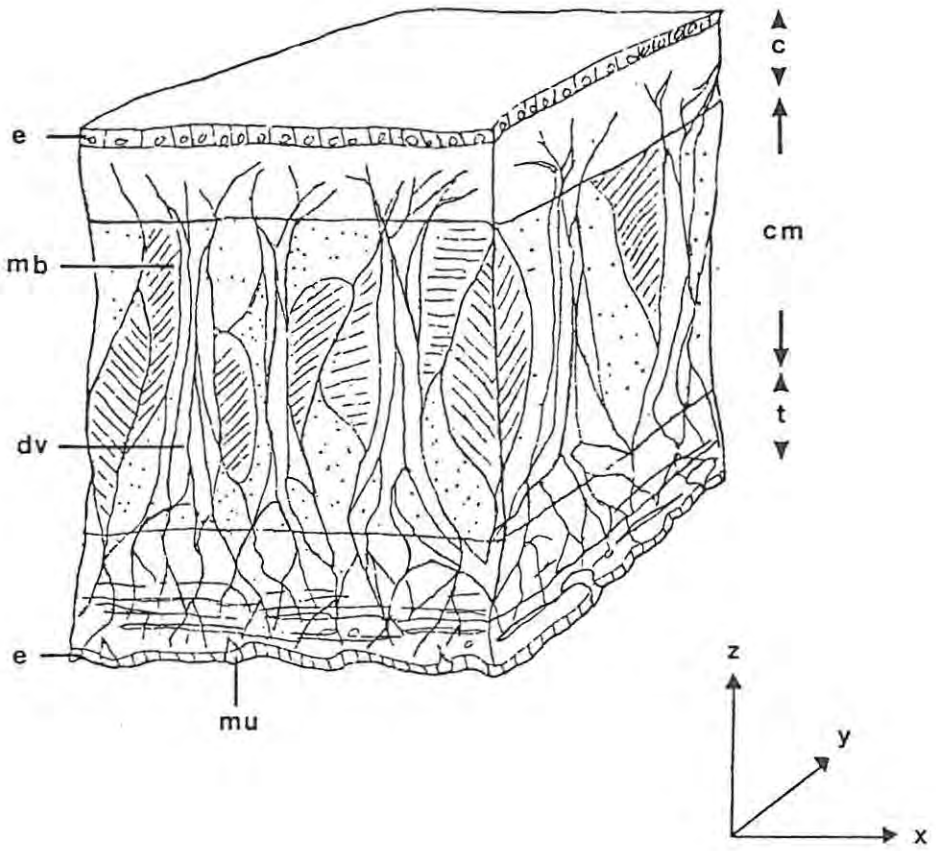

Figure 2.7. 3-dimensional drawing of generalised limpet columellar muscle. Note the thick dorso-ventral muscle bundles (dv), the branching of these bundles in the tarsal region (t) and the tightly packed blocks of muscle (mb) between these bundles. x, y, z dimensions as in Figure 2.1. c: collagenous connective tissue, cm: columellar muscle, e: epithelium, mu: mucocytes. Drawing not to scale.

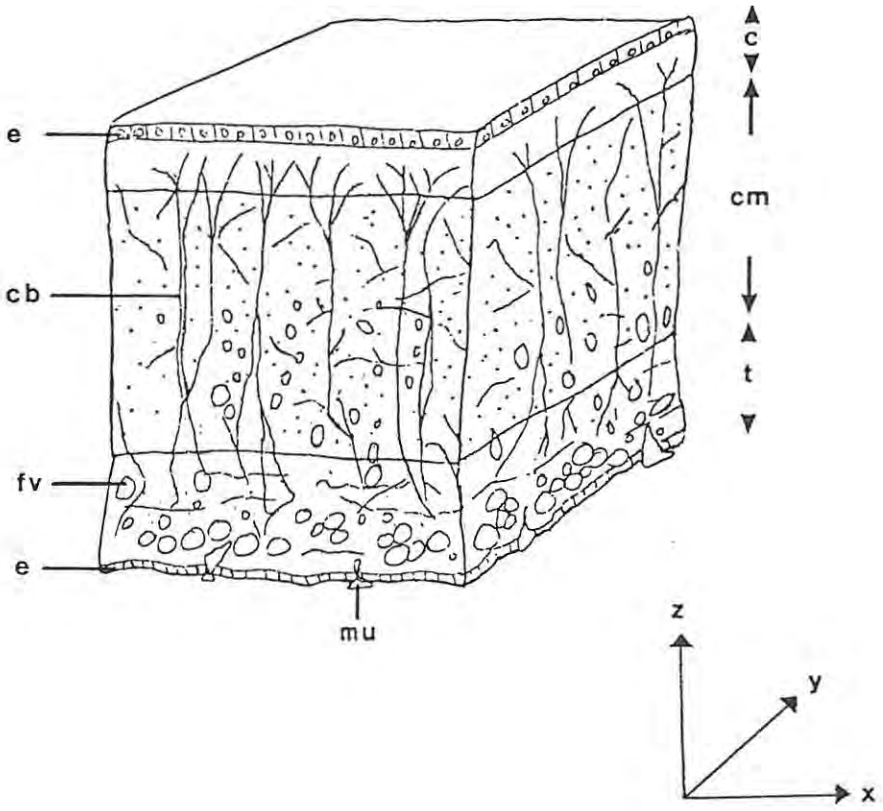

Figure 2.8. 3-dimensional drawing of generalised coiled shell gastropod columellar muscle. Note that the columellar muscle bundles (cb) are finer and packed more loosely relative to limpet columellar muscle and fluid vesicles (fv) are present, particularly concentrated in the tarsus (t). x, y, z, dimensions as in Figure 2.1. c: collagenous connective tissue, cm: columellar muscle, e: epithelium, mu: mucocytes. Drawing not to scale.

2.4 DISCUSSION

2.4.1 Organisation and Function of Columellar and Tarsal Muscle

The arrangement of muscle fibres in the gastropod foot has recently become well documented (Jones & Trueman, 1970; Trueman & Brown, 1976, 1985, 1987; Kier, 1988; Voltzow, 1988, 1990; Frescura & Hodgson, 1990; Trueman & Hodgson, 1990). The limpet columellar musculature is a system of muscle fibre bundles, ensheathed in collagen, (Voltzow, 1988; 1990) that directly antagonise each other without any dependence on a fluid skeleton i.e. a muscular hydrostat (Kier, 1988). It is suggested here that the cross-diagonal muscle bundles seen in limpets at the sides of the foot are involved in rotation of the shell and that when the dorso-ventral muscles contract these may crimp giving rise to what Jones & Trueman (1970) saw as spiral fibres in *Patella vulgata.* The siphonariid columellar muscle looked the same as the patellid form. This is an interesting convergence of morphology demonstrating the power of adaptational forces, since the Patellidae and Siphonariidae are thought to have evolved independently.

This study illustrates a principal feature in the gastropod foot generally, that is, the structural distinction between the tarsal and columellar regions (Frescura & Hodgson, 1990; Voltzow, 1990). The two regions form an inseparable continuum yet are clearly distinguishable both structurally and functionally (see Chapters 3 & 4). Both tarsal and columellar regions consist of intermingling muscle fibres and connective tissue. The difference at the light microscope level, lies in the size of muscle fibres and increased density of connective tissue matrix towards the sole of the foot. Voltzow (1988, 1990) reported that the dorso-ventral bundles ramify at the sole and sides of the foot in her studies of the organisation of the pedal musculature of limpets and *Busycon contrarium* and *Haliotis kamtschatkana.* This study confirms such findings and indicates they are probably true of the gastropod foot in general.

Voltzow (1985) has also shown that the circulatory system of the foot of the marine prosobranch *Busycon contrarium* consists of discrete arteries and veins that anastomose throughout the foot in a manner more closely resembling a "closed" than an "open" circulatory system. Several other marine gastropods have similar blood systems (Voltzow, 1990). What were originally identified as haemocoelic spaces in *P. vulgata* (Jones & Trueman, 1970) have been demonstrated to be mucus glands (Grenon & Walker, 1978, 1981). Voltzow (1985, 1990) suggests that blood probably contributes to overall turgidity of the foot by filling the many fine vascular channels in the tarsus, but she is disposed to consider most of what has been identified as blood sinuses to be shrinkage artifacts from tissue processing. The absence of fluid spaces in the columellar muscle of *Haliotis spadicea* concurs with findings for the species *Haliotis kamtschatkana* (Voltzow, 1990). However, Trueman and Hodgson (1990) found fluid spaces or vesicles in the pedal muscle of *Nassarius kraussianus*. These were especially concentrated just beneath the longitudinal columellar muscle (tarsus) but did not extend down to the very sole of the foot. The fluid spaces in *Burnupena* are very similar to those in *N. kraussianus* indicating that these species may use fluid as an antagonist for muscle contraction, at least in the tarsus. It seems unlikely still, that blood is the principal antagonist for muscle contractions generally in gastropod columellar muscles.

The gastropod foot exhibits a diversity of labour which can conveniently be considered under the headings of two types of muscle contraction: those that involve fine, agile movements such as locomotory waves and prey capture and those that involve gross but more powerful movements like shell clamping and pedal retraction. The complex arrangement of muscle fibres, especially those between limpets and coiled shell gastropods, together with the variations apparent between species, can be understood in terms of these different functions and the degrees to which they are employed by different species.

The thick dorso-ventral muscle bundles which are seen most clearly in

limpets and constitue about 70 % of the total pedal musculature (Jones & Trueman, 1970) draw the shell closer to the substratum. The transverse and circumferential set of muscles antagonise the dorso-ventral muscles (Jones & Trueman, 1970). In coiled gastropods, dorso-ventral muscle bundles as such, are not present. Their counterpart are longitudinal muscle bundles running from the shell columella to the tarsus which may not always run in a dorso-ventral direction. These longitudinal bundles retract the foot into the shell (Trueman & Brown 1976). Relaxation of the same, coupled with contraction of the transverse or circular muscles, results in protrusion of the foot (Trueman & Brown, 1976).

The tarsal region is involved in fine movements as in locomotion (Trueman, 1983; Voltzow, 1988; 1990). The locomotor waves of *Haliotis*, for example, are restricted to the outer edges of the foot and do not pass through the portion of the sole where the columellar muscle inserts (Voltzow, 1990). By recruiting only a few of the small muscle fibres, the finest movements could be accomplished while larger movements could be performed by using several bundles of fibres at a time (Voltzow, 1990).

Brown & Trueman (1982) have examined columellar muscles of a variety of gastropods at the light microscope level including the prosobranchs *Turbo sarmaticus*, *Oxystele variegata*, *Gibbula rosea*, *Argobuccinum argus*, *Burnupena cincta*, the freshwater snail *Planorbis corneus* and the terrestrial pulmonate *Theba pisana*. They have also examined the columellar muscles of *Haliotis midae* (1985) and *Bullia digitalis* (1976). The basic structural organisation was found to be similar in all these gastropods, namely, that of a muscular hydrostat, although some differences pertain with respect to the arrangement of the posterior stem and transverse muscles in *Bullia* (Trueman & Brown, 1976). In the last two examples, it was shown (Trueman and Brown 1976; 1985) that no hydrostatic pressures could be observed in any blood sinuses or mantle cavity during extension of the columellar muscle, thus providing direct evidence that muscular antagonism alone was sufficient to accomplish this movement.

The relative proportion of the foot occupied by muscle and fluid-filled haemocoelic spaces varies widely (Kier, 1988). A tightly packed pedal musculature is reported for the limpet *P. vulgata* (Trueman & Jones, 1977) and the marine snails *Thais rustica* and *Neritina reclivata* (Gainey, 1976). A more intermediate level of packing is described by Voltzow (1985) for *Busycon contrarium*, and a more loosely packed arrangement was observed by Denny (1981) and Jones (1973) in the pedal sole of the terrestrial pulmonates *Agriolimax reticulatus* and *Agriolimax columbianus*. The results of this study indicate a tightly packed musculature is the typical organisation amongst columellar muscles of limpets and coiled shell gastropods generally. On the other hand, a loosely packed arrangement with some fluid spaces is more characteristic of tarsal muscle but is found in some coiled gastropod columellar muscles too (e.g.*Burnupena*).

The presence of mucus storage sites in patellid limpets and their inverse relation to tenacity has been shown and discussed by Branch & Marsh (1978). They have shown that the more tenacious limpets have fewer and smaller storage sites and secrete a thinner layer of mucus. More mobile limpets like *P. oculus* show an opposite trend. It seems perhaps curious that mucocytes were not more in evidence in the soles of coiled shell gastropods.

2.4.2 Collagen and its Role

While elastic fibres have not been shown to be present in the patellid foot (Jones & Trueman, 1970; Kier, 1988) this study and the work of Frescura & Hodgson (1989; 1990) and Voltzow (1990) indicates that collagen is abundant not only in patellids but in all gastropods generally. It has been estimated (Frescura & Hodgson, 1990) that 30-40 % of the muscle tissue in limpets is collagenous connective tissue; for comparison, vertebrate skeletal muscle has 3-30 % (Walls, 1960).

Frescura & Hodgson (1989) and Voltzow (1990) also discuss several possible roles of collagen in gastropod columellar muscles one of which arises

from its ensheathing of muscle bundles. The sheaths form small functional units that not only provide anchorage for the muscles (Frescura & Hodgson, 1989) but also allow localised contractions, a feature typical of muscular hydrostats (Kier, 1988; Frescura & Hodgson, 1989; Voltzow, 1990). Furthermore, the ensheathing collagen may be important mechanically for force transmission as suggested by Voltzow (1990) in her study of the pedal muscles of *Busycon contrarium* and *Haliotis kamtschatkana*. Voltzow (1990) also discusses the role of collagen in conferring a greater degree of plasticity and extensibility to the gastropod pedal musculature which when combined with the finer muscle bundles of the coiled shell gastropods is aptly suited to the more agile movements of their feet.

The prevalence of collagen is confirmed at the ultrastructural level for both limpets and coiled shell gastropods and is given a more quantitative treatment in Chapter 5. Chapter 3 discusses further the subject of collagen and its role in gastropod muscle where data from electron microscopy studies are included. Even at this stage, however, it is clear from histological findings alone, that collagen forms an integral part of the columellar musculature in both limpets and coiled shell gastropods.

2.5 SUMMARY

- The organisation of the columellar muscle of gastropods varies from tightly packed fibres with no fluid spaces to more loosely packed arrangements often with fluid spaces.
- A difference exists between the organisation of the tarsal and columellar regions of the gastropod foot, which reflects the functional difference of these regions.
- Gastropod columellar muscles are based on the muscular hydrostat system.
- Gastropod tarsal muscles are based on systems which range from a

true muscular hydrostat e.g. *Patella* to a fluid-filled cavities system e.g. *Burnupena*.

- Collagen forms an integral part of the muscular hydrostat system of gastropod columellar and tarsal muscles.

Chapter 3

FINE STRUCTURE OF COLUMELLAR AND TARSAL MUSCLE : PROSOBRANCH LIMPETS

3.1 INTRODUCTION

The fine structure of molluscan muscle is extremely diverse (for review see Nicaise & Amsellem, 1983) and muscles from bivalves are probably better documented than any other molluscan muscles. There have been many studies on molluscan cardiac muscle (*Achatina* heart: Nisbet & Plummer, 1968; *Venus* heart: Hayes & Kelly, 1969; Kelly & Hayes, 1969; *Lymnaea* heart: Plesch, 1977; *Crassostrea* heart: Hawkins, Howse & Sarphie, 1980), retractor smooth muscles (*Mytilus* anterior byssus retractor: Twarog, 1967; Sobieszek, 1973; Bairati & Zuccarello, 1976; Bennett & Elliott, 1981, 1989; *Mytilus* posterior byssus retractor: Ishii & Takahashi, 1981; *Lymnaea* head retractor: Plesch, 1977) and adductors (*Crassostrea* translucent adductor: Hanson & Lowy, 1961; *Placopecten* translucent adductor: Morrison & Odense, 1974). Less has been reported on the fine structure of pedal muscles of gastropods, still less on the columellar muscle in particular and little or no study of this muscle has been made in limpets except for work published from this thesis (Frescura & Hodgson, 1990).

Limpet columellar muscle can be distinguished from that of coiled shell gastropods by its function. In limpets the columellar muscle is used primarily to pull the shell down onto the substratum and can withstand large opposing forces (Branch & Marsh, 1978; Grenon & Walker, 1981). In coiled shell gastropods it is used to retract and extend the foot over distances, in some cases, many times the length of the shell. It is this difference in function that suggests that there may be a difference in structure adapting

the limpet columellar muscle to its specialised role. The adductor muscle of bivalves has a similar role in pulling the two halves of the shell together and withstanding opposing forces. It is therefore reasonable to expect some similarity in fine structure between limpet columellar muscle and bivalve adductor muscle. Branch & Marsh (1978) have shown that tenacity (defined as normal force per unit area of muscle) varies, in general, between patellid species according to zonal habitat. This suggests that different species might show fine structural differences in keeping with these different tenacities.

The ultrastructure of the columellar muscle of a range of patellid limpet species is examined and compared with other molluscan smooth muscles. The range of limpets includes species with different habits (e.g. territorial, migratory, generalist grazers, etc. Table 1.1, Chapter 1) from different habitats of the intertidal zone in order to test whether zonal distribution or wave exposure influence the fine structure. In addition, the fine structure of limpet tarsal muscle is examined and compared with the columellar muscle from the species *Patella oculus*.

3.2 MATERIALS AND METHODS

Eight species of South African prosobranch limpet (*Patellidae*), *Patella granularis* (Linné, 1758), *P. oculus* (Born, 1778), *P. miniata* (Born, 1778), *P. longicosta* (Lamarck, 1819), *P. cochlear* (Born, 1778), *P. tabularis* (Krauss, 1848), *Helcion pectunculus* (Gmelin, 1791), *Helcion pruinosus* (Krauss, 1848) were collected from the south east coast of South Africa and three species of North Atlantic prosobranch limpet (*Patellidae*), *Patella vulgata* (L.), *P. intermedia* (Jeffreys), *P. aspera* (Lamarck) were obtained from the south west coast of England. The choice of limpet species was representative of a range of different habits and habitats as shown in (Table 1.1). Limpets were kept in aerated sea water until dissected. Some dissections were done on fresh animals within six hours of collection whilst others within one week on animals retained in an aquarium at a temperature of 18 ^{0}C.

Columellar tissue was prepared for transmission electron microscopy us-

ing the following protocol that maintains an appropriate osmolarity for marine tissue. Pieces of tissue about 1 mm^3 were excised from fresh pedal muscle under sea water and fixed in 2.5 % glutaraldehyde containing filtered sea water and 0.1 M sodium cacodylate buffer, pH 7.0 for 12 hours at 4 ^{0}C. Fixed tissue was washed in 0.1 M cacodylate buffer made up in sea water, post fixed with 1 % osmium tetroxide in the same buffer vehicle for 90 minutes, dehydrated and embedded via propylene oxide in an Araldite CY 212/Taab 812 resin mixture (Cross, 1989). The same protocol was used to examine a portion of the tarsal region of the pedal muscle of *P. oculus*. Silver/gold, sections cut using glass knives were stained either with 5 % aqueous uranyl acetate for 30 minutes, followed by Reynold's lead citrate for 10 minutes both at room temperature, or with 2 to 10 % methanolic uranyl acetate for 30 minutes. Material was viewed on a Jeol JEM 100 CXII electron microscope at 80 or 100 kv.

3.3 RESULTS

3.3.1 Fine Structure of Columellar Muscle

Negligible interspecific variation in the fine structure of the columellar muscle was found and the data is therefore presented in a form that generalises from all species studied. Fusiform smooth muscle fibres with diameters ranging from 4 μm to 15 μm are classified into two types (Fig. 3.1).

3.3.2 Type I Muscle Cells

Type I fibres are the major cell type and are found in all species. Cell components include a random arrangement of thin and very thick filaments (Figs. 3.1, 3.2A & 3.3). Dense bodies, which are sites of thin filament attachment, are seen rarely but this may be due to the fixation procedure. Those present are relatively electron-lucent with a loose granular substructure and diameters of the same order of magnitude as the thick filaments (about 70 nm, Fig. 3.3A). Occasionally electron-dense plaques are seen at

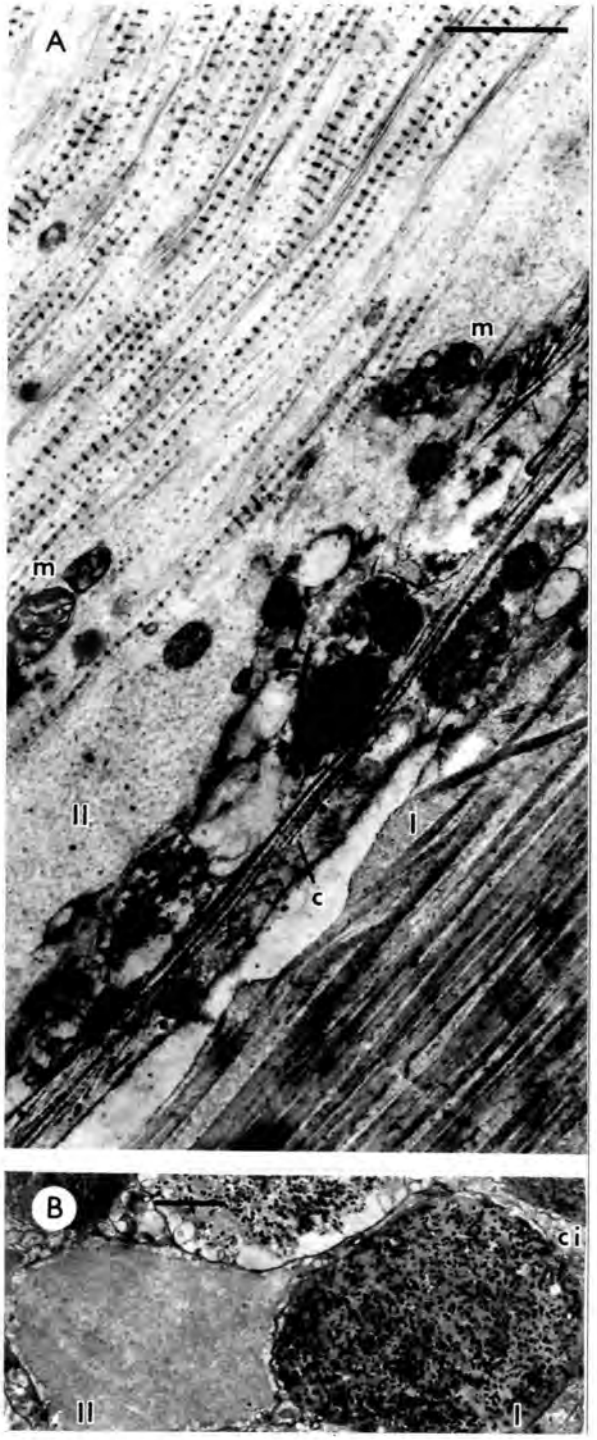

Figure 3.1. Electron micrographs of columellar muscle. A: *P. vulgata* showing longitudinal section of type I muscle cells (I) packed with thick and thin filaments and type II muscle cells (II) with novel striated bundles of thin filaments. Note the absence of thick filaments in type II cells. Scale bar= 1 μm. B: transverse section from *H. pectunculus* showing type I and type II cells. c: collagen, ci: cisternae, m: mitochondria. Scale bar= 2 μm.

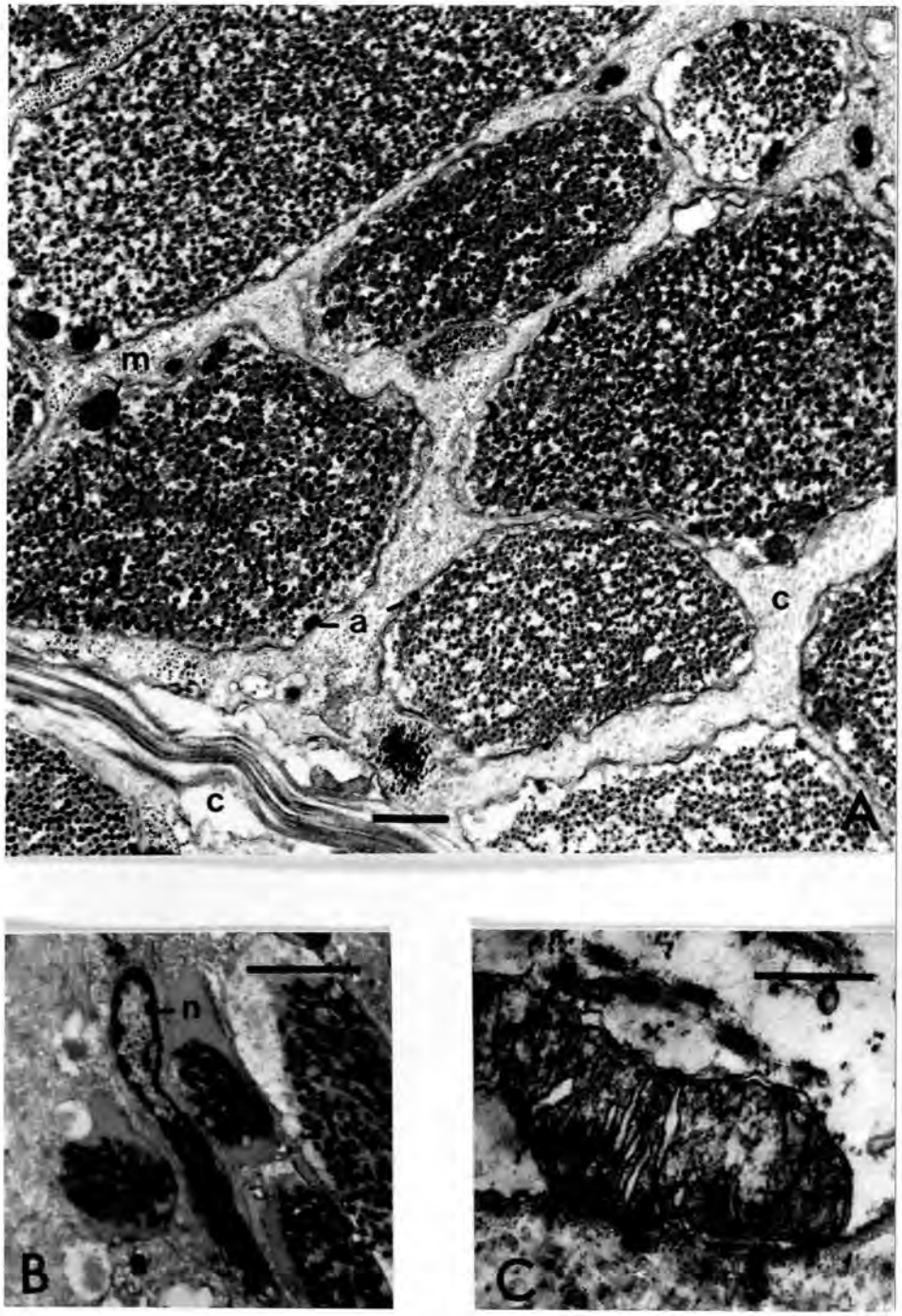

Figure 3.2. Electron micrographs of columellar muscle. A: *P.oculus* showing peripheral mitochondria (m) and tightly packed intercellular collagen fibrils (c) in transverse section lying parallel to the long axis of the muscle cells. Scale bar= 1 μm. B: *P. cochlear* showing distorted nucleus (n) from tightly packed or contracted muscle cells. Scale bar= 2 μm. C: *P oculus* showing peripheral mitochondrion. a: attachment plaque. Scale bar= 0.5 μm.

the cell membrane which may be attachment sites for thin filaments (Fig 3.2A).

Nuclei are often distorted possibly from tightly packed and contracted bundles of muscle (Fig. 3.2B). Mitochondria, which are located peripherally, are scarce, small (500-900 nm diameter) and have long, narrow cristae (Fig. 3.2A & C). A few sarcoplasmic cisternae occur at the periphery of the cells and occasional translucent vesicles are seen within the body of the cell (Fig. 3.1B). Sarcolemmal invaginations are rarely seen.

The diameter of the thin filaments is 6 $\pm$ 0.8 nm (SD, N=30). Length measurements were impracticable because the filaments meander in and out of the plane of the sections. In cross section thick filaments show a partial order in their arrangement forming rows, rings and loops (Fig. 3.3A). When seen in true transverse section thick filaments are surrounded by a complete ring of about 12 thin filaments (Fig. 3.3A). However, most sections show some obliquity so that thin filament rings are often incomplete. Thick filaments exhibit a number of features common to paramyosin containing filaments of other molluscan muscles (Bennett & Elliott, 1987). In cross section, they are diverse in shape and size (Fig. 3.3A) ranging between 20 to 180 nm with a mean diameter of 64 nm (Table 3.1). Reliable measurements of thick filament lengths are difficult to obtain from sectioned material but lengths measured from isolated filaments (Chapter 5) are 15 to 30 μm. Longitudinal sections of thick filaments show that they have a distinct axial periodicity (Fig. 3.3B) of 14 $\pm$ 0.9 nm (SD, N=18) (measured by recording the distance between the first and the last observed period and dividing by the number of periods observed; between 8 to 30 periods were measured per filament). In addition to the 14 nm axial repeat, a checkerboard pattern is seen on some thick filaments (Fig. 3.3B). Close to true transverse section, light and dark bands are seen crossing some filaments with a regular spacing of 22.1 $\pm$ 2.5 nm (SD, N=10) (Fig. 3.3A, inset).

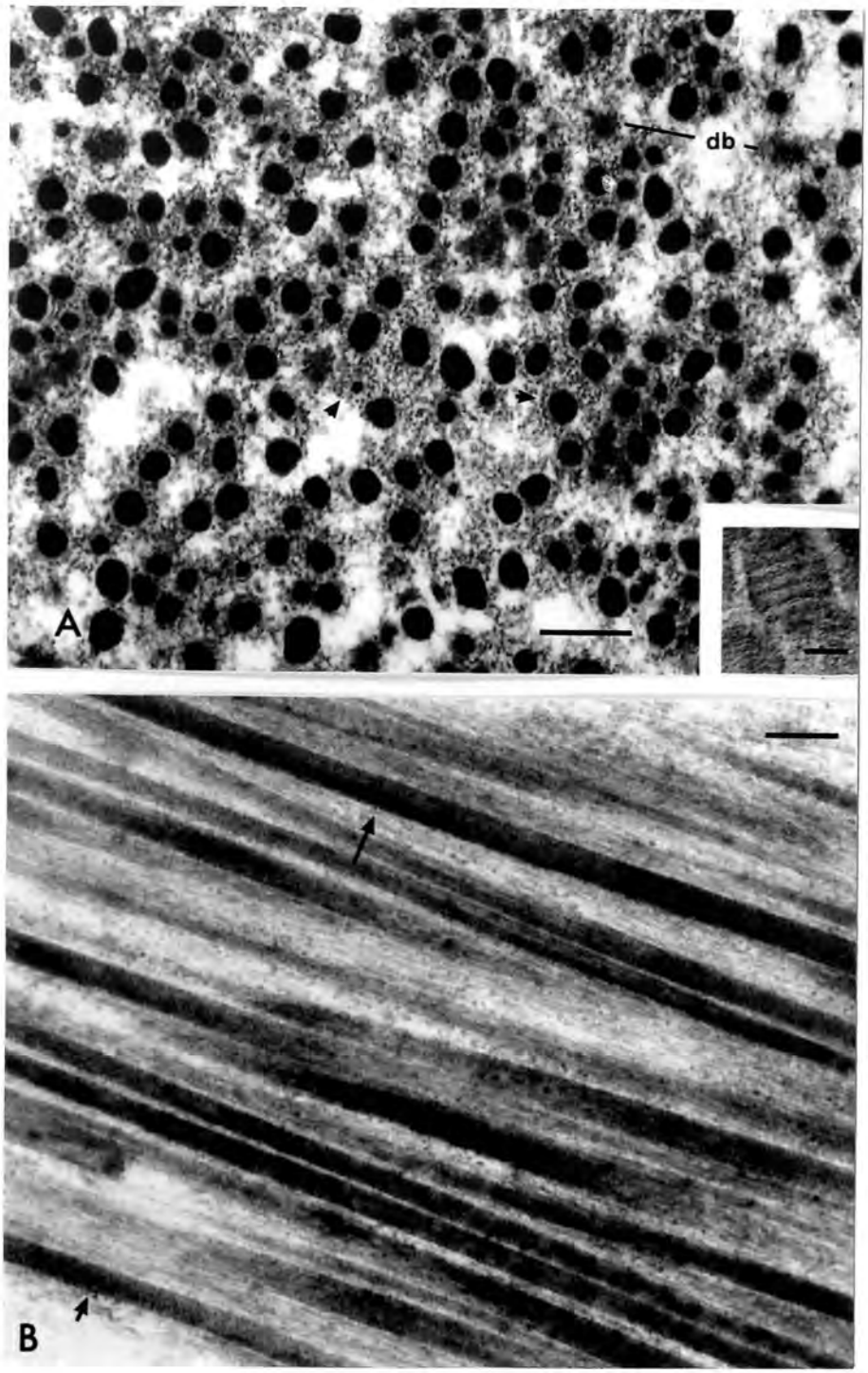

Figure 3.3. Electron micrographs of columellar muscle. A: *P. miniata* showing transverse section of type I cell. Rings of thin filaments are seen round some of the thick filaments (arrowheads). Note the variation in shape and size of the thick filaments. Dense bodies (db) are less electron-dense than thick filaments. Scale bar= 0.2 μm. The inset shows a thick filament in transverse section from *P. granularis*, the banding arises from viewing down the planes of the paracrystalline core of the filament (see Chapter 5). Scale bar= 0.05 μm. B: *P. cochlear* showing longitudinal section of type I cell, filaments show either axial striations (arrow) or the Bear-Selby pattern (arrowhead) both typical of paramyosin filaments. Scale bar= 0.2 μm.

Species	Thick filament diameters (nm) Mean ± SD, Range	N
Patella granularis	67 ± 27, 11-130	44
Patella oculus	69 ± 20, 26-94	65
Patella miniata	60 ± 20, 10-120	188
Patella longicosta *	58 ± - -	3
Patella cochlear	64 ± 20, 11-100	58
Patella tabularis	90 ± 35, 20-180	10
Helcion pectunculus	58 ± 17, 20-90	27
Helcion Pruinosus	54 ± 17, 20-80	29

* too few thick filaments in true transverse section were obtained for reliable measurements

Table 3.1. The diameters of some representative thick filaments from the columellar muscle of patellid limpets.

3.3.3 Type II Muscle Cells

Type II fibres, have been seen in *Patella longicosta*, *P. oculus*, *P. vulgata*, *P. tabularis* and *Helcion pectunculus* where they constitute about 20 to 25 % of cells seen. They contain fascicles of thin filaments of diameter 6 ± 0.8 nm (SD, N=30) many of which show a periodic superstructure of electron-dense bands (Fig. 3.4). The bands have diffuse boundaries with a spread of 30 to 50 nm occurring at a centre-to-centre spacing of between 80 to 150 nm along the length of the filaments (Table 3.2). Some cells contain only a few bundled thin filaments while others are more populated. The background material is amorphous and neither dense bodies nor thick filaments are seen in these fibres although mitochondria have been observed and glycogen granules are often prominent (Fig. 3.4C). The cells tend to be grouped i.e. are not always evenly distributed amongst the type I cells. This explains the difficulty in estimating accurately, the proportion of type I and type II cells from thin sections. It is also not clear if the distribution of the groups is random, which may explain why they have not been seen in every limpet species studied so far. Systematic preparation of sections from several different regions of the muscle did not clarify this. The type II cells seem more closely packed in contrast to type I cells though small amounts of collagen occur between the groupings of type II cells (Fig. 3.4A).

The possibility that the arrangement of bundled thin filaments could be an artifact is unlikely for several reasons. The arrangement has been seen in five species of limpet; it is seen with different embedding and staining protocols; the arrangement occurs in cells adjacent to muscle fibres containing other preserved elements of typical smooth molluscan muscle (Fig. 3.1); and the banding is strictly regular and therefore appears to emphasise some real periodic structure.

3.3.4 Connective Tissue

Intercellular regions can separate muscle cells by as much as 4 to 5 μm. They are packed with collagen fibrils (Fig. 3.2A) with characteristic axial

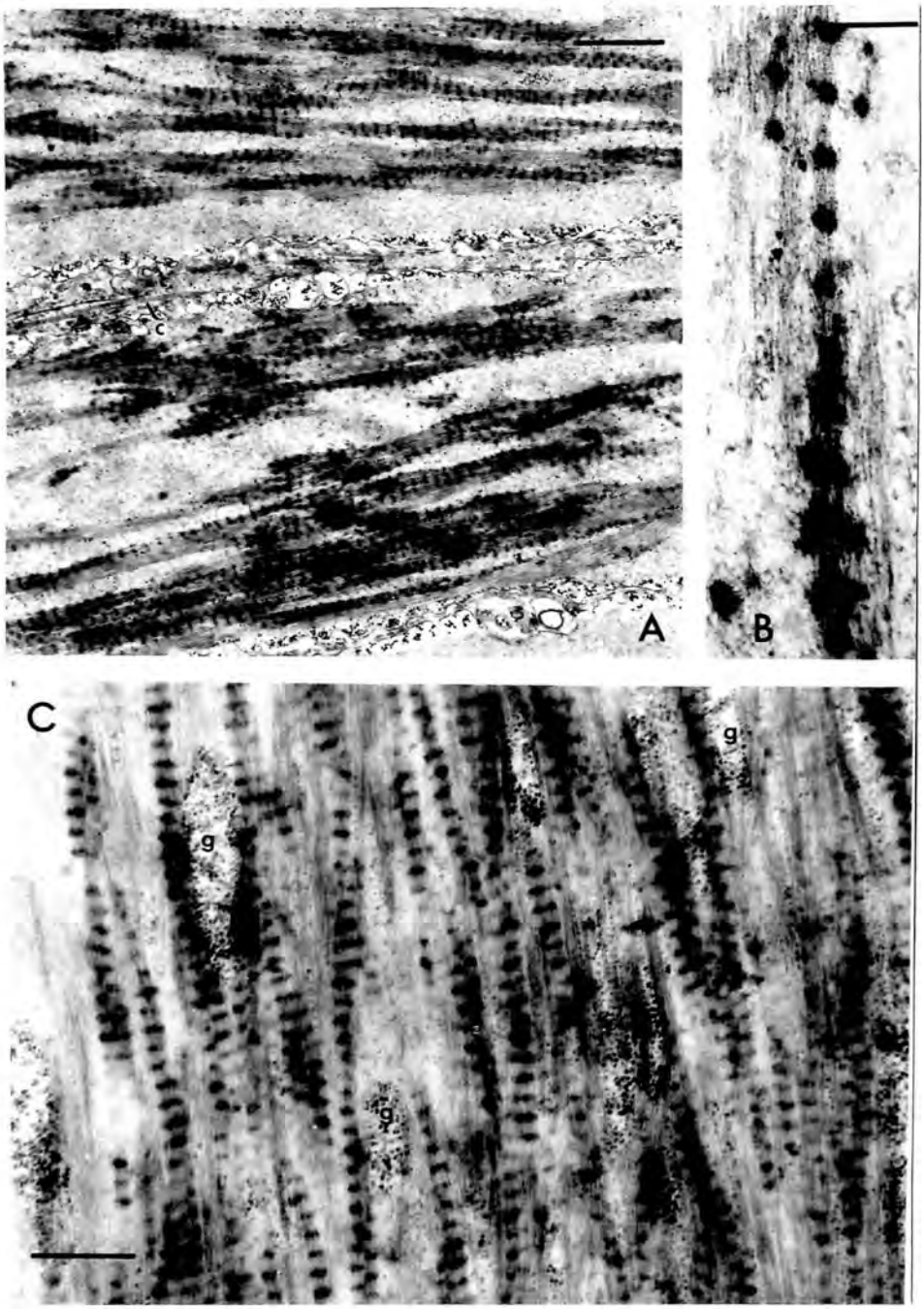

Figure 3.4. A: longitudinal section of two type II cells from *P. longicosta* showing novel striated bundles of thin filaments. Scale bar= 1 μm. B: single striated thin filament bundle from *P. oculus* at a higher magnification than A. Scale bar= 0.2 μm. C: longitudinal section of type II cell from *P. longicosta* showing glycogen granules (g). c: collagen. Scale bar= 0.5 μm.

Species	Spacing (nm)	SD	N
Patella oculus	135	±13.0	10
Patella vulgata	109	± 7.6	16
Patella longicosta	106	± 8.2	10
Patella tabularis	89	± 9.0	3

N=no. independent bundles measured with 3-8 striations per bundle

Table 3.2. Striation spacings of novel bundled thin filaments from type II muscle cells of the columellar muscle of patellid limpets.

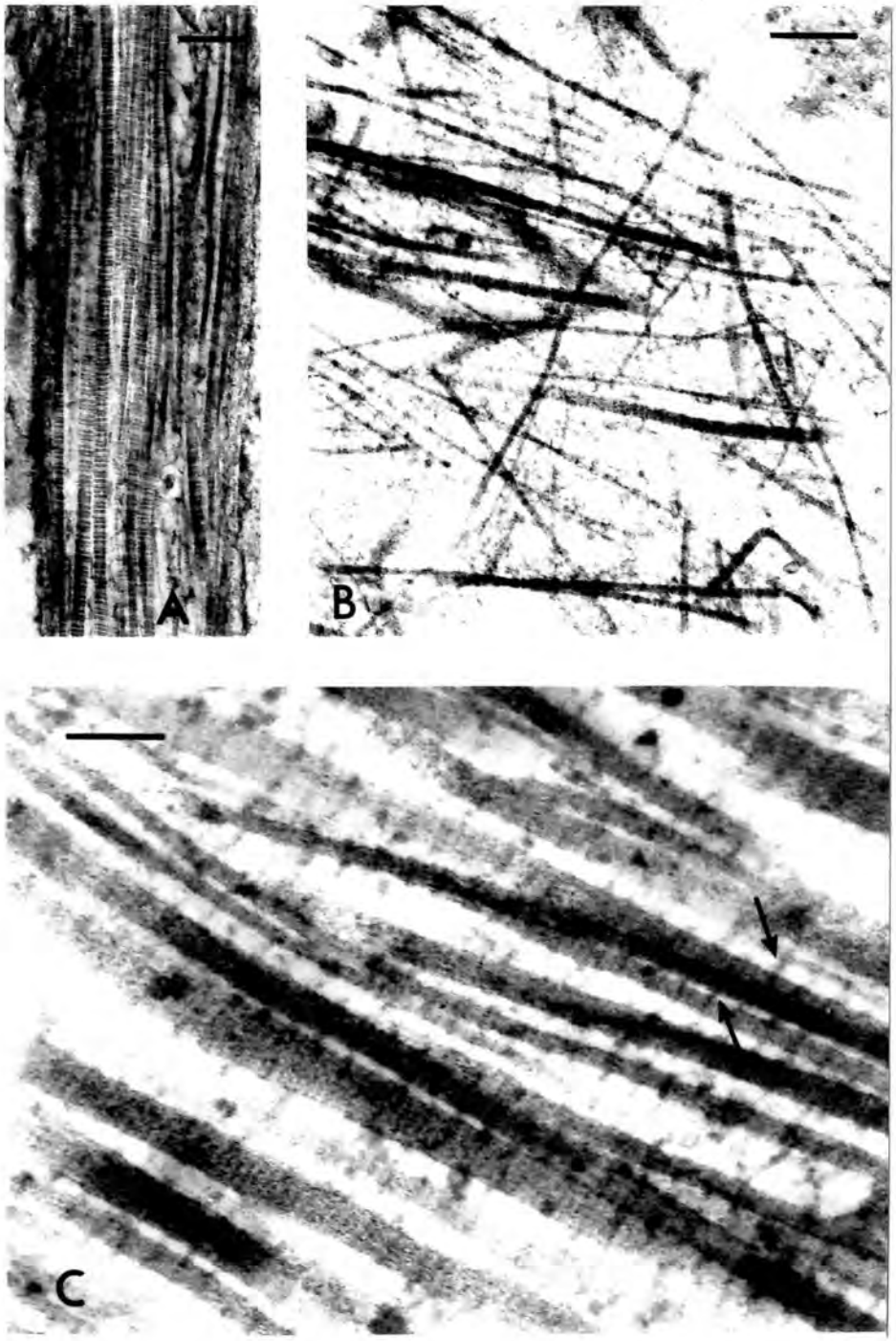

Figure 3.5. A: longitudinal section of close-packed array of collagen fibrils from the columellar muscle of *P. vulgata*. Scale bar= 0.25 μm. B: loose network of collagen fibrils from columellar muscle of *P. tabularis*. Scale bar= 0.2 μm. C: parallel array of collagen fibrils from columellar muscle of *P. tabularis* showing cross-links (arrows). Scale bar= 0.2 μm.

striations, seen most frequently in arrays parallel to the long axis of muscle fibres (Fig. 3.5A) and more rarely in loose networks (Fig. 3.5B). They often appear to cross-link the collagen at intervals (Fig. 3.5C) corresponding to the axial collagen repeat of 62 nm (determined from methods described in Chapter 5). The diameters of the collagen fibrils vary having an upper value of 116 nm (N=22).

3.3.5 Fine Structure of Tarsal Muscle

For comparison, a brief examination of the tarsal region of the pedal muscle of *P. oculus* revealed the following salient features which are summarised in Table 3.3. Muscle cells are all type I cells (Fig. 3.6) which on average, as expected from histological findings (Chapter 2), are of smaller diameter (3.5 μm, Table 3.3). Thick filament diameters are also on average smaller (39 nm, Table 3.3). Sarcolemmal invaginations are seen up to three or four times more frequently than in the columellar muscle (Fig. 3.6). Mitochondria are more prevalent than in the columellar muscle as are glycogen granules (Fig. 3.6). Intercellular distances are larger (up to 9 μm) than in the columellar muscle. Collagen fibrils are more commonly seen in loose networks.

3.4 DISCUSSION

3.4.1 Columellar Muscle

Nicaise & Amsellem (1983) have compiled a system of classification for molluscan muscle. This is a useful means of identifying muscle types by direct comparison. Six morphological parameters have been defined which differentiate between muscle types: length of thick filaments, diameter of thick filaments, presence of Z lines or dense bodies, T tubules, sarcoplasmic reticula and mitochondria. In practice, the system is unwieldy since molluscan muscles show every combination and permutation of these parameters. However, using the criteria of Nicaise & Amsellem, type I cells from limpet columellar muscle do not show the anticipated resemblance to adductor mus-

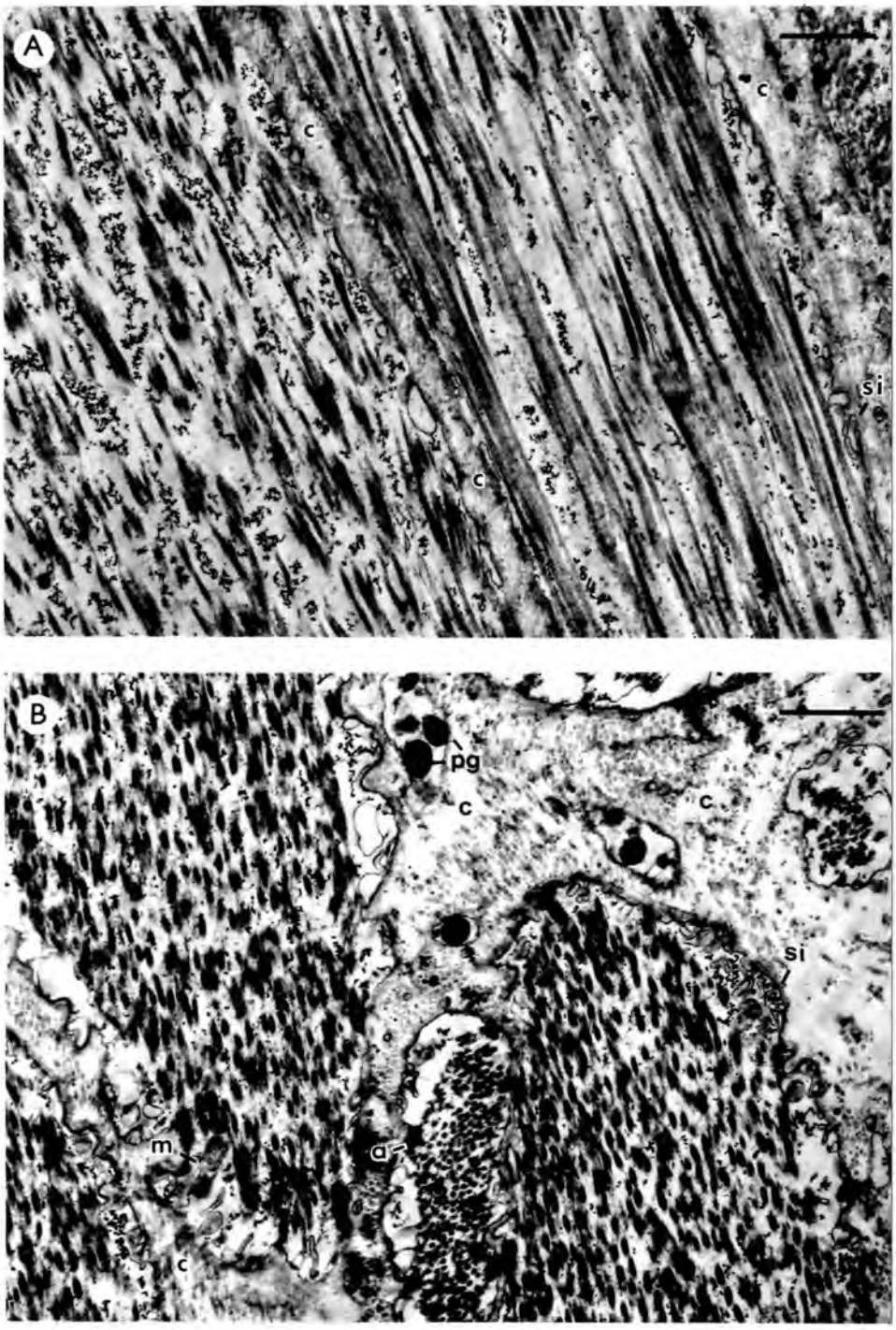

Figure 3.6. Dorso-ventral section of tarsal muscle from *P. oculus*; A: longitudinal section; B: transverse section. Note the sarcolemmal invaginations (si). a: attachment plaque, c:collagen, m: mitochondrion, pg: pigment granules. Scale bars: A = 0.1 μm, B = 0.1 μm.

	D_M (μm) Mean $\pm$ SD, N	Range (μm)	D_T (nm) Mean $\pm$ SD, N	Range (nm)	D_t (nm)	SC	SI	M	DB	II	C
Columellar	8 ± 1.9, 10	4.7-11.0	69 ± 20, 67	33-98	6	+	+	+	rare	present	abundant
Tarsus	3.5 ± 1.6, 26	1.3-6.9	39 ± 10, 40	17-62	6	++	++	++	rare	absent	abundant

D_M average diameter muscle cells
D_T average diameter thick filaments
D_t average diameter thin filaments
SC relative abundance of subsarcolemmal cisternae
SI relative abundance of sarcolemmal invaginations
M relative abundance of mitochondria
DB dense bodies
II type II cells
C collagen

Table 3.3 : Comparison of morphological features of columellar and tarsal muscle from the pedal musculature of *P. oculus*.

cles of bivalves, but are closest in structure to the ABRM of *Mytilus edulis* (Sobieszek, 1973; Nicaise & Amsellem, 1983). The thick filaments of type I cells are typical of large paramyosin containing filaments, characteristically found in muscles with catch properties (Bennett & Elliott, 1987).

While several examinations of molluscan muscles have revealed axially striated thick filaments (Nisbet & Plummer, 1968; Hunt, 1972; Sobieszek, 1973; Plesch, 1977; Frescura & Hodgson, 1990) there has been so much variation in the reported periodicity (Table 3.4), that it is not clear whether the thick filaments in these molluscan muscles are all of the paramyosin type or not (Nisbet & Plummer, 1968; Hunt, 1972). However, Plesch (1977), reported a periodicity of 14 nm for thick filaments from the columellar muscle of *Lymnaea stagnalis*. Differences in reported periodicity in other cases may be due to shrinkage and other effects from preparation for electron microscopy. Optical diffraction studies of isolated thick filaments of limpet columellar muscle have been undertaken to clarify this point (Chapter 5).

Elliott (1979), Bennett & Elliott (1981) and Elliott & Bennett (1982) have shown that the paramyosin core in thick filaments from smooth muscle in bivalves is crystalline and that two appearances seen in negatively stained and sectioned filaments, namely, a 14 nm axial repeat and the checkerboard Bear-Selby net (Bear & Selby, 1956), are two different views of the crystalline structure. This structure also gives rise to the banded appearance seen in approximately transverse views of the filaments when by chance viewed down the crystal planes. It is assumed therefore, that the bands seen in transverse section, and the axial and checkerboard patterns of limpet columellar thick filaments, also arise from a paramyosin crystalline structure like that first deduced by Bear & Selby (1956) from X-ray data of thick filaments of clam adductor muscle (*Mercenaria mercenaria*). Further consideration is given to the architecture of the thick filaments in Chapter 5.

There is still uncertainty whether or not increasing paramyosin content enables a muscle to cope with increasing tensions. The longer the filament, it has been argued (Chantler, 1983), the greater the number of cross- bridge

Genus	Muscle	P (nm)	Reference
Archachatina	collar	12-13	Nisbet & Plummer 1968
Pecten	adductor	10-15	Nisbet & Plummer 1968
Achatina	buccal mass	16	Nisbet & Plummer 1968
Buccinum	hypobranchial gland	17	Hunt 1972
Lymnaea	body wall	14	Plesch 1977
Patella	columellar	13-15	This study

Table 3.4. Comparison of axial periodicities (P) of thick filaments from molluscan muscles.

contacts per half sarcomere. The more cross-bridges made, the larger the tensions attained per unit area of muscle, although, this correlation has not been found in all cases, for example, some non-catch invertebrate muscles (Levine, Elfvin, Dewey, & Walcott, 1976). The advantage of thick filaments with a high tension capacity would be to allow limpets to withstand large forces resulting from wave activity.

The partial order seen in transverse sections of thick filaments of limpet columellar muscle is similar to that observed by Hunt (1972) in the hypobranchial gland muscle of *Buccinum undatum*. It is possibly an indication of the state of the muscle i.e. contracted, not relaxed. The partial organisation seen in transverse sections of cells could be explained by the sharing of thin filaments. This tends to draw neighbouring thick filaments into a more regular order (Bennett & Elliott, 1989). Presumably the extent of contraction or relaxation at the time of fixation affects the order of the filaments, although the high degree of order typical of striated muscle is never seen in molluscan smooth muscle.

Dense bodies in limpet columellar muscle are sparse. Nisbet & Plummer (1968), when examining the collar muscle of *Archachatina marginata*, also found a paucity of dense bodies as did Hunt (1972) in his studies of the hypobranchial gland of the gastropod *Buccinum undatum*. Such scarcity of dense bodies appears more characteristic of gastropod muscle than bivalve muscle (see Nicaise & Amsellem, 1983, for review) but could simply be a result of poor fixation of these structures.

The low number of mitochondria in limpet columellar muscle implies that the muscle is of low endurance (Plesch, 1977) which seems surprising if the muscle is to maintain high tensions for any length of time. Anaerobic respiration might be one explanation. Another is catch, a prolonged state of tension in molluscan smooth muscles which uses little energy (Hanson & Lowy 1960; Lowy & Millman, 1963). The economy of a catch mechanism seems an obvious advantage to a limpet when faced with a need to clamp to rocks for any length of time. The indications of the paramyosin nature of

limpet columellar muscle also suggest catch is employed. However, not all paramyosin muscles have catch properties (Ishii & Takahashi, 1981; Castellani, Vibert & Cohen, 1983), although all catch muscles are paramyosin muscles (Chantler, 1983; Bennett & Elliott, 1987). Only a study of the mechanics of the columellar muscle (Chapter 7) will determine whether or not catch is present.

Type II cells are here referred to as muscle cells, although it would be fair to dispute this term since it is not known if the cells are contractile. However, there are similarities to smooth muscle cells; the cells are elongate and the filaments are similar to those of type I cells both in diameter and appearance.

Rice, Moses, McManus, Brady & Blasik (1970), describe a smooth muscle cell type from vertebrates similar to type II cells having no thick filaments. The similarity with the type II cell of limpet columellar muscle however, ends there because the thin filaments of the vertebrate cells are never reported to be striated or bundled. Furthermore, the vertebrate cell thin filaments occupy the entire fibre, i.e. there are no extensive regions of amorphous material. The bundled thin filaments are similar in appearance to the striated microfilament bundles and stress fibres of non-muscle cells (Giacomelli, Wiener & Spiro, 1970; Goldman, Chojnacki & Yearna, 1979; Sanger, Sanger & Jockusch, 1983), even though their periodicities differ (Table 3.5). They are also similar to the leptomeric organelles in frog and rat intrafusal muscle fibres described by Karlsson & Andersson-Cedergren (1968) and Ovalle (1972) respectively. It is tempting to identify bundled thin filaments with stress fibres for two reasons. Not only do bundled thin filaments have ultrastructural features in common with stress fibres but they both occur in a context where tension is developed. A contractile role has been ascribed to stress fibres and leptomeric organelles. If bundled thin filaments have the same role it seems paradoxical to find them in tissue which is already equipped with a contractile apparatus in the form of type I fibres. It seems better to regard their function as complementary to conventional contrac-

Cell type	Mean periodicity (μm)	Range (μm)	Reference
Fibroblast[a]	1.3	1.00-2.40	Sanger *et al.*, 1983
Epithelial[a]	0.8	0.50-1.10	Sanger *et al.* , 1983
BHK-21[b]	0.6	-	Goldman *et al.* , 1979
Endothelial[b]	0.5	-	Giacomelli *et al.* , 1970
Intrafusal[b]	0.2	0.15-0.30	Karlsson & Andersson-Cedergren, 1968
Limpet columellar[c]	0.1	0.08-0.15	Frescura & Hodgson 1990

Table 3.5. Comparison of striation periodicities between a: stress fibres, b: microfilament bundles c: novel striated thin filaments.

tile apparatus rather than a substitute for it. If they develop in response to high tensions their occurrence should increase or decrease according to the loading imposed on the muscle. Preliminary observations had indicated that the variation in distance between electron-dense regions of the bundled thin filaments may be species-specific (Table 3.2) and related to both tenacity and zonation level of each species. However, their subsequent identification in several other gastropods from different zonal levels (Chapter 4) confers doubt on whether such observations are of any importance. The precise role of the bundled thin filaments is still to be determined.

3.4.2 Tarsal Muscle

Whereas no differences were found in the ultrastructure of the columellar muscle between species of different intertidal zones, ultrastructural differences between the columellar and tarsal muscles do exist (Table 3.3). These differences can be understood in terms of the different functions of the two muscle regions. Sarcolemmal invaginations are analogous to T tubules in vertebrate skeletal muscle which are important in excitation-contraction coupling, while subsarcolemmal cisternae are considered to be equivalent to the sarcoplasmic reticulum, in sequestering calcium (Prosser, 1973). This suggests that the increased prevalence of sarcolemmal cisternae in the tarsal muscle region may enable it to relax more quickly than columellar muscle.

The smaller diameter of muscle cells could increase the agility of the muscle while the smaller diameter of thick filaments may increase the speed of contraction of the muscle. This is because the speed of shortening of muscle is linked to the number of contractile units in series, which, in turn, is related to the length of the filaments (Chantler, 1983). Furthermore, the length of a muscle filament is considered to be a reflection of its diameter (Nicaise & Amsellem, 1983). Since filament lengths are more difficult to evaluate than diameters one may take the diameters as a rough estimate of relative filament length. From this it can be inferred that the speed of contraction of the tarsus, having filaments of smaller diameter, should be faster

than that of the columellar muscle. This ties in with the suggestion that the tarsus relaxes faster thus supporting the conclusion of Chapter 2 that the tarsus is a separate entity functionally, with commensurate structural modifications suited to more finely tuned movements. The larger numbers of mitochondria in the tarsus suggest the muscle is more active, possibly respiring aerobically. Most of the features, in fact, that distinguish tarsal muscle from columellar muscle are typical of muscle with relatively faster and finer graded movements.

The absence of type II cells suggests their function is associated somehow with the role of the columellar muscle in clamping or pedal retraction rather than any role associated with fine movements.

3.4.3 Collagen

Collagen plays an important part in many contractile systems of marine invertebrates (Gosline & Shadwick, 1983; Stott, Hepburn, Joffe & Heffron, 1974; Freinkal & Hepburn, 1975; Kier, 1988; Marshall, Hodgson & Trueman, 1989). For example, there is evidence that intermolecular cross-linking occurs between echinoderm collagen molecules (Freinkal & Hepburn, 1975; Gosline & Shadwick, 1983; Motokawa, 1988). The function of the cross-links is to strengthen mechanically the collagenous tissue by preventing slippage between the fibrils. Freinkal & Hepburn (1975) have shown how the cross-links of collagen increase the stiffness of sea cucumber dermis. In the case of limpets, such stiffening may contribute to rigidity of the foot which is essential to clamping (Fretter & Graham, 1962). Mechanical studies may help to elucidate the connection, if any, of collagen cross-links to limpet shell muscle clamping.

The increased volume of connective tissue in the tarsal region, predominantly in the form of loose collagen networks embedded in a matrix, may allow for greater flexibility and deformability because the action of muscle fibres can be amplified by the passive action of the connective tissue around it (Voltzow, 1990). The loose collagen networks are a more flexible sys-

tem capable perhaps of storing elastic energy. It is proposed here that this could be important to the limpet in allowing the sole of the foot to "spread" (Fretter & Graham, 1962) thereby improving adhesion to the substratum by increased surface area contact. In contrast, collagen in the columellar muscle, found most frequently in arrays ensheathing muscle units is primarily important in providing a tough yet elastic tendon-like anchorage for the large muscle bundles. Hence the muscle antagonism that is fundamental to a muscular hydrostat is dependent on force transmission via the collagen sheaths. Miller (1974) has shown that the limpet foot in comparison to that of most gastropods is very inflexible. Presumably the density and prevalence of collagen fibrils together with the close-packed arrangement of large dorsoventral muscle bundles ensures that the foot is tougher, less deformable but more powerful than the tarsus.

3.5 SUMMARY

- There are negligible differences in fine structure between columellar muscles of patellid limpets occupying different habitat zones.
- Limpet columellar muscle has a fine structure very similar to that of the catch ABRM of *Mytilus edulis*.
- A novel cell type is identified containing striated bundles of thin filaments but no thick filaments.
- The tarsus is distinguished from the columellar by several morphological characteristics which can be directly related to functional differences between these muscles.
- Collagen is seen in the columellar muscle predominantly in the form of arrays or bundles, often cross-linked. It is even more prominent in the tarsus but more frequently seen as loose networks.

Chapter 4

FINE STRUCTURE OF COLUMELLAR AND TARSAL MUSCLE : COILED SHELL GASTROPODS AND *SIPHONARIA*

4.1 INTRODUCTION

There have been several studies on the fine structure of molluscan muscles which are reviewed by Nicaise & Amsellem (1983). Many bivalve muscles have been studied at the ultrastructural level (reviewed in Chapter 3) but less attention has been given to the fine structure of gastropod pedal muscles apart from work on *Helix aspersa* (Rogers, 1969), *Lymnaea stagnalis* (Plesch, 1977) and *Bullia digitalis* (da Silva & Hodgson, 1987). In particular, studies of the fine structure of columellar muscles are poorly represented except for work on *Nassarius kraussianus* (Trueman & Hodgson, 1990) and patellid limpets (Frescura & Hodgson, 1990; Chapter 3).

The principal function of the columellar muscle in coiled shell gastropods differs from that of limpets. In the former, it extends and retracts the foot by several orders of magnitude (Fretter & Graham, 1962; Brown & Trueman, 1982; Hodgson & Trueman, 1985), whereas in limpets, the muscle principally clamps the shell onto the substratum with negligible change in length. It also maintains this state against opposing forces arising from wave action or predation (Fretter & Graham, 1962; Branch, 1981; Frescura & Hodgson, 1989; Chapter 1). These different functions suggest a structural difference might exist which would reflect the different roles of this muscle in coiled shell gastropods and limpets respectively. Furthermore, differences in life-style between coiled shell gastropods and the more sessile limpets are another reason for anticipating some structural modifictions. With this in mind, Chapter 4 is concerned with an examination of the fine structure of

several coiled shell gastropods and one pulmonate limpet in order to compare their columellar muscles with those of patellid limpets (Chapter 3). Gastropods for comparative study have been selected from a range of different habitats and life-styles (Table 1.1 Chapter 1), for example, *Bullia*, which inhabits high energy sandy beaches and has a foot exhibiting a wide range of different movements (Trueman & Brown, 1976); the haliotid *Haliotis spadicea*, a coiled shell gastropod, limpet-like in appearance but without the characteristic horse-shoe arrangement of columellar muscle (Fig.1.1 Chapter 1); *Siphonaria*, a pulmonate limpet which like patellid limpets has a horse-shoe muscle arrangement but unlike patellid limpets does not share the same degree of tenacity (Kilburn & Rippey, 1982) and several examples of rocky-shore coiled shell gastropods with true columellar muscles (Fig. 1.1, Chapter 1).

In addition, the fine structure of the tarsal muscle of *Oxystele sinensis* is also examined and compared with the tarsus of *P. oculus* (Chapter 3).

4.2 MATERIALS AND METHODS

Seven species of coiled shell gastropods, *Oxystele sinensis* (Gmelin, 1791), *O. tigrina* (Anton, 1839), *Turbo sarmaticus* (Linné, 1758), *Burnupena cincta* (Röding, 1798), *Bullia digitalis* (Dillwyn, 1817), *Bullia rhodostoma* (Reeve, 1847), the haliotid *Haliotis spadicea* (Donovan, 1808) and two pulmonate limpets *Siphonaria concinna* (Sowerby, 1824) and *S. aspera* (Krauss, 1848) were collected from the east coast of South Africa. Animals were retained in an aerated sea water aquarium at approximately 18 ^{0}C until dissected (usually within 24 to 48 hours). To access the columellar muscle of coiled shell gastropods, shells were crushed in a vice. Only the right columellar muscle of *Haliotis* was examined. The choice of species was representative of a range of different habits and habitats as shown in Table 1.1. Columellar tissue was prepared for transmission electron microscopy using the following protocol for maintaining appropriate osmolarity for marine tissue. Pieces of tissue about 1 mm^3 were excised from live animals from the region of

columellar muscle close to the shell. Tissue was fixed in 2.5 % glutaraldehyde containing filtered sea water and 0.1 M Na cacodylate buffer, pH 7.0 for 12 hours at 4 ^{0}C. Fixed tissue was washed in 0.1 M Na cacodylate buffer made up in sea water, post fixed with 1 % osmium tetroxide in the same buffer vehicle for 90 minutes, dehydrated and embedded via propylene oxide in an Araldite CY 212/Taab 812 resin mixture (Cross, 1989). The same protocol was used to examine the tarsal muscle from *Oxystele sinensis*. Silver/gold sections, cut using glass knives, were stained either with 5 % aqueous uranyl acetate for 30 minutes, followed by Reynold's lead citrate for 10 minutes both at room temperature, or with 2 to 10 % methanolic uranyl acetate for 30 minutes also at room temperature. Material was viewed on a Jeol JEM 100 CXII electron microscope at 80 kv.

4.3 RESULTS

No differences between species within a single genera were seen. Intergeneric differences will therefore be discussed with reference to generic names only. Most cells are typical smooth muscle cells referred to here as type I cells (Fig. 4.1A) which show little variation between genera. A second category of cells, type II cells (Fig. 4.1B & 4.2B &C), constitutes about 25 % of columellar tissue in *Burnupena* and *Bullia*.

4.3.1 Fine Structure of Columellar Muscle - Type I Cells

Diameters of type I cells range from 3 to 8 μm for *Oxystele*, *Turbo*, *Burnupena* and *Haliotis*, 4 to 10 μm for *Siphonaria* and 8 to 11 μm for *Bullia* (Table 4.1). Cell components include a random arrangement of thin and thick filaments (Fig. 4.2A) and muscle cells seem to vary in electron density (Fig. 4.1). This might be attributable to the density of cell matrix proteins. It is not clear whether this variation in electron density is due to several distinct categories of muscle cells or simply a natural variation in the density of cell components. Dense bodies, which are sites of thin filament attach-

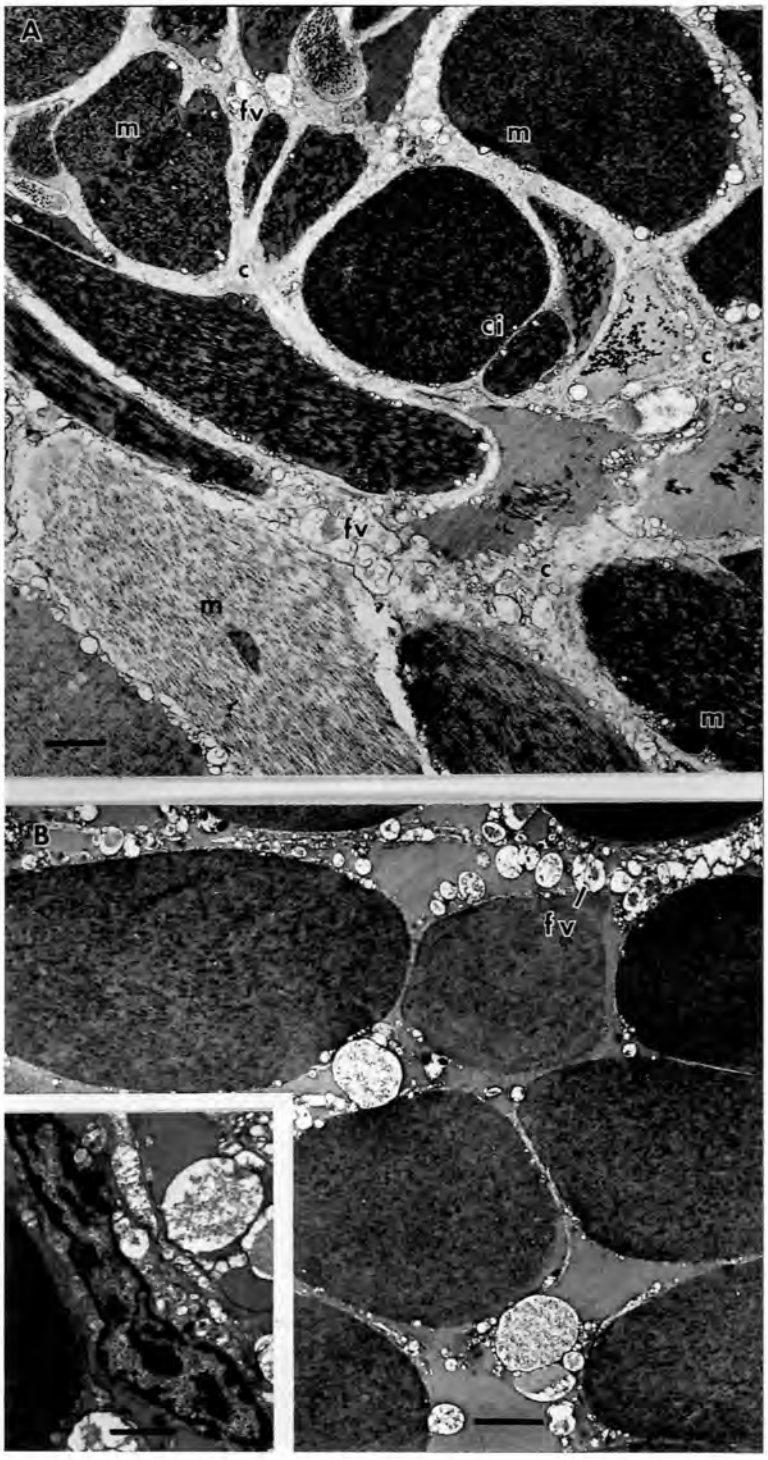

Figure 4.1. Electron micrographs of columellar muscle from *Burnupena cincta* showing A: type I cells; B: type II cells, inset: distorted nucleus. Note the differences in electron density between cells, the fluid vesicles (fv) and peripheral and central mitochondria (m), c: collagenous connective tissue, ci: subsarcolemmal cisternae. Scale bars A & B = 2 μm, inset = 0.2 μm.

Genus	$D_M(\mu m)$	Range (μm)	D_T(nm)	Range (nm)	D_t(nm)	DB	SC	SI	M	FV	II	C
Patella	9	4-13	64	22-180	6-7	scarce	scarce	not seen	peripheral 0.5-0.9 μm	none	present	abundant
Siphonaria	9	4-13	59	22-137	6-7	scarce	scarce	not seen	peripheral 0.5-0.9 μm	none	not seen	abundant
Haliotis	4	3-6	55	33-75	6-7	scarce	rarely seen	rarely seen	peripheral 0.5-0.9μm	none	not seen	abundant
Oxystele	5	3-6	56	38-80	6-7	scarce	seen more often	rarely seen	peripheral and central 1-2μm	present	not seen	abundant
Turbo	6	3-8	26 56	17-30 33-82	6-7	scarce	seen more often	rarely seen	peripheral and central 1-2μm	present	not seen	abundant
Burnupena	6	3-8	69	50-90	6-7	scarce	seen more often	rarely seen	peripheral and central 1-2μm	present	present	abundant
Bullia	9	8-11	40	30-50	6-7	scarce	seen more often	rarely seen	central 1-2μm	present but small	present	abundant

D_M mean diameter of muscle cells
D_T mean diameter of thick filaments
D_t mean diameter of thin filaments
DB dense bodies
SC subsarcolemmal cisternae
SI sarcolemmal invaginations
M mitochondria
FV fluid vesicles
II type II cells
C collagen

Table 4.1. Comparison of morphological features of columellar muscle from a range of gastropods.

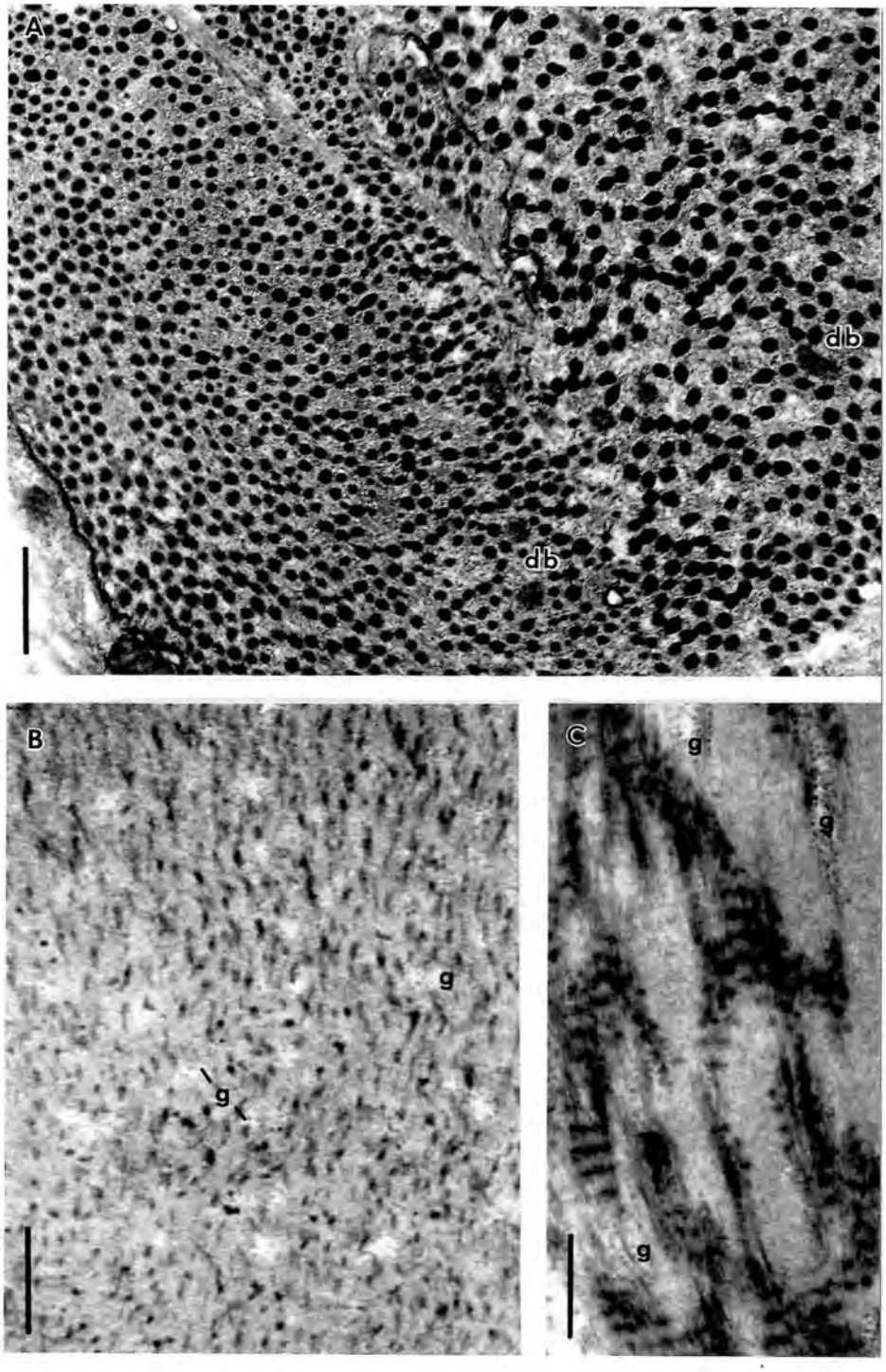

Figure 4.2. Electron micrographs of columellar muscle. A: *Turbo sarmaticus* showing type I cells in transverse section; db: dense bodies. B & C: *Burnupena cincta* showing type II cells in transverse and longitudinal section respectively; g: glycogen granules. Scale bars A, B & C = 0.2 μm.

ment, are relatively electron-lucent with a loose granular substructure and a diameter of 117 ± 24 nm (SD, N=10) (Fig. 4.2A). Electron-dense plaques are sometimes seen at the cell membrane which may be attachment sites for thin filaments. Thick filament diameters measured in transverse section, vary within and between genera from 26 ± 7 nm in *Turbo* (SD, N=24) to 69 ± 20 nm in *Burnupena* (SD, N=21) (Table 4.2). More accurate measurements are obtained from isolated filaments (Chapter 5, Table 5.1) as are thick filament lengths which vary widely but can be as long as 30 μm (Chapter 5). *Turbo* is unusual in appearing to have two classes of type I cells with filaments of different mean diameter, one 26 nm the other 56 nm (Figs. 4.3B & 4.4). Axial striations were seen in longitudinal sections of thick filaments of all genera examined (Fig. 4.3A). These are again, more accurately measured from isolated filaments (Chapter 5, Table 5.1) but were found to range from about 12 to 15 nm. The diameter of the thin filaments in all genera is 6 to 7 nm. Length measurements were impracticable because the filaments meander in and out of the plane of the sections.

Mitochondria are 1 to 2 μm long on average. In *Bullia* they occur along the central axis of the cell, whereas in the other coiled shell gastropods, they occur peripherally or centrally along the muscle fibre (Figs. 4.1A & 4.5A). They are more sparse in *Haliotis* and *Siphonaria* and seen only at the cell periphery (Fig. 4.5A). Subsarcolemmal cisternae are more common in the coiled shell gastropods than *Siphonaria* or *Haliotis* (Figs. 4.1A & 4.5A).

4.3.2 Type II cells

Type II cells, first seen by Frescura & Hodgson (1990) in the columellar muscle of several patellid limpets were seen in two of the coiled shell gastropods examined, *Burnupena* and *Bullia* (Figs. 4.1B, 4.2B & C). They contain no thick filaments or dense bodies but mitochondria, subsarcolemmal cisternae and glycogen granules are present (Fig. 4.2B & C). Thin filaments are bundled together by periodic electron-dense regions which give the structures a striated appearance (Fig. 4.2C). Centre-to-centre spacings between the

Genus	Thick Filament Diameter (nm) Mean ± SD	N
Siphonaria	59 ± 18	65
Haliotis	60 ± 18	21
Oxystele	56 ± 15	20
Turbo	26 ± 7 56 ± 15	16 24
Burnupena	69 ± 20	21
Bullia	40 ± 8	4

Table 4.2. Comparison of thick filament diameters from columellar muscle cells of gastropod species examined in this study. Note that *Turbo* has two distinct populations of thick filaments (compare Figs. 4.3B & 4.4).

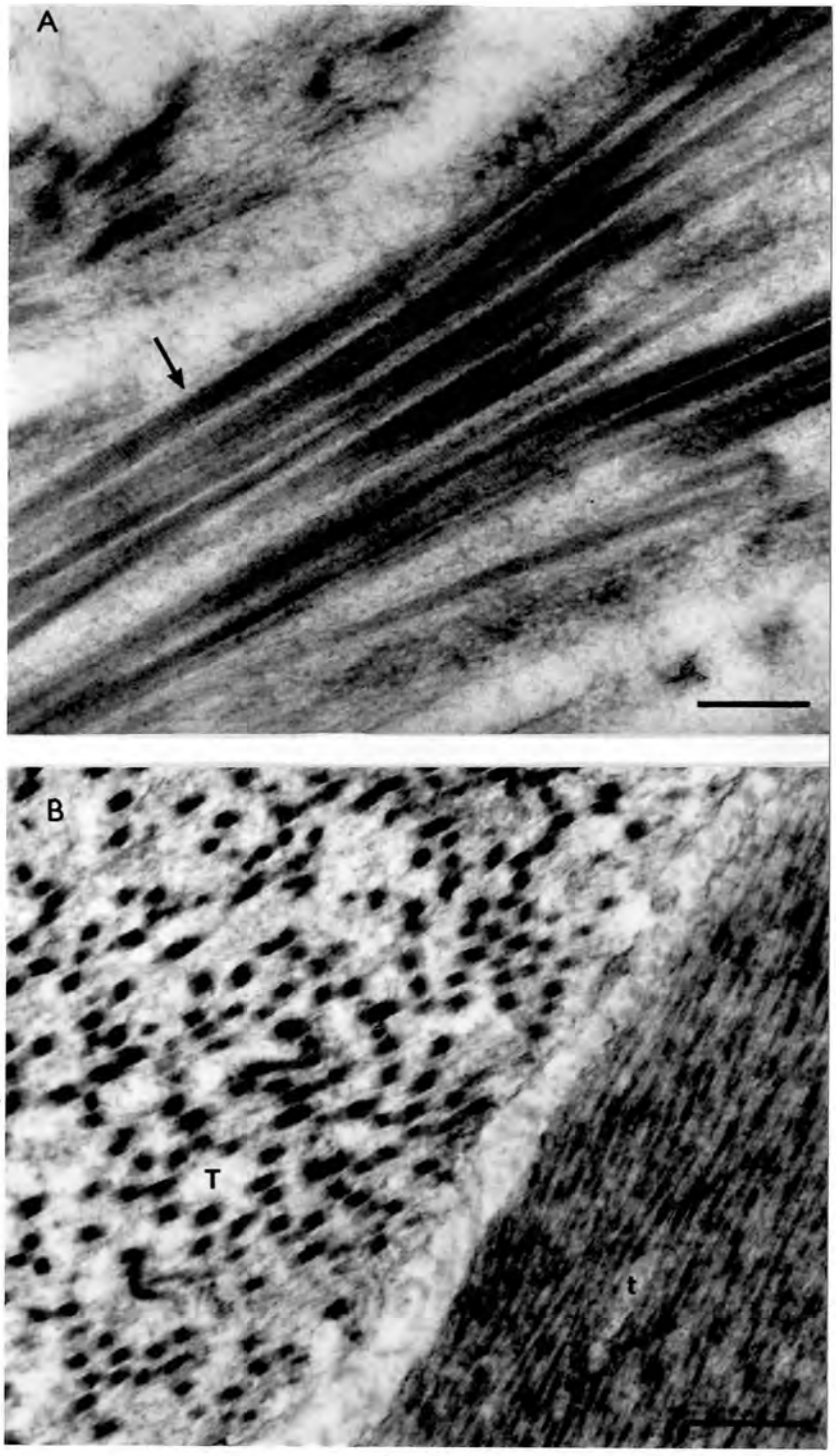

Figure 4.3. Electron micrographs of columellar muscle. A: *Haliotis spadicea* showing longitudinal section of thick filaments. Note the axial striations (arrow). Scale bar = 0.3 μm. B: *Turbo sarmaticus* showing large (T) and small (t) thick filaments in two adjacent cells. Scale bar = 0.5 μm.

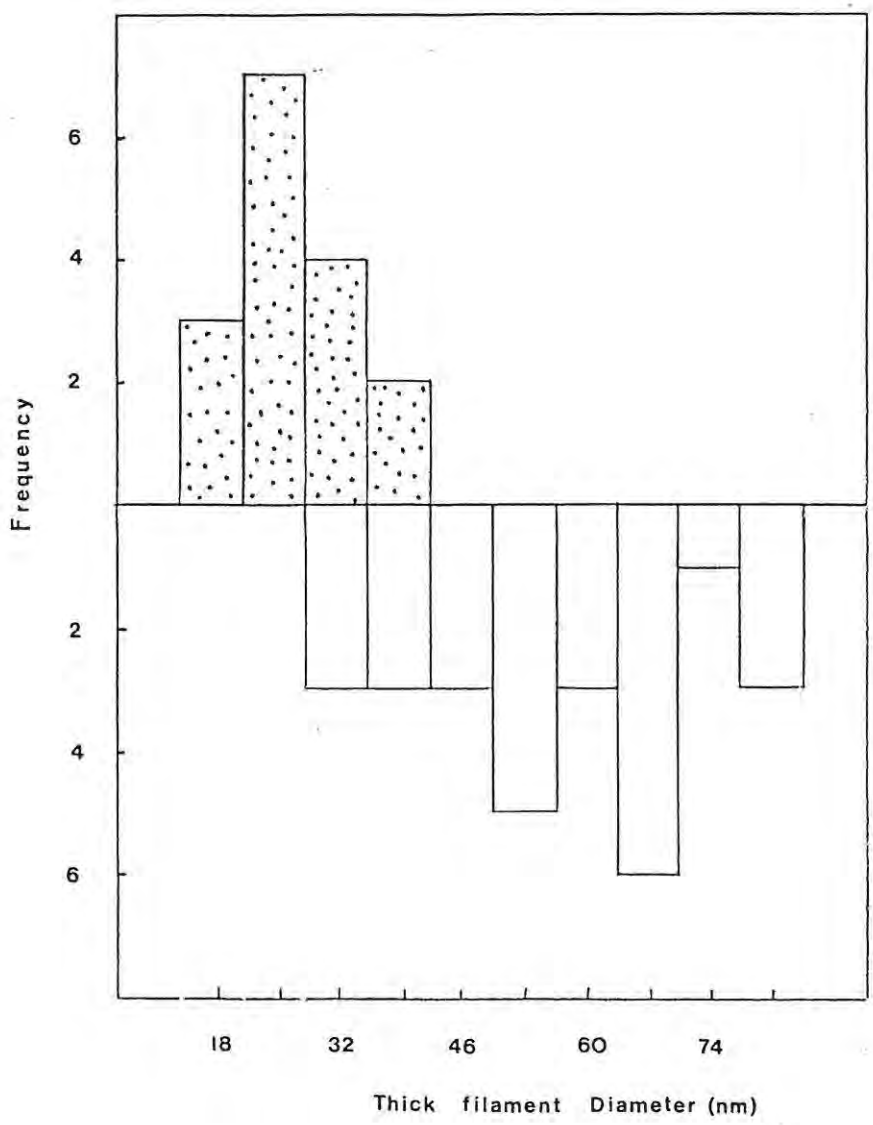

Figure 4.4 Histogram of thick filament diameters from type II cells of the columellar muscle of *Turbo sarmaticus* showing two frequency distributions. Compare stippled distribution with lower right hand cell of Fig. 4.3B and non-stippled distribution with upper left hand cell of same Figure.

electron-dense regions are 92 and 104 nm in *Burnupena* and *Bullia* respectively which are comparable to the range of values reported for those seen in patellid limpets (Frescura & Hodgson, 1990; Table 4.3). Often whole fields of cells consist of type II cells only (Fig. 4.1B). No type II cells were seen in *Turbo*, *Haliotis* or *Siphonaria*.

4.3.3 Intercellular Connective Tissue

Intercellular regions are often packed with collagen fibrils (Figs. 4.1A & 4.5). Sometimes small vesicular fluid spaces are seen amidst the collagenous connective tissue between muscle cells (Fig. 4.1A). However, fluid vesicles were never seen in *Haliotis* or *Siphonaria*. Collagen is usually seen in bundles or cross- linked arrays oriented parallel to the long axis of the muscle cells (Fig. 4.5B) or in looser networks (Fig. 4.5A).

4.3.4 Fine Structure of Tarsal Muscle

A brief examination of the tarsal muscle from *Oxystele sinensis* reveals a similar structure to that described for *P. oculus* (Chapter 3). Muscle cells are generally smaller in diameter, 3 ± 1.7 μm (SD, N=8) (Fig. 4.6A) but are otherwise similar to those described from columellar muscle. Dense bodies and attachment plaques are evident and mitochondria well represented. No type II muscle cells were seen but intercellular fluid vesicles and collagen were profuse (Fig. 4.6).

4.4 DISCUSSION

As expected, many features of the fine structure of the columellar muscles examined here are common to the patellid limpet columellar muscles already investigated (Frescura & Hodgson, 1990; Chapter 3). *Siphonaria* columellar muscle is in most respects like that of patellids except that no type II cells were seen. All other gastropods examined showed varying degrees of differences and similarities, *Haliotis*, being closer to limpets than the other coiled

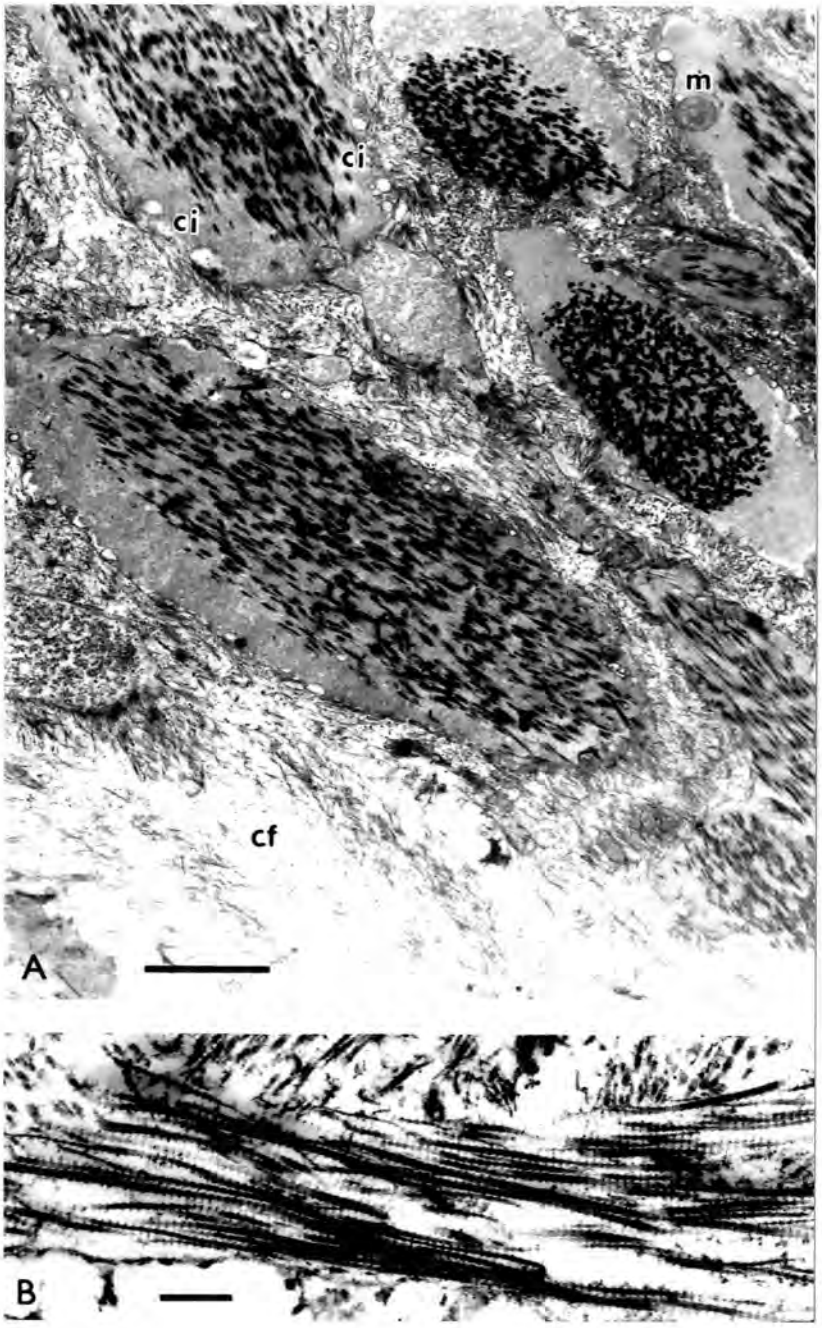

Figure 4.5. Electron micrographs of columellar muscle. A: *Siphonaria concinna* showing intercellular collagen fibrils (cf); note the subsarcolemmal cisternae (ci) and peripheral location of mitochondria (m). Scale bar = 3 μm. B: *Oxystele sinensis* showing bundled collagen fibrils. Scale bar = 0.5 μm.

Genus	Spacing (nm)	SD	N
Patella oculus	135	±13.0	10
Patella vulgata	109	± 7.6	16
Patella longicosta	106	± 8.2	10
Patella tabularis	89	± 9.0	3
Burnupena cincta	92	±15.0	10
Bullia digitalis	104	±16.0	6

N=no. independent bundles measured with 3-8 striations per bundle

Table 4.3. Striation spacings of novel bundled thin filaments from the columellar muscle of two coiled shell gastropods compared with those obtained from patellid limpets.

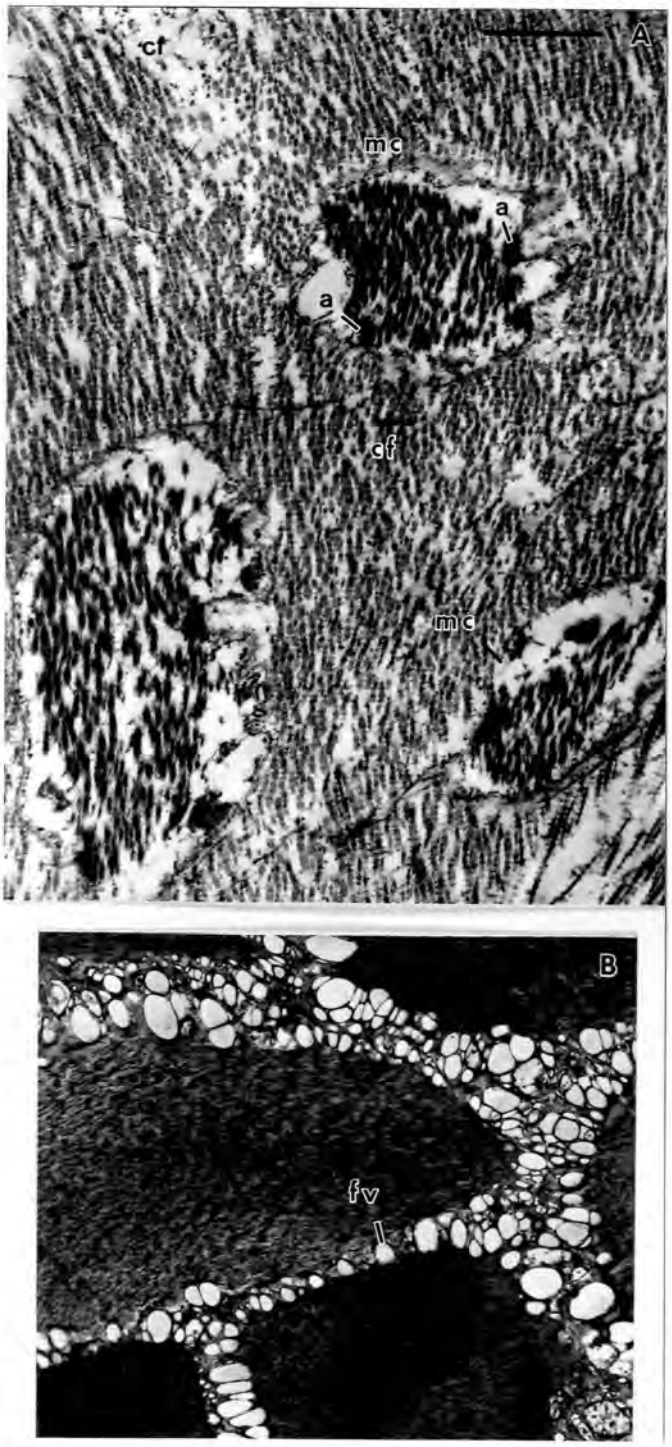

Figure 4.6. Electron micrographs of tarsal muscle. A: *Oxystele sinensis* showing abundance of collagen fibrils (cf) between small diameter muscle cells (mc). a: attachment plaques. Scale bar = 1 μm. B: transitional region between the tarsal and columellar muscles showing profusion of fluid vesicles (fv) between the cells. Scale bar = 2 μm.

shell gastropods. Table 4.1 compares the structural features of each genera examined with those of *Patella*.

Features in common with limpet columellar muscles include:

thin filament appearance and diameter

paucity of dense bodies

presence of type I and type II muscle cells

abundance of collagen.

Features differing from patellid limpet columellar muscles include:

mean muscle cell diameter

mean thick filament diameter

distribution and size of mitochondria

number of sarcolemmal invaginations and cisternae

presence of fluid vesicles.

Many of the above results confirm findings at the histological level. For example, the presence of fluid vesicles and abundance of collagen. These have been discussed at length in Chapter 2. Also, many of the above ultrastructural features and their functional implications have been discussed in Chapter 3. Therefore, only principal points of difference between patellid limpets and coiled shell gastropods will be addressed in the discussion below.

In Chapter 3, limpet columellar fine structure was compared to other molluscan smooth muscle using the morphological criteria of Nicaise & Amsellem (1983). Using the same system, the columellar muscles of coiled shell gastropods from this study, not surprisingly, are most like the shell (columellar) muscle from the coiled shell gastropod *Lymnaea* (Plesch, 1977). Unfortunately, Nicaise & Amsellem make no mention of fluid spaces, a feature which could usefully be added to their system. Trueman & Hodgson (1990) report an abundance of fluid vesicles in the pedal muscle of the coiled gastropod *Nassarius kraussianus* similar to those found in gastropods examined in this study. The vesicles were seen concentrated beneath the columellar muscle, that is, in the tarsal region. They also report a value of 3 μm

for the diameter of muscle cells at the sole of the foot of *Nassarius kraussianus*. Values quoted by these authors for other gastropod pedal muscle cell diameters (*Bullia rhodostoma*, *Helix aspersa*, *Lymnaea stagnalis*) are of a similar size. These fall within the lower range obtained for cell diameters in the columellar muscle of most coiled shell gastropods examined from this study, which are 10 to 12 % smaller than those of patellid limpets (Table 4.1). *Bullia* is an exception with cell diameters measured between 8 and 11 μm which are much larger than values quoted for this genus by Trueman & Hodgson (1990). It is suggested that the diameters reported by these authors may have been obtained from smaller cells of the tarsal region, which would account for the discrepancy. However, another factor that affects the mean cell diameter is the size of the animal. Voltzow (1990) in her studies on the pedal musculature of *Busycon contrarium* and *Haliotis kamtschatkana* found that muscle fibres in the dorso-ventral bundles were 3.1 to 4.4 μm in small specimens of 95 mm long but were 7.4 to 11.1 μm in specimens 150 mm long. Similarly, fibre diameters were 1.7 to 3.4 μm and 2.6 to 4.4 μm in the tarsus for smaller and larger specimens respectively. The relatively smaller muscle cells of coiled shell gastropods may allow more agility and deformability of the muscle.

Thick filament diameters for the species *Helix aspersa*, *Lymnaea stagnalis*, *Bullia rhodostoma* and *Nassarius kraussianus* were reported to vary from 25 to 40 nm (Trueman & Hodgson, 1990). Thick filaments of *Turbo* and *Bullia* obtained from this study are of similar diameter, while the other species have larger filament diameters, though still, on average, less than those of patellids (*Burnupena* is an exception). It is also notable that the widest variation in diameter occurs amongst the limpets, both patellids and *Siphonaria* (Table 4.1). From the arguments of Chapter 3 on the relation between speed of shortening of a muscle and thick filament length, and the relation between thick filament diameter and force developed, it is suggested that thick filaments from columellar muscles of coiled shell gastropods may provide less powerful but more flexible musculatures than those of patellid

limpets, in keeping with their extensible and retractive behaviour.

The two different average thick filament diameters found in *Turbo sarmaticus*, imply that two different populations of filaments exist. Although the functional significance is unknown the performance characteristics of cells with different mean diameters may differ. Morrison and Odense (1974) obtained a bimodal distribution for thick filament diameters from the opaque adductors of *Arctica islandica* and *Astarte undata*. Further work has been done (Chapter 5) in an attempt to isolate the two filament types.

The advantage functionally or otherwise of a central or peripheral distribution of mitochondria remains unclear, but centrally distributed mitochondria are often found in rows along the length of the muscle cell (Da Silva & Hodgson, 1987; Trueman & Hodgson, 1990) and may improve the efficiency of ATP availability to the cell. A central distribution of mitochondria has been shown to be usual in the pedal muscle cells of *Bullia rhodostoma*, *Helix aspersa* (Da Silva & Hodgson, 1987) and *Nassarius kraussianus* (Trueman & Hodgson, 1990). Adding data from this study it appears that a central distribution is common amongst coiled shell gastropod columellar muscles while a peripheral location is more typical of limpets (including *Siphonaria*) and *Haliotis*.

Type II cells are not a feature of patellid limpets only (Frescura & Hodgson, 1990) but seem to be common to a range of marine gastropods. It is surprising that they have not been identified before but this may be because so few electron microscopy studies have been done on columellar muscle *per se*. Bairati & Zuccarello (1976) describe a structure with similar periodic striations (108 nm) in the ABRM of *Mytilus galloprovincialis*, yet still, it is perplexing that they have not been seen by other authors inspite of the extensive work on the ABRM of bivalves (Philpott, Kahlbrock & Szent-Gyorgyi, 1960; Twarog, 1967; Sobieszek, 1973; Bennett & Elliott, 1981; 1989). Their function is unknown, although it has been suggested that they may be important in regulation of tension development rather than contraction (Frescura & Hodgson, 1990).

A distinguishing feature of the coiled shell gastropods is the presence of fluid vesicles. Histological studies have shown that they are present in *Thais* and *Burnupena* (Chapter 2). This study confirms their presence in *Burnupena* and shows that they are also present in *Oxystele*, *Turbo* and *Bullia*. They are not present in *Siphonaria* or *Haliotis* which makes the columellar muscle of these two genera more like the muscular hydrostat of patellid limpets (Kier, 1988; Frescura & Hodgson, 1989, 1990; Chapter 3). Voltzow (1985) has shown, by injecting dye into the bloodstream, that fluid spaces in *Busycon contrarium* were blood spaces. Fluid spaces seen in the gastropods examined in this study are also likely to be blood spaces. In several respects *Haliotis* is like *Patella* in that no vesicles were seen and mitochondria were always seen at the cell periphery. However, the mean muscle cell and thick filament diameters are closer to those of most coiled shell gastropods (Table 4.2). *Haliotis* then has structural features intermediate between the patellids and the coiled shell gastropods.

Vesicular fluid spaces are particularly abundant in the tarsal region. This is the principal difference between the tarsus of *P. oculus* (Chapter 3) and *Oxystele sinensis*. In other respects the structure is very similar having large volumes of collagen fibrils and small diameter muscle cells, indicating that the distinction between columellar and tarsus is general in gastropods. The larger numbers and size of mitochondria together with more developed subsarcolemmal cisternae (Table 3.3, Chapter 3) indicate that tarsal muscle is more active than columellar muscle.

In conclusion, the ultrastructure of coiled shell gastropod columellar muscle indicates that it may use fluid as an antagonist for muscle contraction whereas the patellids, haliotids and *Siphonaria* do not. A scheme, summarising the important differences seen at the ultrastructural level between the columellar and tarsal muscle of a general gastropod is shown in Figure 4.7.

4.5 SUMMARY

- The ultrastructure of *Siphonaria* columellar muscle is very similar to

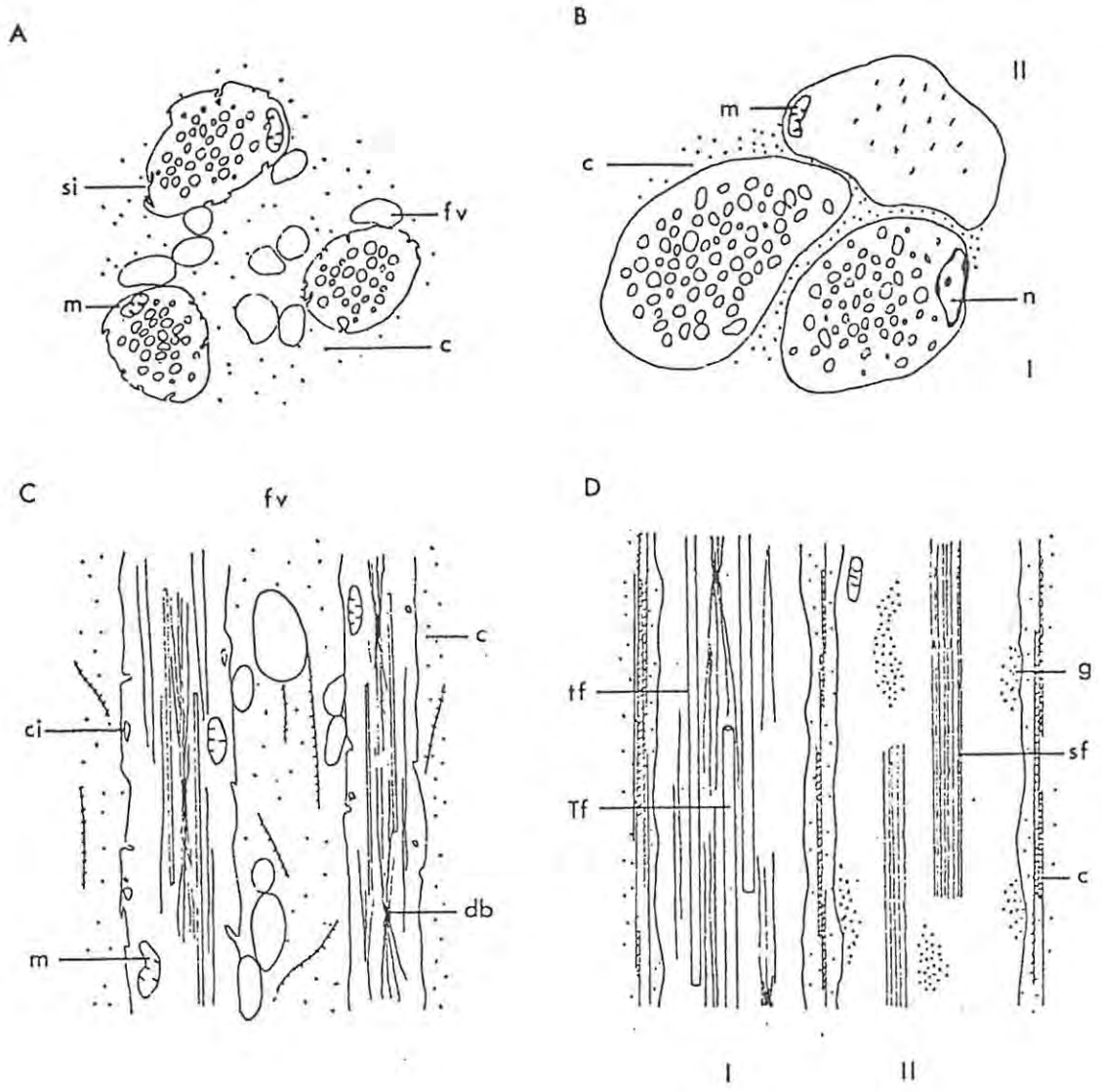

Figure 4.7. A model of a general gastropod foot showing principal ultrastructural differences between the tarsal and columellar muscles. A: transverse section of tarsus; B: transverse section of columellar muscle; C: longitudinal section of tarsus; D: longitudinal section of columellar muscle. c: collagen, ci: subsarcolemmal cisternae, db: dense bodies, fv: fluid vesicles, g: glycogen granules, m: mitochondria, n: nuclei, si: sarcolemmal invaginations, st: striated filament bundles, tf: thin filaments, Tf: thick filaments, I: type I cell, II: type II cell. Drawings not to scale.

that of patellid limpets.

- The ultrastructure of the columellar muscle of *Haliotis* is intermediate between that of patellids and coiled shell gastropods showing a combination of features typical of both.

- The columellar muscles of the coiled shell gastropods have structural characteristics more typical of active and pliable muscle, than those of limpets.

- The fine structure of the tarsus of *Oxystele sinensis* is similar to that of *P. oculus* indicating that the distinction between tarsal and columellar muscle is general in gastropods.

- Type II muscle cells are present not only in patellid limpets (Frescura & Hodgson, 1990) but have now been identified in at least two coiled shell gastropod species as well.

- Fluid spaces are present in varying degrees in the columellar muscles of several coiled shell gastropods, but are usually small vesicles and more abundant towards the tarsus.

Chapter 5

STRUCTURAL ANALYSIS OF FILAMENTS FROM GASTROPOD COLUMELLAR MUSCLES

5.1 INTRODUCTION

Much of the more detailed information in the literature on the fine structure of molluscan muscle filaments has come from studies on the anterior byssus retractor muscle (ABRM) of *Mytilus* (Sobieszek, 1973; Elliott, 1979; Castellani, Vibert & Cohen, 1983; see Chantler, 1983 for review). Bear & Selby (1956), Bennett & Elliott (1981) and Cohen, Szent-Gyorgyi & Kendrick-Jones (1971) have investigated the molecular architecture of the paramyosin core of thick filaments from several bivalve muscles (white adductor, *Ostrea edulis*; white adductor, *Crassostrea gigas*; white and red adductors, *Mercenaria Mercenaria*; ABRM, *Mytilus edulis*; white adductor, *Pecten maximus*). Less work on the more detailed aspects of filament structure have been done for molluscs from other classes. In order to gain information that was unobtainable or less reliable from transmission electron microscopy studies (Chapters 3 & 4), the filaments of several gastropod columellar muscles have been isolated from their surrounding tissue thus allowing their substructure to be determined. Several subsequent methods of analysis (e.g. optical diffraction, gel electrophoresis) confirm the paramyosin nature of the thick filaments.

Bennett & Elliott (1981) have demonstrated that transverse sections of embedded paramyosin thick filaments tilted at an appropriate angle, reveal light and dark bands which arise from viewing down the planes of the crystalline paramyosin core of these filaments. Measurement of these spacings allows the transverse repeat of the paramyosin crystal to be calculated (Ben-

nett & Elliott, 1981) while optical diffraction allows precise calculation of the axial repeat (Sobieszek, 1973; Bennett & Elliott, 1987). Calculations in this study reveal that thick filaments from limpets have a similar paramyosin molecular packing to those of many bivalves.

Structural studies on columellar muscle have shown that a surprisingly large amount of collagen is present in this tissue (Frescura & Hodgson, 1989, 1990, Chapters 2, 3 & 4). Knowledge of the structure and mechanical properties of molluscan collagen is, however, almost entirely based on studies of squid collagen (Ward, 1972; Ward & Wainwright, 1972; Gosline & Shadwick, 1983; Kier, 1988). In order to obtain more quantitative data on the collagen component of columellar tissue, an estimate of the collagen to muscle ratio in a range of gastropods is made by measuring the area of muscle cells and collagenous connective tissue from electron micrographs, and by a second method using microdensitometry to record the relative densities of muscle and collagen proteins from electrophoresis gels.

A novel arrangement of thin filaments has been identified in some of the columellar muscle cells of patellid limpets (Frescura & Hodgson, 1990). Although their ultrastructure has been described, the function of these filaments remains obscure and the protein constituents from which they are made is unknown. A further objective in this Chapter's work therefore, is to isolate these filament bundles from type II cells and identify their protein constituents using methods of gel electrophoresis and immunocytochemistry.

5.2 MATERIALS AND METHODS

5.2.1 Filament Isolation

Filaments were isolated from the columellar muscle of three North Atlantic patellid limpets: *Patella vulgata* (L.), *P. intermedia* (Jeffreys), *P. aspera* (Lamarck, 1819) obtained from the marine laboratories at Plymouth, U. K., and the following South African species of gastropod: the limpets *P.*

granularis (Linné, 1758), *P. oculus* (Born 1778), *P. cochlear* (Born, 1778), *P. tabularis* (Krauss, 1848), *Siphonaria concinna* (Sowerby, 1824), the haliotid, *Haliotis spadicea* (Donovan) and the coiled shell gastropods, *Oxystele sinensis* (Gmelin, 1791), *Turbo sarmaticus* (Linne, 1758), *Burnupena cincta* (Röding, 1798) and *Bullia digitalis* (Dillwyn, 1817) obtained from the south east coast of South Africa. From the North Atlantic species, three separate regions of the columellar muscle: outer circumferential, dorso-ventral and tissue close to the shell, were homogenised using a Virtis blender. From the South African species the dorso-ventral muscle bundles or their equivalent, the central longitudinal columellar muscle bundles, were homogenised using a Sorvall blender. All mechanical parts and tissue were kept at 0 ^{0}C. To remove soluble proteins, tissue was transferred to a buffer solution composed of 60 mM KCl, 5mM $MgCl_2$, NaN_3, 0.5 mM EGTA, 10 mM Imidazole, 5mM DTT, 0.5 % Triton X-100, 2 μg ml^{-1} leupeptin, pH 7.0 at 4 ^{0}C. Tissue was then spun at 2,500 rpm for 5 minutes in the same buffer. The pellet was washed and spun again before being incubated in a medium containing 80 mM KCl, 5 mM $MgCl_2$, 2mM NaN_3, 5mM EGTA, 10 mM ATP, 20 mM MES-KOH, 5mM DTT, 2 μm ml^{-1} leupeptin, pH 6.0 at 4 ^{0}C. for 1/2 hour on ice to dissociate the thick and thin filaments. Isolated filaments were negatively stained with 10 % methanolic uranyl acetate on carbon or formvar coated copper grids and viewed in the electron microscope at 80 or 100 kv. All chemicals were from Sigma Chemical Company, U.S.A.

5.2.2 Optical Diffraction of Isolated Filaments

Optical diffraction patterns of photographic negatives of thick filaments isolated and negatively stained from *P. vulgata* were obtained using a modified Rank-Pullen instrument with 2 mwatt HeNe gas laser red light at a wavelength of 632.8 nm and lens of focal length 150 cm (O'Brien & Bennett, 1972). Catalase crystals were used to calibrate the micrographs.

5.2.3 Calculation of Transverse Repeat of Paramyosin Crystal Core of Muscle Thick Filaments

Specimens of *Patella granularis*, *P. miniata*, *P. cochlear* and *P. tabularis* were collected from the east coast of South Africa. Columellar tissue was prepared for transmission electron microscopy using the following protocol that maintains an appropriate osmolarity for marine tissue. Pieces of tissue about 1 mm^3 were excised from fresh pedal muscle under sea water and fixed in 2.5 % glutaraldehyde containing filtered sea water and 0.1 M sodium cacodylate buffer, pH 7.0 for 12 hours at 4 ^{0}C. Fixed tissue was washed in 0.1 M cacodylate buffer made up in sea water, post fixed with 1 % osmium tetroxide in the same buffer vehicle for 90 minutes, dehydrated and embedded via propylene oxide in an Araldite CY 212/Taab 812 resin mixture (Cross, 1989). Gold/silver sections were cut using glass knives, collected on copper grids and stained with 10 % methanolic uranyl acetate for 1/2 an hour.

5.2.4 SDS Gel Electrophoresis of Filament Isolates

Electrophoresis to identify the proteins present in the isolated filament preparations of *P. vulgata*, *P. oculus*, *P. tabularis*, *Haliotis spadicea*, *Oxystele sinensis*, and *Burnupena cincta* was done on 8 % sodium dodecyl sulphate (SDS) polyacrylamide slab gels stained with Coomassie blue. Protein concentrations were about 1 mg ml^{-1} and loadings 2 μl for the *P. vulgata* preparations except for the circumferential homogenate, which was about 1 μl. Protein concentrations were 0.5 mg ml^{-1} for *Haliotis spadicea* and *P. oculus* and 0.25 mg ml^{-1} for the species *P. tabularis*, *Oxystele sinensis* and *Burnupena cincta* as estimated from spectrophotometric analysis. Protein loadings were approximately 20 μl. Additional 10 μl loadings were made for *Haliotis spadicea* and *P. oculus* because of the higher concentrations of these two preparations.

5.2.5 Estimation of Relative Proportion of Collagen and Muscle in Columellar Muscle

Area Measurement

Electron micrographs encompassing a total area of about 2.5 mm^2 were used in conjunction with a Summagraphics digitising tablet and software (Sigma Scan, Jandel Scientific, U.S.A.) to measure the relative areas of muscle cell and connective tissue present in the columellar muscle of *P. oculus*. Measurement of the muscle cell area was subtracted from the total area to give the proportion of collagenous connective tissue.

Microdensitometry

SDS gels were photographed and contact negatives made (8 x 10 cm). Profiles of band intensities from the gel columns were recorded from the negatives with an Enraf Nonius Microdensitometer (Delft, Holland), fitted with a custom made motor to give a continuous output to a chart recorder. The motor was tested for uniformity of speed to ensure the area under the peaks was proportional to the band intensity of the gel columns. Over a grid of 10 lines, variation was less than 3 %. The ratio of collagen to muscle protein present in the homogenates was then estimated from both relative heights and areas of the peaks. Peak areas were determined using the digitising system and software described previously. Peaks not fully resolved were extrapolated by hand. Where resolution was too poor for extrapolation and area computation, only relative heights of peaks were recorded.

5.2.6 Determination of Protein Constituents of Sriated Thin Filaments from Type II Cells using Immunocytochemistry

Immunocytochemistry was done on the columellar muscle of *P. oculus* and *P. vulgata* using anti-actin rabbit (Bio-Yeda, Israel) and anti-panmyosin mouse (Amersham International, U.K.) anti-bodies with 5 and 10 nm gold conjugates (goat anti-rabbit and goat anti-mouse immunoglobulin, BioClin,

U. K.) prepared in goat serum (courtesy of Prof. B. Fivaz of Tick Research Unit, Rhodes University). *P. oculus* specimens were obtained from the east coast of South Africa and *P. vlugata* specimens from the laboratories of the marine biological association at Plymouth, U.K. Columellar tissue was prepared for transmission electron microscopy as described under section 5.2.3. Silver/gold sections were cut using glass knives and collected onto nickel or gold grids. To stain the tissue, grids were inverted and placed onto a 20 μl drop of each of the following solutions: 1/10 dilution of normal goat serum for 10 minutes, primary antibody made up in 0.1 % PBS plus 0.1 % BSA plus 0.01 % NaN_3 for 120 minutes, 50 mM TRIS pH 7.2 for 1 minute x 4, 50 mM TRIS plus 1 % BSA plus 1 % Triton X, pH 8.2 for 1 minute x 4, 1/10 normal goat serum for 10 minutes, gold made up in TRIS plus BSA, pH 8.2 for 120 minutes, TRIS plus BSA pH 7.2 for 1 minute x 4, TRIS pH 7.2 for 1 minute x 4, distilled H_2O for 1 minute x 4, 2 % methanolic uranyl acetate for 5 minutes, distilled H_2O. The anti-body solution was serially diluted in order to find an acceptable staining concentration. Smooth muscle from the bladder of the bat *Miniopterus schreibersii* was also stained as a control.

5.3 RESULTS

5.3.1 Filament Isolation and Optical Diffraction

Extraction and isolation procedures reveal the presence of collagen fibrils and muscle-derived thick and thin filaments in all homogenates examined (Fig. 5.1 & 5.2). It was found that more collagen filaments were predominant in columellar tissue close to the shell, whereas thick filaments were most prevalent in the dorso-ventral region of the species *P. vulgata*, *P. aspera* and *P. intermedia* and in all preparations from South African species.

Thick filament diameters varied more widely in *Patella*, *Siphonaria*, *Burnupena* and *Bullia*, but less so in *Oxystele* and *Turbo*. Mean diameters of isolated thick filaments varied from 34 to 84 nm (Table 5.1) these values being at least a few % higher than those obtained from transmission elec-

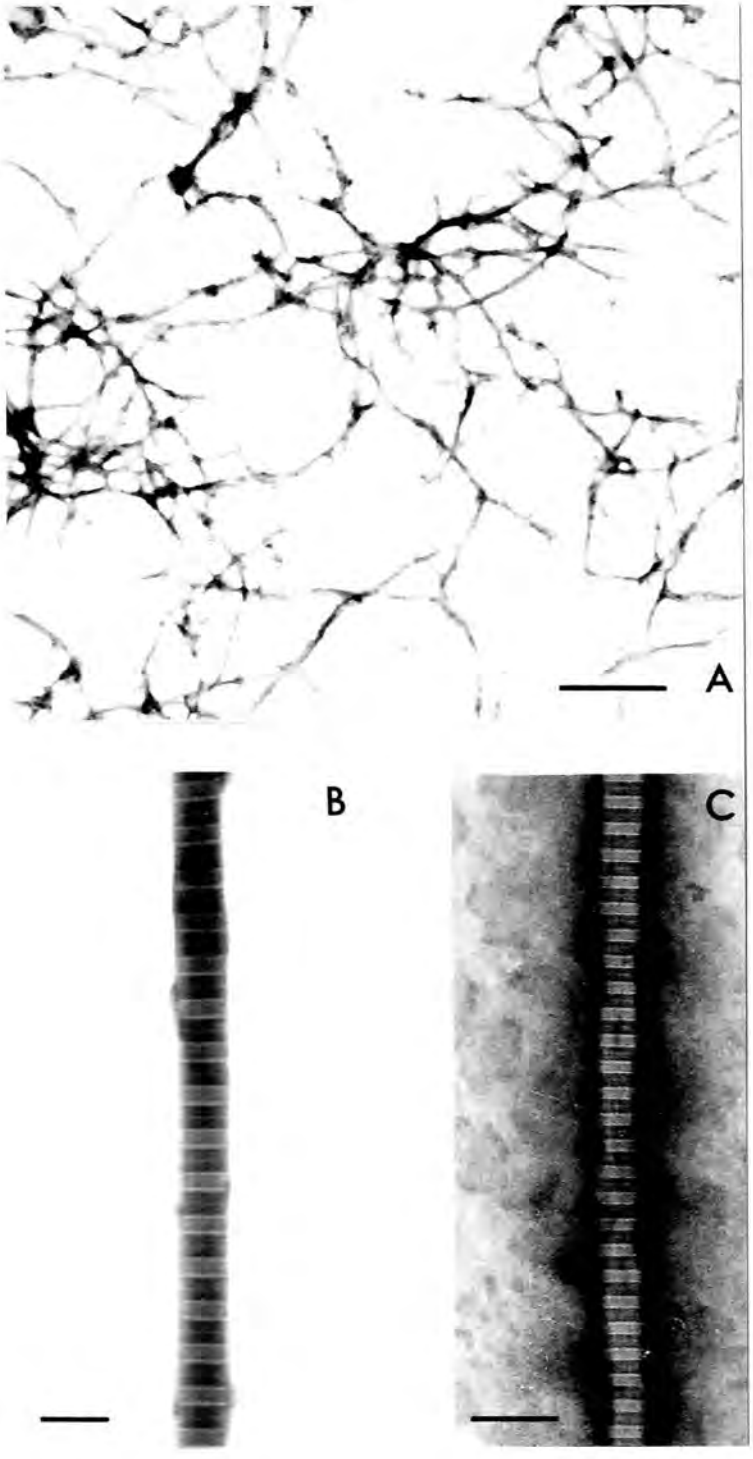

Figure 5.1. A: isolated thick filaments from the columellar muscle of *Bullia digitalis*. Scale bar = 0.5 μm. B: collagen fibril isolated from columellar muscle of *P. cochlear* showing periodic striations of 62 nm. Scale bar = 0.1 μm. C: as for B but from *P. vulgata*. Scale bar = 0.2 μm.

Genus	D (nm)	SD	N	P (nm)	SD	N*
Patella	84	±29	16	14.2_{OD}		
Siphonaria	83	±15	5	14.3	±0.8	4
Haliotis	63	± 8	5	15.2	±0.6	5
Oxystele	61	± 9	8	14.7	±0.3	3
Turbo	34 79	± 5 ± 6	7 4	14.6	±0.3	4
Burnupena	69	±27	12	14.9	±0.5	6
Bullia	78	±15	8	14.5	±0.3	3

N=no. filaments measured
N *=no. filaments with 10-20 periodicities measured per filament
$_{OD}$=optical diffraction data from *P. oculus*

Table 5.1. Diameters (D) and axial periodicities (P) of isolated thick filaments from the columellar muscle of a range of gastropods.

tron microscopy preparations and sometimes as much as 50 % higher (e.g. *Bullia*, c.f. Table 4.2, Chapter 4). The mean diameter of thick filaments isolated from *Bullia* is almost double that obtained from sectioned filaments. Results indicate two classes of thick filament in *Turbo*, one of mean diameter 34 nm the other 79 nm (Table 5.1). The thick filament length for all species ranged between 15 and 30 μm, but some of this variability is probably due to broken filaments. Although typical muscle thin filaments were seen, no striated bundled thin filaments were isolated. The supernatant contained only amorphous material.

Thick filaments showed either the Bear-Selby net (Fig. 5.2D) (Bear & Selby, 1956) or axial striations (Fig. 5.2A,B,C) with periodicity ranging from 14.2 nm to 15.2 nm (Table 5.1). Both patterns are typical of paramyosin filaments (Bennett & Elliott, 1987). Optical diffraction of the thick filaments from *P. vulgata* gave an axial periodicity of 14.2 nm (Fig. 5.3A) which is consistent with a paramyosin crystalline core structure. Five orders of the 1/14 nm^{-1} reflections arising from the Bear-Selby net occur along the meridion (Fig. 5.3B). Collagen fibrils with periodicity 62 nm are prominent in preparations of the shell region (Fig. 5.3C). Optical diffraction of collagen fibrils gave 3rd, 5th and 7th orders of this repeat (Fig. 5.3D).

5.3.2 Calculation of the Transverse Repeat of the Paramyosin Crystal Core of Thick Filaments

The transverse repeat, a, of the crystal lattice (Bear-Selby net) of paramyosin, is calculated using the relation

$$a = h\ s\ (1 - s^2/c^2)^{-1/2}$$

(Bennett & Elliott, 1981; Frescura, 1987; Frescura & Hodgson, 1990) where h is the first digit of the index of the plane giving rise to the spacings seen in transverse section, c is the axial repeat of the Bear-Selby net (Fig. 5.4), and s is the width of the bands which are seen in transverse sections of thick filaments (Fig. 5.2E & F) viewed down one of the crystal planes (Fig.

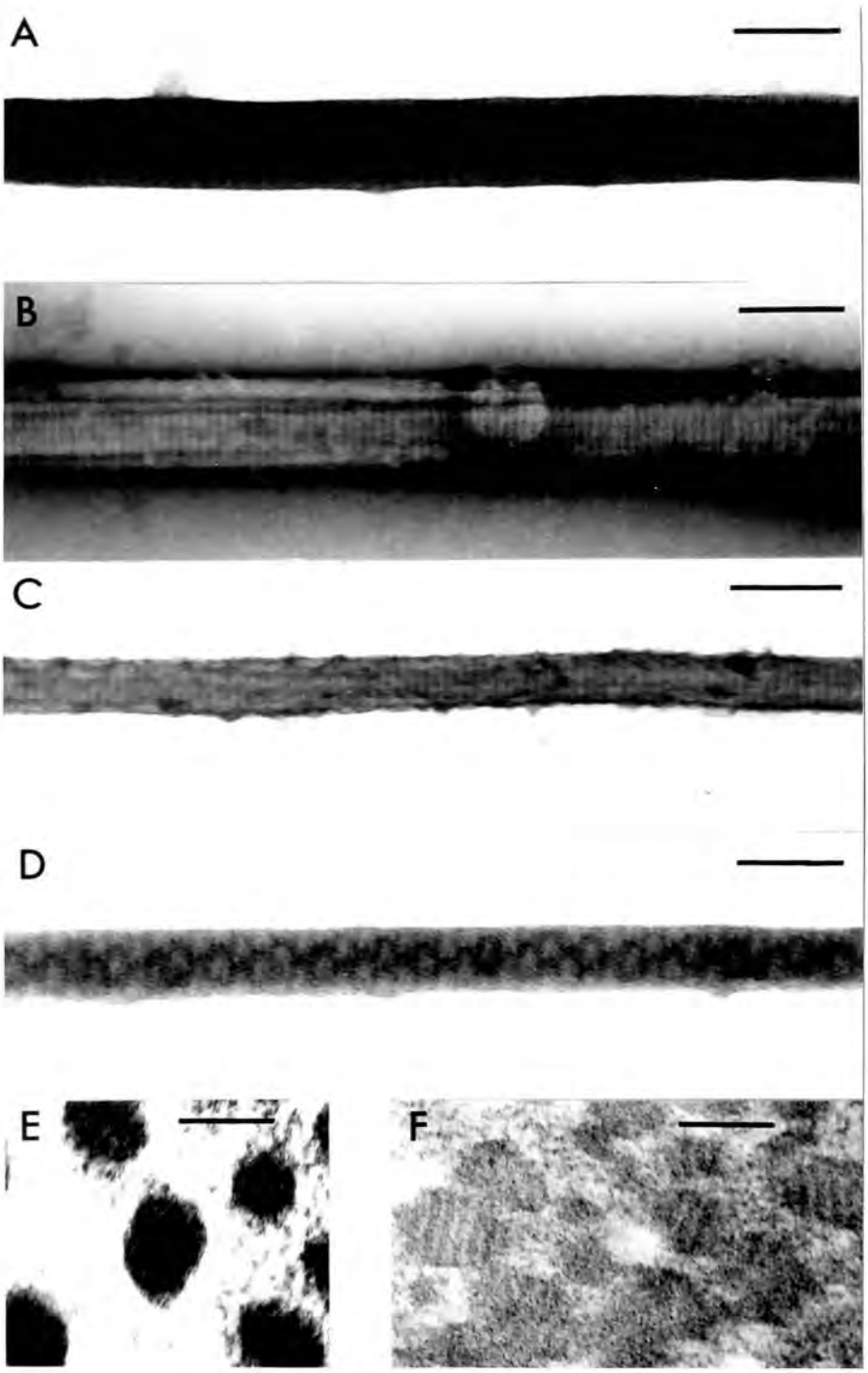

Figure 5.2. Negatively stained thick filaments showing periodic axial striations of about 14 nm isolated from the columellar muscle of A: *Burnupena cincta*, scale bar = 0.1 μm; B: *Siphonaria concinna*, scale bar = 0.2 μm; C: *P.vulgata*, scale bar = 0.15 μm; D: *Turbo sarmaticus* showing Bear-Selby net pattern, scale bar = 0.075 μm; E: transverse section of thick filaments from *P. miniata*, tilted at an angle that allows the crystal planes to be viewed as bands across the axis of the filament, scale bar = 0.1 μm; F: as for E but from from *P. granularis*, scale bar = 0.1 μm.

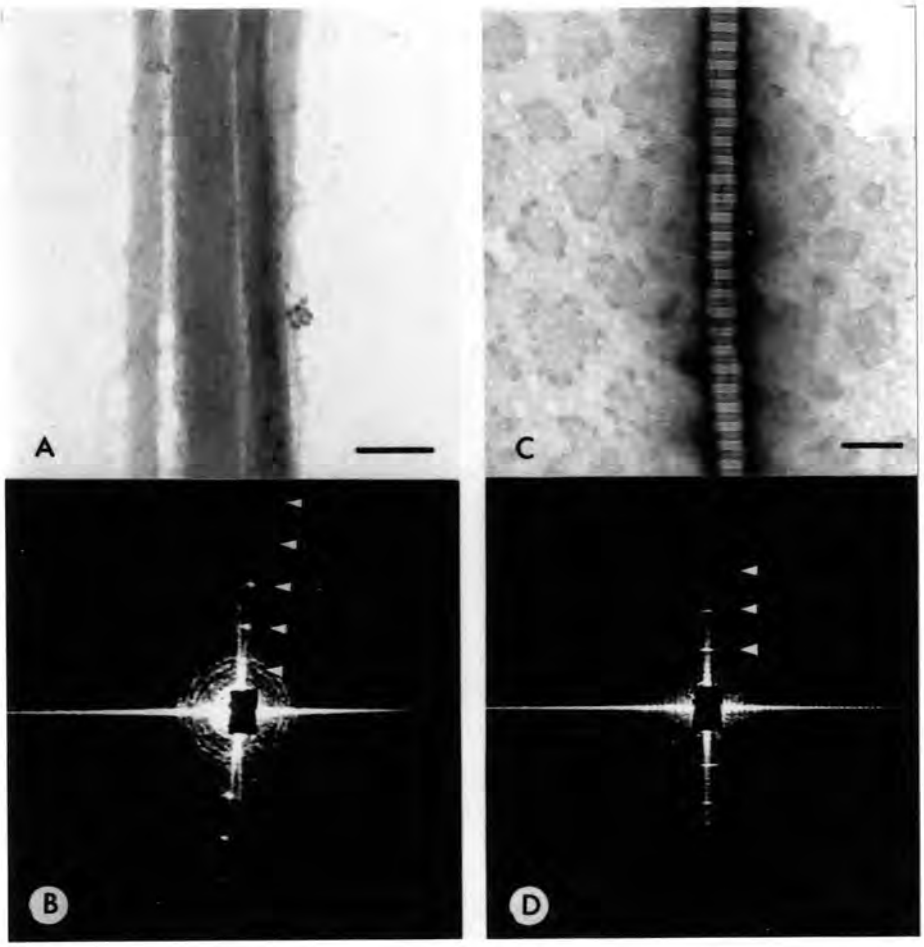

Figure 5.3. A: thick filament isolated from columellar muscle of *P. vulgata* showing an axial periodicity of 14.2 nm, scale bar = ; B: optical diffraction pattern taken from the filament in A, 5 orders of the 1/14 nm^{-1} reflections are visible (arrows) as the 5th, 10th, 15th, 20th and 25th orders of the 1/72 nm^{-1} meridional reflections which are off-centred for greater clarity; C: collagen fibril isolated from columellar muscle of *P. vulgata* showing a periodicity of 62 nm, scale bar = ; D: optical diffraction pattern taken from the filament in C, the 3rd, 5th and 7th orders of the 1/62 nm−1 reflection are visible (arrows).

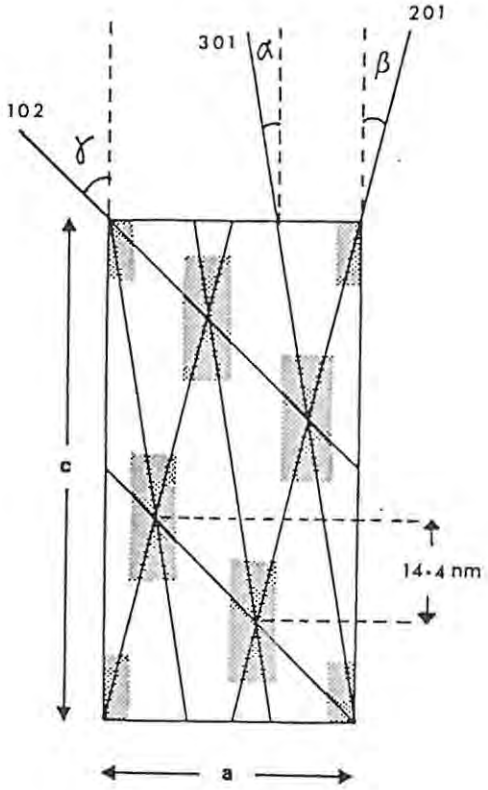

Figure 5.4. Diagram of the Bear-Selby net, modified from Bennett & Elliott, 1981. The 102, 201 and 301 planes are shown. c is the vertical repeat of the net (72 nm) along the axis of the filament. Five axial periodicities (14.4 nm) occur along the filament within one repeat of the net. a is the transverse repeat (19-30 nm) calculated from bands seen in transverse sections of the filament (Fig. 5.2E & F). The boxes are the nodes of the crystal lattice where stain would penetrate the filament which must be tilted at an angle γ from the vertical in order to view the bands as seen in Fig. 5.2E & F.

Species	Muscle	Mean *a* (nm)	Range (nm)	N
Bivalvia				
O. edulis	white adductor	27.3	25.8-29.8	11
P. maximus	white adductor	28.7	21.1-35.6	8
C. gigas	white adductor	34.4	32.3-36.2	9
M. mercenaria	white adductor	26.6	25.2-29.4	5
M. mercenaria	red adductor	24.9	23.1-26.6	3
M. edulis	ABRM	31.1	24.7-36.4	12
Gastropoda : Patellidae				
P. granularis	columellar	25.6	18.9-31.0	7
P. miniata	columellar	22.5	19.9-27.4	5
P. cochlear	columellar	22.3	17.9-25.2	3
P. tabularis	columellar	22.5	22.0-23.1	3

N=no. filaments measured, on average, 3 spacings per filament

Table 5.2. Bear-Selby *a* spacings calculated in this study from thick filaments of patellid columellar muscle compared with those from bivalves (Bennett & Elliott, 1981).

5.4).

Cross sections of limpet columellar thick filaments showing bands in this study were oval with the long axis about 1.5 times longer than the short axis (Fig. 5.2E & F). This suggests there was a large angle of tilt in the filaments and that it is the 102 planes that were visible in this study (Fig. 5.4). Calculated values for a are given in Table 5.2.

5.3.3 SDS Gel Electrophoresis of Filament Isolates

Figure 5.5 shows that paramyosin dominates in all homogenates of columellar tissue examined. It runs as a chain of 100 k molecular weight. Actin and myosin are also present running at 42 k and 220 k molecular weight respectively. Tropocollagen (a sub molecule of collagen) runs on SDS gels as a chain of approximately 95 k molecular weight (Gosline & Shadwick, 1983). It may therefore be merged with paramyosin which would partly account for the wide spread in the band. Partial proteolysis of paramyosin could also contribute to some of the spread. A second protein of smaller molecular weight to paramyosin is just apparent in some columns being most clear in *Burnupena* (Fig. 5.5B, column c). The protein is assumed to be collagen since collagen fibrils were the only other component seen in significant amounts in the filament isolates viewed in the electron microscope.

5.3.4 Estimation of the Relative Proportion of Collagen to Muscle in Columellar Muscle

Over an area of about 2.5 mm^2, 35 % of columellar tissue from *P. oculus* is collagenous connective tissue. Densitometry profiles confirm the presence of a second protein of molecular weight slightly smaller than paramyosin in almost all gel columns which is identified as collagen (Fig. 5.6). The ratios of paramyosin to collagen for a range of gastropod species are given in Table 5.3. The columellar muscles of *P. vulgata* and *Burnupena* have the highest proportion of collagen at 44 % and 47 % respectively.

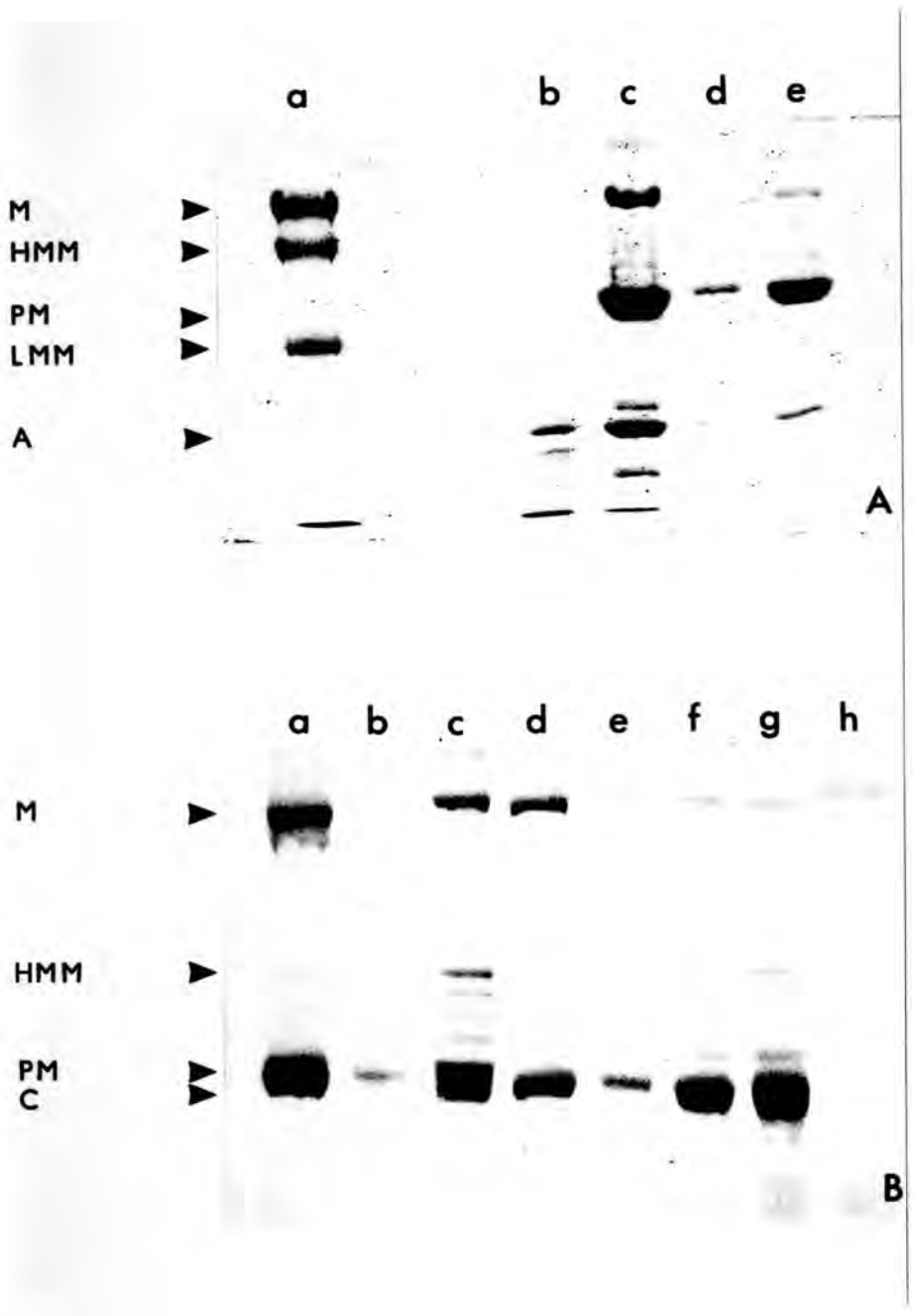

Figure 5.5. A: SDS gel results from columellar muscle of *P. vulgata*, a: standard myosin protein isolated from rabbit psoas muscle, b: supernatant from dorso-ventral muscle region, c: homogenate from dorso-ventral muscle region, d: homogenate from circumferential muscle region, e: homogenate from shell muscle region; B: SDS gel results from columellar muscle of a range of South African gastropods, a: *Haliotis*, b: Haliotis, (small loading, see text), c: *Burnupena*, d: *P. tabularis*, e: *Oxystele*, f: *P. oculus* (small loading, see text), g: *P. oculus*, h: standard protein, lysozyme. HMM: heavy meromyosin, LMM: light meromyosin, PM: paramyosin, M: myosin, A: actin, C: collagen.

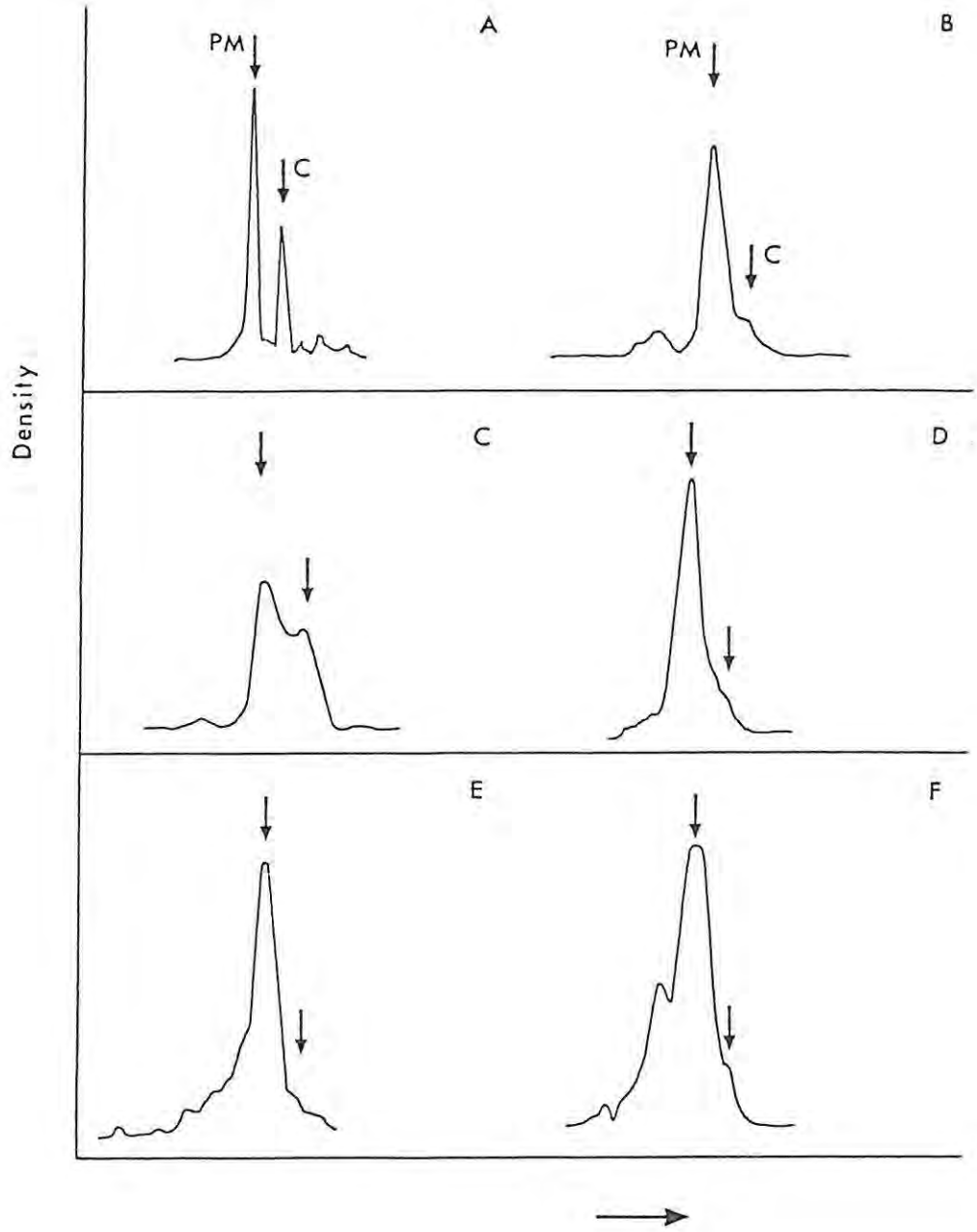

Figure 5.6. Densitometry traces of SDS gel bands, proteins at approx. 100 k mw. A: trace from Fig. 5.5A, column c, *P. vulgata*; B: from Fig. 5.5B, column a, *Haliotis*; C: from Fig. 5.4B, column c, *Burnupena*; D: from Fig. 5.4B, column d, *P. tabularis*; E: from Fig. 5.4B, column e, *Oxystele sinensis*; F: from Fig. 5.4B, column g, *P. oculus*; PM: paramyosin, upper left hand arrows; C: collagen, lower right hand arrows. Large arrow along abscissa indicates direction in which proteins migrate in the columns, smaller molecular weight proteins migrate furthest. Densities not to scale.

Species	Relative Peak Heights		Relative Peak Areas	
	C	PM	C	PM
Patella vulgata	0.44	0.56	0.45	0.55
Patella oculus	0.16	0.84	0.16	0.84
Patella tabularis	0.26	0.74	-	-
Haliotis spadicea	0.16	0.84	0.22	0.78
Oxystele sinensis	0.14	0.86	-	-
Burnupena cincta	0.47	0.53	0.40	0.60

Table 5.3. Ratios of the proteins collagen (C) and paramyosin (PM) from the columellar muscle of a range of gastropods. Values were obtained from relative peak heights or peak areas recorded by densitometry of electrophoresis gel band intensities (c.f. Fig. 5.6).

5.3.5 Determination of Protein Constituents of Striated Thin Filament Bundles using Immunocytochemistry

Anti-actin stained the thin filaments of type I muscle cells and could thus be regarded as an internal control (Fig. 5.7A & B). Some staining occurred on the bundled thin filaments of type II cells (Fig. 5.7C) though not very much even at higher concentrations of antibody. However, where binding did occur it was rarely on the electron-dense regions. Bat smooth muscle bound actin antibody showing that the low level of binding in the invertebrate gastropod muscle was not due to poor or faulty anti-bodies. Pan-myosin did not bind at all.

5.4 DISCUSSION

Diameters measured from isolated thick filaments are overall 10 % or more higher than values obtained from transmission electron micrographs of sectioned thick filaments. The discrepancy is probably due in part to the process of negative staining which causes less shrinkage than preparative procedures for transmission electron microscopy (Bennett & Elliott, 1981; Chantler, 1983). It is also possible that thicker filaments may be isolated more easily than thinner ones giving a small bias in the values obtained. Measurements on diameters of isolated thick filaments proves that the wide variability in diameter (Chapters 3 & 4) is actual and not due solely to tapering ends of filaments. Some of the largest diameter filaments occur in limpets. The presence of such large thick filaments implies that the muscle is capable of sustaining large tensions (Chantler, 1983). This is relevant in view of the large opposing forces limpets can withstand by clamping the columellar muscle.

Measurements from optically diffracted micrographs of isolated thick filaments give a comparable value for the axial repeat (Table 5.4) to those obtained from bivalves using X-ray and optical diffraction techniques (Bear & Selby, 1956; Sobieszek,1973; Bennett & Elliott, 1987). This shows that

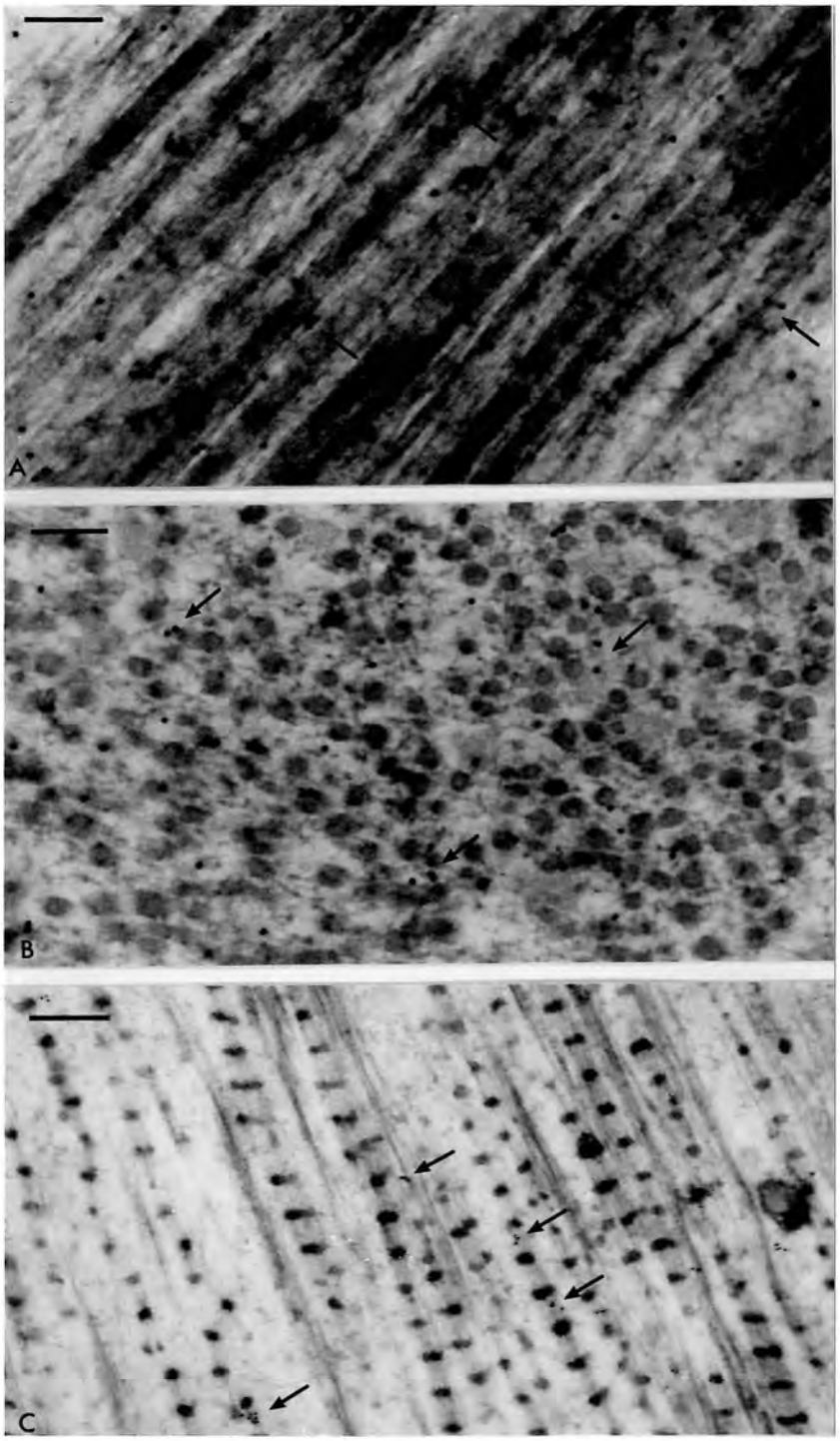

Figure 5.7. Electron micrographs from columellar muscle showing gold particles bound to anti-actin antibody (arrows). A: type I cell in longitudinal section, *P. oculus*, 10 nm gold. Scale bar = 0.1 μm. B: type I cell in transverse section, *P. oculus*, 10 nm gold. Scale bar = 0.1 μm. C: type II cell in longitudinal section, *P. vulgata*, 5 nm gold. Note the gold has bound to the thin filaments only and not the electron-dense regions. Scale bar = 0.3 μm.

Genus	Muscle	P(nm)	Reference
Mercenaria	slow adductor	14.4	Elliott & Bennett 1982
Mytilus	anterior byssus retractor	14.4	Sobieszek 1973 Elliott & Bennett 1982
Ostrea	slow adductor	14.4	Elliott & Bennett 1982
Patella	columellar	14.2	Frescura & Hodgson 1990

Table 5.4. Comparison of axial periodicities (P) of isolated thick filaments from some molluscan muscles. Values obtained from optically diffracted negatives of electron micrographs.

if filament measurements were made only on isolated filaments, rather than sectioned filaments, using the more precise X-ray or optical diffraction techniques, the wide variation in periodicity discussed in Chapter 3 would be resolved.

Table 5.2 compares calculated *a* values from this study to those from the literature obtained for bivalves. The variability between patellid species is small with values falling consistently within the X-ray data range of 19 to 30 nm for bivalve data (Bennett & Elliott, 1981). This consistency indicates that the paramyosin crystal core is fairly stable with a constant packing arrangement for the paramyosin molecules. It is also further evidence that the crystal-like form is the usual structure of the paramyosin core of thick filaments in smooth molluscan muscles generally and not bivalves only (Frescura & Hodgson, 1990).

SDS gel results show that paramyosin is the predominant protein in both North Atlantic and South African species of patellid limpet. This compares favourably with results obtained for several bivalves (Bennett & Elliott, 1987; white and yellow adductor of *Crassostrea*, posterior adductor and ABRM of *Mytilus*, white and red adductor from *Mercenaria*, white adductor from *Pecten*).

The combined evidence from transmission electron microscopy of sectioned thick filaments, transmission electron microscopy of isolated thick filaments, optical diffraction studies and SDS gel electrophoresis demonstrates the paramyosin nature of the thick filaments in patellid limpets. Furthermore, filament isolates from *Haliotis*, *Siphonaria* and coiled shell gastropods all contain thick filaments with periodicities and appearance typical of paramyosin filaments suggesting that paramyosin thick filaments are characteristic of gastropod muscles in general.

There is insufficient evidence to attach any functional significance to the two ranges of filament diameters found in *Turbo*. It is tentatively suggested that such filament variations between cells may naturally occur in other species and genera but not always be evident because of a more continuous

gradation in the diameter differences.

The failure to isolate the bundled striated thin filaments of the type II cells could imply that they are easily degraded, or that a different set of conditions is required to maintain their integrity. The electrophoresis results suggest that they are made from familiar muscle proteins, possibly actin, since there are no indications of any major novel proteins present in the gel.

Immunocytochemistry was used in an attempt to determine from which proteins the striated thin filaments of the type II cells were made. The drawback of having used commercially available anti-bodies raised in vertebrates, however, is that from the beginning it was not known how well they would bind, if at all, to marine invertebrate smooth muscle proteins. Actin differences between vertebrates and invertebrates consist of highly conservative substitutions in amino-acid sequence (Chantler. 1983), so that it is not surprising to find some actin binding. Molluscan myosins by contrast, vary considerably from those of vertebrates, and within vertebrates, in their light chain structure (Chantler, 1983). Hence a greater requirement for compatibility of antibodies exists when dealing with myosin. Ideally monoclonal anti-bodies should be raised in the same muscle type to which they are ultimately to bind. It would be useful to determine whether any paramyosin protein is present in the striated thin filaments and since molluscan paramyosins are very similar to each other both immunologically and in amino-acid sequence (Chantler, 1983) this is an obvious investigation to make, the only limitation being that paramyosin antibody is not commercially available. Lastly, work by Langanger, Moeremans, Daneels, Sobieszek, De Brabander & De Mey, (1986) to identify the proteins from which stress-fibres are made, have revealed the presence of α-actinin at the electron-dense regions. Since it has been shown that the striated thin filaments of type II cells are smilar in appearance to stress-fibres (Frescura & Hodgson, 1990; Chapter 3) it would be informative to see if antibodies to the protein α-actinin bind at the electron-dense regions of the type II

striated thin filaments found in this study.

Knowledge of the structure and mechanical poperties of molluscan collagen is almost entirely based on studies of squid collagen (Ward, 1972; Ward & Wainwright, 1972; Gosline & Shadwick, 1983). Gosline & Shadwick (1983) point out that there has been some variation in the collagen periodicities of invertebrates. Collagen isolated from squid mantle is reported to have a periodicity of 68 nm (Hunt, Grant & Liebovich, 1970) whereas the 62 nm periodicity of isolated limpet columellar collagen compares favourably with the 64 nm periodicity of vertebrate collagen (Wainwright, Biggs, Currey & Gosline, 1976; Gosline & Shadwick, 1983). Since the axial repeat is a direct reflection of the packing of tropocollagen molecules we can infer that limpet columellar collagen fibrils probably have a similar structure to collagen from vertebrate skeletal muscle collagen. Histology (Chapter 2) shows the distribution and organisation of collagenous connective tissue and electron microscopy (Chapters 3 & 4) shows the abundance of collagen fibrils in the columellar muscle. It is useful to attempt to quantify how much collagen is present in order to make comparisons with other muscles. Walls (1968) states that the proportion of total protein formed by collagenous connective tissues in voluntary muscle of vertebrates ranges from 3 to 30 %. However, he adds that although reticular fibres are present collagen fibres are often very scant. It is not clear therefore, how much of this protein is collagen alone. This is in contrast to gastropod columellar muscles which are packed with collagen fibrils, especially in patellid limpet columellar muscle. This highlights an important point; direct comparisons with data from other sources are not always valid unless one is certain that the same quantities are being compared. The figure of 35 % connective tissue per cross sectional area of limpet columellar muscle is probably more comparable with Walls's data than the results from microdensitometry which give an estimate of the collagen to paramyosin protein ratio, paramyosin being the most predominant muscle protein present.

Evaluation of areas under peaks is not very accurate by hand. The less

resolved the peaks the more uncertain is the extrapolation. More accurate determinations of areas under such peaks, could be obtained, in principle, as follows. A Gaussian curve can be fitted over the two peaks (only the two parameters width and height are required). Two further Gaussians (having different parameters to the ones above) must be found which when added together give the first Gaussian function. The areas under these two last Gaussians then give the protein estimates. However, in practice, this can be difficult to compute reliably so that the final decision rests on whether the means justifies the end.

It should also be pointed out that different protein densities being recorded in this technique are actually different dye densities. In order to make comparisons, the different proteins must be similar in their binding properties (Gordan, 1975). This point is difficult to ascertain and leaves one with some degree of unreliability in the result unless substantiated by results from other techniques. In this study, the results, which indicate a high collagen to muscle ratio in most gastropods, are supported by both histology and electron microscopy examination and by area measurements. As discussed previously (Chapters 3 & 4), collagen forms a large part of the columellar tissue in gastropods and probably has a hitherto underestimated but important role to play in many functions of the foot (Frescura & Hodgson, 1989).

5.5 SUMMARY

- A wide variation is shown in the large diameter of thick filaments from gastropod columellar muscles.
- Isolated thick filaments from a range of different gastropod columellar muscles show either the axial periodicity or Bear-Selby net characteristic of paramyosin filaments.
- Calculations for the transverse repeat of the paramyosin crystal and axial periodicities from optically diffracted micrographs of thick filaments from limpet columellar muscles,show they have the same dimen-

sions and similar molecular packing to thick filaments from bivalves.

- Gel electrophoresis shows that paramyosin is the predominant protein in the columellar muscle of a range of gastropods.
- Densitometry of the gel bands together with an estimation of the percentage of collagenous connective tissue present from micrographs, indicates that a high ratio of collagen to muscle is present in most of the gastropod columellar muscles studied.
- Collagen fibrils are similar in appearance to those from vertebrate skeletal muscle but have a slightly smaller axial repeat.
- Novel proteins that might have been associated with the novel striated thin filaments were not evident in any of the electrophoresis studies.
- Preliminary immunocytochemistry indicates that the novel striated thin filaments may be composed of actin, but protein constituents of the electron-dense regions of these filaments remain unidentified.

Chapter 6

AN EXAMINATION OF THE COLUMELLAR MUSCLE-SHELL ATTACHMENT SITE OF LIMPETS

6.1 INTRODUCTION

The ability to clamp is crucial to the survival of limpets on their wave beaten rocky shore habitat. Adhesion of the foot to the substratum has been shown to be sufficiently strong to withstand forces as great as 5.18 kg cm^2 (Branch & Marsh, 1978). However, for this adhesion to be effective in maintaining the shell against the substratum, it is essential that the bond between columellar muscle and the shell to which it is attached, be of equivalent strength. If it is not, it limits the maximum tenacity attainable.

The attachment of the soft parts of the body to the shell in a variety of molluscs has been investigated by several authors (Hubendick, 1958, *Acroloxus lacustris*; Nakahara & Bevelander, 1970, *Pinctada radiata*; Plesch, 1976, *Lymnaea stagnalis*; Tompa & Watabe, 1976, *Helix aspersa*, *Anguispira alternata*, *Laevipex* sp., *Pomacea paludosa* and Smith, 1983, *Margaritiferidae* sp.). In addition, a generalised description of attachment of muscles to the shell in gastropods is given by Bubel (1984) who shows that in all the molluscs examined, a modified adhesive epithelium exists at the shell surface. However, the details of the mechanism of attachment often differ (Plesch, 1976).

This chapter considers the possibility that the mechanism of muscle shell attachment in patellid limpets may differ from the generalised gastropod case because of the large opposing forces it is able to withstand. To date, no studies of attachment sites have been made on limpets except for the freshwater pulmonate *Acroloxus lacustris* (Hubendick, 1958).

Furthermore, Branch & Marsh (1978) in examining six species of patellid limpet have shown that tenacities of limpets can be correlated to a number of different parameters. One of these is the area of muscle attachment to the shell. Unfortunately, no method is given by which this parameter was estimated. This study enlarges on the work of Branch & Marsh by examination of additional species and larger samples of data.

6.2 MATERIALS AND METHODS

The structure of the columellar tissue at the muscle-shell attachment site and shell surface of muscle scar areas was studied using histology and scanning electron microscopy methods respectively.

6.2.1 Histology of Muscle Attachment Site

The following species of gastropod were obtained from the east coast of South Africa: *Oxystele sinensis*, (Gmelin, 1791), *Patella oculus* (Born, 1778) and *Helcion pruinosus* (Krauss, 1848). The shell of *Oxystele sinensis* was crushed in a vice to access the columellar muscle. The muscle was gently peeled off the shell of all three species and prepared for histological observation as follows. Tissue was fixed either in 10 % formalin or Bouin's aqueous fixative. After being dehydrated through a series of alcohols and cleared in xylene, tissue was embedded in Paraplast, sectioned in the dorso-ventral plane at a thickness of about 6 μm and stained with Milligan's trichrome (staining of blood cells with Orange G omitted, Humason, 1967), Mallory's trichrome stain or haematoxylin counterstained with eosin (Humason, 1967).

6.2.2 Surface Structure of Shell Muscle Scar

Three different regions of the interior surface of the shell, the muscle scar region and the shell to the inside and to the outside of this region, from six species of patellid limpet and one pulmonate limpet were compared using a Jeol JSM 840 scanning electron microscope at 5 kv. The species exam-

ined included representatives from high to mid shore: *Patella granularis*, (Linné, 1758), *P. oculus*, (Born, 1778), the pulmonate *Siphonaria aspera* (Krauss, 1848); mid to low shore: *P. miniata*, (Born, 1778), *P. longicosta*, (Lamarck, 1819), *P. cochlear*, (Born, 1778) and the subtidal region: *P. tabularis*, (Krauss, 1848) (Table 1.1, Chapter 1). In addition, the shell of *P. oculus* with a portion of muscle tissue still attached was rapidly frozen in liquid nitrogen, fractured and viewed in profile in the same scanning electron miscroscope with CT 1000 cryo-attachment facilities.

6.2.3 Estimation of Muscle Scar Area

Shells of a range of sizes of six species of *Patella*: *P. granularis*, *P. oculus*, *P. miniata*, *P. longicosta*, *P. cochlear* and *P. tabularis*, were collected from the south east Cape coast of South Africa. Shells showing severe erosion were discarded.

Several methods of estimating the muscle scar area were tried but the most consistent results were obtained by making photocopies of shell interiors (Fig. 6.1) and using a Summagraphics computer digitising tablet and Sigma Scan software (Jandel Scientific, U.S.A.) to record areas. In some species the contrast between scar and surrounding shell had to be enhanced either by painting the area or outlining the area with a marker pen (Fig. 6.1). Where shell edges were very crenulated e.g. *P. longicosta*, the projected shell area was taken as the area defined by the inner edges of the crenulations. The muscle scar measurements, to be exact, should take into account a correction factor for the slight angle at which the scars occur on the inside of the shell i.e. they do not lie parallel to the horizontal. However, variation in this angle between species is sufficiently small to allow it to be considered a constant and can therefore be disregarded in a comparative analysis.

The muscle attachment site, mA, was plotted against the projected shell area, sA, (taken as an approximation of foot area) for a range of shell sizes. Using computer software by Statistical Graphics Corporation regres-

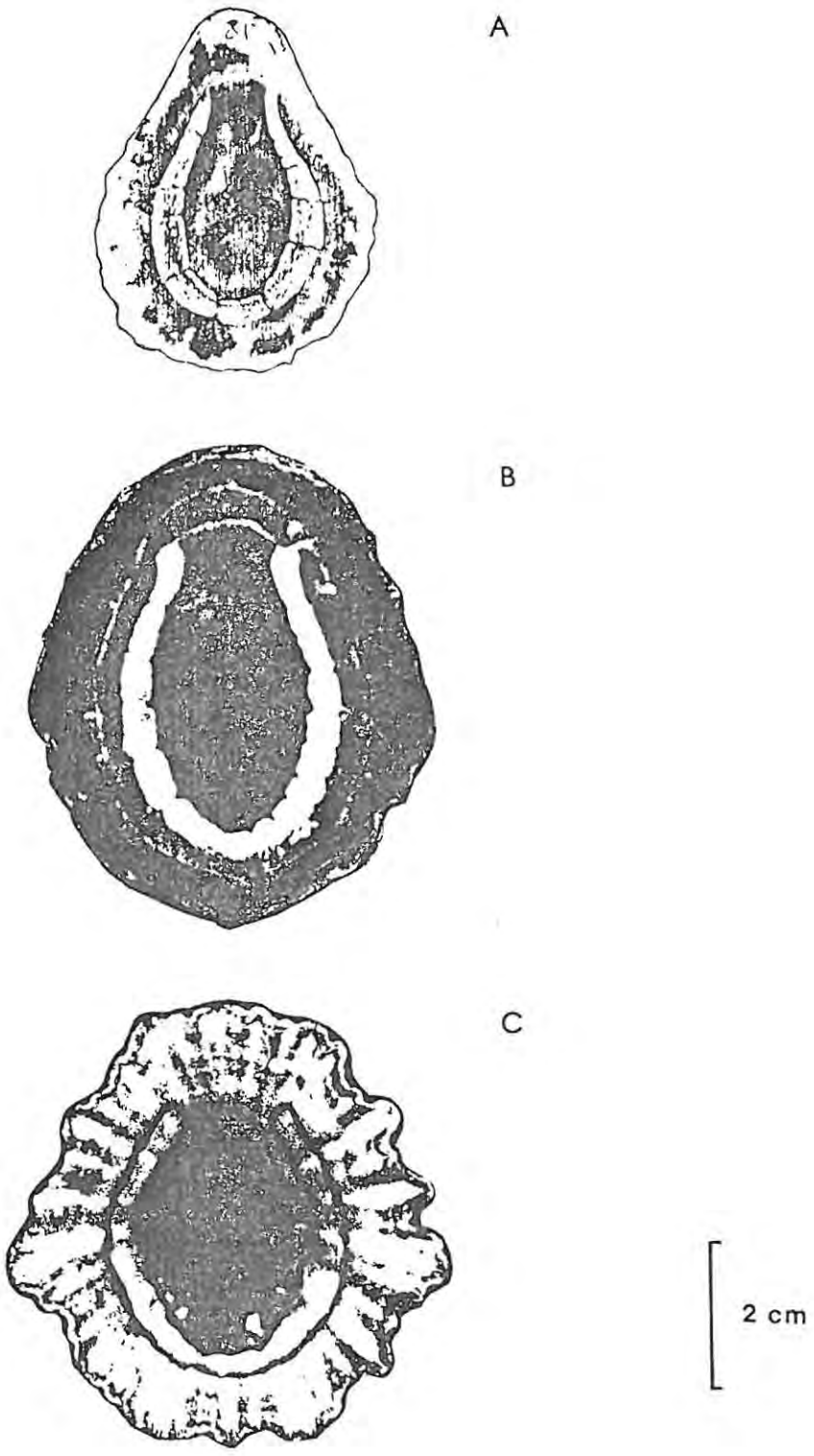

Figure 6.1. Examples of photocopied shell interiors to show muscle scar areas; A: *P. cochlear*, natural scar; B: *P. oculus*, painted scar; C: *P. longicosta*, area delineated by marker pen.

sion analyses were made to ascertain whether the ratio mA to sA was a constant for each species. An analysis of variance test was applied to assess the significance of variation between the means of the ratio mA to sA for each species.

6.3 RESULTS

6.3.1 Histology of Muscle Attachment Site

A single layer of epithelial cells is situated at the columellar muscle-shell interface of all three species of gastropod examined (Fig. 6.2). For comparison, Figure 6.2 also shows the epithelial layer at the sole of the foot for *Patella oculus* and *Helcion pruinosus*. The difference between the two types of epithelia is immediately evident. The muscle attachment site epithelial cells are cuboidal (about 6-8 μm high) with large densely-staining basal nuclei (Fig. 6.2A,C &E). The cells are often separated like the teeth of a comb (Fig. 6.2C), a condition which may have arisen when the tissue was gently peeled away from the shell scar.

The prevalence of collagen adjacent to both types of epithelia is evident in Figures 6. 2A & B where Milligan's trichrome stain shows the differentiation between muscle and collagen. A collagenous region of about 25 μm extends into the columellar musculature (Fig. 6.2A, see also Fig. 2.3A & C, Chapter 2). The muscle cells insert into this collagenous layer but do not extend into the epithelial layer. In contrast, muscle cells of the tarsus occur in close proximity to the epithelial sole of the foot (Fig. 6.2B).

At the sole, mucus-secreting cells abound (Fig. 6.2B & D) and the basic epithelial cell is about 6 μm and 10 μm high in *Helcion pruinosus* and *P. oculus* respectively. The basal lamina is also clearly seen (Fig. 6.2B &D).

6.3.2 Surface Structure of Shell Muscle Scar

In all species of *Patella* studied the shell surface either side of the muscle scar region is relatively smoother (Figs. 6.3 to 6.8). In contrast, the opposite

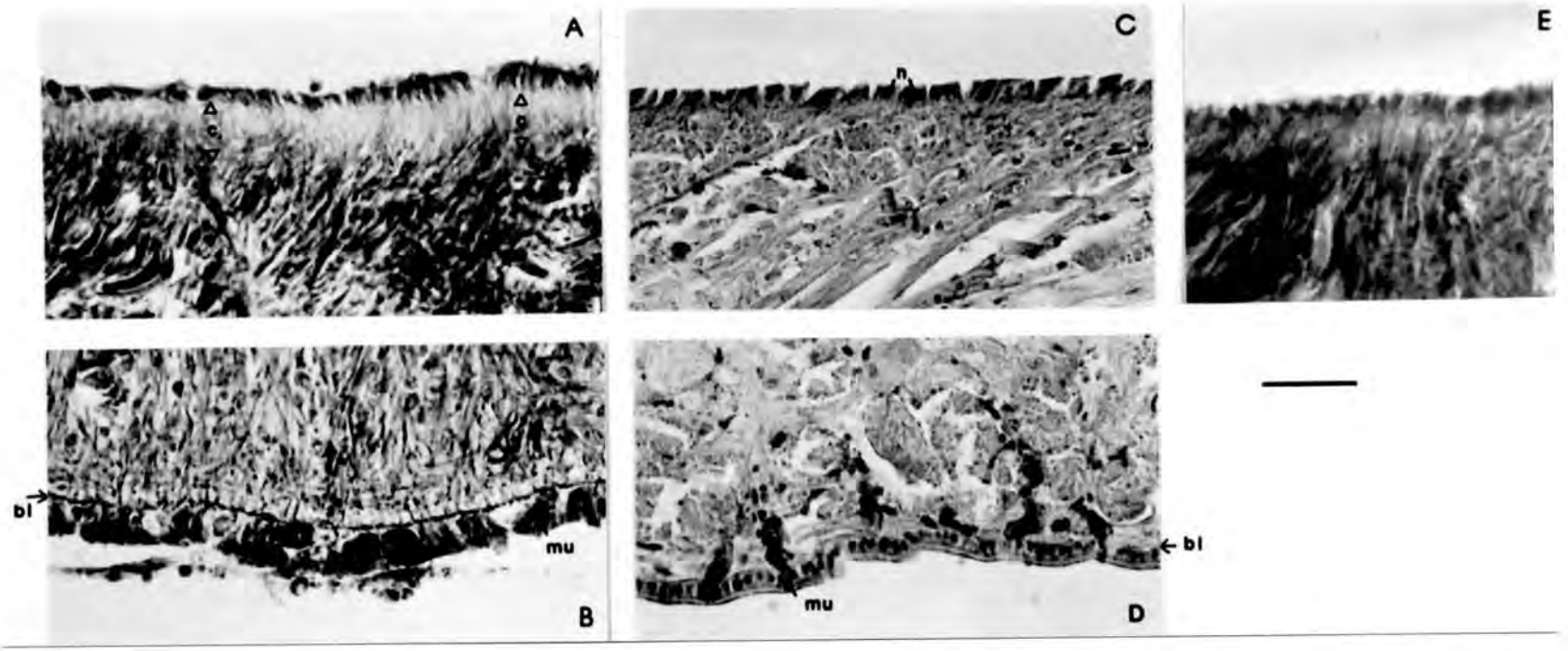

Figure 6.2. A: dorso-ventral section of columellar tissue from *P. oculus* at muscle-shell interface, shell removed, stained with Milligan's trichrome, muscle stains red, collagen stains green. Note the dense collagen matrix (c). B: tarsal tissue from *P. oculus* at sole of foot (ts), stained with Milligan's trichrome. Note the mucocytes (mu) and distinct basal lamina (bl). C: as for A but from *Helcion pruinosus* and stained with haemotoxylin and eosin. Note the densely-stained nuclei (n) in the epithelial layer. D: as for B but from *Helcion pruinosus* and stained with Haemotoxylin and eosin. E: as for A but from *Oxystele sinensis* and stained with Mallory's trichrome. Scale bars = 30 μm.

is found in *Siphonaria* where the muscle scar region appears smoother (Fig. 6.9). *P. tabularis* has the smoothest surface of all patellid species (Fig. 6.8). Pores or pits in the shell surface were not seen. It should be noted, however, that the apparent pores in Figs. 6.4C & 6.7C are due to some erosion of the shell which also explains the similar appearance of these two specimens. The muscle scar of *Siphonaria aspera* has a different gross appearance to patellid scars having a crenulated inner boundary (Fig. 6.9).

Freezing limpet shells in liquid nitrogen with the shell muscle still attached always resulted in a complete detachment of the muscle from the shell, leaving an apparently "clean" scar surface. This indicates a natural cleavage site at this interface. The scanned profile revealed a layer of mucus-like material at the shell scar surface (myostracum) (Watabe, 1988) in a number of places (Fig. 6.10). Again, no pits were evident and no muscle fibres were seen inserted into the shell.

6.3.3 Estimation of Muscle Scar Area

Figures 6.11 to 6.13 show the relationship of shell area to muscle scar area for each species studied. Several models, namely, reciprocal, exponential and multiplicative were fitted to the data but a linear model fitted best in all species except *P. miniata*, with 95 % confidence. This indicates that the ratio muscle area (mA) to shell area (sA), in general, is a constant for each species and allows interspecific comparisons to be made. The data for *P. miniata* could also be fitted to an exponential function of the form

$$y = e^{(a+bx)}$$

(Fig. 6.14A). The correlation coefficients for both linear and exponential models differ by only a few percent but are also both lower than those of other species. Of the species analysed in this study, *P. cochlear* had the largest mA to sA ratio (0.206) and *P. miniata* the smallest (0.098) (Table 6.1). Analysis of variance indicates that most of the means of the ratio mA to sA are significantly different between species at the 95 % confidence level

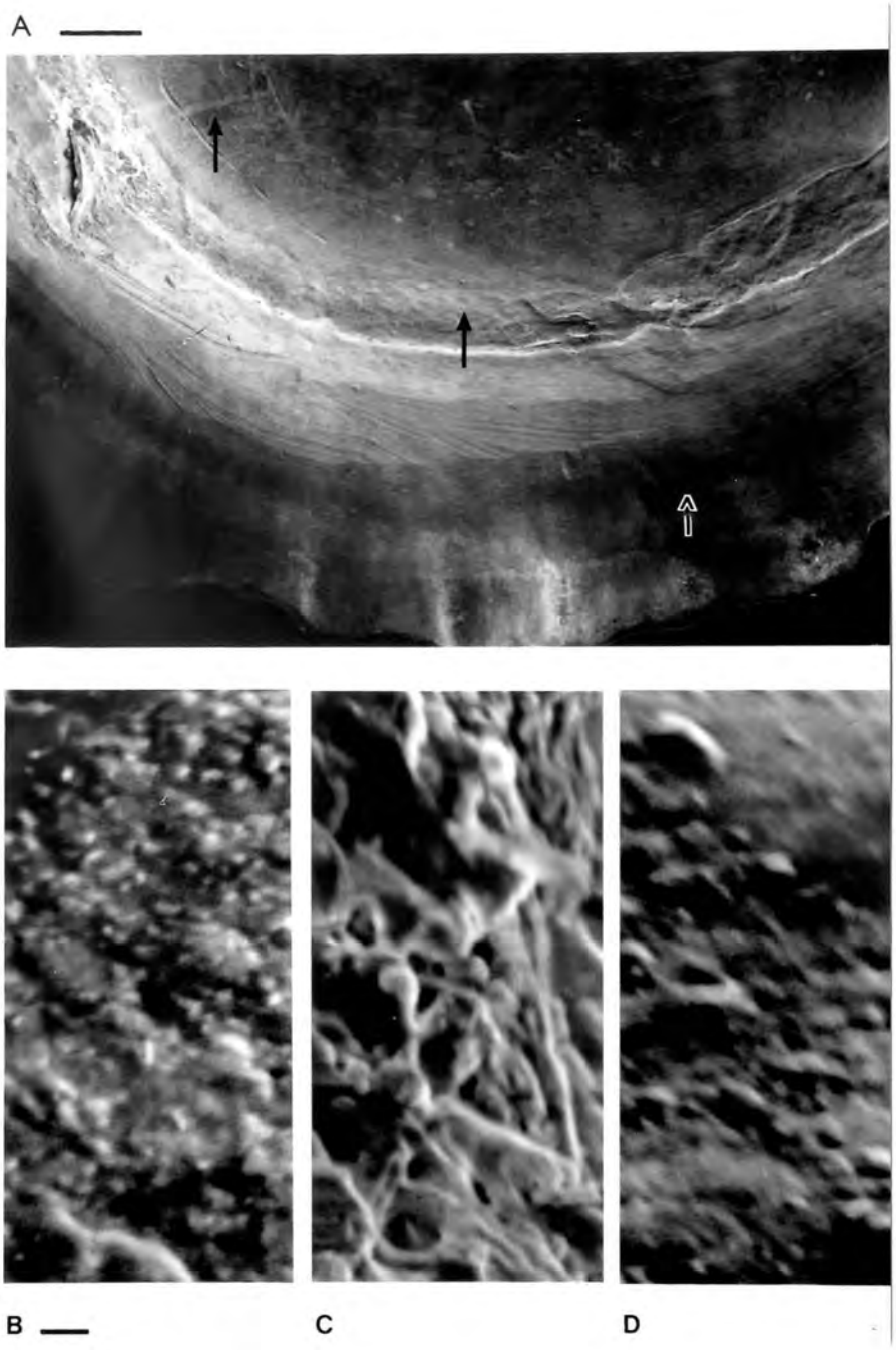

Figure 6.3. Scanning electron micrographs of uncoated shell interior from *P. granularis*. Arrows indicate region of shell interior from which micrographs B, C & D have been taken. A: muscle scar specimen area. Scale bar = 1 mm. B: shell interior to inside of scar. Scale bar = 1 μm. C: muscle scar area. Scale bar = 1 μm. D: shell interior to outside of scar. Scale bar = 1 μm.

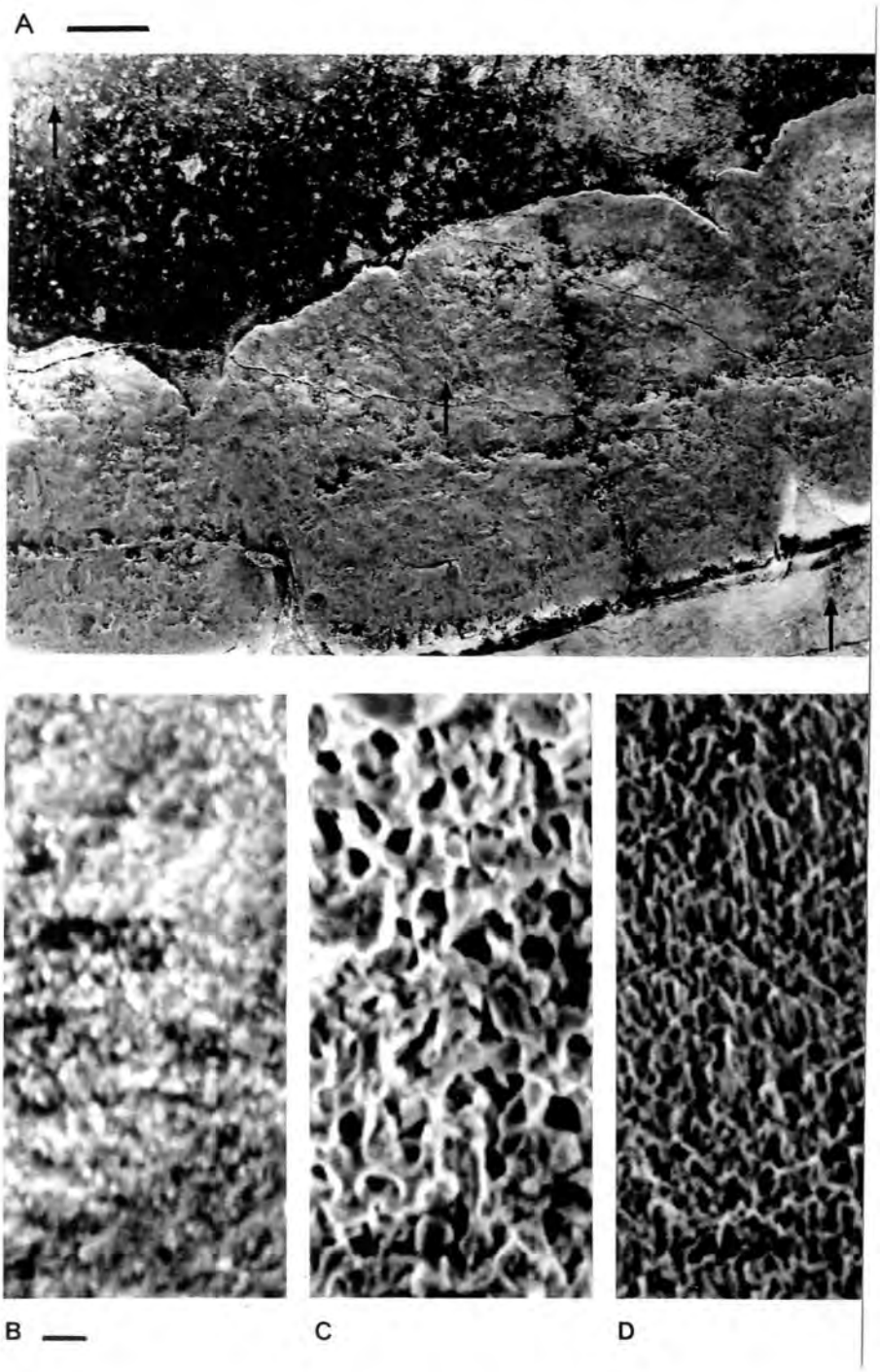

Figure 6.4. As for Figure 6.3 but from *P. oculus*.

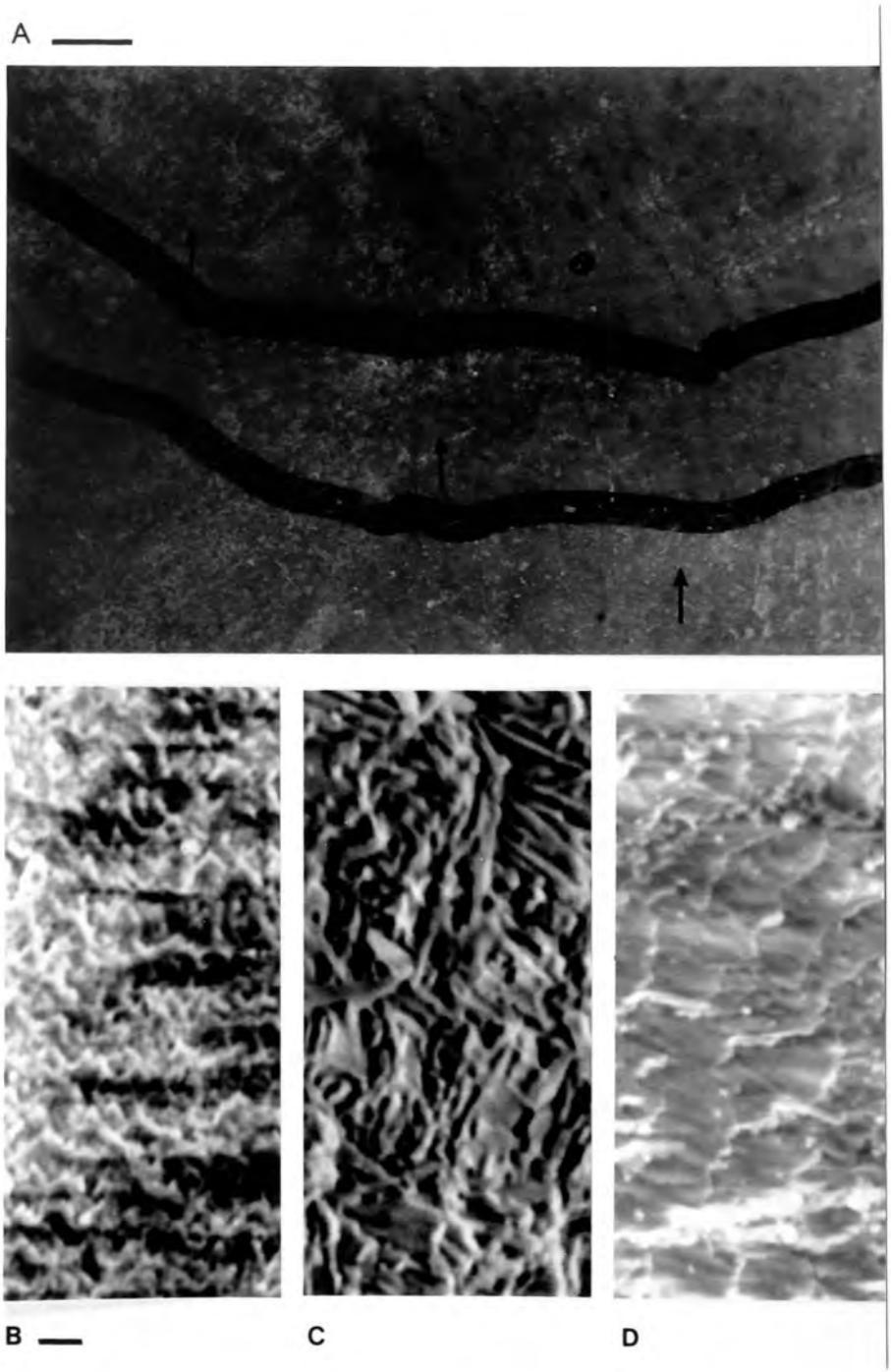

Figure 6.5. As for Figure 6.6 but from *P. miniata*. Note the muscle scar in this specimen has been demarcated by pen.

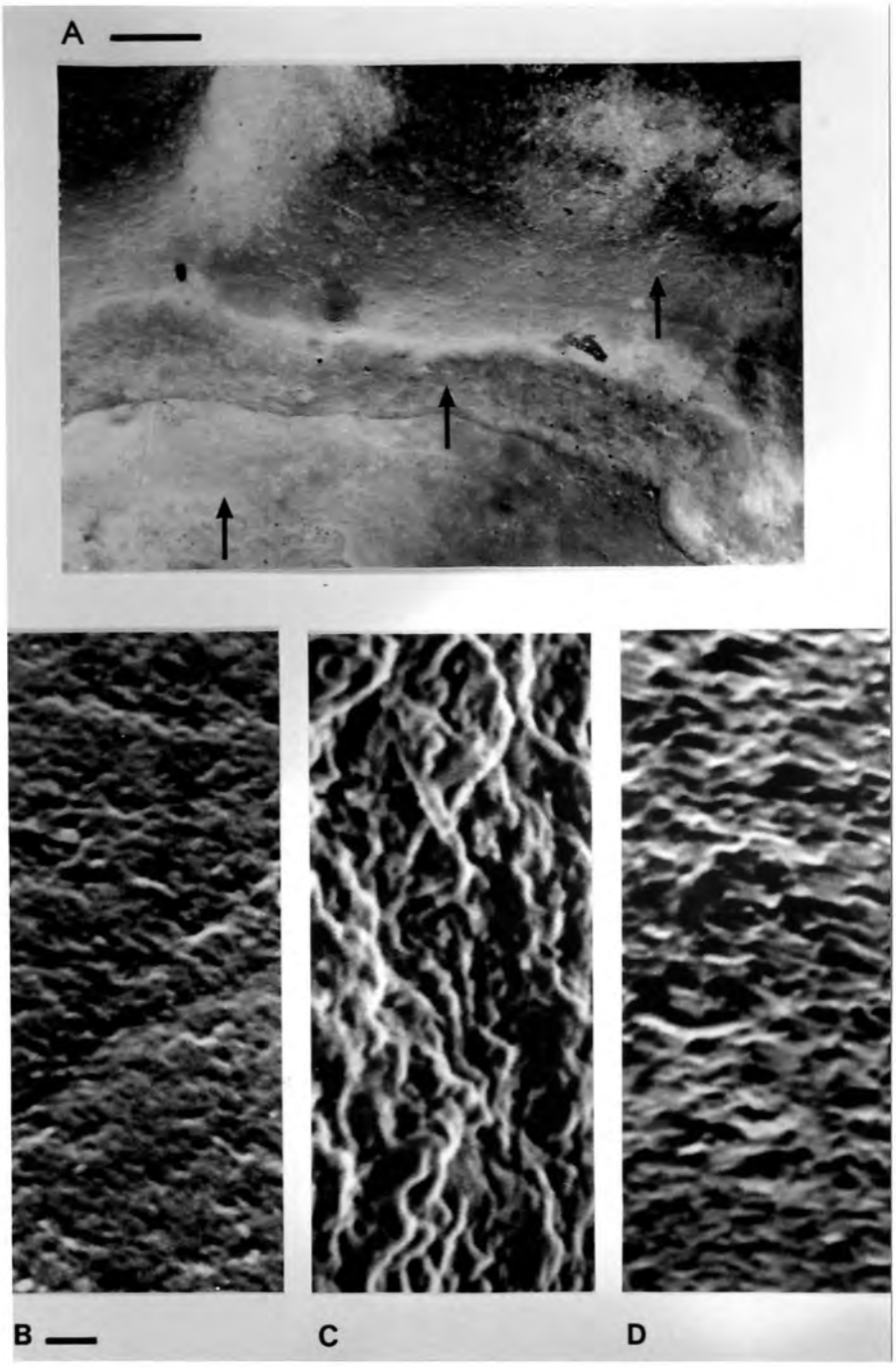

Figure 6.6. As for Figure 6.7 but from *P. longicosta.*

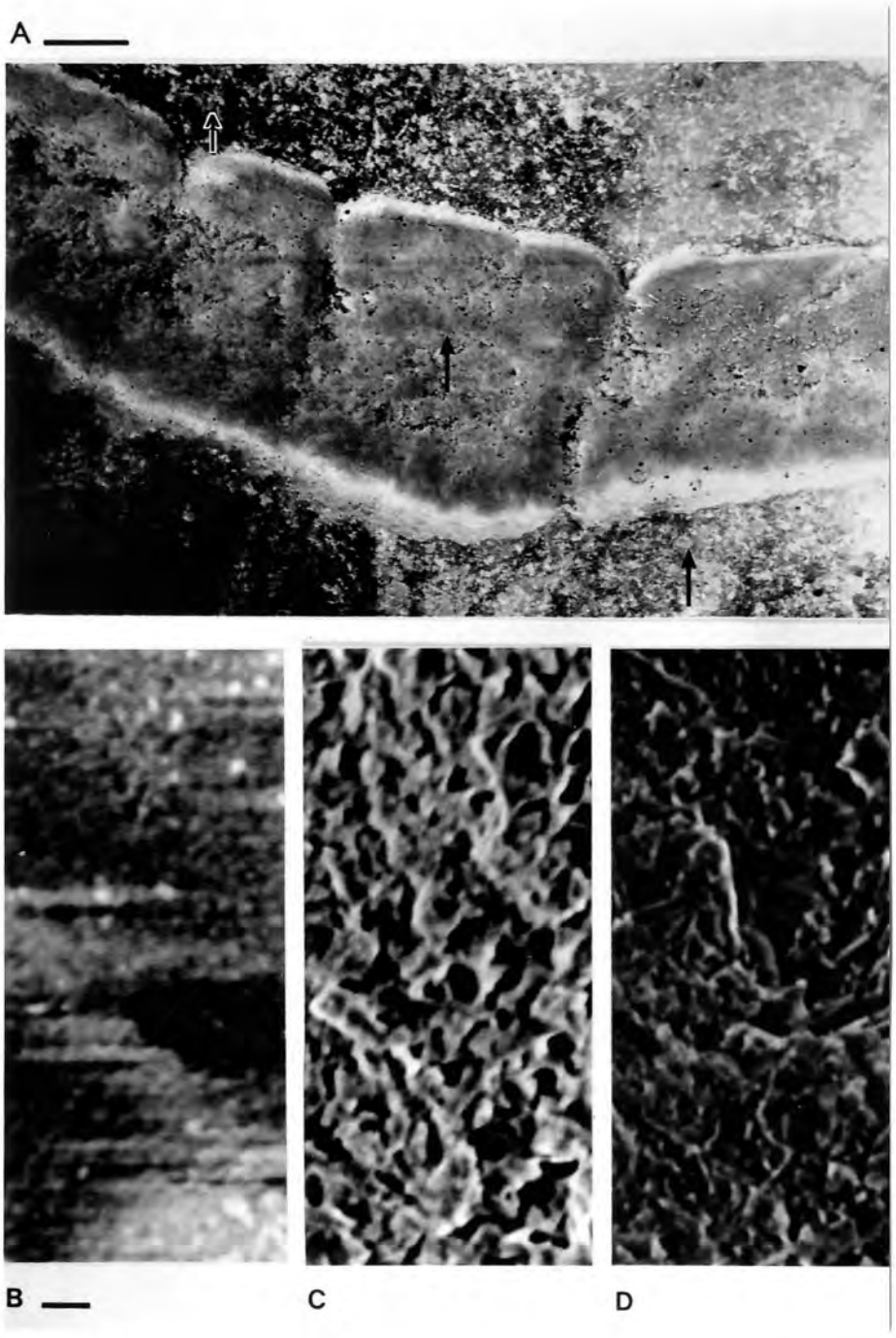

Figure 6.7. As for Figure 6.8 but from *P. cochlear.*

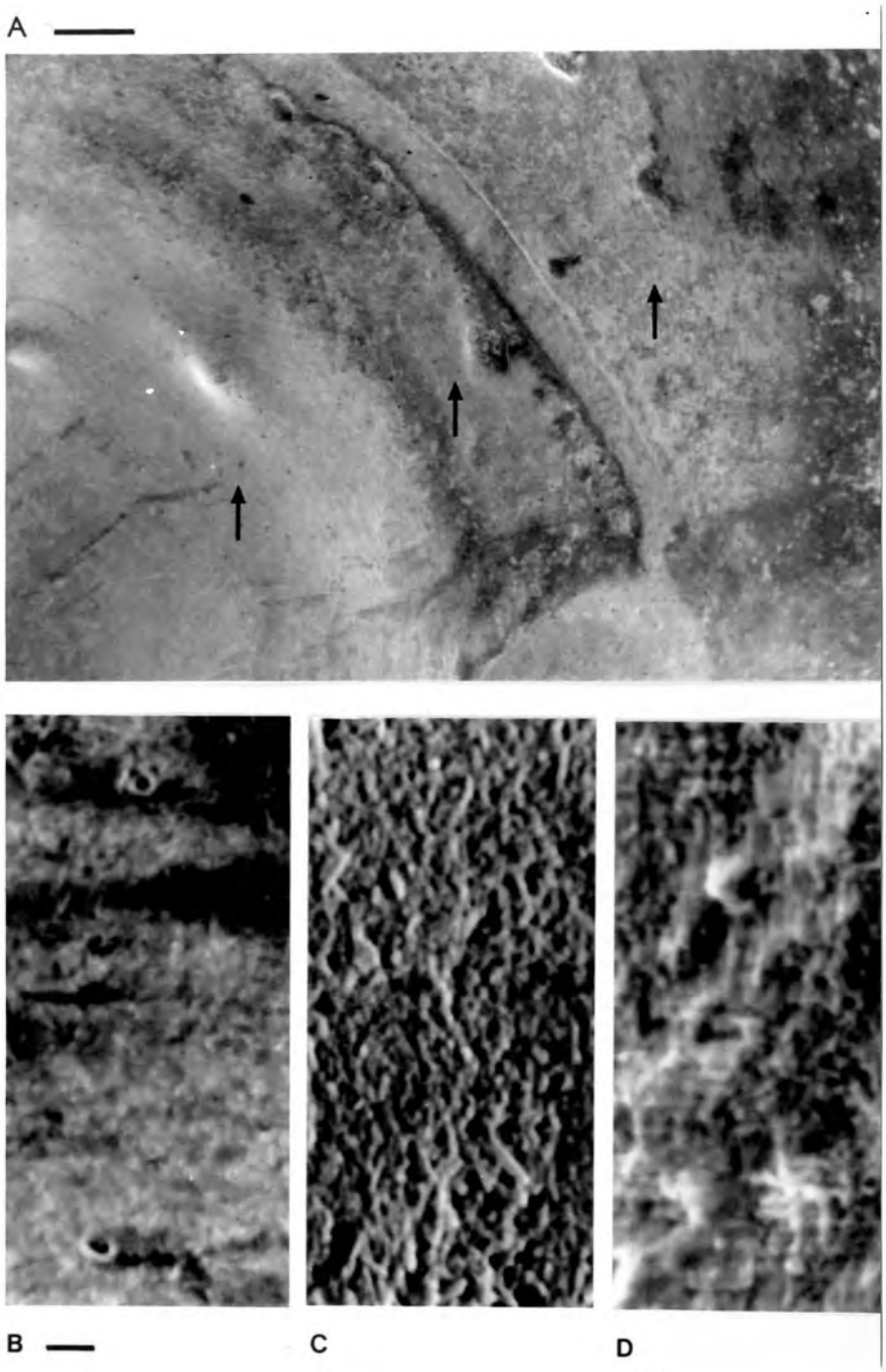

Figure 6.8. As for Figure 6.3 but from *P. tabularis*.

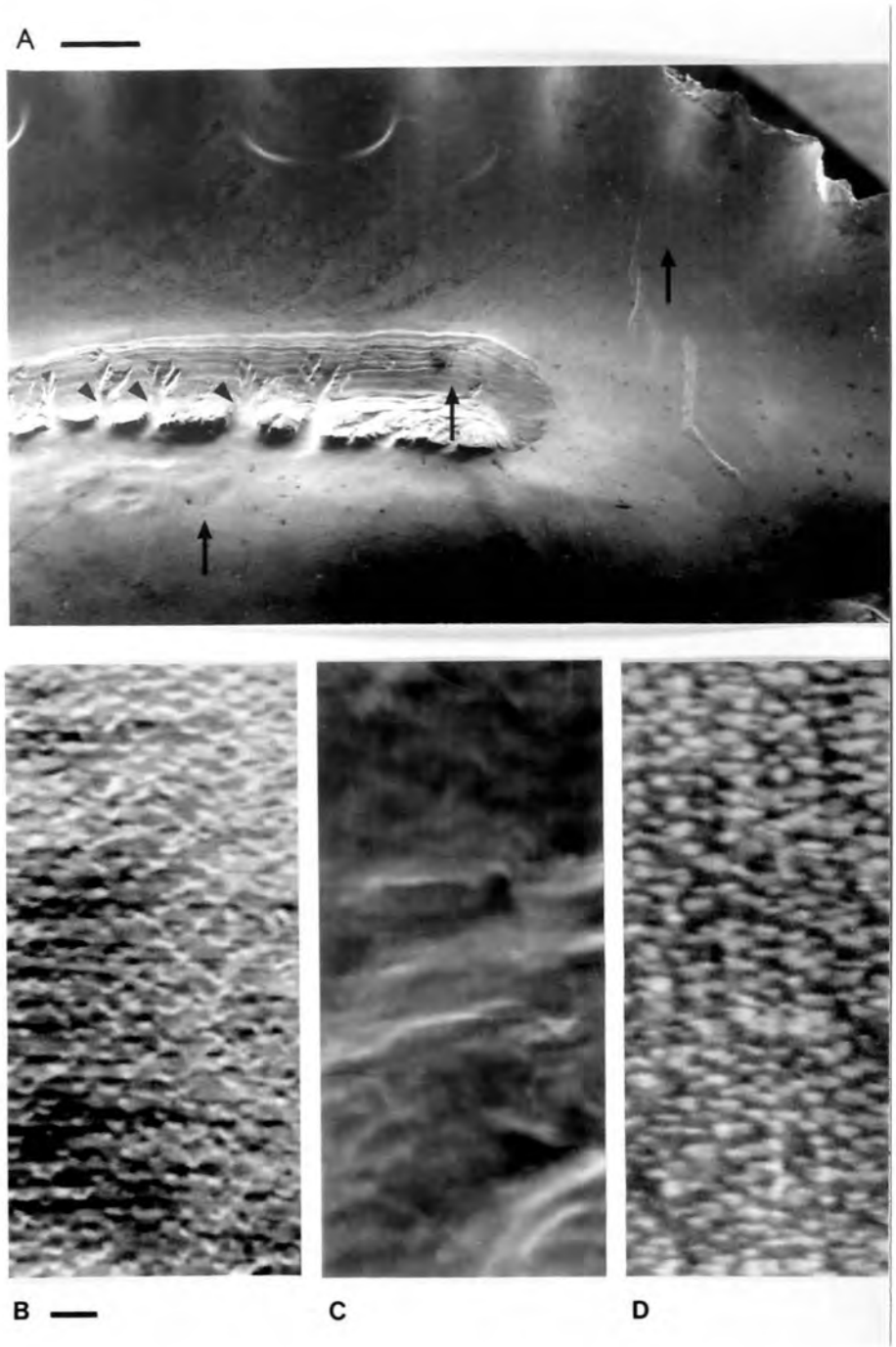

Figure 6.9. As for Figure 6.3 but from *Siphonaria aspera*. Note the crenulated scar edge (arrowheads).

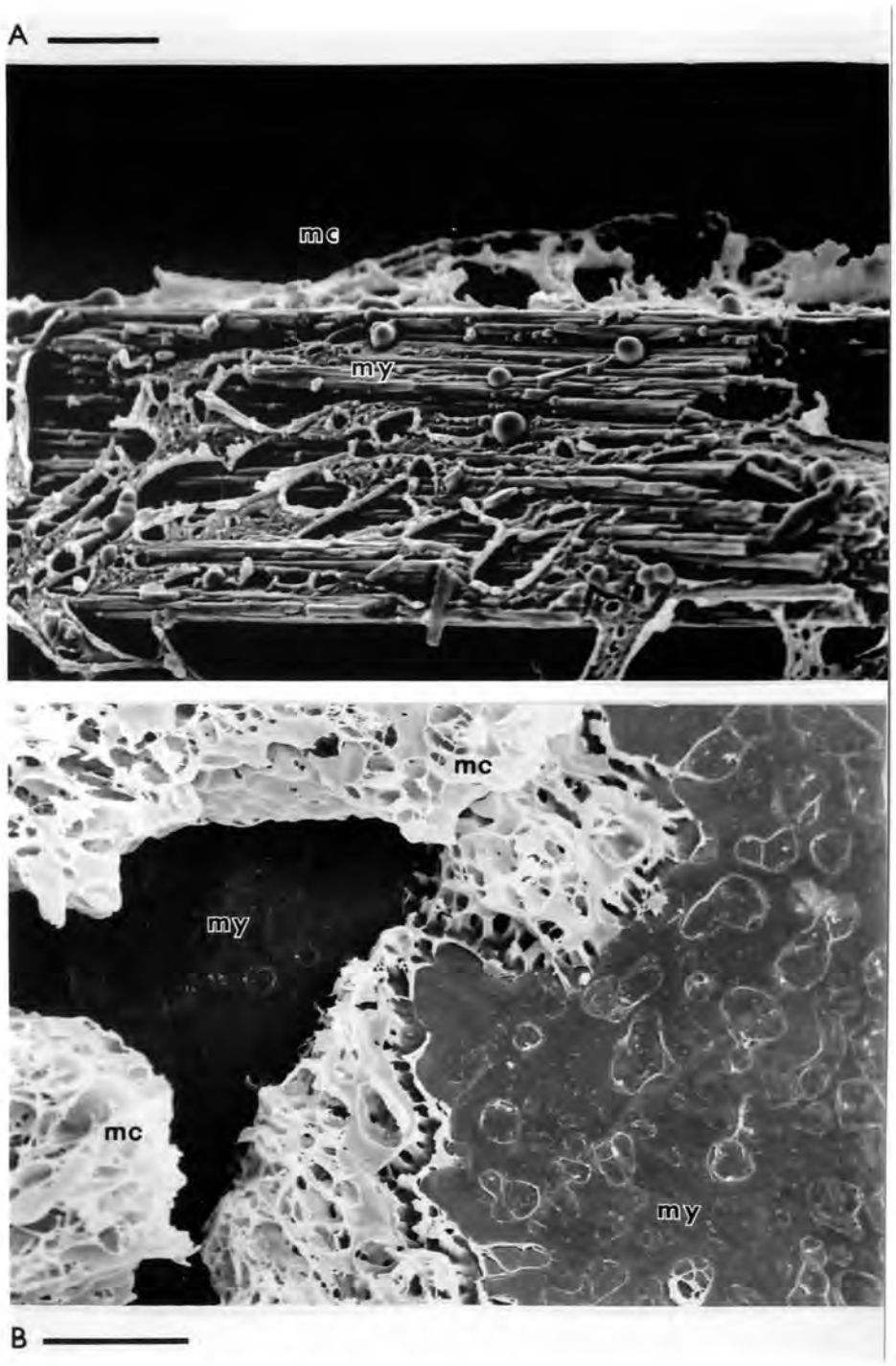

Figure 6.10. Scanning electron micrographs of shell muscle attachment site from *P. oculus*. After freeze fracturing the shell with muscle attached, the muscle cleaves off. There is no evidence of muscle fibres inserting into the shell myostracum (my), but a layer of mucus material (mc) is present. A: ventro-dorsal section. Scale bar = 10 μm. B: direct view of myostracum surface showing mucus (mc). Scale bar = 10 μm.

but for a small overlap between *P. tabularis* and *P. longicosta* (Fig. 6.14B) and are all significantly different at the 90 % level.

6.4 DISCUSSION

This study shows that near the shell attachment site, the columellar muscle of *Oxystele sinensis*, *Patella oculus* and *Helcion pruinosus* is composed of a dense matrix of collagen which is lined by a single layer of epithelial cells at the muscle-shell interface. This arrangement is similar to that described for other molluscs (Hubendick, 1958; Nakahara & Bevelander, 1970; Tompa & Watabe, 1976; Plesch, 1976; Smith, 1983). Hubendick (1958) described in addition, the presence of microvilli at the surface of the epithelial layer of the pulmonate limpet *Acroloxus lacustris* which insert into pits in the shell. However, in her study of the shell attachment of *Lymnaea* shell muscle (1976), Plesch showed that no pits or pores of a size suitable to accommodate these microvilli (0.15-0.2 μm diameter) were present at the shell surface. The results of this study show that no such pits are present in the shell muscle scar surface of limpets.

It has been shown however, that smaller organic fibres (3-4 nm diameter) extend into the shell in the bivalve mollusc *Pinctada radiata* (Nakahara & Bevelander, 1970) and the land snails *Helix aspersa* and *Anguispira alternata*, the feshwater pulmonate *Laevipex* sp. and the freshwater prosobranch *Pomacea paludosa* (Tompa & Watabe, 1976). However, these fibres are only visible in the shell after decalcification. Plesch (1976) makes no mention of fine organic fibres but did observe that a gel layer left at the attachment site after removal of the columellar, reacted positively for acid mucopolysaccharides and proteins. The composition of the gel was shown to be similar to that of other mucoid glues. She suggests that the adhesive epithelium attaches to the shell by means of this gel. The frozen mucus-like material observed at the muscle-shell interface of *P. oculus* may correspond to such an adhesive gel (Fig. 6.10).

Plesch (1976) suggests further, that the more robust attachment con-

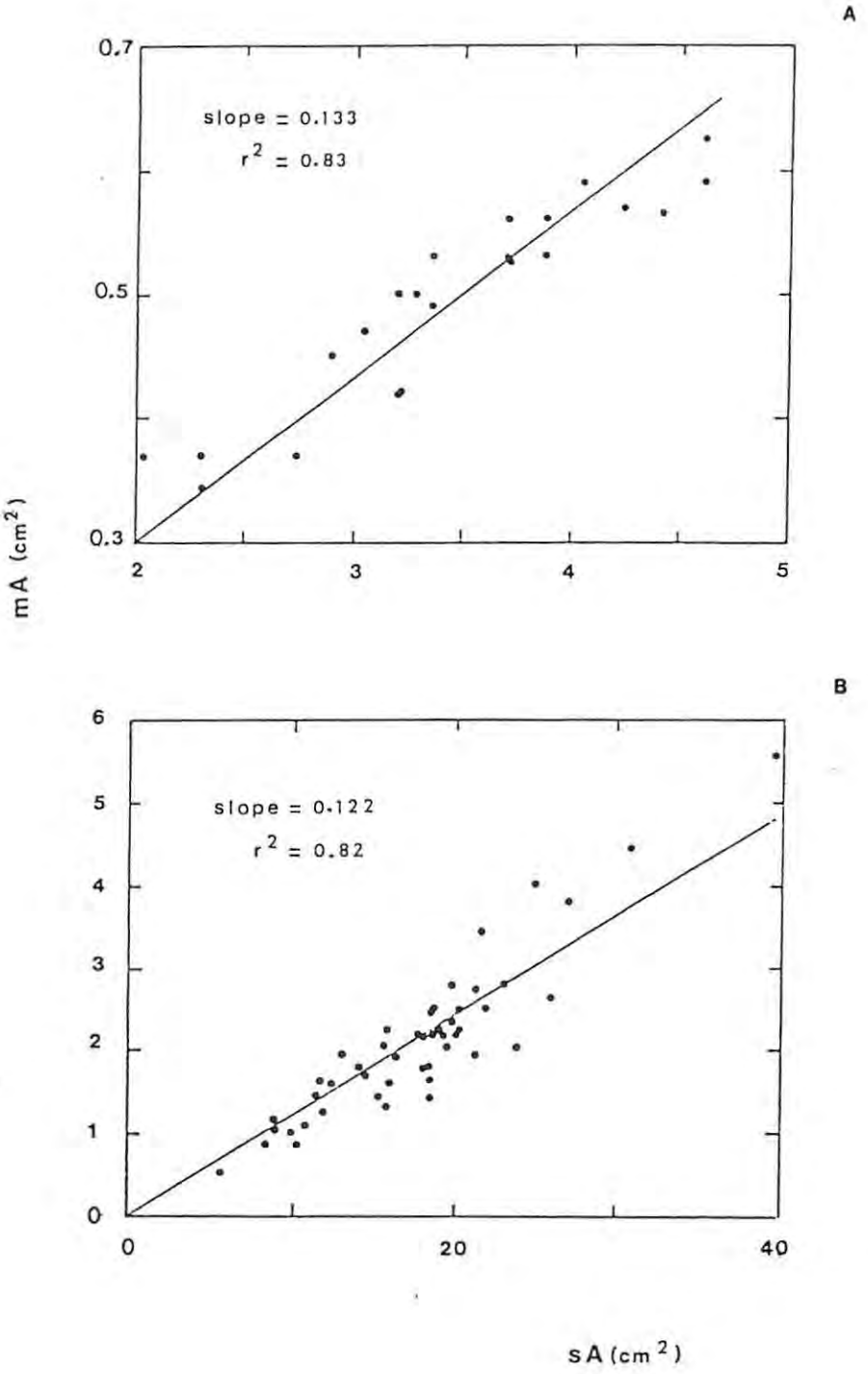

Figure 6.11. Linear regressions of mA against sA, the slope and r^2 values are given; A: *P. granularis*; B: *P. oculus*.

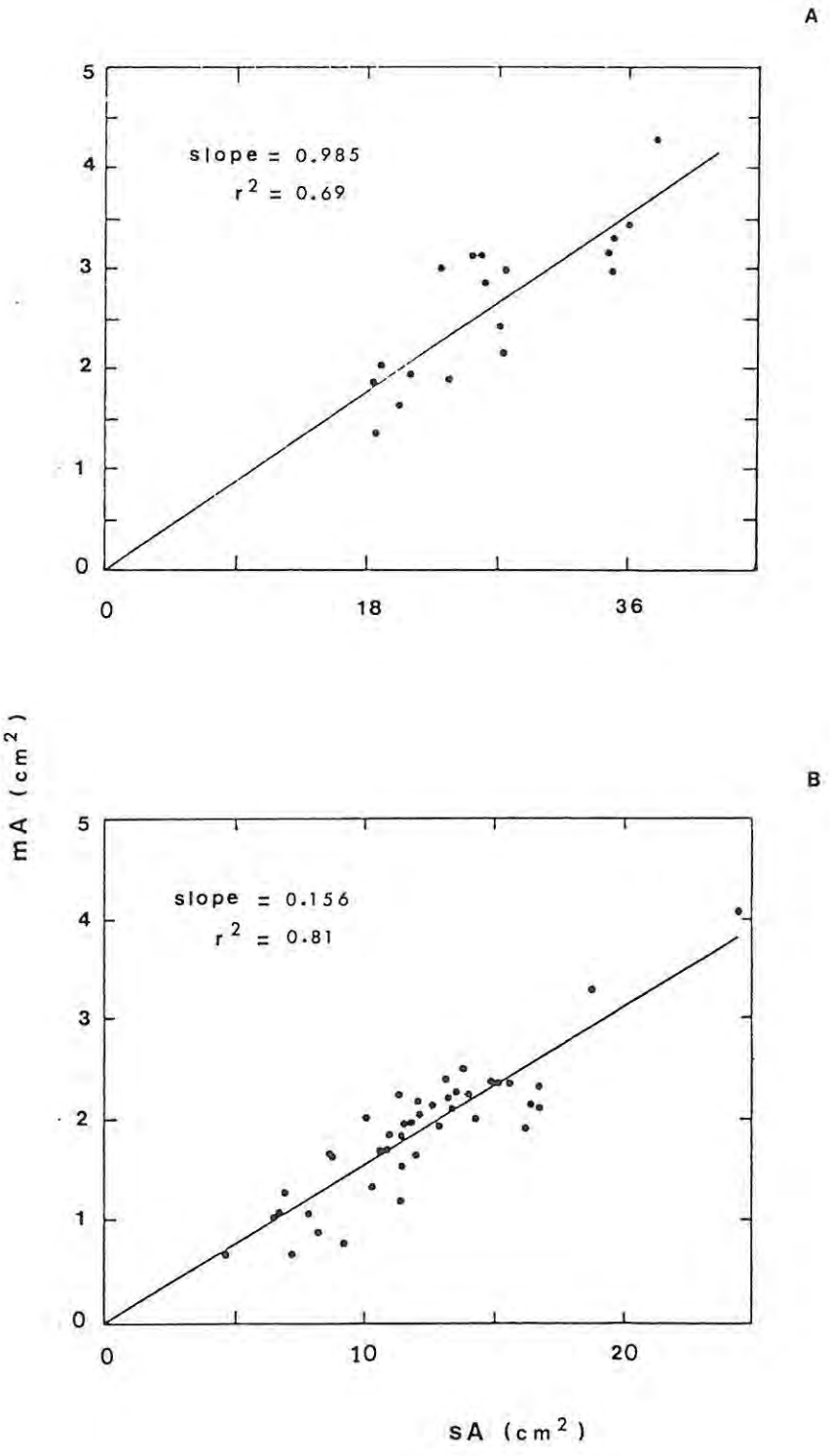

Figure 6.12. As for Figure 6.11 but from A: *P. miniata*; B: *P. longicosta.*

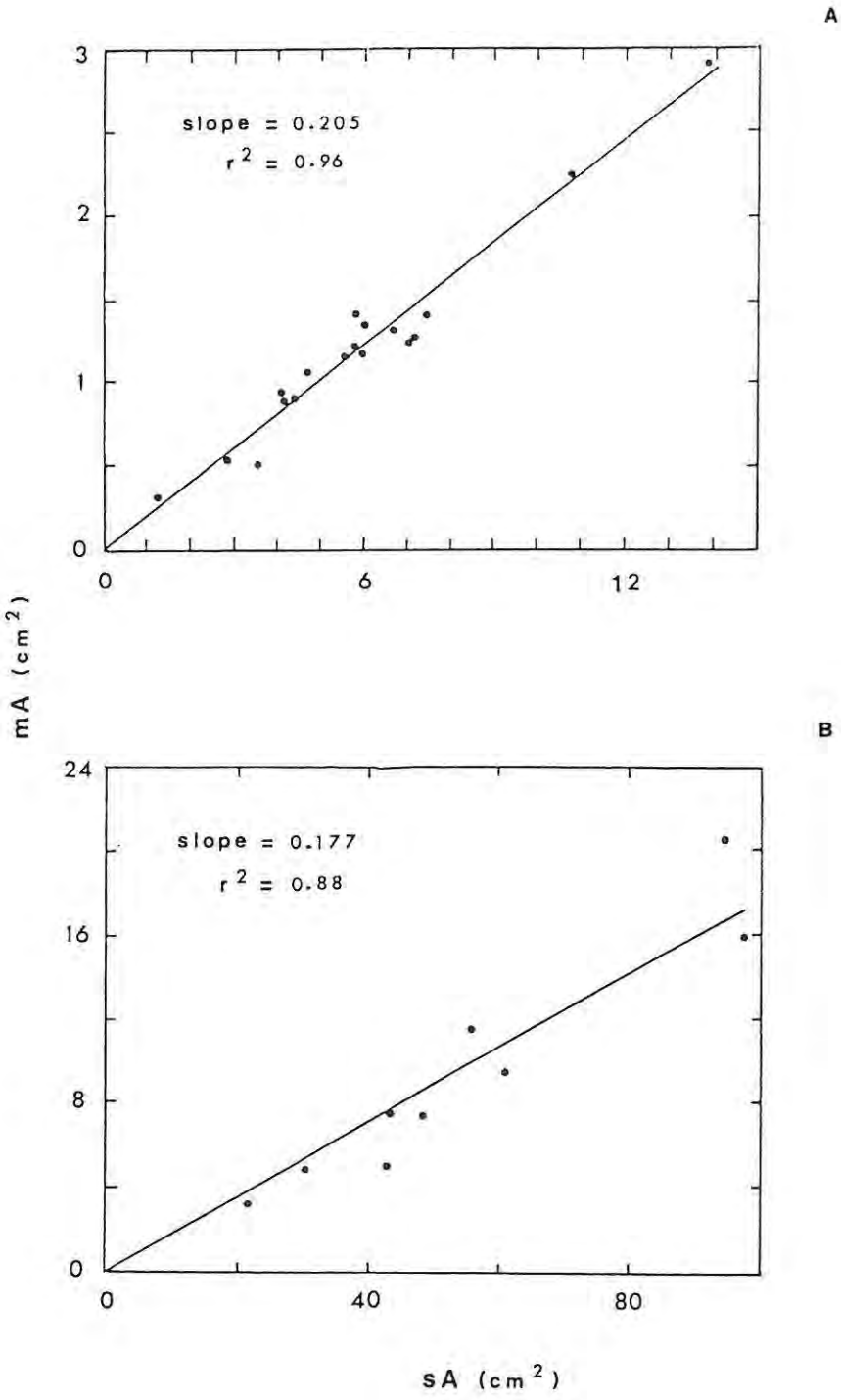

Figure 6.13. As for Figure 6.11 but from A: *P. cochlear*; B: *P. tabularis*.

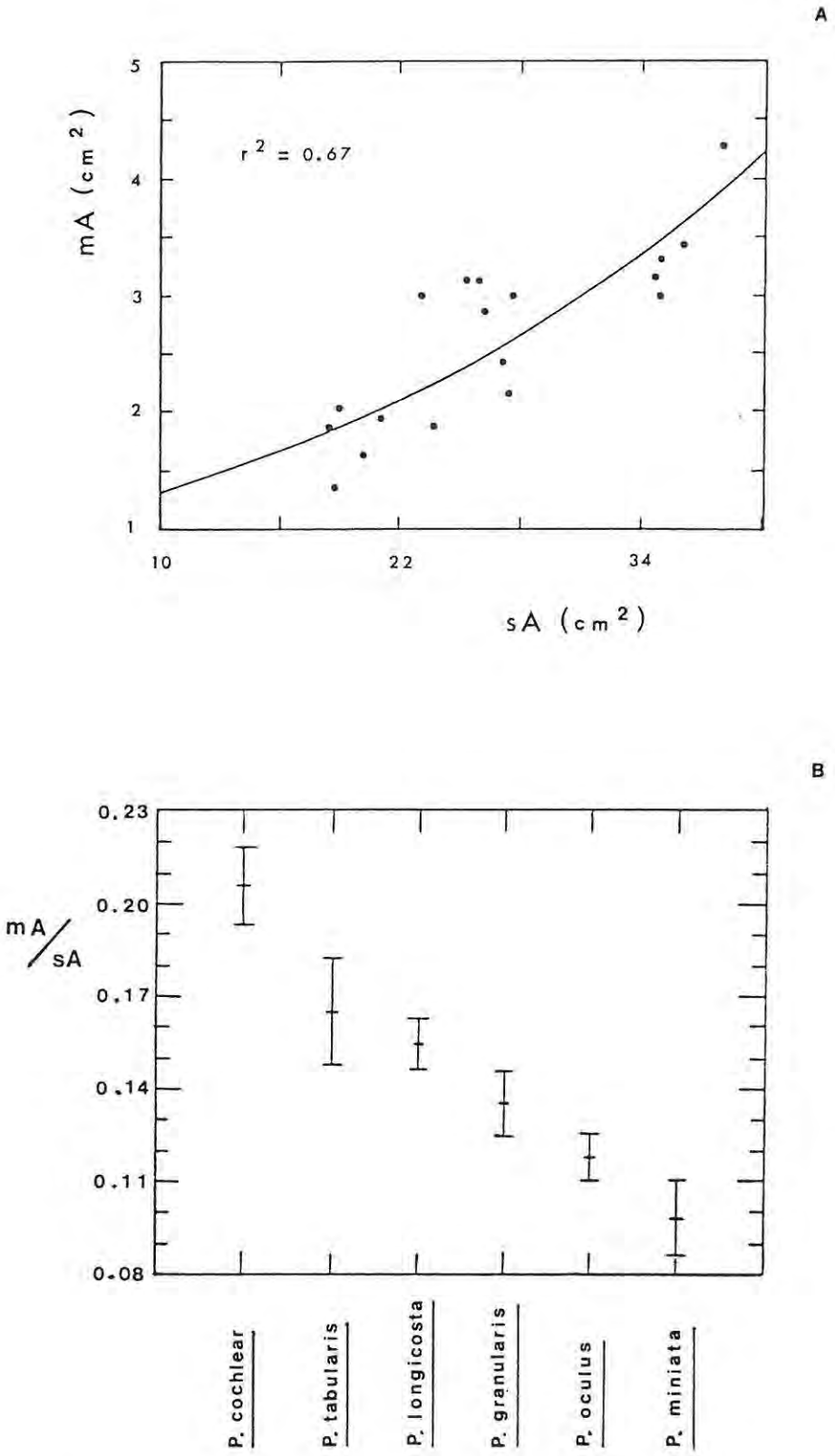

Figure 6.14. A: exponential plot of mA against sA for data from *P. miniata*; B: A plot of the means of the ratio mA/sA for six species of patellid limpet. Vertical bars represent 95 % confidence intervals. No significant overlap between species is shown at this confidence level except for a small amount between *P. tabularis* and *P. longicosta*.

Species	mA/sA	Wave Exposure	Tenacity*	Habitat Zone
P. miniata	0.098	+++	-	low to subtidal
P. oculus	0.118	+	1.95	high to mid
*P. granatina***	0.131	++	2.71	high to mid
P. granularis	0.136	++	3.25	high to mid
P. longicosta	0.155	+++	4.40	mid to low
P. tabularis	0.165	+++	-	subtidal
P. cochlear	0.206	++++	5.18	low
*P. argenvillei***	0.310	++++	4.67	low to subtidal

*(Kg/cm^2) Data from Branch & Marsh, 1978

** Insufficient specimen shells of these species were available for analysis in this study. However, rough estimates of mA/sA values from 2 - 3 specimens have been included in the Table for comparison.

Table 6.1. The ratio of area of shell muscle attachment (mA) to projected shell area (sA) compared to tenacity, habitat zone and wave exposure for a range of patellid limpets. Species are ranked according to increasing mA/sA values.

struction in *Pinctada* may be related to the larger forces exerted on the attachments by the adductor muscles. Hence she argues that the comparatively weaker"glue" construction is adequate for *Lymnaea* living in a quiet habitat of stagnant water. Following this reasoning one might expect limpets which inhabit high energy rocky shore environments to have the more robust construction. Although no organic fibres were seen in this study they cannot be discounted since high magnifications and shell decalcification are necessary to visualise them. It is worth pointing out however, that the very much larger muscle attachment area probably enhances the glue mechanism and would add to the strength of the muscle to shell attachment even if ultimately, fibrous connections are also shown to be present.

Plunging the shell into liquid nitrogen always resulted in detachment of the columellar muscle from the shell surface. This observation suggests that the properties of the adhesive gel are destroyed or at least chemically altered at this temperature (about - 150 °C) giving rise to a weakening of adhesion followed by a clean break. Perhaps also pertinent here, is the observation that the columellar muscle of newly dead or moribund limpets as well as other gastropods, is readily peeled off the attachment site, leaving a clean scar with no remaining tissue. Presumably, the adhesive properties of the gel are biologically maintained only as long as the animal is alive and healthy, but are lost in sick, dying or dead animals.

This work shows that the relation between the variables muscle attachment area and projected shell area (an approximation of foot area), is linear in most species and that the ratio mA to sA is therefore a species-specific quantity (Fig. 6.14B). Comparisons can thus be made between species whatever the variation in size due to age or natural variation. Table 6.1 shows that there is a positive correlation between the ratio mA to sA and the parameters tenacity, tidal height and wave exposure. Branch & Marsh (1978) have data on muscle attachment areas but give no indication of how these were measured. They show that muscle attachment area and base of foot area are two of several variables related to tenacity in several species of

patellid limpet. That is, the larger the tenacity and foot area the larger the area of muscle attachment site. Data from this study with larger N values and results from additional species, support most of Branch & Marsh's findings (1978) (Table 6.1). The data for *P. miniata* however, do not fit the correlation trends shown in Table 6.1. It could be argued that *P. miniata* minimises the wave action it experiences because of its low flat shell and so does not need a large area of muscle insertion. But Branch & Marsh (1978) have shown that shell shape is not correlated to tenacity. Alternatively, it could be argued that juveniles of this species shelter (Branch, 1981) because they are less well accommodated to withstand wave action, and therefore have a reduced mA to sA ratio. In fact the tendency towards a non linear curve (Fig. 6.14A) is some evidence that this is so. But the curvature is not very great so this alone is unlikely to account for the unexpectedly low mA to sA value. The lower R-squared value in any case indicates that the real trend in *P. miniata* could be obscured and that an increased sample size might help to clarify this.

P. tabularis which is a subtidal species gives an mA to sA ratio that is smaller than *P. cochlear*, a low shore species. Although this does not follow the trend of correlation between habitat zone and muscle scar area, it is argued that subtidal species experience less wave action than low shore species. Hence a correlation does exist between the ratio mA to sA and wave exposure (Table 6.1).

P. granularis being a high shore species where little wave action occurs might have been expected to have the smallest ratio; however, it has a larger mA to sA value than *P. oculus*. Branch & Marsh (1978) obtained a similar result but stated that the migratory habit of this species causes it to experience a wide range of wave action from very sheltered to moderately strong. Thus a compromise is made between a smaller area of muscle insertion which is common to more mobile species (Branch & Marsh, 1978) and a larger area which is common to species with higher tenacities.

6.5 SUMMARY

- Histological studies show that the columellar muscles at the muscle-shell attachment site of limpets and *Oxystele* bears a special epithelial layer similar to that described in other molluscs.
- Scanning electron microscopy indicates that a layer of mucus material lies at the interface between this epithelial layer and the shell which may function as an adhesive glue.
- The surface structure of the shell muscle scar of patellids is relatively more coarse than the surface elsewhere in the shell interior, but is relatively smoother in *Siphonaria*.
- The ratio of muscle area attachment to projected shell area of limpets is shown to be a species-specific parameter which can be correlated with tenacity and wave exposure in patellid limpets.

Chapter 7

CONTRACTION CHARACTERISTICS OF LIMPET COLUMELLAR MUSCLE : A TEST FOR CATCH

7.1 INTRODUCTION

Catch is a state found in many invertebrate muscles containing paramyosin in which muscle tension is maintained with minimum energy expenditure (for reviews see Ruëgg, 1971; Twarog, 1979; Muneoka & Twarog, 1983). Extensive work has been done on the mechanical behaviour of bivalve muscles which offer some of the best examples of catch muscle. The anterior byssus retractor muscle (ABRM) of the bivalve *Mytilus* has been particularly well studied in this respect being especially suited to experimental investigation (Winton, 1937; Singh, 1938a, 1938b; Twarog, 1954, 1959, 1967, 1973; Jewell, 1959; Cornelius & Lowy, 1978; Bennett & Elliott, 1989). By contrast, very few studies have been done on the mechanical behaviour of gastropod muscle (one example is the pharynx retractor of *Helix*, Abbott & Lowy, 1957). Since the ultrastructure of limpet columellar muscle has been shown to be very similar to that of the ABRM (Frescura & Hodgson, 1990) it is reasonable to hypothesise that the mechanical behaviour might also be similar. A catch state in the columellar muscle of limpets would be advantageous to limpets in allowing them to clamp economically for long periods of time.

Any interpretation of the mechanical properties of muscle must take into account the structure of the subject material. A thorough knowledge of fine structure is therefore an essential step to understanding the mechanical properties of muscle, as is the uniformity of the cells or groups of cells on which measurements are made (Hill, 1950; Ishii & Takahashi, 1981). Confronted with the comparatively disorganised muscular hydrostat of a limpet columellar where muscle fibres are inextricably mixed with collagen

(Frescura & Hodgson, 1990, Chapters 2 & 3) the problems may seem insuperable. Nevertheless, by taking suitable measures, it has been possible to obtain some useful information about the mechanical behaviour of less amenable muscle systems (e.g. pharynx retractor of snail (*Helix*), Abbott & Lowy, 1956; mammalian vascular smooth muscle, Dobrin, 1973; body wall of leech (*Haemopis*), Miller & Aidley, 1973; Miller, 1975). It is demonstrated in both this Chapter and Chapter 8, that by judicious isolation of muscle from the limpet columellar region, many obstacles can be sufficiently overcome to allow several mechanical properties to be determined.

Muscles are composed of a contractile component and an elastic component which is present both in series and in parallel with the contractile material (Hill, 1950). If the mechanical properties of the muscle investigated are to reflect the behaviour of its individual contractile fibres the complication of the non-contractile elastic material must be avoided or at least minimised (Hill, 1950). In the case of limpet columellar muscle this material forms an essential part of the muscle system and is largely unavoidable. Thus the mechanical properties investigated in this work reflect the behaviour of the entire complex of limpet columellar muscle and not only the contractile component.

The ultimate objective in this Chapter is to test the hypothesis that limpet columellar muscle is a catch muscle. The rationale for this is first, the similarity of the ultrastructure of the muscle with that of the classic example of catch muscle, the ABRM (Chapter 3); and second, the established paramyosin nature of the thick filaments (Chapter 5), a prerequisite for catch muscle systems (Bennett & Elliott, 1987). Although catch, activated by excitatory nerves which release acetylcholine (ACh) has been observed in phyla other than molluscs (Muneoka & Twarog, 1983), control of catch by relaxing neurons is a feature unique to molluscs (Muneoka & Twarog, 1983). The monoamine 5-hydroxytryptamine (5-HT), shown to be present in the ABRM (Twarog, 1954), not only relaxes catch but also modulates the response to ACh (Muneoka & Twarog, 1983). In view of this, a preliminary

pharmacological study was performed, firstly to test which neurotransmitters initiated a response in whole limpet columellar muscle; secondly the mechanical properties of isolated limpet columellar muscle bundles were determined and compared with those of the ABRM and other invertebrate muscles; thirdly, tests were performed to determine whether or not 5-HT caused modulation of ACh-induced muscle contractions.

7.2 MATERIALS AND METHODS

Three species of *Patella* have been investigated. These are: two South African species, *P. oculus* and *P. barbara* and one North Atlantic species, *P. vulgata*. Anatomical investigations and histology showed that the most suitable regions of columellar muscle for isolation were the circumferential bundles (Fig. 2.1 & 2.2B; Chapter 2). These have relatively long parallel muscle fibres and thus uniformity of behaviour could be expected. An additional advantage was that at least one side would not be damaged by the dissection procedure. Since the circumferential sphincter bundle arguably does not have the same behavioural characteristics as the columellar muscle, some dorso-ventral bundle specimens were also prepared so that any differences between the mechanical properties of each muscle region could be assessed. In practice, after removal of the tarsal muscle, the dorso-ventral bundles were inconveniently short to work with. A further complication lay in the fact that the dorso-ventral bundles branch greatly (Chapter 2) and thus seemed likely to behave in a more complicated manner.

The apparatus to measure muscle tension from South African limpets consisted of an isometric transducer coupled to a strain gauge (Bioscience, Palmer & George Washington, U.K.). The output, which was proportional to the tension developed, was displayed on a chart recorder (George Washington, U.K., linearity better than 10 % , sensitivity 1 g cm^{-1} deflection) calibrated by suspending a 1 g weight from the transducer. A glass chamber with tap was attached to a clamp stand to house the muscle preparation and solutions in which the muscle was bathed (Fig. 7.1). The apparatus

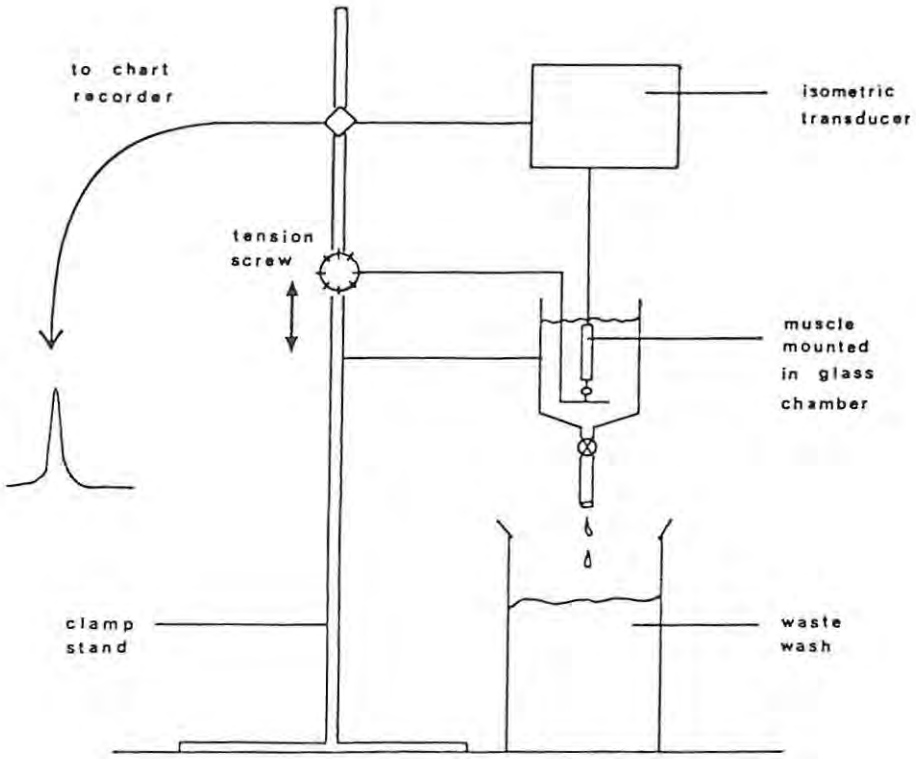

Figure 7.1. Schematic representation of apparatus for investigating the mechanical behaviour of isolated bundles of columellar muscle.

used to examine *P. vulgata* (courtesy of the Medical Research Council's Cell Biophysics Unit. U.K.) was essentially the same. Minor differences were a moveable perspex chamber for emptying solutions, an arrangement that held the muscle preparation horizontal instead of vertical and a calibrated potentiometer for recording the tensions developed (for more detail see Bennett & Elliott, 1989). All measurements on *P. vulgata* were made at 0 ^{0}C while measurements on South African species were made at room temperature and 0 ^{0}C (explained in Results Section 7.3.2).

7.2.1 Investigation of Response to Neurotransmitters

To determine which neurotransmitters would induce muscle contraction, the dorsal portion of the shell of live limpets was sawn off with a coping saw and the viscera removed leaving the pedal musculature attached to the shell (Fig. 7.2). The head, heart and ganglia were left intact. The neurotransmitters L-glutamate, dopamine, 5-hydroxytryptamine (5-HT) and acetylcholine (ACh) (Sigma Chemical Company, U.S.A.) were made up in sea water at concentrations ranging from 5.5 x 10^{-6} M to 5.5 x 10^{-4} M. These final concentrations have been shown to be effective in ABRM preparations (Cornelius & Lowy, 1978; Bennett & Elliott, 1989). The musculature was bathed by solutions containing the drugs and the response observed under a dissecting microscope. Sea water was used to wash out the drugs. The only neurotransmitter found to induce muscle contraction was ACh, which was accordingly used in all subsequent experiments. To examine whether an intact nervous system was necessary for contraction, the pleural and pedal ganglia which innervate the tarsal and columellar muscles respectively, were removed and ACh was again washed over the muscle.

7.2.2 Dissection and Muscle Preparation

Some dissections were done on fresh animals within a few hours of collection whilst others within one week on animals retained in an aquarium at a temperature of about 20 ^{0}C. To determine the characteristics of the muscle

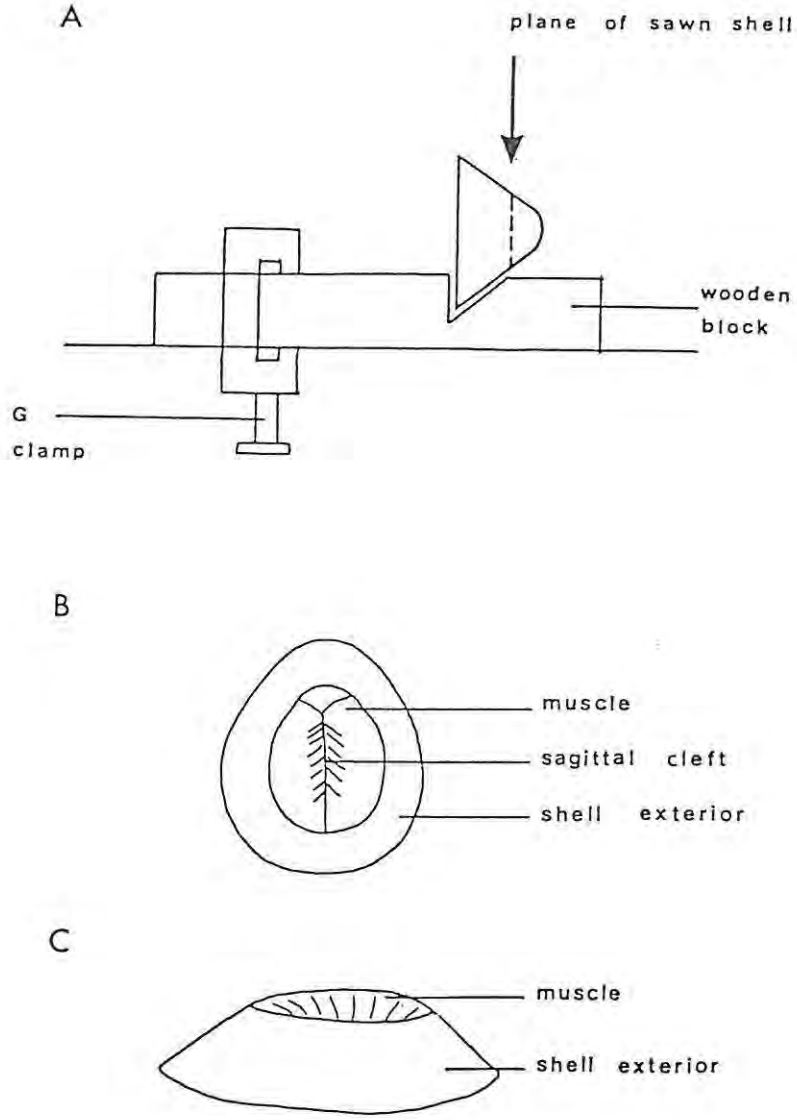

Figure 7.2. A: arrangement for removing the dorsal half of a limpet shell. B: dorsal view of limpet with dorsal portion of shell and viscera removed. C: sagittal section of limpet with dorsal portion of shell and viscera removed. Pharmacological agents can be applied to the pedal muscle and direct observation made.

contraction, pieces of circumferential tissue about 1 mm in diameter and with an *in situ* length of about 10 mm long were excised from the circumferential region and mounted as follows. Cotton loops were tied securely to both ends of the tissue pieces. One loop was attached to the isometric transducer the other to a fixed hook inside the water chamber (Fig. 7.1). The chamber was filled with sea water. Muscle preparations remained responsive between 12 and 30 hours without the need for an aerator in the chamber. A tension screw was turned until the tissue gave a small reading on the chart recorder to remove any slack (about 1 g) and then left to equilibrate for 1 hour.

To test if the muscle filaments within the fibres were well-oriented and homogeneous, circumferential muscle bundles were fixed while mounted and under tension as described above, the sea water being substituted with 5 % glutaraldehyde made up in 50 % sea water and 0.1 M sodium cacodylate, pH 7.0. The chart recorder and transducer were left in operation during this procedure to determine if any contraction occurred during fixation. None occurred. Tissue was transferred after half an hour to a fresh solution of 5 % glutaraldehyde. Fixed tissue was washed in 0.1 M cacodylate buffer made up in sea water, post fixed with 1 % osmium tetroxide in the same buffer vehicle for 90 minutes, dehydrated and embedded via propylene oxide in an Araldite CY 212/Taab 812 resin mixture (Cross, 1989). Silver/gold sections cut using glass knives were stained with 2 to 10 % methanolic uranyl acetate for 30 minutes. Material was viewed on a Jeol JEM 100 CXII electron microscope at 80 kv.

7.2.3 Contractions and Dose-Response

Using fresh muscle preparations the chamber was emptied of sea water and a solution of acetylcholine added so that it just covered the muscle. When the muscle contraction reached its peak, the chamber was emptied and refilled with sea water. This was followed by two more changes of sea water to ensure no traces of ACh were left. All contractions were induced by single dose

exposure. Examples of contractions left to run their course to full relaxation before removal of ACh were also obtained (the continued presence of ACh is not required to maintain the catch state) (Twarog, 1954; Bennett & Elliott, 1989). However, it is not entirely clear what this means physiologically, as *in vivo*, the action of ACh is rapidly destroyed by cholinesterase (Katz, 1966). The tissue was left to recover for 10-20 minutes between contractions. Shorter recovery periods led to progressively weaker contractions. Tissue life-time varied from sample to sample but usually 12-15 full contractions could be obtained before the sample began to fatigue or deteriorate, as shown by poor or inconsistent response to ACh. Contractions were repeated for a range of ACh concentrations with two to three contractions at each dose. A check was made on possible muscle fatigue or damage by repeating initial doses at the end of the experimental run to ensure that contractions were still reproducible. Dose-response curves were obtained for each species investigated by plotting the mean of the tension maxima obtained from contractions at different ACh concentrations. The optimum ACh dose was taken as that at which 90 % full contraction occurred, since above this concentration, responses were not always reproducible.

7.2.4 Catch

A series of contractions was initiated over a period of several hours using ACh at the optimum dose range as determined above. The reason for this is that the rate of relaxation is often slower after successive treatments with ACh for the ABRM (Jewell, 1959; Bennett & Elliott, 1989) so that several cycles of treatment may be necessary to "prime" the muscle before it will go into the catch state. To test for catch, 5-HT (10^{-4} to 10^{-9} M) which is the most effective of several monoamines known to relax catch muscles (Muneoka & Twarog, 1983) was used in three ways: first, it was applied at the peak of contraction; second, it was applied in combination with 10^{-6} M ACh; third, the muscle was pretreated with a solution of 5-HT alone, before application of ACh.

7.3 RESULTS

7.3.1 Effects of Acetylcholine on Whole Pedal Muscle

Of the four pharmacological agents investigated only ACh elicited a response in the whole muscle preparation. Removal of both pleural and pedal ganglia had no visible effect on the ACh-induced contractions which were typically slow and asynchronous and caused distortion of discrete units of muscle on alternate sides of the saggital cleft. This phenomenon was observed with every preparation (n = 7). The alternating contractions appeared almost as a train of reflex responses gradually subsiding over a period of about 3 minutes.

7.3.2 Dose-Response from Isolated Columellar Muscle Bundles

Hoyle (1964) states that spontaneous activity (a feature typical of smooth muscle) seldom occurs in very small bundles of muscle as these are less likely to contain nerve elements. Spontaneous bursts of activity in isolated limpet columellar muscle bundles were, in fact, observed only twice over many months experimentation. Moreover, circumferential muscle bundles fixed under tension confirmed the suitability of choice of this region of muscle by showing that not only were the muscle fibres well oriented parallel to the long axis of the muscle but so were the filaments within the fibres (Fig. 7.3). Few type II cells were seen so the cells were reasonably homogeneous. Furthermore, results from the dorso-ventral and circumferential bundles were very similar (see Section 7.3.3); the more convenient circumferential bundles were accordingly used for all subsequent experiments. Thus, having minimised many of the obstacles previously outlined there appeared to be some prospect of determining aspects of the mechanical behaviour of limpet columellar muscle *bona fide*.

Initial trials were done on *P. vulgata* with tissue on ice in order to minimise the rate of deterioration of the muscle. However, the species *P. oculus*

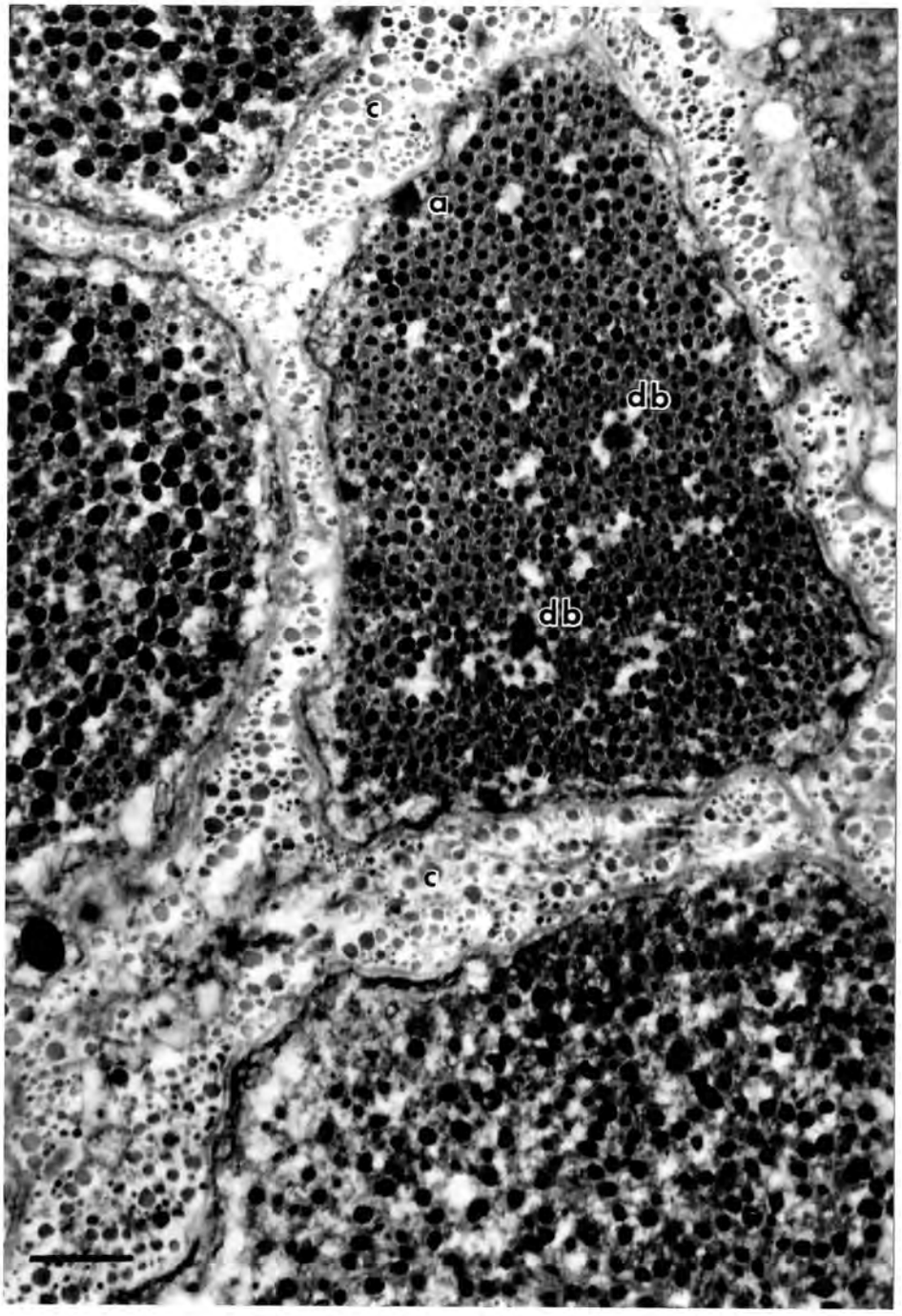

Figure 7.3. Electron micrograph of transverse section of circumferential muscle bundle from *P. oculus*, fixed while under 1 g passive tension. Note how the cells and thick filaments within the cells are all in true transverse alignment. Scale bar = 0.5 μm. a: attachment plaque, c: collagen, db: dense body.

and *P. barbara* gave such weak responses at this temperature that subsequent recordings were made at room temperature; tissue remained active at this temperature without any deleterious effects. It is possible that South African species respond better at higher temperatures than *P. vulgata* because of the warmer climate in which they live.

The optimum ACh concentration as determined from the dose-response study (Fig. 7.4) occurs between 5.5 x 10^{-4} and 5.5 x 10^{-5} M. Although Cornelius & Lowy (1978) found the ABRM was damaged at doses not much higher than 5.5 x 10^{-5} M, limpet columellar responses were still reproducible at 5.5 x 10^{-4} M. However, preparations of limpet columellar muscle stimulated at doses above this began to plateau and were unreliable in that they were not uniform, declined rapidly or became erratic. The optimum ACh concentration did not vary considerably between species, but the maximum tensions attained did (Fig. 7.4) because of variations in the initial length of the muscle; length-tension relations are examined further in Chapter 8. In addition, any variation in the cross-sectional area between muscle bundles will produce different tensions, larger cross-sections yielding larger tensions.

7.3.3 Contraction Characteristics and Tests for Catch

ACh at 5.5 x 10^{-4} to 5.5 x 10^{-5} M typically induced a slow contraction with a rising phase of about 55 s and a relaxation time of 210 s for *P. vulgata* from both dorso-ventral and circumferential preparations (Fig. 7. 5A & B) and a rising phase of 10-13 s with a relaxation of 64-70 s for South African limpets (Fig.7.6; Table 7.1). Under the equilibration conditions described in Section 7.2 at an ACh dose of 5.5 x 10^{-4} M, maximum active tensions were about 3 g mm^{-2} (Table 7.1). However, it is shown in Chapter 8 that higher tensions can be obtained depending on the length of the muscle. Figure 7.5D shows the response of the muscle when ACh is not removed at the peak of the contraction. No response to 5-HT in any of the three modes of application was found. Furthermore, neither higher concentrations of 5-HT (10^{-4} M) nor lower (10^{-9} M) made any difference to the absence of response to 5-HT.

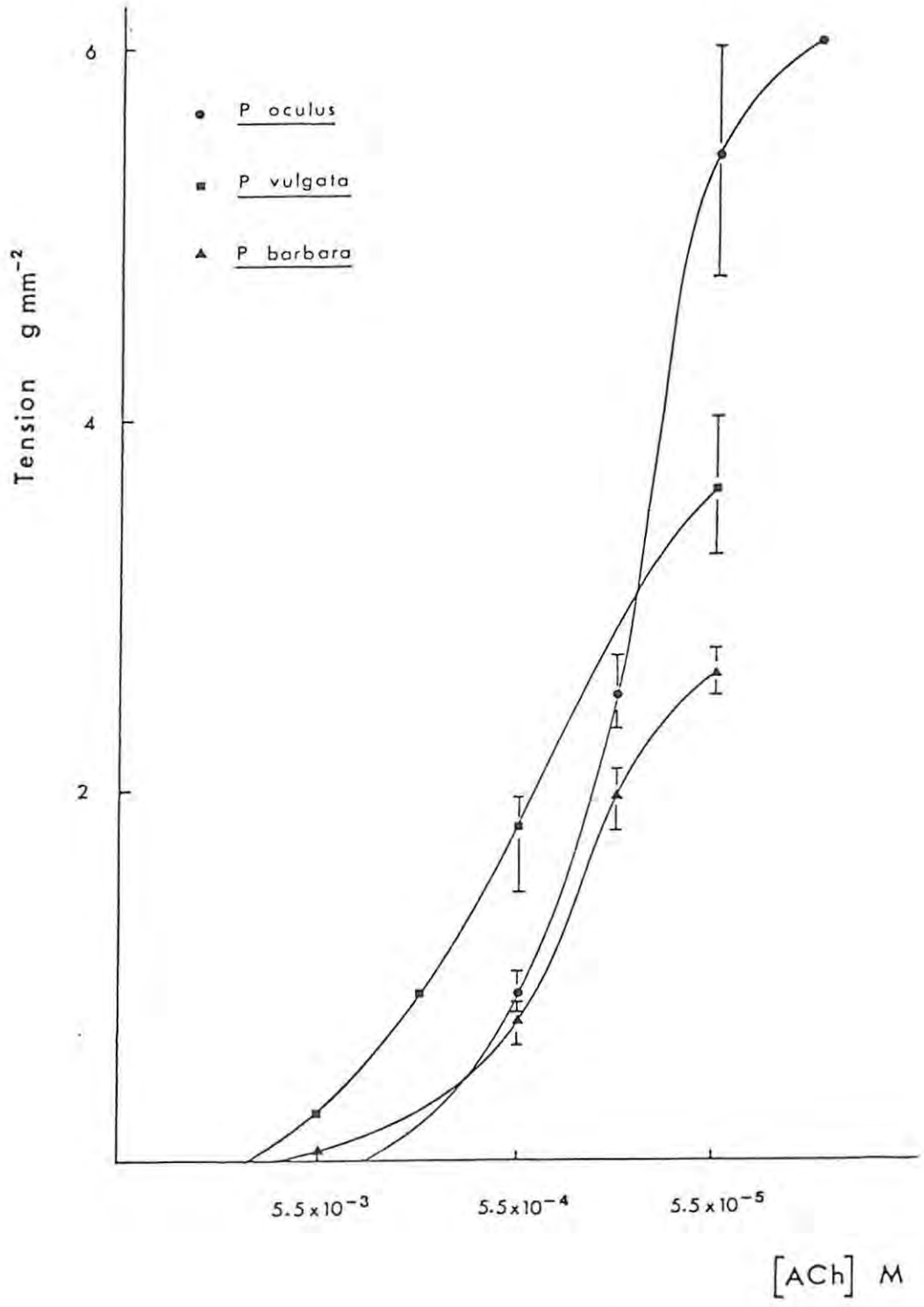

Figure 7.4. Dose-response curves obtained from the means of the maximum tensions of ACh-induced contractions exposed to single doses from the columellar muscle of *P. oculus*, *P. vulgata* and *P. barbara*. Vertical bars = Range (between 2 & 4 readings). Where no bars are present readings were identical.

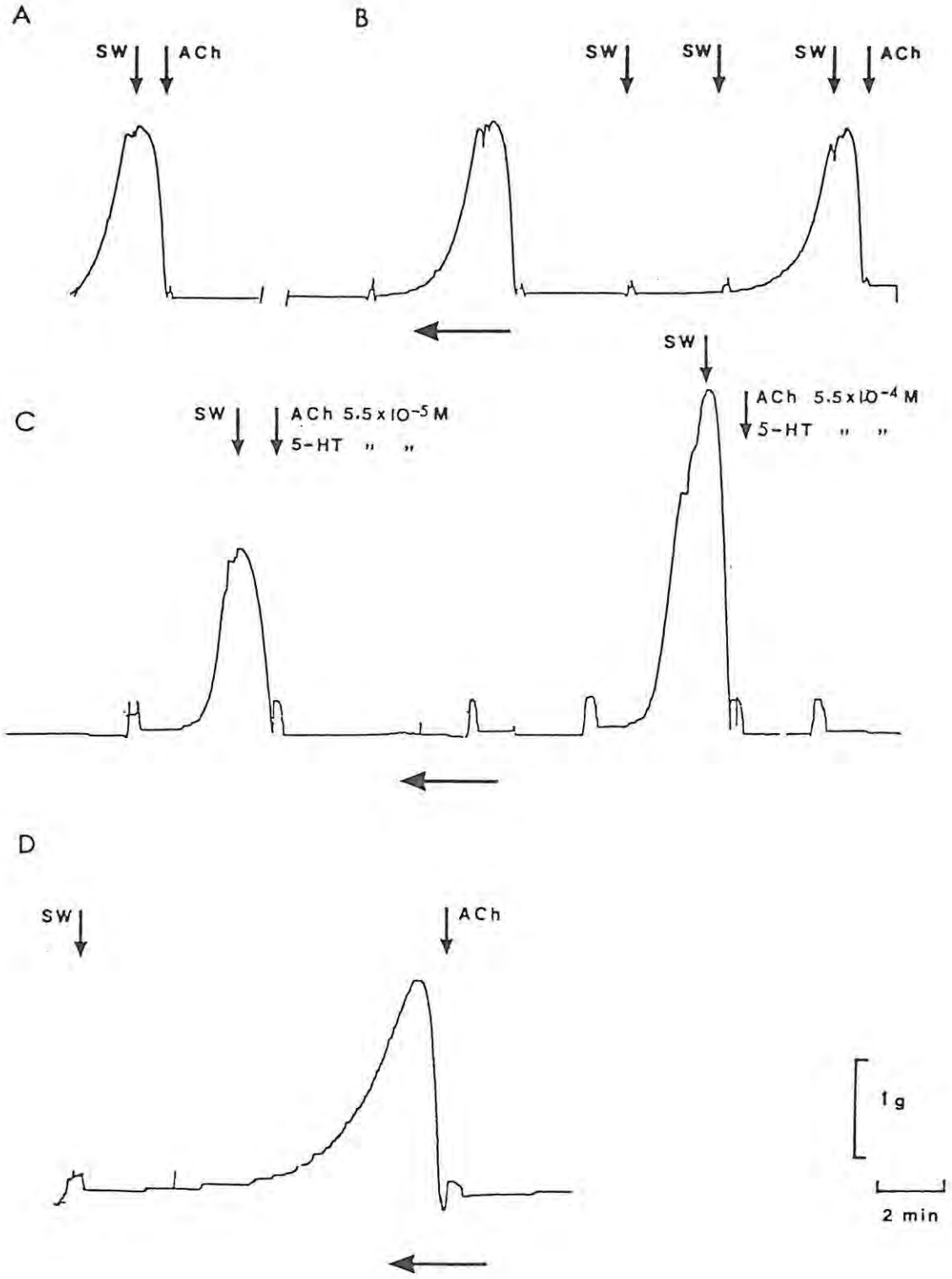

Figure 7.5. Traces of ACh-induced contractions from isolated columellar muscle bundles of *P. vulgata*. A: trace from dorso-ventral muscle bundle; SW: sea water wash; ACh: Acetylcholine (5.5×10^{-5} M unless otherwise stated). B: trace from circumferential muscle bundle. Note the similar response to that in A. C: trace from circumferential muscle bundle, ACh combined with 5-HT. D: trace from circumferential muscle bundle, ACh not washed out until full relaxation attained. Large horizontal arrows indicate direction in which trace is to be read.

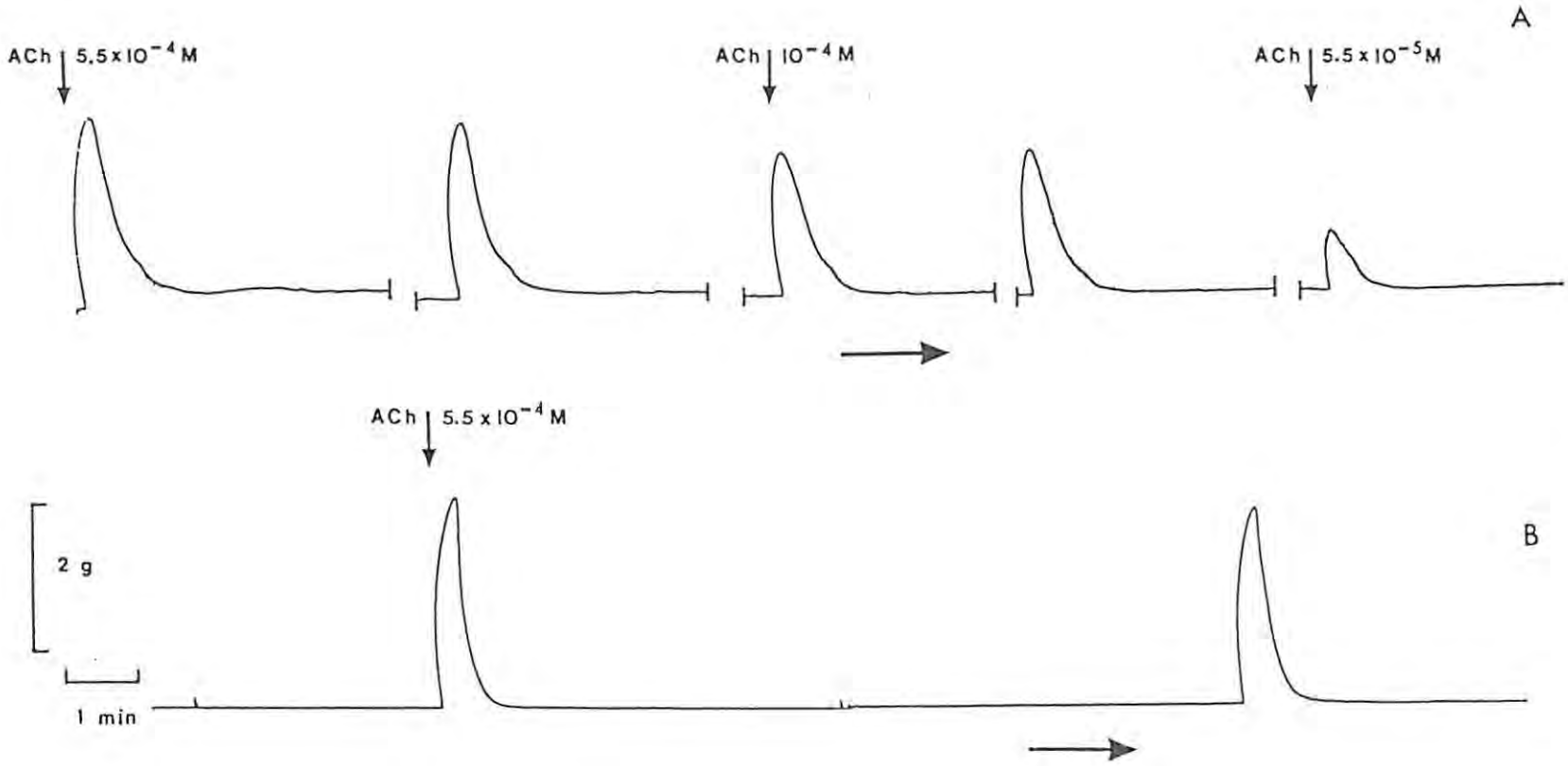

Figure 7.6. Traces of ACh-induced contractions from isolated columellar muscle bundles from A: *P. barbara*, B: *P. oculus*. Large arrows indicate direction in which traces are to be read.

Species	Temperature ^{0} C	Time to maximum contraction (s) Mean ± SD	Time to half relaxation (s) Mean ± SD	Time to full relaxation (s) Mean ± SD	N	[ACh] M	Maximum tension developed (g mm^{-2}) Mean ± SD	N
P. vulgata	0 ^{0} C	55 ± 17	67 ± 16	210 ± 55	18	5.5 x 10^{-5}	1.7 ± 0.2	14
P. oculus	20 ^{0} C	13 ± 5	15 ± 5	70 ± 20	10	5.5 x 10^{-4}	2.8 ± 0.5	10
P. barbara	20 ^{0} C	10 ± 4	27 ± 5	64 ± 15	15	5.5 x 10^{-4}	2.2 ± 0.5	6

Table 7.1. Properties of ACh-induced contractions of isolated bundles of columellar muscle from three patellid limpets. Maximum tensions are dependent on initial length (Ch. 8). Since length was not standardised in this study, only the largest values were measured. More accurate values are obtained from investigations in Chapter 8.

Figure 7.5C shows a response to 5-HT and ACh combined. The response is as if ACh alone was applied.

7.4 DISCUSSION

Takahashi (1957) demonstrated that ACh applied to the pedal ganglia of *Mytilus* caused inhibition of the tonic catch contraction evoked by applying the same stimulant to the muscle. However, no difference could be detected in the response of whole limpet columellar muscle when the pedal and pleural ganglia were intact or absent. The fact that intact pleural and pedal ganglia were not necessary for contraction of the whole muscle indicates that ACh is acting directly on the the muscle via postsynaptic receptors, perhaps by effecting release of Ca^{++} into the sarcoplasm via the ACh receptor (Muneoka & Twarog, 1983). Twarog (1954) deduced that the action of ACh was direct on the ABRM. The fact that contractions can be induced in small preparations of isolated limpet columellar muscle with negligible neuronal tissue supports this idea which may be more widely applicable to molluscan smooth muscles.

The asynchronous contraction of discrete muscle units in the whole muscle might be understood in terms of the muscular hydrostat organisation if it could be shown that each collagen-ensheathed muscle bundle (Chapters 2 & 3) constitutes a pharmacologic unit. Twarog, Dewey & Hidaka (1973) invalidated the assumption that muscle fibres of the ABRM run the whole length of the muscle (up to 4 cm) and showed that muscle fibres were organised into bundles, only some of which ran the entire length of the muscle. Fibres were not connected mechanically but formed attachments with connective tissue fibres. However, fibres within a bundle did maintain electrical continuity via nexal junctions. In fact, electrical coupling between fibres has been shown or implicated in many molluscan muscles (Muneoka & Twarog, 1983) and this seems a particularly appropriate method of neuromuscular control for the local contractions characteristic of a muscular hydrostat (Kier, 1988; Frescura & Hodgson, 1989).

Gastropods have been particularly useful subjects of neurobiological studies (Willows, 1985). However, the Prosobranchia are still somewhat neglected especially with respect to pharmacological study. Notable contributions to pharmacological knowledge of the ABRM have come from the work of Twarog (1954; 1959) and Takahashi (1957; 1960) amongst others (reviewed by Muneoka & Twarog, 1983). ACh is known to be effective as a neurotransmitter in a wide range of molluscan muscles, thus most are cholinergic innervated muscles, but not all. The striated part of the adductor from the bivalve *Pecten*, for example, does not respond to ACh (Hoyle, 1964).

Several workers (Twarog, 1954; Jewell, 1959; Cornelius & Lowy, 1978; Bennett & Elliott, 1989) have shown that the muscle catch phenomenon (Fig. 7.7A) in the ABRM responds to 5-HT in the following ways. First, catch is rapidly relaxed if 5-HT is applied during the slow relaxation phase (Fig. 7.7B); second, a phasic twitch is induced in response to an application of ACh and 5-HT combined (Fig. 7.7C); third, a phasic twitch response is potentiated after preliminary application of 5-HT (Fig. 7.7D) (Muneoka & Twarog, 1983). These applications of 5-HT have been used to test for catch in other muscles (Ishii & Takahashi, 1978; Bennett & Elliott; 1989). However, limpet columellar muscle showed no response to 5-HT.

The ABRM responds to various stimuli including ACh but the nature of the response can always be divided into two categories: phasic, in which the rising phase lasts 10-20 s and relaxation is complete within about 30-60 s, and tonic, or catch, in which the rising phase is similar but relaxation may take from several minutes to several hours (Twarog, 1954; Jewell, 1959). The difference between the phasic and tonic responses of the ABRM can be explained in terms of stimulation of both inhibitory and excitatory neurons at the same time (Hoyle, 1964). Strictly, stimulation of the inhibitory neuron does not inhibit the action of the excitatory neuron but has a plasticising or relaxing effect on a tonic contraction thus giving rise to the so-called phasic response.

Although a catch-like state has been observed in invertebrates other than

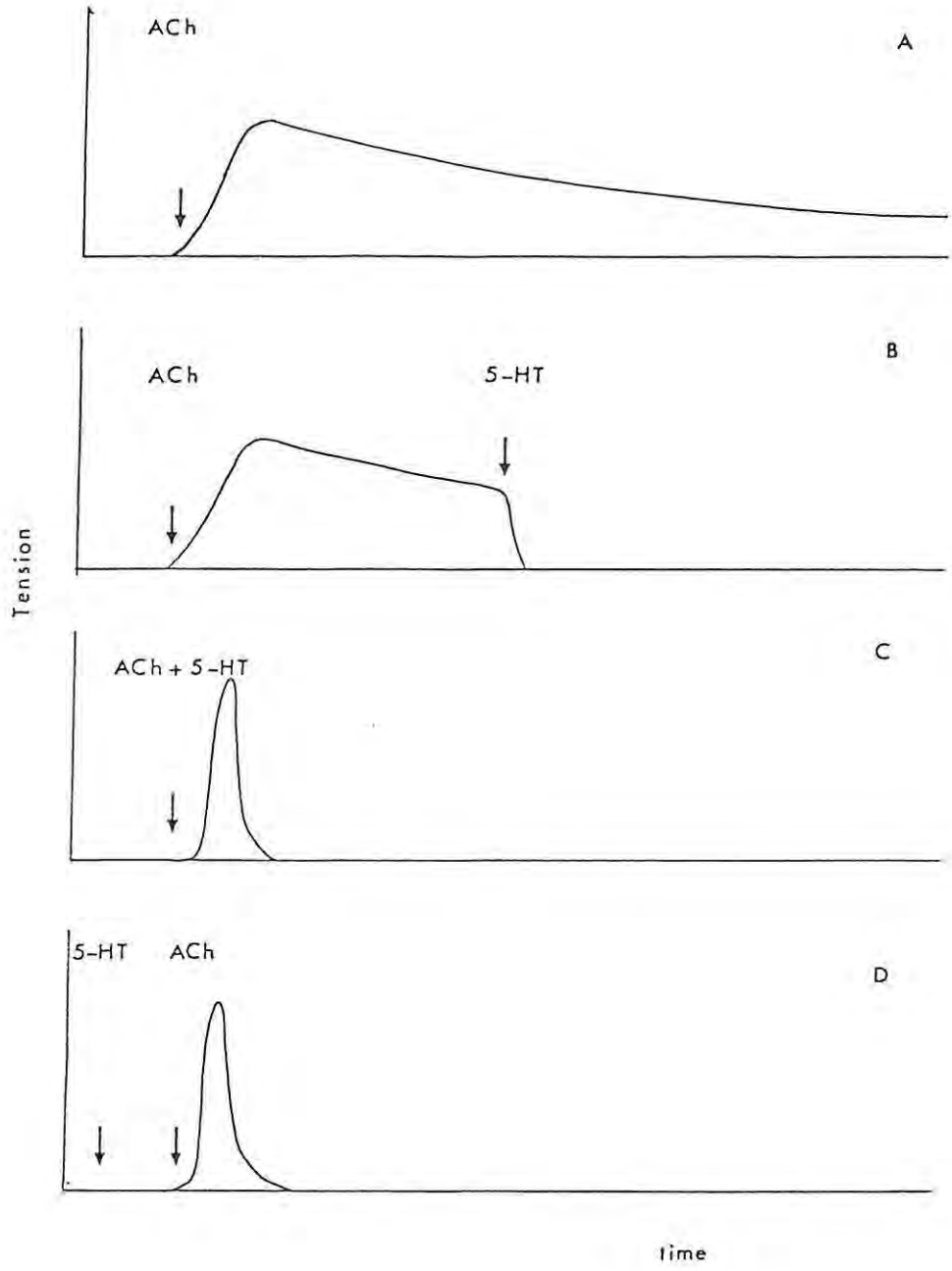

Figure 7.7. Diagrammatic illustrations of A: typical muscle catch response to ACh. B: typical response to 5-HT during catch tension, i.e. rapid relaxation. C: typical response to ACh in combination with 5-HT i.e. a twitch-like contraction. D: typical response to 5-HT applied prior to ACh i.e. a twitch-like contraction instead of catch (A).

molluscs such as the earthworm *Lumbricus* (Tashiro & Yamamoto, 1971) and the worm *Paragordius* (Swanson, 1971), control of catch by relaxing neurons seems unique to molluscs. The most well known relaxing agent, 5-HT, shown by Twarog (1954) to be present in the ABRM not only releases catch but modulates its excitability (Muneoka & Twarog, 1983). The contractions induced in limpet columellar muscle are slow when compared with those of striated muscles (Prosser, 1973) and are more like those of many smooth muscles (see Prosser, 1973). The slow contraction time is due to the compliance of the series elastic component (principally collagen sheaths). The rate of tension development is much reduced in muscles with less series elastic element (Prosser, 1973). But there is no evidence that a catch contraction as typified by the tonic response of the ABRM to ACh is employed by limpet columellar muscle under the experimental conditions of this study. Neither did 5-HT have any modulating effect on the muscle. However, it cannot be conclusively inferred that the muscle is not a catch muscle since it may be that a catch state is induced by different neurotransmitters to those considered in this study.

Catch muscles always have paramyosin thick filaments (Bennett & Elliott, 1987; Chantler, 1983) and are thus often assumed to have a role in the catch mechanism (Cohen, Szent-Gyorgyi & Kendrick-Jones, 1971; Ruëgg, 1971; Castellani & Cohen, 1987). The converse, however, is not always true. Ishii & Takahashi (1981), studying the posterior byssus retractor muscle (PBRM) and Castellani, Vibert and Cohen (1983), studying the pedal retractor muscle (both muscles from *Mytilus edulis*), showed that thick filaments were of the paramyosin type. However, neither muscle has been shown to have catch properties. It could be concluded that the performance characteristics of limpet columellar muscle are more akin to the PBRM and pedal retractors of *Mytilus edulis* than the ABRM. However, it will be shown from the Length-Tension investigations of Chapter 8 that such a conclusion may need qualification.

Abbott & Lowy (1958) studying the mechanical properties of the snail

Helix aspersa found the decay of isometric tension to be dependent on temperature. This would explain the difference in time scale between the columellar muscle contractions of *P. vulgata* and the South African limpets *P. oculus* and *P. barbara* made at different temperatures (Table 7.1). This was confirmed by comparing the time scale of responses from *P. oculus* at 0 ^{0}C as well as room temperature.

The maximum tensions attained in limpet columellar muscle seem very weak (0.2 to 0.3 kg cm^{-2}) when compared with those attained by the ABRM, which are at least an order of magnitude higher (up to 8 kg cm^{-2}, Hanson & Lowy, 1960; 10-12 kg cm^{-2}, Prosser, 1973; see Hoyle, 1964 & Prosser, 1973 for tables of maximum force developed in different muscles). This is perhaps surprising in view of the fact that such large loads are required to detach a limpet from its substratum (Branch & Marsh, 1978; Grenon & Walker, 1981). However, the recorded tensions in limpet columellar may be underestimated because of a contribution from all or some of the following factors:

1. the muscle is not a complete entity unlike the ABRM so that fibres at the periphery damaged by dissection may not contribute to tension development
2. some fibres may lie at angles other than axial which, when contracting, will make a smaller contribution to tension development by an amount proportional to the cosine of the angle at which they lie to the direction of tension development
3. still other fibres may lie perpendicular to the tension axis and thus make no contribution to tension development at all
4. some cells may be type II cells which have not been shown to be contractile
5. the presence of collagen fibrils and epithelia in parallel with the muscle fibres.

Precautions have been taken to minimise most of the above obstacles. However, those present to whatever extent, and this may well vary between each preparation, will contribute to a reduction in the number of contrac-

tile elements per cross-sectional area and thus lower the maximum tension attained.

It had been hoped that comparisons of maximum force could be made for a range of species and compared with habit and habitat. However, the above elements probably introduce more variation between preparations than already exist as natural variation between species. This makes comparisons meaningless until some method of standardising the source of variation can be found.

7.5 SUMMARY

- ACh induces contractions in limpet columellar muscle, whilst dopamine, L-glutamate and 5-HT do not. In addition, 5-HT does not induce reversal of ACh-induced contractions.
- The optimum ACh concentration for muscle contraction is 5.5 x 10^{-4} to 5.5 x 10^{-5} M.
- The mode of action of ACh is possibly direct on the postsynaptic ACh receptors of the muscle fibres, producing asynchronous local contractures in whole muscle which are probably related to the organisation of a muscular hydrostat.
- Typical responses are slow with a time scale of about one minute to full contraction and 2 minutes to full relaxation and with a maximum tension development of about 0.2 kg cm^{-2} for muscle at resting body length and ambient temperature.
- Results show no evidence of the classical muscle catch phenomenon in limpet columellar muscle under the experimental conditions of this investigation.

Chapter 8

LENGTH-TENSION RELATIONS OF LIMPET COLUMELLAR MUSCLE

8.1 INTRODUCTION

It has been found that under isometric conditions, the active tension (tension due to the contractile component of muscle) developed by all muscles, is a function of muscle length (Hanson & Lowy, 1960). In all the muscles studied, active tension reaches a maximum at a length, L_0, which is close to *in situ* lengths for most muscles and decreases on either side (in smooth muscles the maximum active tension often occurs at longer lengths, Prosser, 1973). The resting tension (tension due to the non-contractile component of muscle) rises non-linearly and is apparent usually at or soon after L_0 is exceeded (Hanson & Lowy, 1960; Prosser, 1973).

The length-tension relation has been determined for mammalian striated muscle (Gordon, Huxley & Julian, 1966) some oblique muscles (*Lumbricus* body wall, Hidaka, Kuriyama & Yamamoto, 1969; *Haemopis* body wall, Miller, 1975) and some molluscan smooth muscles (*Helix* pharynx retractor, Abbott & Lowy, 1956; ABRM of *Mytilus*, Cornelius & Lowy, 1978; PBRM of *Mytilus*, Ishii & Takahashi, 1981). The main differences are to be found in the working range of the muscles, that is, the range of lengths over which the muscle can develop active tension. Typically, smooth muscles have a wide working range (PBRM, 0.35-1.8 of L_0; snail pharynx, 0.3-2.0 of L_0; *Taenia coli*, 1.3-1.8 of L_0; see Prosser 1973 for review) while striated muscle, for instance, insect flight muscle, has a very short working range (about 0.9-1.1 of L_0). These differences are due primarily to the non-contractile elastic component in parallel with the contractile component of the muscle (Hill, 1950; Prosser, 1973). This is also reflected in the shape of the resting tension

curve which rises steeply in muscles with little or a very inextensible parallel elastic material (insect flight muscle) and rises less sharply in muscles with relatively more extensible connective tissue in parallel (Wilkie, 1976).

Since the structure of limpet columellar muscle is like that of the ABRM (Chapter 3 & 5) the length-tension relation might also be expected to show similarities. It is shown in this Chapter that the length-tension behaviour of limpet columellar muscle does not conform to that of the ABRM or any other molluscan smooth muscle. Rather, it displays distinctive characteristics that can be related to its function in the whole animal. In addition, a "catch-like" state is shown to be induced by acetylcholine at lengths close to L_0 only.

8.2 MATERIALS AND METHODS

Specimens of *P. oculus* were collected from the east coast of South Africa and retained live in an aerated aquarium at about 20 ^{0}C until dissected.

Investigations were made on circumferential bundles of the columellar muscle from *P. oculus*. This region has been shown to be the most convenient for studies on the mechanical behaviour of limpet columellar muscle (Chapter 7). Muscle bundles were isolated by making two parallel incisions in the muscle tissue 1mm apart and 10 mm long. A needle tip was inserted about 1mm below the surface of the tissue and used to tease the muscle bundle away from the rest of the muscle, the two extreme ends still attached to the columellar musculature. Cotton thread was passed under the bundle, ligatured and loops made at each end before the muscle bundle was excised. The distance between the 2 cotton ligatures *in situ* was taken as the resting body length of the muscle L_r (measured under a dissecting microscope). The isolated muscle bundle was mounted in a chamber filled with sea water as shown in Fig. 8.1. The tension screw was turned just enough to take up any slack and the muscle left to equilibrate for about one hour. A calibrated graticule was used in the eye-piece of the microscope for measuring the length of the mounted muscle.

Acetylcholine (ACh) (Sigma Chemical Company USA), shown previously

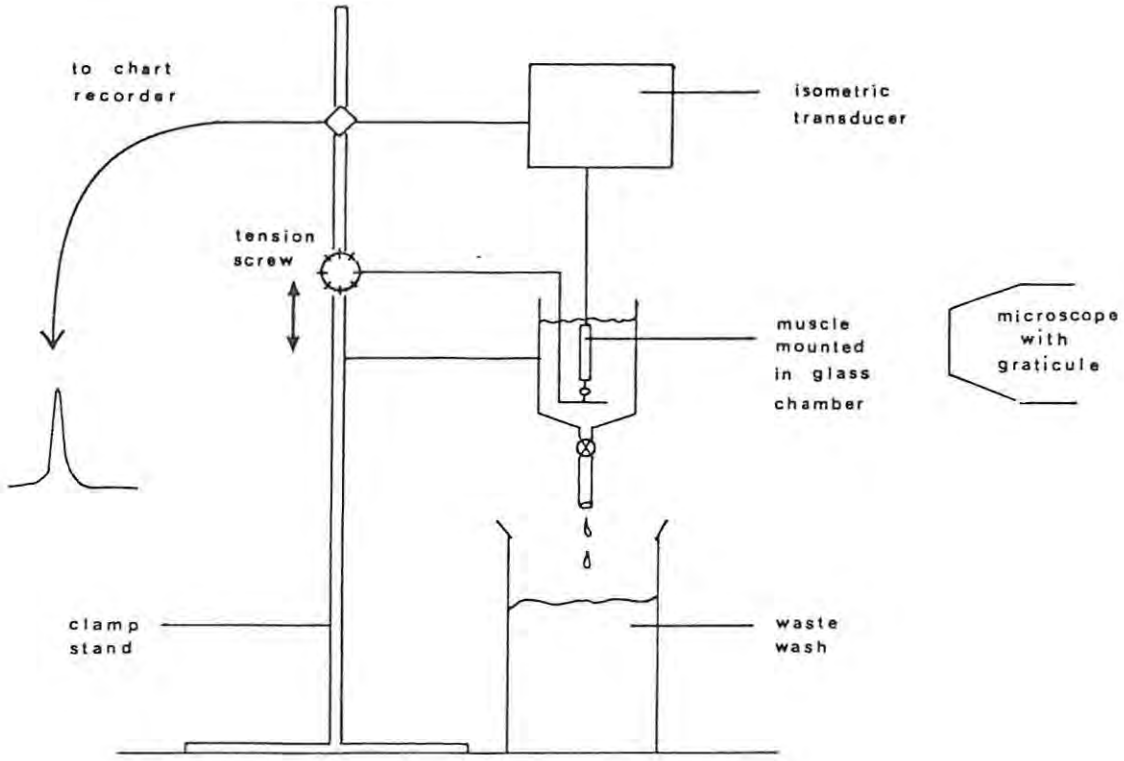

Figure 8.1. Schematic representation of apparatus for investigating length-tension behaviour of isolated bundles of limpet columellar muscle.

to induce contractions in limpet columellar muscle (Chapter7) was made up in sea water at a concentration of 5.5 x 10^{-4} M which is within the optimum concentration range for contraction of this muscle (Chapter 7). Contractions were induced by exposing the tissue to ACh. At the peak of the contraction, ACh was washed out with two to three changes of sea water.

Two types of investigations were performed. In the first, the mounted muscle bundle was extended by units of approximately 0.5 mm between ACh-induced contractions. Each extension was performed manually by slowly turning the tension screw over a period of a few seconds and was followed by a 5 minute stress-relaxation period. The maximum active tension T_a and the resting tension T_r attained was recorded after each increment in length and followed by a 10-15 minute recovery period. Contractions and extensions were continued until the muscle no longer responded to ACh or until the responses were no longer reproducible.

A curve showing the active tension at each length was plotted from the peak tension developed at each ACh-induced contraction. A resting tension curve was plotted from the tension developed above the base line in the muscle at rest between stretches and the total tension $T_a + T_r$ was also plotted. The data were normalised and re-plotted using the maximum tension, T_0, and the length at which this tension develops, L_0, as references to allow comparisons to be made with other muscles.

In the second series of investigations the procedure was as described for the first except that the muscle was extended, relaxed then re-extended by increments of about 0.2 mm to 0.5 mm continuing with ACh-induced contractions at each incremented length. The sequence of changes in muscle length was traced and plotted against the tension developed at the peak of each contraction.

8.3 RESULTS

It is found that at ACh doses of 5.5 x 10^{-4} M and at lengths below and above the range 0.9-1.1 of L_0 contractions are relatively twitch-like (Fig.

8.2A). But within the range 0.9-1.1 of L_0 contractions become consistently "catch-like", that is, acquire prolonged tensions after peak contraction (Fig. 8.2B, Chapter 7). However, this is only evident if ACh is not washed out at the peak of the contraction. Figure 7.5D (Chapter 7) shows what the response looks like when the muscle remains exposed to ACh at lengths outside the range 0.9-1.1 of L_0. If ACh is replaced by sea water at the peak of a "catch-like" contraction or at any time during the course of its relaxation, the tension rapidly decays (Fig. 8.2B). Application of 5-HT at about 10^{-4} to 10^{-6} M (a drug known to relax muscle catch; Twarog, 1954; Jewell, 1959) had no effect on the contraction. When administered during the protracted tension, 5-HT had the same effect as sea water alone. When 5-HT was administered prior to contraction or in combination with ACh, the response was unchanged. The time constants of the "catch-like" contractions are given in Table 8.1 and compared with those of twitch-like contractions obtained from the same muscle preparation. The mean maximum tensions are also compared.

Figure 8.3 shows the total tension $T_a + T_r$ obtained from contractions of a single muscle bundle preparation from *P. oculus*. The resting tension T_r and the active tension T_a are obtained by subtraction from the total tension. The most outstanding features are the narrow bell-shaped active tension curve and the rapid increase in the resting tension soon after L_r is reached. Either side of this region, active tension falls rapidly and symmetrically though low levels of tension are still apparent. Figure 8.4 is a plot of three sets of data normalised for comparison.

Great care was needed to obtain a set of data over the entire working range of the muscle because of sudden erratic behaviour, damage and breakage of the tissue due to high resting tensions developed. For this reason, the extension-relaxation cycle was kept within the length range where the resting tension did not become too high (Fig. 8.5 & 8.6) that is, for lengths L not greater than L_0.

A hysteresis effect is clearly evident in both the active and resting tension

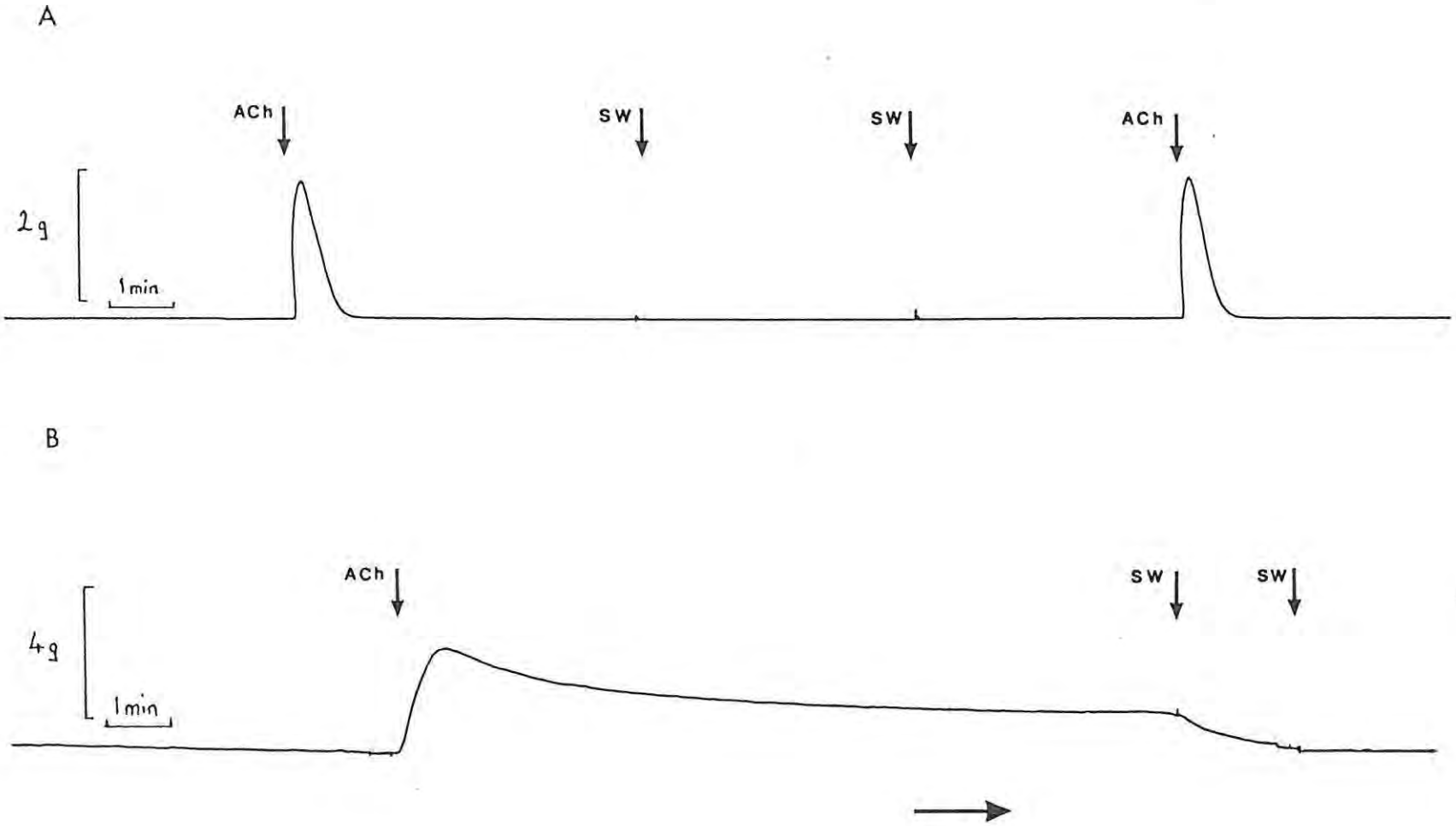

Figure 8.2. Traces of ACh-induced contractions (5.5×10^{-4} M) from isolated columellar muscle bundles of *P. oculus*. A: muscle length close to L_r (resting body length), muscle remains exposed to ACh until relaxation complete. B: as for A but muscle length slightly greater than L_r and close to L_o (length at which maximum tension occurs, Fig. 8.3). Contractions are "catch-like" at this length.

Response	Temperature ^{0} C	[ACh] M	Time to full contraction (s) Mean ± SD	Time to half relaxation (s) Mean ± SD	Time to full relaxation (s) Mean ± SD	Maximum tension developed (g mm^{-2}) Mean ± SD	N
"Catch-like"	20	5.5×10^{-4}	32 ± 9	160 ± 70	1073 ± 399	3.4 ± 0.7	6
Twitch-like	20	5.5×10^{-4}	13 ± 5	15 ± 5	70 ± 20	2.8 ± 0.5	10

Table 8.1. Comparison of properties of ACh-induced "catch-like" and twitch-like contractions from isolated bundles of the columellar muscle of *P. oculus*.

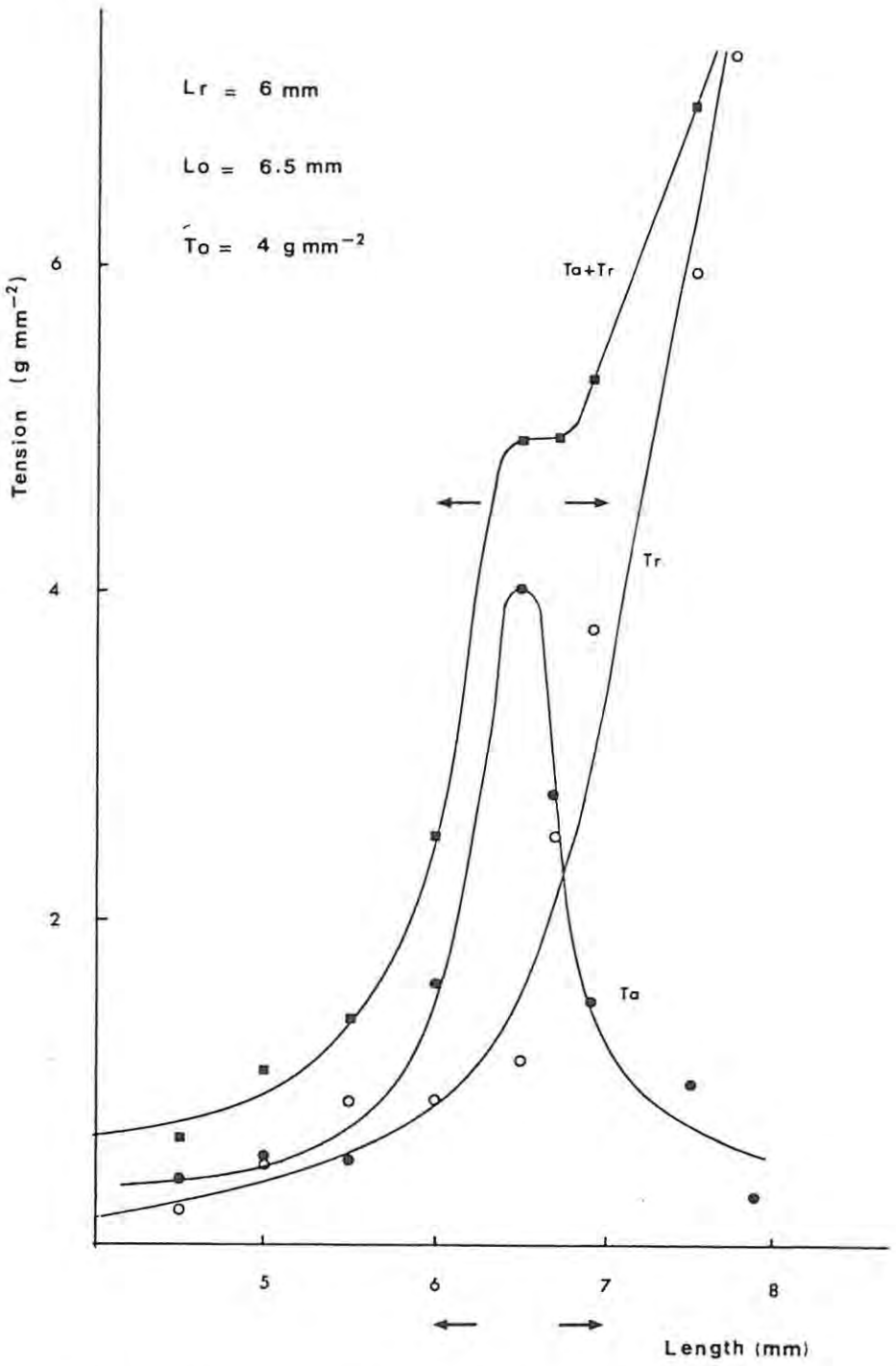

Figure 8.3. Curve T_a (●) shows peak tensions from ACh- induced contractions of isolated columellar muscle bundles (*P. oculus*) plotted against increasing lengths of the muscle. Curve T_r (o) shows the resting tension developed at each length increment i.e. passive tension developed due to extension of muscle alone (no contraction). Curve $T_a + T_r$ (■) shows the total tension developed at each contraction for different muscle lengths. L_r = the length of the muscle when it is at rest in the body (*in situ*). L_o = the length at which maximum tension is developed. T_o = maximum tension developed. The arrows indicate the length range over which "catch-like" contractions occur (Fig. 8.2B). It should be noted that this range co-incides with the region where maximum tensions develop. All doses of ACh were at 5.5×10^{-4} M.

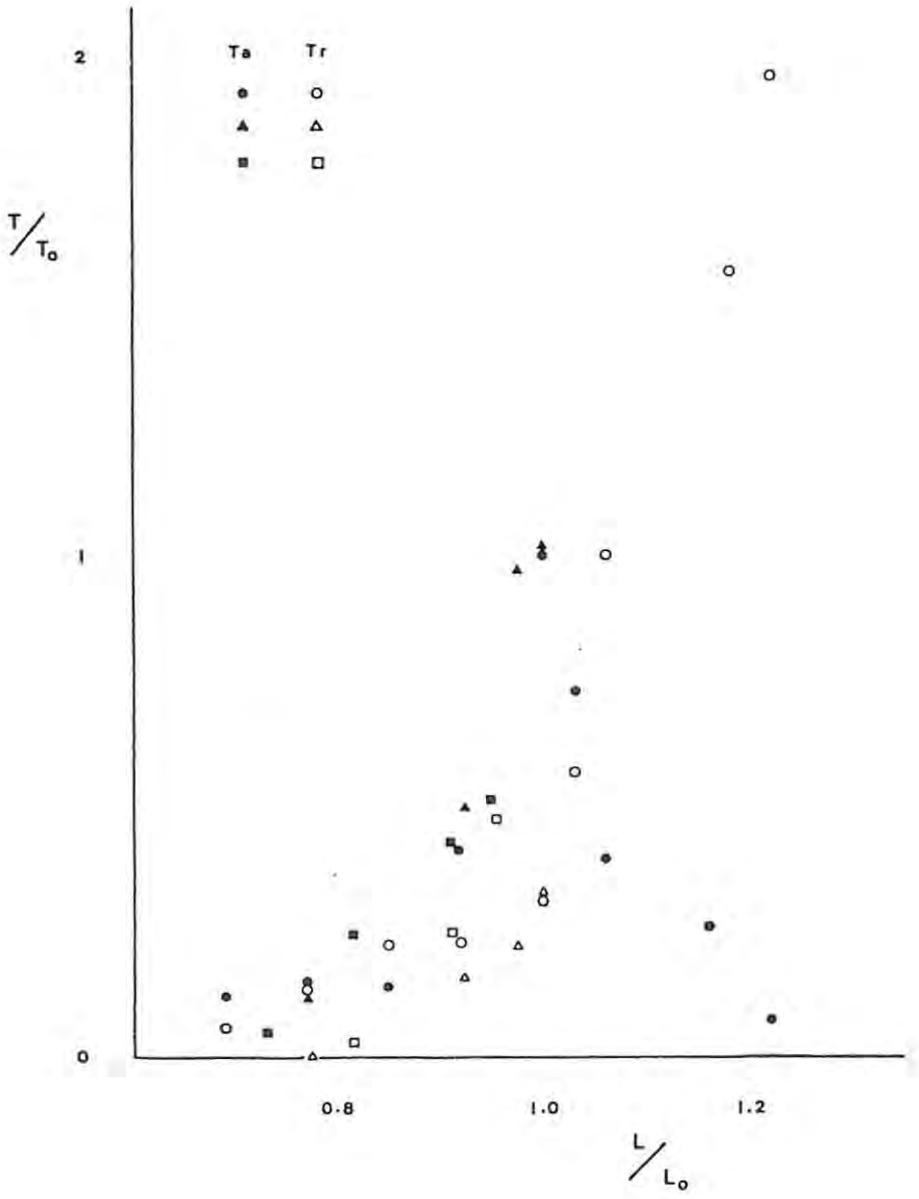

Figure 8.4. Three independent sets of length-tension data are plotted from ACh-induced contractions of isolated columellar muscle bundles from *P. oculus*. T_a = active tension curve (Fig. 8.3), T_r = resting tension curve (Fig. 8.3). Length (L) and tension (T) are normalised with respect to the reference values L_o (length at which maximum tension develops) and T_o (maximum tension developed). All ACh doses were at 5.5×10^{-4} M.

curves in (Figs. 8.5 & 8.6). There is some recovery on the second cycle of extension but it is not total. It should be noted that the limited length range of this investigation means that the contractions from which these data are obtained were all phasic and that the resting tension T_r is not very steep, or even apparent, until the muscle length reaches approximately L_0.

8.4 DISCUSSION

8.4.1 Hysteresis

The hysteresis effect in the extension-relaxation cycles of both the active and resting tension curves indicates that there has been some energy loss after stretching the muscle, most of which is unrecoverable. This is shown by the drop in active tension and a shift in the curve to the right along the length axis (Figs. 8.5 & 8.6). Furthermore, there was no loss of active tension after repeated stimuli at fixed but different lengths. Miller, (1975) investigating the leech body wall muscle, Cornelius & Lowy (1978) and Ishii & Takahashi (1981) investigating the ABRM and PBRM of *Mytilus edulis* all found a similar phenomenon. Plasticity is a characteristic feature of smooth muscles (Prosser, 1973). In principle, plasticity could arise from slippage between contractile fibres or fibres of the intercellular material (e.g. collagen) or from viscous shear of the connective tissue (Ishii & Takahashi, 1981). Since most of the slip is unrecoverable, the last of these two possibilities seems most likely especially since it has been shown that a large proportion of muscle tissue in limpets is collagenous connective tissue (Frescura & Hodgson,1989, 1990). Slip between contractile fibres may account for the minor portion that is reversible.

8.4.2 "Catch-Like" Contractions

Several invertebrate muscles other than the ABRM have catch properties, for example, the adductors of several bivalves (Chantler, 1983; Bennett & Elliott, 1987) and *Lumbricus* body wall (Miller, 1975). Catch muscles re-

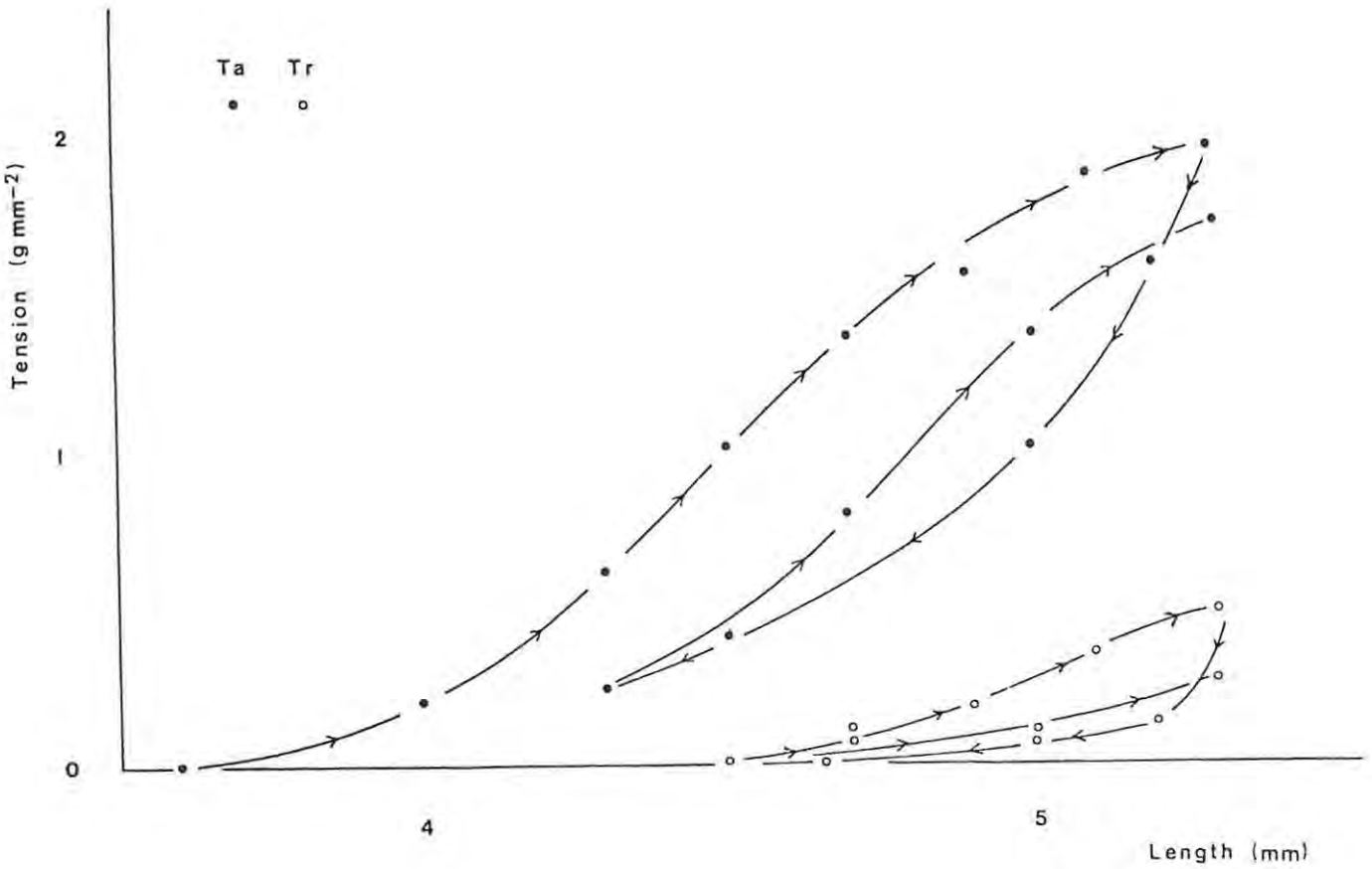

Figure 8.5. A cycle of extension, relaxation and re-extension of an isolated columellar muscle bundle from *P. oculus*. Contractions were induced by ACh at 5.5 x 10^{-5} M. T_a = active tension curve, T_r = resting tension curve. The hysteresis is clearly evident in both curves.

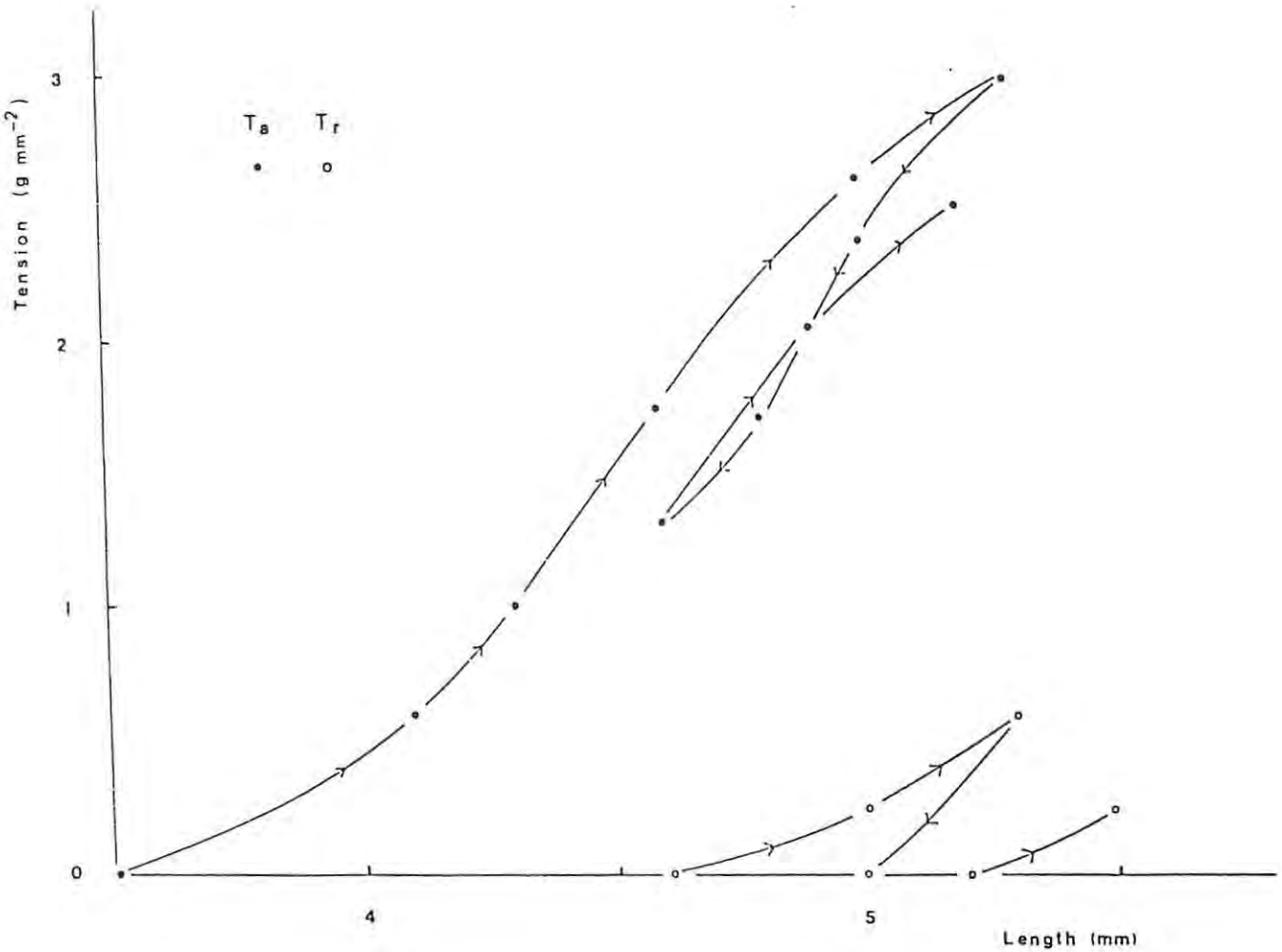

Figure 8.6. As for Figure 8.5 showing hysteresis in both active tension T_a and resting tension T_r curves; *P. oculus*, ACh at 5.5 x 10^{-5} M.

spond charateristically to 5-HT, a compound found to be present in the ABRM that not only relaxes the catch state but modifies the ACh response (Twarog, 1954; Hoyle, 1964; Chapter 7). Other invertebrate smooth muscles show a "catch-like" state (posterior byssus retractor: Ishii & Takahashi, 1981) but do not respond to 5-HT in the expected manner for a typical catch muscle. The "catch-like" state of limpet columellar muscle is not like any described in the literature for the following reasons: the prolonged tension is not maintained in the absence of ACh; it is present only over a limited length range; it is not affected by 5-HT in any way. Echinodermata have an unusual connective tissue that stiffens in the presence of ACh and is plasticised by cholinergic antagonists like epinephrine (Ruëgg, 1971; Motokawa, 1988). Freinkal and Hepburn (1975) showed that the mechanism of connective tissue stiffening in the dermis of *Holothuria scabra* may be due to cross-link formation between collagen fibres. It is suggested here that the connective tissue component of limpet columellar muscle stiffens in a similar manner in response to ACh so that when ACh induces contraction of the contractile apparatus the connective tissue component stiffens at the same time. Mechanical relaxation might then be dependent on the rate of breakage of connective tissue cross-links.

8.4.3 Length-Tension Relations

Any interpretation of the length-tension relation must take into account the structure of the muscle. The length-tension behaviour of limpet columellar muscle is a reflection of the mechanical properties of the characteristically inseparable combination of connective tissue and contractile material (Frescura & Hodgson, 1989, 1990).

The narrow symmetrical bell shape of the length-tension curve where the largest active tensions occur over the principal working range 0.9 - 1.1 (L_0) is not like that of any other molluscan smooth muscle. Typical curves for molluscan smooth muscles are broad and flat (Fig. 8.7A) with a relatively small resting tension (Fig. 8.7B). The following functional considerations

show how the curve relates to the manner in which the muscle functions in a limpet. The working range of the muscle is restricted effectively to its *in situ* length i.e. when the limpet is at rest or moving and the shell is elevated no more than a few mm above the substratum, and when it is clamped and the shell is closely apposed to the substratum. Rarely does the muscle need to shorten or extend beyond these limits. Thus the principal contraction mode is almost isometric. This is in contrast to the columellar muscles of coiled shell gastropods (and the ABRM of *Mytilus*) which have a large working range to allow extensions of the muscles many times their resting lengths. The principal working mode of the muscle in these cases is isotonic. Occasionally the columellar muscle in limpets is extended further to lift the shell off the substratum as in the aggressive behaviour of adult *P. oculus* (Branch & Marsh, 1978), or in attempts to right itself after inversion. It is suggested that some of this extension might arise from changes in orientation of cells and unfolding of crimped muscle fibres. Whether or not this is so, the extension is still relatively small compared to the coiled shell gastropod columellar muscles and thus explains the narrow T_a curve.

Another example of a muscle which functions under near isometric conditions is insect flight muscle. This too has a narrow working range (Fig. 8.7A) (Hanson & Lowy, 1960). However, inspite of a similar functional operation and length-tension behaviour the structure of insect flight and limpet columellar muscles is different and this is reflected in turn in the different rates of contractions of these two muscles (Prosser, 1973; Chapter, 7).

In structural terms the length-tension curve of mammalian striated muscle has been explained by the sliding filament hypothesis (Gordon, Huxley & Julian, 1966). Maximum active tension develops at maximum filament overlap and decreases sharply on either side. Molluscan smooth muscles however, generally have a much wider working range than striated muscles as evidenced by a flatter and broader active tension curve (Fig. 8.7A). Although the ultrastructure of molluscan smooth muscles shows no well-defined sarcomeres, so that almost unlimited sliding between filaments is theoretically

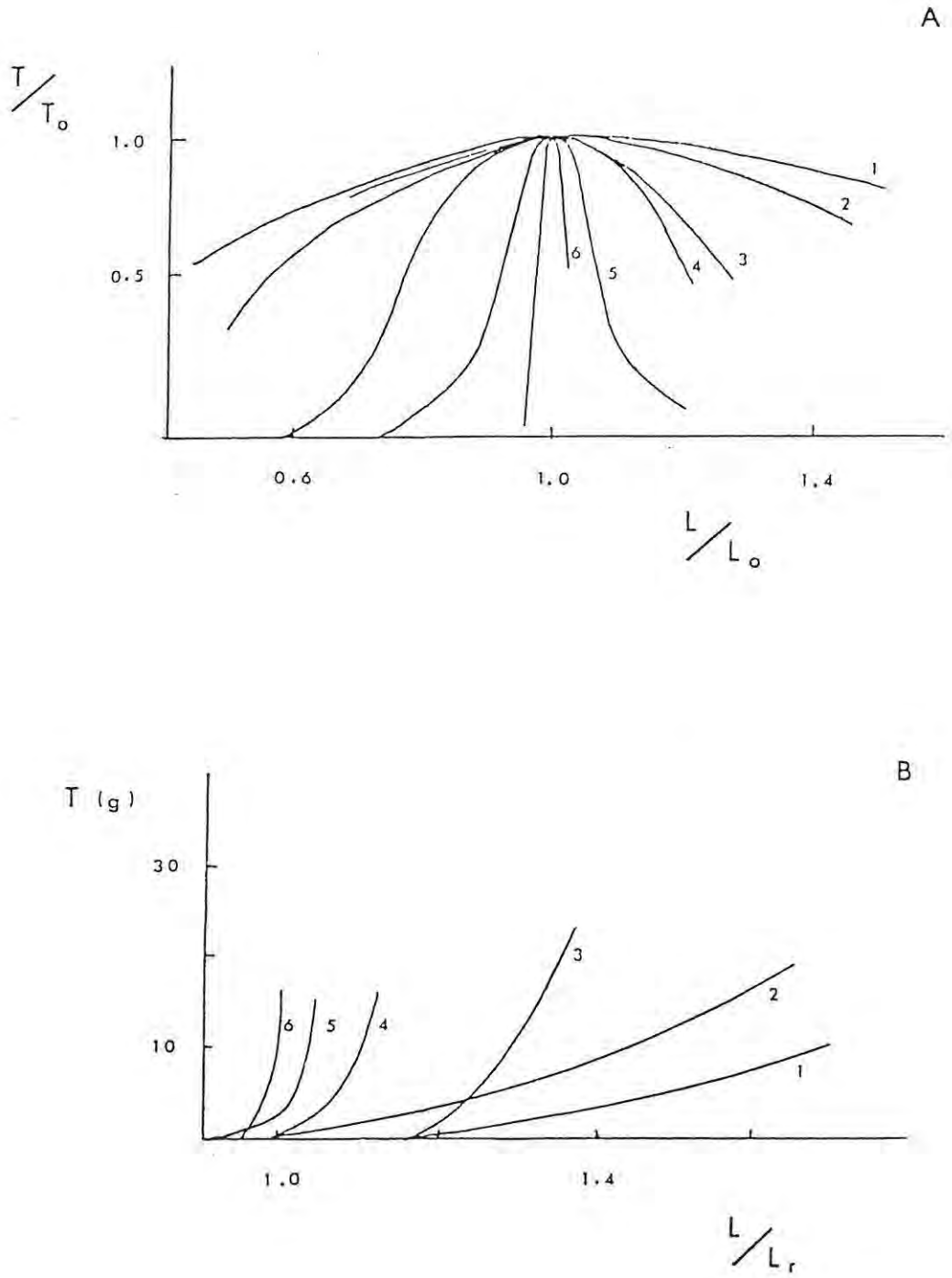

Figure 8.7. A: the active tension curve of the columellar muscle of *P. oculus* compared to those of other invertebrate muscles. Data is normalised for comparison. B: as for A but comparing resting tension curves. 1: snail pharynx retractor (*Helix*); 2: mussel anterior byssus retractor (*Mytilus*); 3: leech body wall (*Haemopis*); 4: frog sartorius (*Xenopus*); 5: limpet columellar (*Patella*); 6: bumblebee flight muscle (*Bombus*).

possible, several models of contraction have been proposed (similarly for oblique muscles) based on the sliding filament mechanism of contraction (Miller, 1975; Cornelius & Lowy, 1978; Ishii & Takahashi, 1981). None of the models however, would satisfactorily account for the length-tension behaviour of limpet columellar muscle and the appearance of "catch-like" contractions over a limited range.

The elasticity of the connective tissue in parallel with the muscle cells is responsible for the shape of the resting tension curve (Prosser, 1973). In the striated flight muscles of insects where there is very little parallel connective tissue the resting tension curve rises sharply when L exceeds L_r (Fig. 8.7B) because the parallel elastic component consists only of relatively inextensible sarcolemma (Hanson & Lowy, 1960). The resting tension curve of smooth muscles typically only rises after the muscle is stretched considerably (Fig. 8.7B) and is not very steep (Abbott & Lowy, 1957; Hanson & Lowy, 1960). The resting tension curve in limpet columellar muscle is very steep, like that of striated muscles but it differs in that it begins to rise even before the maximum active tension has been reached. A similar resting tension curve occurs in the earthworm (Miller, 1975). If the connective tissue in limpet columellar muscle has a catch mechansim as previously suggested, it could account for the steep and early onset of the resting tension curve since the muscle would become stiffened by collagen cross- linking and therefore resist extension. In addition, it may account for the ACh-induced "catch-like" contractions, which give rise to the steep bell-shaped active tension curve. Moreover, this explanation would not require any alteration to existing sliding filament models for explaining contraction of the contractile component of smooth molluscan muscles.

A connective tissue catch model could provide a mechanism of clamping that does not require much energy expenditure after initial muscle contraction and would thus provide an economical means of maintaining the clamped state in limpets throughout periods of desiccation, strong wave activity or prolonged predatory attacks. It is interesting that the catch

contractions occur only at the muscle length where the limpet would benefit from such a mechanism biologically i.e. at or slightly greater than *in situ* resting body length. It remains to investigate further, the nature of the "catch- like" state and to test the possible collagen cross-link mechanism for stiffening the muscle. This could be done by applying the chemical beta-amino proprionitrile (BAPN), which inhibits the enzyme responsible for cross-link formation (Freinkal & Hepburn, 1975) , before application of ACh. This should prevent collagen cross-links forming and in turn, prevent the occurrence of "catch-like" contractions if this model is correct. However, there is also evidence that the catch connective tissue in echinoderms may operate by a change in the ionic environment of the connective tissue matrix (Motokawa, 1988). Thus an investigation into the effects of various ions on the muscle might also be informative.

Finally, a study to elucidate which receptor sites on the muscle are in operation may also help to understand what regulates the twitch-like and "catch-like" contractions.

8.5 Summary

- The mechanical behaviour of limpet columellar muscle shows plasticity similar to that of other molluscan smooth muscles.
- Resting tension develops at low levels and rises steeply after L_r is reached.
- ACh induces "catch-like" contractions in limpet columellar muscle over a narrow length range of approximately 0.9 - 1.1 (L_0).
- Active tension is present at low levels over a wide working range but reaches substantially higher levels only over the narrow range coincident with "catch-like" contractions.
- It is proposed that the "catch-like" contractions arise in conjunction with a stiffened connective tissue from cross-linked collagen fibres.

Chapter 9

GENERAL DISCUSSION

It has been shown (Branch & Marsh, 1978; Branch, 1981) that limpets are well adapted to survive the environmental conditions of wave swept rocky shores. One such adaptation is the development of a large foot with a thick columellar muscle which enables the limpet to cling tenaciously to rocks (Branch & Marsh, 1978). This is in contrast to the smaller but very extensible columellar muscle of coiled shell gastropods (Trueman & Brown, 1976). The possibility that modifications of internal muscle structure exist that underly the different roles of the columellar muscle in coiled shell gastropods and limpets has been tested by comparing the structure at the histological and ultrastructural level. First, comparisons were made between patellid limpet species from different regions of the intertidal zone and second, between the columellar muscle of a range of non-patellid and patellid gastropods of various habits and habitats.

Several structural features were found that distinguish the general columellar muscle of a coiled shell gastropod from that of a limpet at both the light and electron microscope level (Chapters 2, 3 & 4). These are shown to be important modifications that are finely tuned to the respective roles of the columellar muscle in each of these categories of gastropod. Limpet columellar muscles (including that of *Haliotis*) are not antagonised by fluid, but function in the manner of a muscular hydrostat with tightly packed muscle bundles of different orientation which antagonise each other (Chapter 3). Columellar muscle cells and thick filaments are larger overall in limpets than in coiled shell gastropods. Dense arrays of collagen fibrils further typify limpet columellar muscle. In contrast, the organisation of coiled shell gastropod columellar muscles is intermediate between muscular and classical fluid hydrostat systems, having a more loose arrangement of muscle bundles interspersed between fluid vesicles and networks of collagen fibrils (Chapters

2 & 4). These differences relate to the different roles of the muscle. A more loose and fluid arrangement allows for large extensions and retractions that typify the function of columellar muscle in coiled shell gastropods, whereas the more compact arrangement in limpets produces a sturdy, rigid but more powerful musculature suited to holding the shell against the substratum in opposition to dislodging forces. In *Haliotis* the columellar muscle shows a combination of characteristics from that of both limpets and typical coiled shell gastropods. The lack of any significant differences between *Siphonaria* and *Patella* is interesting, illustrating a convergence of form in two limpet genera from different gastropod classes.

The role of type II cells, shown to be present in some coiled shell gastropods as well as patellid limpets, is not yet identified. It is possibly analogous to the proprioceptor role of intrafusal cells where structures similar in appearance to the striated thin filaments of type II cells occur (Karlsson & Andersson-Cedergren, 1968; Ovalle, 1972). Such a role, namely, monitoring tension and extension in the columellar muscle, would than have application in both limpet and coiled shell gastropods. The possibility that the filaments are themselves contractile has not yet been investigated. One approach would be to compare the periodicity between electron-dense regions under a relaxed and a contracted state. This could perhaps best be accomplished using fast-freeze techniques.

Although no differences were found in the ultrastructure of patellid limpets from different tidal heights and wave exposure, there was, nevertheless, a significant difference in the area of muscle attachment to the shell. In general, this parameter is correlated to both wave exposure and tenacity (Chapter 6). Studies on the muscle attachment area have shown that where environmental demands for clamping are smaller in terms of wave activity and exposure, the trend is for limpets to have a correspondingly smaller area of muscle attachment to the shell. Limpets of different intertidal habitat then, have adapted their clamping mode to the level of wave action they experience, not by modification of the structure of the columellar muscle,

but by a finely tuned and economical variation in the cross sectional area of the columellar muscle. The mechanism of attachment of the columellar muscle to the shell is probably by means of adhesive mucopolysaccharides (Chapter 6). Although at first this system may seem to be weak considering the large forces limpets can withstand during attempts to dislodge them (Branch & Marsh, 1978), the large surface area of attachment increases the efficacy of the system.

The paramyosin nature of the thick filaments of patellid limpet columellar muscle was confirmed by optical diffraction analysis, gel electrophoresis and calculation of crystal parameters (Chapter 5). The paramyosin crystal core dimensions have been shown to be similar to those of the anterior byssus retractor muscle (ABRM) of *Mytilus* (Chapter 5). The catch property of the ABRM and some other paramyosin muscles enable them to remain in tension for long periods of time (several minutes to hours) (Ruëgg, 1971) with a minimum of energy expenditure. The obvious economy that this muscle property would bestow on limpets in clamped mode, exposed to the dangers of dislodgement in their wave swept environment, led to an investigation of the mechanical properties of the columellar muscle in order to determine if the catch property was present. No true muscle catch state like that of the ABRM (Twarog, 1954) was found (Chapter 7). Instead, a "catch-like" state was shown to occur in the natural working length range of the muscle which is postulated to be a connective tissue catch phenomenon (Chapter 8). Length-tension relations for limpet columellar muscle clearly show that the range where highest muscle tensions develop is very critical (Chapter 8) but also very economical in functional terms, since it coincides precisely with the *in situ* length at which clamping occurs. This is in contrast to the more extensible muscle like the ABRM of *Mytilus* and the pharynx retractor of *Helix* which have been shown to have correspondingly wide working ranges (Hanson & Lowy, 1960). Although the ultrastructure of the ABRM and limpet columellar muscles are very similar, it is suggested that the organisation of the collagen component of the musculature is an important

factor in determining the different behaviour of the muscles.

Structural studies have revealed that a large percentage of collagen is present in limpet columellar tissue in the form of tightly-packed arrays of cross- linked fibrils (Chapters 3 & 5). This accounts for the high resting tensions observed (Chapter 8) since cross-linked fibrils would be more resistant to stretch. It is thus in contrast to the relatively low resting tensions of other smooth muscles (Hanson & Lowy, 1960). It is postulated that the connective tissue "catches" or stiffens in the manner described for Holothuroideans (Freinkal & Hepburn, 1975) involving the formation and breakage of collagen cross-links. This hypothesis could be tested using the agent β-aminoproprionitrile which alters the cross-linking ability of the collagen. Furthermore, Motokawa (1988) has shown that the ionic environment affects the connective tissue catch in echinoderms. Thus mechanical studies under altered ionic conditions may also help to further our understanding of this system. Moreover, if the cross-links break slowly, this could give rise to the slow relaxation phase of the "catch-like" state observed (Chapter 7). A future research priority then, is further investigation of the "catch-like" property of limpet columellar muscle to determine more precisely under what conditions it is present. It would also be of value to study the neuromuscular control of limpet columellar muscle, since different receptor sites (nicotinic and muscarinic) may also contribute to the complexity of the mechanical behaviour.

A structural difference between the columellar and tarsal muscles of a variety of gastropods at both the light and electron microscope levels has been correlated with the respective functions of the two muscle regions (Chapters 2, 3 & 4). An interesting question is whether or not a physiological difference also exists. The larger mitochondrial numbers and smaller, finer movements of the tarsus suggest the muscle may be more oxidative than the columellar muscle. Retracting the foot inside the shell in the case of coiled shell gastropods and clamping in the case of limpets are more spasmodic activities, whereas production of locomotory waves in the tarsus re-

quires continuous activity. Gäde (1988) has shown tissue specific differences in energy metabolism between the tarsus and columellar muscle of *Haliotis lamellosa* (the terms used by Gäde are foot and shell adductor respectively). The columellar muscle is metabolically more active when performing short burst contractions which, in general, rely on anaerobiosis, and contains four times higher levels of high energy phosphates. After continuous exercise of the columellar muscle a ten-fold increase in energy demand occurs but only a two-fold increase occurs in the tarsus. Recovery is by enhanced glycolysis. Under both environmental and internal anoxia (the latter induced by exercise), the end products of anaerobiosis are different in the tarsus and the columellar muscles. Also the enzymes involved in metabolism differ. In both tissues, 70 to 80 % of energy is provided by anaerobiosis, the rest by phosphagens. Gäde concludes that both tissue types respire by means of anaerobic metabolism but the columellar muscle more so. It would be interesting to investigate and compare a range of gastropod columellar muscles, particularly those of patellid limpets, to see if such a physiological distinction is present between the tarsal and columellar muscles.

In conclusion, the gastropod columellar muscle is adapted structurally to the different roles it plays in coiled shell gastropods and limpets. The gastropod tarsal muscle also shows important adaptations commensurate with its role in producing finer, more agile movements such as locomotory waves. Collagen forms a large part of the gastropod foot. It makes an important contribution to strength and stiffness of the muscle in limpets by its cross-linked arrays of fibrils, but contributes to pliability and extensibility in the tarsus and coiled shell gastropods by its presence as loose networks. Fluid spaces in the columellar muscle of coiled shell gastropods probably contribute to extensions and overall turgidity but work in conjunction with muscular antagonism. The mechanical behaviour of limpet columellar muscle shows an optimum muscle tension at precisely the narrow working range where clamping occurs. Such economic elegance is again apparent in measurements of muscle cross sections where limpets exposed to more wave ac-

tivity have acquired marginally, but significantly larger muscle attachment areas compared to those exposed to less wave action. Finally, a "catch-like" phenomenon, distinct from the classic muscle catch of the ABRM of *Mytilus* is present at muscle lengths where an isometric contraction produces clamping. This may be evidence of a stiffening mechanism that maintains the muscle rigidity essential for clamping with minimum energy expenditure. If proved correct it would provide yet another elegant example of economy in limpet design.

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Publications

On collagen and its potential role in the columellar muscle of some gastropod molluscs

M. Frescura and A.N. Hodgson

The shell of gastropod molluscs is attached to the body by the columellar (or shell) muscle. Most studies of this tissue have concentrated on describing the organization of the muscle bundles,[1-5] and the role that the muscle plays in pushing the animal out of,[2] pulling it into,[6,7] or elevating the shell.[3] In addition it is well known that in limpets the shell muscle has the ability to clamp the shell firmly against the substratum. Attention has recently been drawn to the occurrence of collagen in molluscan tissue[4] and to the possibility that connective tissues may have important mechanical and structural roles in these animals.[5] Knowledge of the structure and mechanical properties of molluscan collagen is, however, almost entirely based on studies of squid collagen.[8-10] Little is known of the distribution, abundance and role of collagen in gastropod tissues. In this article we present some observations on the prevalence of collagen in the columellar muscle of some gastropods and discuss the roles that collagen may have in this tissue.

We have examined eleven species of patellid limpet, as well as the prosobranch *Oxystele sinensis* and the pulmonate limpet *Siphonaria concinna*. Histology and electron microscopy have revealed that collagenous connective tissue permeates the entire pedal and columellar musculature and is particularly abundant towards the shell attachment site, and in the sole and sides of the foot (Figs 1, 2 and 3). Although an accurate quantitative assay of the collagen to muscle ratio is not yet complete, we estimate that 30–40% of the total columellar muscle of the limpets investigated is collagen and more than 50% in *O. sinensis*. By contrast, vertebrate muscle tissue has only 3–30% collagen.[11] Furthermore, electron microscopy has revealed that intermyocellular material separates the muscle cells by as much as 4–5 μm in limpets and 9 μm in *O. sinensis* and that these regions are packed with collagen fibrils. These fibrils (which have a 62 nm axial striation) occur either as a loose network (Fig. 1) or, more frequently, in parallel arrays with cross-bridging between them (Figs 2 and 3).

It has been proposed that the intercellular collagen in gastropods not only serves to anchor muscle and delimit body shape[7] but also to support blood vessels and nerves.[4] The abundance of collagen in columellar and limpet pedal muscle (virtually unrecorded in previous structural studies[5]), as well as squid mantle[10] and limpet cephalic tentacles,[12] suggests that collagen is particularly important in these muscular-hydrostat systems. A muscular hydrostat is an arrangement of antagonistic muscles in which local contractions cause small perturbations in the adjacent muscles without the participation of large, fluid-filled spaces.[3,5] Collagen is essential for partitioning groups of muscle cells into small functional units. The efficient operation of this system is very likely enhanced by cross-bridging in the collagen, which provides a rigid anchor against which the muscle cells can pull.

Another possible and, as yet, unconsidered role for collagen is as a stiffening agent. Before a limpet clamps to a rock the foot, although firm, is pliable, but during clamping the tissue becomes very rigid, enabling the limpet to resist lateral and rotational forces imposed on the shell. Although muscular contraction undoubted-

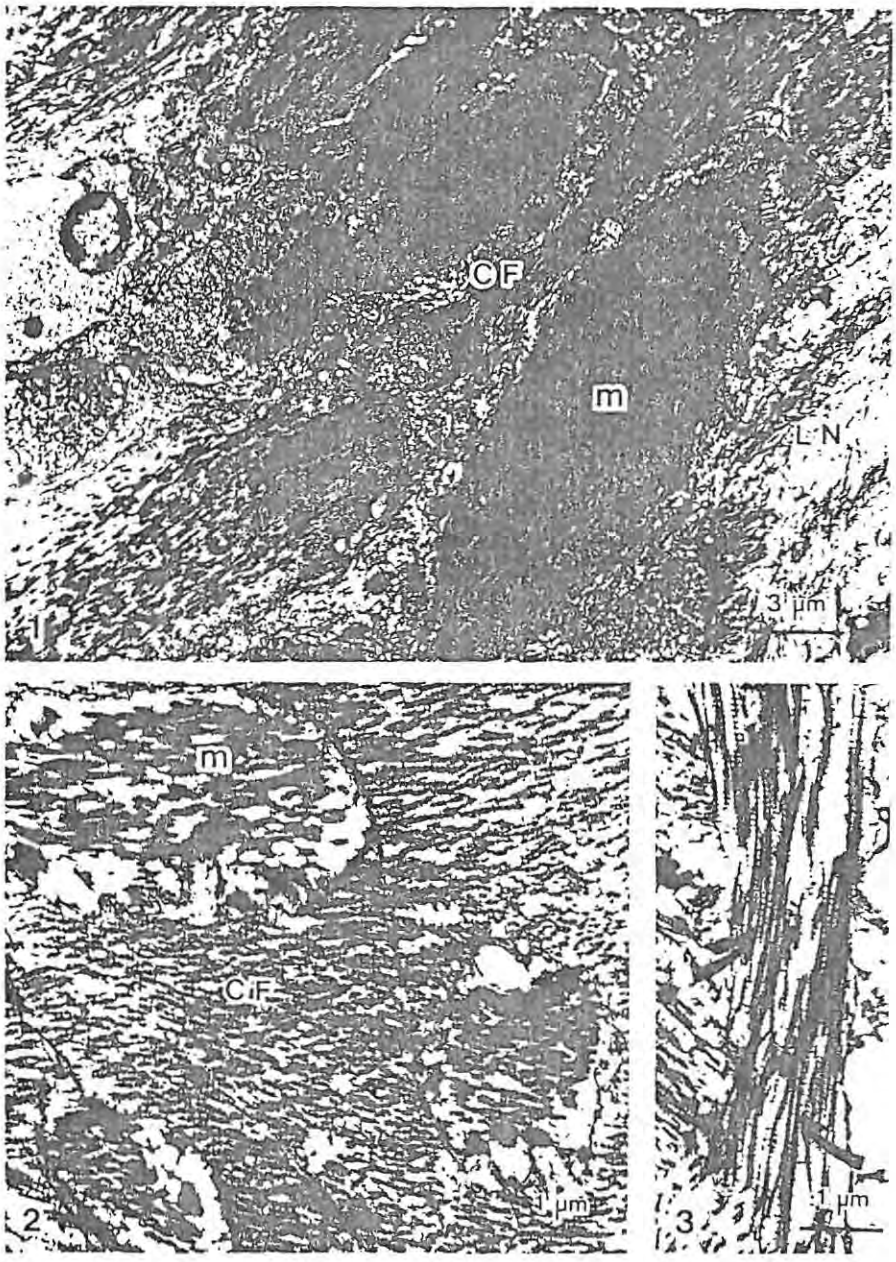

Fig. 1. Transverse section of muscle fibres from the columellar muscle of *Siphonaria concinna*, showing large intermyofibre spaces filled with collagen fibres (CF). A loose network of fibrils is evident (LN). m, muscle. Fig. 2. Transverse section of muscle fibres from the columellar muscle of *Oxystele sinensis*, showing very large intermyofibre spaces filled with arrays of collagen fibres (CF). m, muscle. Fig. 3. Longitudinal section of a parallel array of collagen fibrils from the columellar muscle of *Oxystele sinensis*, showing cross-links (arrows).

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ly contributes to this resistance, we propose that the collagenous tissue is also important for tissue rigidity. An analogous situation is to be found in echinoderms, for which the mechanical importance of collagen is well documented.[13] Stiffening of the body wall of sea cucumbers occurs by cross-linking of parallel arrays of collagen fibres[13] which have been termed 'catch-connective tissue'. This phenomenon allows sea cucumbers to maintain body rigidity for long periods with minimal energy expenditure.[13]

Whereas catch-connective tissue has been reported in only echinoderms, it is possible that cross-linking of the abundant parallel collagen fibres in the columellar and pedal muscle of limpets causes tissue to go rigid.

Collagen may also play a role in shell support. Jones[6] has suggested that blood pressure is the means by which snails support their shells. If collagen is found in large amounts in other columellar muscles, however, it may also contribute to shell support. We are currently investigating the structure and properties of the columellar muscle of gastropods.

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Setting priorities for minimum flow assessments in Southern Africa

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As demand for clean, potable water increases in Southern Africa, more waters will be withdrawn or transferred from the scarce and relatively pristine rivers of this semi-arid region. Although human consumers may have legal standing in respect of rights to riverine waters, the lotic ecosystems themselves do not when it comes to securing the flow that allows the maintenance of ecosystem integrity. Southern African limnologists have lately become concerned about the viability of their river ecosystems and have established working groups to assess the techniques available to predict and evaluate minimum flow needs.[1,2] No formal decisions have been made, but sentiment leans towards models which are based on the response of aquatic organisms to changes in river flow.

It must be remembered that the process of securing a minimum flow requirement involves the negotiations of many user representatives and must take into account water quality, geomorphological stability (flood and flushing events), aesthetic and recreational needs, as well as biological requirements. Some of these demands are closely interrelated. A consensus on acceptable flow allocations is mandatory. With the exception of biological needs (unless a fishery is concerned), all of these demands have a direct impact on the human economy, and water supply models for the necessary water allocations have long been available. Only recently have there been methods to assess the instream flow needs of riverine biota. I have been visiting South Africa, under a Fulbright fellowship, to introduce scientists to one of these response-based models.

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In the United States, the most successful regulated-flow management model for aquatic biota has been the Instream Flow Incremental Methodology (IFIM).[3] It has been used to adjudicate a number of flow reservations and has resulted in providing legal standing for a number of North American rivers as primary water consumers.[4] IFIM is based on the assumption that biota in running water have their distribution and certain phases of their life cycles controlled by the hydraulic conditions within the water column. Although lotic organisms appear to be highly adapted to flow and regulated by some other conditions (say, organic energy state or interspecific interactions), changing morphology and hydrodynamic conditions 'force' aquatic organisms to migrate to different hydraulic conditions throughout their life cycles.[5–7] Indeed, the distribution of both fish and benthic organisms can be predicted from the flow patterns of the river channel and corresponding hydraulic fitness,[8] breeding behaviour,[9] or the complex conditions of shear stresses across the substratum.[10]

IFIM uses the biota's preferences for velocity, depth and channel conditions to predict habitat availability at various discharge levels. The essence of IFIM is the computer simulation of the physical habitat, PHABSIM, which predicts changes in velocity, depth and channel roughness in each habitat element surveyed. These conditions are compared to the habitat preferences of target species or communities determined (in most cases) in the field. Usable habitat areas are then predicted in relation to each discharge. The inflection point on the curve of habitat availability (that is, where the habitat values decline rapidly with decreasing discharge) is usually considered to be the point of minimum acceptable flow to maintain ecosystem integrity. These values of habitat quality for biota are then compared against the highest minimum flow criteria for other riverine demands in order to establish a minimum-flow allocation.

Ideally, the negotiations at which annual flow allocations are made should involve members of all user groups as well as the regulatory agencies. All participants must be committed to the idea of an initial evaluation of monthly allotments and to the notion that these can be re-evaluated as new data are obtained.

As with demands from industry, irrigation and recreation, the flow requirements of biota will probably change during the yearly cycle of life. The salmonids, for example, spawn only in clear, fast flowing riffles, yet adult maintenance is in slower pools with dense overhead cover.[11] In contrast, fry and juveniles require slow moving, shallow, edge habitat. Similary, breeding crayfish[9] need to change their habitat, as do species of aquatic insects as they progress through larval instars to adult forms.[10] Thus, river flow that suits spawning will be different from that appropriate to adult maintenance. In other months, flushing flows may take precedence over biological concerns. Thus, the total annual allotment of water for a river system is a combination of variable monthly allocations.

Elektronmikroskopievereniging van Suidelike Afrika — GRAHAMSTAD (1990)

A COMPARATIVE STUDY OF GASTROPOD COLUMELLAR MUSCLE WITH SPECIAL REFERENCE TO LIMPETS

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The columellar muscle of gastropods is a complex structure composed of muscle cells and collagen[1,2,3,4]. This muscle is used by gastropods with coiled shells to extend or retract the foot whereas gastropods such as limpets and abalones use it to clamp the shell against the substratum[2,4]. This difference in function suggests a difference in ultrastructure. The subject of this paper is a comparison of the columellar muscle of a number of species of gastropods.

Tissues from five species of gastropod with coiled shells, 11 species of patellid limpet and one species of abalone were prepared for light and electron microscopy by standard techniques. In addition, preparations of isolated muscle filaments were obtained and further examined by optical diffraction[2] and SDS gel electrophoresis using techniques described elsewhere[2].

One difference between the columellar muscle of coiled shell gastropods and limpets is the presence of blood sinuses in the former and their complete absence in the latter. A second difference is the average thick filament and muscle cell diameters of the coiled shell gastropods and abalone (51 nm and 6 µm respectively) contrasting with large diameter thick filaments and muscle cells in limpets (89 nm and 15 µm). The novel muscle cell type[2] containing bundled thin filaments previously described for five species of limpet has been observed in most of the gastropods studied (Fig. 1).

Optical diffraction and SDS gel electrophoresis together firmly indicate[2] that the thick filaments are made from paramyosin not only in limpets but also in coiled shell gastropods. In all gastropods examined collagen is abundant[1] (Fig. 2). Microdensitometry of the gel bands has been used to quantify the collagen to muscle ratio which is as high as 2:3 in some species (Fig. 3).

Coiled shell columellar muscles are seen to vary between a muscular hydrostat system where muscles antagonise each other, and a hydrostatic skeleton system where fluid provides the antagonist for the muscles. The prevalence of collagen and finer muscle bundles gives a plasticity and extensibility to the coiled shell gastropod foot which is compatible with its more flexible behaviour. Limpet columellar muscles, in contrast, are true muscular hydrostats. Fine muscle bundles occur more in the sole of the foot lending agility to the tarsus for locomotory movements. In contrast, thick dorso-ventral muscle bundles in the shell muscle, ensheathed with cross-linked arrays of

collagen fibrils [1,2], produce a tougher more stiff shell muscle which is essential to clamping [2,4]. Furthermore, the large thick filament diameters of limpet shell muscle can be understood in terms of providing greater power for muscle clamping.

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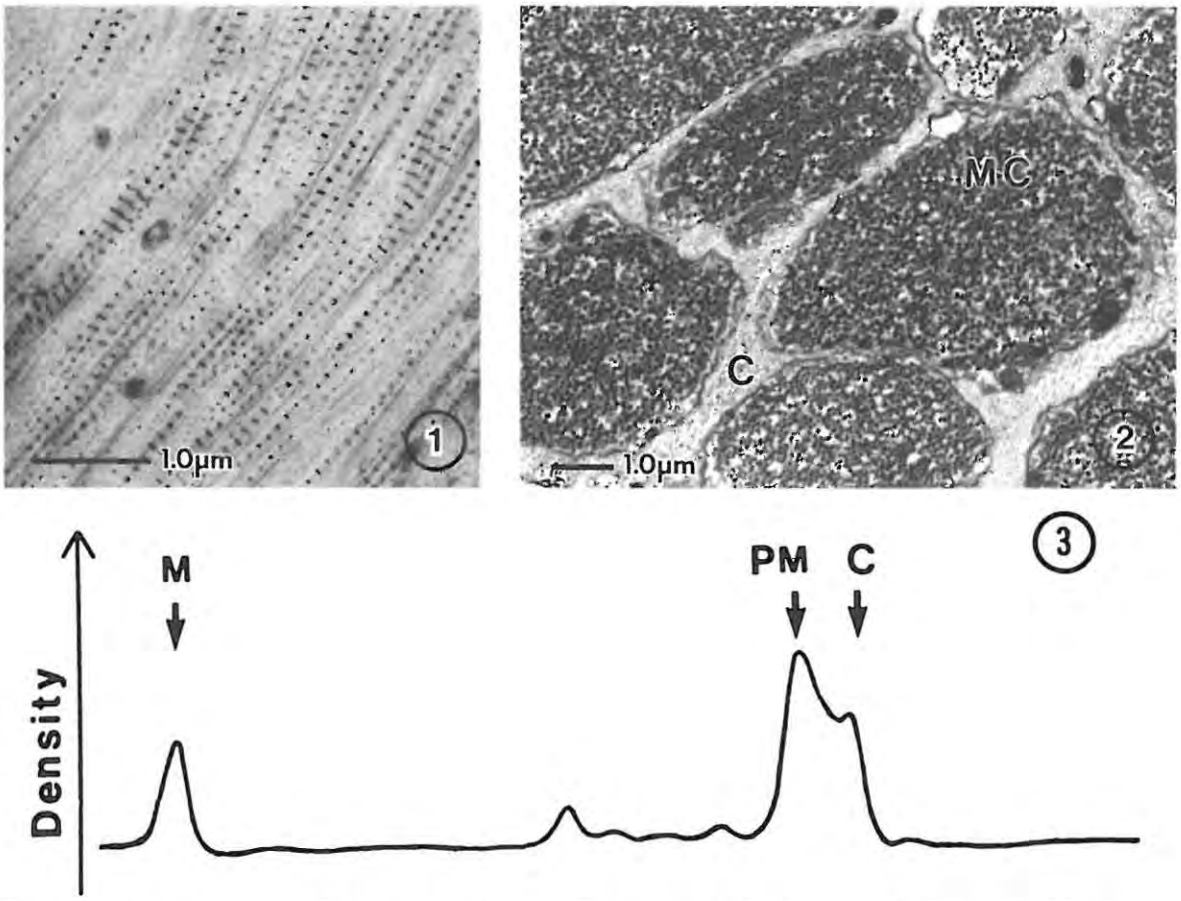

Fig. 1. Micrograph from the columellar muscle of Patella vulgata showing longitudinal section of bundled thin filaments with electron-dense repeats of about 100 nm.
Fig. 2. Micrograph from the columellar of Patella oculus showing transverse section of several muscle cells (MC) with large diameter thick filaments. Note the abundant collagen (C).
Fig. 3. Microdensitometry trace of SDS gel bands obtained from a filament isolate of the columellar muscle of Burnupena sp. showing relative proportions of collagen (C), paramyosin (PM) and myosin (M).

Elektronmikroskopievereniging van Suidelike Afrika JOHANNESBURG (1987)

AN ULTRASTRUCTURAL STUDY OF LIMPET SHELL MUSCLE

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The mechanism of contraction of smooth molluscan muscle is still poorly understood. Furthermore, though some bivalves have been investigated [1,2], very little work has been done on gastropods. The shell muscle of Prosobranch Limpets, Family Patellidae, like all smooth muscle, is capable of remaining tense for considerable periods of time. This can be observed when the limpet shell clamps down onto its substratum.

The ultrastructure of most smooth molluscan muscle, although less ordered than striated muscle, nevertheless, still allows definition of a contractile unit, with dense bodies analagous to Z lines and thick and thin filaments present in orderly arrays. Transmission electron microscopy of limpet shell muscle reveals a complex and relatively unorganised tissue (Fig. 1), the structure of which is difficult to accomodate wholly within the sliding filament hypothesis of muscle contraction.

The thin filaments often fill cross-sectional areas of up to 300 nm, with no thick filaments in the vicinity (Fig. 2). Moreover, well-defined orbital arrays of thin filaments surrounding thick filaments are absent in many cases. Thick filaments appear to be of the smooth paramyosin type with axial spacings of approx. 14,5 nm (Fig. 3), and diameters ranging between 20-120 nm for most species studied, and up to 180 nm in *Patella tabularis*. The filaments are often seen winding and twisting in several orientations within a single myofibre. Dense bodies are few and far between.

Transverse sections of thick filaments reveal regularly spaced bands (Fig. 4), which are due to sectioning of the material at an orientation that enables planes of the crystal lattice of paramyosin to be viewed. The transverse repeat of the lattice can be calculated from the band spacing using the relation[2]

$$a = ns/\cos(\sin^{-1} s/c) = ns\ (1 - s/c^2)^{-1/2}$$

Patella tabularis has a spacing value which does not accord with the values cited for other molluscan paramyosin filaments[2]. This perhaps suggests that either the crystal packing or the paramyosin molecule is different in this species. Controlled goniometer studies should confirm or disprove this by allowing the transverse repeat to be computed from a wider range of data.

An unusually large component of cross-linked extra-myocellular collagen may contribute signficantly to the functional properties of

limpet shell muscle. The axial spacing of the collagen fibres in the nine species studied is consistently about 17 % smaller than mammalian collagen.

Cryofracture and S.E.M. techniques are being used to investigate the sites of muscle attachment to the shell.

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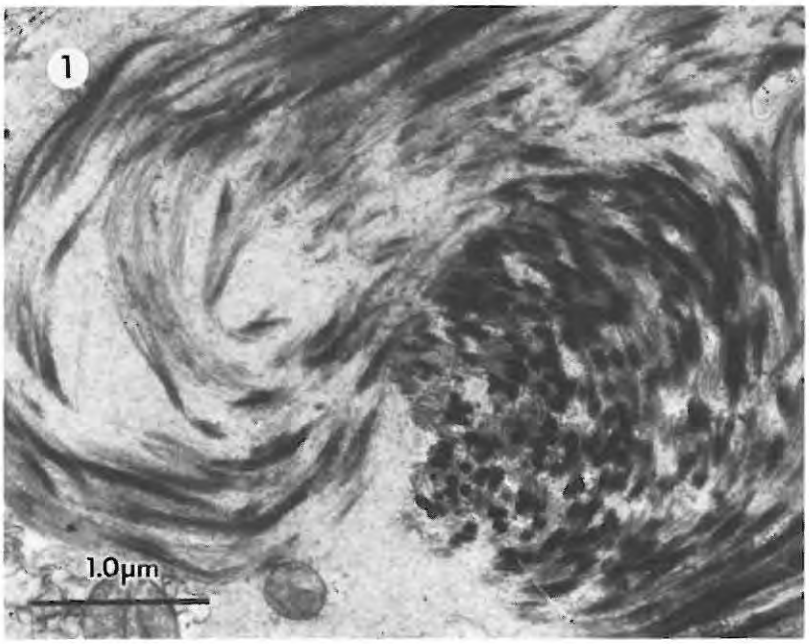

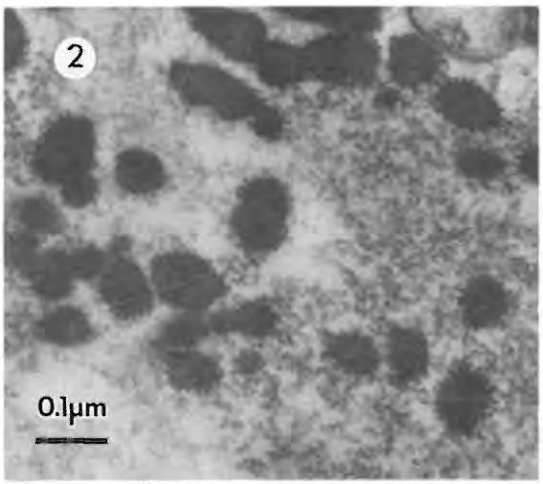

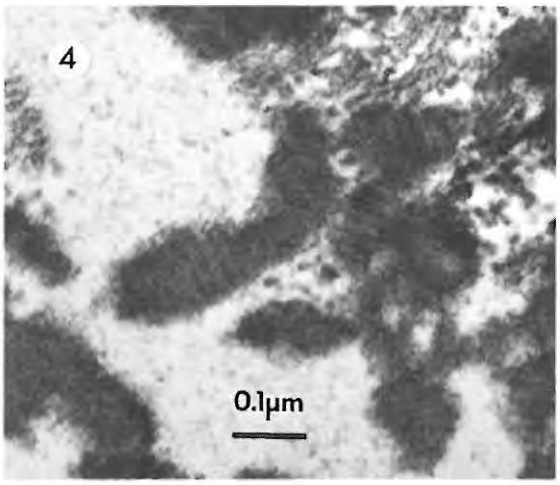

Fig. 1 A spiral of filaments from Limpet shell muscle.
Fig. 2 Transverse section of thick and thin filaments.
Fig. 3 Longitudinal section of a thick filament showing an axial periodicity of 14,5 nm.
Fig. 4 Transverse section of thick filaments showing spacings.

J. Moll. Stud. (1990), **56**, 435–447.

THE FINE STRUCTURE OF THE SHELL MUSCLE OF PATELLID PROSOBRANCH LIMPETS

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ABSTRACT

The structure of the shell muscle of eleven species of patellid limpet is described from light and transmission electron microscope studies. Although the muscle has many structural characteristics typical of molluscan smooth muscle, it also has a number of unusual features. At the electron microscope level two myofibre types are distinguishable. Type I cells, present in all species, contain conventional contractile apparatus in the form of thick and thin filaments. Thick filaments contain paramyosin and vary in diameter between 20-180 nm. An axial striation with a repeat of 14.2 nm is calculated from optically diffracted micrographs of isolated thick filaments. Transverse sections of thick filaments reveal bands from which the transverse repeat of the paramyosin crystal lattice is calculated. Type II myofibres, which are present in five species, contain a novel arrangement of thin filaments with electron-dense regions at intervals of 80-150 nm. The striated thin filaments are similar in appearance to the microfilament bundles and stress-fibres of non-muscle cells. They also have similarities to the leptomeric organelles of some vertebrate muscle tissues. Associated with the muscle is an unusually large amount of collagen which has a periodicity of 62 nm calculated from optical diffraction patterns of isolated collagen fibrils.

INTRODUCTION

The tenacious clamping of limpets to rocks is essential to prevent desiccation on exposed rocky shores and to avoid dislodgement by predators and wave activity. The unusual degree of tenacity, approaching 7×10^5 Pa in some species (Branch, 1981), is produced by a thin layer of mucus between the sole of the foot and the substratum (Denny, 1983; Grenon & Walker, 1980, 1981) together with a sustained contraction of the shell (columellar) and pedal musculature. Although the actual tenacity of limpets has been thoroughly investigated (Aubin, 1892; Miller, 1974; Branch & Marsh, 1978; Grenon & Walker, 1981; Branch, 1981; Davenport, 1988), little is known about the fine structure of limpet shell muscle and even less about its performance. To date only the general arrangement of muscle fibres is known from light microscopy (for reviews see Fretter & Graham, 1962 and Kier, 1988). The fibres are tightly packed (Voltzow, 1988; Kier, 1988) in the manner of a muscular hydrostat (Kier, 1988; Jones & Trueman, 1970). Before the mechanical properties of limpet columellar muscle can be fully appreciated, a knowledge of the ultrastructure of this tissue is essential.

The aim of this study is to examine the fine structure of the columellar muscle of prosobranch limpets and to compare it with other molluscan muscles. Furthermore, as it is possible that muscle structure might be related to habitat level on the shore, limpets from different zonal levels are examined.

MATERIALS AND METHODS

We define the shell muscle as being the entire shell-pedal musculature except for a region immediately above the sole of the foot (Fig. 1). This region differs morphologically (Voltzow, 1988) and ultrastructurally (Frescura, personal observation) from the rest of the muscle.

Eight species of limpet (Prosobranchia: Patellidae), *Patella cochlear Born, P. granularis Linnaeus, P. longicosta Lamarck, P. miniata Born, P. oculus Born, P. tabularis Krauss, Helcion pectunculus (Gmelin), H. pruinosus (Krauss)*, were collected from the east coast of South Africa and three species, *P. vulgata Linnaeus, P. intermedia Murray, P. aspera Röding*, from the south west coast of England. These were kept in aerated sea water until dissected. Some dissections were done on fresh animals within six hours of collection and others within one week on animals retained in an aquarium. For light microscopy, tissue was fixed in either 10% formalin or Bouin's aqueous fixative, embedded in Paraplast, sectioned at 6 µm and stained by two methods: Milligan's trichrome, omitting orange G stain, and Mallory's trichrome (Humason, 1967). Both methods differentiate between collagenous connective tissue and muscle.

For electron microscopy, pieces of tissue about 1 mm^3 were excised from living tissue under sea water and fixed in 2.5% glutaraldehyde containing filtered

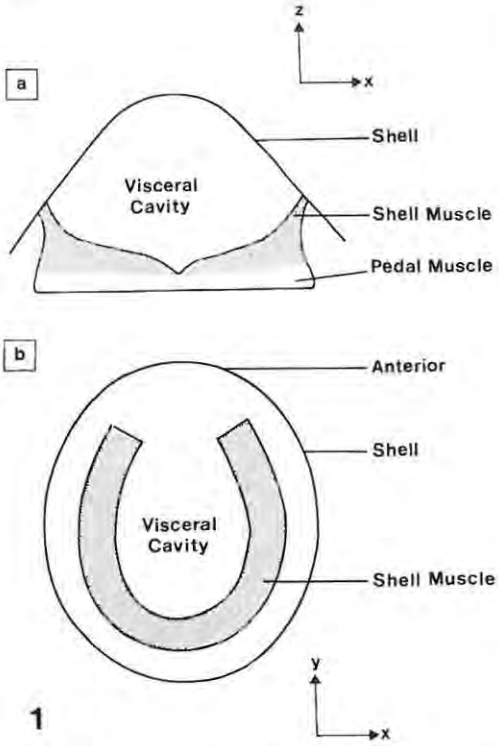


Fig. 1. Diagram to indicate the location of the shell and pedal muscle of a limpet. a. Antero-posterior section. b. Ventral view showing horse-shoe-shaped muscle attachment site to the shell.

sea water and 0.1 M sodium cacodylate buffer (pH 7.0) for 12 hours at 4°C. Fixed tissue was washed in 0.1 M cacodylate buffer made up in sea water, post-fixed with 1% osmium tetroxide in the same buffer for 90 minutes, dehydrated and embedded via propylene oxide in an Araldite CY212/Taab 812 resin mixture (Cross, 1989).

Silver/gold sections cut using glass knives were stained either with 5% aqueous uranyl acetate for 30 minutes, followed by Reynold's lead citrate for 10 minutes, both at room temperature, or with 10% methanolic uranyl acetate for 30 minutes. Material was viewed on a Jeol JEM 100 CXII electron microscope at 80-100 kv. Catalase crystals were used for calibration of micrographs.

Filaments were isolated from *Patella vulgata*, *Patella intermedia* and *Patella aspera* by homogenising tissue in a Virtis blender from three regions of the musculature: outer circumferential fibres, dorso-ventral fibres and tissue close to the shell. All parts were kept cool and all specimen material kept on ice. Tissues were homogenised to remove soluble proteins in a buffer solution composed of 60 mM KCl, 5 mM $MgCl_2$, 1 mM NaN_3, 0.5 mM EGTA, 10 mM Imidazole, 5 mM DTT, 0.5% Triton X-100, 2 μg/ml leupeptin, pH 7.0 at 4°C. It was then spun at 2,500 rpm for 5 minutes in the same buffer. The pellet was washed and spun again before being incubated in a medium containing 80 mM KCl, 5 mM $MgCl_2$, 2 mM NaN_3, 5 mM EGTA, 10 mM ATP, 20 mM MES-KOH, 5 mM DTT, 2 μm/ml leupeptin, pH 6.0 at 4°C for ½ hour on ice to dissociate the thick and thin filaments. Isolated filaments were negatively stained with 10% methanolic uranyl acetate for 30 minutes and viewed in the electron microscope at 100 kv.

Optical diffraction patterns of isolated thick filaments were obtained using a modified Rank-Pullen instrument with 2 mwatt HeNe gas laser red light at a wavelength of 632.8 nm and lens of focal length 150 cm (O'Brien & Bennett, 1972).

Electrophoresis to identify the proteins present in the filament preparations was done on an 8% sodium dodecyl sulphate (SDS) polyacrylamide slab gel that was stained with Coomassie blue. The protein concentration was estimated to be 1 mg/ml buffer and protein loadings were 2 μl, except for the circumferential homogenate which was about 1 μl.

RESULTS

Since negligible interspecific variation in muscle structure was found we present the data in a form that generalises from all species studied.

Organisation of the shell and pedal musculature

The shell and pedal musculature of limpets has a complex arrangement of muscle fibres (Fig.

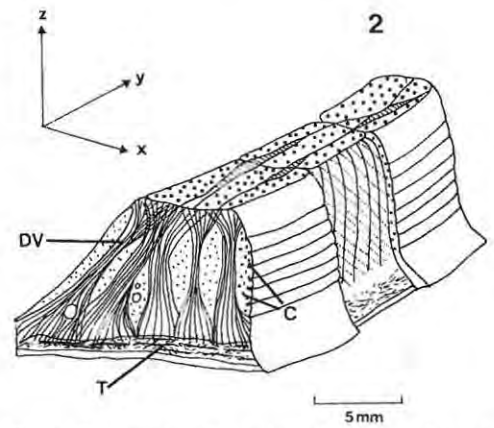


Fig. 2. 3-dimensional drawing of section of limpet shell muscle. Notice the large dorso-ventral (DV) muscle bundles (z-direction) which ramify at the sole and sides of the foot. C, circumferential fibres; t, transverse fibres; x-z plane, cross-section; x-y plane, anterior-posterior section; y-z plane, dorso-ventral section.

2). There is an outer layer of muscle consisting of circumferential bundles of fibres that form a thin sphincter around the entire foot. The tissue medial to the inside of the sphincter is composed of fibres running cross-diagonally from shell to pedal muscle. The majority of muscle fibres within the foot are found in large dorso-ventral bundles (z-direction) while a few run circumferentially (y-direction) and near the sole of the foot a few run transversely (x-direction) (Figs. 2 & 3). Voltzow (1988) has shown that dorso-ventral fibres ramify into the sole and

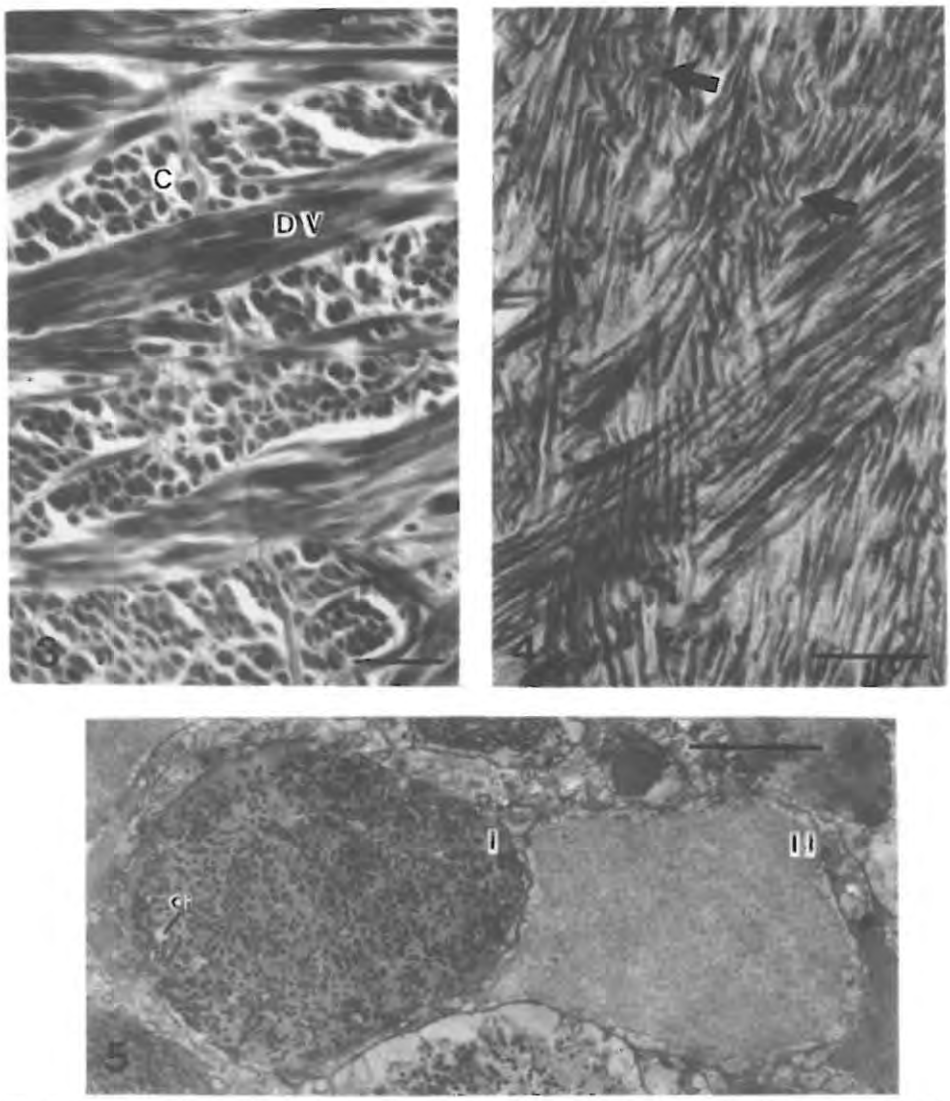


Fig. 3. Longitudinal (antero-posterior) section of the columellar muscle of *Patella longicosta* showing dorso-ventral (DV) and circumferential (C) muscle fibres. Scale bar = 50 μm.

Fig. 4. Longitudinal section of the columellar muscle of *Helcion pectunculus* showing several crimped muscle fibres (arrows). Scale bar = 100 μm.

Fig. 5. Transverse sections of type I (I) and type II (II) myofibres from *Helcion pectunculus*. Note the abundance of thick filaments in the type I fibres and the total absence in type II. Small cisternae (ci) are visible in the type I myofibre. Scale bar = 3 μm.

sides of the foot of patellid limpets forming a network of very fine muscle fibres. In addition we have found some crimped fibres at the sides of the foot (Fig. 4).

Histological observations also show that collagenous connective tissue permeates the entire musculature and constitutes about 30-40% of the shell and pedal musculature (as estimated from relative areas of differentially stained collagen and muscle). Collagen is particularly conspicuous near the shell attachment site, the sole and sides of the foot.

Fine structure of the shell muscle

The fusiform muscle fibres, which have a maximum observed diameter of 13 μm, can be classified into 2 types (Fig. 5). Type I fibres are the predominant cell type and are found in all species. Cell components consist of a random arrangement of thin and very thick filaments (Figs 6 and 7). Dense bodies, which are sites of thin filament attachment, are seen rarely, but this may be due to the fixation procedure. Those present are relatively electron-lucent with a loose granular substructure and diameters (about 70 nm, Fig. 6) of the same order of magnitude as the thick filaments. Occasionally electron-dense plaques are seen at the cell membrane. These may be attachment sites for thin filaments.

Mitochondria, which are located peripherally, are scarce, small (500-900 nm diameter) and have long, narrow cristae. A few sarcoplasmic cisternae occur at the periphery of the cells and occasional translucent vesicles are seen within the body of the cell (Fig. 5). No sarcolemmal invaginations were observed.

The diameter of the thin filaments is 6 ± 0.8 nm (standard deviation, n = 30) as is typical for actin-containing filaments. Length measurements were impracticable because the filaments meander in and out of the plane of the sections.

In cross section the thick filaments show a partial order in their arrangement forming rows, rings and loops. This is analogous to the arrangement observed by Hunt (1972) in the hypobranchial gland muscle of *Buccinum undatum*. A few often incomplete rings of approximately 12 thin filaments are sometimes observed surrounding the thick filaments (Fig. 6).

Thick filaments exhibit a number of features common to paramyosin-containing filaments of other molluscan muscles (Bennett & Elliott, 1987). In cross section, they are diverse in shape and size (Fig. 6) ranging between 20-180 nm although the most frequently observed diameter is approximately 70 nm. Some of this variation may be attributable to the filaments being tapered. Reliable measurements of thick filament lengths are difficult to obtain but lengths greater than 5 μm are observed.

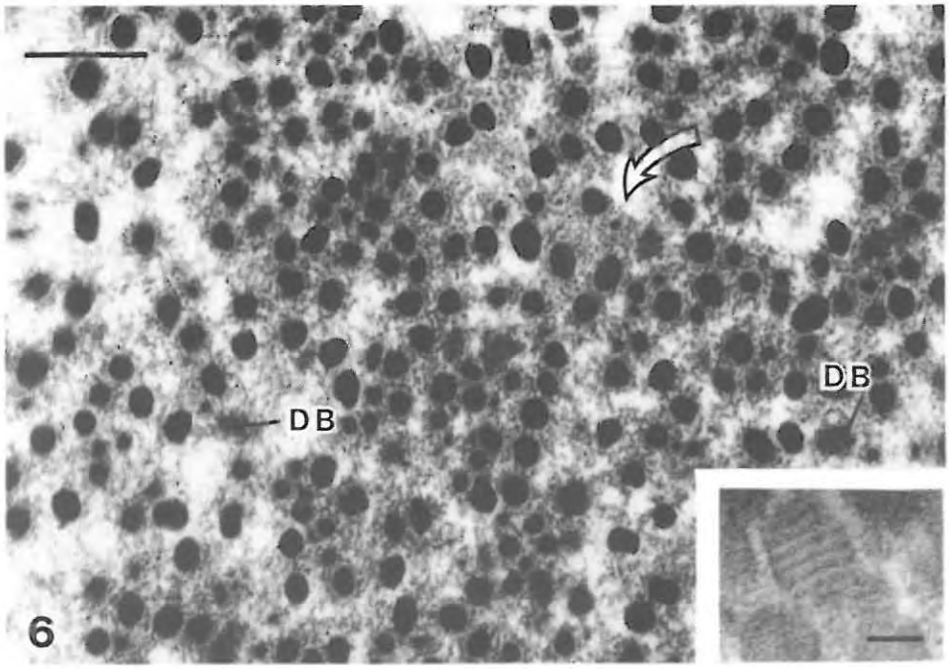
Longitudinal sections of thick filaments show that they have a distinct axial periodicity (Fig. 7) of 14 ± 0.9 nm (standard deviation, n = 18) (estimated by measuring the distance between the first and the last observed period and dividing by the number of periods observed; between 8-30 periods were measured per filament). In addition to the 14 nm axial repeat, a chequerboard pattern is seen on some thick filaments (Fig. 7). This is the crystalline lattice of paramyosin that was first deduced from X-ray data of thick filaments of clam adductor muscle by Bear and Selby (1956).

When observed in nearly transverse section, electron-lucent and electron-dense bands are seen crossing some filaments with a regular spacing of 22.1 ± 2.5 nm (for n values see Table 1) (Fig. 6, inset).

Type II fibres have so far only been seen in *Patella longicosta, P. oculus, P. vulgata, Helcion pectunculus* and *P. tabularis*, where they constitute about 25% of cells seen. They contain fascicles of thin filaments, many of which show a periodic superstructure of electron-dense bands (Fig. 8). The bands have diffuse boundaries and occur at a centre to centre spacing of 80-150 nm. Some cells contain only a few bundled thin filaments while others are more populated. The background material is amorphous and neither dense bodies nor thick filaments are seen in these fibres, although mitochondria have been observed. The cells tend to be grouped, i.e. are not always evenly distributed among the type I cells. This explains the difficulty in estimating accurately the proportion of type I and type II cells from thin sections. It is not clear if the distribution of the groups is random, which may explain why they have not been seen in all limpet species studied so far.

The possibility that the arrangement of bundled thin filaments could be an artifact is unlikely for several reasons. The arrangement has been seen in five species; it is reproducible with different embedding and staining protocols; the arrangement occurs in cells adjacent to muscle fibres containing other preserved elements of typical smooth molluscan muscle; and the banding is strictly regular and therefore appears to emphasise some periodic structure.

The type II cells seem more closely packed than type I cells, but small amounts of collagen occur between the groupings of type II cells.



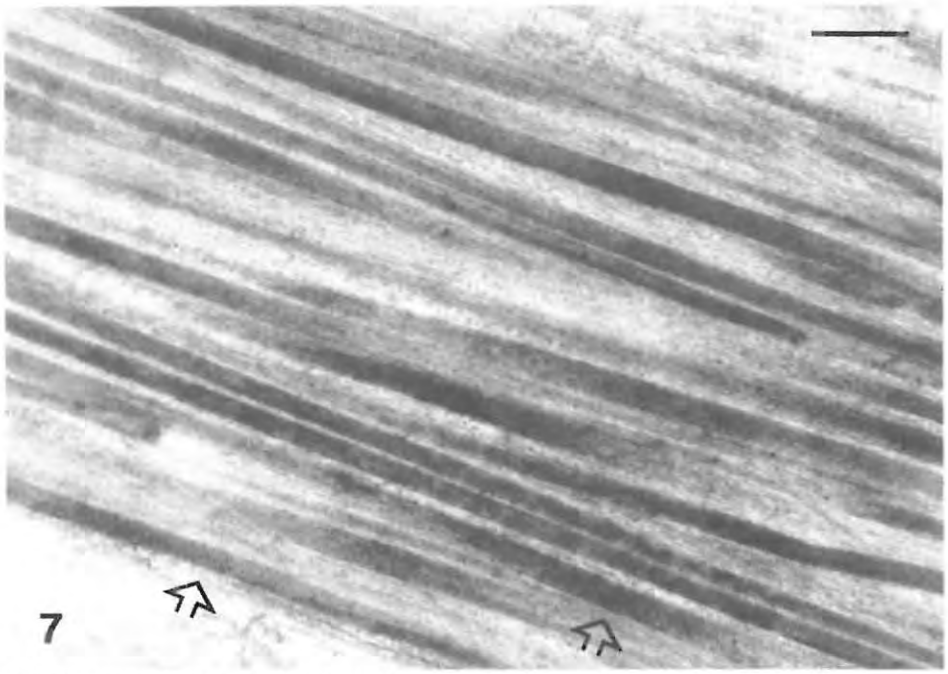
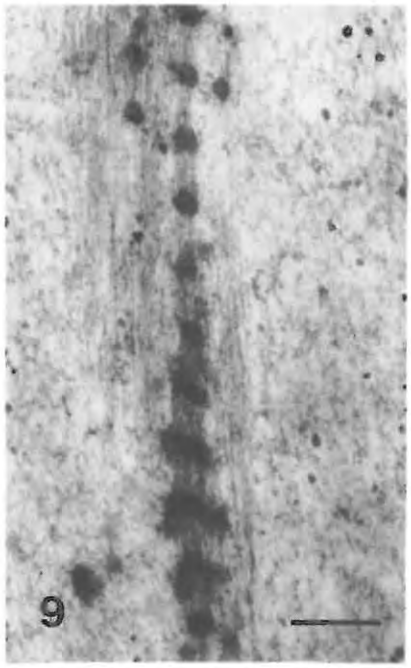


Fig. 6. Transverse section of a type I myofibre from *Patella miniata*. Some thick filaments have rings of thin filaments (arrow). Note the variation in size and shape of the thick filaments and the lack of preferred order in the arrangement of the filaments. Possible candidates for dense bodies (DB) are also shown. They are relatively less electron dense than the thick filaments and are of a comparable size. Scale bar = 0.3 μm. The inset shows a thick filament in transverse section from *Patella granularis*. The banding arises from viewing down the planes of the Bear-Selby net. Scale bar = 0.05 μm.

Fig. 7. Longitudinal section of a type I myofibre from *Patella cochlear*. Most thick filaments have a regular axial striation and some thick filaments show the Bear-Selby net pattern (arrowheads). Scale bar = 0.3 μm.



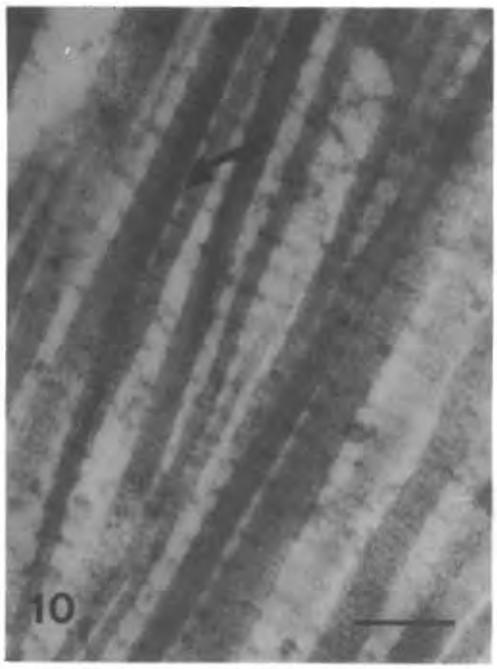


Fig. 8. Longitudinal section of type II myofibres from *Patella longicosta*. The fibre contains thin filaments bundled together periodically by electron dense regions. Scale bar = 1 μm.

Fig. 9. Longitudinal section of a type II myofibre from *Patella oculus* at a high magnification. Scale bar = 0.2 μm.

Fig. 10. Parallel arrays of collagen from *Patella tabularis* showing apparent cross bridging between the fibrils (arrow). Scale bar = 0.2 μm.

Table 1. Bear-Selby net *a* spacings from thick filaments of patellid limpet shell muscle compared with those from thick filaments of bivalve adductor and ABRM muscle from Bennett & Elliott (1981).

Species	Muscle	n	Mean *a* (nm)	Range (nm)
Bivalvia				
O. edulis	White adductor	11	27.3	25.8-29.8
C. gigas	White adductor	9	34.4	32.3-36.2
M. mercenaria	White adductor	5	26.6	25.2-29.4
M. mercenaria	Red adductor	3	24.9	23.1-26.6
M. edulis	ABRM	12	31.3	24.7-36.4
P. maximus	White adductor	8	28.7	21.1-35.6
Gastropoda: Patellidae				
P. tabularis	Shell muscle	3	22.5	22.0-23.1
P. granularis	Shell muscle	7	25.6	18.9-31.0
P. miniata	Shell muscle	5	22.5	19.9-27.4
P. cochlear	Shell muscle	3	22.3	17.9-25.2

n = no. filaments measured, on average 3 spacings per filament.

Intercellular regions can separate muscle cells by as much as 4-5 μm. They are packed with collagen fibrils that occur either as a loose network or, more frequently, as longer filaments in parallel arrays with side-projections which appear to cross-link the collagen at intervals corresponding to the axial collagen repeat (Fig. 10). The diameters of the collagen fibrils vary, having an upper value of 116 nm (n = 22).

Isolated filaments

Extraction and isolation procedures reveal the presence of collagen fibrils and muscle-derived thick and thin filaments in all three tissue homogenates examined. Thick filaments are most prevalent in the dorso-ventral tissue and have an axial periodicity of 14.2 nm (Fig. 11). The optical diffraction pattern of the thick filaments is consistent with a paramyosin structure (Fig. 11b). Five orders of the 1/14 nm reflections occur along the meridion and off-meridional reflections arise from the Bear-Selby net. Collagen fibrils with periodicity 62 nm are prominent in preparations of the shell region (Fig. 12). Their optical diffraction pattern shows 3rd, 5th and 7th orders of this repeat (Fig. 12b). Although typical muscle thin filaments were seen no striated bundled thin filaments were isolated. The supernatant contained only amorphous material.

SDS gel electrophoresis

Figure 13 suggests that paramyosin dominates in all 3 homogenates examined. It typically runs as a chain of 100 k molecular weight. Actin and myosin are also present running at 42 k and 220 k molecular weight respectively. Tropocollagen (a sub-molecule of collagen) runs on SDS gels as a chain of approximately 95 k molecular weight (Gosline & Shadwick, 1983). It may therefore be merged with the paramyosin which could partly account for the wide spread in this band. On the other hand, partial proteolysis of paramyosin or overloading of the gel could also account for the spread. If collagen molecules were covalently cross-linked, SDS would not break these bonds and collagen would not be seen on the gel. The standard protein is myosin isolated from rabbit psoas. There is no large quantity of unidentified protein except perhaps that running behind actin at approximately 50 k in the shell and dorsal tissue homogenates.

DISCUSSION

The arrangement of muscle fibres in the patellid foot is now known (Hyman, 1967; Fretter & Graham, 1962; Jones & Trueman, 1970; Kier, 1988; Voltzow, 1985, 1988). Muscular contraction in limpets is not accomplished in conjunction with a classical hydrostatic skeleton and the theory of fluid-filled cavities, essentially a modified hydrostatic skeleton, now seems unlikely (Grenon & Walker, 1978; Kier, 1988). Our results confirm that no fluid-filled cavities are present. The limpet columellar and pedal musculature is a system of muscle fibre bundles ensheathed in collagen (Voltzow, 1988) that work in antagonism, i.e. a muscular hydrostat (Kier, 1988).

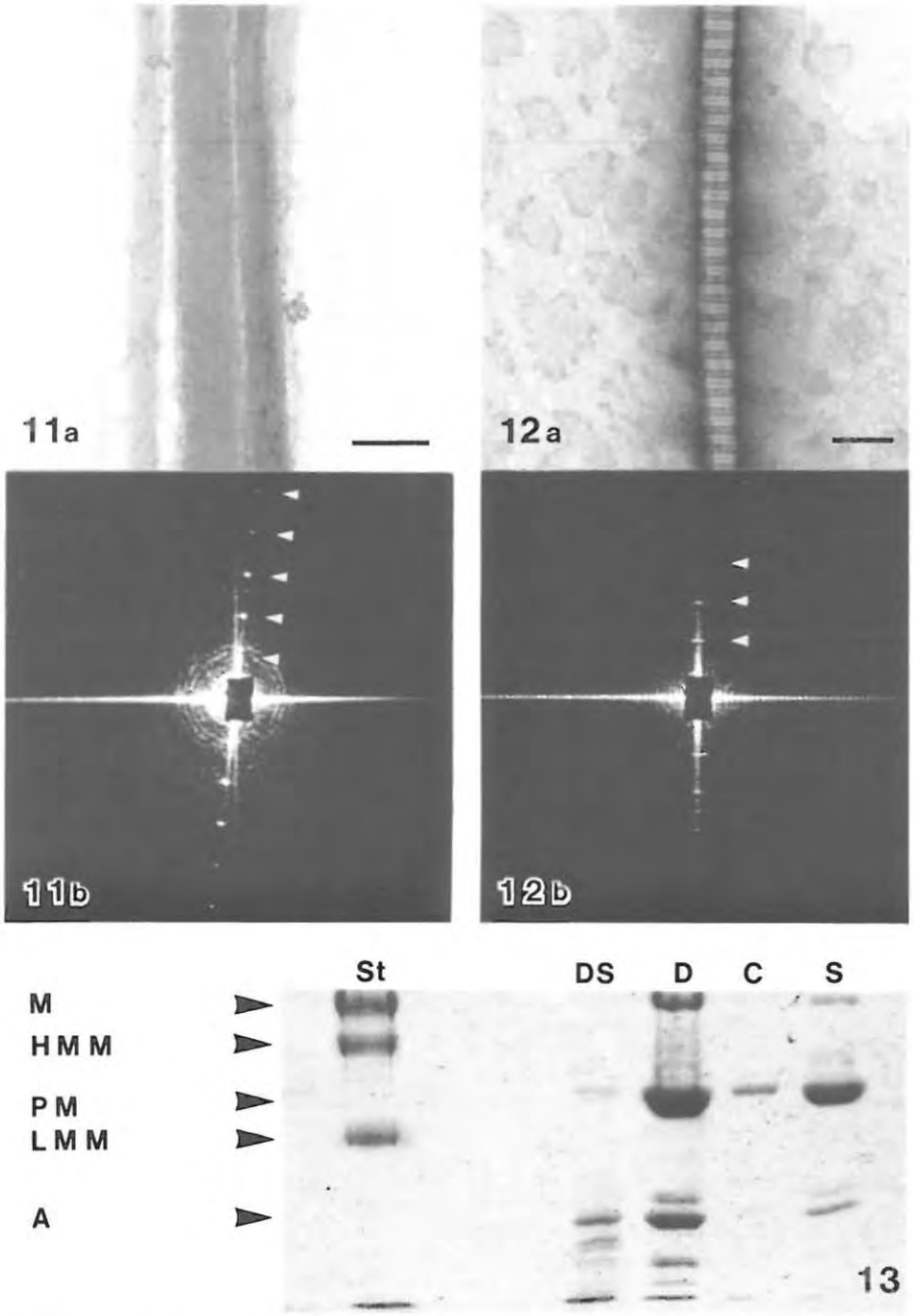


Fig. 11a. Isolated paramyosin thick filament with a 14.2 nm repeat from the dorso-ventral tissue of *Patella vulgata*. Scale bar = 0.2 μm. b. Optical diffraction pattern taken from the same filament. 5 orders of the 1/14 nm^{-1} reflections are visible (arrows) as the 5th, 10th, 15th, 20th and 25th orders of the 1/72 nm^{-1} reflection. They run along the meridion but are off-centred for greater clarity.

The crimped fibres revealed by our study may be the spiral fibres described by Jones & Trueman (1970), who suggested that these control rotation of the shell. We suggest that the cross-diagonal muscle bundles are involved in shell rotation and that crimping of these may arise when the dorso-ventral muscles contract.

While elastic fibres are not present in patellids (Jones & Trueman, 1970; Kier, 1988) collagen nevertheless occurs (Voltzow, 1988). The histological findings of this study show the distribution of collagen in the patellid foot and that it is present in high proportions comprising about 30-40% of the tissue. The prevalence of collagen is confirmed at the ultrastructural level. Gosline & Shadwick (1983) note that variation in invertebrate collagen periodicities has been observed. Collagen isolated from squid mantle is reported to have a periodicity of 68 nm (Hunt, Grant & Liebovich, 1970) whereas the 62 nm periodicity of isolated limpet shell muscle collagen compares more favourably with the 64 nm periodicity of mammalian collagen (Wainwright, Biggs, Currey & Gosline, 1976; Gosline & Shadwick 1983). Since the axial repeat is a direct reflection of the packing of tropocollagen molecules it can be inferred that limpet collagen fibrils have a similar structure to mammalian collagen.

There is evidence that transient intermolecular cross-linking occurs between echinoderm collagen molecules (Freinkal & Hepburn, 1975; Gosline & Shadwick, 1983; Motokawa, 1988). The function of the cross-links is to strengthen mechanically the collagenous tissue by preventing slippage between the fibrils. Freinkal & Hepburn (1975) have shown how the cross-links of collagen increase the stiffness of sea cucumber dermis. In the case of limpets such stiffening may contribute to rigidity of the foot which is essential to clamping (Fretter & Graham, 1962). Mechanical studies would be required to test this suggestion.

Another role of collagen is related to its ensheathing of muscle bundles. This forms small functional units that provide anchorage for the muscles and allow localised contractions, a feature typical of muscular hydrostats (Kier, 1988). Furthermore, the ensheathing collagen may be important mechanically for force transmission as suggested by Voltzow (1985) in her study of the pedal muscles of *Busycon contrarium* and *Haliotis kamtschatkana*. Since collagen plays an important part in many contractile systems of marine invertebrates (Gosline & Shadwick, 1983; Stott, Hepburn, Joffe & Heffron, 1974; Freinkal & Hepburn, 1975; Kier, 1988) we suggest that collagen has a more significant role in the functioning of gastropod muscular hydrostats than has previously been supposed.

Type I cells most resemble muscle cells from the ABRM of the bivalve *Mytilus edulis* (Sobieszek, 1973; Nicaise & Amsellem, 1983), the shell muscle of the gastropod *Lymnaea stagnalis* (Plesch, 1977; Nicaise & Amsellem, 1983) and muscle cells from the hypobranchial gland of *Buccinum undatum* (Hunt, 1972; Nicaise & Amsellem, 1983). Nisbet & Plummer (1968), when examining the collar muscle of *Archachatina marginata*, found a paucity of dense bodies. Hunt (1972) also saw few dense bodies when studying the hypobranchial gland of the gastropod *Buccinum undatum*. Such scarcity of dense bodies appears to be more characteristic of gastropod muscle than bivalve muscle (see Nicaise & Amsellem, 1983 for review) but could simply be a result of poor fixation of these structures. The thick filaments of limpet shell muscle are characteristic of large paramyosin-containing filaments like those from bivalves (Bennett & Elliott, 1987). The partial organisation found among the thick filaments could be explained by the sharing of thin filaments in contracted muscle. This tends to draw neighbouring thick filaments into a more regular order (Bennett & Elliott, in press).

X-ray and optical diffraction data for bivalves give a value of 14.4 nm and 14.6 nm respectively for the axial periodicity of thick filaments (Bear & Selby, 1956; Sobieszek, 1973). Measurements from optically diffracted micrographs and direct measurement of negatively stained isolated limpet shell muscle thick filaments give a comparable value to the bivalve data. While several examinations of molluscan muscles have revealed axially-striated thick filaments, so much variation has been observed in the periodicity, that it is not clear whether the thick filaments in these molluscan muscles are of the paramyosin type or not (Nisbet & Plummer,

Fig. 12a. Collagen fibril isolated from *Patella vulgata* showing a periodicity of 62 nm. Scale bar = 0.2 μm. b. Optical diffraction pattern of the fibril. The 3rd, 5th and 7th orders of the 1/62 nm are seen (arrows).

Fig. 13. Gel results from *Patella vulgata*. M, myosin; HMM, heavy meromyosin; PM, paramyosin; LMM, light meromyosin; A, actin; St, standard protein; DS, supernatant of dorso-ventral homogenate; D, dorso-ventral homogenate; C, circumferential homogenate; S, shell tissue homogenate.

1968; Hunt, 1972). However, Plesch (1977), reported a periodicity of 14 nm for the thick filaments of the columellar muscle from *Lymnaea stagnalis*. Differences in reported periodicity in other cases may be due to shrinkage and other effects of preparation for electron microscopy. Our optical diffraction results verify that limpet columellar muscle thick filaments are of the paramyosin type.

Elliott (1979), Bennett & Elliott (1981) and Elliott & Bennett (1982) have shown that the paramyosin core in thick filaments from smooth muscle in bivalves is crystalline and that the two appearances seen in negatively stained filaments and sections, namely, a 14 nm axial repeat and the Bear Selby net, are two different views of the crystalline structure. Since both these appearances are seen in the limpet thick filaments it can be assumed that they have a similar structure to the bivalve thick filaments. This crystalline structure would also explain the banded appearance seen in approximately transverse views of the filaments. Bennett & Elliott (1981) have shown that such banding can be seen in the thick filaments of a variety of bivalves when the paramyosin crystals are by chance viewed down the crystal planes. The transverse repeat, a, of the crystal lattice (Bear-Selby net) of paramyosin, is calculated using the relation

$$a = sh(1-s^2/c^2)^{-\frac{1}{2}}$$

where h is the first digit of the index of the plane giving rise to the spacings and c is the axial repeat of the Bear-Selby net (Fig. 14). (Bennett & Elliott, 1981; Frescura, 1987). (Note that the equation appears here in a slightly modified form amended by Bennett). Cross sections of thick filaments revealing banding are oval with the long axis about 1.5 times longer than the short axis (Fig. 6, inset). This suggests a large angle of tilt in the filaments. It is thus assumed that the 102 planes are the ones visible in this study (Fig. 14).

Table 1 compares calculated values from this study for the transverse repeat, a, assuming the 102 planes are visible, to those from the literature for bivalves. The variability between patellid species is small with values falling consistently within the X-ray data range of 19-30 nm for bivalve data (Bennett & Elliott, 1981). This consistency indicates that the paramyosin crystal core is fairly stable, with a constant packing arrangement for the paramyosin molecules. It is also further evidence that the crystal-like form is the usual structure of the paramyosin core of thick filaments in smooth molluscan muscles

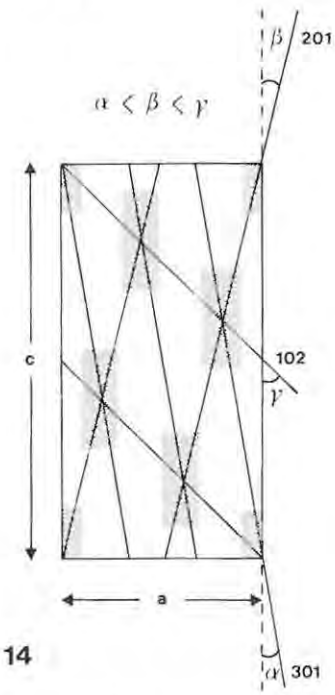


Fig. 14. Diagram of the Bear-Selby net. The 102, 201 and 301 planes are shown. c is the vertical repeat of the net (72nm) along the filament axis; a is the transverse repeat of the net (19-30nm). The boxes are the nodes of the crystal lattice where stain would penetrate the thick filament. A thick filament must be tilted at an angle gamma from the vertical axis in order to reveal the bands as seen in Fig. 6, inset. Modified from Bennett and Elliott (1981).

generally and not in bivalves only (Bennett & Elliott, 1981).

A low mitochondrial density implies the muscle is of low endurance (Plesch, 1977), which seems surprising if the muscle is to maintain high tensions for any length of time. Anaerobic respiration is one explanation. Another is catch, a prolonged state of tension in molluscan smooth muscles which uses little energy (Hanson & Lowy 1960; Lowy & Millman, 1963). Catch muscles always have paramyosin thick filaments (Bennett & Elliott, 1987; Chantler, 1983) and such thick filaments are thus often assumed to have a role in the catch mechanism (Cohen, Szent-Gyorgyi & Kendrick-Jones, 1971; Ruegg, 1971; Castellani & Cohen, 1987). The converse however, is not always true. Ishii & Takahashi (1981) studying the posterior byssus retractor muscle and Castellani, Vibert & Cohen (1983) studying the pedal retractor muscle (both

Table 2. Comparison of periodicities of stress fibres, a; microfilament bundles, b; bundled thin filaments, c.

Cell type	Periodicities (μm)	Range (μm)	Reference
Fibroblast[a]	1.3	1.00-2.40	Sanger *et al.*, 1983
Epithelial[a]	0.8	0.50-1.10	Sanger *et al.*, 1983
BHK-21[b]	0.6	—	Goldman *et al.*, 1979
Endothelial[b]	0.5	—	Giacomelli *et al.*, 1970
Intrafusal[b]	0.2	0.15-0.30	Karlsson & Andersson-Cedergren, 1968
Limpet shell muscle[c]	0.1	0.08-0.15	This study

muscles from *Mytilus edulis*) showed that the thick filaments were of the paramyosin type. However, neither muscle has catch properties. The economy of a catch mechanism seems an obvious advantage to a limpet when faced with a need to clamp to rocks for any length of time. A study of the mechanics of the shell muscle should disclose whether catch is present or not.

Although type II cells are here referred to as muscle cells, it would be fair to dispute this terminology since we do not know if the cells are contractile. However, there are similarities to smooth muscle cells; the shape is elongate and the filaments are similar to type I thin filaments both in diameter and appearance.

Standard muscle filament extraction procedures did not isolate the bundled thin filaments of the type II muscle fibres. This implies either that they are easily degraded or that a different set of conditions is required to maintain their integrity. The electrophoresis results suggest that they are made from conventional muscle proteins, probably actin, since there are no indications of any major quantities of other proteins. Work is currently in progress to identify the constituent proteins.

Rice, Moses, McManus, Brady & Blasik (1970) described a smooth muscle cell type from vertebrates which has no thick filaments. However, the similarity with the type II cell of limpet columellar muscle ends there, because the thin filaments of the vertebrate cells are not striated or bundled. Furthermore, the vertebrate cell thin filaments occupy the entire fibre, i.e. there are no extensive regions of amorphous material. The bundled thin filaments are similar in appearance to the striated microfilament bundles and stress fibres of non-muscle cells, even though their periodicities differ (Table 2). They are also similar to the leptomeric organelles described by Karlsson and Andersson-Cedergren (1968) and Ovalle (1972).

It is tempting to relate bundled thin filaments to stress fibres for two reasons. Not only do bundled thin filaments have ultrastructural features in common with stress fibres, but they both occur in a context where tension is developed. A contractile role has been ascribed to stress fibres and leptomeric organelles. If bundled thin filaments have the same role it seems paradoxical to find them in tissue which is already equipped with a contractile apparatus in the form of type I fibres. It seems better to regard their function as complementary to conventional contractile apparatus rather than a substitute for it. On the other hand if they are contractile, one would expect the periodicity to vary under contracted and relaxed states. If they develop in response to high tensions one might expect their occurrence to increase or decrease according to the loading imposed on the muscle. It is worth noting that preliminary observations indicate that the variation in distance between electron-dense regions of the bundled thin filaments may be species-specific and related to both tenacity and zonation level of each species. The precise role of the bundled thin filaments is yet to be determined.

In conclusion, since many of the ideas arising from this discussion lend themselves to experimental investigation, further studies, particularly of the mechanical properties of the muscle, should enable us to define more precisely the correlations between structural components of limpet shell muscle and their role in clamping. Furthermore, it will be important to see whether such correlates are present or absent in related species exhibiting strong or weak clamping abilities respectively.

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