POST MORTEM BREAKDOWN OF THE MYOTENDINOUS JUNCTION IN FISH

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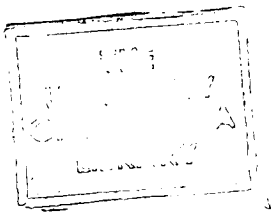

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Statement

This thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution and that, to the best of the candidates knowledge and belief, the thesis contains no material previously published or written by another person, except when due reference is made in the text of the thesis.

H. Allan Bremner

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Abbreviations

1^{0}Ab	Primary antibody
Ala	Alanine
Arg	Arginine
Asp	Aspartic acid
BLOTTO	Non-fat dried milk powder
BSA	Bovine serum albumin
CM	Carboxymethyl-
CNBR	Cyanogen bromide
Cys	Cystine
DAB	Diaminobenzaldehyde
DEAE	Diethylaminoethyl-
DHLNL	Dihydroxylysinorleucine
DPX	Section mounting glue
ELISA	Enzyme linked immunoabsorption assay
Glu	Glutamic acid
Gly	Glycine
His	Histidine
HLONL	Hydroxylysino-5-oxo-norleucine
HLNL	Hydroxylysinorleucine
HO-Lys	Hydroxylysine
Ho-Pro	Hydroxyproline
HPLC	High Performance Liquid Chromatography

Hyl	Hydroxylysine
IgG	Immunoglobulin G
Ile	Isoleucine
Kd	Kilodalton
Leu	Leucine
Lys	Lysine
Met	Methionine
PAG	Protein A gold conjugate
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
Phe	Phenylalanine
Pro	Proline
PVA	Polyvinyl acetate
RAM	Rabbit antimouse serum
RT	Room temperature (nominally near 20^0C)
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
Ser	Serine
TEM	Transmission electron microscope
Thr	Threonine
Tris	Tris(hydroxymethyl)aminomethane
Trp	Tryptophan

Tyr	Tyrosine
Val	Valine

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Papers Published from Work Covered in this Thesis

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Abstract

Abstract

The myotendinous junction of fish muscle was investigated in detail using both scanning and transmission electron microscopy (SEM, TEM respectively). The properties of the main connective tissue structure, type I collagen, were characterised and the purified collagen was used to prepare antibodies to examine structural aspects by immunogold procedures.

The SEM studies showed that a network of fibrous connective tissue surrounds each muscle fibre, linking it into a socket-like indentation in the myocomma. These connective tissue fibres were degraded in chill storage, leading to the detachment of muscle fibres from the myocommata and subsequent loss of tissue integrity. Detailed study by TEM demonstrated grooves and invaginations in the terminal ends of the muscle fibres that were filled with fine collagen fibres from the myocomma. These collagen fibres were linked by fine connections to the basal lamina which in turn was linked to the sarcolemma.

During chill storage, the basal lamina, the fine connections and the fine collagen fibres progressively degraded and significant deterioration occurred in the myotendinous junction. This deterioration preceded any obvious changes within the muscle fibre structure.

The major structural collagen of the skin and muscle and other organs in the fish blue grenadier is an heterotrimer of type I collagen. This collagen was highly soluble in dilute acid and the proportion of insoluble collagen

increased with the age of the fish. The collagen possessed three alpha chains in its molecular structure and the amino acid composition of the α-3 chain indicated its derivation from the α-1 chain. The melting and shrinkage temperatures obtained for this collagen were consistent with the imino acid levels and the environment of the fish.

Immunogold labelling procedures were developed which confirmed the presence of type I collagen fibres in the myocomma adjacent to the muscle fibre cell but which were inadequate to define individual collagen fibre types.

These studies illustrate the complex and intricate nature of the myotendinous junction in commercial fish species. They show that post mortem degradation occurs external to the muscle fibre cell in the extracellular matrix and, in particular, in the fine collagen fibres that form the muscle cell envelope and fill the interstitial muscle space. The study has thus shown that the initial problems of post mortem softening and gaping have their origin in the myotendinous junction not within the muscle fibre itself. The TEM work confirms the SEM work that the breakdown occurs at the interface between the muscle fibres and the connective tissue of the myocomma. It has highlighted the need for further work on the nature and properties of the interstitial collagens and on the nature, activity, specificity and location of the enzymes responsible for the degradation. It has also pointed out the need to establish whether the marine equivalents of minor

components of mammalian muscle and its extracellular matrix occur in fish.

Foreword

Foreword

In the post mortem state, the flesh of many species of fish softens and 'gapes'. Gaping is the name given to the phenomena when the muscle bundles, the myotomes, separate from the myocomma, the sheet of connective tissue that separates them. Fissures may also appear along the myotomes between the muscle bundles. This undesirable phenomenon may occur within a day or two post mortem even in fish that have been adequately chilled (near 0^0C). The result is poorer products of inferior appearance, that are more difficult to handle and process. Gaping is of considerable economic importance. The problem is common in many commercially important species, but is particularly prevalent in the merluccid hakes of which some 1.5 million tonnes are caught annually worldwide. The blue grenadier (*Macruronus novaezelandiae* Hector) is an important trawlfish caught in Australia that is related to the hakes, and its flesh is prone to softening and gaping. The fishery is in a rapid developmental stage and the current total allowable catch is 5000 tonnes per annum.

The breakdown in integrity of the muscle structure that leads to softening and gaping occurs in the region of the myotendinous junction but it is not

clear whether this change is within the structure of the junction itself, or in the adjacent muscle tissue, or in the connective tissue of the myocomma; or, in all these structures. Since the detail of the structure of the myotendinous junction in fish is not well elucidated it had not been possible to identify which components deteriorate and by what mechanisms these changes occur.

Aim

This study was initiated to establish detail of the fine structure of the myotendinous junction in fish, to examine which parts of this structure deteriorate and to explore the mechanisms involved.

Changes in components in tissue, particularly proteins, are often detected using electrophoresis or similar separative techniques on extracts of the tissue. Changes in structure of the tissue are then inferred from these results. In this study elucidation of the structures *in situ* was considered to be more important initially.

The following approach was taken - muscle was examined under the scanning electron microscope (SEM), which indicated that fine connective tissue structures of the myocomma and the muscle cell envelope were being degraded. This was followed by studying similar muscle samples under the transmission electron microscope (TEM) which showed detail of the

elements of the fine structure being degraded during storage. In order to identify the nature of the collagen involved, this structural element was extracted and purified by chromatography and electrophoresis and chemically characterised. To locate and identify the particular collagen fibrils in situ, antibodies to the collagen were raised and used with an immunogold method to explore the ultrastructure.

Chapter 1

Introduction

1.0 Introduction

1.1 Structure of fish flesh

The flesh of teleost fish is constructed of adjacent muscles blocks, called myotomes, separated from each other by sheets of collagenous tissue called myocommata (Nursall, 1956). The myotomes on both sides of the axial skeleton each take the form of double cones which fit together along the long axis of the body to give the appearance at the surface of a series of W-shaped sections resting on their sides (Figure 1.1). Within each myotome the muscle fibres (myomeres or myofibrils) run approximately parallel to each other but at varying angles to the myocommatal sheet to accomodate the juxtapositional rhythmical contractions that occur during swimming so that all the fibres in the myomere contract to a similar extent when the fish bends. This results in maximum power output at a given rate of contraction (Alexander, 1969). The myocommata are connected internally to the skin and to the skeletal system and are also linked to the membrane dividing the fish into epaxial and hypaxial planes and to the median vertical septum. The overall geometry of the muscle fibres to the connective tissue is most complex making it extremely difficult to prepare samples with favoured orientation. Further detail on the overall structure can be found in the reviews by Nursall (1956) and Alexander (1969).

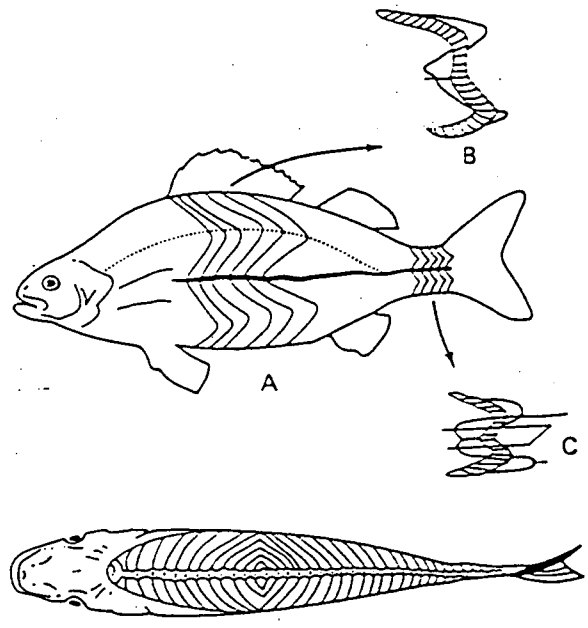

Figure 1.1 Diagramatic representation of the musculature of a typical teleost showing A the myocommata separating the muscle blocks (myomeres or myotomes) and B, C the approximate orientation of the muscle fibres within the myomeres and their tapering cone-like structures which interlock to form the muscle mass. (Adapted from Ellis *et al.* 1978).

The junctions between the myomeres and the myocommata in fish are equivalent to the myotendinous junction in mammalian muscle and this term will be used to describe them in this thesis. The myotendinous junction is reported to be formed by fine collagenous processses which have their origin in the myocomma; these then proceed as sheaths to surround each muscle fibre (Love 1970, Love *et al* .1969).

There is, however, remarkably little detail known of the fine structure of the junction in fish muscle even though it plays a prime role in the transfer of muscular contractions *in vivo* and in maintaining flesh integrity post mortem. Pre-eminence has been given to understanding the structure of myotendinous junction of mammalian muscle.

1.2 General aspects of the myotendinous junction

Early this century, general texts on anatomy described the fibres of the tendon bundle as ending abruptly on reaching the rounded or obliquely truncated extremity of a muscular fibre (Schaffer,1912). Under the light microscope, the muscle fibre end was not smooth and rounded, but appeared scalloped where it embedded in the connective tissue. Until the advent of the electron microscope (EM) little progress was made in elucidating this fine structure. The early EM studies by Porter (1954) and Ruska (1954) established that the muscle fibre terminated in finger like

projections which were bounded by the cell membrane; the actin filaments terminated inside the cell. Collagen fibres likewise did not penetrate the cell membrane and were never found within the muscle cell (Porter 1954; Ruska 1954). Thus, the continuity theory which was held by many early workers (Schultze 1912; Sobotta 1924; Carr 1931; Butcher 1933; Schmidt 1936), that muscle fibres merged into tendon fibrils, was rejected in favour of the view that the sarcolemma formed a boundary between muscle cells and the connective tissue, as reported by several early workers (Baldwin 1913; Haggqvist 1931; Goss 1944; Long 1947).

A major feature which is evident in all species so far investigated is the complicated folds and invaginations which occur at the myotendinous junction. This complex geometry increases the area of contact between the end of the muscle cell and the external matrix by a factor of about 20 to 30 times for fast twitch muscles and 50 times for tonic cells (Tidball and Daniel 1986). The load on the cell membrane caused by muscular contraction is thus reduced by an order of magnitude (Trotter, Hsi, Samora and Wofsy 1985). Further, the geometry of the invaginations, relative to the force transmitting actin filaments attached to the sarcolemma ensures that the forces are transmitted in shear not tensile mode (Tidball 1983).

1.2.1 Three structural domains

The myotendinous junction has three major structural domains which are in close proximity (i) the membranes and fine processes at the junction (ii) the external connective tissue stroma which eventually links to the tendon and (iii) the internal elements of the muscle cell and the sub- sarcolemmal surface.

1.2.1.1 The basement membrane

The basement membrane, sometimes called the basal lamina, occurs adjacent to the sarcolemma. It has commonly been considered part of an integral structure marking the boundaries of cells, even though it may not always follow exactly all the convolutions of the sarcolemma. The basement membrane serves a variety of functions - mediation of interactions between specific cell layers and their underlying stroma, molecular ultrafiltration and tissue organization.

The basement membrane is an extracellular matrix in which two obvious features are discernible. The outer area, the lamina densa, is an electron dense zone that is comprised mainly of collagen, mostly type IV, and the lamina lucida (rara). The latter lies between the lamina densa and the sarcolemma and although it is more transparent to electrons some structural

details are recognised.

In the lamina densa the collagen type IV molecules form unique end to end associations to construct a network of molecules which comprise the structural framework of the membrane (Timpl and Dziadek 1986). There are several other components, in particular laminin and heparin sulphate proteoglycan. The large molecular weight glycoprotein laminin has been localised to the basement membranes of skeletal muscle and is distributed thoughout the lamina densa and the lamina lucida (Stephens, Bendayan and Silver 1982; Mayne and Sanderson 1985). The proteoglycans are formed of a central protein core with glycosoaminoglycan side chains that are multiply linked by covalent bonds; these molecules affect permeability and cell attachment (Mayne and Sanderson 1985).

Collagen fibres from the connective tissue were thought to attach to the basement membrane and the analogy of a rope untangling at its end to provide the fibres which were woven into the carpet of the basal lamina was employed to convey the concept of how force may be transmitted from the muscle to the tendon (Mackay *et al.* 1969). It was assumed that tropocollagen molecules provide the linkage between the sarcolemma and the basal lamina.

Low (1961,1962) described a set of fibrous structures with a diameter in the range of 4 to 12 nm which were finer than the collagen fibrils and which appeared to link between the collagen fibrils. He termed them

'microfibrils', although this term has other connotations in collagen chemistry. Hanak and Böck (1971) examined muscle-tendon transitions in the papillary muscle of the heart, muscle from the tip of the tongue, the diaphragm and the gastrocnemius of the guinea pig and reported the presence of 'microfibrils'. They proposed that these elements actually passed through the basal membrane and fused with the outer electron-dense layer of the sarcolemmal membrane. They interpreted this from their electron micrographs which showed a "ladderlike" structure formed between the sarcolemma and the basement membrane but which in some parts of the sections was at 45^0 angles to the sarcolemma and had the appearance of a solid fibre. In some instances the 'microfibrils' had the appearance of beaded threads rather than solid structures. Similar structures were demonstrated by Korneliussen (1973) in the myotendinous junctions of muscles of the hagfish *Myxine glutinosa* L. where spine-like structures about 6nm in diameter at 15-25 nm intervals extended from the external leaflet of the plasma membrane to the internal surface of the lamina densa. These structures were interpreted as being consistent with arrays of circular ridges between the sarcolemma and the lamina densa of the basement membrane but they were not as predominant or noticeable in the rat diaphragm muscle which was studied at the same time. Studies on tadpole muscle (Nakao 1974) also demonstrated the presence of a connecting 'intermediary layer' and further work by the same author (Nakao 1975)

showed similar structures in the myotendinous junctions of the lamprey. These beaded threads were also noted by Ajiri *et al.* (1978) in the myotendon of the rectus abdominus muscle of the bullfrog in the extracellular space of the lamina rara in invaginations at the end of the muscle cell. Similar connections were seen linking the lamina densa of the basement membrane to the fine collagen fibrils.

Trotter, Corbett and Avner (1981) used detergent and EGTA solvents to disrupt the sarcolemma and found that the small filamentous structures that cross the lamina lucida remained intact and that tension could still be transmitted across the myotendon, indicating that these filaments were attached to elements of the contractile structure not just to the sarcolemma alone. From this and other evidence they concluded there were three important structural facets, one that binds actin near the sarcolemma to transmit the contraction, another that crosses the hydrophobic portion of the membrane and a third that transmits tension from the membrane to the lamina densa. Further work indicated that the filaments of the lamina lucida are composed of two subdomains one closely associated with the sarcolemma and the other with the lamina densa; the connection between them is ionic not covalent (Trotter, Eberhard and Samora 1983a).

Very recent work has shown that the beaded microfibrillar structures in the extracellular matrix of many tissues are composed of fibrillin (Keene *et al.* 1991; Kielty *et al.* 1991). The method of preparation and the degree of

tension in the tissue affects whether the fibrillin structures appear in the TEM in the beaded form or as microfibrils, thus explaining the previous confusion as to their morphology (Keene *et al.* 1991). Fibrillin is a large glycoprotein (350Kd), containing approximately 14% cysteine, which has the appearance under TEM of an extended flexible molecule 148 nm long and 2.2 nm in diameter (Sakai *et al.* 1991). The ultrastructural and immunohistochemical evidence indicate parallel, head-to-tail alignment of fibrillin into microfibrils.

Nakao (1975) seems to be the first to have observed that the basement membrane, which otherwise is continuous, is absent in the terminal ends of the invaginations of muscle cells in the lamprey and the tadpole (Nakao 1976). This absence is unusual; no breaks occur in the sarcolemma. The ends of the invaginations often coincide with the cisterns of the sarcoplasmic reticulum and there are specific couplings between the sarcolema and these terminal cisterns. In these invaginations only the sarcolemma forms a barrier between the muscle cell and the connective tissue and the external milieu. Some fine collagen fibres proceed beyond the limits of the basement membrane but there is no evidence that they join with or penetrate the sarcolemma.

1.2.1.2 The extracellular matrix

The major feature of the extracellular matrix is the fibrous collagenous network that surrounds each cell and which joins to form the tendons and ligaments that attach to the skeletal system. The structure of collagens, their types and properties is discussed in detail in a subsequent section. In the endomysium the fibres are fine and tend to form a lace network around the muscle fibre (Rowe 1981). Branched reticular fibres which form rows arranged obliquely or perpendicularly to the long axis of the muscle are also present in mammalian muscle (Orcutt *et al.* 1986). These reticular fibres were once considered to be another form of proteinaceous fibre, but have since been shown to be type III collagen. Larger diameter collagen fibres of the perimysium surround the muscle fibre bundles and these run into the major sheets of collagen of the epimysium which cover individual muscles to become tendons.

Other cells and components are present in the collagen matrix along with the ground substance in which it embeds. Fibroblasts with their flattened dendritic processes are present, often at boundaries between adjacent layers of collagen fibres. These fibroblasts are derived from mesenchymal cells and they produce the collagen molecules. Adipose cells, mast cells and macrophages containing lysosomes are regularly noted in the looser connective tissues as well as eosinophilic leukocytes and plasma cells.

The collagen is produced by the fibroblasts which are evident adjacent to the muscle cell ends and from which flattened dendritic processes emerge. Elastin fibres, which play a role in providing some of the elastic properties, are also present. These fibres are comprised of two separate protein components, elastin itself and microfibrils of approximately 10 - 12 nm in diameter, which occur mostly around the periphery of the elastin and comprise about 10% of its weight (Gosline and Rosenbloom 1984). The microfibrils are glycoproteins rich in polar amino acids (Bailey and Etherington 1980), somewhat similar in composition to the extension peptides of procollagen (Scherr *et al.* 1973). They appear to be laid down before the elastin and influence elastin deposition.

Elastin is an amorphous protein with a molecular weight near 70 kD and an amino acid composition with some similarities to collagen in that glycine comprises one third of its residues. Some 40% of its amino acids have hydrophobic side chains and its molecules are cross-linked by two characteristic and unusual amino acids, desmosine and isodesmosine formed from the conjunction of four lysine molecules (Partridge *et al.* 1963) mediated by the enzyme lysyl oxidase. Structurally it is quite different to collagen being arranged as a kinetically free random coil network which helps impart the elastic properties. Elastin occurs in the blood vessels of fish (Sage and Gray 1979) but there are no reports of it in the extracellular matrix of the muscle tissue. Elastin is only degraded by the group of

enzymes known as elastases which can also act on a whole range of other proteins (Bailey and Etherington 1980).

The ground substance of the extracellular matrix is composed mostly of glycoproteins and proteoglycans. The proteoglycans are complexes of a protein core with glycosaminoglycans chains attached covalently. These chains are linear polymers of about fifty repeating disaccharides, often O-sulphated N- acetylhexosamine and uronic acid. They are commonly of high molecular weight, up to millions of daltons, and may occur in aggregates over 100 million dalton. As a result they are large, highly charged and disperse molecules which form extended structures (Heinegard and Paulson 1984).

Proteoglycans in connective tissues attach to collagen fibrils at regular binding sites. In this way, the collagen controls its external environment. Furthermore there is evidence of proteoglycans present within collagen fibrils attached to the protofibrils that make up the fibrillar construction (Scott 1991). This presents a considerable advantage in tissue reconstruction or remodelling since the only the proteoglycans attached to the protofibrils need to be broken down, not the whole collagen molecule. In fish muscle the only reports of the presence of proteoglycans and sulphated proteoglycans is that of Kim and Haard (1992) who detected these substances in low levels in rockfish (Sebastes sp).

Hyaluronan is the largest proteoglycan. One of its main roles is in retention

and regulation of water flow. The chondroitin sulphates contain only glucuronic acid and are a common component in cartilage and intervertebral discs. Dermatan sulphates are distinguished by the presence of L-iduronic acid and are variable in composition being common in tendon, skin, aorta etc but not in cartilage; they bind to type IV collagen (Laurie *et al.* 1986). Heparin and heparin sulphate are complex copolymers of two types of disaccharides and are interactive components of the extracellular matrix. Keratan sulphate is distinguished by having sialic acid present at nonreducing terminal segments and by possessing oligosaccharide branches. Tissues such as cornea and nasal cartilage are rich in keratan sulphate and in fish it has been reported in the skin, cornea and caudal fin (Ito *et al.* 1982), but there are no reports of it in intramuscular tissue.

Two other glycoproteins that occur in connective tissue and basement membranes are the fibronectins and laminin (Hakomori *et al.* 1984). Fibronectin occurs in pericellular tissue and, among other roles, it binds to collagen, proteoglycans and cell surface receptors so that it is involved in the stability of the myotendinous junction. Its structure is made up of two unequal subunits joined by a disuphide bond in which the arms are free to link with other structures (Hynes 1986). Individual domains in each of the subunits impart to fibronectin the ability to bind with a wide range of substrates. There are no reports of fibronectin in the muscle of fish but it has been separated from the plasma of torpedo fish (Engvall *et al.* 1978),

carp (Uchida *et al.* 1990) and recently a fibronectin-like protein has been found in the plasma of rainbow trout (Takahashi *et al.* 1992).

Laminin contains no hydroxy amino acids, but many half-cystine residues, and so is quite distinct from collagen. It is known to mediate adhesions of epithelial and endothelial structures at basement membranes. Its attachment to collagen type IV occurs 81 nm from the carboxy terminal end of this molecule (Laurie *et al.* 1986).

In muscle tissue the large collagen fibres are almost invariably type I collagen. Smaller proportions of type III occur and type V is associated with the type IV of the basement membrane. The collagen fibres are embedded in a matrix of proteoglycans. They are often crimped and run in layers in different directions to provide a strong flexible structure. The fibres have a range of diameters according to their position and function, from fine endomysial fibres of about 30 nm to over 100 nm in diameter.

Low (1961) indicated that finer connections appeared to join the collagen fibres. More recent studies have shown that in some epithelial tissues 'anchoring fibrils' contain a recently discovered collagen, type VII (Bentz *et al.* 1983, Sakai *et al.* 1986), as the primary structural agent. This collagen forms an extended network of fibrils between anchoring plaques in the lamina lucida of many epithelial tissues (Keene, Sakai, Lunstrum, Morris and Burgesson 1987) but to date type VII has not been reported in muscle or other internal organs.

A new group of collagens that provide molecular bridges in the extracellular matrix has now been described comprising collagen types IX, XII and XIV. They are named the FACIT collagens (Fibril-Associated Collagens with Interrupted Triple helices) since they contain quite large non-triple helical regions (Shaw and Olsen 1991). Their multi-domain structure and their location and protrusions into the perifibrillar space suggests that they are important elements in providing the structural diversity of connective tissue collagen scaffolds. Type IX locates its long arm along the surface of cartilage type II collagen while its short arm, which terminates in a large non helical domain, protrudes and is capable of interactions with other matrix components such as proteoglycans.

Type XII molecules have been demonstrated by antibody staining to localize in dense type I containing tissues such as tendons and ligaments but not in bone and skin. Another collagen type designated as type XIV which contains some sequences homologous to type XII collagen occurs in skin and tendon. Different forms of type IX collagen are found in different tissues and hence their interaction properties are likely to be different. None of these FACIT molecules have yet been reported in fish.

1.2.1.3 The internal structure

Muscle fibres are comprised of bundles of fibrils (myofibrils) arranged

longitudinally within the muscle cell and organised within a cytoskeletal framework of desmin- containing intermediate filaments (Lazarides 1980). The basic repeat unit of the myofibril is the sarcomere (often termed myomere in fish) and each sarcomere is bounded at each end of its long axis by the electron dense structure known as a Z disc (or Z band, or Z line). Each sarcomere has a central protein-dense region, the A band, and the isotropic I band situated between the A band and the Z disc (Figure 1.2). The contractile apparatus is comprised of the major internal components of the sarcomere, the interdigitating proteins actin and myosin. Actin filaments extend from one Z disc to the next and, in cross section, are arranged in an hexagonal array parallel to and around the myosin rods which themselves do not attach to the Z disc, but which occur in the mid portion of the sarcomere. The rod-shaped protein tropomyosin occupies the grooves of the actin helix providing a structure for the globular protein troponin to attach at regular intervals. The third most abundant protein in muscle is titin (also called connectin) which as 'gap filaments' join the thick myosin filaments from their ends to the Z disc and thus stabilize the myosin in the centre of the sarcomere (Horowits and Podolsky 1987). Another protein, nebulin is associated with titin in these 'gap filaments'.

The protein components of the Z disc are unresolved but alpha-actinin is a protein with actin-bundling properties and it comprises about 50% of the Z disc. Actin filaments from adjacent sarcomeres overlap into this structure.

Other proteins that have been suggested as components are Z-protein, amorphin, Eu-actinin, Z-nin, filamin, zeugmatin and another 220 Kd protein (Yamaguchi *et al.* 1986). Zeugmatin is present in the early formation of Z disc structures grown in cell culture, before alpha-actinin is observed, and hence probably plays some organisational role in development and organisation (Maher, Cox and Singer 1985). At the edge of each fibre, elements of the cytoskeleton link the filaments from the terminal Z disc to an electron dense meshwork at the sub-sarcolemmal surface. The terminal Z discs near the sarcolemma have been found to contain much less alpha actinin than those in the body of the fibrils. Accordingly it is assumed that this indicates that thin filaments are not bundled by alpha actinin near the sarcolemma (Tidball 1987) and that Z discs in different locations are not homogeneous in composition.

Talin, a 225 Kd protein has also been located at myotendinous junctions and is a component of the digitlike processes that extend into the tendons and may be involved in force transmission (Tidball, O'Halloran and Burridge 1986). The cytoskeletal proteins, the skelemins, are located at the periphery of the M discs while vinculin is organised along the sarcolemma in an array of rib-like bands termed costameres (Pardo, Siciliano and Craig 1983). Transverse sarcomeric filamentous systems organised at the Z and M bands levels are evident linking these structures to the sarcolemma (Pierobon-Bormioli 1981). Other proteins, C-protein and an X-protein, are

also found at the A bands. C-protein has a molecular weight near 135 Kd and it occurs bound to the light meromyosin region of the myosin tail where it appears to be able to interact with three to five actin sub-units. It is presumed to have a function in the interaction of actin and myosin (Pearson and Young 1989). The function of X-protein (152 Kd) is not known but it is found closely associated with C-protein, and may be bound to it (Pearson and Young 1989).

Each fibril is bathed in cytoplasm and is partly enshrouded by the membranous sarcoplasmic reticulum (SR) containing the sarcoplasm (Figure 1.3). The fenestrated network of the reticulum has two notable features, the tubular system (t-tubules) and the sarcoplasmic reticulum itself. The t-tubules are interconnected forming a network across the fibre surrounding each fibril at the level of the Z disc and they open out through the sarcolemma to the extracellular space. The SR system is continuous transversely but is discontinuous longitudinally and each sarcomere has its own reticulum. The SR swells out to form the terminal cisternae which in apposition with those of the adjacent sarcomere and the intermediate element, the T tubule, make up the triad structure. In fish the triads are situated external to the Z disc, but in mammals the triad occurs in the midportion of the sarcomere near the junction of the A and I bands.

Near the end of a muscle fibre the sarcomeres anastomose around the invaginations of the sarcolemma and actin fibres reach from the last

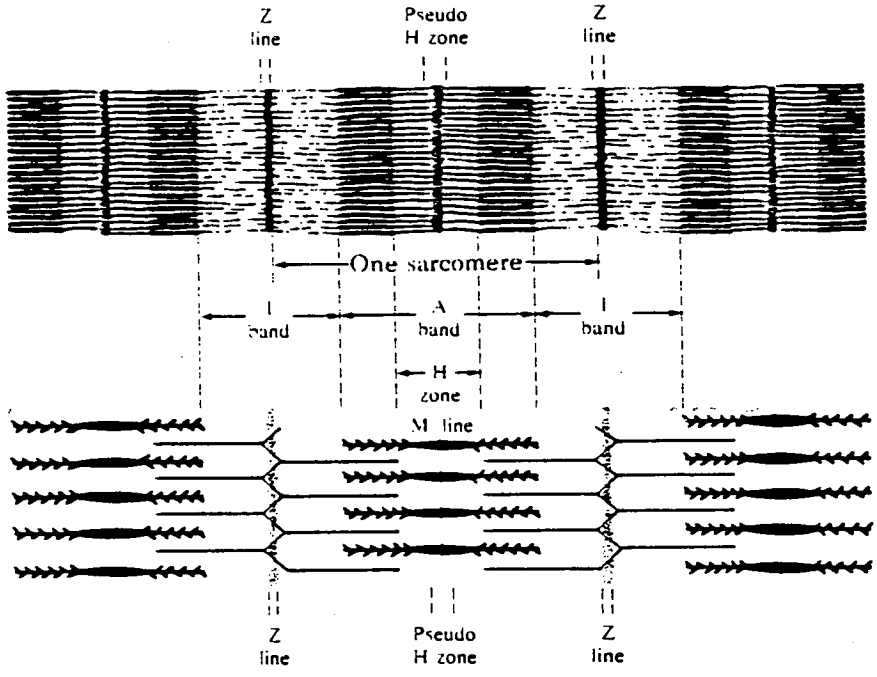

Figure 1.2. A diagrammatic representation of sarcomere structure identifying bands, zones and lines. (Adapted from Huxley 1965).

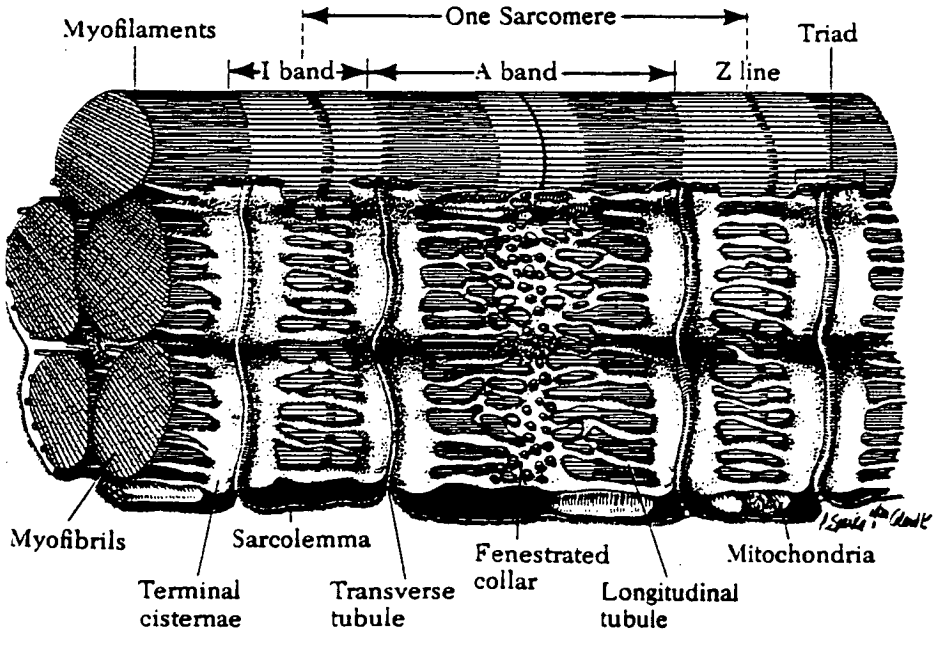

Figure 1.3. Diagrammatic representation of the sarcoplasmic reticulum and t-tubules and their relation to the myofibrils of skeletal muscle. (Adapted from Bloom & Fawcett 1968).

complete Z disc to the internal surface of the sarcolemma, where they attach in an electron dense sub-sarcolemmal layer. This layer is comprised of both globular densities and fine linear elements which run approximately parallel to the major actin fibres (Trotter, Eberhard and Samora 1983b). It is assumed that these structures are involved in anchoring the actin to the interior of the sarcolemma and the region has been referred to as the internal lamina by Trotter *et al.* (1983a). The proteins talin and vinculin are found at this location and it is considered that these may be the force transmitting and attaching proteins (Tidball, O'Halloran and Burridge 1986).

Longitudinal muscle growth occurs at the fibre ends (Williams and Goldspink 1971) and glycogen granules, mitochondria, polysomes and ribosomes occur with notable frequency near the fibril ends (Schattenberg 1973).

1.2.2 The myotendinous junction in fish

Shwarzacher (1960) examined muscle fibre-tendon junctions in the seahorse in comparison to those from the cat, rat, mouse and frog. There were considerable similarities and all the junctions revealed folds and finger-like projections at the ends enveloped by the basement membrane. Similarly, Schippel and Reisig (1969) indicated that the myotendon junction of the

flowing muscle of the spinal cord of the pipefish was similar to the appearance of that in the tadpole tail. In the primitive hagfish (*Myxine glutinosa*) Korneliussen (1973) found spine-like projections and threadlike cones between the lamina densa and the external surface of the sarcolemma which he concluded had the same feature, namely ring like structures that, according to the angle of section, could appear either as spines or threads. He also showed the fibres from the I band (actin fibres) attaching to the sub-sarcolemmal surface in electron dense areas and he suggested these were analogous to Z discs.

The sequence of events in the longitudinal growth of myofibrils of the skeletal muscle of Macropodus opercularis was reported by Schattenberg (1973) as follows. High ribosomal concentrations occur in the short terminal myofibril region along which actin filaments are formed. As the terminal portion enlarges myosin filaments are evident and A and I bands appear followed by the Z disc which forms in close contact with the sarcolemma and gradually detaches from it. Nakao (1975) showed that in the lamprey the sarcolemma at the terminal end of the invagination frequently showed specific coupling with the cisterns of the sarcoplasmic reticulum; the basal lamina was partially or completely deficient in this area. Although this allowed for the possibility of collagen fibres to be in direct contact with the sarcolemma no definite relationship between them was found. Thus the myotendinous junction in fish is similar in general

structure to that found in mammalian muscle. However, many details of the structure and composition have yet to be ascertained. Species of commercial importance have been neglected.

1.3 Collagen

The protein collagen is perhaps the most important structural tissue in the animal kingdom. It occurs in a diverse range of tissues, provides the framework for bone, is the major component of skin and it enshrouds muscle fibres and groups of fibres to provide the elastic components of tendon and cartilage. Several different genetic types exist, with the most ubiquitous and most studied being type I. This is taken as the model for discussion of collagen chemistry and biochemistry.

Collagen type I is a fibrous protein comprised of rod like molecules each 300nm long, with a molecular weight near 300 Kd structured in a triple α-helical arrangement of three chains of amino acids, two of which are generally identical in composition. The low molecular weight amino acid glycine occurs at every third location within the chains in a repeat sequence Gly-X-Y where X and Y can be any amino acids but, are often proline and hydroxyproline. This imposes steric restrictions and in order to meet the tight steric requirements for the alpha helix the side groups of other amino acids protude from the long axis of the molecule and are important in

determining intermolecular crosslinks. The three α chains are held together by hydrogen bonds, one to every three residues, and each chain may be joined to one or both of the other chains in the molecule by covalent bonds formed by aldol condensation of allysine groups on adjacent chains with subsequent dehydration. There are two separate functional parts to the molecule: the central triple helical region and the non-helical region at each end of the α chains comprising a short telopeptide ranging from 9 to 50 amino acid residues. It is in these C-terminal and N-terminal peptides that cross linking occurs. Each molecule is joined to its neighbouring molecule at a point one quarter along its length by intermolecular reactions to produce a quarter staggered effect. The quarter stagger occurs every 67 nm; is observable under the electron microscope, and is known as the D period of which 4.4 D occur in the length of one molecule (Figure 1.4). This results in a regular almost head to tail arrangement since the crosslinks are formed at a specific position in the molecule and a regular pattern occurs which is evident as a cross-striations in stained fibres (Bailey and Light 1989).

1.3.1 Formation

Collagen fibres are formed from the tropocollagen molecules assembled on the polyribosomes of the endoplasmic reticulum within the fibrolasts. The

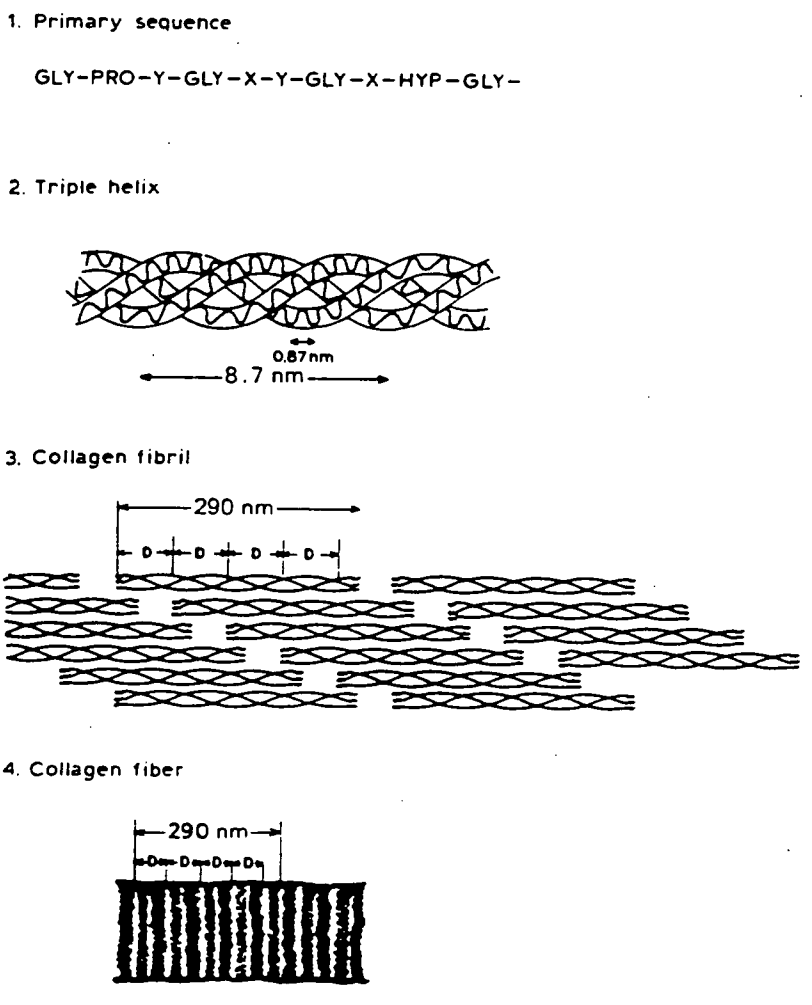

Figure 1.4 Diagram showing (1) the primary amino acid sequence of collagen (2) its triple helical structure (3) the quarter stagger arrangement of the fibril and (4) the collagen fibre with repeating 68 nm D-period.(Adapted from Pearson and Young 1989).

tropocollagen molecule is the α chain with a peptide at the N-terminal and the C-terminal end. These peptides are snipped off as the chains are aggregated into the collagen molecule proper at the time of its release from the Golgi complex of the cell into the ground substance. The collagen then polymerises into its fibrous form (Bailey and Light 1989)(Figure 1.5).

1.3.2 Crosslinking

Both intra and inter molecular crosslinks are found in collagen and are either derived from lysine residues or, in the case of types II and IV collagen, also from disulphide bridges.

1.3.2.1 Intramolecular links derived from lysine

The intramolecular link arising from lysine occurs from the action of the extracellular enzyme lysyl oxidase on the lysine residues in the telopeptides at the N-terminal and C-terminal ends of the molecules to produce lysine aldehyde (1-amino-1-carboxy-pentan-5-al). The aldehyde groups in the neighbouring chains are aligned by the enzyme with a set active lysine site held opposite a binding site which in types I, II and III collagen contains the amino acid sequence Hyl-Gly-His-Arg. The intramolecular aldol group then is formed between two lysine aldehyde groups on adjacent chains in

the N-terminal telopeptides. No intramolecular aldol links have been found at the C-terminal ends (Bailey and Light 1989).

1.3.2.2 Intermolecular groups derived from lysine

There are two types of intermolecular links derived from lysine: aldimines and oxo-imines, formerly called keto-imines. The covalent aldimine link dehydro-hydroxylysinorleucine (dehydro-HLNL) is formed between the allysine group in the telopeptide and the hydroxylysine in the triple helical portion of the neighbouring molecule at the binding site of the lysyl oxidase enzyme (Yamauchi and Mechanic 1988). In type I collagen this occurs at residue 103 in the α1 chain. This bond is stable under physiological conditions but is both heat and acid labile. Its double bond can be reduced in vitro to yield hydroxylysinonorleucine (HLNL), which is stable to acid hydrolysis and thus can be isolated chromatographically (Bailey and Light 1989; Yamauchi and Mechanic 1988).

The oxo-imine crosslink is similarly formed from the reaction of the hydroxylysine aldehyde produced by the action of lysyl oxidase on hydroxylysine groups on the telopeptides with the hydroxylysine of the enzyme-binding sequence on the triple helical section of the neighbouring aligned molecule. This forms the Schiff's base dehydro-dihydroxylysinorleucine which *in vivo* undergoes an Amadori

rearrangement to form the acid and heat stable oxo-imine hydroxylysino-5-oxo-norleucine (HLONL). In vitro reduction eg with $NaBH_4$ can reduce the HLONL to dihydroxylysinorleucine (DHLNL)- which can be separated chromatographically after acid hydrolysis (Bracho and Haard 1990). Tissues such as skin type I collagen have few hydroxylated groups and tend to contain more aldimine than oxo-imine groups. Types II and IV which are more highly hydroxylated are rich in oxo-imine crosslinks.

1.3.2.3 Disulphide crosslinks

In type IV collagen both intra and intermolecular disulphide crosslinks are known to occur. The molecules occur as tetramers with extensive disulphide crosslinks between and within molecules at their N-terminal regions where the four molecules join in an X shape. Other disulphide bonds occur at the C-terminal non-collagenous end at the apex of an arm of the X between it and an arm of the adjoining X unit. In this way a "chicken wire like" structure is built up (Bailey and Light 1989).

To date only intramolecular disulphide bonds have been found in type III collagen but it is assumed likely that intermolecular ones must exist. The intramolecular bonds are found at the C-terminal end in the last triplet before the non helical portion. It is thought that the bond is formed by the enzyme protein disulphide isomerase during assembly in the endoplasmic

reticulum.

1.3.3 Crosslinks and ageing

The intramolecular crosslinks are structurally important for the molecule but it is the intermolecular links that are responsible for the considerable tensile strength of collagen fibres. In most tissues there is an increase in the number of the heat and acid stable oxo-imine crosslinks with age. This yields a ready explanation for the fact that the cooked flesh of young animals is more tender than that from older ones since collagen fibres become progressively stronger and more rigid with age. Factors other than an increase in the number of aldimine or oxo-imine crosslinks are involved since lysyl oxidase acts on newly formed fibres; these crosslinks form only in the non-helical regions. Also a decrease in the number of reducible crosslinks occurs with age and this has led to speculation that some rearrangements have taken place to form tri or tetravalent crosslinks. Candidates for the actual crosslink are pyridinoline, histidino-hydroxylysinorleucine and a third compound which has been isolated but not yet characterised. The situation is by no means resolved (Bailey and Light 1989).

The very presence of the crosslinks means that a fibre of considerable tensile strength is built up. Further intermolecular bonds that increase in

number with age have been postulated to account for the intractability of mature tissue. The nature of these bonds is a subject for active investigation, but it has also been proposed that a network of existing cross links could be built up to explain the increase in cross linking and other age-related changes.

1.3.4 Genetic types

There were at least ten well described genetic types of collagen (Martin *et al.* 1985), but with the recent addition of the FACIT group (Fibril-Associated Collagens with Interrupted Triple helices; Shaw and Olsen 1991), fourteen are now reasonably well known. Types, I II and III are fibre forming in nature and are as described above for type I molecules 300 nm in length with short non-helical peptides at the N-terminal and the C-terminal ends. Type I is ubiquitous in connective tissues, type II is a major structural collagen in cartilage and type III is now known to comprise the silver staining reticulin fibres and to be a minor component in the endomysium. Collagen type IV is found in basement membranes where the 400 nm long molecules aggregate only with their identical ends. The C-terminal end holds two molecules together while four are joined at the N-terminus: the result is an open basket weave or chicken-wire structure (Figure 1.6). Type V is widely distributed in different tissues and is also

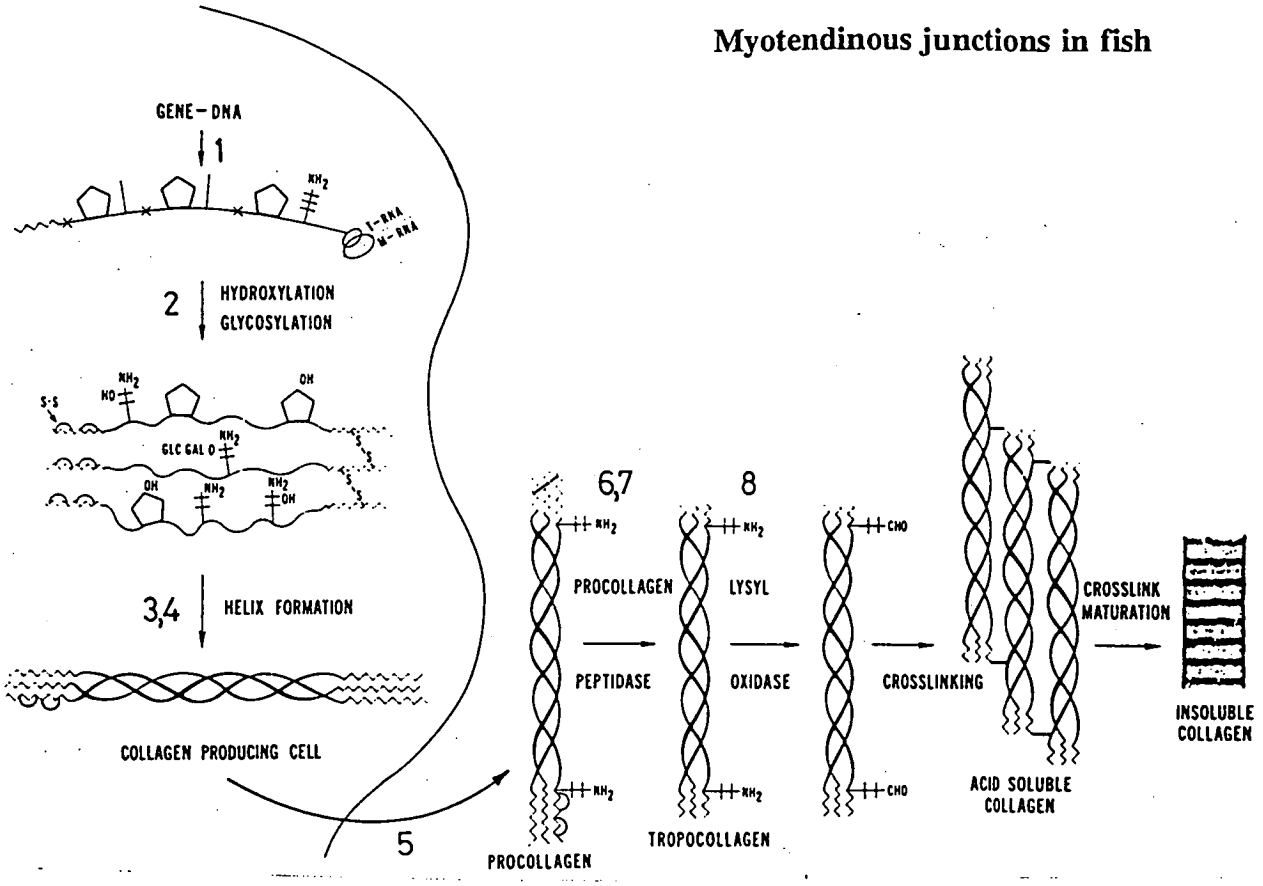

Figure 1.5 The flow chart shows an overview of collagen synthesis modification and secretion. (From Bailey and Light 1989).

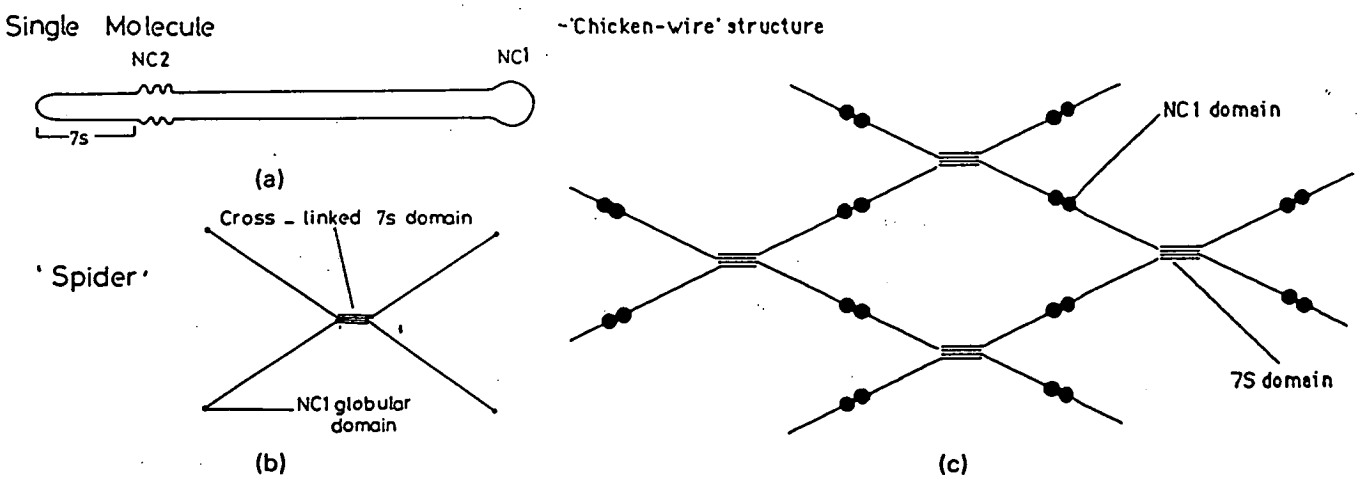

Figure 1.6. Single Type IV collagen molecule (a), tetramer 'spider'(b) and the proposed 'chicken wire' network (c). (From Bailey and Light 1989).

found in the endomysial layer of muscle (Duance *et al.* 1977). Type VI collagen occurs as microfibrils in interstitial connective tissues but the collagen is only one third of the mass with globules on either end comprising the rest. It is made up of dimers formed from 105 nm long monomers, tetramers and polymers which exhibit end to end aggregation and are linked through disulphide bonds. Type VII is an anti- parallel dimer with a 60 nm overlap region stabilised by disulphide bonds and is comprised of three identical subunit α chains within a triple helical domain 424 nm in length (Morris *et al.* 1986). It is the major protein component of anchoring fibrils that serve to anchor the basement membrane zone to the underlying connective tissue matrix in epithelial tissue (Lunstrum *et al.* 1986).

Type VIII is not well characterised and has been produced in culture by epithelial cells and may be composed of short helical domains interspersed with non-helical, protease labile segments. Type IX collagen is found as a minor constituent of collagens in cartilage through which it is uniformly distributed. The gene which codes for one of the chains is somewhat different to the normal pattern found for the interstitial collagen genes. Type X has a relatively short helix (150 nm) with a globular domain at one end and is found in hypertrophic and mineralizing cartilage. Types II, IX and X are produced and associated with chondrocytes while type IV is produced by epithelial cells. Types XII and XIV collagens are localised in

type I collagen containing tissues. They both contain amino acid sequences homologous to those of type IX collagen but are otherwise unique in their structure and expression pattern (Shaw and Olsen 1991).

The amino acid sequences of the different α chains of collagen are under genetic control. The chains are designated α 1, α 2 or α 3. In types II, III, VIII and X the chains are identical and are written in shorthand as $[\alpha 1(II)]_3$, $[\alpha 1(III)]_3$ and $[\alpha 1(VIII)]_3$ respectively, whereas in types I and IV there are two identical chains and one different $[\alpha(I)]_2\alpha 2(I)$, $[\alpha 1(IV)]_2\alpha 2(IV)$ respectively and in types V, VI and IX the three chains are different $[\alpha 1(V)][\alpha 2(V)][\alpha 3(V)]$, $[\alpha 1(VI)][\alpha 2(VI)][\alpha 3(VI)]$ and $[\alpha 1(IX)][\alpha 2(IX)][\alpha 3(IX)]$ respectively. The genes coding for collagen are complex with the pro-α 2 gene for type I chicken collagen being the most highly interrupted gene yet identified. Its 5 Kb of coding is located in over 40 Kb of genomic DNA interrupted by 50 introns. Examination of the amino acid sequences suggests that the three fibrous collagens diverged some 500 million years ago (Boedtker and Aho 1985).

1.3.5 Separation of collagen molecules and chains

Soluble collagens are extracted from tissue by dilute acid or neutral salt solutions and these treatments are sufficient for embryonic and newborn tissues. For most adult tissues predigestion with pepsin is necessary and

this releases the α chains since digestion occurs in the non-helical telopeptides where crosslinks occur. Hard tissues may require the acid removal of bone. Materials such as cartilage are often extracted with guanidinium chloride to remove the proteoglycan.

Separation of the collagen types can be effected by differential precipitation in salt solution of differing strength and pH (Trelstad *et al.* 1972). Further purification is achieved by repeated precipitation and/or by chromatography on DEAE or CM cellulose using either repeated passes or varying conditions of ionic strength and pH to elicit separation.

The separate collagen chains can be resolved from each other by chromatography on CM cellulose, or by electrophoresis, or by HPLC using appropriate conditions. As well as the α chains, β dimer and γ trimer chains can be distinguished. The β chains arise through linkage of two α chains to give the respective dimer and all combinations can occur depending on the number of α chains present in the molecule. For example in type V collagen where three chains may occur an α1 chain with an α3 chain gives the β1,3 dimer. Similarly β1,2 dimer, β2,2 dimer, β1,1 dimer, β2,3 dimer and β3,3 dimer can all occur. Likewise the γ trimers can occur from combinations of the α chains. The relative proportions of these dimers and trimers can give an indication of the composition of the collagen under investigation.

Controlled treatment with proteases to achieve reproducible cleavage

patterns is also used as an aid to proving the identity of collagens. A further refinement is the use of cyanogen bromide (CNBr), which splits the molecule at specific locations into characteristic peptide fragments for identification. This technique is convenient, sensitive and reproducible (Scott and Veis 1976).

The amino acid composition of collagens is used as a further means of identification and ratios of the proportions of the various amino acids can be used as an indicator of similarity (Metzger *et al.* 1968). Recently, hierarchical cluster analysis and principal components have been used to provide a natural taxonomy of collagen based on amino acid composition (MacFie, Light and Bailey 1988).

1.3.6 Thermal properties of collagen

The temperatures at which collagen shrinks (T_s) and at which it melts (denatures) in solution (T_m, sometimes called T_d) are important indicators of its stability. The values depend to an extent on the method of determination. However the value T_s - T_d is remarkably constant at 27^{0}C for a wide variety of collagens. The shrinkage temperature relates to the energy required to overcome the intermolecular interactions of the closely packed molecules in the fibres and is affected by other endogenous components and by external conditions. The melting temperature is a

measure of the energy required to disrupt intramolecular cross links and is more indicative of the properties of the collagen molecule itself since it is less affected by superimposed intermolecular interactions. Thus, T_d represents the temperature of collapse of the triple helix whereas T_s represents denaturation of the highly organised long rigid molecules of collagen to the random chains of gelatin. This collapse of structure is accompanied by significant shrinkage to about one quarter of the original length of the fibre. If the fibres are anchored, considerable tension can be generated during shrinkage which can be measured instrumentally. The melting temperature is related to the imino acid content of the collagen since the interchain hydrogen bonds at the overlapping pyrrole groups of proline and hydroxy proline are largely responsible for its stabilization.

1.3.7 Immunochemistry of collagen

There are three classes of antigenic determinants in type I collagen (von der Mark 1981) (i) terminal, nonhelical antigeniic determinants (ii) helical, conformation dependent antigenic determinants and (iii) central antigenic determinants.

The terminal nonhelical determinants are short sequences of amino acids at either the C-terminal or the N-terminal ends of the molecule in the telopeptide region and are generally best raised in rabbits. The helical,

conformation dependent determinants are generally in the native triple helical section of the molecule and may not cross react with the denatured collagen (Beil, Timpl and Furthmayr 1973). Antibodies raised to triple helical trimers of the same chain show only weak cross reactivity against a mixed chain molecule. They are generally raised in rats, mice, guinea pigs and chickens. The central determinants are only exposed after some denaturation treatment of the molecule and are less well characterised than the helical or terminal antigenic sites; antibodies are generally raised in chickens. Peptides from CNBr treatment of collagen are often used and high cross reactivity across diverse species often results (von der Mark 1981).

In general, collagen has low immunogenicity and high doses of antigen are often required, administered subcutaneously in Freunds adjuvant followed by booster injections intraperitoneally or subcutaneously. Since relatively high doses of antigen are administered, which are hard to completely purify, it is difficult to guard against the production of antibodies against contaminants. As a result it is necessary to purify collagen-specific antibodies by affinity chromatography on an homologous collagen type to remove non-specific effects. This would also remove antibodies to other structurally related collagens which may have similar antigenic determinants. The production of monoclonal antibodies can overcome some of the difficulties but even here the monoclonal may not be specific since it

may be reacting to an amino acid sequence common to more than one type. Use of the antibodies to collagen has tremendous potential in locating the various types in particular tissues using immunofluorescence and immunoperoxidase techniques. Reactions with ferritin or gold particles have made it possible to visualise fine structures by electron microscopy. There are, however, potential limitations and sources of error in these techniques (von der Mark 1981) such as (1) nonspecificity of the antibody (2) non specific reactions of fluorescein-labelled antibodies (3) masking or denaturing of the antigen and (4) autofluorescence of tissues. With preparations for electron microscopy, masking or denaturing of the antigen can represent a significant problem since fixation techniques may also destroy antigenicity. Even if suitable fixation is used which retains antigenicity, then definition of tissue structure can be insufficient to enable identification of the features which have been labelled.

1.3.8 Proteases and collagenases

Collagen is degraded by a number of enzymes acting in concert, with the initial attack on undenatured molecules performed by a collagenase acting at a specific site, followed by other enzymes acting on the fragments (Nimni and Harkness 1988). Historically, the mammalian collagenases are defined by their ability to cleave the triple helical region of the native

collagen molecule at a specific locus. The discovery of a broader range of collagens with globular domains and discontinuities in the helix has required that this simple definition is modified. Furthermore, serine proteases and an heterogeneous group of proteases can degrade some collagen types (Figure 1.7). Two main groups of collagenases have emerged - the 'classical' collagenases which degrade types I, II and III collagens and type-specific interstitial and basement membrane collagenases which attack types IV and V (Stricklin and Hibbs 1988). Collagenases are mostly metallo-endoproteases having a pH optimum in the range 7 to 8, which are activated and stabilised by calcium ions. Consequently, they are inhibited by metal chelators such as EDTA. They have been isolated from a wide variety of tissues, but not as yet from fish muscle tissue although collagenase from fish caeca and pancreas has been known for some years (Yoshinaka *et al.* 1978).

The collagenases that degrade types I, II and III collagen do not affect types IV and V collagen. Specific collagenases which degrade type IV have been isolated from tumours, but it can be degraded by other metalloproteinases including gelatinase, proteoglycanase, serine proteases, neutrophil elastase and mast cell chymase. Type V is attacked by metalloproteinases which are also gelatinases of a molecular weight greater than the classical collagenases (Stricklin and Hibbs 1988, Liotta *et al.* 1981). Type V collagen isolated from bovine bone is susceptible to attack

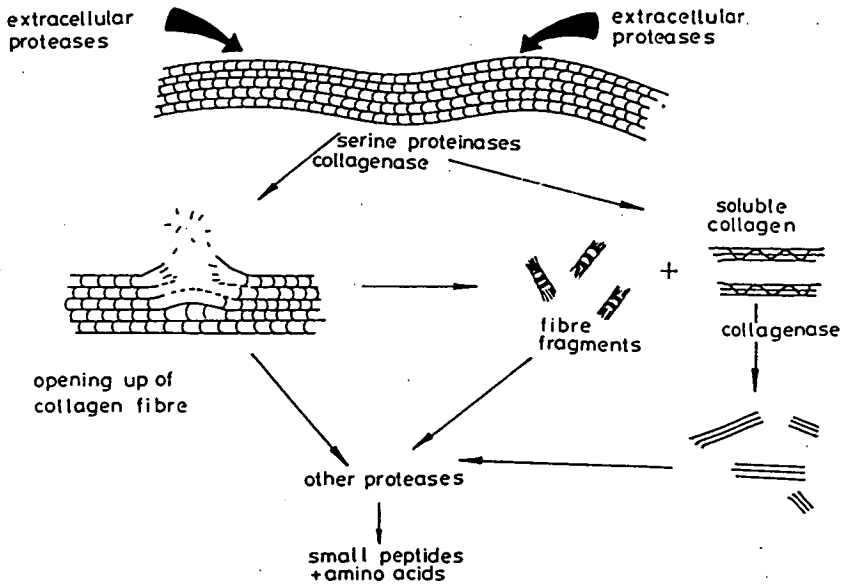

Figure 1.7. Collagenases and proteases exhibit concerted action on collagen fibres to degrade them. (From Bailey and Light 1989).

by trypsin at 35°C (Niyibizi and Eyre 1989), at a site which may represent the natural target domain for cleavage *in vivo* and which is at the opposite end of the molecule to the site at which type I is attacked by collagenase. This indicates that, *in vivo*, separate enzyme systems are necessary for types I and V to be copolymers in the same fibril.

After synthesis, collagenases are secreted into the extracellular matrix and it is obvious there must be specific inhibitory mechanisms to prevent spontaneous tissue destruction. The collagenases, present in the tissue in latent form as zymogens, are activated by a wide variety of proteolytic enzymes such as trypsin, plasma kallikrein, cathepsin B, plasmin (Unemori and Werb 1988), but once activated they must be controlled. In human tissue, the main control mechanism appears to be small cationic glycoproteins known as Tissue Inhibitors of Metallo Proteinases, or TIMP. They are ubiquitous and their relatively low molecular weight, 28.5 Kd, allows them ready access within the extracellular matrix where they play a major role in collagenase inhibition (Stricklin and Hibbs 1988). Other control mechanisms such as the α_2-macroglobulin molecule in plasma are too large to penetrate the tissue.

The basement membrane degrading enzyme, collagenase IV/V -gelatinase, is more easily released by kallikrein and becomes active in the extracellular space before other lysosomal proteinases are released (Tschesche *et al.* 1989). Tissue kallikrein, a serine protease (specifically an arginyl

esteropeptidase), also activates type I collagenase and is a likely candidate to perform these functions *in vivo*. It may also be an activating factor in post-mortem tissue. There are about twenty kallikreins which seem to be ubiquitous. So far they have only been reported in the skeletal muscle of the rat. The lysosomes in fish muscle are commonly associated with the connective tissue (Steiner *et al.* 1984) so that cathepsin B, which could also activate this collagenase, would be released by post-mortem disruption of the lysosomes. The concentration of calcium ions would not be a factor limiting collagenase activity due to leakage from the sarcoplasmic reticulum. Indeed, it has been shown that the introduction of Ca^{++} into fibroblasts promotes a cascade of proteolytic events culminating in activation of collagenase (Unemori and Werb 1988).

The question is whether these mechanisms are present in fish tissue and whether they are active at post-mortem pH and at chill temperatures. The type IV/V basement membrane collagenase isolated from human leukocytes has a pH optimum between 7 and 8, with about 50% of this optimum activity at pH 6 (Tschesche *et al.* 1986). No information relating to kallikrein in fish has been found but it is probably present and, since it is active at physiological pH, it is likely to retain activity at post-mortem pH. However, it too is subject to the effect of inhibitors in the serum, similar to trypsin inhibitors. Cathepsin B may not be very active at post-rigor pH.

1.4 Fish collagens

The collagens of fish are, in general, much more easily solubilised than those of mammals and for this reason they were the subject of many of the early studies that helped ascertain general collagen properties. Apart from a continued interest in Japan there has been very little effort over the last two decades in furthering the knowledge of the structure, chemistry, properties and uses of marine collagens.

The major collagen present is type I (Sikorski, Scott and Buisson 1984, Kimura 1985), type II has been found in the cartilage and notochord of lampreys (Miller and Matthews 1974, Kimura and Kamimura 1982) and in lamprey skin (Kelly *et al.* 1988), type V has been reported in carp and spotted mackerel (Sato *et al.* 1988) and in lamprey along with type IX (Kelly *et al.* 1988). Evidence for the fibre forming type III common in mammals has not been found in any investigation to date. Type IV has not been reported either, probably because it has not been specifically sought, but fish have quite clearly defined basement membranes so it is reasonable to assume that type IV is present. In the intramuscular tissue only types I and V have been demonstrated.

Piez (1965) was the first to demonstrate the presence of three α chains in the skin of cod. Bogason (1984) reported three α chains in the intramuscular connective tissue of rockfish and more recently Kimura

(1985) and Kimura and Ohno (1987) found the $\alpha 1$, $\alpha 2$, $\alpha 3$ hetrotrimer in the skin of carp and Alaska pollock. The swimbladders of these species contained the dimer $[\alpha 1(I)]_2\alpha 1(I)$. This indicated tissue specific existence of the two molecular forms. Furthermore Kimura *et al.* (1987) examined the skin collagen in teleost fish from ten different orders and established that the occurence of the three α chains in the type I collagen is common, but was not consistent within an order. This widespread occurrence led the authors to suggest that the gene for the $\alpha 3$ chain may be universally present in teleosts, having arisen about the time of the adaptive variation of the bony fish. However, in some species it may be quiescent or only active at very low levels. In contrast the collagens of the lower vertebrate species such as lamprey and shark do not exhibit the $\alpha 3$ chain at all. Ramshaw *et al.* (1988) also reported the occurrence of the $\alpha 1\alpha 2\alpha 3$ trimer in the skin of the blue grenadier.

Studies of the type I collagen of the myocommata from fish muscle indicate that eel and mackerel have the $\alpha 1$, $\alpha 2$, $\alpha 3$ heterotrimer. Saury contains only $\alpha 1$, $\alpha 2$ chains and carp and chum salmon seem to possess two different heterotrimers, $(\alpha 1)_2\alpha 1$ as a major component and $\alpha 1\alpha 2\alpha 3$ as a minor component (Kimura *et al.* 1988).

Sato *et al.* (1988) recently reported the presence of type V in the white muscle of carp in a higher proportion than it occurs in mammalian muscle. Electrophoresis of the fractions after treatment in reducing conditions with

2-mercaptoethanol did not change the band pattern indicating the absence of reducible thiol bonds and hence the absence of type III collagen. Further studies have shown the presence of type V in lizard fish, japanese eel, sturgeon, spotted shark and lamprey indicating widespread occurrence of type V in both elasmobranchs and teleosts (Sato *et al.* 1989). The relative concentration of type V collagen to type I was higher in the endomysial than in the myocommatal fraction in carp and spotted mackerel. Both the type I and the type V were less soluble in the endomysial than in the myocommatal fraction. Three distinct chains of type V were identified from two molecular forms stated as $[\alpha 1(V)]_2\alpha 2(V)$ and $\alpha 1(V)\alpha 2(V)\alpha 3(V)$, with a higher proportion of the latter occurring in the endomysium in comparison to the myocommata.

This recent evidence indicates that, not only can there be differences between major tissue groups eg muscle, skin or swimbladder, but that differences in the chain structure of collagen can occur within different domains in the one tissue. These differences in chain composition are likely to result in slightly different properties and stabilities in the collagen.

In fish muscle, type V collagen probably plays a role similar to that of type III in mammalian muscle in that it forms copolymers with type I and acts to control fibril diameter (Keene, Sakai, Bachinger and Burgeson 1987, Birk *et al.* 1990, Adachi and Hayashi 1986). Collagen fibrils of different diameters are to be found in the myocommata near muscle fibre ends and

in the invaginations of the myotendinous junction. Børresen (1976) developed a method for preparing the muscle cell envelope from cod (Gadus morhua). These envelopes were tubular structures with an outer three-dimensional network of fibres (30-60 nm in diameter) and an inner membrane about 2000 nm thick. Further work using this method showed that the cell membrane was composed of three layers (Almås 1982). The innermost layer, the sarcoplasmic membrane, was 8-16 nm thick. The middle layer, the basement membrane, was 50-70 nm thick and the outer layer, which was mostly collagen, was approximately 600-1100 nm thick. This collagen was shown to be type I, possessing a chain structure $\alpha 1_2 \alpha 2$ containing the acid- and heat-stable cross-link hydroxy-lysino-5-keto-norleucine.

1.4.1 Properties of fish collagens

The content of collagen in fish muscle varies considerably from species to species and is found in increasing proportion in the tail region. In the main edible portion concentrations of 0.3% to 3% are common (Sato *et al.* 1986, Sikorski *et al.* 1984) but even within species there is seasonal variation as the body reserves are depleted during spawning and migration. Since muscle is not conserved there is greater reliance on the connective tissues to hold the fish together.

While it would appear obvious that the content of collagen should affect the textural properties of the muscle this relationship is not straightforward because of the seasonal turnover and other species effects. Sato *et al.* (1986) investigated the collagen content and the texture of twenty two species of fish and concluded that there was a broad relationship between collagen content, swimming motion and the raw texture of the flesh prepared for sashimi. A high collagen content near 2% indicated that the species were too tough for sashimi. Hatae *et al.* (1986) also reported a significant correlation between collagen and raw texture. In cooked fish the reverse is true, the collagen softens and the myofibrillar component toughens and is the dominant component (Dunajski 1979). The gelatinised collagen, however, still contributes to the mouthfeel. Indeed a model has been proposed to explain textural differences between species on the basis of fibre diameter and the amount of coagulated sarcoplasmic material that sticks the fibres together and impedes them sliding across one another when chewed (Hatae *et al.* 1990). Other workers eg Feinstein and Buck (1984) have found no relationship between collagen and the raw texture in a limited number of species. Bogason (1984) reported high levels of acid insoluble collagen in three species of rockfish that he attributed to the relative longevity of the fish of commercial size and that the thickness of the skin collagen layer provided a crude indicator of flesh toughness. The thicker the skin the more robust the flesh.

The other major influential factor is the age of the fish. It is well established that in mammalian tissues collagen crosslinking increases with the age of the animal and that the tissue increases in toughness. Because of the seasonal changes occurring in most fish species this relationship is less clear. It is not just the amount or proportion of collagen present but the degree to which it is crosslinked that affects texture. Montero and Borderias (1990a) measured collagen content, the proportions of α, β and γ chains and the shear force in the muscle of trout (*Salmo irideus* Gibb) from four different size (age) groups. Although there was a higher proportion of connective tissue in the oldest fish, it had slightly higher acid solubility and fewer cross-links and did not give higher shear strength values. It is generally regarded that there are higher levels of connective tissue near the tail region (Love 1970) and this was confirmed recently for trout and hake, with higher levels of connective tissue in the ventral than the dorsal portions for the trout (Montero and Borderias 1989a). Shear strength values were highest nearer the tail as was a higher proportion of insoluble collagen. The amino acid composition, chain structure and type of collagen from trout and hake were also characterised in samples from the skin, myotomes, fasciae and myocommata. The collagen from all sites in both species was mainly type I, type III was not detected and the extraction conditions used would not have allowed the separation of type V (Sato *et al.* 1991). The amino acid compositions differed from mammalian sources

and the fish skin collagen was less cross-linked than the collagen from the fish muscle.

Bailey (1970) reported that the same crosslinks that occurred in mammalian muscle were also to be found in the skin, scales and swimbladder of cod. Recently Bracho and Haard (1990) investigated the crosslinks in the intramuscular collagen of rockfish after removing extraneous protein with alkali, stabilising the crosslinks with NaB^3H_4 and separating the compounds by HPLC after hydrolysis. They found 0.2, 0.34 and 0. 15 moles per mole of collagen of the links DHLNL, HLNL and LNL in brown rockfish (Refer section 1.3.2.2). Whereas in chilipepper rockfish the levels were 0.19 and 0.14 and 0.49 moles respectively per mole of collagen. In contrast rat tail tendon contained 0.15, 0.87 and 0.22 moles per mole of collagen of DHLN, HLNL and LNL respectively. Although there were differences between the fish species they both had about half the total number of crosslinks and entirely different levels of HLNL from the rat tail tendon.

1.5 Post mortem changes in fish flesh texture

During post-harvest storage, textural changes occur in many fish species long before they are spoilt. The result is that the flesh softens and gapes, trimming losses occur, products have a poorer appearance and are downgraded. In extreme cases, mechanical processing becomes impractical

since the fillets fall apart in the skinning operation. Softening and gaping are common problems in the merluccid hakes (Burt 1978; Huss and Asenjo 1978; Kordyl and Karnicki 1969; Schroeder *et al.* 1978) and in the related species blue grenadier (hoki, *Macruronus novaezelandiae*) (Bremner 1980). Farmed species also have this problem (Lavety *et al.* 1988). It is clear that there are differences between related species and that circumstances affect whether gaping occurs. Whole cod stored in ice showed no gaping whatsoever even after 6 weeks storage when the fish were thoroughly spoilt (Love 1968). This lack of change was further borne out with measurement of the forces need to pull samples apart at the myocommata. No change in cohesiveness was found for cod stored in ice for up to 26 days. Whole muscles loaded to failure fail at or near the myotendinous junction in the region between the cell membrane and the lamina densa of the basement membrane (Tidball and Chan 1989).

1.5.1 Post-mortem change attributed to changes in the collagen

Unfortunately, there are few published investigations into the nature of the specific changes that occur. Using goldfish (*Carassius auratus*) as a model species, changes in the structure could be observed in post-rigor fish held in ice for 3-4 days, in comparison to pre-rigor fish (Bello *et al.* 1982). Shrinkage and distortion of the myofibres, an increase in the extracellular

space, loss of configuration of the endomysium and breakdown of the connective tissue occurred. Further changes to these structures occurred with subsequent storage. Similar structural changes in the flesh of the major carp (*Labeo calbasu*) during chilled storage were also observed histologically (Menon and Nair 1988). Disorganisation of the structure was evident when the samples were examined after 7 days. By 14 days, fissures appeared in the cells and partial disappearance of the connective tissue structure was apparent.

Texture, determined as shear force using the Kramer shear press, was related to an increase in heat-soluble collagen in rockfish stored in ice (Cepeda *et al.* 1990). A significant decrease in the solubility of collagen from trout muscle occurs during rigor, followed by an increase in solubility during storage post-rigor (Montero and Borderias 1990b). The proportions of heat- and acid-soluble collagen increased during and after rigor, while the levels of insoluble collagen and shear strength progressively decreased. Proteolytic activity increased post-rigor. Using a compression test as a means of applying a force to muscle segments, Ando *et al.* (1991) demonstrated by light microscopy and SEM that a gradual disintegration of the extracellular matrix occurred during chilled storage of rainbow trout *(Oncorhynchus mykiss)*.

1.5.2 Freezing damage

Although a comprehensive coverage of frozen storage changes is outside the scope of this thesis, it is pertinent to note here some of the recent observations that changes in the collagen contribute to changes in texture which occur in frozen stored fish.

Both trout (*S. irideus*) and hake (*Merluccius merluccius* Linnaeus) collagens exhibited a decrease in the proportion of α-chains and a concomitant increase in γ- chains during frozen storage at -18°C after only 25 days, with further changes occurring progressively. The amount of insoluble collagen in the hake samples also increased with the period of storage (Borderias and Montero 1985). This increase in collagen insolubility in hake flesh and decrease in the percentage of heat-soluble collagen with period of frozen storage was confirmed in further studies. It was suggested that aggregation of hake collagen was due to reaction with formaldehyde produced by breakdown of trimethylamine oxide (Montero and Borderias 1989b, 1990c). This is also consistent with the other reports (Howgate 1980, Connell 1962) suggesting that the remnants of the sarcoplasmic reticulum could serve as a glue to cement the fibres to produce a tougher product, the opposite of the situation that occurs in chilled storage.

Walton and Gill (1989) suggest that the collagen layer of the endomysium may be the cementing agent responsible for cell strength. They found that

the level of salt soluble collagen of Atlantic cod (*G. morhua*) decreased with frozen storage and high molecular weight complexes of both myocommatal and endomysial collagens were formed. They also suggested the possibility of complexes between sarcoplasmic proteins and the endomysial collagen. If such complexes were not denatured during cooking they would increase the toughness of the flesh in a manner consistent with the model proposed by Hatae *et al.* (1990).

1.5.3 Changes in the muscle

It has long been known that fish flesh has greater catheptic activity than mammalian muscle (Siebert 1958) and that it possesses higher concentrations of enzymes responsible for proteolysis and amino acid metabolism (Siebert *et al.* 1965). Neutral proteases are found in many species (Makinodan *et al.* 1983). Recent work, stimulated by the need to understand the softening (modori) phenomenon that occurs during the setting of fish protein gels (surimi), has uncovered a number of proteases. These are mostly serine proteases, bound to both the sarcoplasmic and myofibrillar fractions of the muscle in a variety of species (Shimizu and Wendakoon 1990, Yanagahira *et al.* 1991, Toyohara *et al.* 1990, Kinoshita 1990). These enzymes are active during the heating step in forming fish gels and it is unclear what role they may play in the live fish; it seems

unlikely that they are active in the same way in chill stored fish.

Cathepsin L from lysosomes has been implicated in the extensive muscle softening observed in chum salmon (Yamashita and Konagaya 1990). This enzyme has increased activity in the muscle of ayu (sweet fish, *Plecoglossus altivelis*) as the fish approaches maturity. Lysosomes have been located within fish muscle cells mostly concentrated at the periphery (Steiner *et al.* 1984, Ueno *et al.* 1986). These lysosomes break down post-rigor. Other cathepsins (e.g. cathepsins A and B) require a pH lower than occurs in post-mortem fish muscle. Even the pepstatin-sensitive cathepsins (mainly cathepsin D) are not active at pH 6.5 (Jiang *et al.* 1990). However, in fish such as tuna, where the pH is often lower than this, it is likely to be one of the factors responsible for the honeycomb problem in the flesh that is sometimes encountered (Frank *et al.* 1984).

White croaker (*Micropogon opercularis*) contains an endogenous serine proteinase which degrades the cytoskeletal network (Busconi, Folco, Martone and Sanchez 1989; Busconi, Folco, Martone, Trucco and Sanchez 1989). It also initiates protein turnover *in vivo* and can completely disrupt the myofibrils (at 37°C) and degrade the major proteins (Busconi *et al.* 1987). When croaker were stored at 0°C for 7 days, after dipping in azide to prevent bacterial growth, only minimal changes were found in the major proteins of the myofibril. There was considerable breakdown of nebulin, a major cytoskeletal protein of the trabecular network (Busconi, Folco,

Martone and Sanchez 1989). Desmin, troponin and Z lines were shown to be stable under these conditions, whereas these entities are known to be degraded in post-mortem storage of beef muscle along with titin and alpha-actinin (Hwan and Bandman 1989). The connectin fraction of carp muscle also exhibits change when extracted from fish that have been stored chilled for 7 days at 25°C (Seki and Watanabe 1984).

There are two Ca^{++}-activated neutral proteinases that are considered to be responsible for post-mortem softening of sheep muscle (Koohmaraie, Babiker, Merkel and Dutson 1988; Koohmaraie, Babiker, Schroeder, Merkel and Dutson 1988). Calpain I requires only 0.1 mM Ca^{++} for activation while Calpain II is active at higher concentrations of calcium. In rat muscle calpain I is predominantly located intracellularly at the I band region (Yoshimura *et al.* 1986). Both enzymes are subject to inhibition by calpstatin. Calpain II has been found in both carp [(*Cyprinus carpio*)(Toyohara *et al.* 1985)] and tilapia (Tilapia nilotica x Tilapia aurea) (Jiang *et al.* 1991). Calpstatin and a trypsin inhibitor are also present in carp (Toyohara *et al.* 1983).

1.6 Concluding comments

From a series of experiments in which they (i) examined electrophoretic patterns of sarcoplasmic alkali-soluble and stroma proteins of five species

of fish stored for 14 days at 4°C, (ii) physically measured a range of textural properties using a General Foods type Texturometer Hatae *et al.* (1985) concluded that post-mortem softening of the flesh was "more affected by the changes of the muscle structure than by the changes of the component proteins". Similarly, Toyohara and Shimizu (1988) stated that "the weakening of muscle may be explained not as a proteolytic breakdown of myofibrils, but as a decomposition of the muscle structure". These observations, in conjunction with those reviewed here, lead to the conclusion that muscle integrity is due to minor components which link the major components together. Proportionately small changes in these minor components can have disproportionately large effects on the structure, and hence the texture, of the flesh.

There are three possible explanatory mechanisms for post mortem softening:-

(a) some major components within either the myofibrils or in the extracellular connective tissue degrade, or

(b) links, bonds and connections that organise and stabilize the structure between the muscle components degrade, or

(c) both of these mechanisms occur.

Hypothesis (b) has considerable attraction as an explanation of the changes occurring early in the storage period before they are sufficiently gross to be

detected by such means as alteration in an electrophoretic pattern.

Chapter 2

Methods and materials

2.0 Methods and materials

The methods used in this investigation are set out in this chapter.

2.1 Sampling

A wide range of fish species and samples were used in this study (Tables 2.1 & 2.2) plus other samples as described in following sections for SEM and TEM. All fish flesh samples comprised white muscle only taken from the anterior dorsal area. For TEM studies on blue grenadier, samples were taken from the region of the twelfth myotome, posterior to the gill slit.

2.2 Fixation

The fixatives used were as follows:-*

1. Phosphate buffered formaldehyde (10%) (PBF)

Fixative

a.	2.26% Monosodium phosphate ($NaH_2PO_4H_20$)	62.2 ml
b.	2.52% Sodium hydroxide (NaOH)	12.8 ml
c.	40% Formaldehyde	25.0 ml
		100.0

Constituents

a. 2.26g $NaH_2PO_44H_2O$ dissolved in 100 ml distilled water.
b. 2.52 g NaOH dissolved in 100 ml distilled water.
c. 40 g paraformaldehyde dissolved in 50 ml water heated to 60°C. Dropwise addition of 0.1 M NaOH used to clear solution (approx 12 drops give clear solution with pH near 7.2) then made up to 100 ml.

* M. A. Hayat. Principles and techniques of electron microscopy: biological applications. Van Nostrand Reinholt, New York, 1970

2. Fixative A

81 ml	0.1M Phosphate buffer (Sorenson's)
4 ml	25% purified Glutaraldehyde
15 ml	Saturated aqueous Picric acid
100	

3. Fixative B

81 ml	0.1M Phosphate buffer
2.4 g	Nacl
15 ml	Aqueous saturated picric acid

4. Glut/fix (Kryvi 1977)

Fixative

	8.3 ml	Solution A
	1 ml	Glutaraldehyde
Solution A	53.6 ml	0.1 M Na_2HPO4
	20.8 ml	0.1 M NaH_2PO4
	8.4 ml	5% sucrose
	1.7 g	NaCl

5. Glut/form fixative

83 ml	Solution A
7 ml	40% formaldehyde
10 ml	2.5% glutaraldehyde

6 GF/cac fixative

2g	paraformaldehyde
50 ml	0.2M sodium cacodylate
1g	NaC1
10 ml	25% glutaraldehyde

made up to 100 ml, filtered through Whatman No. 4 paper.

2.3 Light microscopy

Tissue samples were embedded in paraffin by standard methods using the Tissue Tek II* apparatus. Sections were stained with haemotoxylin and eosin, Massons trichrome and modified Van Gieson's stain. A list of samples examined is given in Table 2.1.

*Ames Laboratories

2.3.1 Preliminary histology

A live trumpeter (*Latris lineata*) was killed by a blow on the head and flesh samples from the anterior dorsal muscle were placed immediately in fixative (Sample code 5.1, Table 2.1). Other samples were taken and fixed from the same fish five and nine days after it had been stored chilled [(0°C), Samples 5.2 and 5.3, respectively)]. The samples were processed into wax and sections were stained with van Gieson's stain which stains collagen fibres purple and myofibrils a light pinkish brown.

2.4 Sampling and fixation for SEM

Blue grenadier (*Macruronus novaezelandiae* Hector) caught in Tasmania were sampled immediately after catch then were iced and stored for subsequent sampling. Muscle samples were fixed in Glut/fix (3.2%

Table 2.1 List of samples for light microscopy and histochemistry

Date caught	Date fixed	Date processed	Sample code	Fixative	Description
6.12.84	11.12.84	12.12.84	1.1 2.1 3.1 1.2 2.2 3.2	Glut/form Glut/form Glut/form PBF PBF PBF	Blue grenadier caught by Petuna endeavour stored in ice. Three fish samples.
6.12.84	19.12.84	19.12.84	4.1 4.2 4.3	PBF PBF PBF	Three previously untouched fish (iced) sampled.
9.1.85	9.1.85	10.1.85	5.1	PBF	Pre-rigor samples from live trumpeter.
	14.1.85	15.1.85	5.2	PBF	Trumpeter stored in ice 5 days.
	18.1.85	22.1.85	5.3	PBF	Trumpeter stored in ice 9 days.
14.12.84		22.1.85	6.1	Seawater Formalin	Juvenile blue grenadier caught by Soela cruise SO6/84/125
22.10.84			7.1	Seawater Formalin	Larval blue grenadier caught by Soela cruise SO5/84/68
30.1.85	30.1.85	31.1.85	8.1 9.1 10.1	PBF PBF PBF	Juvenile blue grenadier pre-rigor, caught by Challenger
17.8.88	29.8.88	29.8.88	11.2	PBF	Myocomma of blue grenadier frozen 17.8.88 then fixed.
	17.8.88	29.8.88	11.2	Glut/fix	Myocomma of blue grenadier.
	29.8.88	29.8.88	11.3	Glut/fix	Myocomma of blue grenadier.

glutaraldehyde in 0.16M phosphate buffer, 2% NaCl and 0.5% sucrose, Kryvi 1977) and despatched to the DSIR, Mount Albert Research Centre, Auckland, New Zealand. After a short wash in buffer, the samples were rapidly frozen in dichlorodifluoromethane at -158°C then transferred to liquid nitrogen at -196°C. They were freeze fractured under liquid nitrogen by striking them with a cooled sharp blade. The frozen samples were then freeze dried in a modified vacuum evaporator (Ladd Research Industries, Vermont, USA). Other samples of blue grenadier caught and held in ice by commercial boats were sampled at a fish process factory in New Zealand and were similarly fixed, freeze fractured and freeze-dried.

Juvenile samples of spotted trevalla (*Seriolella punctata*) were caught by hand line in the Derwent river estuary, Tasmania, and were immediately killed by a blow on the head and muscle samples taken for fixation in glut/fix. Further samples were taken from fish stored on ice. These samples of spotted trevalla were also sent to New Zealand where they were freeze fractured as for the above samples of blue grenadier.

Freeze fractured samples were mounted on stubs, sputter coated with gold and examined with a Philips 505 scanning electron microscope.

2.5 Structural studies using transmission electron microscopy

2.5.1 Sampling and fixation

A number of methods were used to prepare samples for the TEM studies. Some of the work was done while the author was on sabbatical at the then Department of Scientific and Industrial Research, Mount Albert Research Centre, Auckland, New Zealand and was continued in collaboration with Dr Ian Hallett of that laboratory. Samples of fish muscle were obtained from fish caught in New Zealand and from fish caught in Tasmania and sent fixed to Auckland. Other samples were processed and examined in Hobart.

New Zealand caught fish were sampled immediately after catching, at the onset of rigor (24h) and after storage on ice for 8 days. Tissue was fixed in 30 g litre $^{-1}$ glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2. Samples were post-fixed in 10 g litre $^{-1}$ osmium tetroxide in 0.2 M sodium cacodylate buffer, dehydrated in an ethanol series and embedded in Spurr's* low-viscosity resin. Tasmanian caught fish were sampled at catching and after 11 days of storage on ice. Tissue samples were fixed in a mixture of 25 g litre $^{-1}$ glutaraldehyde, 40 g litre $^{-1}$ paraformaldehyde, 20 g litre $^{-1}$ tannic acid, and 0.8 g litre $^{-1}$ sucrose in 0.2 M phosphate buffer, pH 7.2 These samples were post-fixed in 10 g litre $^{-1}$ osmium tetroxide in 0.2 M

*(R. A. Spurr, J. Ultrastruct. Res. 26 (1969) 31.)

phosphate buffer, dehydrated in an ethanol series and embedded in epoxy resin. Ultrathin sections were cut using an LKB Ultrotome III and stained sequentially with a saturated solution of uranyl acetate in ethanol/water (1:1v) and an aqueous solution of lead citrate. Sections were observed in a JEOL 100B transmission electron microscope. Despite differences in fixation procedure and timing of processing similar results were obtained.

Other fish muscle samples processed and examined in Tasmania (Table 2.2) were processed from the fixation stage by rinsing in phosphate buffer, postfixing in 0.1% osmium tetroxide in 0.2 M phosphate buffer, dehydrated in an alcohol series then embedded in Epon-Araldite resin*. Ultrathin sections were stained in saturated uranyl acetate in ethanol water (1:1 v/v) and aqueous lead citrate. Sections were observed in a Phillips transmission electron microscope.

* British United Formulators Pty Ltd./Ciba Geigy Pty Ltd.

Table 2.2 List of samples for immunogold procedure

Date caught	Date fixed	Date processed	Fixative	Code	Description
26/11/87	26/11/87	24/2/88	Glut/form	88002	Blue grenadier prerigor, fixed immediately
		25/7/88	Glut/form	88025	Stored in dilute fixative
	8/12/87	28/3/88	Glut/form	88007	Blue grenadier, 12 days in ice
		25/7/88	Glut/form	88026	Stored in dilute fixative
18/1/89	18/1/89	18/1/89	Fix A	88007	Prerigor flathead fixed immediately
		26/2/89	Fix A	89020	Stored in dilute fixative
		18/1/89	Fix B	89008	Prerigor flathead fixed immediately
19/7/89	19/7/89	19/7/89	Fix B	89026	Prerigor jack mackerel
10/8/89	10/8/89	10/8/89	Fix B	89027	Rock cod prerigor
18/10/89	19/10/89	19/10/89	Fix B	89029	Rainbow trout fixed after treatment with 1^0Ab
		19/10/89		89030	Fixed after treatment in PBS
7/11/89	7/11/89	7/11/89	GF/CAC	89042 89043 89044	Gurnard muscle fixed prerigor PBS treated Antibody treated
7/11/89	7/11/89	7/11/89	GF/CAC	89045 89046 89047	Gurnard myocomma fixed prerigor PBS treated Antibody treated.

2.5.2 Methods used for immunocytological studies

In order to preserve immunogenic structures, samples comparable to those used in the structural studies (above) were prepared by omitting post fixation in osmium tetroxide. These samples were also embedded in Epon-Araldite. Other samples in which this post fixation step was likewise omitted were embedded in the hydrophilic resin LR White medium grade (London Resins). Fixed samples of tissue were rinsed in phosphate buffer, then 50% ethanol, 70% ethanol, in resin diluted with ethanol (1:1), in resin (100%) followed by an overnight soak in fresh resin before being finally set in fresh resin by heating for 24 hours at 50^{0}C in gelatin capsules as recommended for immunocytochemistry by the manufacturer. The following procedure gave a clear resin:-

Embedding in LR White Resin

1. Fish muscle in buffer from appropriate fixative procedure was trimmed into small pieces approx 7 x 3 mm.

2. Washed in 0.1 M phosphate buffer, 3 x 1 min then 3 x 10 min.

3. Dehydrated through an alcohol series.

   - 3 x 3 min in 50% alcohol
   - 3 x 15 min in 70 % alcohol.

4. Impregnated with resin

   - 2 parts LR white resin: 1 part 70% alcohol for 1 hour
   - 100% resin 1 hour

- 100% resin overnight
- 100% resin 1 hour.

5. Section embedded in fresh resin in gelatin capsule by placing 2-3 drops of resin in capsule, inserting sample, then topping up with resin and pushing on the cap.

6. Placed in oven set at 50°C. 24 hours.

Other samples of unfixed tissue, or lightly fixed tissue were prepared in LR white resin by similar methods.

2.6 Measurement of collagen properties

2.6.1 Isolation of collagen.

Blue grenadier (*Macruronus novaezelandiae*, Hector, 1871) were obtained from the CSIRO research vessel, RV Soela. The age of specimens was estimated from length and weight measurements (Kenchington and Augustine 1987). Skin samples, cut from the dorsal region and dissected free of other adhering tissue, were diced and suspended in 0.1 M acetic acid, adjusted to pH 2.5 with HCl, for 20 h. After removal of insoluble material by centrifugation, 800 G for 1 h, the soluble collagen was purified using differential salt precipitation by adding a cold solution of 4.4M NaCl to the collagen solution slowly with stirring (Trelstad, Catanese and Rubin 1976; Trelstad 1982). The precipitate was removed by centrifuging (800 G

for 1 h), then resuspended in 0.1M acetic acid and reprecipitated with salt solution with gentle stirring overnight. This procedure was repeated and the resulting precipitate was centrifuged then dialysed against double distilled water before lyophilization.

2.6.2 SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970), using 5% (w/v) polyacrylamide running gels for intact collagen chains and 12.5% polyacrylamide running gels for cyanogen bromide fragments, both with 3.5% (w/v) polyacrylamide stacking gels. After electrophoresis, gels were fixed and stained for 14 h in 0.1% Coomassie blue R-250 in methanol:acetic acid:water (5:1:5, v/v) and then destained in methanol:acetic acid:water (2:3:35, v/v).

2.6.3 Purification of collagen α-chains

Purified type I collagen was separated into its component α-chains by chromatography on CM-52 cellulose (Whatman CM-52) at 42°C, in 60 mM sodium acetate, pH 4.8, with elution by a linear gradient of 0-100 mM NaCl (Piez, Eigner and Lewis 1963) followed by re-chromatography of separated chain fractions under the same conditions. The α-chains were

then separated from contaminating β-components by chromatography on Superose 6 (Pharmacia) in 50 mM sodium phosphate, pH 6.8, containing 150 mM NaCl and 2 M urea (Bateman *et al.* 1986). The separated alpha chain fractions were retained for subsequent electrophoretic examination, amino acid analysis and characterisation after treatment with cyanogen bromide.

2.6.4 Chemical characterization of collagen α-chains

Purified α-chains were cleaved by 50 mg/ml CNBr in 70% formic acid for 4 h at room temperature, followed by lyophilization (Scott and Veis 1976). Resulting fragments were analyzed by SDS-PAGE. For amino acid analysis, samples were hydrolyzed in 6 M HCl containing 0.01% phenol in evacuated tubes for 24 h at 108°C, and analyses were performed with a Waters HPLC amino acid analysis system using ninhydrin detection.

2.6.5 Collagen melting temperature determination

The melting temperature of collagen in intact skin was determined in 0.2 M NaCl, 10 mM sodium phosphate, in apparatus described by Bavinton (1969). Small strips of skin were attached between a central Invar rod, which passes through a displacement transducer, and an adjacent location

on the wall of an enclosing brass tube. This tube was located inside a buffer chamber jacketed with a heating system to raise the temperature in the chamber at the rate of 1°C/min. As the temperature rises the collagen shrinks and movement in the sample is sensed from outside the vessel by the transducer. A continuous line record of shrinkage versus temperature was obtained from the voltage output of the transducer and from a thermocouple positioned at the centre of the sample. A sharp inflexion in the record represents the shrinkage temperature. The melting temperature of purified collagen in the same buffer was determined by measurement of circular dichroism at 221 nm (Hayashi, Curran-Patel and Prockop 1979), with the same rate of temperature increase.

2.6.6 Collagen solubility determination

Neutral-salt-soluble, acetic acid-soluble, and insoluble residue collagen fractions were prepared from powdered skin (Jimenez and Bashéy 1978). The skin was scraped free of adhering tissue then frozen in liquid nitrogen and crushed to powder. The powdered skin was homogenised in buffer containing 1M NaCl in 0.02 M Tris-HCl, pH 7.45 at 4°C three times with 20 volumes of buffer. The remaining tissue was then subjected to three extractions with 0.5 M acetic acid at 4°C. The insoluble residue and the extracts pooled from each step were then freeze-dried prior to amino acid

analysis. The collagen content for each fraction was calculated as a percentage of the total collagen recovered in all three fractions. Collagen quantities were based on hydroxyproline values determined by amino acid analysis.

2.6.7 Antigenic characterization of collagen

Murine polyclonal antibodies to the purified collagen were obtained courtesy of the (then) CSIRO Division of Protein Chemistry, Parkville, Victoria produced by Dr. Jerome Werkmeister on the purified type I collagen isolated from blue grenadier skin. This was done in 12-week-old female SJL/J mice that had been immunized intraperitoneally with 200 μg collagen emulsified in Freund's complete adjuvant. After three weeks they were further immunized intraperitoneally with 200μg of the same antigen in Freund's incomplete adjuvant. Mice were bled 7 days after the last immunization and sera were tested for reactivity to the blue 'grenadier collagen by standard ELISA. Monoclonal antibodies were prepared from the spleens of mice from the same batch according to the methods of Ramshaw and Werkmeister (1988). Mice were boosted intravenously with 100μg of purified fish skin type I collagen in phosphate buffered saline 3 days before collection of cells for fusion. Immunized spleen cells were fused with NS-1 myeloma cells in a ratio of 4:1 using 40% (w/v)

polyethylene glycol. Hybrid cells were grown in selective media and, after 14 days, supernatant from the hybridoma culture was screened for binding to collagen by a standard ELISA using rabbit antimouse IgG coupled to alkaline phosphatase and *p*-nitrophenyl phosphate, 1mg/ml in 10% (w/v) diethanolamine, pH 9.6, as substrate. Hybrids that secreted antibodies against type I collagen were cloned.

The specificity of the polyclonal antibody was further examined after separation of collagen chains by SDS-PAGE and transfer to nitrocellulose by electroblotting (Towbin, Staehelin and Gordon 1979). Nitrocellulose sheets were then stained for protein with 0.1% amido black in methanol:acetic acid:water (5:1:5, v/v) for 3 min. For staining with antibodies, nitrocellulose membranes were blocked for 1 h in 5% Blotto [(nonfat, dried milkpowder), (Johnson *et al.* 1984)] and then reacted with antibody diluted 1:1000 in Blotto. Goat anti-mouse Ig (Bio-Rad)*, conjugated to horseradish peroxidase, was diluted 1:1000 in Blotto and used as the secondary antibody. Binding of antibodies was visualized using 0.3% 4-chloro-1-naphthol in 20 ml methanol containing 0.06% H_2O_2 added to 100 ml Tris-buffered saline, pH 7.4. The antibody was allotted the code blue grenadier anticollagen SB10(2)-F6 Polyclonal.

* Cat. No. 172-1011

2.7 Immunohistology

2.7.1 Immunofluorescence against blue grenadier tissue

Sections, 6 μm thick, were cut from frozen tissue using a freezing microtome. They were stained with the polyclonal antibody, diluted 1:100 in 0.15 M NaCl, 5 mM sodium phosphate, pH 7.4 (PBS), washed twice for 10 min in PBS, and then visualized with affinity-purified, fluorescein isothiocyanate-conjugated, sheep anti-mouse antibody (Silenus Laboratories, Melbourne) diluted 1:50 in PBS. After a further two washes for 10 min in PBS, sections were mounted in glycerol:water (9:1, v/v) containing 1 mM 1,4-phenylenediamine. Control slides were made either using preimmune serum instead of the mouse anti-collagen antibody or by omitting the mouse antibody.

2.7.2 Immunofluorescence against other tissues

Sections were cut on a freezing microtome of human skin, chicken skin, chicken tendon, fresh blue grenadier skin, fresh blue grenadier and trevally myocomma and from blue grenadier and trevally myocomma taken from fish stored chilled (0^0) for 5 days. The reactivity of the murine anticollagen antibody was assessed at various concentrations using the method

essentially the same as that given in 2.7.1

2.7.3 Immunoperoxidase

The technique of immunoperoxidase was used to establish that the fixed tissue retained antigenicity before proceeding to TEM work. In essence the sections were dewaxed, etched with protease, blocked for endogenous peroxidase, treated with antibody, covered with peroxidase conjugated rabbit antimouse serum, treated with DAB, then rinsed counterstained and mounted. The details were as follows:-

Immunoperoxidase method

1. 4 micron paraffin sections on glass slides coated with a dried thin film of polyvinyl acetate wood glue obtained by dipping in a 1% solution

2. Dry slides overnight at 37°C. Melt wax in 56°C oven for 5 minutes prior to dewax.

3. Dewax in xylene (2 x 5 min), hydrate in absolute alcohol (2 min) 95% alcohol (2 min) and 70% alcohol (2 mins). Running tap water (2 min).

4. Place sections in PBS.

5. Prewarm PBS in coplin jar x 2 at 37°C.

   (Place slides in prewarmed PBS)
   (Place 30 ml PBS in coplin jar)

   Add 10 ml PBS to test tube and prewarm to 37°C.

6. Digest slide sections in protease for 8 minutes.

(Protease solution: Dissolve 0.02 g protease in prewarmed 10 ml PBS in tube at 37°C, add to the 30 ml PBS at 37°C in coplin jar.)

Protease digestion for formation fixed tissue 'opens' crosslinked antigen binding sites.

7. Terminate digestion in tap water and wash in running tap water for 5 minutes.

8. Wash in PBS 3 x 2 mins.

9. Block endogenous peroxidase using 3% hydrogen peroxide in distilled water (FRESH) 30 min.

10. Rinse with PBS 3 x 2 min.

11. Place slides in humidity rack.

    Wipe off excess PBS and add 1:5 dilution normal swine serum 30 min.

12. Drain off excess and apply primary antibody to test slides dilution of 1:50 ie α Blue Grenadier Coll. SB10(2) - F6 Polyclonal.

    Negative control slides add PBS instead of 1° Ab. Leave for 30 minutes.

13. Wash with PBS 2 X 5 min..

    Wipe off excess PBS.

14. Cover sections with diluted Peroxidase - conjugated rabbit antimouse serum ie Add 0.05 g Albumen to 1.5 ml PBS.

    Leave for 30 minutes. Add 0.05 ml of peroxidase conjugate rabbit antimouse.

15. Wash 2 x 5 min with PBS.

16. Cover with freshly made up DAB and incubate in dark in coplin jar 10 minutes.

(Make up fresh 0.04 g DAB in 1 ml PBS add to 79 ml PBS. Add 8 drops H_2O_2. Use gloves).

17. Rinse in distilled and wash in running tap water 5 minutes.

18. Stain in Mayers Haematoxylin 4 minutes.

19. Wash in running tap water 2 minutes.

20. Blue in Scotts tap water (NH_3 water).

21. Wash in tap water 2 min.

22.

Dehydrate	70% alcohol	30 sec.
	95% alcohol	30 sec.
	Abs alcohol	30 sec.
	Abs alcohol	30 sec.
Clear	Xylene	2 mins.
	Xylene	2 mins.
Mount	DPX	

2.7.4 Reactivity between antibody and protein A gold

The method chosen to define collagenous structures under the TEM was to visualize the attached antibody by reacting it with a bacterial protein (Protein A) to which are conjugated electron-dense gold particles (Romano and Romano 1977). Before proceeding to lengthy TEM procedures, it was necessary to establish that the protein A gold complex (PAG) would react with the antibody attached to the tissue. The gold particles are only 15 nm in diameter and are not visible under the light microscope. Silver enhancement was used to make the deposition visible as follows.

Silver enhancement method

1. 4 micron paraffin sections on PVA woodglue coated slides

2. Dry slides overnight at 37°C. Melt wax in 56°C oven for 5 minutes prior to dewax.

3. Dewax in xylene (2 x 5 min), hydrate in abs. alcohol (2 min), 95% alcohol (2 min) then 70% alcohol (2 mins). Wash in running tap water (2 min).

4. Place slides in PBS.

5. Block protein binding sites with 5 mg/ml BSA in PBS for 10 minutes at 37°C (or 30 minutes RT).

6. Drain off excess and apply 1° Ab to test slides with dilution 1:50 ie α Blue Grenadier Coll.

   SB10(2) - F6 Polyclonal 30 minutes at RT
   (PBS 30 min for control slides)

7. Wash in PBS 3 x 5 min washes on rocker.

8. Incubate slides in either rabbit antimouse antiserum dilution 1:50 for 30 minutes or PBS for 30 minutes as blank reference slides.

9. Wash in PBS three times x 5 min on rocker.

10. Rinse in PBS/PEG/BSA

    PBS + 0.05% PEG (mw 20,000) + 5 mg/ml BSA

11. Incubate in Protein A gold diluted in PBS/PEG/BSA

    dilutions 1:10 + 1:40 for 30 minutes at room temp.

12. Wash 3 x 5 min in PBS on rocker.

13. Wash 2 x 5 min in H_2O (Rinse slides in H_2O, then wash on rocker).

14. Make up silver intensification solution and stain sections in darkroom for 10-15 minutes (30 minutes was used in this procedure)

15. Wash thoroughly in H_2O.

16. Stain nuclei in Mayers Haematoxylin and Blue in Scotts tap water.

17. Dehydrate, clear and mount in Eukitt.

Silver enhancement solution (made up fresh immediately before use)

A. 15 ml - 10 g gum acacia in 20 ml of deionised water. Stirred for 1 day and centrifuged 30 min before use.

B. 2.5 ml - 2.55 g citric acid + 2.35 g Trisodium citrate made up to 10 ml in distilled water. (Buffer).

C. 3.75 ml - 0.11 g silver lactate in 15 ml distilled water. (Silver Ion).

D. 3.75 ml - 0.85 g hydroquinone in 15 ml distilled water (make up fresh and add quickly).

Initially, silver enhancement proved unsuccessful until it was found that protein A gold does not react well itself with murine antibodies (the primary anticollagen was raised in mice) and that it was necessary to conjugate the protein A gold with the primary antibody through an intermediate rabbit antimouse (RAM) antibody since protein A gold reacts well against rabbit. The results are given in Table 2.3.

Table 2.3 Reaction between primary antibody, RAM, PAG and silver reagent

Presence of reagent				Reaction
1° Ab	RAM	PAG	Silver	
+	-	-	+	-ve
+	+	-	+	-ve
+	+	+	+	+ve
+	-	+	+	-ve
-	+	+	+	-ve
+	+	-	+	-ve

A positive staining reaction is indicated thus +ve
1^0Ab = primary antibody blue grenadier anticollagen SB10(2)-F6 polyclonal
RAM = rabbit antimouse serum
PAG = protein A gold reagent
Silver = silver stain reagent (see above)

No blank reagent reactions occurred and no reaction of the primary antibody with protein A gold was noted unless RAM was used. The RAM alone without the protein A gold did not yield silver enhancement.'

2.7.5 Immunogold TEM

Ultrathin sections cut from blocks of tissue embedded in LR white resin were incubated on Nickel grids with the anticollagen antibody after blocking for non-specific binding with bovine serum albumin. The sections

were rinsed then incubated with rabbit antimouse serum, rinsed to remove excess RAM and incubated with protein A gold conjugate. Control sections consisted of substituting normal mouse serum or PBS for the primary antibody. Sections were then rinsed and stained in uranyl acetate and lead citrate. The procedure used was mainly that of Stephens, Bendayan and Silver (1982) and is given in detail as follows:-

Protein A Gold procedure

Performed in moist chamber

1. Thick sections (100 nm) were cut from the LR white blocks and mounted on nickel grids.

2. Float grids on PBS.

3. Block protein binding sites with 5 mg/ml BSA in PBS at room temperature for 20 min.

4. Remove excess BSA/PBS and place test grids on a 1:50 dilution of 1° Ab in PBS (SB10(2) - F6 Polyclonal)

   Control slides placed on a 1:50 dilution normal mouse serum.

5. Wash three times x 5 min with PBS on rocker 1 hour at RT.

6. Incubate grids on drops of rabbit antimouse serum dilution 1:50 in PBS 30 minutes.

7. Wash in PBS three times x 5 min on rocker.

8. Rinse in PBS/PEG/BSA

   PBS + 0.05% PEG (mw 20,000) + 5 mg/ml BSA (0.5 ml of 1% PEG in 10 mls BSA/PBS)

9. Incubate in Protein A gold dilution 1:100 with PBS/BSA/PEG. 30 min at RT.

10. Wash 1 x 5 min PBS/BSA/PEG.

11. Wash 3 x 5 min PBS.

12. Rinse in Milli-Q water and blot dry.

13. Examine under EM.

14. Stain with saturated uranyl acetate in 0.9% $KmnO_4$ in phoshate buffer, aqueous lead citrate.

15. Carbon coat grids for 3 min at 70kV - 25 Amp

Note BSA was used as a non-specific blocking agent instead of gelatin used in the original method (Stephens, Bendayan and Silver 1982) since this may have reacted with the antibody.

Chapter 3

SEM studies on fish muscle

3.1 SEM studies of fish muscle

There have been few studies of fish muscle using the SEM. Schaller and Powrie (1971) published SEM micrographs of trout muscle at various stages of post mortem storage (3^0C). These demonstrated progressive shrinkage of the transverse elements of the SR and T system such that from being quite pronounced in appearance above the surface they shrank beneath it after 4 days storage. Collagen fibres were not evident in their preparations. Giddings and Hill (1976,1978) studied effects of freezing and of frozen storage on crab and shrimp muscle but again the emphasis was placed on the muscle rather than the connective tissue.

By using enzyme treatments Børresen (1976) was able to develop a method for isolation of the muscle cell envelope of cod. Under the SEM it was a lace-like network of collagen fibres (Almäs 1982).

Lampila *et al.* (1985) used SEM to study the effects of different freezing procedures on rockfish (*Sebastes sp.*) by preparing samples using isothermal freeze fixation. Using this technique they were able to show the distortion and damage to the cell membrane that occurs in the freezing process. The effects of thermal processing on tuna muscle were studied by Lampila and Brown (1986) using SEM to record the progressive disruption that occurs. Hatae, Yoshimatsu and Matsumoto (1990) examined sections of cooked muscle from five species of fish and related their results to

textural characteristics. In a comprehensive study, Schrubring and Sandau (1989) used SEM to study the effects of salting and heating on fresh and frozen cod and herring muscle and minces.

Ando *et al.* (1991) subjected samples of rainbow trout muscle (*Oncorhynchus mykiss*) to a controlled compression test which opened the flesh structure to reveal the connective tissue fibres of the intermuscular space. They also observed disintegration of the collagen fibres in the pericellular connective tissue after storage of the muscle for only 24 hours post mortem at 5^{0}C (Ando *et al.* 1992).

In none of these studies was the myotendinous junction examined. Until recently, the myotendinous junction had only been studied using light microscopy and a three dimensional representation of the area had been lacking. This situation changed with our initial studies (Bremner and Hallett 1985, 1986). This chapter describes these investigations which formed the basis on which this thesis was formulated.

3.1.1 The myotendinous junction of blue grenadier (*Macruronus novaezelandiae* Holthius) and spotted trevalla (*Seriolella punctata* Forster)

In broad outline, samples taken from the dorsal musculature of the fish pre-rigor, during rigor and at intervals post rigor were fixed, freeze-fractured

and freeze dried and gold coated before examination under the SEM. The freeze fracture process exposed sites at the junction of the muscle fibres with the connective tissues of the myocommata.

Similar structures were observed to occur in both blue grenadier and spotted trevalla. The muscle fibres were seen to join to the myocomma and were connected to it by a sheath of fine collagen fibrils which arose from the myocomma to wrap around each muscle fibre (Figure 3.1, 3.2).The muscle fibres fitted into socket like indentations on the myocomma (Figure 3.3). When the muscle fibres were part dissolved from these structures with salt solution, the sheath of fine collagen fibrils holding the muscle fibre to the myocomma could clearly be seen. This network of collagen fibrils was evident on the surface of the muscle fibres (Figure 3.4). At a fractured end, the structure of the muscle fibril had the typical elongated myofibrils situated around the periphery of the fibre with polygonal myofibrils in the interior. The connective tissue network peeled back, revealed the plasmalemma and endomysium on the fibre surface (Figure 3.5).

3.1.2 Post rigor changes in the myotendinous junction

After storage at 0^0C obvious changes occurred. Holes appeared in the endomysial surface (Figure 3.6) and the fine collagen fibrils were

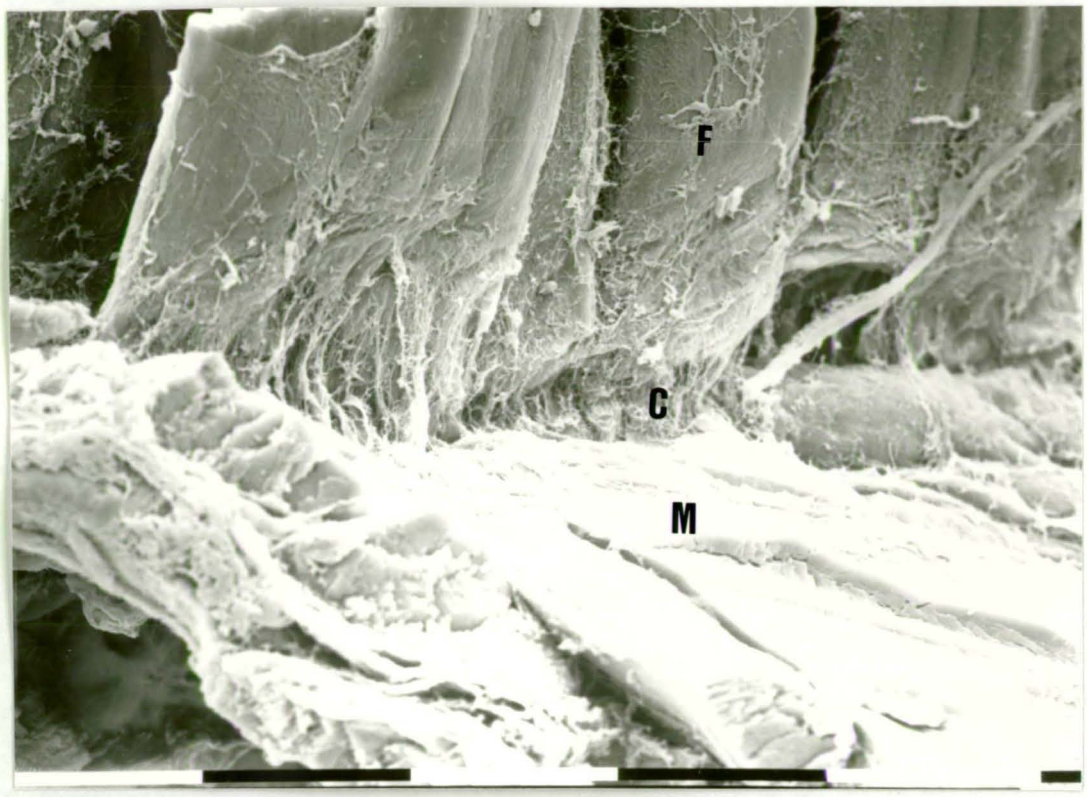

Figure 3.1 Pre-rigor blue grenadier, fine collagen fibrils **C** connect the myomeres **F** to the myocomma **M**. Bar 0.1mm.

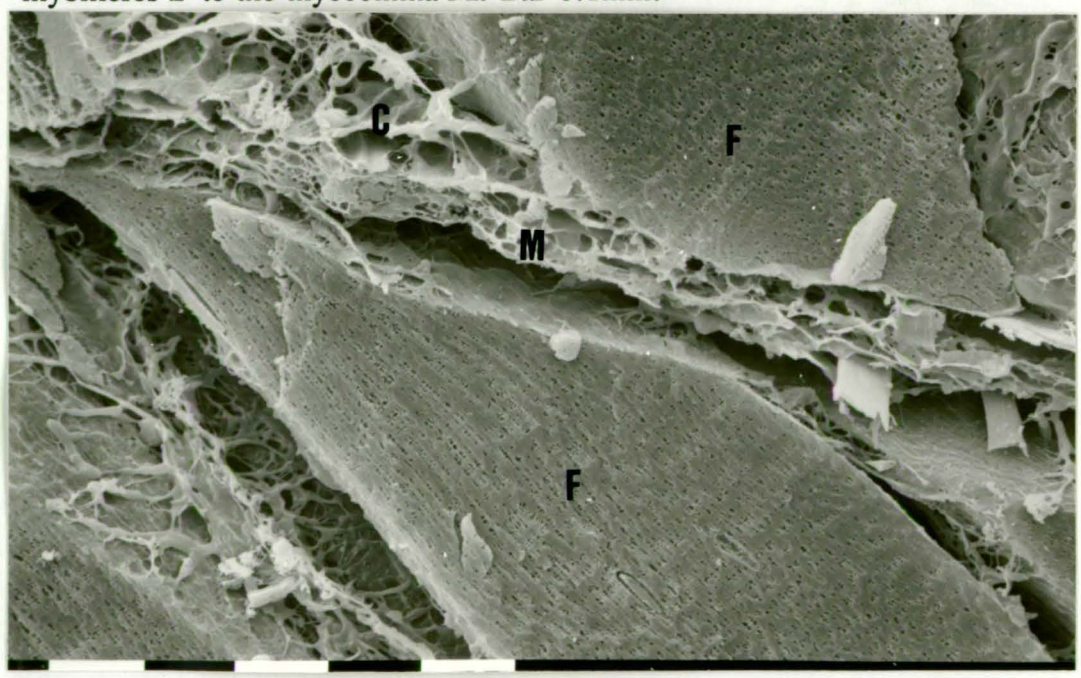

Figure 3.2. Pre-rigor spotted trevalla. The interface of muscle fibres with the myocomma exposed by freeze-fracture. The fine collagenous fibrils **C** run from the muscle fibres to the myocomma which lies horizontally across the figure. The ends of the muscle fibres **F** are intimately connected to the surface layers of the myocomma **M** which shows signs of being pulled apart in the fracturing process. Bar 0.1 mm.

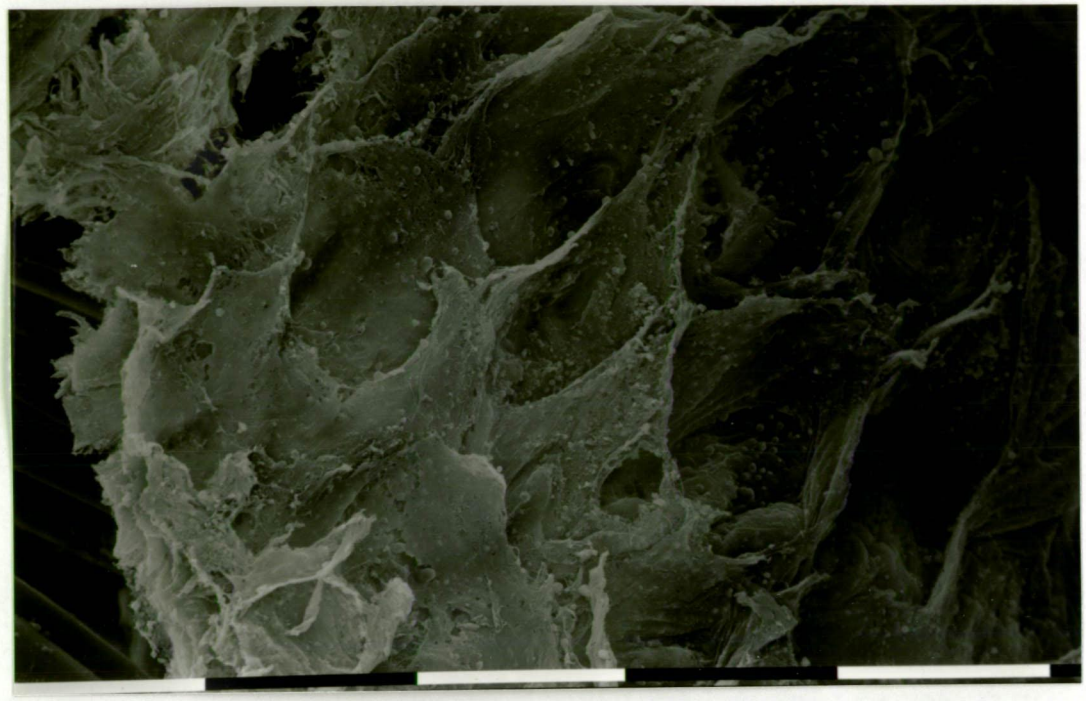

Figure 3.3. Post-rigor blue grenadier. Muscle fibres have been teased out of the myocomma to reveal socket-like indentations. Bar 0.1 mm.

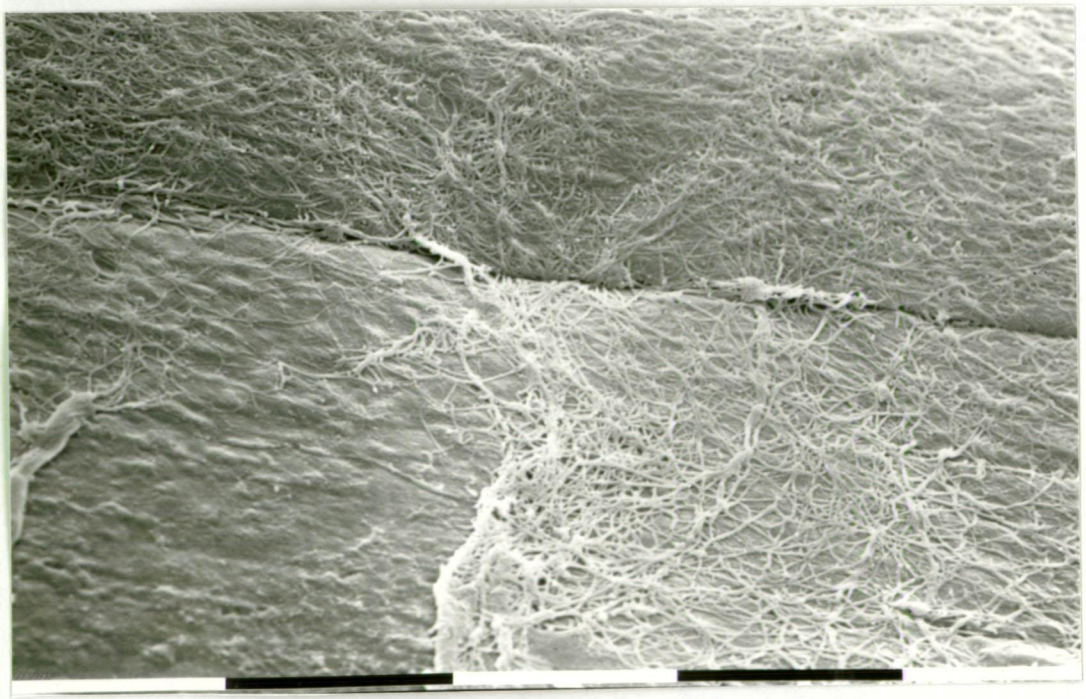

Figure 3.4. Pre-rigor blue grenadier. Adjacent muscle fibres, showing network of fine collagen fibrils and intact pericellular layer. Bar 0.01 mm.

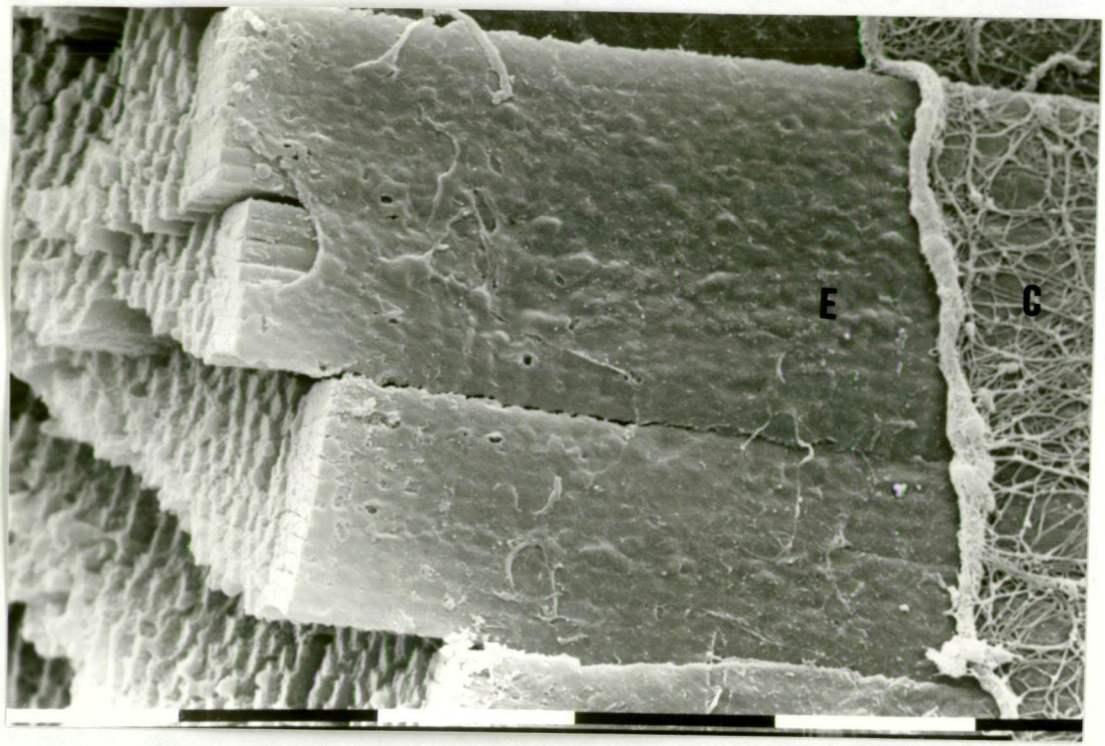

Figure 3.5. Fractured muscle fibre end showing the fine pericellular collagen fibrils **C** peeling back and the intact endomysial layer **E**. The myofibrils on the perimeter are flattened in cross section. Bar 0.01 mm.

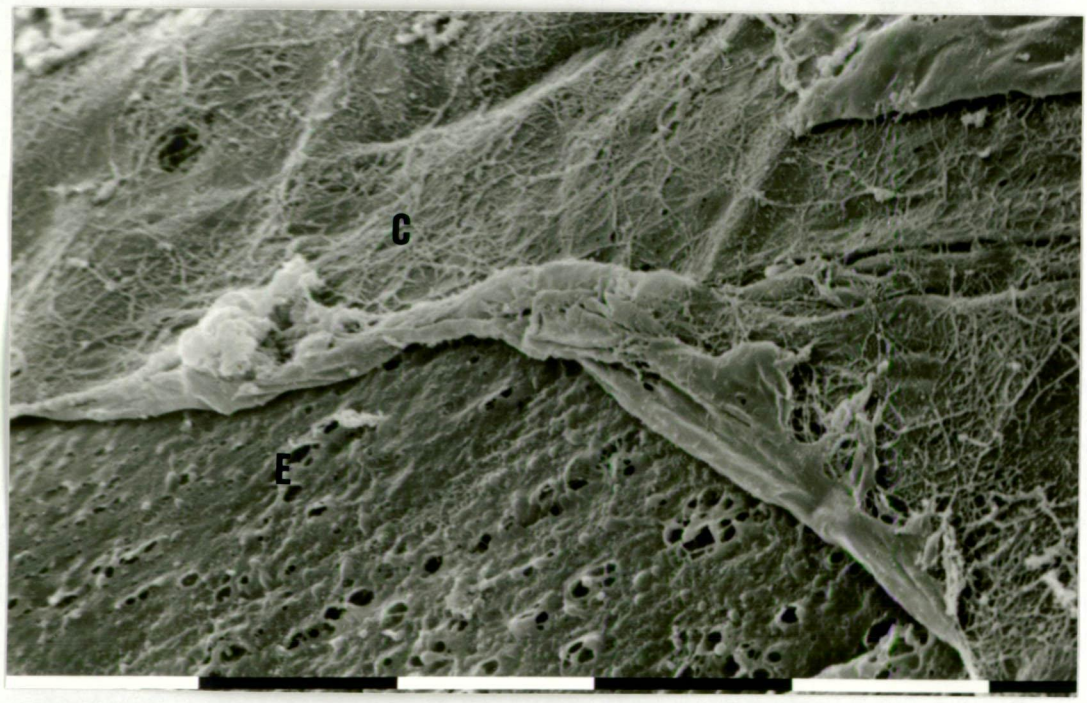

Figure 3.6. Blue grenadier after 5 days ice-storage. The muscle fibre surface exhibits deterioration of the pericellular collagen fibres **C** and the endomysial/plasmalemmal layer **E**. Bar 0.01 mm.

disrupted. The connections between the muscle fibres and the myocomma degraded (Figure 3.7), the pericellular collagen fibrils appeared to be digested and the muscle fibre ends detached from the myocomma (Figure 3.8, 3.9).

3.2 Discussion

There were similar structures observed in both these unrelated species. The muscle fibres were surrounded by a pericellular layer of fine collagen fibres which attached the muscle fibres to sockets in the myocomma. During post mortem storage the interface between the muscle fibres and the myocomma deteriorated and, in some areas, the fine collagen fibres appeared to be completely degraded. This resulted in muscle fibres completely detached from the myocommata. Unattached fibres were never seen in pre-rigor preparations. The changes in the muscle fibres were only evident on the fibre surfaces where deterioration of the endomysial/plasmalemmal area could be seen.

3.3 Conclusion

It was concluded that deterioration of the collagen of the connective tissues of the extracellular matrix of fish muscle occurred in stored fish. This could be a significant factor in post mortem softening of fish flesh. The

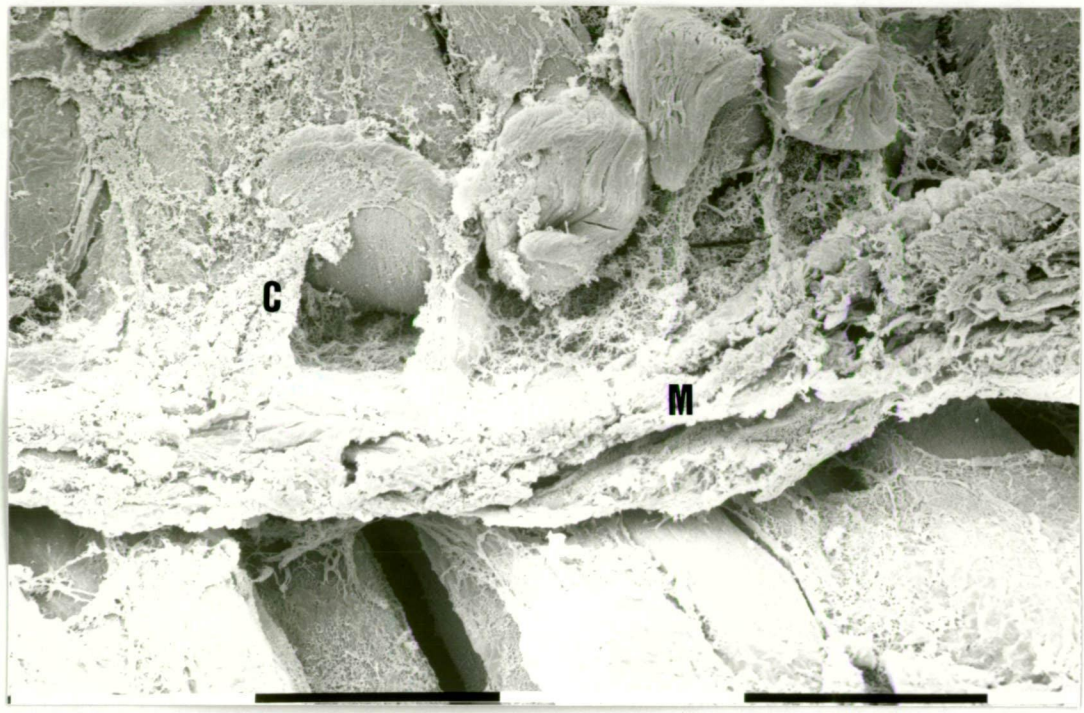

Figure 3.7 Spotted trevalla stored 4 days in ice. The fine collagenous fibrils **C** of the pericellular area and the myocomma **M** have been degraded allowing loosening of attachment of the muscle fibres. Bar 0.1 mm.

Figure 3.8. Blue grenadier stored 11 days in ice. A muscle fibre **F** shows complete detachment from the myocomma and absence of fine connecting collagen fibrils. Bar 0.1 mm.

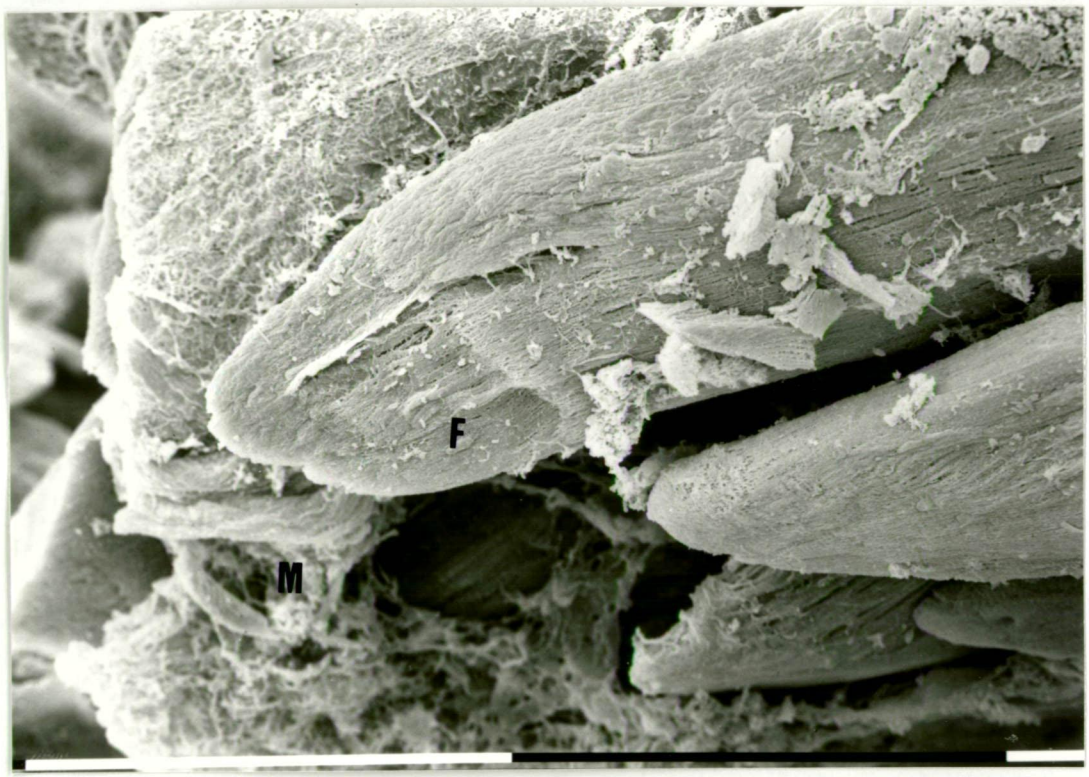

Figure 3.9. Spotted trevalla stored 8 days in ice. Muscle fibres **F** have been detached from the myocomma **M**. General degradation in the perimysial and sarcolemmal area. Bar 0.1 mm.

indications were that the deterioration was enzymic in nature. However, it was unclear which structures were being attacked and it was obviously necessary to examine the fine structure in more detail. These studies also indicated breakdown in the fine collagen fibrils.

Collagen is normally regarded as a fairly stable tissue that is resistant to enzymic attack - hence this aspect called for further investigation into the nature and type of collagen present in the pericellular region and at the myotendinous junction and the susceptibility of this collagen to attack by enzymes.

These initial studies provided the major focus for the work reported in this thesis. Research was initiated to elucidate the fine structure at the myotendinous junction and to characterize the collagen.

Chapter 4

Transmission electron microscope studies

4.0 Transmission electron microscope studies

4.1 Introduction

In Chapter three evidence was presented from SEM studies that deterioration in the region of the myotendinous junction and in the pericellular region occurred during the post mortem storage of chilled fish. It appeared that the fine collagen fibres and other components of the extracellular matrix were being degraded. The myotendinous junction in fish has been reported to be similar in structure to that occurring in mammalian muscle (Schwarzacher 1960; Schippel and Reisig 1969; Korneliussen 1973; Schattenberg 1973 and Nakao 1975). None of these studies had examined post mortem changes, nor had commercial fish been examined. Rather the investigations were performed on unusual specimens such as the hagfish, seahorse or lamprey. Therefore the main thrust of the present study was placed on establishing in greater detail the myotendinous junction of a commercial species and in following the changes that occurred in the junction during the course of chilled storage.

4.1.1 Preliminary histology

In the pre-rigor samples of trumpeter the muscle fibres were packed closely

together but evidence of fine collagen fibres could be seen between some myofibres (Figure 4.1), similar to results reported by Love 1970. Some separation of the collagen sheets within the myocommata due to processing was observed (Figure 4.2), but the epimysial collagen was intimately attached and associated with the terminal ends of the fibres. Samples taken after five days showed quite distinct gaps between muscle fibres with some loosening of the epimysial collagen from the muscle fibre ends (Figure 4.3). In those samples taken from fish stored 12 days, large gaps between muscle fibre bundles were evident and there appeared to be some loss of continuity within the myocomma (Figure 4.4) and some muscle fibre ends had separated from the collagen (Figure 4.5).

These results are similar to those described by Menon and Nair (1988) for storage changes observed in major carp (*Labeo calbasu*) and are consistent with the observations presented in Chapter 3.

4.2 Prerigor samples

Examination of the pre-rigor blue grenadier samples in the transmission electron microscope revealed an obvious regular myofibrillar banding pattern and a well defined sarcoplasmic reticulum and tubule system, which in fish is found at the level of the Z-line (Figure 4.6; cf Figures 1.2 and 1.3). The T system can be seen opening into the extracellular space in

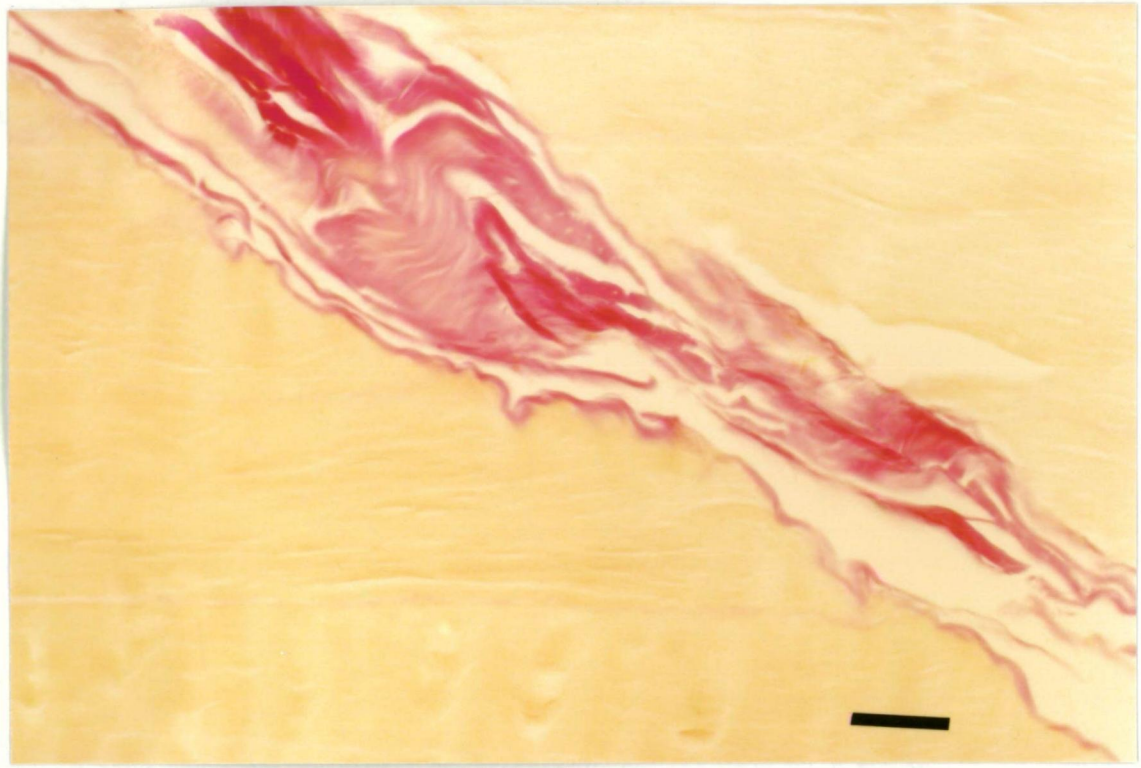

Figure 4.1 Pre-rigor trumpeter muscle stained with Van Gieson's, showing network of purple collagen fibres of the myocomma adhering closely to the muscle fibre base. Muscle fibres are closely packed. Magnification X 100. Bar 10 µm

Figure 4.2 Fine collagen fibres can be seen between closely packed muscle fibres of the pre-rigor trumpeter. Magnification X 100. Bar 10 µm

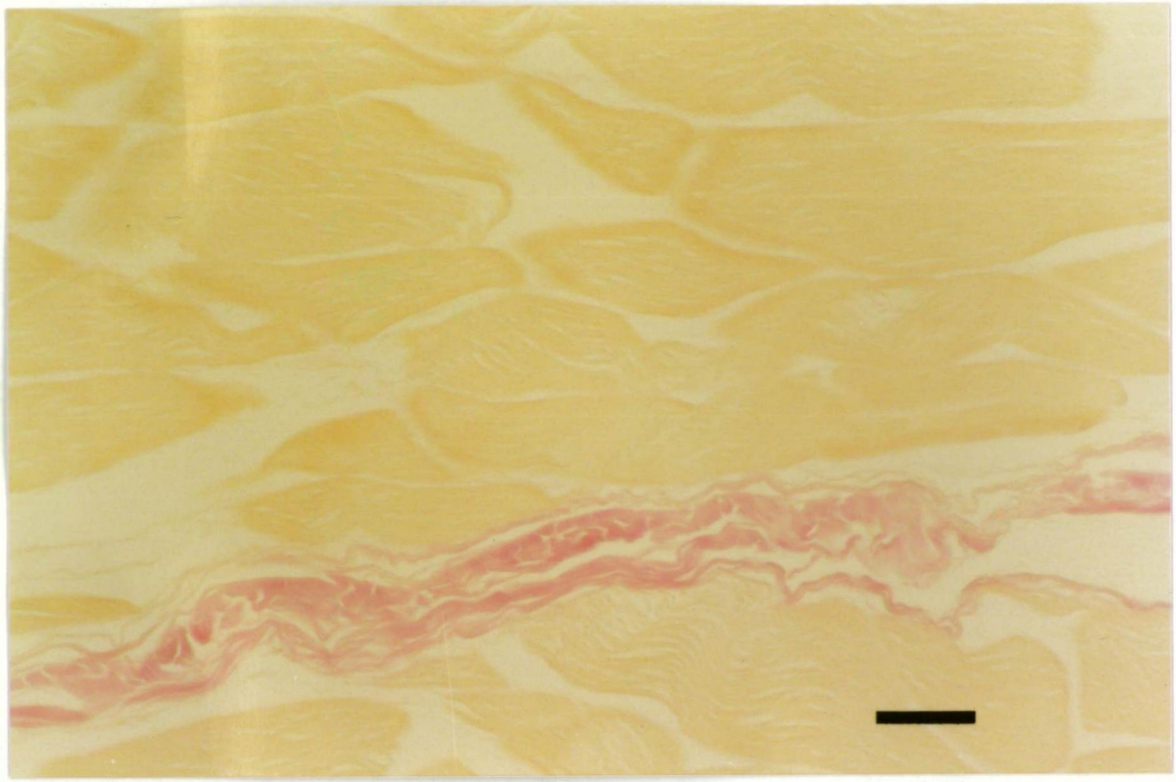

Figure 4.3 The same fish as in 4.1 after 5 days chill storage in ice. Spaces between muscle fibres are appearing and the myocomma is starting to part into layers. Magnification X 100. Bar 10 µm

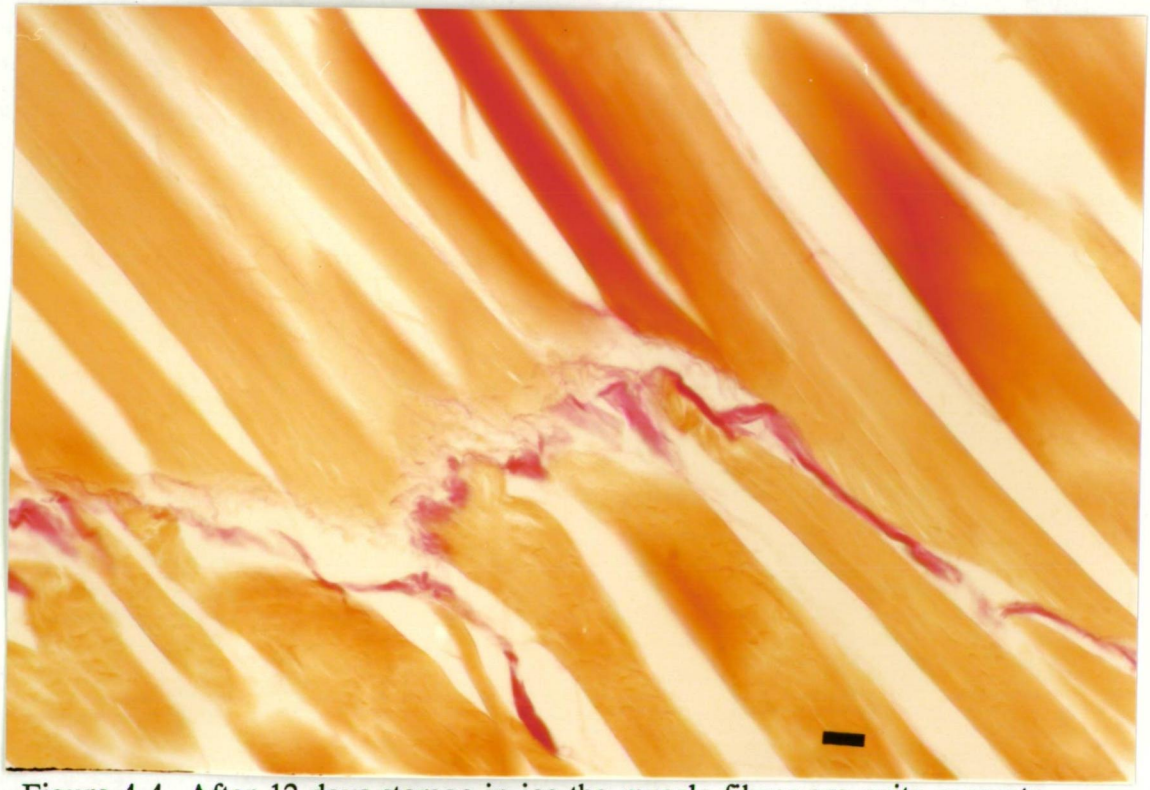

Figure 4.4 After 12 days storage in ice the muscle fibres are quite separate and the collagen has pulled away from the muscle fibre ends. Magnification X 40. Bar 10 µm

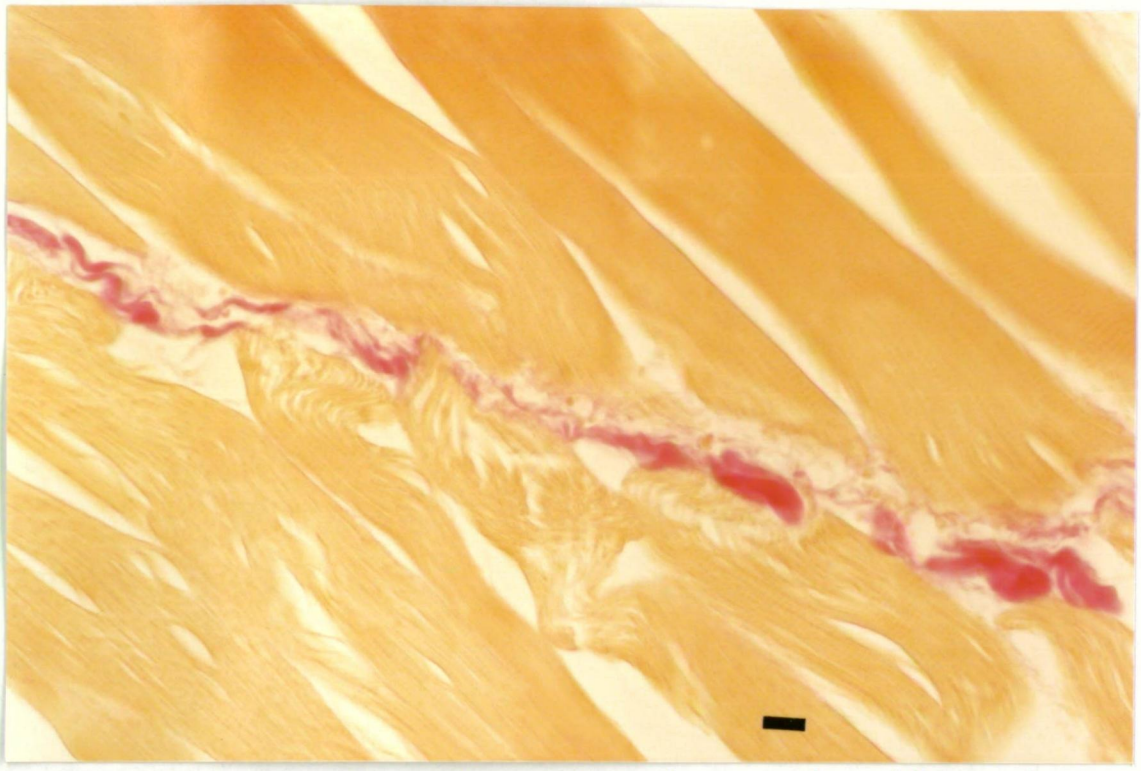

Figure 4.5 Similar section to Figure 4.4 showing separation of the muscle fibres and loosening of the collagen from the muscle fibre base. Magnification X 40. Bar 10 µm

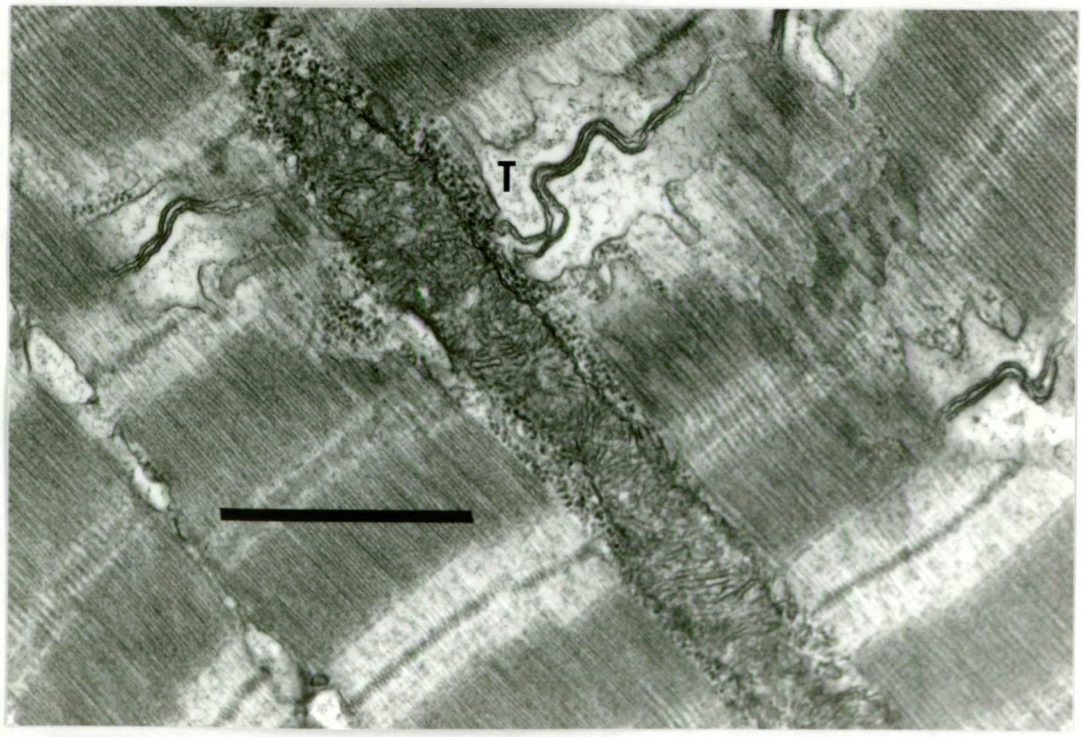

Figure 4.6 Pre-rigor blue grenadier showing a well defined sarcoplasmic reticulum and tubule system with tubule **T** opening on to the extracellular space. Bar 1μm.

which there are numerous glycogen granules and an elongated mitochondrion. A similar system was described for cod by Howgate (1980). In the bulk of the muscle the myofibrils were in register (not shown, but see also Figure 4.11). The Z lines were well defined and finer features of the structure such as the N-lines in the I band were evident.

At the fibre surface a distinct sarcolemma, the basal lamina and endomysial collagen layer were present (Figure 4.7). In some sections (not shown) the surface had a periodic undulating appearance giving the impression of attachment between the basal lamina and the M line (Pierobon-Bormioli 1981).

At the fibre end, the sarcolemma occurred in close proximity to the bounding membrane of the sarcoplasmic reticulum of an invagination (Figure 4.8). The basal lamina was absent from this region.

Collagen fibres of two classes of diameter were associated with the tapering ends of individual muscle fibres (Figure 4.9). The endomysial fibres were of much smaller diameter (approx 18 nm) and had a much less defined banding pattern than the larger (approx 80 nm) fibres. The flattened dendritic processes of a fibroblast formed boundaries near this fibre end and one of the collagen fibres along the upper process appeared to be in the latter stage of formation since its banding was not well defined and it appeared to consist of long strands, which may have been protofibrils.

A more general view of a muscle fibre base is shown in Figure 4.10

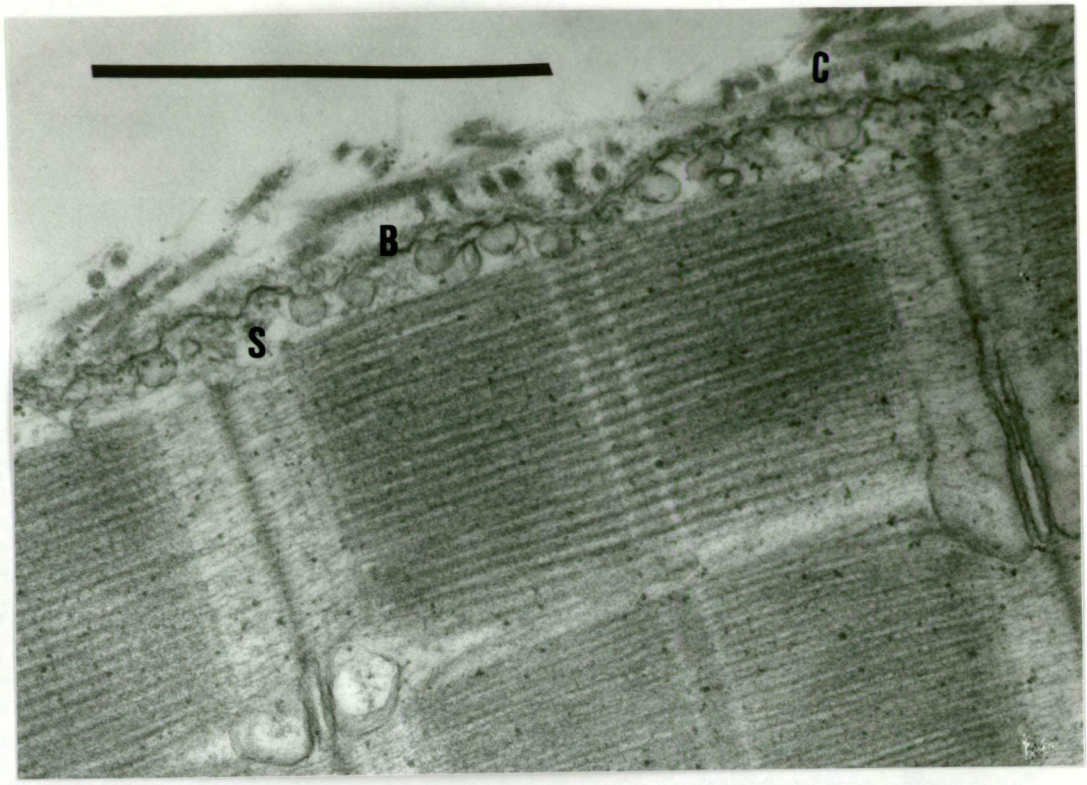

Figure 4.7 Pre-rigor blue grenadier at a muscle fibre edge distict sarcolemma **S**, basal lamina **B** and endomysial collagen fibres **C**. Bar 1μm.

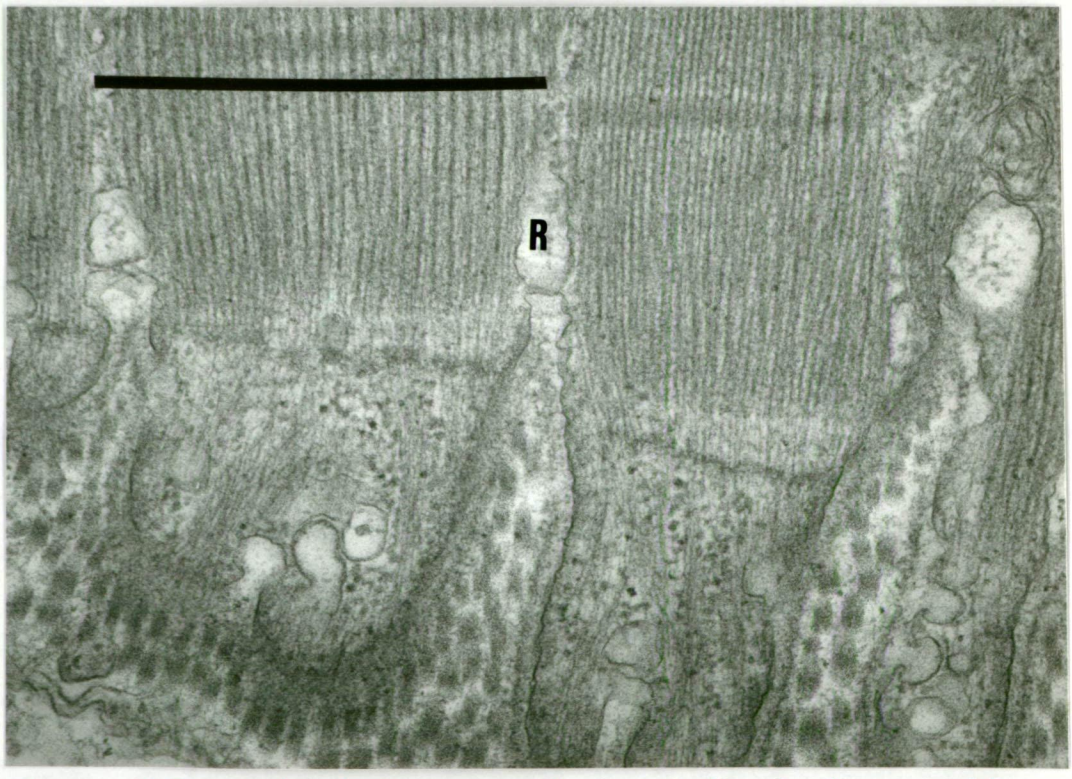

Figure 4.8 Terminal end of a groove-like invagination in close apposition with a vesicle of the sarcoplasmic reticulum **R**. The sarcolemma is well defined and complete, but the basal lamina is not continuous at the end of the groove. Bar 1 μm.

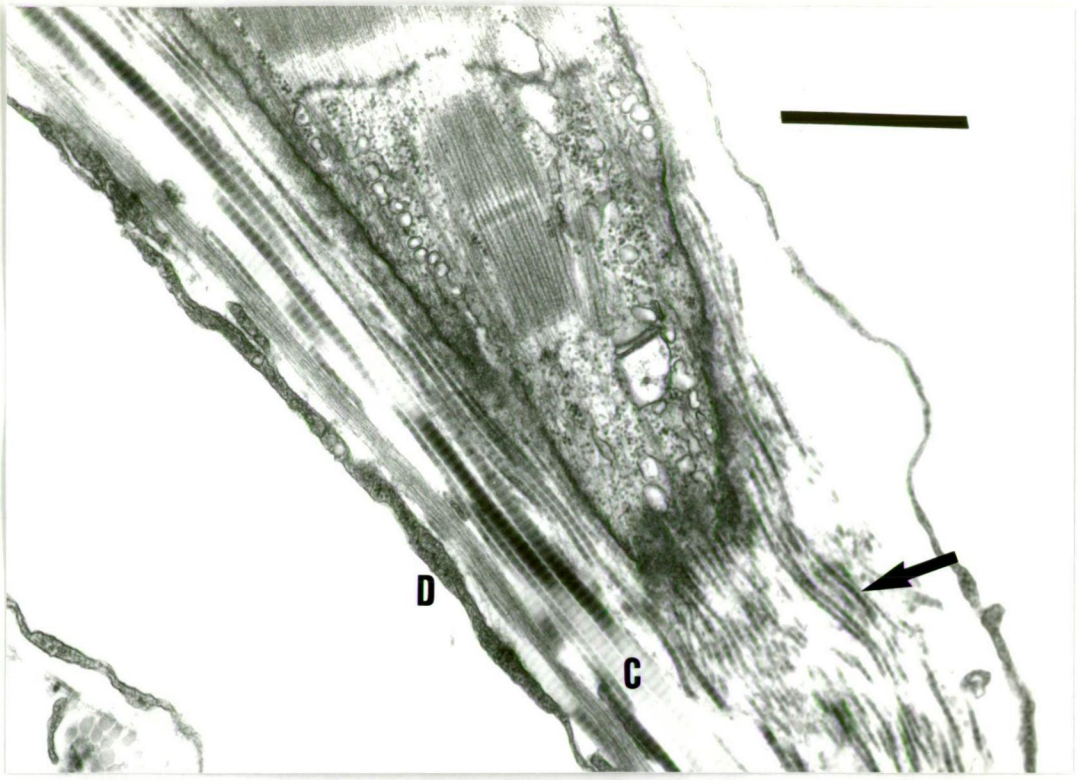

Figure 4.9 Collagen fibrils of two different diameters, large **C** and small **arrow**, can be seen at the end of a muscle fibre which is bounded by the flattened processes of a dendritic cell **D**. Bar 1 μm.

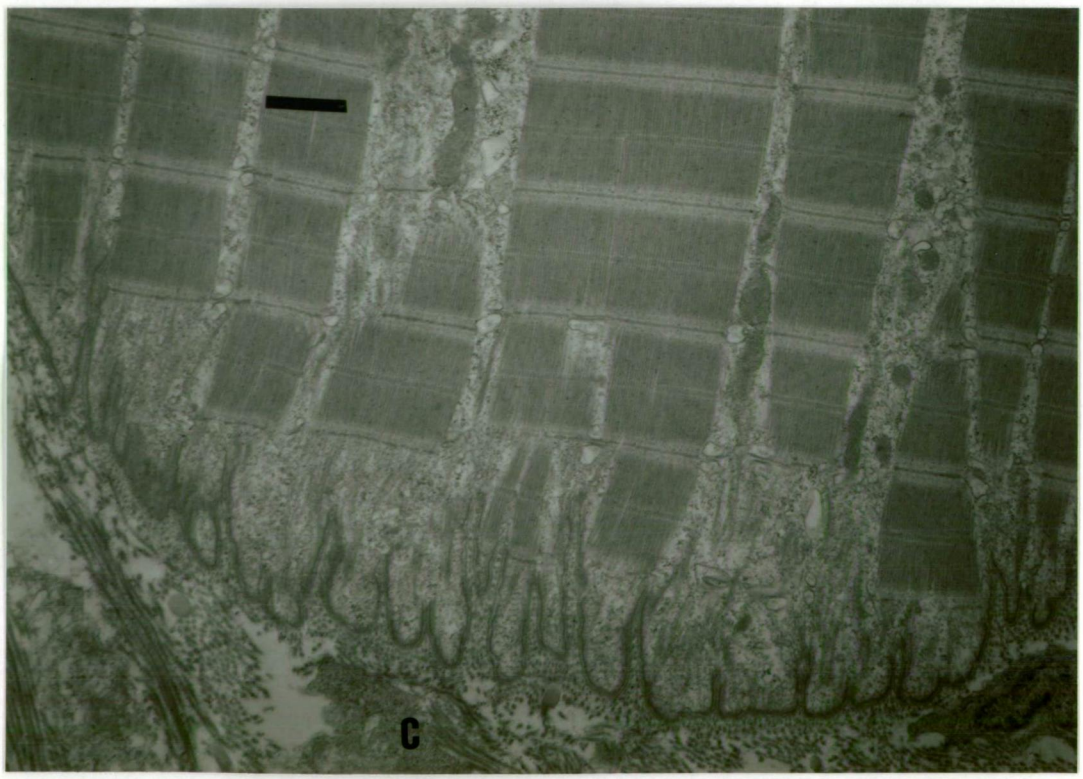

Figure 4.10 Muscle fibre base from pre-rigor blue grenadier showing the intimate association between it and the collagenous myocomma **C**.Bar 1μm.

illustrating the intimate relationship between the collagen of the myocomma and the fibre itself. The grooves and invaginations in the fibre end were filled with collagen fibres and these extended considerable distances, up to 10 μm, into the fibre base between the myofibrils (Figure 4.11). External to the muscle fibre there was a dense network of collagen fibres which ran in apparent layers arranged at different angles. Fibroblasts were often evident and the processes of the dendritic cells tended to form boundary layers that were apparent weaknesses in the total structure since it is often along these lines that sections were seen to separate. Some invaginations were finger-like (Figure 4.11) and others were more like grooves (Figure 4.12), or were shallow tube-like structures (Figure 4.13). Each had a clearly defined sarcolemma and a basal lamina which in most cases was complete (Figure 4.14, cf Figure 4.8). Vesicle-like indentations of the sarcolemma were often present (Figure 4.14) and these may represent the genesis of the sarcoplasmic reticulum at these sites since they often coincided with the existence of rudimentary Z lines. Connecting structures between the basal lamina and the sarcolemma were evident (Figure 4.12, 4.14). Similarly there was evidence of connecting structures between the collagen fibrils (Figure 4.12, 4.14) and between the collagen and the basal lamina (Figure 4.15).

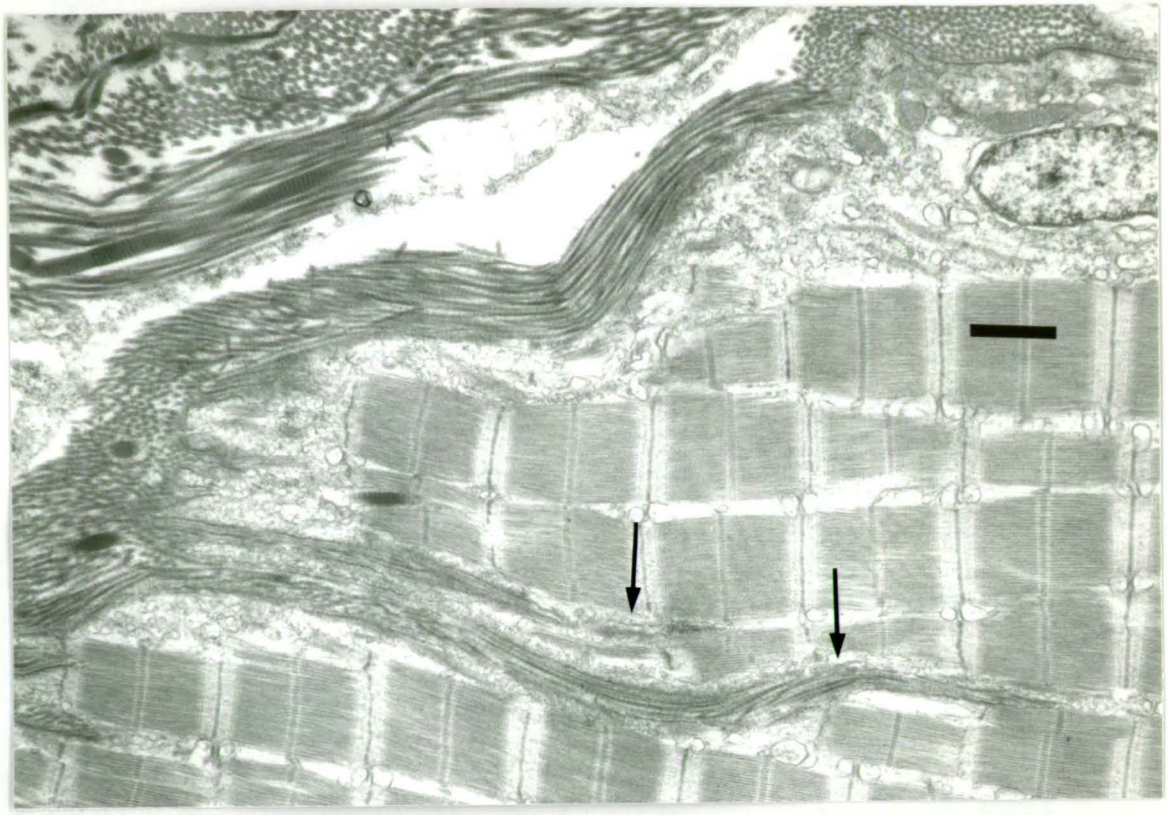

Figure 4.11 An elongated invagination filled with collagen fibres (**arrows**) penetrating between myofibrils. Bar 1 μm.

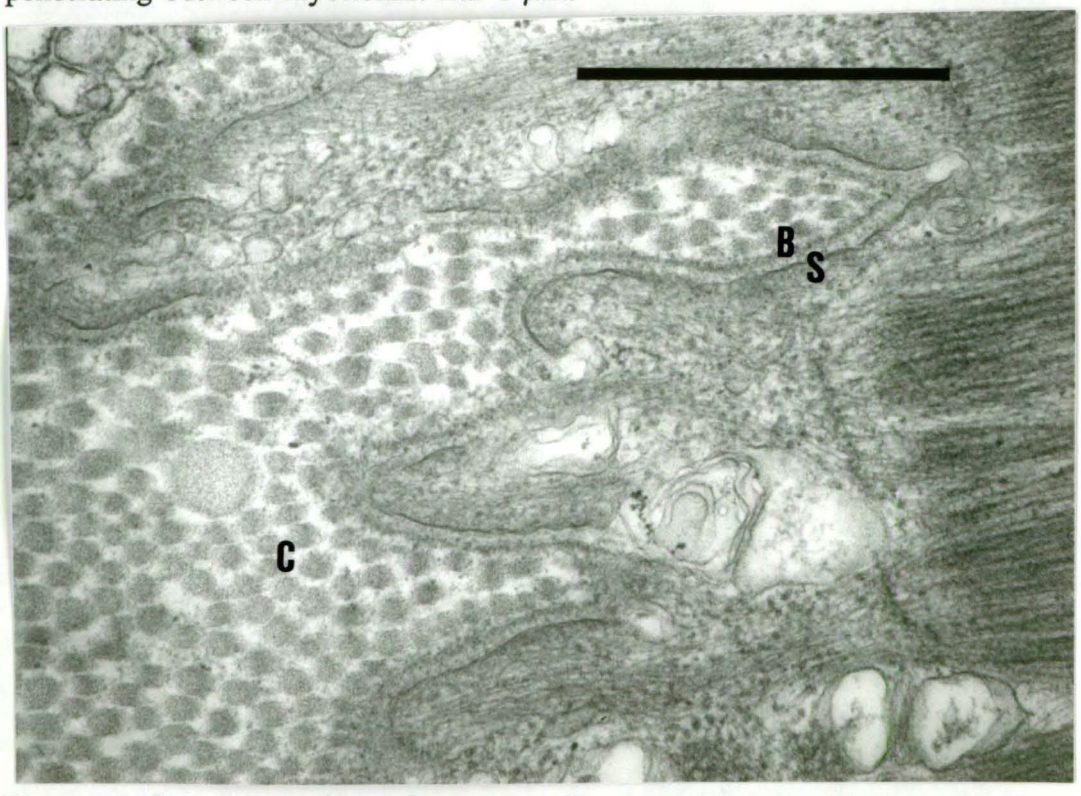

Figure 4.12 Detailed view of a groove-like invagination with the , basal lamina **B**, sarcolemma **S** and collagen fibres **C** clearly defined. Bar 1 μm.

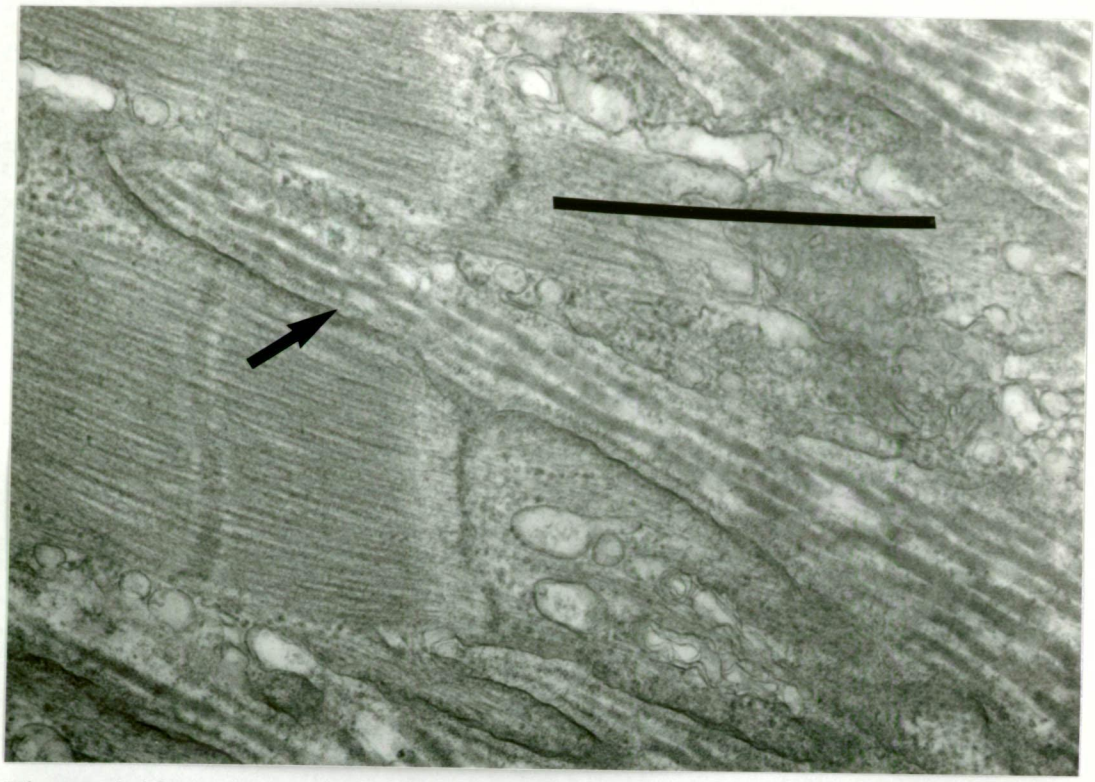

Figure 4.13 A short tubular invagination **arrow** at the base of a pre-rigor muscle fibre filled with collagen fibres. Bar 1μm.

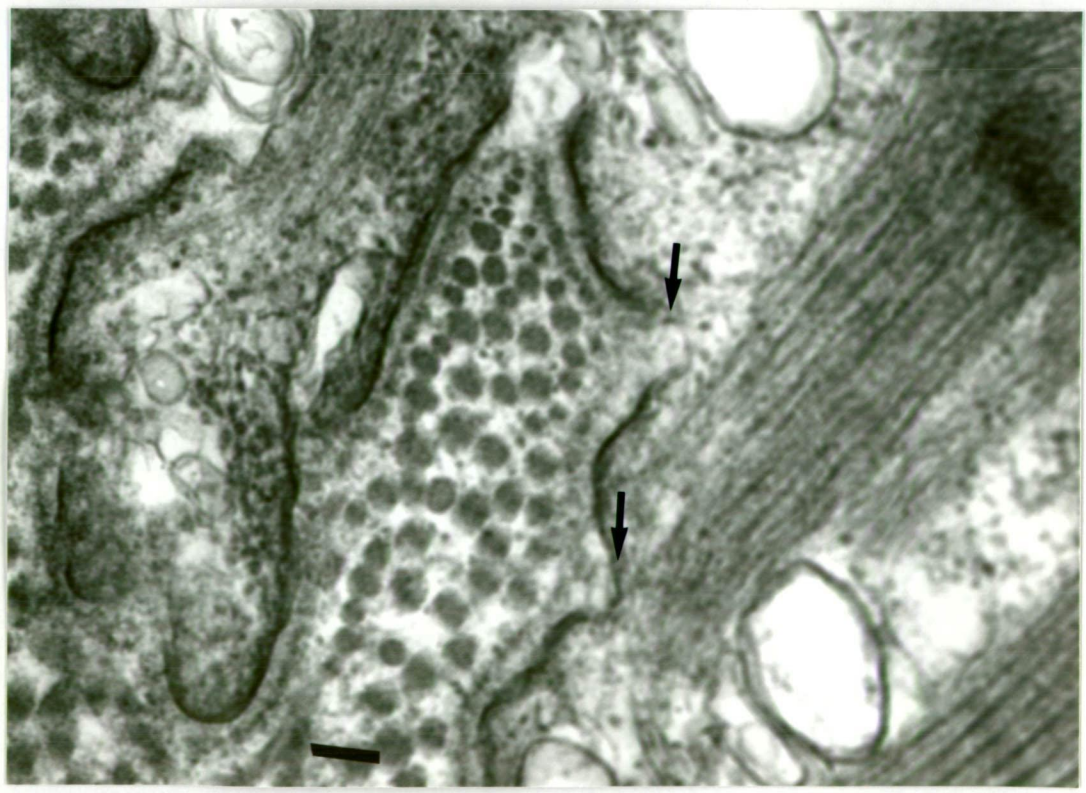

Figure 4.14. A groove near the end of a muscle fibre. The sarcolemma exhibits vesicle-like indentations (**arrow**). Connections between the basal lamina and the sarcolemma are evident. Bar 100 nm.

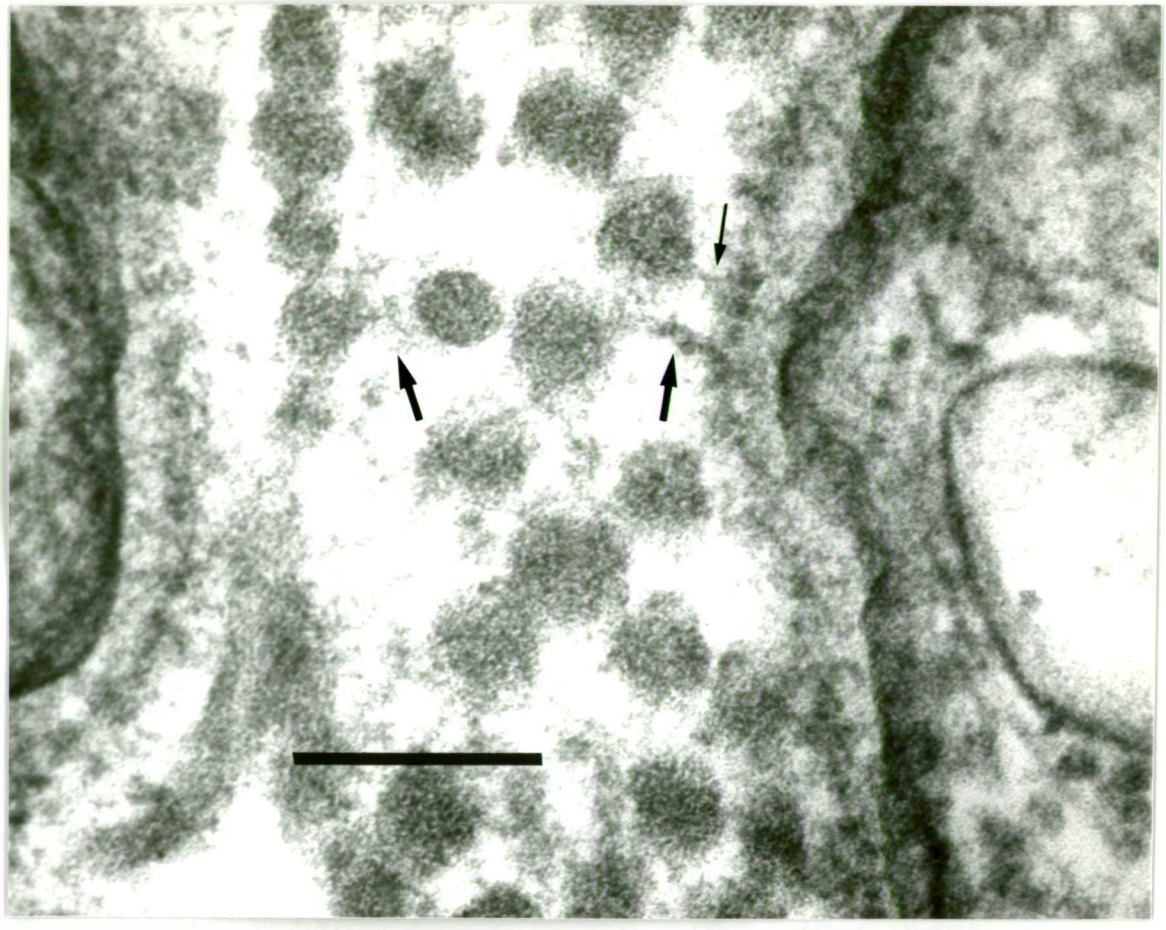

Figure 4.15 Connecting structures (**arrows**) occur between the collagen fibres and between collagen fibres and the basal lamina. Bar 100 nm.

Within the muscle cell fine fibres run from the last complete Z line to connect at the inner surface of the sarcolemma in an electron dense area (Figure 4.16, 4.17). The fibres were most likely to be actin since it is known that they are laid down as part of the cytoskeletal network (Schattenberg 1973). In general this region also contained large numbers of glycogen granules and mitochondria consistent with the fact that growth in fish muscle occurs at the fibre ends. Hence a higher concentration of metabolic apparatus is required in this region.

4.3 Fish in rigor

The overall structure of the muscle of fish in rigor was similar to that seen in pre-rigor fish (Figure 4.18). However, there was a notable loss of contrast between the background matrix, in which there was a distinct increase in the amount of staining, and the basal lamina and the collagen fibres within the invaginations.

4.4 Post-rigor fish

In muscle from post-rigor fish (8 days) the interface region showed considerable variation in structure, even within the same fibre. Some invaginations (Figure 4.19) retained both collagen fibres and a discernible

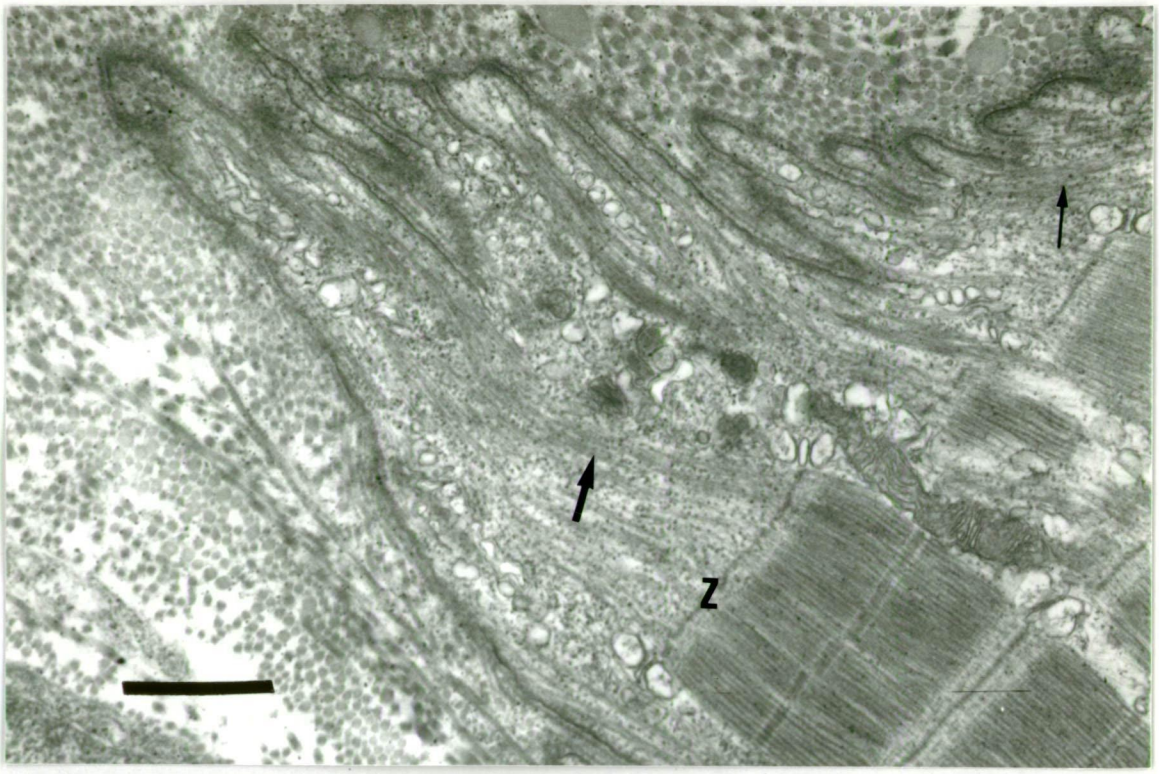

Figure 4.16 Section of a muscle fibre base, fine filaments (**arrow**) extend from the last intact Z line **Z** to attach to the inner surface of the sarcolemma in an electron dense layer. Bar 1 μm.

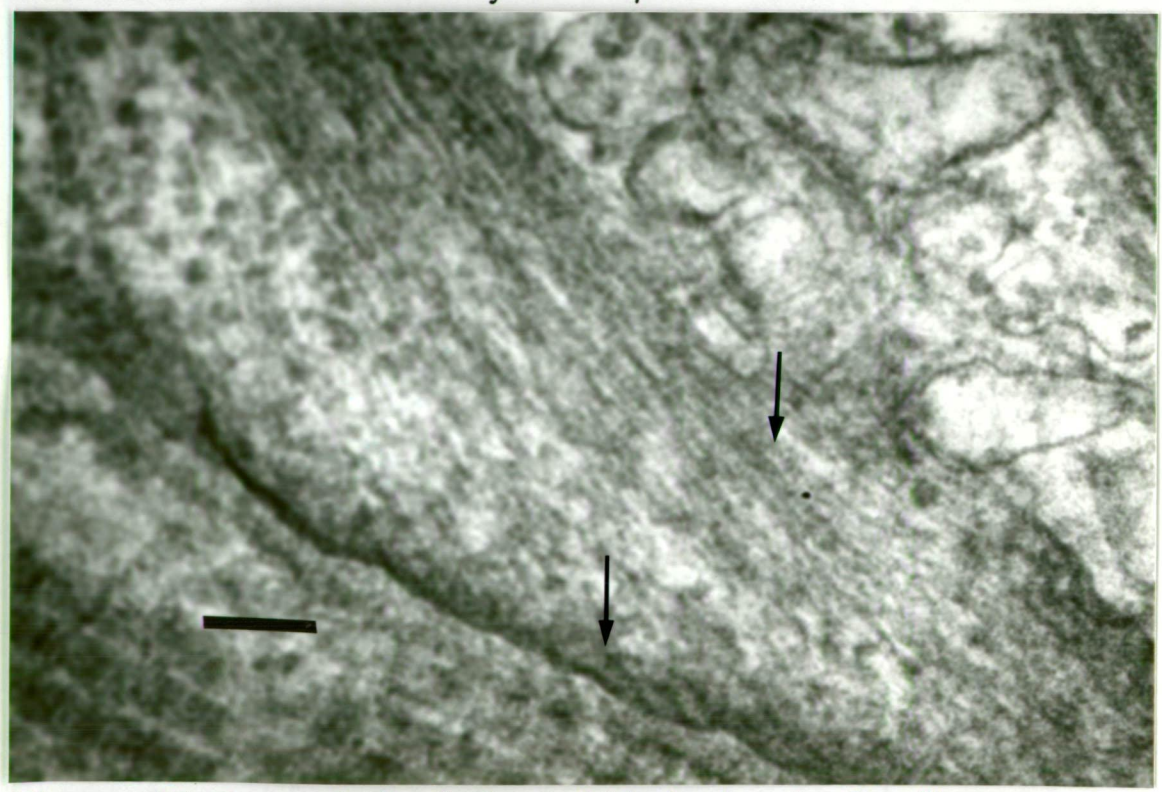

Figure 4.17 Greater detail of Figure 4.16 showing the fine filaments (arrow) in apparent bundles attaching to an amorphous electron dense area on the inner surface of the sarcolemma. Bar 100nm.

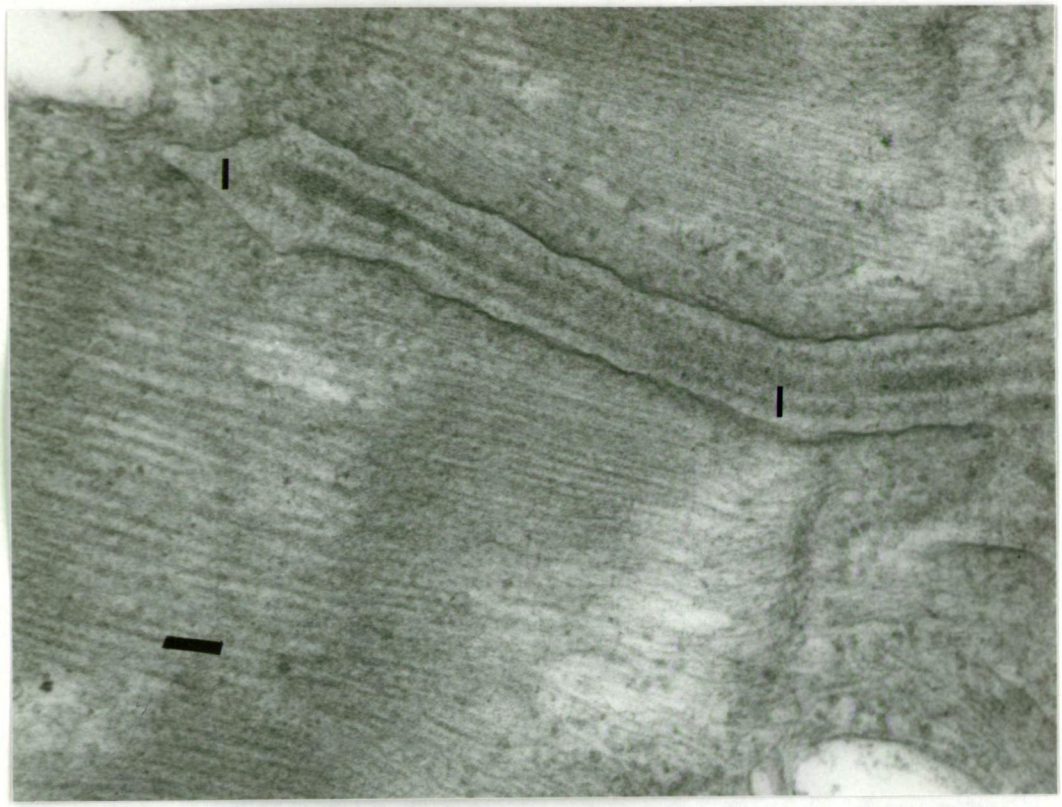

Figure 4.18. Fish in rigor. A tubular invagination **I** near a muscle fibre base showing the same structure as in pre-rigor fish, although definition is less clear due, to greater background staining. Bar 100 nm.

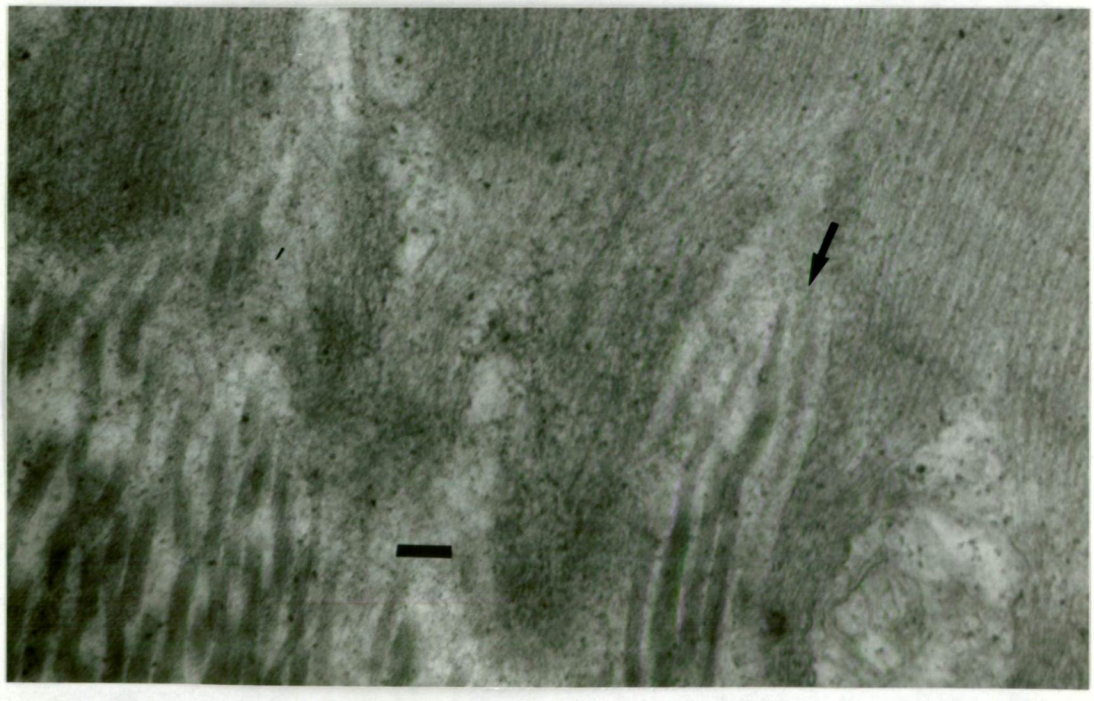

Figure 4.19. Invagination into the muscle fibre base in a post-rigor fish (stored 8 days). The basal lamina is discernible, but indistinct and blurred. Small vesicles (**arrow**) are visible in the terminal end of the invagination. There is considerable staining of the background matrix. Bar 100 nm.

basal lamina. However, this latter was usually blurred. In some invaginations loss of the basal lamina and of collagen fibres had occurred to a greater or lesser extent (Figure 4.20). This appeared to start at the terminal region of the invagination (Figure 4.21). In many regions the fibre base and collagen sheet had parted and empty invaginations were apparent (Figure 4.22). Regions of empty and almost intact invaginations could be present close on the same fibre base; breakdown of the basal lamina could be seen in between. Invaginations of detached fibre base contained neither the basal lamina nor collagen. However, the sarcolemma often remained intact at this stage (Figure 4.23).

In fish stored for 11 days the majority of fibres were more or less completely detached. Invaginations were not apparent at the muscle fibre base even when this was still in contact with the myocommatal sheets (Figure 4.24). In many instances massive vesicular deposits appeared between the fibre end and the collagenous sheets, and the sarcolemma had disappeared (Figure 4.25). However, despite this, the overall integrity of the muscle fibres, including the fine filaments to the fibre base region, remained intact (Figure 4.24, 4.25).

In all samples the overall background matrix of the collagen appeared much more densely stained than that of pre-rigor fish. Within the muscle cell the main structural features were still evident. Z lines were still intact although the banding pattern was much less distinct. The structure of the sarcoplasm

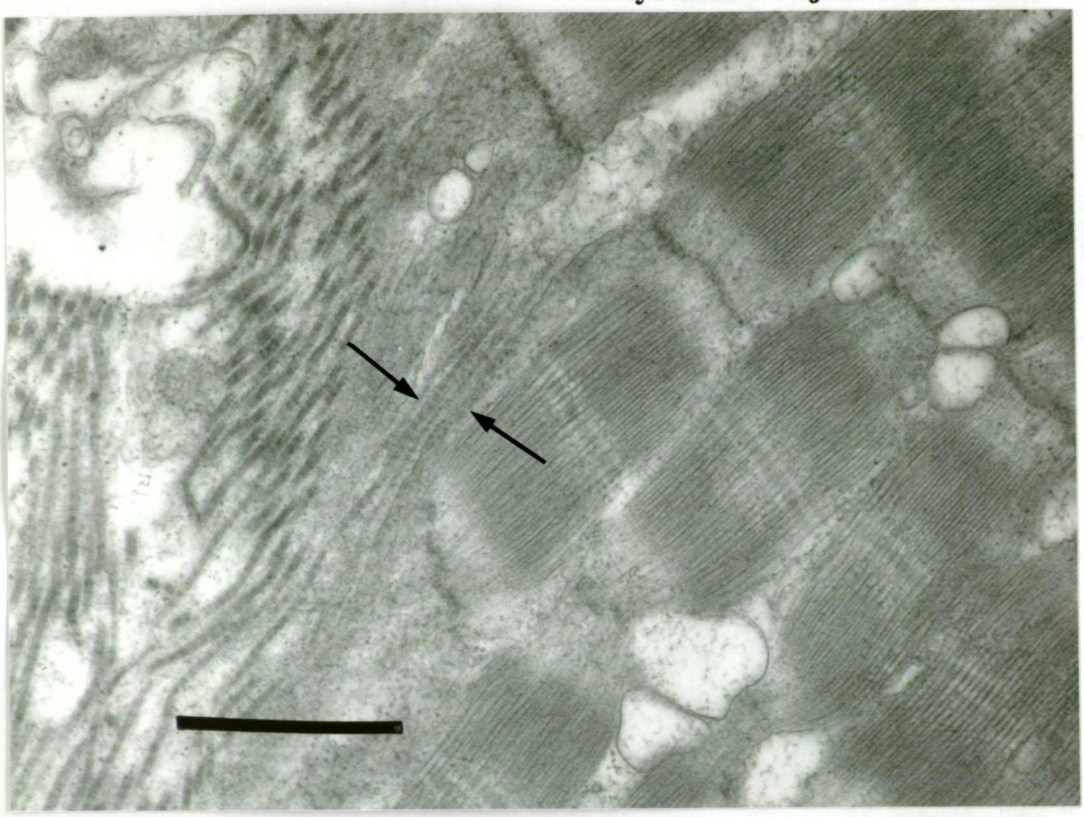

Figure 4.20. A tubular, but indistinct, invagination (**arrows**) in a post rigor fish stored 8 days. Bar 100 nm.

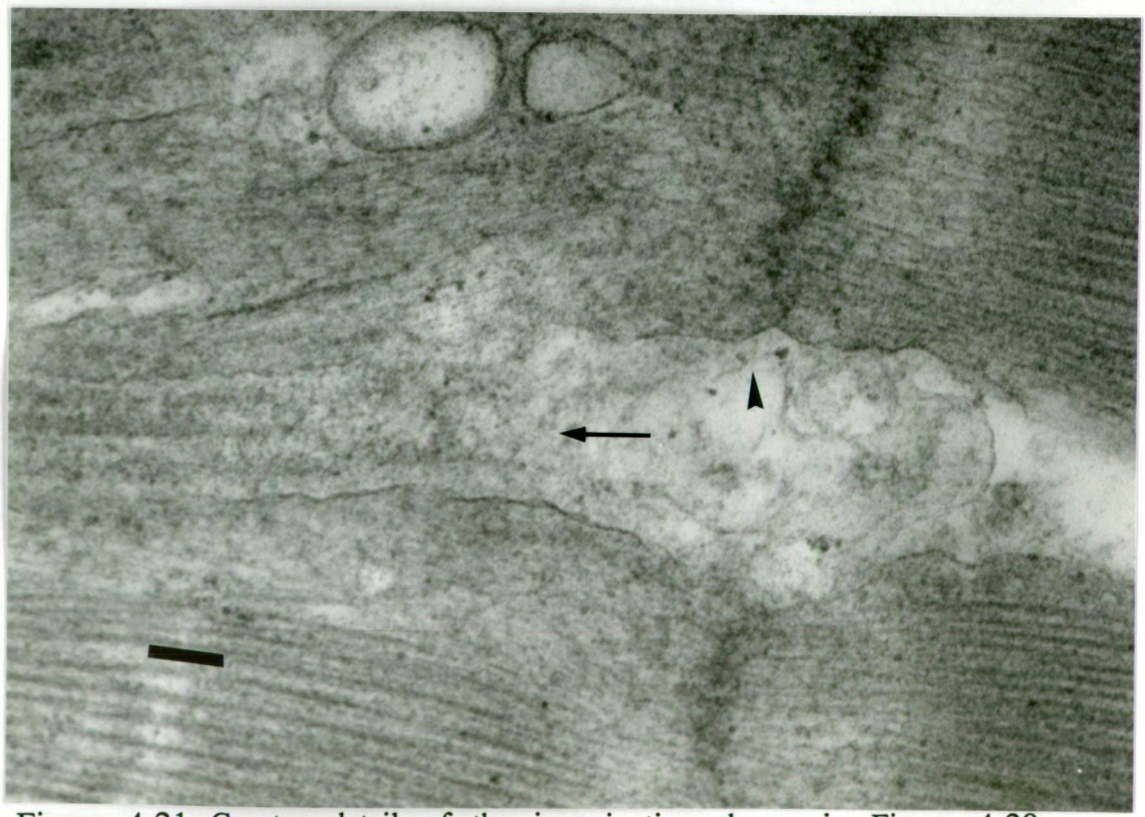

Figure 4.21 Greater detail of the invagination shown in Figure 4.20 indicating apparent loss of collagen and basal lamina from the terminal end of the invagination (**arrows**). Bar 100 nm.

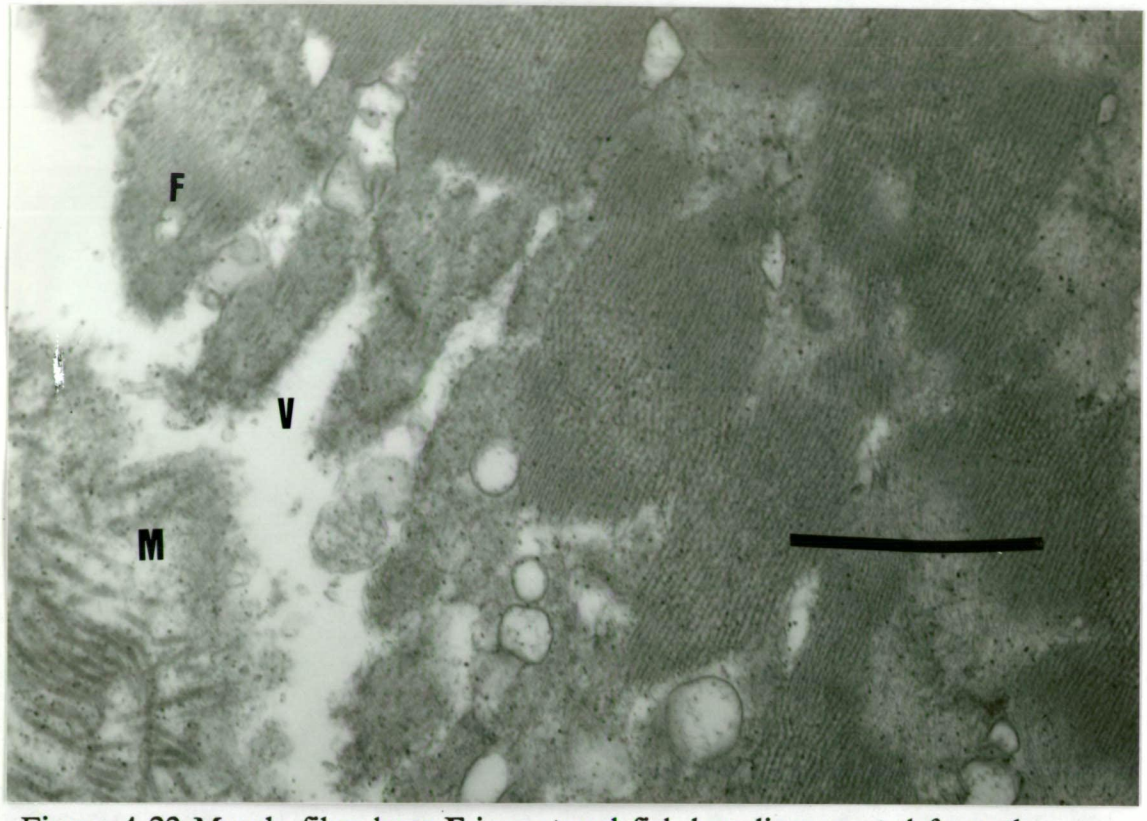

Figure 4.22 Muscle fibre base **F** in a stored fish has disconnected from the myocomma **M** and the invaginations **V** are empty of content. The region of the myocommata closest to the fibre base shows an area of amorphous stained material beneath which are collagen fibres. Bar 1 um.

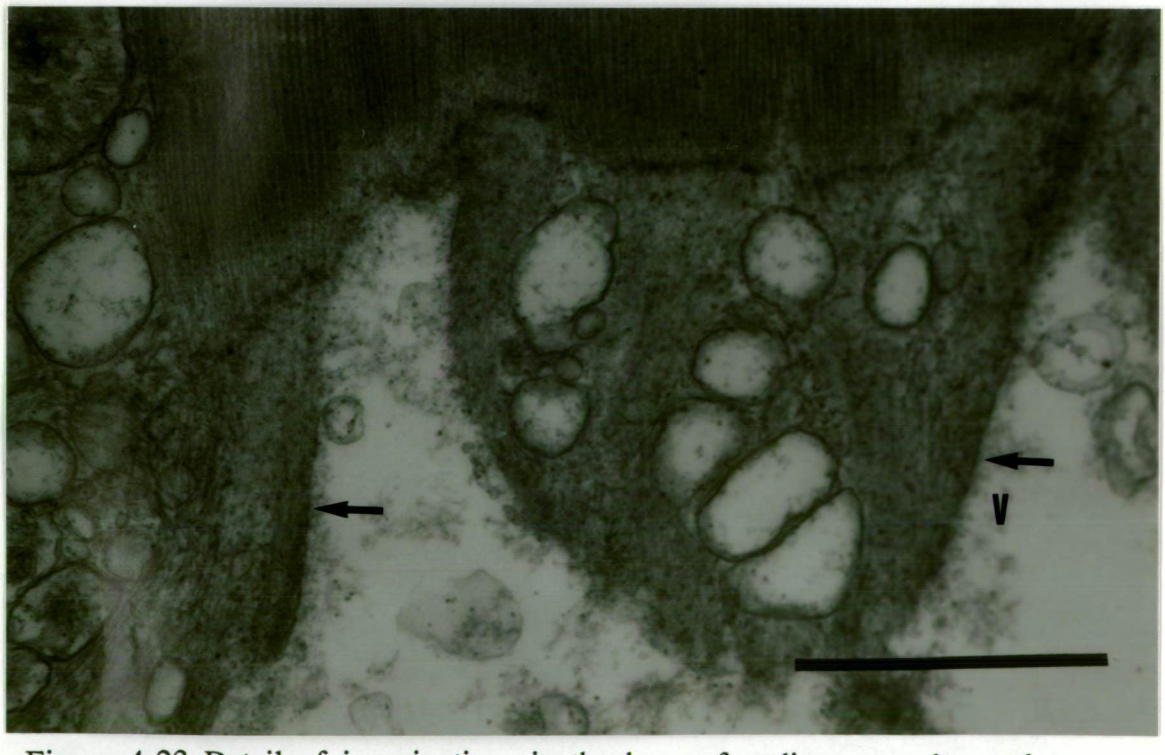

Figure 4.23 Detail of invaginations in the base of a disconnected muscle fibre. Apart from debris and a few vesicles the invagination **V** is empty but the sarcolemma (**arrow**) still lines the invagination wall. Bar 1 um.

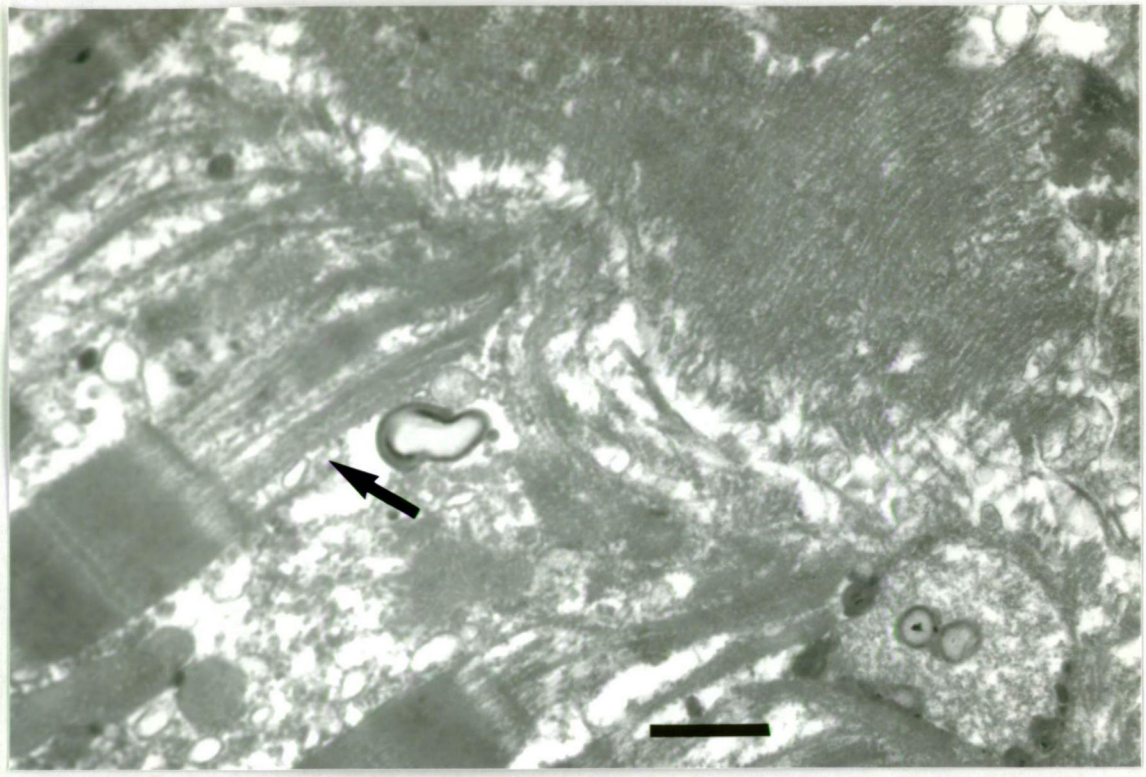

Figure 4.24 The muscle fibre base region in a fish stored for 11 days. Invaginations are not distinct although the fibrebase appears to remain close to the myocommatal sheet. The fine filaments from the Z line (**arrow**) still extend to the basal region of the fibre. Bar 1 um.

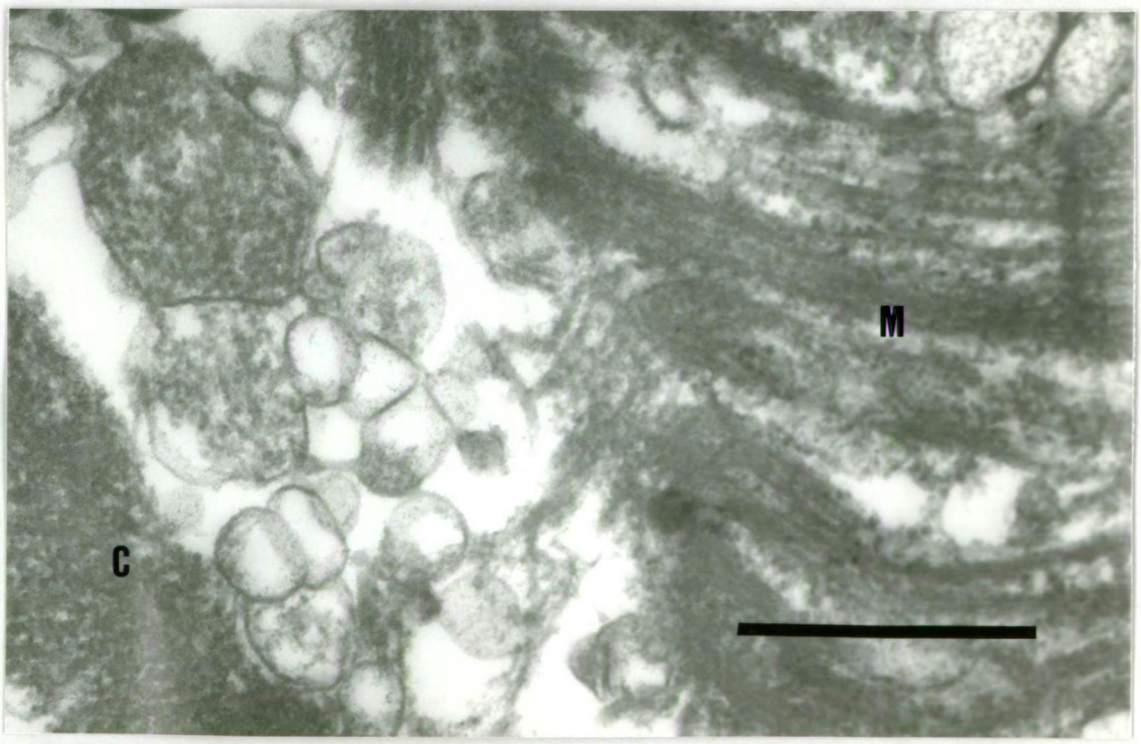

Figure 4.25 Similar muscle fibre base region to Figure 4.24. The region between the fibre base **M** and the myocommata **C** contains large number of vesicles and the sarcolemma is no longer identifiable. Bar 1 um.

had deteriorated and there was generally a lack of cohesion between muscle fibrils and between the filaments composing the fibres (Figure 4.26). Elements of the sarcoplasm were retained at the fibre margin but the collagenous surround in the invagination was much less distinct (Figure 4.27).

4.5 Discussion

4.5.1 The myotendinous junction

The structure of the myotendinous junction is similar to that observed in other vertebrates (Gelber *et al.* 1960; Schwarzacher 1960; Schippel and Reisig 1969; Hanak and Böck 1971; Korneliussen 1973; Schattenberg 1973; Nakao 1975,1976; Ajiri *et al.* 1978; Demell *et al.* 1979; Trotter *et al.* 1985). Descriptions of the structure of the interface area in the seahorse (Schwarzacher 1960), pipefish (Schippel and Reisig 1969),' hagfish (Korneliussen 1973), and lamprey (Nakao 1975) accord with the present observations that, in fish, the muscle fibres terminate at a myocomma with indentations and invaginations of the sarcolemma that are filled with fine collagen fibrils. These finger-like invaginations, often anastamose, and project longitudinally between adjacent myofibrils for depths ranging from 2 to 10 μm. Hanak and Böck (1971) reported invaginations 6-7 μm in

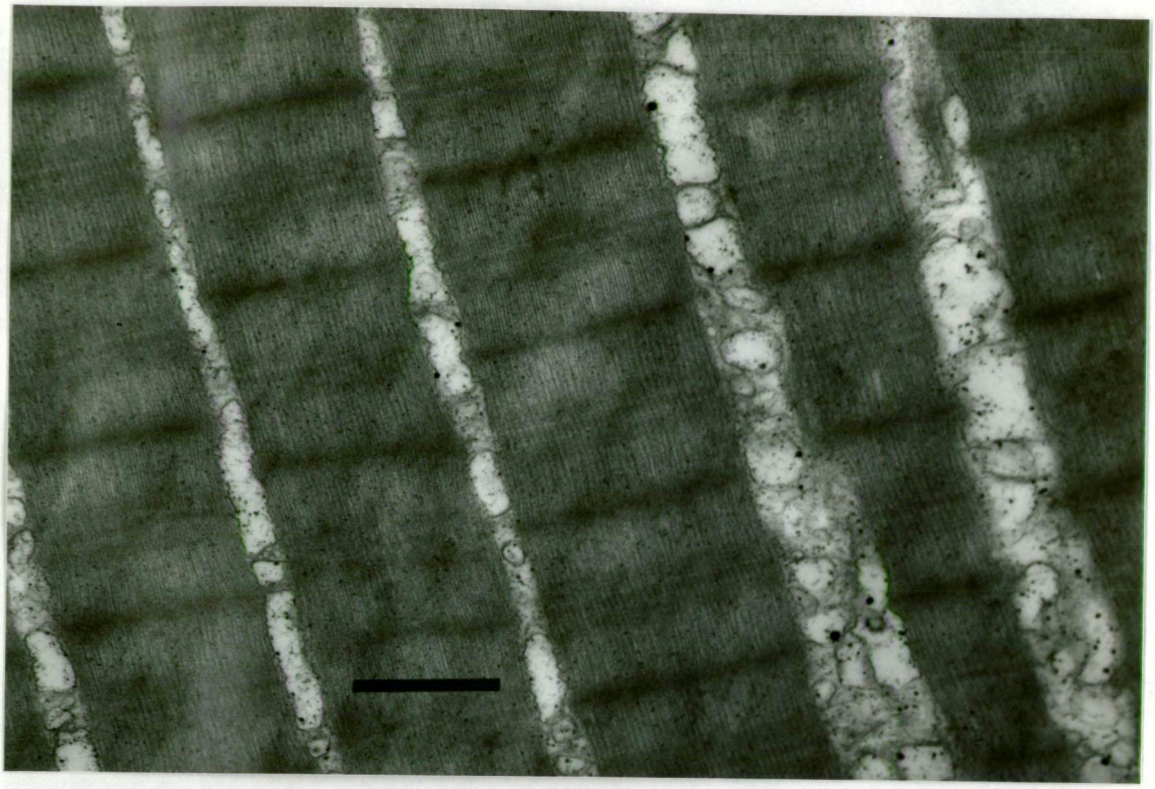

Figure 4.26 Within the muscle fibre of a fish stored 8 days there is evidence of breakdown of the sarcoplasmic layer between the myofibrils into unconnected vesicles and banding is much less distinct although the Z lines appear to be intact. Bar 1 um.

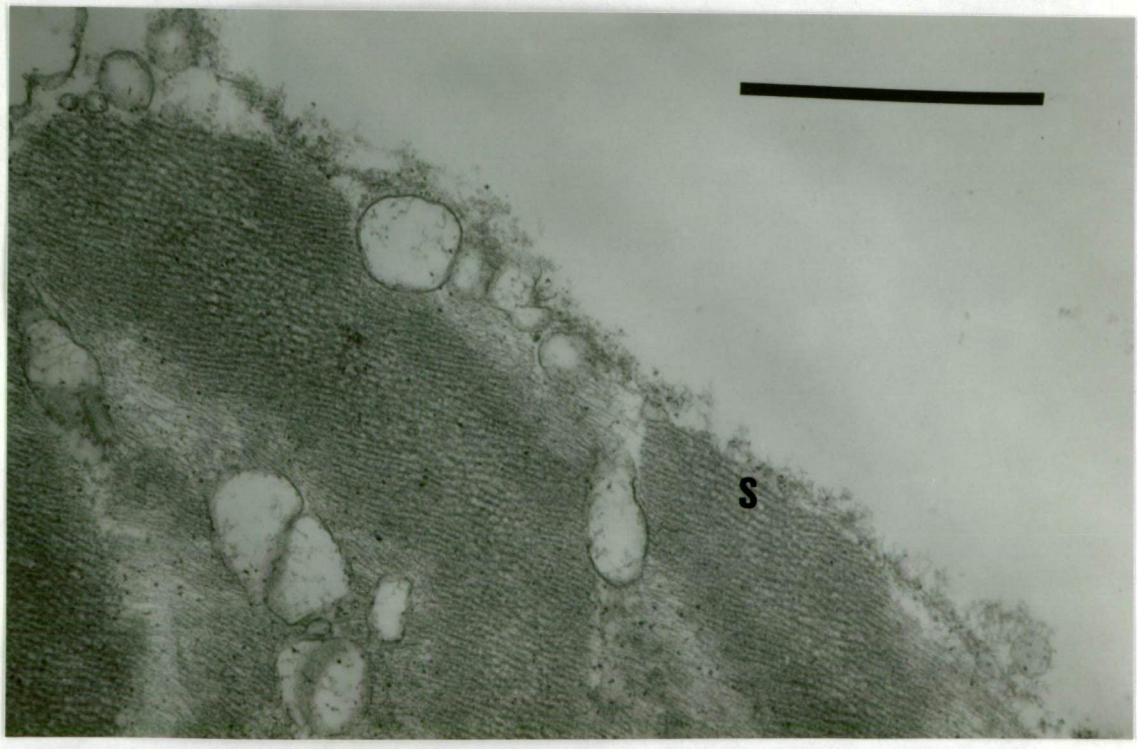

Figure 4.27. At the muscle fibre edge the sarcolemma **S** is retained but the endomysial collagen and the basal lamina have degenerated. Bar 1 um.

length in the guinea pig while for lamprey Nakao (1975) recorded lengths near 1-2 μm. Large numbers of groove-like invaginations are also present which are similar to features shown in the terminal ends of mouse plantaris muscle (Trotter *et al.* 1985) and rat sternothyroid muscle (Ishikawa *et al.* 1983). Serial sectioning indicated that the grooves and invaginations were real features and not artefacts of different orientations of the block face. In some sections the finger-like processes appear to arise from the grooves but much more work beyond the scope of this investigation would be required to fully delineate the geometry and to establish whether the different types of invaginations are found with greater frequency in different parts of the fibre base. This is a possibility since the myofibrils around the muscle fibre periphery are flat in cross section (Figure 3.5), whereas those in the interior are polygonal.

The structures external to the muscle fibre resemble those reported for other organisms in that there are fine connections between the sarcolemma, basal lamina and the ingressing collagen fibres (Hanak and Böck 1971; Nakao 1975, 1976; Ajiri *et al.* 1978 and Trotter *et al.* 1981). Recent work indicates that the connections are exceptionally intricate (Trotter *et al.* 1983 a,b; Keene, Sakai, Lunstrum, Morris and Burgeson 1987). In the basement membrane zone of human skin and cornea an extended network of connecting plaques comprised of type IV collagen and anchoring fibrils of collagen type VII have been reported. The type VII collagen fibrils arise

from the epithelial layer to loop over the larger diameter type I fibrils and attach to a connecting plaque comprised of type IV collagen (Keene, Sakai, Lunstrum, Morris and Burgeson 1987). Other type VII fibrils proceed between plaques or to the cell surface. In this way an entangling network in which the type I collagen fibres are embedded is built up to form a strong connecting structure. In some respects there is an analogy with "Velcro" the fibre and loop fastening material used on clothing, footwear and display materials. A structure of this nature is consistent with the 'beaded threads' reported by Ajiri *et al.* 1978 and the structures shown by Nakao (1975, 1976). However this proposed network of anchoring plaques and fibrils has not yet been demonstrated in muscle. In addition type VII collagen has not yet been reported in fish and neither has type IV. Type IV collagen is a major component in basement membranes and since fish muscle has well defined basement membranes (Figure 4.12, 4.13) it is certain to be present. Fibronectin (Hantai, Gautron and Labat-Robert 1983) has been shown to be present on the sarcolemma extending from the cell membrane to the intercellular collagen fibres beyond the basal lamina lucida externa in muscles of the rat. This was interpreted as demonstrating a role for fibronectin in providing contact between the cell membrane and the intercellular matrix, at least along the periphery of the muscle fibres. They also found laminin to be a constituent of the basal lamina delineating each muscle fibre, but the myotendinous junction was not studied.

In the myocomma and in the invaginations the collagen fibrils are embedded in, and connected by, the proteoglycans of the 'ground substance' (Figure 4.12, 4.14, 4.15). These proteoglycan structures are much more than just amorphous elements and have a critical role to play in organising and supporting the whole structure (Muir 1990, Scott 1991), even though the reported levels in fish (pacific rockfish *Sebastes sp.*) are quite low (Kim and Haard 1992).

Coupling of the sarcolemma of the invaginations and vesicles, presumably of the sarcoplasmic reticulum, was seen in some sections consistent with the reports of Nakao (1975,1976). The basal lamina was observed to be absent from the terminal ends of the finger-like processes similar to the situation for lamprey and frog muscle (Nakao 1975, 1976). Presumably this increases the opportunity for biochemical or ion flux at this point during formation of new sarcomeres (Schippel and Reisig 1969), or during the normal operations of the cell. In contrast, the basal lamina was always present at the bases of the groove-like processes.

Variability in the distance between the final Z line and the fibre base was observed and this was attributed, in part, to differences in the orientation of the sections and, in part, to actual differences in fibril growth. Fish muscle fibres continue to grow throughout the life of the animal (Schattenberg 1973) so that any section of myotendinous junction is likely to contain areas of formation of new myofibrils. The sarcolemmal region of attachment

probably has elements in common with the membranous Z line (Franzini-Armstrong and Porter 1964) and it is from this region that a new Z line arises as a new sarcomere forms. However, Yamaguchi *et al.* 1990 reported the presence of bands in the terminal segment of the muscle of guppy (*Lebistes reticularis*) which they concluded were not Z lines even though they were electron dense and had a similar appearance. Inside the muscle cell, fine filaments proceed from the terminal Z line to the interior surface of the sarcolemma. On the basis of their appearance and position it is assumed that these are actin filaments, consistent with previous reports (Schattenberg 1973; Maruyama and Shimada 1978; Trotter *et al.* 1983b; Yamaguchi *et al.* 1990). This could be confirmed by 'decoration', a procedure that involves treating the tissues with heavy meromyosin which reacts with actin filaments to delineate the arrowhead structures which are then readily observable with TEM (Ishikawa, Bischoff and Holtzer 1969; Maruyama and Shimada 1978). These fibres attach on the internal surface of the sarcolemma in an electron-dense layer. Other proteins of the cytoskeletal system are probably part of this layer. Alpha-actinin, an actin 'bundling' protein (Tidball 1987) has been found in myotendinous junctions and these actin filaments appear in bundles (Figure 4.16, 4.17). Vinculin (Geiger 1979; Geiger *et al.* 1980), talin (Tidball, O'Halloran and Burridge 1986), and zeugmatin (Maher *et al.* 1985) are also likely to be present as part of the attachment mechanism although none of these has ever been

reported in fish muscle. Fibronectin not only binds to cell surfaces but also interacts with actin filaments in the cell interior (Hynes 1986), mediated through a structure in the cell wall that spans the membrane between the fibronectin fibrils outside the cell and the internal actin filaments. These mediating elements are three cell-surface glycoproteins known as the 140K complex, because of their molecular weights of 140 Kd (Hynes 1986). Thus the electron-dense area on the internal surface of the sarcolemma contains a complex range of elements each contributing to the integrity of the structure. Furthermore this series of linkages must be maintained through the sarcolemma to the basal lamina through to the collagen fibres on to the myocommata and, in turn, to the skeletal structure in order that the forces of muscular contraction result in locomotion. Trotter *et al.* (1981, 1983b) have shown that even when the membranous nature of the sarcolemma has been disrupted by the use of lipid solvents, the connection and tension of the system is unaltered. This suggests that structures pass through, or connect through the sarcolemma and that firm linkages exist between the fibrils of the muscle fibre and the collagen of the myocommata. The invaginations of the myocommata increase the surface contact area by factors of between 20 to 30 for fast twitch muscles (Trotter *et al.* 1985) and 50 times for tonic cells (Tidball and Daniel 1986); the geometry of these junctions means that the forces are applied in shear rather than tensile mode (Tidball 1983). This effectively decreases the load

at any individual point but the degree of membrane folding found is not determined solely by the magnitude of the stress at the junction (Tidball and Daniel 1986).

The structure of the myotendinous junction in these samples appears to be consistent with the evidence reported for both mammals and for vertebrate fish species.

4.5.2 Post-rigor changes

The disconnection of the muscle fibre ends from the myocommata requires the breakage of at least one of the critical links in the structure of the myotendinous junction. The fact that in the blue grenadier this structure is no different in its essentials from that of other fish species (or indeed from other higher vertebrates), plus the absence of any major structural change during rigor, indicates that mechanical stress is not the cause of gaping. Mechanical stresses will exacerbate and make evident underlying weakening and deterioration that has already occurred in the structure. There is no evidence to suggest that the cause of change is bacterial in origin.

The observations here support the hypothesis that gaping and softening is due to the action of enzymes with proteolytic and collagenolytic activity. This activity seems to be localised within the invaginations and the

immediate vicinity of the basal lamina. In most of the samples examined neither the bulk of the collagen in the myocommata nor the filamentous connections between the Z lines and the sarcolemma showed major effects due to enzyme activity. The enzymic breakdown thus appears to commence with the basal lamina and extends to ingressing collagen from the terminal end of the invagination while the sarcolemma itself is only disrupted and lost in the later stages. The fine fibrils of collagen in the invaginations appear to be completely destroyed in some instances although collagen is normally regarded as being a fairly stable tissue somewhat resistant to enzymic attack. This either indicates the presence of a powerful collagenase active at chill temperatures, or a labile form of collagen or both these factors. In terms of disruption to the structure, however, dissolution of the basal lamina and disruption of the microfilamentous connections to the sarcolemma may be as important.

To the best of the authors knowledge there is no other detailed study of these post rigor changes in fish muscle or in mammalian muscle but it would seem that similar processes occur but mostly to a lesser extent in other species.

4.6 Conclusions

The myotendinous junction in the blue grenadier is similar to that reported for other marine species (Schwarzacher 1960; Schippel and Reisig 1969; Korneliussen 1973; and Nakao 1975). This study shows fine detail of the complex and intricate connections that occur between the fine collagen fibres that fill the grooves and invaginations in the terminal ends of the muscle fibre with the basal lamina and the sarcolemma. It also demonstrates there are at least two classes of collagen fibres of different diameters present. The finer fibres of approximately 18 nm diameter occur adjacent to the muscle fibre ends and in the endomysial layer.

The histological evidence and the observations with TEM demonstrate, for the first time, the progressive deterioration that occurs in this region during chilled storage. The deterioration occurs near the basal lamina and in the invaginations at the muscle fibre ends. The basal lamina degrades and the fine collagen fibres appear to be digested from the invaginations. Deterioration is progressive and muscle fibres become completely disconnected from their adjacent myocommata. The process is not bacterial, nor is it mechanical, and it is inferred that it must be enzymic. This implies the presence of enzyme(s) with collagenolytic activity in the chill range and /or very labile collagen.

Chapter 5

Characterisation of blue grenadier collagen

5.0 Introduction

The observations made by electron microscopy described in chapters 3 and 4 indicated that the fine collagen fibres in the myocommatal area and endomysium were being degraded during post mortem storage. Since much of the deteriorative process was observed to occur in the connective tissue, it was obvious that characterization of the blue grenadier collagen would be necessary in order to further understand the breakdown phenomenon. It was thus necessary to determine the properties of the collagen of blue grenadier and to relate these to the observed changes. Therefore collagen was selectively extracted from blue grenadier skin; its solubility and melting temperature were determined; it was purified and its amino acid and chain composition ascertained. Purified collagen was then used to produce antibodies in mice for use in immunofluorescence, immunoperoxidase and immunogold studies to characterize the collagen fibrils in the invaginations of the muscle fibre base.

5.1 Solubility and extraction

The fish skin collagen was readily soluble in dilute acetic acid and pepsin treatment was not required. The results for solubility of skin collagen from fish of different age groups are shown in Table 5.1.

Table 5.1 Solubility of Skin Collagen From Blue Grenadier of Various Ages

	Length (mm)	Weight (g)	Age (years)	Neutral salt soluble (%)	Acid soluble (%)	Insoluble (%)
Juvenile	310	106	1-1.2	8.2	87.8	4.0
Juvenile	315	106	1-1.2	10.0	87.7	3.3
Young adult	490	748	2	3.3	91.8	4.9
Young adult	610	798	3	3.0	91.5	5.5
Mature adult	960	3 750	>8	2.3	89.3	8.4
Mature adult	1 020	4 450	>8	2.1	91.0	6.9

Note: The collagen in each fraction is given as a percentage of the total collagen of all three fractions (Neutral salt soluble + Acid soluble + Insoluble). Collagen was taken from the same position in each fish, as determined from myotome number, analysed in duplicate and averaged. The age of fish was judged from fish length, using the data of Kenchington and Augustine (1988).

The method of ageing is not very precise but it was clear that the youngest samples had the greatest quantity of neutral-salt soluble collagen, while the amount of acid-insoluble collagen increased with the age of the fish. High solubility in acid occured even in fish at least eight years old. This high acid-solubility in mature fish, has been observed in other species (Sikorski, Scott and Buisson 1984) and it may reflect a slow rate of development of mature crosslink formations, which render collagen less acid-soluble. Instead, acid-labile aldimine crosslinks occur. This deficiency in mature crosslinks may, in part, result from a continuing lower availability of oxygen in fish skin in comparison to mammalian tissues (Rigby, Mitchell

and Robinson 1977, Bone and Marshall 1982) since the formation of crosslinks is an oxidative process (Rigby *et al.* 1977). One of the factors leading to the lack of post-mortem structural integrity in blue grenadier may result from the low amount of mature crosslinks in this species.

The acetic acid soluble collagen was readily purified by fractional salt precipitation under the same conditions as those used for purification of mammalian type I collagen (Trelstad *et al.* 1976; Trelstad 1982). There was no evidence of significant quantities of other collagens precipitating at these salt concentrations and it was inferred that the skin contained only type I collagen as a major constituent.

5.2 Chain composition

Separation of the precipitated fraction of purified type I collagen by chromatography on CM-cellulose (Figure 5.1) gave a pattern of alpha-chains that were distinct from those obtained for mammalian type I collagens (Piez, Eigner and Lewis 1963). This pattern indicated the presence of a third α-chain which was very difficult to separate from the α1-chain. The presence of this third alpha-chain was confirmed after repeated chromatography on CM-cellulose, followed by gel-permeation chromatography on Superose 6 to obtain components free of the β-chains.

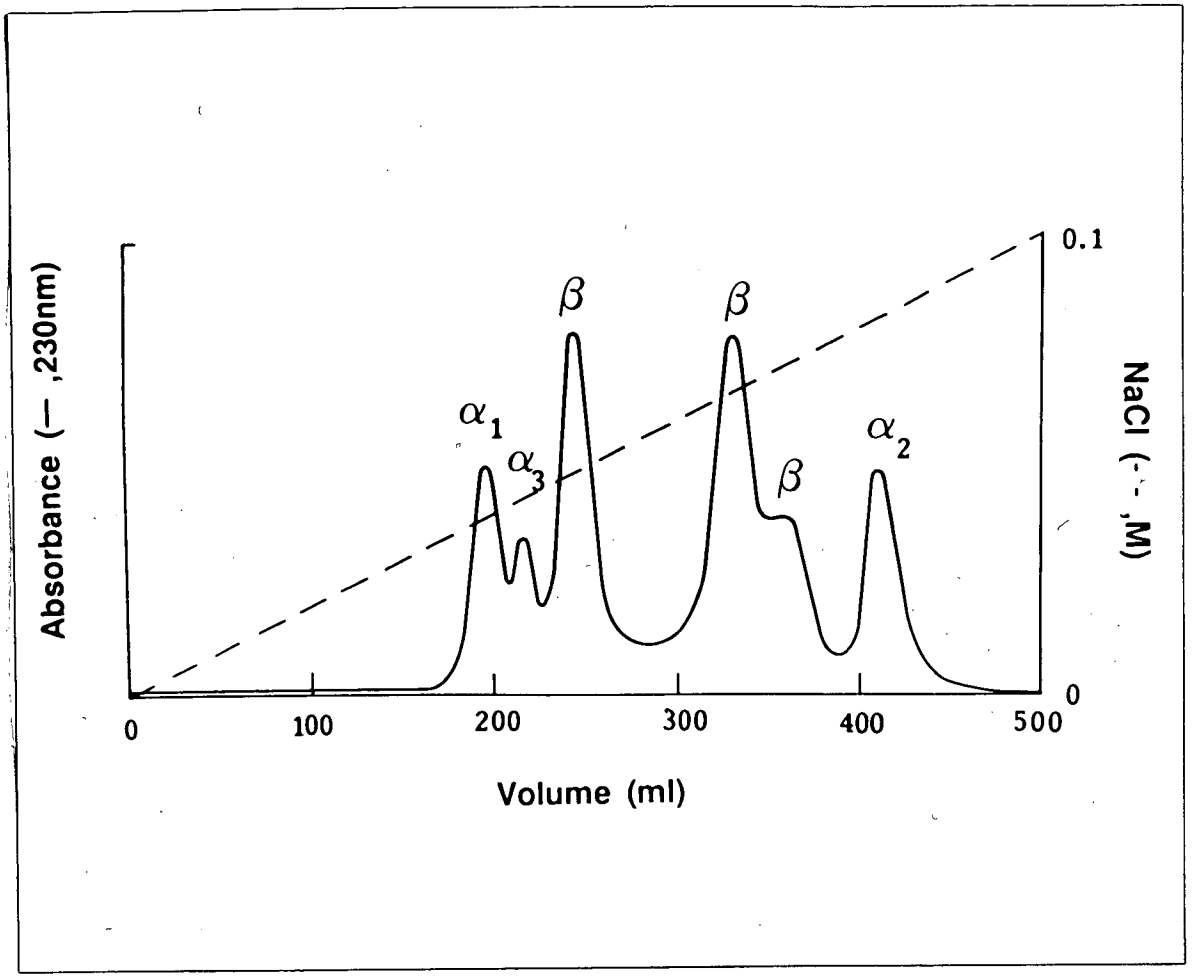

Figure 5.1 Separation of the alpha chains of blue grenadier skin collagen by chromatography on CM-cellulose (Whatman CM 52), 25 X 80 mm, in 60 mM sodium acetate, pH 4.8, with elution by a linear gradient of 0-100 mM NaCl, in a total elution volume of 500 ml. The peaks from which purified α-chains were prepared by further chromatography are indicated. The peaks labelled β each contained a range of β and higher polymer components.

5.3 Electrophoresis

The separated fractions from the CM-cellulose chromatography were subjected to SDS-PAGE and this technique demonstrated the three distinct α-chains in the collagen and in the purified fraction (Figure 5.2), even though the α1 and α3 chains were not well resolved. At least four distinct β components were also resolved by SDS-PAGE in fractions from the CM-cellulose column of the broad zones which contained these β-components and higher polymer forms. Repeated chromatography was not sufficiently effective to enable individual β components to be prepared with sufficient purity to allow for accurate analysis of their chain composition. This probably occurred because of the great apparent similarity between the α1 and α3-chains.

5.4 Chain analysis

The amino acid composition of purified α-chains indicated that each chain fraction had a distinct composition, with the α3- chain more closely resembling the α1-chain than the α2-chain (Table 5.2).

The fragments obtained by treatment with CNBr were separated by SDS-PAGE to display differences between all three chain fractions (Figure 5.3).

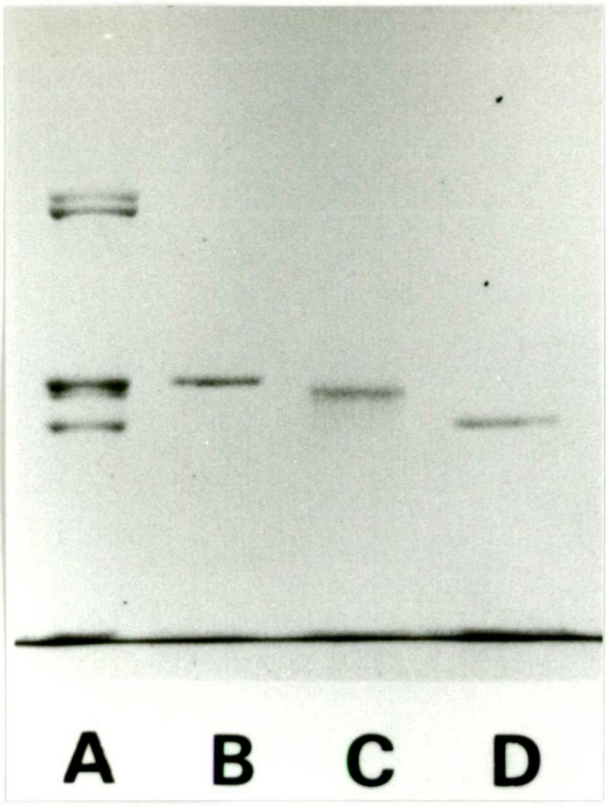

Figure 5.2 SDS-PAGE of purified blue grenadier skin collagen (lane A) and the purified α 1 (lane B), α 3 (lane C) and α 2 (lane D).

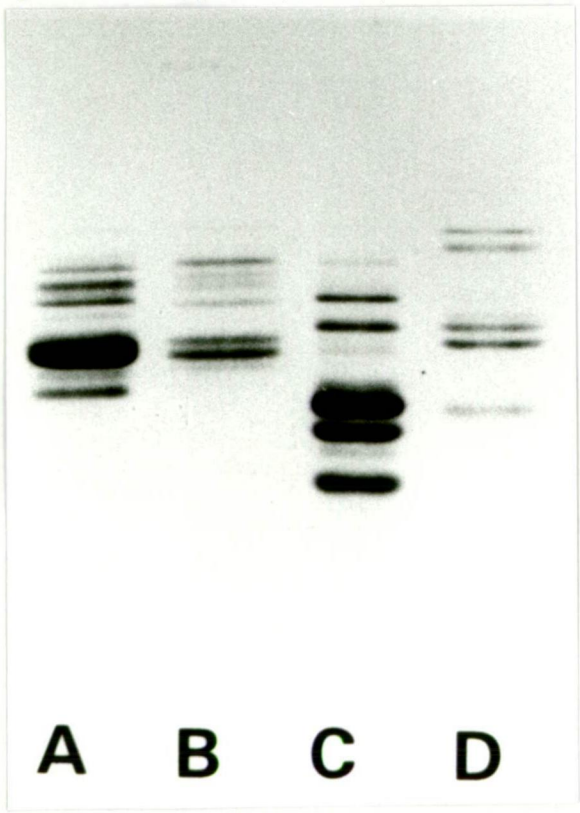

Figure 5.3 Separation by SDS-PAGE of CNBr fragments from purified blue grenadier (A) α 1 chain, (B) α 3 chain, (C) α 2 chain, and (D) bovine α 1(I) chain for comparison.

Table 5.2 Amino Acid Analysis of Purified α-Chains

	α1	α2	α3
HO-Pro	69	60	60
Asp	47	52	49
Thr	26	26	24
Ser	46	53	49
Glu	82	65	88
Pro	96	99	94
Gly	345	347	347
Ala	135	121	138
Cys	0	0	0
Val	17	23	17
Met	15	13	10
Ile	8	11	10
Leu	14	23	14
Tyr	1	5	1
Phe	15	9	15
HO-Lys	4	7	4
His	3	11	12
Lys	29	22	26
Arg	48	53	42
Trp	nd	nd	nd

Note: Values are given as residues/1000. Tryptophan was not determined (nd).

The α3-chain showed a similar separation to the α1-chain but a distinct faster moving band was present only in the α1-chain. Both the α1-chain and the α3-chain showed patterns that were quite distinct from the α2-chain. Fewer CNBr fractions were observed than might be expected from the amino acid composition. This may indicate that several small fragments were formed which were not resolved by electrophoresis.

Nevertheless it is clear that three distinct α-chains are present in the type I collagen of blue grenadier.

5.5 Collagen properties

The melting temperature of the collagen of intact skin was determined on a hydrothermal shrinkage apparatus (Bavinton 1969) to be 48 ^{0}C and that of the purified collagen was 22 ^{0}C. This is lower than observed for avian and mammalian collagens (36-41^{0}C) and is consistent with a lower imino acid content of fish collagen (Matthews 1975). There is a correlation between the approximate temperature of the environment of an animal and the melting point of its collagen. This relationship was originally noted for fish (Gustavson 1956) and was later extended to other animals (Matthews 1975; Rigby 1971). Blue grenadier are found mostly in waters off the continental shelf, 400-700 m deep, where temperatures range from 7 to 12^{0}C. However at night they may rise closer to the surface to feed at depths nearer 50-

100m where the temperature in summer may rise to near 20°C. The melting temperatures of the skin and the collagen of blue grenadier are higher than those for cold water fish but lower than those for warm water fish (Matthews 1975), consistent with this behaviour pattern.

5.6 Tissue distribution of collagen and reactivity of the antibody

The antibody prepared in mice from purified collagen was shown to be active against all three α-chains using standard ELISA. Most non-collagenous components are highly antigenic when compared to collagen (Steffen, Timpl and Wolff 1967; von der Mark 1981; Wick, Furthmayr and Timpl 1975), but electroblotting (Towbin, Staehelin and Gordon 1979) showed that the antibody was highly specific for the collagen antigen and did not exhibit non-specific binding.

Frozen sections of tissue from various body parts dissected from a fresh blue grenadier were reacted with the polyclonal antibody prepared from purified blue grenadier collagen and stained using immunofluorescence. The immunofluorescence technique demonstrated a broad distribution of collagen in skin, myocomma, blood vessels, intestine and swim bladder all of which are consistent with the structural role of type I collagen and its distribution (Figure 5.4). Control sections showed negligible fluorescence. It is well known that collagen is a major structural element in many tissues

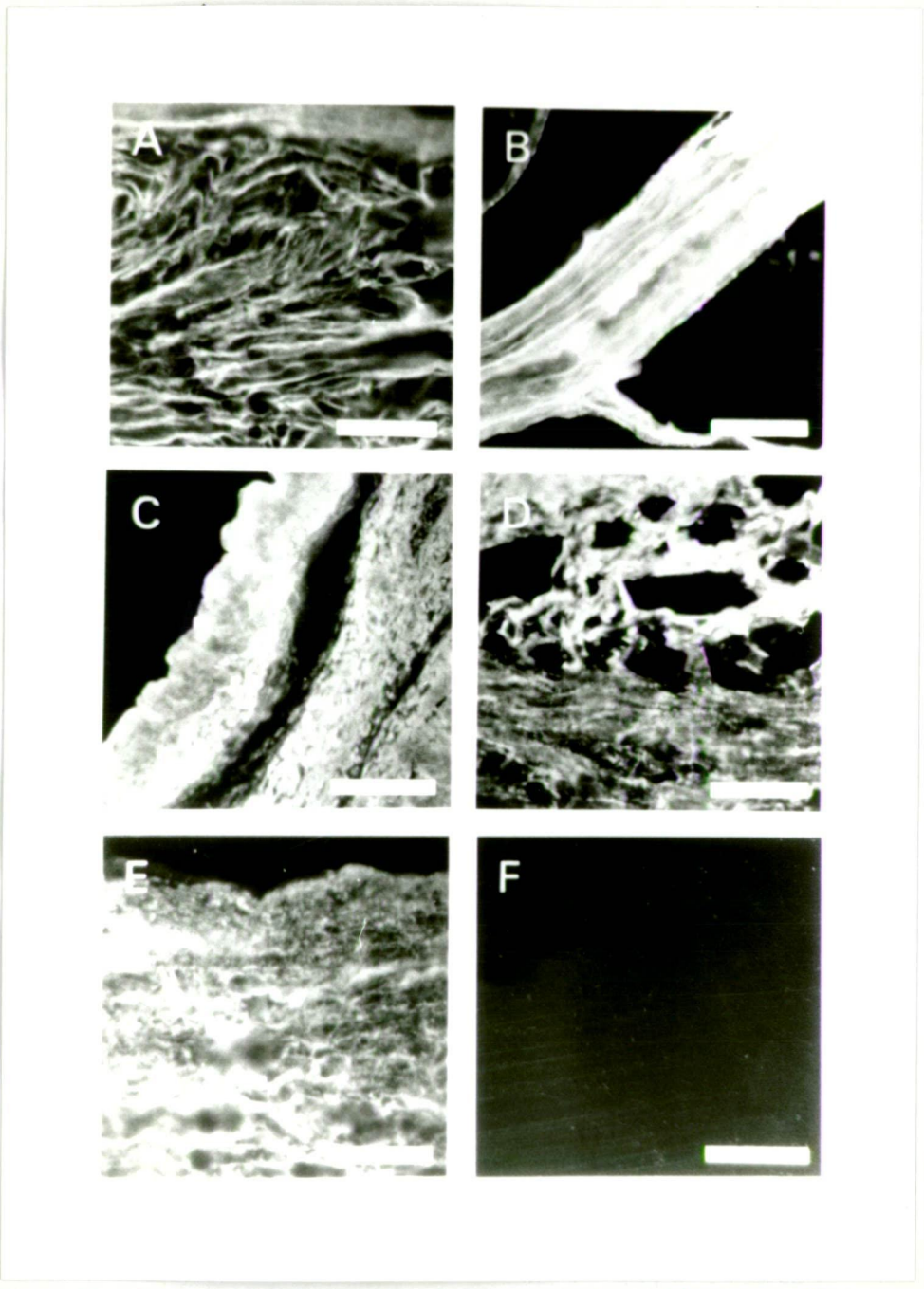

Figure 5.4 Immunohistology of various tissues from blue grenadier using a murine polyclonal antibody against the purified skin collagen and fluorescein isothiocyanate labelled, affinity purified, sheep anti-mouse antibody. Tissues examined: (A) skin, (B) myocomma, (C) blood vessel, (D) intestine and (E) swim bladder. Control sections, examined without the murine polyclonal antibody, indicated little non-specific staining, as shown for skin (F). Bars 50 μm.

particularly in muscle (Sikorski *et al.*. 1984). The polyclonal antibody prepared from purified type I collagen extracted from blue grenadier skin was reactive with a wide range of tissues indicating the presence of type I collagen in these organs identical to, or at least homologous with, the type I present in skin. Since no evidence of type III collagen had been found in the extraction and precipitation procedures and, at that time, type V collagen had not been reported in fish then it was inferred that type I collagen was the only major collagen species present.

5.7 Discussion

The aim of this section of the work was to establish some of the properties of the collagen of blue grenadier which would help understand the nature of the degradative changes which had been observed in the post-mortem fish. To do this collagen from the most accessible part of the blue grenadier, the skin, was extracted and characterised. In both mammalian and fish skin and muscle, type I collagen is by far the major species reported (Sikorski *et al.* 1984). The collagen extracted from the blue grenadier was consistent with type I in its properties of extractability and salt solubility. The ready solubility of the collagen in acid indicated the low degree of crosslinking even in mature specimens.

The melting temperature of the intact skin and of the purified collagen was

consistent with that of other fish collagens from species occupying the same thermal range in their habitat. These factors indicate there is nothing unusual about the collagen from this particular fish species which would explain its lability post-mortem.

The fact that the collagen possesssed a third alpha-chain was not unusual either. Kimura (1985) and Kimura *et al.* (1987) reported the presence of an α3-chain in the collagen of carp skin, whereas it was known to be absent in the swim bladder (Piez *et al.* 1963). This suggested that multiple forms of type I collagen may be present in fish and that they may have different tissue distributions. The α3-chain may be present as a homotrimer but the number of observed β components indicated that it forms heterotrimers with the α1 and α2-chains. Piez (1965) first reported the presence of an α3-chain in the skin of cod and Kimura (1985) and Kimura *et al.* (1987) have subsequently reported its occurrence and tissue distribution in a number of other species. These species are all members of the subdivision Teleostei of the bony fish (class Osteichthyes). Since chromatographic studies on fish skin collagens from taxonomically different groups (Piez 1965; Kimura *et al.* 1987) failed to show an α3-chain this additional chain may be restricted to within one class of fish and hence may have some taxonomic or phylogenetic value.

The polyclonal antibody prepared from the purified type I collagen of blue grenadier skin showed high affinity and reactivity to each of the three alpha

chains and was used to demonstrate the wide distribution of type I collagen in a variety of structures.

5.8 Conclusion

The collagen of the blue grenadier had a melting temperature and a shrinkage temperature consistent with the temperature range of its habitat and its imino acid composition. There was a slight but progressive increase in the proportion of insoluble collagen with increasing age of the fish.

The main type of collagen in the blue grenadier is an heterotrimer of type I collagen. It is widely distributed throughout the skin and major structural organisms as indicated by immunofluorescence using a specific polyclonal antibody. It was inferred from these results that the specificity of the antibody was sufficiently high to allow further immunocytochemical work to proceed. This further work had the aim of establishing whether the fine fibres found in the myotendinous junction of the blue grenadier were type I collagen.

Chapter 6

Collagen localisation using immunogold technique

6.0 Introduction

The structure of the myotendinous junction consists of invaginations in the muscle fibre base filled with fine collagen fibres from the myocomma. In post mortem storage these fine fibres are degraded and the junction loses its integrity. Type I collagen is the major structural collagen in fish but it is normally considered to be a relatively stable molecule. Therefore the aim of the experiments in this chapter was to ascertain whether these fine collagen fibres were composed of type I collagen. To do this it was necessary to select a technique that would allow for identification of individual fibres under the electron microscope.

The technique chosen was that of immunogold labelling (Beesley 1989). In this technique the tissue should undergo fixation under mild conditions to conserve as far as possible, the antigenic sites. Hydrophilic resin should be used as the embedding medium to allow penetration of the aqueous antibody and reagents (Newman and Jasani 1984). In principle, thin sections are treated with antibody which is then reacted with a bacterial protein (Protein A) linked to gold particles. Protein A is a cell wall component produced by strains of the bacterium *Staphylococcus aureus* that binds selectively to the Fc region of IgG immunoglobins from most mammalian species (Langone 1982). The protein A gold complex was developed by Romano and Romano (1977) for immunocytochemical

localisation of cell components. It is assumed that the negatively charged surface of the gold particles are electrostatically bound to the positive charged groups of the protein. The gold particles are electron dense and serve to outline the structures to which they are bound, via the protein A and the antibody attached to a specific antigen in the tissue under study. It should be noted, however, that the stoichimetry of these reactions is generally unknown and the whole procedure is dependent on pH.

The immunogold method has been used widely (Bendayan 1984) for locating collagen in dentine and other tissues (Stephens *et al.* 1982, Magloire *et al.* 1988). Most investigators have employed the antibody protein A gold sequence to fixed tissue but it has been used pre-fixation to maximise reactivity of the antibody-antigen complex (Magloire *et al.* 1988). The sequence followed in this chapter outlines the preliminary steps necessary to ensure that the primary antibody would react with the fixed tissue (in the preceding chapter it was shown that it was reactive to frozen unfixed tissue) and that the protein A gold reagent would react with the primary anticollagen antibody. It was also necessary to develop suitable fixation techniques for use with the primary antibody to conserve antigenic sites.

Preliminary experiments were also performed to examine the structure omitting the usual post-fixation step in osmium tetroxide (Roth, Bendayan and Carlemalm 1981; Beesley 1989) in order to conserve the antigenic

sites. In the investigations outlined in chapter 4 postfixation with osmium was an essential step to obtain definition of the finer details of structure. To enhance ultrastructural definition a number of other procedures were also tried such as etching the sections with $KMnO_4$ solution and using the counterstains uranyl acetate and lead citrate at elevated temperatures.

6.1 Experiments to establish dilutions for use of the primary antibody

Immunofluorescence was used to determine the appropriate dilution at which to employ the antibody. Sections of human skin, chicken skin, chicken tendon, fresh blue grenadier skin, fresh blue grenadier and trevally myocomma and from blue grenadier and trevally myocomma taken from fish stored chilled (0^0) for 5 days were cut on a freezing microtome. The samples were examined under the light microscope for fluorescent staining of the collagen fibres.

The results (Table 6.1) indicated that a dilution of 1 in 50 of the anticollagen antibody should be used for future work. It was also evident that this antibody against type I fish collagen could cross react with type I collagen from a wide range of other sources. Reaction was less strong on the fish skin than on the myocomma due to smearing of subcutaneous oil on the section surface. Background staining of the human skin occurred since the murine FITC reacts with human tissue.

Table 6.1 Reactivity of fish anti-collagen type I with various tissues

Source	Antibody dilution	Reactivity	Comments
Human skin	1:50	+ ve	some non specific staining
	1:80	+ ve	some non specific staining
	control	slight + ve	some non specific staining
Chicken skin	1:50	+ ve	fine fibres evident
	1:80	+ ve	
	control	- ve	no staining
Chicken tendon	1:50	+ ve	fine fibres evident
	1:80	+ ve	fine fibres evident
	control	- ve	fine fibres evident
Blue grenadier skin (fresh)	1:50	+ ve	strong staining
	1:80	+ ve	clear staining of fibres
	1:100	+ ve	
	control	- ve	no staining
Blue grenadier myocomma (fresh)	1:50	+ ve	strong
	1:80	+ ve	strong
	1:100	+ ve	very weak, apparent
	control	- ve	no staining
(stored 5 days)	1:50	+ ve	
	1:80	+ ve	weak
	control	- ve	no staining
Trevally myocomma (fresh)	1:50	+ ve	strong
	1:80	+ ve	clearly positive
	1:100	+ ve	very weak
	control	- ve	no staining
(stored 5 days)	1:50	+ ve	patchy
	1:80	+ ve	patchy
	control	- ve	no staining

6.2 Immunoperoxidase reaction on frozen and on fixed tissue

To assess whether the anticollagen antibody would react with tissue after fixation, sections of samples of blue grenadier myocomma and chicken tendon were cut into frozen sections and fixed in 10% formalin for 4h before processing and embedding in wax. Other sections were cut from blue grenadier after treatment in glutaraldehyde/formalin fixative (Glut/form fixative) then processing and embedding in wax. The sections were reacted with antibody and peroxidase reagent to stain the collagen according to the method described in chapter 2.7.3.

The fixation of the frozen tissue in 10% formalin resulted in faint staining of the collagen in the pericellular area (Figure 6.1) in contrast to the control sample (Figure 6.2). A similar result was achieved for chicken tendon (Figure 6.3 and 6.4). However, strong positive staining was observed when the fresh blue grenadier was treated in glut/form fixative (Figure 6.5) in comparison to the control (Figure 6.6). The intensity of the reaction did not decrease in samples taken from fish stored 12 days (Figure 6.7, control Figure 6.8) indicating no loss of reactivity even though degeneration may have occurred during the storage period. Thus the tissue remained antigenic to the primary antibody after the normal fixation process required for TEM.

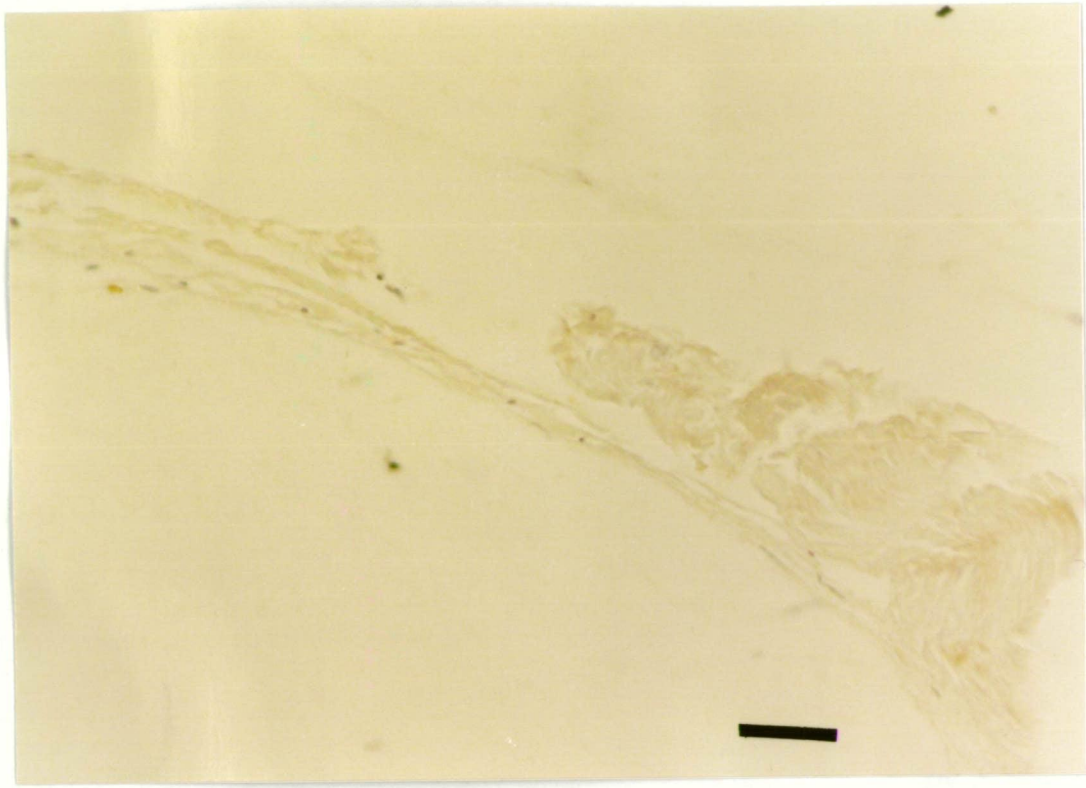

Figure 6.1 Blue grenadier tissue, frozen section treated in 10% formalin, reacted with antibody to collagen and stained by the immunoperoxidase method. A weak positive peroxidase reaction can be seen as brown staining in the pericellular area. Magnification X 100. Bar 10 µm

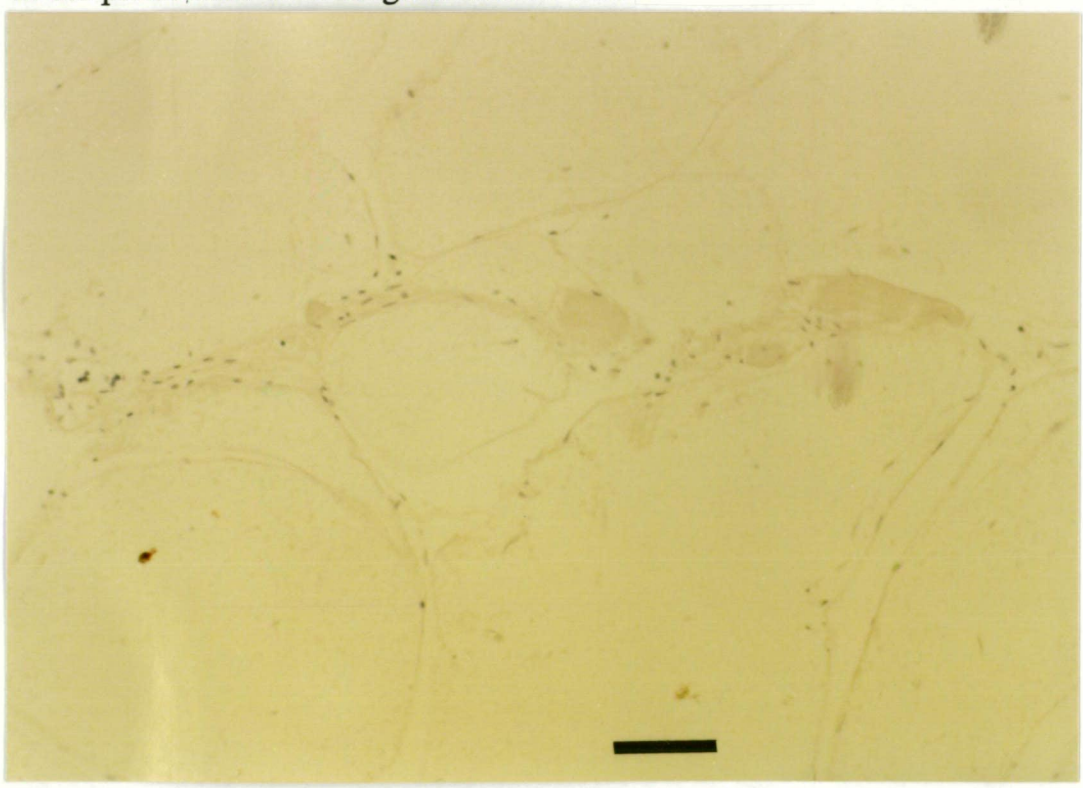

Figure 6.2 Control section to above not treated with antibody to collagen. Magnification X 100. Bar 10 µm

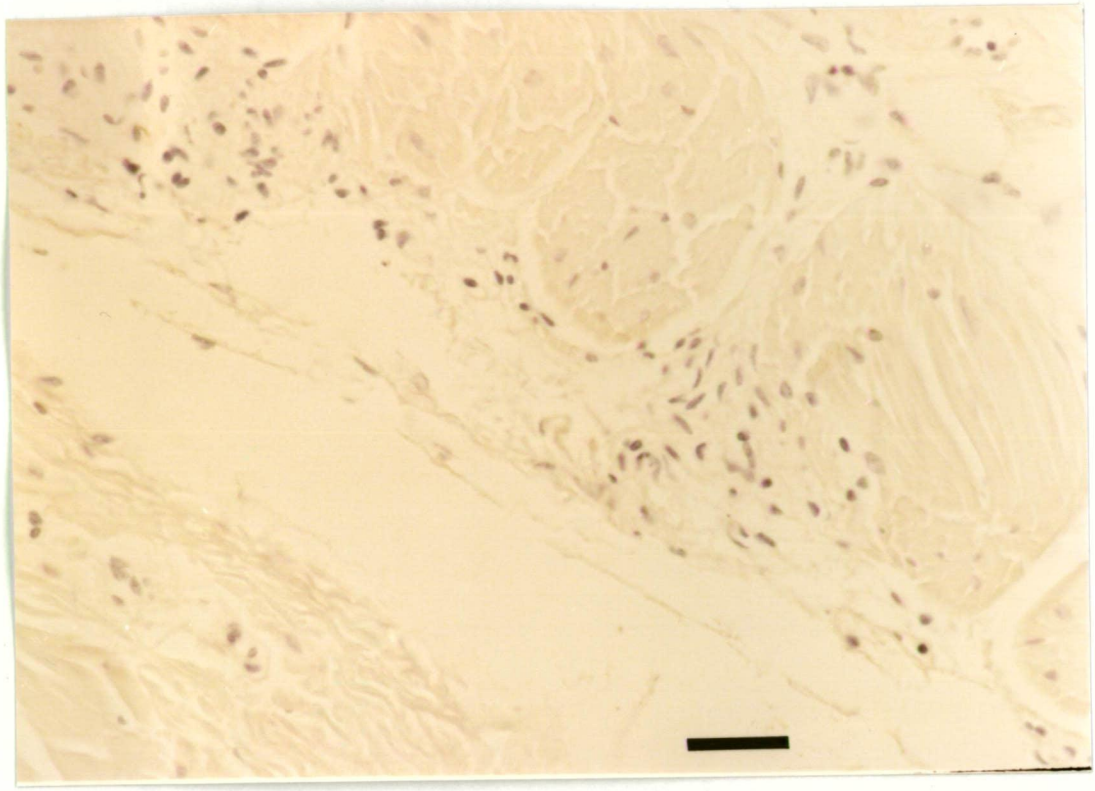

Figure 6.3 Chicken tendon, frozen section fixed with 10% formalin, reacted with antibody to collagen and stained by the immunoperoxidase method. Faint reaction with peroxidase. Magnification X 100. Bar 10 µm

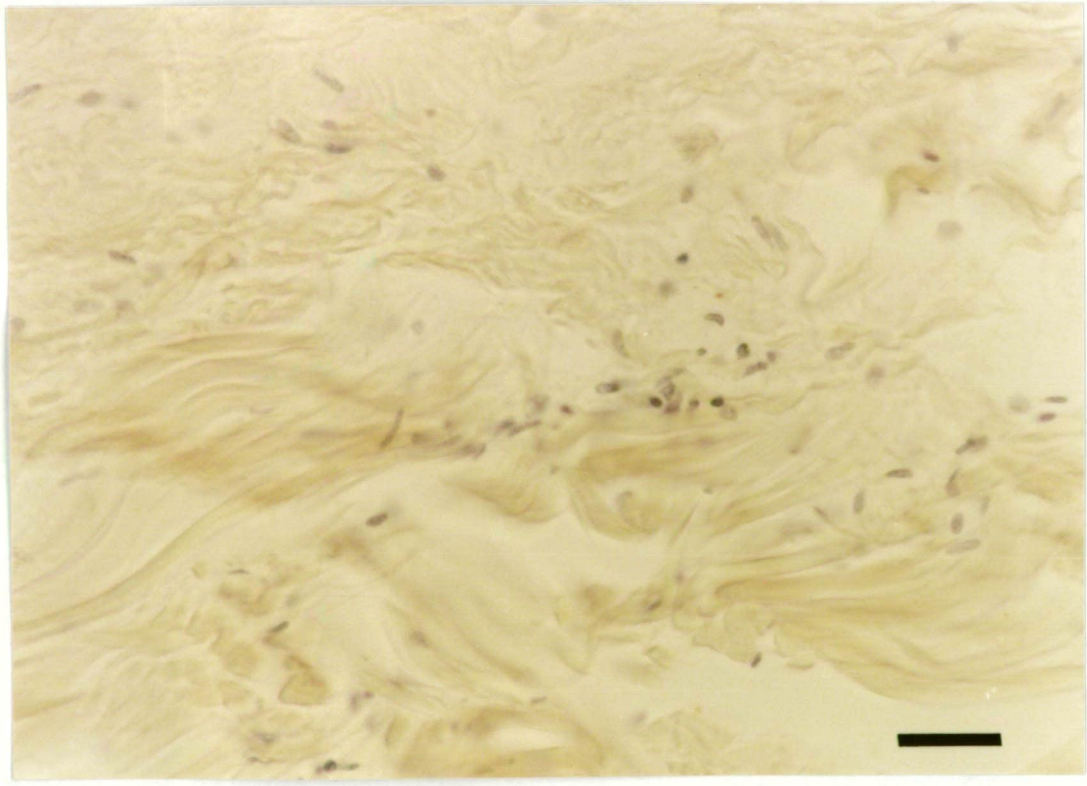

Figure 6.4 Control section for above not treated with antibody to collagen. Magnification X 100. Bar 10 µm

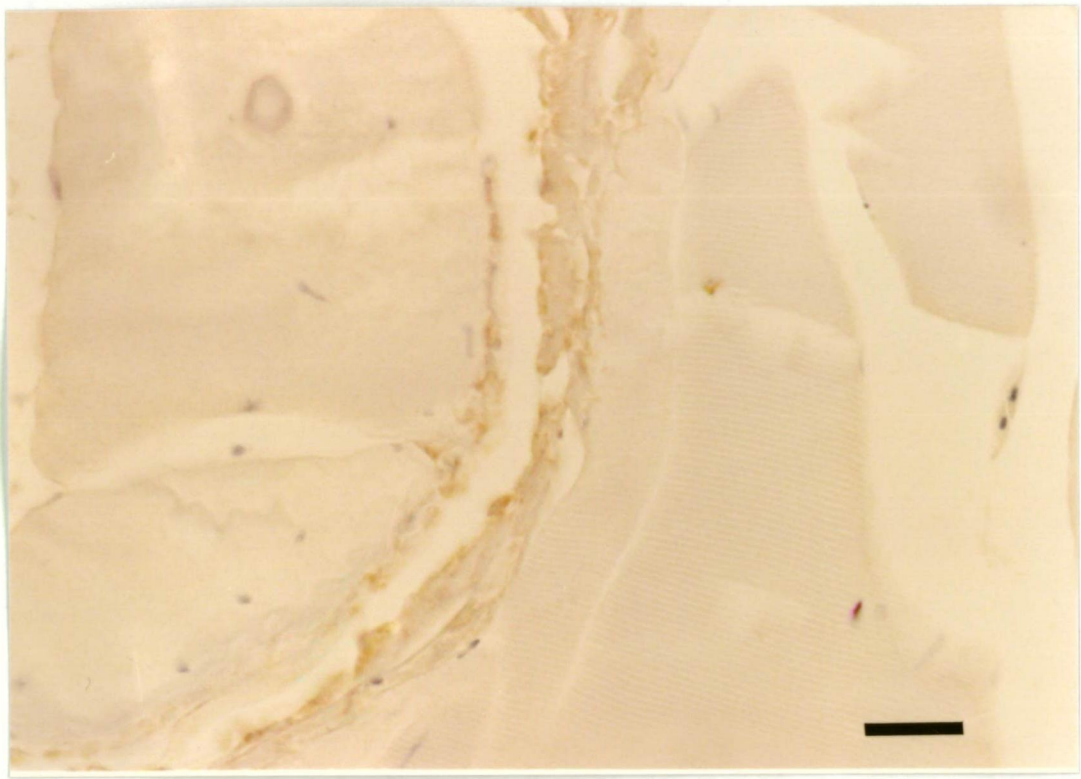

Figure 6.5 Fresh blue grenadier fixed in glut/form fixative reacted with antibody to collagen and stained by the immunoperoxidase method. Strong positive brown staining of the collagen in the myocomma. Magnification X 100. Bar 10 µm

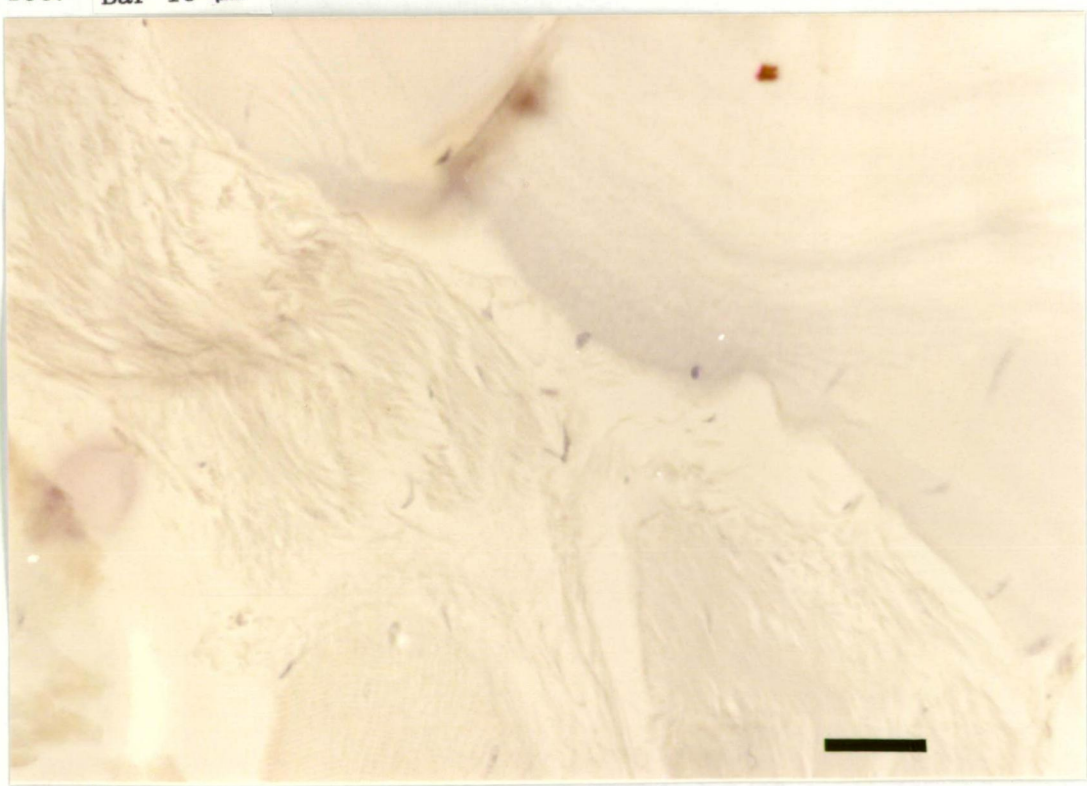

Figure 6.6 Control section for above, no brown immunoperoxidase staining. Magnification X 100. Bar 10 µm

Figure 6.7 Blue grenadier stored chilled for 12 days. Samples fixed in glut/form fixative, treated with antibody to collagen then stained by the immunoperoxidase method. There is strong positive staining of the myocommatal collagen. Magnification X 100. Bar 10 µm

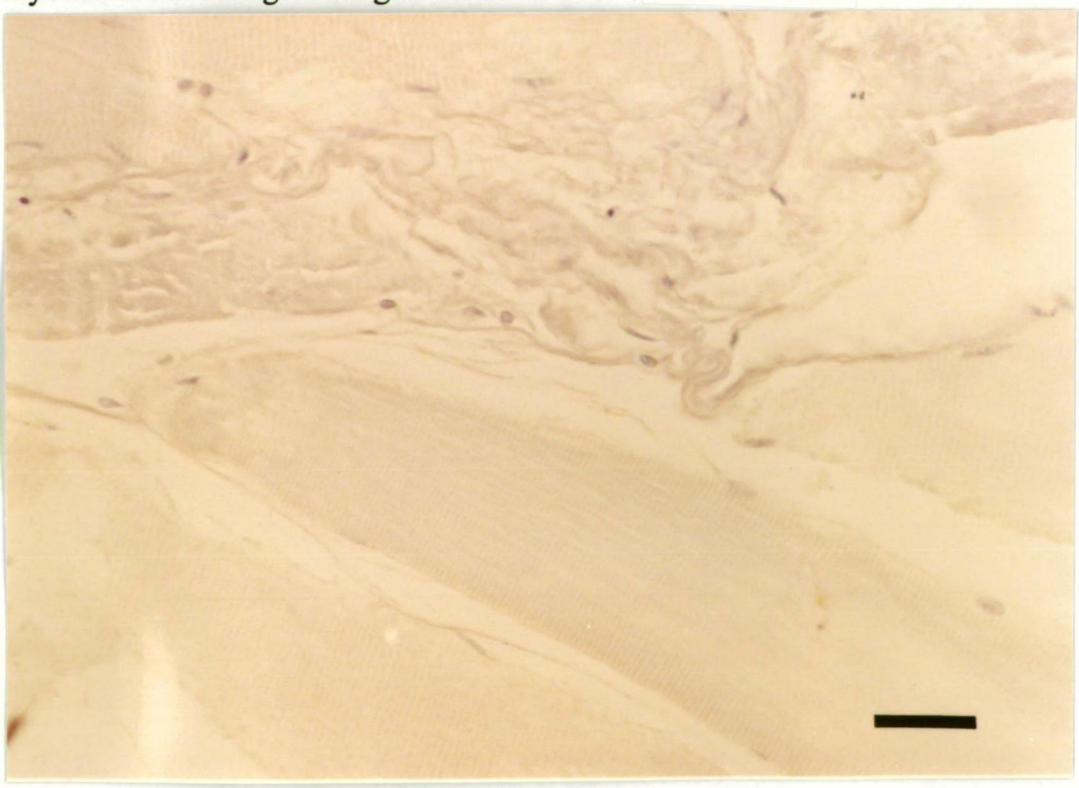

Figure 6.8 Control section for above not treated with antibody to collagen. No staining evident. Magnification X 100. Bar 10 µm

6.3 Reaction with Protein A gold

No blank reagent reactions occurred and no reaction of the primary antibody with protein A gold was noted unless a rabbit antimouse serum (RAM) was used as a conjugate. The RAM alone without the protein A gold did not yield silver enhancement (Chapter 2.7.4, Table 2.3). The conclusion from these preliminary trials was that the protein A gold reacted with the primary antibody provided RAM was used as a conjugate. This system was then employed in the TEM studies.

6.4 Osmium post staining

Since the post staining step with osmium may result in loss of immunogenicity (Roth *et al.* 1981), sections were prepared without this step in order to establish whether sufficient definition could be obtained. This was done initially using Epon-Araldite resin.

The resulting micrographs showed less clarity and definition, but many of the fine structural features were retained (Figure 6.9). It was decided to proceed with the next stage and embed samples in the hydrophilic LR White resin (Newman and Jasani 1984) .

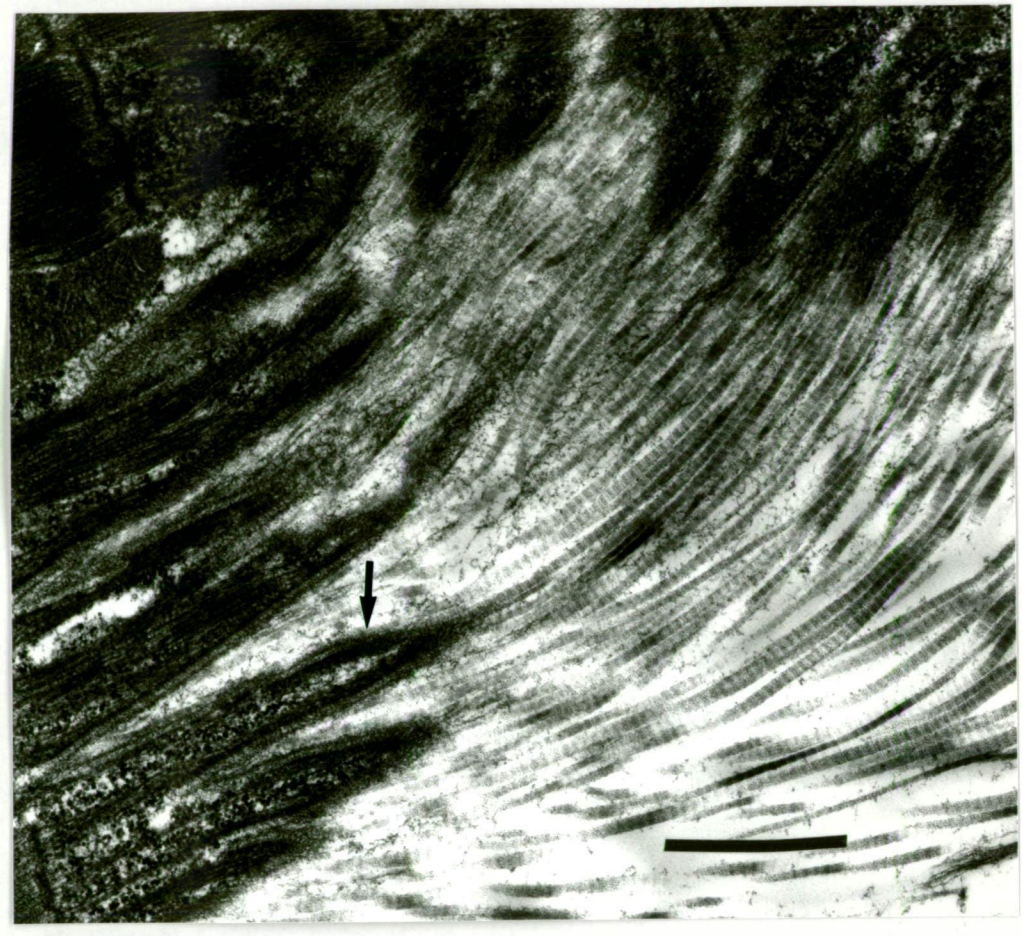

Figure 6.9 Sample 88002, blue grenadier muscle fixed immediately in glut/form fix but not post-fixed in osmium, embedded in Epon-Araldite. End of a muscle fibre showing finger-like tapering invaginations and fine collagen fibres. Sarcolemma and basal lamina (**arrow**) obvious. cf. Figure 4.11. Bar 1μm.

6.5 Sections in LR white resin

Considerable difficulties were encountered with the use of this resin. Firstly the blocks required refacing on the microtome, even after short intervals between cutting sections. Secondly the electrostatic properties of the resin often resulted in the wetting of the block face from the water in the receptacle used to float the cut sections. This was partly overcome by firing an antistatic gun at the block face to decrease static electricity. Thirdly, the resin proved to be very unstable in the uranyl acetate solution used as counterstain and it was necessary to use an aqueous rather than an alcoholic solution to minimise this effect. However, this gave poorer staining which was partly improved by either staining at 30°C or by etching the sections on the grid by immersion for 2 min in 0.9% potassium permanganate solution. Fourthly, the resin proved to be unstable in the electron beam. The use of Formvar and pioloform coated grids improved the stability but decreased the definition to an unnacceptable level. Coating with carbon improved the stability in the beam but it was still less than desirable. A fresh delivery of resin and resin borrowed from another laboratory were similarly unstable. Curing for longer periods, or at 60°C instead of 50°C, did not result in more stable sections.

For immunocytochemistry, curing the resin with an accelerator is not recommended in the manufacturers leaflet. However, it is possible that

curing at low temperatures with UV light may have yielded a more stable resin, but this option was not available.

6.6 Initial results on blue grenadier

Initially, excessive background staining was observed which was decreased by using lower dilutions of bovine serum albumen (BSA) as the non specific blocking agent. Several mouse serums were tried as a control in place of the murine primary anticollagen. All exhibited some nonspecific binding to the protein A gold and PBS treated sections were used as comparative controls.

There was binding of the gold particles to the collagen of the blue grenadier whether it had been immediately fixed (Figure 6.10) or fixed after 12 days in ice (Figure 6.11). The muscle structure was reasonably well defined and so too were the sarcolemma and basal lamina in the immediately fixed section (Figure 6.10). Some nonspecific attachment of gold particles to the muscle occurred, but counts of particles indicated that they were to be found in the myocommatal area at a much higher frequency. Attempts were made to quantitate the difference in deposition by counting the number of gold particles in adjacent fields of both muscle and myocomma on several grids for both test and control sections. It proved impossible to obtain a balanced set of fields for a statistical comparison of

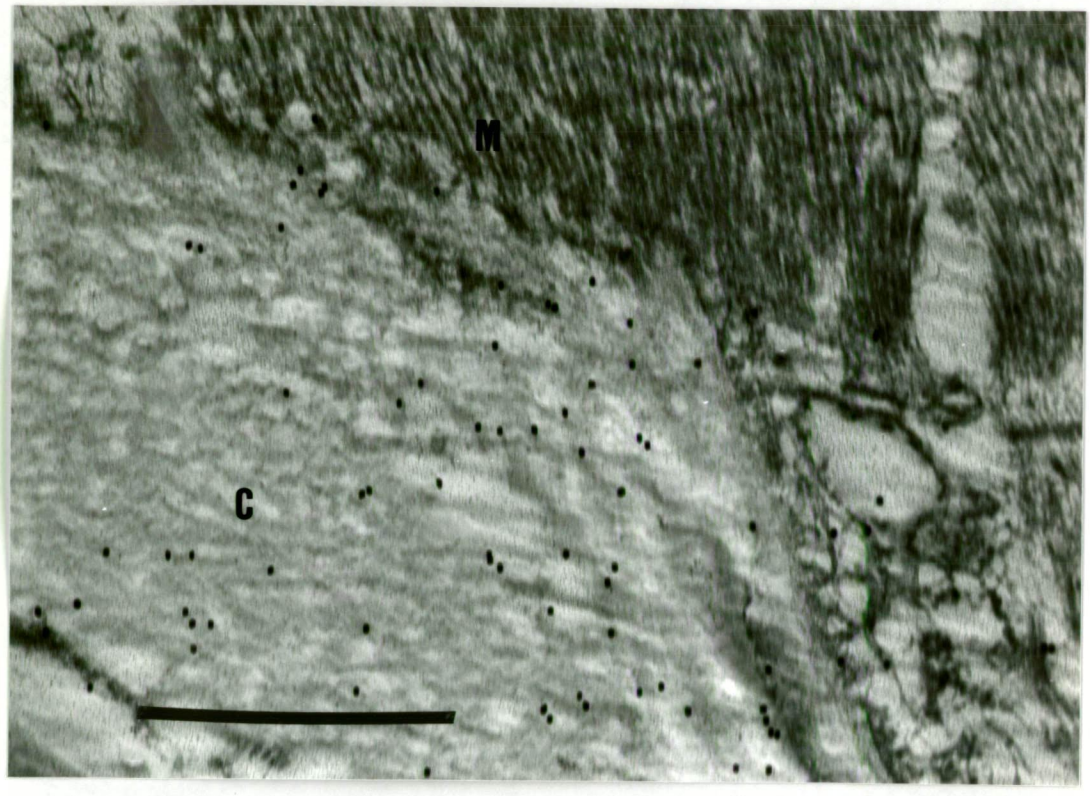

Figure 6.10 Sample 88025, blue grenadier as in Figure 7.9 but embedded in LR White resin and section processed through the immunogold procedure. Note gold particles on the collagen **C**, few in the muscle **M**, structural features less obvious. Bar 1μm.

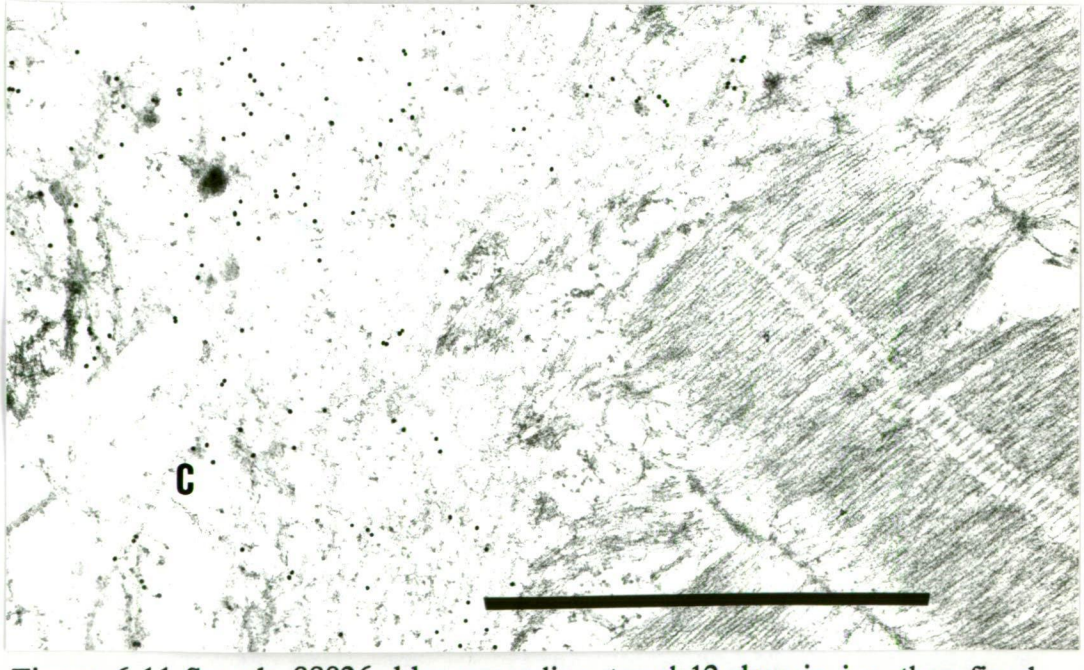

Figure 6.11 Sample 88026, blue grenadier stored 12 days in ice, then fixed in glut/form, no post fixation in osmium, embedded in LR White. Deposition of gold particles on the collagen **C**. No distinct sarcolemma and basal lamina but muscle appears reasonably intact. Bar 1μm.

counts because of uneven densities of occurrence of the collagen fibres in areas close to the muscle. However comparisons within individual test grids indicated that deposition on the collagen varied from as low as 1.5 up to 15 times greater on the muscle. In control grids the deposition was similar on the muscle, the collagen and on the exposed resin. However, the definition of the collagen fibres was too poor to ascertain whether particular fibres (type I) were being labelled in preference to other fibres. Therefore steps were taken to assess whether the definition could be improved. Pretreatment with 0.9% $KMnO_4$ in 0.1M phosphate buffer for two min at 37^0C to etch the section before counterstaining with aqueous uranyl acetate (37^0C) improved the density of staining but still did not provide sufficient definition.

6.7 Conclusions from the above studies

Omission of the post fixation step in osmium tetroxide resulted in poorer ultrastructural definition. This poorer definition was more pronounced in samples embedded in LR white resin. The LR white resin proved difficult to section and sections cut from it were not stable in alcoholic uranyl acetate counterstain, nor were they very stable in the electron beam. Etching the sections in 0.9% $KMno_4$ before counterstaining in warm aqueous uranyl acetate improved the ultrastructural definition. Coating the

sections with carbon improved the stability in the beam.

A dilution of 1 : 50 for the anticollagen antibody was demonstrated as a suitable working level. The anticollagen antibody was shown to react with tissue fixed for TEM studies. The protein A gold reagent was shown to react with the anticollagen antibody provided RAM was present to act as a conjugate. Collagen fibres in sections of stored fixed blue grenadier muscle were labelled with gold particles after reaction firstly with the primary anticollagen antibody, then with RAM followed by the protein A gold reagent. The ultrastructural definition was insufficiently clear to enable differentiation between individually labelled fibres. It was concluded that the procedure should be tried on fresh material that had only been lightly fixed to retain maximum antigenicity and hence to optimise labelling.

6.8 Ultrastructural localisation of collagen

6.8.1 Flathead

Attempts to obtain pre-rigor blue grenadier were thwarted by bad weather and the lack of a commercial fishery at the time. Live flathead (*Neoplatycephalus richardsoni*) were landed and muscle sections were fixed within one minute of the fish being killed by a blow to the skull.

Similar results were obtained to those with the blue grenadier in that the

resin was unstable in the beam. The muscle structure was not as well defined and the striations on the collagen fibres were indistinct (Figure 6.12). There was demonstrably higher deposition of gold particles on the myocomma than in the muscle, but it was not possible to determine the degree of labelling of individual collagen fibres.

6.8.2 Jack mackerel

A live jack mackerel (*Trachurus declivis* Jenyns) was killed by a blow on the head and muscle samples were placed immediately in fixative B for 1¾ hours after which they were washed in buffer, dehydrated in an alcohol series then embedded in a new batch of LR White resin.

Before proceeding to treat sections by the antibody protein A gold procedure some were examined after counterstaining with uranium and lead to check section orientation and for appropriate definition. The muscle fibre boundaries were well delineated but the collagen fibres were not well defined and even the detail in the muscle was inadequate. It was concluded that the fixation was possibly too short in duration. Nevertheless other sections were put through the immunogold procedure. They too exhibited poor definition (not shown) and although there was greater deposition of gold on the collagen than on the muscle it was not possible to sufficiently differentiate between structures.

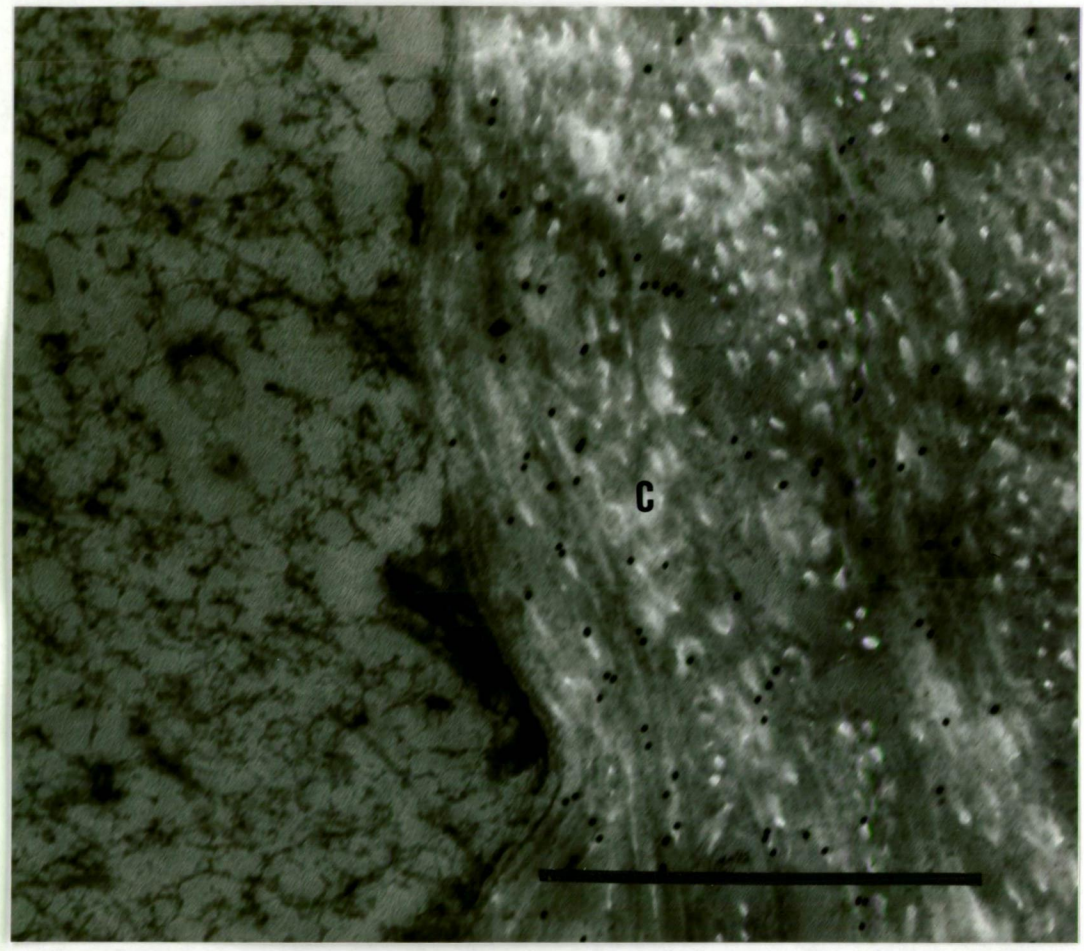

Figure 6.12 Sample 89008, flathead fixed immediately in fix B embedded in LR White. Disruption and distortion at end of muscle cell, poorly defined collagen fibres **C** labelled with gold. Poor resin integrity. Bar 1μm.

6.8.3 Rock cod

It was decided to use a longer fixation time and to try the process of post-fixing the sections after the immunogold treatment to endeavour to obtain better definition. A live rock cod (*Physiculus barbatus*) was spiked through the brain and muscle samples were fixed in Fix B for three hours after which they were processed through buffer, an alcohol series then into LR White resin. The longer fixation gave slightly better defined structures and these were further enhanced by employing a post-fixation step for 10 min. in 2% osmium tetroxide after the immunogold procedure and before counterstaining with uranium and lead. While this seemed to improve definition, the fragility of the sections through the multistep procedures and the instability of the resin in the beam resulted in few sections surviving for examination. Those that did, showed deposition of gold particles on the collagen (Figure 6.13), but again it was impossible to attribute labelling to individual collagen fibres particularly in the invaginations of the muscle fibre base.

6.8.4 Rainbow trout-direct

The investigations using LR White resin so far had all resulted in poor definition of structure due to the omission of osmium as a post staining

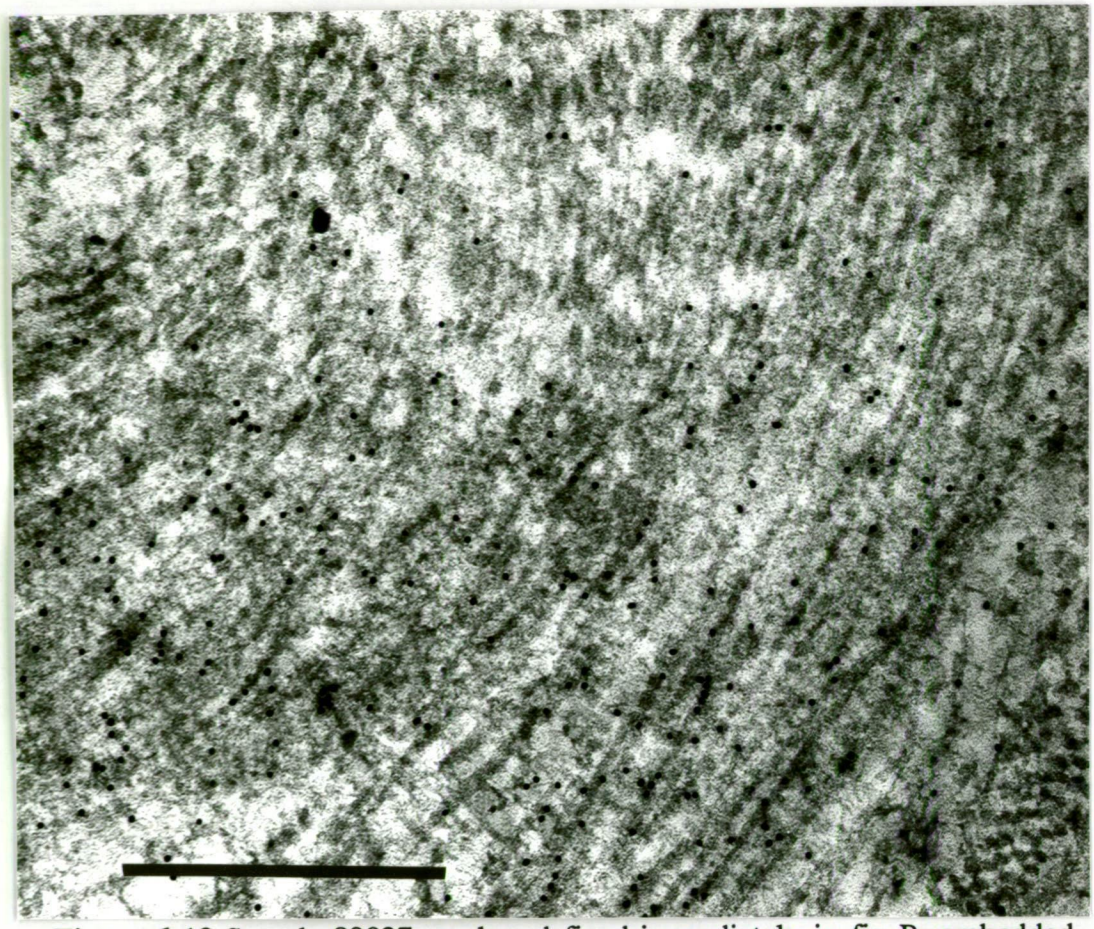

Figure 6.13 Sample 89027, rock cod fixed immediately in fix B, embedded in LR White, section post-fixed in osmium after the immunogold procedure. Poorly defined collagen fibres labelled with gold. Bar 1μm.

procedure before the embedding step. Even the use of osmium after the immunogold procedure post-embedding did not give well defined structures. As an alternative approach it was decided to try direct reaction of the antibody with the tissue then to process through the normal steps of fixation, osmium postfixation and embedding in Epon-Araldite resin. This was similar to the procedure of Keene, Sakai, Burgeson and Bachinger (1987) who used a monoclonal IgM antibody to type III collagen to identify its location on collagen fibrils reformed in vitro and in human skin and foreskin sections.

Samples of muscle were taken from a rainbow trout immediately after killing by spiking the brain. These samples were rinsed in PBS for 3 hours then incubated overnight in primary monoclonal antibody diluted 1 in 3 in PBS. They were rinsed in PBS the following morning then fixed for one hour in Karnovsky's cacodylate (GF/Cac, Section 2.2), after which they were rinsed in buffer, dehydrated through an alcohol series and embedded in Epon-Araldite.

The collagen fibres were quite well defined, but the overnight incubation in PBS or in primary antibody had softened the muscle fibres (Figure 6.14) and they had broken up showing extensive formation of vesicles. There were insufficient fields of collagen fibres to see if there were structures on them at regular intervals that could be attributed to the antibody (Figure 6.15).

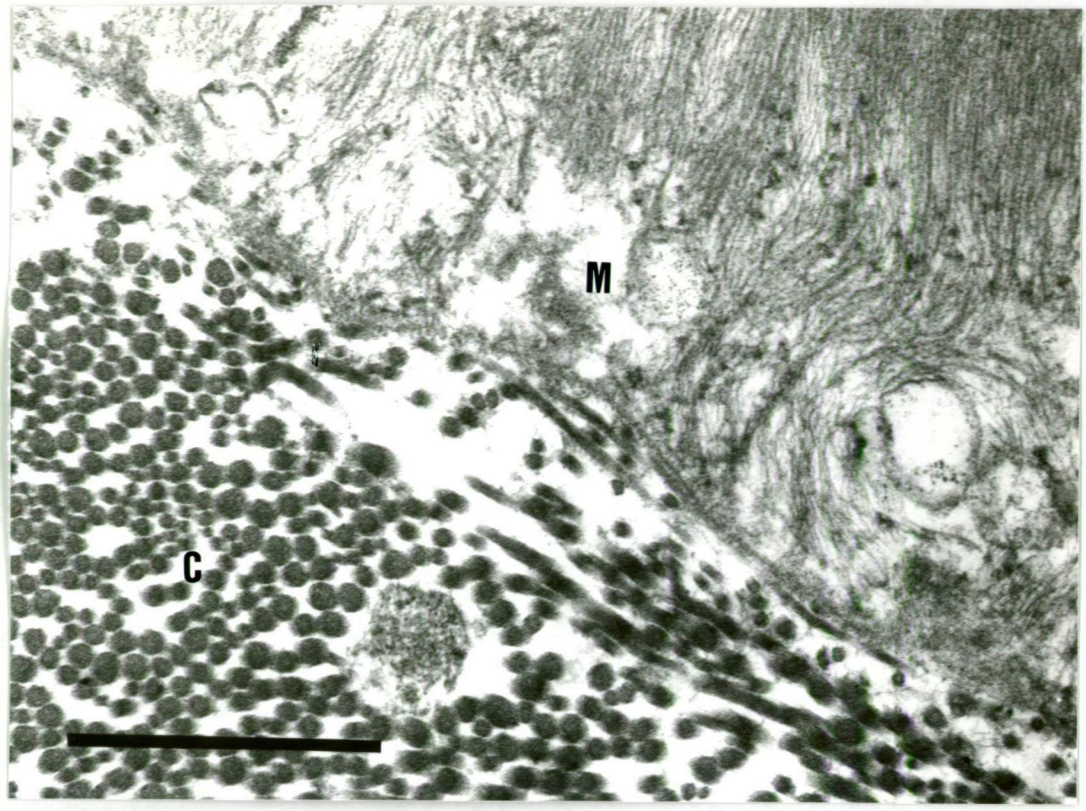

Figure 6.14 Sample 89030, rainbow trout treated 12 h in PBS then fixed in fix B, post stained with osmium then embedded in Epon-Araldite. Fairly well defined collagen fibres **C** in cross section, sarcolemma and basal lamina evident but degeneration evident within the muscle **M**. Bar 1μm.

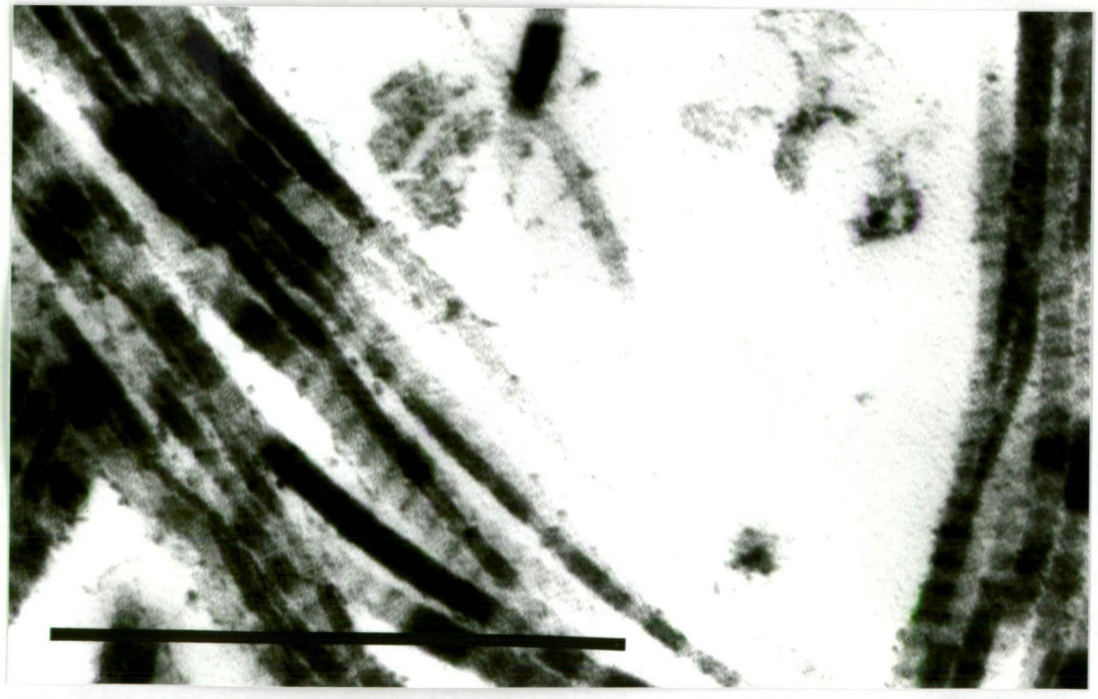

Figure 6.15 Sample 89029, rainbow trout incubated in IgM monoclonal anticollagen antibody 12 h then fixed in fix B, post-fixed in osmium then embedded in Epon-Araldite. Collagen fibres are reasonably well defined but there is no evidence of direct reaction of the antibody. Bar 1 μm.

Keene, Sakai, Burgeson and Bachinger (1987) had used this technique on tissues rich in collagen and it was decided to repeat the procedure using myocomma rather than muscle tissue.

6.8.5 Myocomma of gurnard

A live gurnard (*Helicolenus sp.*) was obtained and sections of muscle and myocomma were taken from it after spiking it through the brain. Samples were fixed in Karnovsky's cacodylate, and others were incubated overnight in PBS and fixed the following day. A third series were rinsed in PBS then reacted overnight with the monoclonal anticollagen antibody at a 1 in 5 dilution in PBS before fixation the following day. The fixed samples were then rinsed in PBS, dehydrated and embedded in Epon-Araldite.

The muscle and myocomma that were immediately fixed showed features similar to those outlined in chapter 5 (Figure 6.16, 6.17). The muscle structure was well delineated and the basal lamina and sarcolemma and the structures joining them were evident. Those samples that were incubated in buffer and in antibody showed softening and breakdown in the muscle and disruption of the field. There were no evident structures or changes on the collagen fibrils that could be attributed to direct reaction of the antibody with the collagen (Figure 6.18). The banding pattern on the collagen (Figure 6.18) was slightly less well defined than in those samples that had

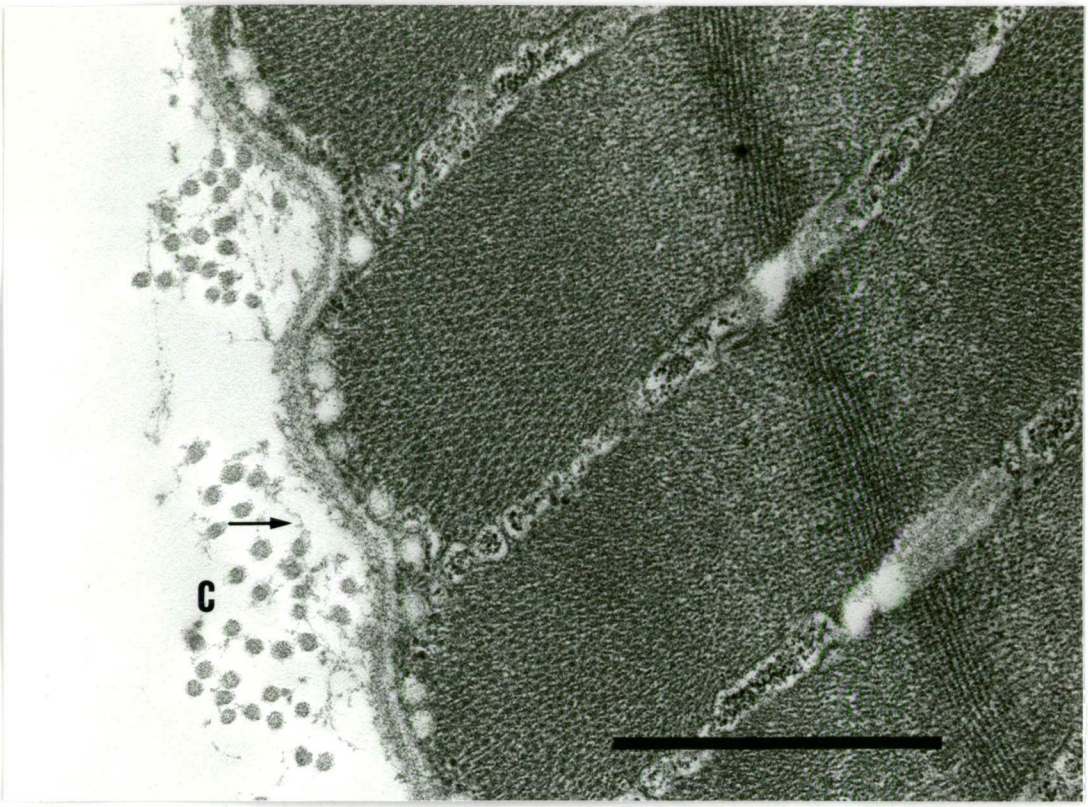

Figure 6.16 Sample 89042, gurnard muscle fixed prerigor in Gf/Cac, post-fixed in osmium then embedded in Epon-Araldite. Muscle fibre edge showing collagen **C** and linking structures (**arrow**) external to a well defined basal lamina. Bar 1 μm.

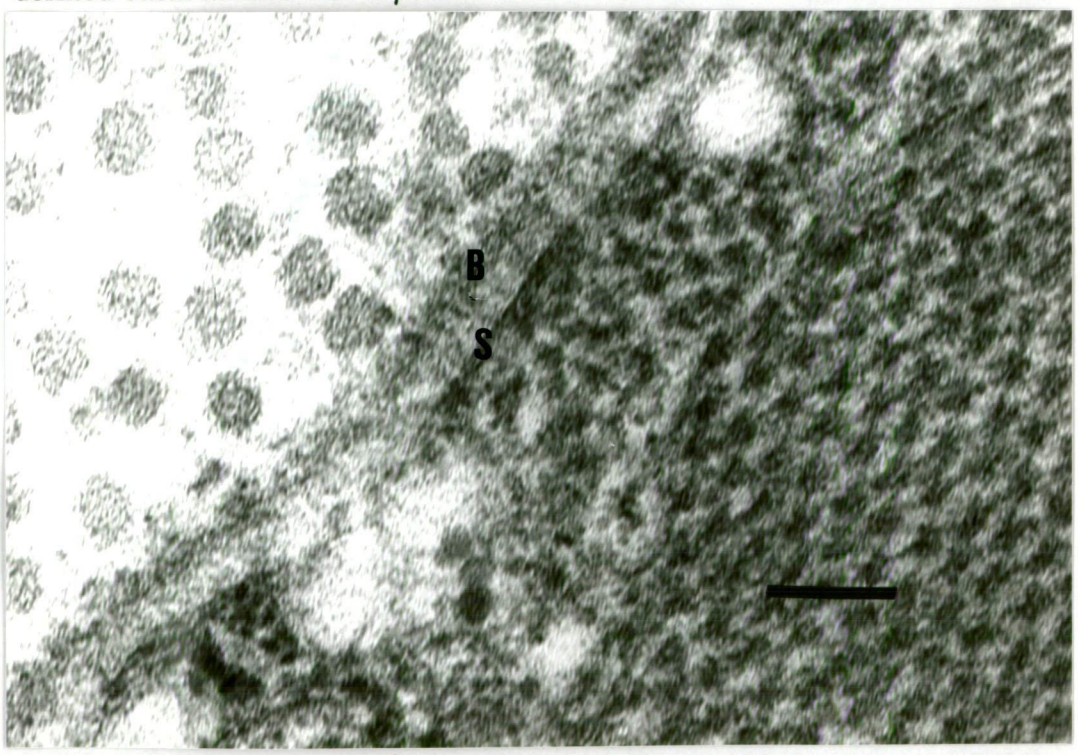

Figure 6.17 Sample 89042, as above (Figure 6.16) in slightly higher magnification. Sarcolemma **S** and basal lamina **B** evident, transverse section near an M-line. Bar 100 nm.

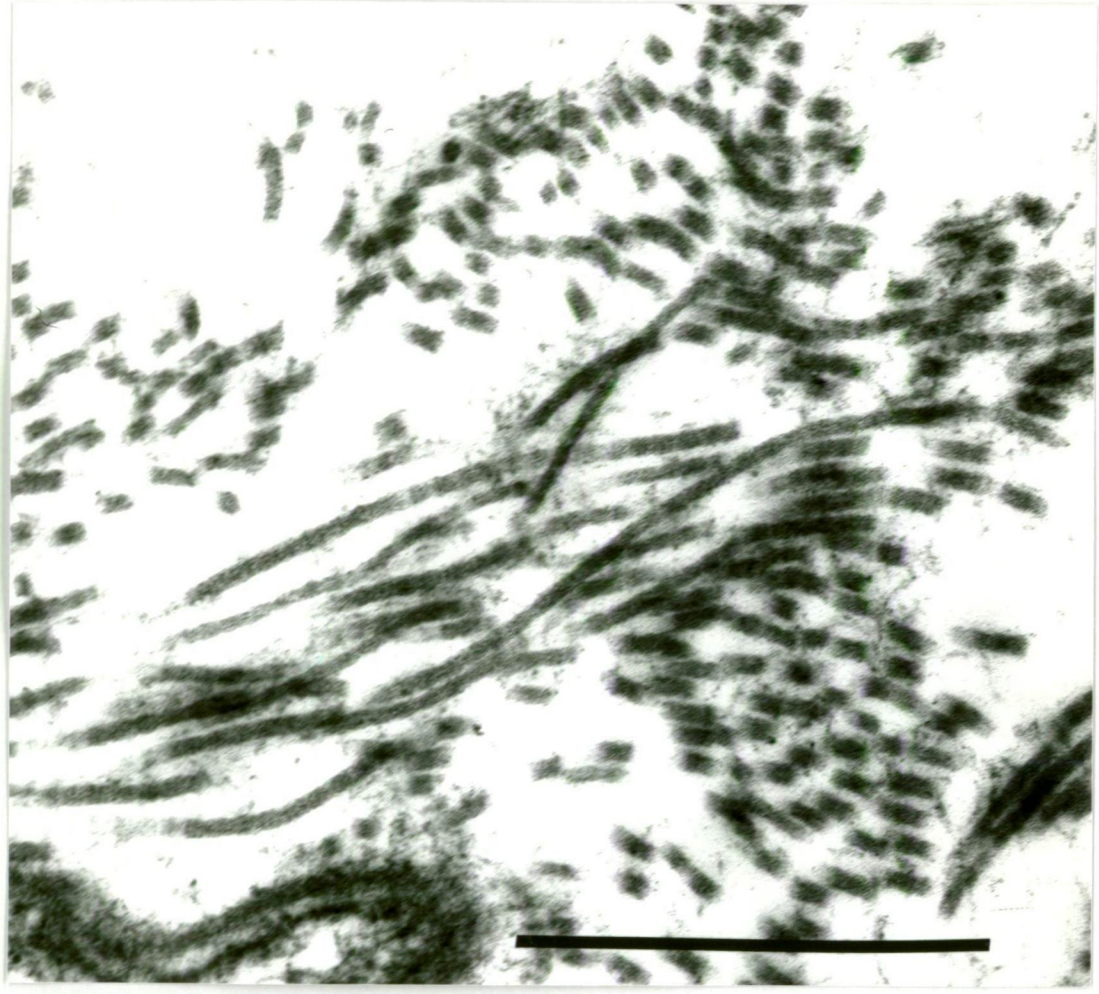

Figure 6.18. Sample 89047, gurnard myocomma incubated 12 h in IgM monoclonal anticollagen antibody then fixed in Gf\Cac post-fixed in osmium and embedded in Epon-Araldite. Collagen fibres slightly blurred no evidence of direct reaction with the antibody. Bar 1μm.

been immediately fixed (not shown). This approach using direct reaction was not pursued further since, in the meantime, Dr Werkmiester had ascertained that this antibody exhibited cross-reaction with other substrates. In hindsight it would have been prudent to have included enzyme inhibitors in the incubation medium to have prevented the degeneration during the incubation period. Indirectly, these results confirm the lability of the fish muscle/collagen system in chill storage.

6.9 Discussion

The use of the immunogold technique was not successful in further elucidating the fine structure at the myotendinous junction and identifying the collagen fibrils in the invaginations of the muscle fibre base. The aim of the approach had been to characterize those fine fibrils in the endomysium and invaginations. When this work was initiated it seemed likely that type I collagen would be present and, by analogy with mammalian muscle, that type III may also be present as a minor constituent. It now appears that type V collagen is the minor collagen present in the endomysium of fish (Sato *et al.* 1988,1989,1991), and that type III does not occur in fish muscle. Since type I is the major collagen it was appropriate to raise antibodies to it and to use these to develop the immunogold system for identifying the collagen fibres at the myotendinous

junction. Once the system had been proven it would then have been applied to detect the presence of other collagen types after raising the appropriate antibodies. Since different diameter gold particles can be linked with protein A it is feasible to label more than one structure in the same section (Roth 1982).

Furthermore it was assumed that the anticollagen antibody would be reactive to a site or sites on the triple helical portion of the intact collagen molecule. Hence it could have been used to seek evidence of breakdown in the collagen since it should have been less reactive to degenerated collagen observed in stored fish (Chapter 4) which would have resulted in lower gold counts on the sections. However, since the body temperature of the mice used to produce the antibody was above the melting temperature found for the collagen molecule (Chapter 5), it is inferred that the antibody was either active against an amino acid sequence or a telopeptide common to all three α-chains rather than to an intact triple helix.

Two major practical difficulties prevented the successful development of the immunogold approach. The LR White resin proved to be very fragile and sensitive to alcohol in reagents eg uranyl acetate counterstain, and to vapours of other solvents. It was also unstable in the electron beam, a problem which was only partly solved by coating the sections with carbon.

The major difficulty was the lack of definition of the collagen fibres under the conditions necessary to retain antigenicity (i.e. omission of post-fixation

treatment with osmium). Although the immunogold procedure worked, it proved impossible to differentiate between those collagen fibres to which gold particles were attached and those to which they were not. Furthermore the definition of the fine collagen fibrils in the invaginations at the muscle base made it impossible to state with certainty that they were type I fibrils or not.

Other workers have used immunogold to locate collagen types. Inspection of these reports also showed fairly poor definition of the fibre structure with similar frequencies of deposition of gold particles to those obtained in this work although it is not possible to quantitate this. Similar apparent degrees of labelling were seen by Stephens *et al.* (1982) and Stephens, Bendayan and Gisiger (1985). However, Magloire *et al.* (1988) and Keene, Sakai, Burgeson and Bachinger (1987) achieved a higher degree of labelling. Again the main problem was not lack of label, but lack of ultrastructural definition. While it still seems that the immunogold labelling may present an approach superior to immunoferritin labelling the problem of lack of staining of the collagen fibrils remains a challenge to be overcome.

6.10 Conclusion

Fish type I collagen can be labelled with gold particles for visualisation in

the TEM by treatment with an anticollagen antibody followed by reaction with protein A gold. However, the present methodology does not allow confident differentiation between individual collagen fibres due to poor ultrastructural definition. Significant care needs to be exercised in the multistep procedure and modified procedures are necessary to obtain stable sections with the best possible definition.

Chapter 7

General discussion

7.0 Conclusions and discussion

There are several major points that arise from this study concerning the myotendinous junction in fish and its post mortem deterioration.

7.1 Summary of key findings

The key findings of this study can be summarised as follows.

SEM studies:-

* confirm that a network of fibrous connective tissue surrounds each muscle fibre linking it into a socket-like indentation in the myocomma

* demonstrate these connective tissue fibres are degraded in chill storage to the extent that muscle fibres are detached from the myocomma

TEM studies:-

* demonstrate grooves and invaginations filled with fine collagen fibres from the myocomma at the muscle fibre ends

* reveal the fine connections occuring in these invaginations linking the collagen fibres to the basal lamina and the basal lamina to the sarcolemma

* show that during chill storage the basal lamina, the fine

connections and the fine collagen fibres are degraded

* imply that significant deterioration occurs in the myotendinous junction which precedes any obvious changes within the muscle fibre structure

Collagen characterisation studies:-

* confirm the major structural collagen of blue grenadier skin and muscle and other organs is an heterotrimer of type I collagen

* show the collagen is highly soluble in dilute acid and the proportion of insoluble collagen increases with the age of the fish

* suggest the amino acid composition of the $\alpha 3$ chain indicates its derivation from the α-1 chain

* demonstrate the melting and shrinkage temperatures for this collagen are consistent with the imino acid levels and the environment of the fish

Immunogold studies:-

* confirm the presence of type I collagen fibres in the myocomma adjacent to the muscle fibre cell

7.2 SEM observations

It was concluded that deterioration of the collagen of the connective tissues of the extracellular matrix of fish muscle occurred in stored fish. This could be a significant factor in post mortem softening of fish flesh. The indications were that the deterioration was enzymic in nature.

However, it was unclear which structures were being attacked and it was obviously necessary to examine the fine structure in more detail. These studies also indicated breakdown in the fine collagen fibrils of the muscle cell envelope (Figures 3.6,3.7 & 3.8).

Collagen is normally regarded as a fairly stable tissue that is resistant to enzymic attack - hence this aspect called for further investigation into the nature and type of the collagen present in the pericellular region and at the myotendinous junction and the susceptibility of this collagen to attack by enzymes.

7.3 TEM observations

The myotendinous junction of commercial species of fish examined eg. blue grenadier is similar in its major structural elements to observations obtained on other vertebrates and in other marine species such as the seahorse (Schwarzacher 1960), pipefish (Schippel and Reisig 1969), hagfish

(Korneliussen 1973) and lamprey (Nakao 1975). The muscle fibres terminate in a series of folds, grooves and invaginations into which protrude fine collagen filaments from the myocomma. (Figure 4.10, Figure 4.11). These fine filaments also form part of the muscle cell envelope and are in general of finer diameter (18-20 nm) than the collagen fibres of the myocomma (~80 nm). The distinct impression was gained that these finer collagen fibrils did not stain as densely as those of larger diameter (Figure 4.9) nor was their banding pattern as well-defined.

In the myotendinous junction fine connections exist between the collagen fibres, between the collagen and the basal lamina and between the basal lamina and the sarcolemma (Figure 4.12, 4.14, 4.15). These are of unknown identity but it is reasonable to assume proteoglycans are involved (Scott 1991) and that fibrillin is a possible constituent (Keene *et al.* 1991). The possibility that other collagens may be involved, such as type VII in connecting plaques (Keene, Sakai, Lunstrum, Morris and Burgeson 1987), must be also considered although these structures have not yet been reported in muscle.

During post mortem storage there is notable degradation of the collagen fibrils in the invaginations at the muscle fibre ends. This appears to proceed before there are significant changes within the muscle cell. It suggests that either the collagen is a very labile form or that a very potent enzyme system is present which is active at temperatures near 0°C.

Alternatively, both these factors could occur.

Post mortem degradation occurs external to the muscle fibre cell in the extracellular matrix in the fine collagen fibres that form the muscle cell envelope and fill the interstitial muscle space before changes within the muscle fibre are particularly evident. This study has thus shown that problems of post mortem softening and gaping have their origin in the myotendinous junction not within the muscle fibre cell itself. Breakdown occurs at the interface between the muscle fibres and the connective tissue of the myocomma. The TEM work confirms the earlier SEM work in this regard and has highlighted the need for further work on the mixture and structure of the collagen types present and on the mechanisms by which the fine fibres are degraded. The result of this rapid breakdown is a softening of texture that in some cases leads to gaping of the fish muscle. This focuses attention on the identity and location of the fine collagen fibres observed in the invaginations and in the muscle cell envelope.

7.4 Collagen studies

The collagen of the blue grenadier had a melting temperature and a shrinkage temperature consistent with the temperature range of its habitat and its imino acid composition. There was a slight but progressive increase in the proportion of insoluble collagen with increasing age of the fish.

The main type of collagen in the blue grenadier is an heterotrimer of type I collagen. It is widely distributed throughout the skin and major organs as indicated by immunofluorescence using a specific polyclonal antibody. It was inferred from these results that the specificity of the antibody was sufficiently high to allow further immunocytochemical work to proceed. This further work had the aim of establishing whether the fine fibres found in the myotendinous junction of the blue grenadier were type I collagen.

7.5 Immunogold labelling studies

Fish type I collagen can be labelled with gold particles for examination in the TEM but the lack of definition of the collagen fibres under the conditions required to retain antigenicity severely limited this approach. The difficulties encountered with the stability of the resin added to this problem. There is a need for further developmental work on methods of fixing collagenous tissues in suitable resins to allow for adequate depth of staining to delineate separate fibrils. At the time when the work was initiated the presence of type V collagen in fish muscle had not been reported. The polyclonal antibody to the fish collagen was not tested for crossreaction to fish type V collagen since none was available. None is still available. It is possible that the labelling with immunogold seen here may be due to reaction of the polyclonal antibody with both type V and

type I collagens. Although this is unlikely it would completely negate the approach used here which relied on the labelling, or not, of individual fibres.

Another complicating factor is that if both collagen types coexist in the one fibril then it may be labelled even when there is no cross reaction of the antibodies between the collagen types. In addition it has also been recently reported that types I and V form copolymers in which the type V is 'hidden' inside the type I and antibodies to the triple helical regions of the type V do not gain access to react with it (Birk *et al.* 1988; Birk *et al* 1990). In this case antibodies to the peptide regions which can protrude from the fibril are required. If the range of antibodies were available then it should be possible to label the collagen fibres in the interstitial area and invaginations using different size gold particles for each antibody. Assuming fibre definition was adequate, this would provide some degree of estimation of fibre type and an indication if both types coexist in a single fibre.

7.6 Technical constraints

Constraints on this work were the difficulties in obtaining prerigor and fresh samples of blue grenadier. This commercial species is only obtainable by going to sea on trawlers. Juveniles sometimes can be obtained by beach

seining operations in shallow waters in estuaries but mainly the fish are caught at depths of 400m or greater. Adult fish have not yet proved capable of being taken live and juveniles have not survived in ponds or aquaria. From an experimental viewpoint this is more than inconvenient and considerable difficulties were encountered in obtaining suitable samples, which caused delays in the studies. As a result most of the immunogold studies had to be done on other species that could be obtained live.

The difficulties that were encountered with the LR white resin caused further delays to the main thrust of the immunogold studies. This resin has been used by other workers for similar studies but there is no mention of its instability to solvents or in the electron beam. The instability in the beam was partly overcome by minimising exposure and by coating the sections with carbon.

The other major constraint on this study was in the difficulties encountered in obtaining sufficient ultrastructural definition to differentiate individual collagen fibres when samples were not post-stained with Osmium tetroxide, a step which was omitted in order to retain optimum level of antigenicity to maximise staining with the immunogold procedure. Techniques were adopted to improve counterstaining of the collagen fibres to enhance definition in the TEM but clarity and differentiation of individual fibres was still insufficient to provide the desired information of whether an individual collagen fibre was, or was not, labelled.

7.7 Degradative processes

The nature of the degradation at the myotendinous junction has been inferrred to be enzymic in nature with major degradation occurring in the collagen. Collagenases and control and activating mechanisms have been discussed in detail in section 1.3.8. Collagenases are metallo-endoproteases activated and stabilised by calcium ions operating in the range pH 6 to 8 but none have yet been identified in fish muscle tissue. In the post rigor state normal control mechanisms no longer operate and calcium ions leak from the sarcoplasmic reticulum in amounts sufficient to activate latent collagenases. Introduction of Ca^{++} into fibroblasts promotes a cascade of proteolytic events culminating in activation of collagenase (Unemori and Werb 1988) and this may provide one initiating mechanism but it is likely that a whole series of events are set in motion post mortem.

The type IV collagen of the basal lamina can be degraded by metalloproteinases including gelatinase, proteoglycanase, serine proteases, neutrophil elastase and mast cell chymase. If type V collagen is present in the endomysial layer and the invaginations, this too can be attacked by metalloproteinases which are also gelatinases of a molecular weight greater than the classical collagenases (Stricklin and Hibbs 1988, Liotta *et al.* 1981).

In vivo, separate enzyme systems are necessary for types I and V to be

copolymers in the same fibril (Niyibizi and Eyre 1989) and this makes it likely, but not obligatory, that separate systems are required for their degradation post rigor.

One of the recently recognised control mechanism for collagenases are the TIMPs (Stricklin and Hibbs 1988), but whether they are active post-mortem is unknown. Basement membrane degrading enzyme, collagenase IV/V - gelatinase, is readily released by kallikrein and becomes active in the extracellular space before other lysosomal proteinases are released (Tschesche *et al.* 1989). Tissue kallikrein, a serine protease (specifically an arginyl esteropeptidase), also activates type I collagenase and is a likely candidate to perform these functions *in vivo*. It may also be an activating factor in post-mortem tissue, since it is active at the pH of postmortem fish muscle (Tsesche *et al.* 1986). Lysosomes in fish muscle are found near connective tissue (Steiner *et al.* 1984) and cathepsin B released by post-mortem disruption of the lysosomes could activate latent collagenases.

Thus there are several ways in which collagenases may be activated to effect the tissue disruption seen in this study. Neutral proteases are found in the flesh of many fish species (Makinodan *et al.* 1983) and a number of proteases, mostly serine proteases, bound to both the sarcoplasmic and myofibrillar fractions of the muscle occur in a variety of species (Shimizu and Wendakoon 1990, Yanagahira *et al.* 1991, Toyohara *et al.* 1990, Kinoshita 1990), but it seems unlikely that they are active in chill stored

fish. Cathepsin L from lysosomes has been implicated in the extensive muscle softening observed in chum salmon and ayu (Yamashita and Konagaya 1990) during migration and maturation. An endogenous serine proteinase which degrades the cytoskeletal network (Busconi *et al.* 1989, 1989), which initiates protein turnover *in vivo* and which can completely disrupt the myofibrils (at 37°C) and degrade the major proteins (Busconi *et al.* 1987) has been reported in white croaker (*Micropogon opercularis*). When croaker were stored at 0°C for 7 days, after dipping in azide to prevent bacterial growth, only minimal changes were found in the major proteins of the myofibril but there was considerable breakdown of nebulin, a major cytoskeletal protein of the trabecular network (Busconi *et al.* 1989, 1989). In the present samples similar activity would be likely to weaken the integrity of the structure. Busconi, Folco, Martone,Trucco and Sanchez (1989) reported no changes in the proteins desmin, troponin and Z lines were shown to be stable under these conditions (0^0C, 7 days), whereas these entities are known to be degraded in post-mortem storage of beef muscle along with titin and alpha-actinin (Hwan and Bandman 1989).

Calpain II is found in both carp (*Cyprinus carpio*)(Toyohara *et al.* 1985) and hybrid tilapia (*Tilapia nilotica* x *Tilapia aurea*) (Jiang *et al.* 1991). Calpstatin and a trypsin inhibitor is also present in carp (Toyohara *et al.* 1983) but it is not clear whether the calpains would be a likely cause of the deterioration seen in this study.

Following their studies using SEM, Bremner and Hallett (1985) suggested that the fine collagen fibres of the endomysial layer in fish muscle which were being degraded may have been comprised of type III collagen that was susceptible to enzymic attack. No evidence for type III collagen in fish muscle has been found (Ramshaw *et al.* 1988; Sato *et al.* 1988, Sato *et al.* 1989), but type V is widely distributed in fish as the intramuscular connective tissue (Sato *et al.* 1988; Sato *et al.* 1989). It appears that type V in fish may perform the function of type III in mammals. Sato *et al.* (1991) have demonstrated that the type V collagen in trout muscle degraded in post mortem storage, as indicated by increased solubility, whereas no change was seen in the solubility of type I collagen. Their evidence pointed to cleavage of non helical regions. In a very recent publication Ando, Toyohara and Sakaguchi (1992) using TEM demonstrated breakdown of fine interstitial collagen fibres in the muscle of rainbow trout in less than 24 hours storage post mortem at a temperature of 5°C. This was accompanied by a concomitant drop in the breaking strength of the muscle. The thicker collagen fibrils remained intact.

K. Sato, Kyoto Prefectural University Japan, (personal communication) has also noted breakdown in intramuscular collagen of the sardine within three hours post mortem storage at a temperature of 0°C. Electrophoretic evidence shows an increase in acid soluble type V collagen with intact helical fragments of type V. It appears that rapid cleavage is occurring at

the nonhelical peptide regions. Type V collagen has a greater proportion of nonhelical regions than type I and these may be more susceptible to attack by general tissue proteases that do not specifically possess collagenase activity ie they do not attack the triple helix.

Taken as a whole, there is consistent evidence to suggest that rapid post mortem breakdown of type V collagen in the interstitial muscle tissue and at the myotendinous junction can occur in fish. This breakdown is responsible for some of the effects seen in the present study using TEM and in the initial studies using SEM.

The evidence in the TEM indicates that the sarcolemmal cell membrane remains intact until later stages of degradation. This is consistent with the recent work of Ando *et al.* (1992). It is reasonable to conclude that the enzymes responsible are therefore extracellular in nature and are most likely located in close proximity to the collagen or are intimately associated with it. It is likely they are part of the normal mechanism of collagen formation and catabolism.

Identification of the enzymes responsible first relies on purification of quantities of suitable substrate, eg fish type V collagen, for screening muscle extracts for enzyme activity. Type V is only present at a level of about 0.2% of the wet weight of fish muscle (Sato *et al.* 1989). Once the enzyme or enzymes responsible are prepared by extraction and purification by gel filtration or affinity chromatography it may be possible to prepare

antibodies to them (it) to locate them in the tissue.

No type V collagen was found by the method used. It is now known that higher salt concentrations are needed for the precipitation of type V fish collagen (Sato *et al.* 1991), so it is possible that it occurs in blue grenadier. The antibody prepared from the type I fish collagen was specific for type I and this is the major structural collagen type in the muscle and organs of the blue grenadier. It was not tested for crossreaction with fish type V collagen since none was available. Further work is required to examine the blue grenadier muscle for the presence of type V.

At the time of the investigation the conditions for purification of type V from fish muscle had not been published, neither had the evidence for its existence in specific fish tissues. Blue grenadier skin was chosen as a substrate for extraction of collagen since skin is commonly used for this in other fields. It would appear also that it is necessary to reinvestigate the nature of the muscle cell envelope of other important commercial species such as cod. The conclusions of Almås (1982) and Børresen (1976) that the collagen of the muscle cell envelope is solely type I collagen may need revision. Their technique involved 'ageing' the muscle cells, swelling of the myofibrillar contents of the cell then digestion of the myofibrillar protein gel with trypsin. These conditions would probably have resulted in the loss of any type V collagen present. Since softening and gaping can be a serious problem in cod, and cod is a major commercial species, then

reinvestigation of its muscle cell envelope is warranted.

7.8 Implications in other fields

This investigation has provided evidence for the post mortem degradation of collagen at chill temperatures. In addition, the work of Sato *et al.* (1991) and Ando *et al.* (1992) now indicates that type V collagen is degraded. This may well be an important phenomenon in species other than fish where type V collagen has an important structural role in maintaining tissue integrity and function.

In meats from beef and sheep type V collagen has only been regarded as a minor constituent of the epimysium and perimysium and it has not been considered to have a major influence on texture but its influence on postmortem tenderization should not be disregarded.

More importantly in the medical field, hidden degradation of type V collagen may occur in tissues in the time between removal from the donor and transplant into the recipient.

If a tissue or organ has type V collagen as an important part of its structure, albeit in low proportion in compositional terms, then its stability should be investigated.

7.9 Further directions

This investigation has opened up many possible areas for further research related to both the extra, intra and intercellular components of fish muscle. In the extracellular matrix there is a need to

- confirm the location of type V collagen;
- confirm the presence and location of type IV collagen;
- establish the location of proteoglycans in the myotendinous junction;
- establish the presence of other collagen types eg types VII, IX or other members of the FACIT group;
- establish the presence and location of fibrillin, elastin, fibronectin and laminin.

In the intercellular tissue there are similar needs to

- confirm the location of type V collagen;
- examine activity of muscle extracts for enzymic activity against type V;
- locate proteoglycan structures.

In the intracellular muscle there is a need to identify whether components that have been reported in mammalian muscle also occur in fish muscle in the same locations.

Components which could be identified are:

fibronectin, zeugmatin, filamin, talin, skelemin, vinculin and other components of the Z line and trabecular structure of the fish muscle cell.

Further investigation of the internal structure of fish muscle cells is warranted to elucidate the sites of attachment to the inner surface of the sarcolemma at M and Z lines as well as at the terminal end of the fibre.

The current focus of the work now resides in investigating the breakdown of type V collagen. For further elucidation of the mechanism involved, preparation of type V fish collagen must be undertaken to provide amounts sufficient for further investigations such as the raising of specific antibodies for the detection of enzymic activity.

7.10 Final comment

In conclusion, this work with the TEM to elucidate the structure of the myotendinous junction and the description of its degradation (Hallett and Bremner 1988; Bremner and Hallett 1989; Bremner 1992) has stimulated research on the collagen in the interstitial tissues of fish and the concomitant post mortem changes in texture associated with its degradation (Professor K. Sato, Kyoto Prefectural University Japan, Professor M. Sakaguchi, Kyoto University, Japan - personal communications). It has prompted others to look at post mortem textural changes and softening of fish muscle in a new light, not solely as biochemical changes which occur within the muscle cell.

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Myotendinous junctions in fish

Publications

H.H. Huss et al., (eds.) Quality Assurance in the Fish Industry

Fish flesh structure and the role of collagen - its post-mortem aspects and implications for fish processing

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Abstract

Structural links between the muscle cells and the connective tissues of fish provide the necessary integrity for the flesh to withstand the effects of post-harvest handling, processing and storage. Therefore, a fundamental knowledge of the components of the delicate and complex structure of fish flesh is essential in order to understand the changes that occur post-harvest. This understanding can then lead to techniques and processes to minimise change, or, to use it to advantage.

Minor components which form the interfaces and adhesive links are as important in this regard as are the major components. Within the muscle cell, elements of the cytoskeleton serve to link the major proteins, actin and myosin, into an ordered structure. External to the cell, collagen is the major connective tissue in the structure and it is dominant in determining the textural attributes of the raw flesh. The links between the muscle cells and the external connective tissue occur mainly at the myotendinous junction where the forces of muscular contraction are transmitted from within each cell to the connective tissue of the myocommata.

Post-mortem changes occur within the muscle cell in the elements of the cytoskeleton, in interactions between the proteins in the cell and externally in the links between the cell and its envelope and in the myotendinous junction. The relative importance of these phenomena varies with species and circumstance, but each affects the characteristics of the flesh during processing.

This review concentrates on the external structures and on changes occurring at the myotendinous junction and in the fine collagen fibrils of the endomysium.

INTRODUCTION

Sikorski *et al.* 1984 [1] have reviewed collagen in fish, Howgate [2] has summarised work on fish muscle structure up until 1980 and the continued output of Love and his colleagues in a series of investigations spanning many years, most recently reviewed in 1988 [3], provide the background for this paper and reference is mostly restricted to recent work. In order to discuss the nature of post-mortem change and the mechanisms and

possible agents causing these changes, it is necessary to first describe the flesh structure including the myotendinous junction and the muscle cell constituents, the basement membrane and the extracellular matrix.

FISH FLESH STRUCTURE

The flesh of teleost fish is constructed of adjacent muscle blocks, called myotomes, separated from each other by sheets of collagenous tissue called myocommata [4]. Within each myotome, the muscle fibres (myomeres, sarcomeres or myofibrils in the literature on mammalian muscle) run approximately parallel to each other but at varying angles to the myocommatal sheet to accomodate the juxtapositional rhythmical contractions that occur during swimming so that all the fibres in the myomere contract to a similar extent when the fish bends. This results in maximum power output at a given rate of contraction [5]. The myocommata are connected internally to the skin and to the skeletal system and are also linked to the membrane dividing the fish into epaxial and hypaxial planes and to the median vertical septum.

The junctions between the myomeres and the myocommata in fish are equivalent to the myotendinous junction in mammalian muscle. In fish, this junction is formed by fine collagenous processses which have their origin in the myocomma and which then proceed as sheaths to surround each muscle fibre [6,7].

GENERAL ASPECTS OF THE MYOTENDINOUS JUNCTION

Early work with the electron microscope established that the muscle fibre terminated in finger-like projections which were bounded by the cell membrane and that the actin filaments terminated inside the cell and that collagen fibres did not penetrate the cell membrane and were never found within the muscle cell [8,9]. A complicated series of folds and invaginations at the myotendinous junction increase the area of contact by a factor of about 20 to 30 times for fast twitch muscles [10] and 50 times for tonic cells [11]. This reduces the load on the cell membrane by an order of magnitude [12]. The geometry of attachment of the actin filaments to the sarcolemma ensures that the forces are transmitted in shear rather than in tensile mode [13].

THREE STRUCTURAL DOMAINS

Muscle cells are bounded by a continuous cell membrane called the sarcolemma. External to this is the basement membrane and the endomysial layer of fine collagen fibres. The myotendinous junction can be considered as three major structural domains which are in close proximity: (i) the internal elements of the muscle cell and the sub-sarcolemmal surface; (ii) the membranes and fine processes at the junction; and (iii) the external connective tissue stroma which eventually attaches to the tendon.

The internal structure

Most of the detailed work on muscle components has been done on mammalian muscle and a recent comprehensive review of muscle structure and biochemistry is given by Pearson and Young [14]. The presence of many of the minor proteins of the mammalian cytoskeletal system has not yet been established for fish muscle.

Muscle fibres are comprised of bundles of fibrils (myofibrils) arranged longitudinally within the muscle cell and organised within a cytoskeletal framework of desmin-containing intermediate filaments [15]. The basic repeat unit of the myofibril is the sarcomere (often termed myomere in fish) and each sarcomere is bounded at each end of its long axis by the electron-dense structure known as a Z disc (or Z band, or Z line) (see Figure 2,3 later). Actin filaments extend from one Z disc to the next and, in cross-section, are arranged in an hexagonal array parallel to and around the myosin rods. The myosin rods themselves do not attach to the Z disc but occur in the mid-portion of the sarcomere. The rod-shaped protein tropomyosin occupies the grooves of the actin helix providing a structure for the globular protein troponin to attach to at regular intervals. Titin (originally named connectin, when first isolated in a slightly impure form [16]), together with nebulin, forms 'gap filaments' which join the thick myosin filaments from their ends to the Z disc and which thus stabilize the myosin in the centre of the sarcomere [17].

The protein components of the Z disc have not been fully resolved, but alpha-actinin, a protein with actin-bundling properties, comprises about 50%. Actin filaments from adjacent sarcomeres overlap into the structure. Other proteins that have been suggested as components are Z-protein, amorphin, Eu-actinin, Z-nin, filamin, zeugmatin and another 220 Kdalton protein [18]. Zeugmatin is present in the early formation of Z disc structures grown in cell culture, before alpha-actinin is observed, and hence probably plays some organisational role in development and organisation [19]. At the edge of each fibre, elements of the cytoskeleton link the filaments from the terminal Z disc to an electron-dense meshwork at the sub-sarcolemmal surface. The terminal Z discs near the sarcolemma have been found to contain much less alpha-actinin and it is assumed that this indicates that thin filaments are not bundled by alpha-actinin near the sarcolemma [20] and that Z discs in different locations are not homogeneous in composition. Talin, a 225 Kdalton protein has also been located at myotendinous junctions and is a component of the digit-like processes that extend into the tendons there and may be involved in force transmission [21].

The cytoskeletal proteins, the skelemins, are located at the periphery of the M discs while vinculin is organised along the sarcolemma in an array of rib-like bands termed costameres [22]. Transverse sarcomeric filamentous systems organised at the Z and M band levels link these structures to the sarcolemma [23].

Each fibril is bathed in cytoplasm and is partly enshrouded by the membranous sarcoplasmic reticulum containing the sarcoplasm. This sarcoplasmic reticulum swells out to form the terminal cisternae which in apposition with those of the adjacent sarcomere and the intermediate element, the T tubule, make up the triad structure. In fish, the triads are situated external to the Z disc but in mammals the triad occurs in the midportion of the sarcomere near the junction of the A and I bands.

Near the end of a muscle fibre, the sarcomeres anastomose around the invaginations of the sarcolemma. Actin fibres reach from the last complete Z disc to the internal surface

of the sarcolemma where they attach in an electron dense sub-sarcolemmal layer. This layer is comprised of both globular densities and fine linear elements which run approximately parallel to the major actin fibres [24] and is involved in anchoring the actin to the interior of the sarcolemma, referred to as the internal lamina [25]. The proteins talin and vinculin are found at this location and it is considered that these may be the force-transmitting and attaching proteins [21]. Longitudinal muscle growth occurs at the fibre ends [26] and glycogen granules, mitochondria, polysomes and ribosomes occur with notable frequency near the fibril ends [27].

The basement membrane

The basement membrane, sometimes called the basal lamina, occurs adjacent to the sarcolemma as part of the integral structure marking the boundaries of cells, although it may not follow exactly all the convolutions of the sarcolemma. The basement membrane serves a variety of functions such as molecular ultrafiltration and tissue organization and mediation of interactions between specific cell layers and their underlying stroma. The outer area of the basement membrane, the *lamina densa*, is an electron-dense area that is comprised mainly of collagen, mostly type IV. The *lamina lucida (rara)* lies between the *lamina densa* and the sarcolemma and is more transparent to electrons. In the *lamina densa*, the type IV collagen molecules form unique end-to-end associations to construct a network of molecules which comprise the structural framework of the membrane [28]. Several other components, in particular laminin and heparin sulphate proteoglycan, form parts of the overall structure. The large molecular weight glycoprotein laminin has been localised to the basement membranes of skeletal muscle and is distributed throughout the *lamina densa* and the *lamina lucida* [29,30]. The proteoglycans are comprised of a central protein core with covalently bound glycosaminoglycan side chains which affect permeability and cell attachment [30].

It was proposed that collagen fibres from the connective tissue were attached to the basement membrane. The analogy of a rope untangling at its end to provide the fibres which were woven into the carpet of the basal lamina was employed to convey the concept of how force may be transmitted from the muscle to the tendon [31]. Tropocollagen molecules were proposed as the links between the sarcolemma and the basal lamina.

Low [32,33] described a set of fibrous structures with a diameter in the range of 4 to 12 nm. These structures, which were finer than the collagen fibrils, appeared to link the collagen fibrils. He termed them 'microfibrils', although this term has other connotations in collagen chemistry. 'Microfibrils' were also reported in muscle-tendon transitions in the papillary muscle of the heart, muscle from the tip of the tongue, the diaphragm and the gastrocnemius of the guinea pig [34] and it was proposed that these elements actually passed through the basal membrane and fused with the outer electron-dense layer of the sarcolemmal membrane. Similar structures were demonstrated [35] in the myotendinous junctions of muscles of the hagfish (*Myxine glutinosa* Linnaeus) where spine-like structures about 6 nm in diameter at 15-25 nm intervals extended from the external leaflet of the plasma membrane to the internal surface of the *lamina densa*. Further work [36] showed similar 'intermediary' structures in the myotendinous junctions of the lamprey [37]. When detergent and EGTA solvents were used to disrupt the sarcolemma, the small filamentous structures that cross the *lamina lucida* remained intact and tension could still be transmitted

across the myotendon, indicating that these filaments were attached to elements of the contractile structure not just to the sarcolemma alone [38]. From this and other evidence, three important structural facets were proposed: one that binds actin near the sarcolemma to transmit the contraction; another that crosses the hydrophobic portion of the membrane; and a third that transmits tension from the membrane to the *lamina densa* [38]. Further work indicated that the filaments of the *lamina lucida* are composed of two subdomains: one closely associated with the sarcolemma the other with the *lamina densa*. The connection between the layers is ionic, not covalent [38]. There does not appear to have been any further advances in ascertaining the identity and structure of these filaments.

Nakao [36] first observed that the basement membrane, which otherwise is continuous, is absent in the terminal ends of the invaginations in the finger-like projections of the ends of the muscle cells in the lamprey and the tadpole. This absence is unusual since no breaks occur in the sarcolemma.

The extracellular matrix

The major structural feature of the extracellular matrix is the collagenous network that surrounds each cell and which forms the tendons and ligaments that attach to the skeletal system. In the endomysium, the fibres are fine and tend to form a lace network around the muscle fibre [39]. Branched reticular fibres, now considered to be type III collagen, form rows arranged obliquely or perpendicularly to the long axis of the muscle [40]. Larger diameter collagen fibres of the perimysium surround the muscle fibre bundles and these merge into the major sheets of collagen of the epimysium which cover individual muscles to become tendons.

Fibroblasts, with their flattened dendritic processes, are often present at boundaries between adjacent layers of collagen fibres. These fibroblasts, derived from mesenchymal cells, produce most of the collagen adjacent to the muscle cell ends. Adipose cells, mast cells and macrophages containing lysosomes are regularly noted in the looser connective tissues. Eosinophilic leukocytes and other plasma cells occur nearby. Elastin fibres, which play a role in the providing some of the elastic properties of the tissue are also present.

In muscle tissue, the large collagen fibres are almost invariably type I collagen. In mammalian muscle, smaller proportions of type III collagen occur, while types IV and V are associated with the basement membrane. The collagen fibres embed in a matrix of proteoglycans. They are often crimped and run in layers in different directions to provide a strong flexible structure. The fibres have a range of diameters according to their position and function (from 30 nm to over 100 nm).

Early studies [31] indicated that finer connections appeared to join the collagen fibres. Some external tissues contain 'anchoring fibrils' with the recently discovered type VII collagen [41,42] as the primary structural agent. This collagen forms an extended network of these fibrils between anchoring plaques in the *lamina lucida* of many epithelial tissues [43]. Collagen type VII has not been found in muscle or other internal organs.

THE MYOTENDINOUS JUNCTION IN FISH

Schwarzacher [44] compared muscle fibre-tendon junctions in the seahorse with those from the cat, rat, mouse and frog. There were considerable similarities and all the junctions revealed folds and finger-like projections at the ends enveloped by the basement membrane [45]. The myotendon junction of the flowing muscle of the spinal cord of the pipefish was similar to the appearance of that in the tadpole tail. In the primitive hagfish (*M. glutinosa* L.), spine-like projections and thread-like cones between the *lamina densa* and the external surface of the sarcolemma were found [35]. They were considered to be the same feature, namely ring-like structures that, according to the angle of section, could appear either as spines or threads. Fibres from the I band (actin fibres) attaching to the sub-sarcolemmal surface in electron-dense areas were observed and were considered to be analogous to Z discs [35]. The sequence of events in the longitudinal growth of myofibrils of the skeletal muscle of *Macropodus opercularis* is that high ribosomal concentrations occur in the short terminal myofibril region along which actin filaments are formed [27]. As the terminal portion enlarges, myosin filaments are evident and A and I bands appear, followed by the Z disc which forms in close contact with the sarcolemma and gradually detaches from it. In the lamprey, the sarcolemma at the terminal end of the invagination frequently showed specific coupling with the cisterns of the sarcoplasmic reticulum. The basal lamina was partially or completely deficient in this area [36]. Although this allowed for the possibility of collagen fibres to be in direct contact with the sarcolemma, no definite relationship between them was found.

Recent studies

The three-dimensional structure of the junction and the effects of post-mortem storage have recently been reported [46-48]. In the blue grenadier (*Macruronus novaezelandiae* Hector), a network of fine collagen fibres emerges from the myocomma to envelope the muscle fibre along its length (Figure 1) [cf. 49,50]. The muscle fibres fit into socket-like indentations in the myocomma which are revealed when the muscle fibres are removed [48].

The microstructure at the myotendinous junction at the base of a fibre reveals folds and invaginations in the fibre ends filled with fine collagen fibres (Figure 2,3) which may protrude up to 10 μm into the fibre end. The sarcolemma forms a continuous boundary to the cell (Figures 2,3,4), but the basement membrane lying outside this appears to be discontinuous near the ends of the invaginations which often occur in close apposition to a vesicle of the sarcoplasmic reticulum [48]. Fine connections of an unknown nature exist between the collagen fibres, between these fibres and the basement membrane, and between the basement membrane and the sarcolemma (Figure 5,6). Within the muscle cell, the regular banding pattern typical of skeletal muscle is evident (Figure 2,3,4) and fine filaments, resembling actin, proceed from the what appears to be the last Z disc to the inner surface of the sarcolemma, where they appear to be attached by some electron-dense material (Figure 4). In all respects, these studies confirm the nature of the myotendinous junction as discussed above. The structure at the periphery of the fibre is also typical [48].

It has recently been shown in guppy muscle (*Lebistes reticulates*) that the structure of what appears to be the terminal Z disc is not a Z disc but some as yet uncharacterized

electron-dense band. The actin bundling protein alpha-actinin was absent and actin filaments were observed to pass through the band without any alteration in orientation as indicated by decoration with heavy meromyosin [51].

FISH COLLAGENS

The collagens of fish are, in general, much more easily solubilised than those of mammals. The major collagen present is type I [1,52]; type II has been found in the cartilage, skin and notochord of lampreys [53-55]; type V has been reported in carp and spotted mackerel [57] and in lamprey along with type IX [56]. Evidence for the fibre-forming type III collagen common in mammals has not been found in any investigation to date. Type IV has not been reported either, probably because it has not been specifically sought. Nonetheless, fish have quite clearly delineated basement membranes and it is reasonable to assume that type IV collagen is present. In the intramuscular tissue of teleost fish only types I and V have been demonstrated to be present.

Piez [57] was the first to demonstrate the presence of three different α-chains in the skin of cod and more recently they were also reported in rockfish [58]. Kimura [52] and Kimura and Ohno [59] found the $\alpha 1\alpha 2\alpha 3$ heterotrimer of type I collagen in the skin of carp and alaska pollock, whereas the swimbladders of these species contained the dimer $\alpha 1_2\alpha 2$. This indicated tissue-specific localisation of the two molecular forms. The three different α-chains in the type I collagen of the skin of fish from ten different orders was reported, but the occurence was not consistent within an order. The widespread occurrence of three different α-chains led the authors [60] to suggest that the gene for the $\alpha 3$ chain may be universally present in teleosts, having arisen about the time of the adaptive variation of the bony fish, but that it may be quiescent or only poorly expressed in some species. In contrast, the collagens of the lower vertebrate species, such as lamprey and shark, do not exhibit the $\alpha 3$ chain at all. The occurrence of the $\alpha 1\alpha 2\alpha 3$ trimer in the type I collagen from the skin of the blue grenadier was also reported [61]. Within the muscles of fish, studies of the type I collagen of the myocommata indicate that eel and mackerel have the $\alpha 1\alpha 2\alpha 3$ heterotrimer, that saury contains only $\alpha 1$ and $\alpha 2$ chains and that carp and chum salmon seem to possess two different heterotrimers, with $\alpha 1_2\alpha 2$ as a major component and $\alpha 1\alpha 2\alpha 3$ as a minor component [62].

The presence of type V collagen in the white muscle of carp in a higher proportion than it occurs in mammalian muscle has recently been reported [56]. Electrophoresis of the fractions, after treatment in reducing conditions with 2-mercaptoethanol, did not change the band pattern, indicating the absence of reducible thiol bonds and hence the absence of type III collagen. Further studies showed the presence of type V in lizard fish, japanese eel, sturgeon, spotted shark and lamprey, suggesting widespread occurrence of type V in both elasmobranchs and teleosts [63]. The relative concentration of type V collagen to type I was higher in the endomysial fraction than in the myocommatal fraction from carp and spotted mackerel. Both the type I and the type V were less soluble in the endomysial fraction than in the myocommatal fraction. Three distinct chains of type V were reported to occur in the molecular forms $\alpha 1_2\alpha 2$ and $\alpha 1\alpha 2\alpha 3$. A higher proportion of the heterotrimer was found in the endomysium in comparison to the myocommata. This recent

evidence indicates that, not only can there be differences between major tissue groups (e.g. muscle, skin or swimbladder), but that differences in the chain structure can occur within different domains in the one tissue. These differences in chain composition are likely to result in slightly different properties and stabilities in the collagen.

In fish muscle, type V collagen probably plays a role similar to that of type III in mammalian muscle in that it forms copolymers with type I and acts to control fibril diameter [64-66]. Collagen fibrils of different diameters are to be found in the myocommata near muscle fibre ends and in the invaginations of the myotendinous junction. Borresen [67] developed a method for preparing the muscle cell envelope from cod (*Gadus morhua*). These envelopes were tubular structures with an outer three-dimensional network of fibres (30-60 nm in diameter) and an inner membrane about 2000 nm thick. Further work using this method showed that the cell membrane was composed of three layers [49]. The innermost layer, the sarcoplasmic membrane, was 8-16 nm thick. The middle layer, the basement membrane, was 50-70 nm thick and the outer layer, which was mostly collagen, was approximately 600-1100 nm thick. This collagen was shown to be type I, possessing a chain structure $\alpha 1_2 \alpha 2$ containing the acid- and heat-stable cross-link hydroxylysino-5-keto-norleucine.

Collagen content and texture

The content of collagen in fish muscle varies considerably from species to species and is found in increasing proportion in the tail region. In the main edible portion, concentrations of 0.3% to 3% are common [1,52,68], but even within species there is seasonal variation as the body reserves are depleted during spawning and migration. Since muscle is not conserved there is greater reliance on the connective tissues to hold the fish together.

Figure 1. Pre-rigor blue grenadier muscle. Fine collagen fibres (**C**) connect the muscle fibres (**F**) to the myocomma (**M**). Bar 100 μm.

Figure 2. Muscle fibre end from a pre-rigor fish showing collagen-filled (**C**) grooves and folds bounded by the basement membrane. Bar 5 μm.

Figure 3. Pre-rigor fish at a muscle fibre end with an elongated collagenous invagination penetrating into the myofibrils. Bar 5 μm.

Figure 4. Muscle fibre base showing fine filamentous fibres (**arrow**) extending from the terminal segment to the inner surface of the sarcolemma (**S**). Bar 2 μm.

Figure 5. Cross-section of a groove at the muscle fibre end showing well-defined sarcolemma (**S**) and basement membrane (**arrow**). Bar 500 nm.

Figure 6. Detail of Figure 5 showing fine connections (**arrow**) between collagen fibres, in cross-section, and basement membrane. Other connections across the *lamina lucida* between the basement membrane and the sarcolemma and between collagen fibres are evident. Bar 100 nm.

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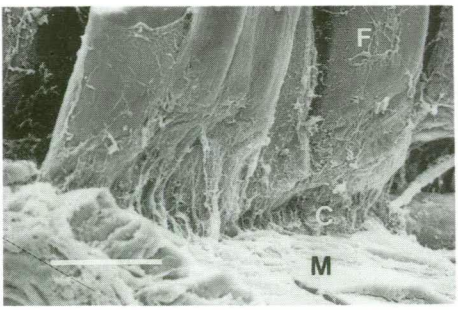

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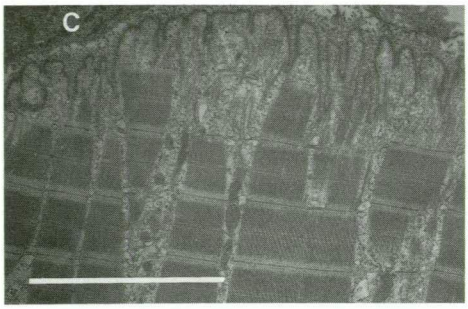

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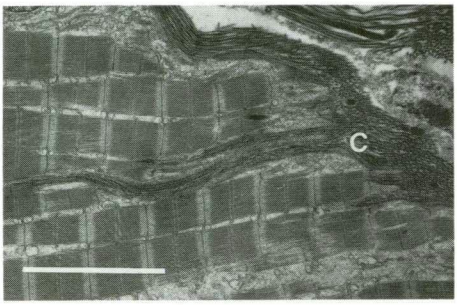

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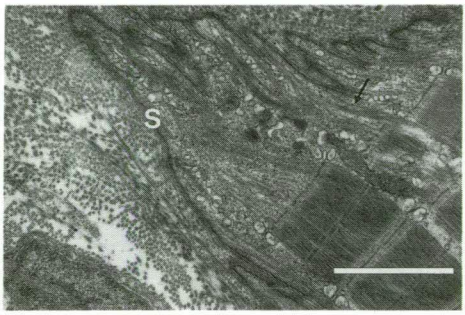

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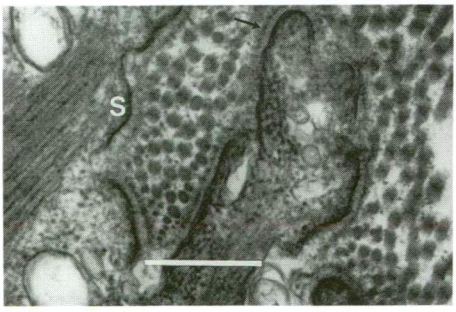

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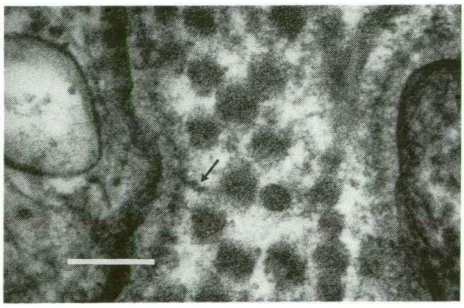

Figure 6.

Collagen content should affect the textural properties of the muscle but this relationship is not straightforward because of the seasonal turnover and other species effects. Sato *et al.* [68] investigated the collagen content and the texture of twenty two species of fish and concluded that there was a broad relationship between collagen content, swimming motion and the raw texture of the flesh prepared for sashimi. A high collagen content (near 2%) meant that the species were too tough for sashimi. Hatae *et al.* [69] also reported a significant correlation between collagen and raw texture. In cooked fish, the reverse is true and the collagen softens and the myofibrillar component toughens to become the dominant component [70]. Indeed, a model has been proposed to explain textural differences between species on the basis of fibre diameter and the amount of coagulated sarcoplasmic material that sticks the fibres together and impedes them sliding across on another when chewed [71]. Other workers have found no relationship between collagen and the raw texture in a limited number of species [72].

It is well established, that in mammalian tissues, collagen cross-linking increases with the age of the animal and that the tissue increases in toughness. Because of the seasonal changes occurring in most fish species, this relationship is less clear. It is not just the amount or proportion of collagen present but the degree to which it is crosslinked that affects texture. Montero and Borderias [73] measured collagen content, the proportions of α, β and γ chains and the shear force in the muscle of trout (*Salmo irideus* Gibb) from four different size (age) groups. Although there was a higher proportion of connective tissue in the oldest fish, it had slightly higher acid solubility and fewer cross-links and did not give higher shear strength values. It is generally regarded that there are higher levels of connective tissue near the tail region [6] and this was confirmed recently for trout and hake, with higher levels of connective tissue in the ventral than the dorsal portions for the trout [74]. Shear strength values were highest nearer the tail as was a higher proportion of insoluble collagen. The amino acid composition, chain structure and type of collagen from trout and hake were also characterised in samples from the skin, myotomes, fasciae and myocommata. The collagen from all sites in both species was mainly type I and type III was not detected. The amino acid compositions differed from mammalian sources and the fish skin collagen was less cross-linked than the collagen from the fish muscle.

POST-MORTEM CHANGES

During post-harvest storage, textural changes occur in many fish species long before they are spoilt. The result is that the flesh softens and gapes, trimming losses occur, products have a poorer appearance and are downgraded, and, in extreme cases, mechanical processing becomes impractical since the fillets fall apart in the skinning operation. Softening and gaping is a common problem in the merluccid hakes [75-78] and in the related species blue grenadier (hoki, *M. novaezelandiae*) [79]. Farmed species also have this problem [80]. It is clear that there are differences between related species and that circumstances affect whether gaping occurs. Whole cod stored in ice showed no gaping whatsoever, even after 6 weeks storage when the fish were thoroughly spoilt [81]. This lack of change was further borne out with measurement of the forces need to pull samples

apart at the myocommata. No change in cohesiveness was found for cod stored in ice for up to 26 days. Whole muscles loaded to failure fail at or near the myotendinous junction in the region between the cell membrane and the *lamina densa* of the basement membrane [82].

Post-mortem change attributed to changes in the collagen.

Unfortunately, there are few published investigations into the nature of the specific changes that occur. Using goldfish (*Carassius auratus*) as a model species, changes in the structure could be observed in post-rigor fish held in ice for 3-4 days, in comparison to pre-rigor fish [83]. Shrinkage and distortion of the myofibres, an increase in the extracellular space, loss of configuration of the endomysium and breakdown of the connective tissue occurred. Further changes to these structures occurred with subsequent storage. Similar structural changes in the flesh of the major carp (*Labeo calbasu*) during chilled storage were also observed histologically [84]. Disorganisation of the structure was evident when the samples were examined after 7 days. By 14 days, fissures appeared in the cells and partial disappearance of the connective tissue structure was apparent.

Texture, determined as shear force using the Kramer shear press, was related to an increase in heat-soluble collagen in rockfish stored in ice [85]. A significant decrease in the solubility of collagen from trout muscle occurs during rigor, followed by an increase in solubility during storage post-rigor [86]. The proportions of heat- and acid-soluble collagen increased during and after rigor while the levels of insoluble collagen and shear strength progressively decreased. Proteolytic activity increased post-rigor.

Changes in the myotendinous junction observed with SEM and TEM

The structure of the myotendinous junction of the blue grenadier did not change during rigor mortis, but after 8 days storage in ice the basement membrane and the collagen fibrils in the tubular invagination were degraded [48]. Progressive deterioration of the fine collagen results in detachment of the muscle fibre from the myocomma (Figure 7) and notable deterioration within the muscle fibre end leading to the production of vesicles (Figure 8). This is in accord with the results shown by SEM where the fine collagen fibres of the cell envelope are degraded (Figure 9) and muscle fibres are shown detached from the myocomma (Figure 10).

The progressive deterioration and disruption of the structure is consistent with it being attacked by enzymes. Collagen is normally considered to be quite a stable protein, yet these micrographs indicate digestion within a few days at 0°C. This implies that either these collagens are very susceptible to attack, or that the enzymes present are very active. Even if the extent of disruption seen in Figures 7 & 8 is partly an artefact of preparation, the structure must have been severely weakened to allow this to occur [46-48].

Use of antibodies to identify collagen fibre types and to detect changes

An immunogold method [29, Bremner unpublished] using an antibody to purified fish type I collagen [61] and protein A-gold complex was used to label the myocommatal collagen (Figures 11,12). The individual collagen fibres were not defined well enough to be able to state unequivocally whether, or not, they were labelled with the gold. Collagen *in situ* can be difficult to stain and, in these preparations, the normal post-fixation with

osmium could not be included since this would have destroyed the antigenic sites. Had the technique proved adequate, then antibodies to other collagen types would have been used to localize them in the tissue and identify the fine fibrils in the invaginations. These fibrils may be copolymers of more than one type with the minor component buried in the interior as occurs in other tissues [64-66] making them inaccessible for reaction with antibody unless suitably treated.

Although rockfish soften in storage and their collagen becomes more heat-soluble, no collagen breakdown products were detected by an antibody to bovine type I collagen when extracts were run on electrophoretic gels [87]. This antibody reacted with intact collagen, with cyanogen bromide peptides and with peptides produced by collagenase treatment of purified rockfish collagen. If the collagen was degraded, either the fragments were degraded so rapidly once they were released from the fibrils that they were lost in the extraction procedure, or they were of such nature that they did not react with the antibody. If the detected increase in heat-soluble collagen was due to another collagen type present in minor proportion, such as type V, then reaction of the fragments with the antibody could not be expected.

Proteases and collagenases

Collagen is degraded by a number of enzymes acting in concert, with the initial attack on undenatured molecules performed by a collagenase acting at a specific site, followed by other enzymes acting on the fragments [88]. Historically, the mammalian collagenases are defined by their ability to cleave the triple helical region of the native collagen molecule at a specific locus. The discovery of a broader range of collagens with globular

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Figure 7. Muscle fibre base has separated from the myocommata, the invaginations are empty and amorphous material occurs near the collagen fibres in blue grenadier stored 8 days in ice. Bar 1 μm.

Figure 8. Vesicles have formed in the ends of the myofibrils. The sarcolemma is mostly intact (**arrows**) but only degraded material remains in the invaginations. Bar 1 μm.

Figure 9. Disruption of fine collagen fibres (**C**) of muscle cell envelope, allowing muscle fibres (**F**) to part from myocomma (**M**) in spotted trevally stored 4 days in ice. Bar 100 μm.

Figure 10. Muscle fibre (**F**) completely detached from myocomma in blue grenadier stored 11 days in ice. Bar 100 μm.

Figure 11. Gold particles (15 nm diam.) label myocomma (**M**) of pre-rigor blue grenadier, with only a few random labels in the muscle fibre (**F**). Bar 1 μm.

Figure 12. Gold particles label myocommatal collagen fibres of rock cod fixed within 30 seconds of death. Bar 1 μm.

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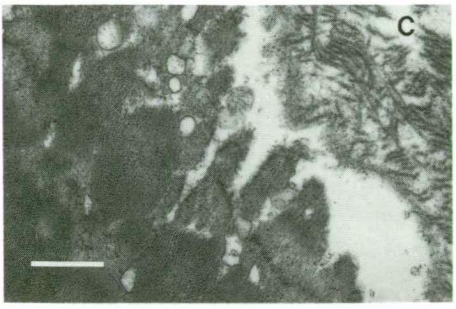

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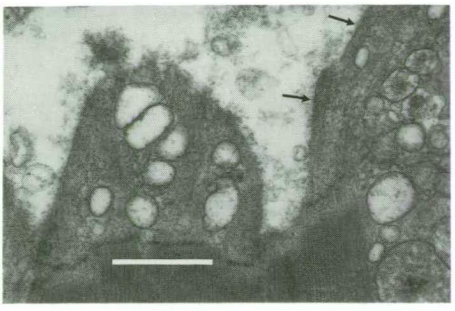

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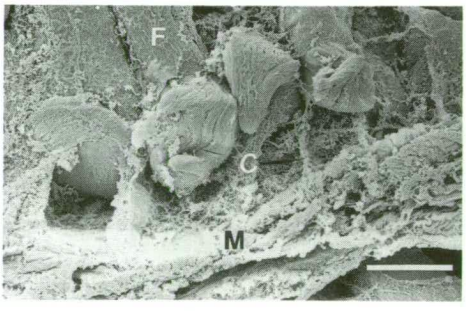

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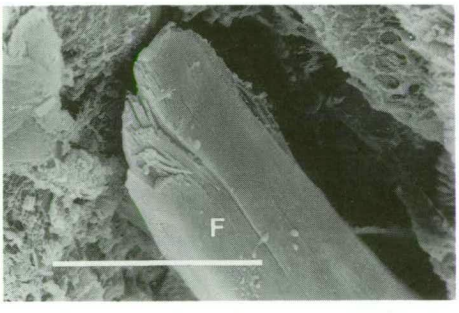

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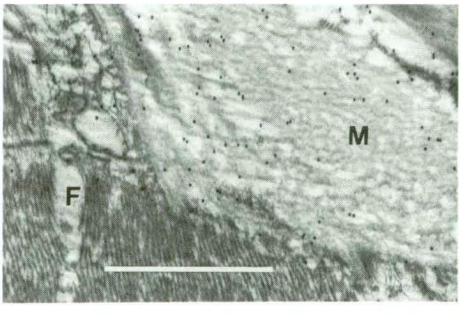

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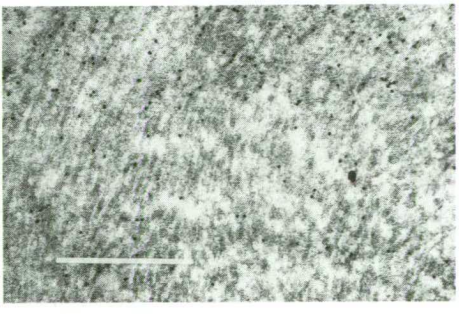

Figure 12.

domains and discontinuities in the helix has required that this simple definition be modified. Furthermore, serine proteases and a heterogeneous group of proteases can degrade some collagen types. Two main groups of collagenases have emerged - the 'classical' collagenases which degrade types I, II and III collagens and type-specific interstitial and basement membrane collagenases which attack types IV and V [89]. Collagenases are mostly metallo-endoproteases having a pH optimum in the range 7 to 8 which are activated and stabilised by calcium ions. Consequently, they are inhibited by metal chelators such as EDTA. They have been isolated from a wide variety of tissues, but not as yet from fish muscle tissue although collagenase from fish caeca and pancreas has been known for some years [90].

The collagenases that degrade types I, II and III collagen do not affect types IV and V collagen. Specific collagenases which degrade type IV have been isolated from tumours, but it can be degraded by other metalloproteinases including gelatinase, proteoglycanase, serine proteases, neutrophil elastase and mast cell chymase. Type V is attacked by metalloproteinases which are also gelatinases of a molecular weight greater than the classical collagenases [89,91].

Type V collagen isolated from bovine bone is susceptible to attack by trypsin at 35°C [92] at a site which may represent the natural target domain for cleavage *in vivo* and which is at the opposite end of the molecule to the site at which type I is attacked by collagenase. This indicates that, *in vivo*, separate enzyme systems are necessary for types I and V to be copolymers in the same fibril.

After synthesis, collagenases are secreted into the extracellular matrix and it is obvious there must be specific inhibitory mechanisms to prevent spontaneous tissue destruction. The collagenases, present in the tissue in latent form as zymogens, are activated by a wide variety of proteolytic enzymes such as trypsin, plasma kallikrein, cathepsin B, plasmin [93], but once activated they must be controlled. In human tissue, the main control mechanism seems to be through small cationic glycoproteins known as Tissue Inhibitors of Metallo Proteinases, or TIMP. They are ubiquitous and their relatively low molecular weight, 28.5 Kdalton, allows them ready access within the extracellular matrix where they play a major role in collagenase inhibition [89]. Other control mechanisms such as the α_2-macroglobulin molecule in plasma are too large to penetrate the tissue.

The basement membrane degrading enzyme, collagenase IV/V -gelatinase, is more easily released by kallikrein and becomes active in the extracellular space before other lysosomal proteinases are released [94]. Tissue kallikrein, a serine protease (specifically an arginyl esteropeptidase), also activates type I collagenase and is a likely candidate to perform these functions *in vivo*. It may also be an activating factor in post-mortem tissue. There are about twenty kallikreins which seem to be ubiquitous. So far they have only been reported in the skeletal muscle of the rat. The lysosomes in fish muscle are commonly associated with the connective tissue [94] so that cathepsin B, which could also activate this collagenase, would be released by post-mortem disruption of the lysosomes. The concentration of calcium ions would not be a factor limiting collagenase activity due to leakage from the sarcoplasmic reticulum. Indeed, it has been shown that the introduction of Ca^{++} into fibroblasts promotes a cascade of proteolytic events culminating in activation of collagenase [93].

The question is whether these mechanisms are present in fish tissue and whether they

are active at post-mortem pH and at chill temperatures. The type IV/V basement membrane collagenase isolated from human leukocytes has a pH optimum between 7 and 8, with about 50% of this optimum activity at pH 6 [96]. No information relating to kallikrein in fish has been found but it is likely to be present and, since it is active at physiological pH, it is likely to retain activity at post-mortem pH. However, it too is subject to the effect of inhibitors in the serum, similar to trypsin inhibitors. Cathepsin B may not be very active at post-rigor pH.

Freezing damage

Although a comprehensive coverage of frozen storage changes is outside the scope of this paper, it is pertinent to note here some of the recent observations that changes in the collagen contribute to changes in texture which occur in frozen stored fish.

Both trout (*S. irideus*) and hake (*Merluccius merluccius* Linnaeus) collagens exhibited a decrease in the proportion of α-chains and a concomitant increase in γ- chains during frozen storage at -18°C after only 25 days, with further changes occurring progressively. The amount of insoluble collagen in the hake samples also increased with the period of storage [97]. This increase in collagen insolubility in hake flesh and decrease in the percentage of heat-soluble collagen with period of frozen storage was confirmed in further studies and it was suggested that aggregation of hake collagen was due to reaction with formaldehyde produced by breakdown of trimethylamine oxide [74,98]. This is also consistent with the other reports [2,100] that suggested that the remnants of the sarcoplasmic reticulum could serve as a glue to cement the fibres to produce a tougher product, the opposite of the situation that occurs in chilled storage.

Walton and Gill [50] suggest that the collagen layer of the endomysium may be the cementing agent responsible for cell strength. They found that the level of salt soluble collagen of Atlantic cod (*G. morhua*) decreased with frozen storage and high molecular weight complexes of both myocommatal and endomysial collagens were formed. They also suggested the possibility of complexes between sarcoplasmic proteins and the endomysial collagen. If such complexes were not denatured during cooking they would increase the toughness of the flesh in a manner consistent with the model proposed by Hatae *et al.* [71].

Changes in the muscle

It has long been known that fish flesh has greater catheptic activity than mammalian muscle [101] and that it possesses higher concentrations of enzymes responsible for proteolysis and amino acid metabolism [102]. Neutral proteases are found in many species [103]. Recent work, stimulated by the need to understand the modori phenomenon during the setting of surimi gels, has uncovered a number of proteases, mostly serine proteases, bound to both the sarcoplasmic and myofibrillar fractions of the muscle in a variety of species [104-107]. These enzymes are active during the heating step in forming fish gels and it is not clear what role they may play in the live fish, but it seems unlikely that they are active in the same way in chill stored fish. Cathepsin L from lysosomes has been implicated in the extensive muscle softening observed in chum salmon [108] and has been shown to increase in activity in the muscle of ayu (sweet fish, *Plecoglossus altivelis*) as the fish approaches maturity. Lysosomes have been located within fish muscle cells mostly

concentrated at the periphery [95]. These lysosomes break down post-rigor. Other cathepsins (such as cathepsins A and B) require a pH lower than occurs in post-mortem fish muscle. Even the pepstatin-sensitive cathepsins (mainly cathepsin D) are not active at pH 6.5 [109]. However, in fish such as tuna, where the pH is often lower than this, it is likely to be one of the factors responsible for the honeycomb problem in the flesh that is sometimes encountered [110]. White croaker (*Micropogon opercularis*) contains an endogenous serine proteinase which degrades the cytoskeletal network [111,112], which initiates protein turnover *in vivo* and which can completely disrupt the myofibrils (at 37°C) and degrade the major proteins [113]. When croaker were stored at 0°C for 7 days, after dipping in azide to prevent bacterial growth, only minimal changes were found in the major proteins of the myofibril. There was considerable breakdown of nebulin, a major cytoskeletal protein of the trabecular network [111]. Desmin, troponin and Z lines were shown to be stable under these conditions, whereas these entities are known to be degraded in post-mortem storage of beef muscle along with titin and alpha-actinin [114]. The connectin fraction of carp muscle also exhibits change when extracted from fish that have been stored chilled for 7 days at 25°C [115].

There are two Ca^{++}-activated neutral proteinases that are considered to be responsible for post-mortem softening of sheep muscle [116,117]. Calpain I requires only 0.1 mM Ca^{++} for activation while Calpain II is active at higher concentrations of calcium. Both enzymes are subject to inhibition by calpstatin. Calpain II has been found in both carp (*Cyprinus carpio* [118] and tilapia (*Tilapia nilotica* x *Tilapia aurea*) [119]. Calpstatin and a trypsin inhibitor is also present in carp [120].

OVERALL MECHANISMS

Hatae *et al.* [121] examined electrophoretic patterns of sarcoplasmic alkali-soluble and stroma proteins of five species of fish stored for 14 days at 4°C as well as physically measuring a range of textural properties using a General Foods type Texturometer. They concluded that post-mortem softening of the flesh was "more affected by the changes of the muscle structure than by the changes of the component proteins". Similarly, Toyohara and Shimizu [122] stated that "the weakening of muscle may be explained not as a proteolytic breakdown of myofibrils, but as a decomposition of the muscle structure". These observations, in conjunction with those presented here, lead to the conclusion that integrity is due to minor components which link the major components together and that proportionately small changes in these minor components can have disproportionately large effects on the structure, and hence the texture, of the flesh.

There are three possible explanatory mechanisms for post mortem softening:-

(a) some major components within either the myofibrils or in the extracellular connective tissue degrade, or
(b) links, bonds and connections that organise and stabilize the structure between the muscle components degrade, or
(c) both of these mechanisms occur.

Hypothesis (b) has considerable attraction as an explanation of the changes occurring early in the storage period before they are sufficiently gross to be detected by such means as alteration in an electrophoretic pattern.

CONCLUSION

The structure of fish flesh is complicated and intricate and the interrelationships between all the components is, as yet, obscure. Topics for further research include:-

- Establishing the type and location of minor collagens in fish muscle
- Establishing the nature of the fine connections in the myotendinous junction
- Determining the presence, location and activity of kallikrein
- Extracting and characterizing collagenases and proteases that act on the extracellular matrix
- Further identification of enzymes that degrade the cytoskeleton and their occurrence in commercial species
- Examining the use of suitable inhibitors to both extra and intracellular enzymes
- Exploring further uses of these enzymes in food processing.

The initial softening in texture that occurs in many species is due to changes in the cytoskeleton and in the collagen produced by enzymes acting on structural links and bonds. Internally, serine proteases and cathepsin L are the most likely agents, while the extracellular matrix collagenases active against collagen types I, IV and V are implicated as initiators of breakdown.

There is a need for considerably more work to elucidate the components of fish muscle and the processes of deterioration. Only then can specific methods to minimise these effects be placed on a rational basis.

Acknowledgment

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Muscle Fiber—Connective Tissue Junctions in the Fish Blue Grenadier (*Macruronus novaezelandiae*). A Scanning Electron Microscope Study

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ABSTRACT

The junctions between the muscle fibers and the connective tissue of blue grenadier (*Macruronus novaezelandiae* Hector) were studied using scanning electron microscopy. In fish before, during, and just after rigor mortis, the muscle fibers are attached to the myocommatal connective tissue sheets by fine collagenous fibrils. After chilled storage these fibrils deteriorate and the muscle fibers gradually detach from the myocommata. It appears that the fibrils are destroyed by endogenous collagenases and/or other proteinases.

INTRODUCTION

THE FLESH of teleost fish is constructed of adjacent muscle blocks called myotomes separated from each other by collagenous sheets called myocommata (Nursall, 1956). The myotomes on both sides of the axial skeleton each take the form of double cones which fit together along the long axis of the fish body to give the appearance at the surface of a series of W-shaped sections resting on their sides (Fig. 1). Within each myotome the muscle fibers (myomeres) run approximately parallel to each other but at varying angles to the myocommatal sheets to give the necessary moment for swimming during contraction. The myocommata are connected internally to the skin and the skeletal system and are also linked to the membrane dividing the fish into epaxial and hypaxial planes and to the median vertical septum. Further details of the structure and function of the system as a whole can be found in Nursall (1956) and Alexander (1969).

The junctions between the myomeres and the myocommata are reported to be formed by fine collagenous processes which have their origin in the myocomma and which then form the sheaths surrounding each muscle fiber (Love, 1970, Love et al., 1969). The microscopy of fish flesh has been reviewed by Howgate (1979). Most electron microscopy on fish muscle has been done on the fibers themselves using the transmission mode (TEM) (Jarenbäck and Liljemark, 1975; Liljemark, 1969). Relatively few reports have included scanning electron microscope (SEM) data. Almaas (1982) published SEM studies on isolated cod muscle cell envelopes and Schaller and Powrie (1971) published SEM micrographs of trout muscle fibers at various stages postmortem. Despite the importance of the myomere/myocommata interface both in vivo and postmortem, its study has been neglected. One reason for this could be the difficulty in preparing suitable samples.

Although there are differences in the gross organization between fish muscle and that of terrestrial animals, in which the muscles terminate in tendons which attach to the skeletal framework, the ultrastructures have been found to be similar. For this reason, and because no text describing fish muscle specifically could be found, the terminology applied to muscle by Gould (1973) is used in this paper. Thus the term plasmalemma (sarcolemma) applies to the cell membrane of the muscle fiber. External to this is the basal lamina (basement membrane) to which are attached the collagenous (reticular) fibrils of the endomysium that run into the surrounding perimysium which in fish is continguous to the myocomma.

Blue grenadier, known in New Zealand as hoki, is an increasingly important trawlfish (Patchell, 1982) related to the merluccid hakes (Bremner, 1980; Nelson, 1976). Freezing of fish in rigor is well known as a cause of gaping, the name given to the phenomenon when the connective tissues fail to hold the muscle blocks (and fibers) together (Love et al., 1969). In some species such as blue grenadier the incidence of gaping occurs progressively postrigor even during chilled storage. Fillets taken from these fish prerigor, or soon after rigor has resolved, generally do not gape whereas fillets from fish stored longer than about 4 to 5 days on ice, gape readily either when first cut or during handling. Blue grenadier is thus a suitable subject for the study of postmortem changes in the myomere/myocommata junction. This paper reports the development of techniques used to expose the myomere/myocommata interface for SEM and shows the results obtained with blue grenadier (*Macruronus novaezelandiae* Hector) muscle prerigor, during rigor, postrigor and after chilled storage.

MATERIALS & METHODS

BLUE GRENADIER from a number of sources were used in this study. Muscle samples were taken from the anterior dorsal area at approximately the 13th, 14th and 15th myotome, counting from the head.

Fish L were sampled immediately after catch on board the Tasmanian Fisheries Development Authority vessel FV 'Challenger', and were iced and used to study changes occurring during rigor mortis and subsequent storage in ice (Table 1). Samples from these fish were fixed in 3.2% glutaraldehyde in 0.16M phosphate buffer containing 2% NaCl and 0.5% sucrose to increase osmolarity (Kryvi, 1977). They were sent in fixative to the DSIR Mt. Albert Research Centre, Auckland, N.Z. After a short wash in buffer, samples were frozen, in dichlorodifluoromethane (CCl_2F_2) at −158°C and transferred to liquid nitrogen (LN_2) at −196°C. Frozen samples were fractured under liquid nitrogen by striking a cooled sharp blade strategically positioned over the sample. The frozen samples were then freeze dried in a modified vacuum evaporator with a copper block system cooled by LN_2 (Ladd Research Industries, Vermont, USA.)

Table 1—Sampling times for blue grenadier caught by F.V 'Challenger', April 1983

Sample	Time sampled (hr)	Time elapsed	Comments
L1	1909	0	Fresh prerigor fish straight out of the net.
L2	2045	66 min	Fish starting to go firm, packed in ice.
L3	2248	3 hr 40 min	Fish firm, in rigor.
L4	1100	4 day 16 hr	Post rigor
L5	1000	8 day 13 hr	Post rigor
L6	1000	10 day 13 hr	Fish starting to deteriorate

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Fish M and Fish N, obtained from Sanfords Ltd., Auckland, N.Z. and from Sealords, Nelson, N.Z. respectively were held in ice. Samples from these fish were fixed, freeze-fractured then either freeze-dried or dehydrated in an ethanol series and then dried at the critical point from CO_2 (Samdri 780, Tousimis Research Corp., Maryland, USA.).

Fish O were frozen pre-rigor on board the N.Z. Ministry of Agriculture and Fish research vessel FV 'James Cook', and samples were held frozen at −30°C at the Mt. Albert Research Center. Samples were taken by allowing the fish to rise in temperature to the point where they could be cut, refreezing the sample in LN_2 or CCl_2F_2 before fracturing and subsequent freeze drying.

Solvent treatments

Samples from Fish O were placed in stainless steel mesh boats in glass vials and using gentle agitation on a rotating wheel (6 rpm) at 20°C were treated with various solvent solutions for either 1, 4 or 6 hr. These treatments were chosen to preferentially solubilize or disperse different flesh components in order to expose inner surfaces. The solutions used were: 1M calcium chloride to extract soluble components of the connective tissue (Robert and Comte, 1968); 6M urea to disrupt hydrogen and nonpolar bonds (Tsuchiya et al., 1980); 1% sodium dodecyl sulphate (SDS) as a secondary bond breaker to disrupt hydrogen and hydrophobic bonds (Connell, 1965); buffered 0.5M KCl to break ionic bonds (Anderson and Ravesi, 1968); or 1 mg/mL trypsin solution pH 7 in 0.14M NaCl to effect proteolysis. Treatment with distilled water was included as a comparison.

After fixation (Kryvi, 1977) these treated samples were dehydrated in an ethanol series then critical-point dried. None of these treated specimens was freeze fractured. After drying, all the prepared samples were mounted on stubs, sputter-coated with gold and examined with a Phillips 505 scanning electron microscope at the Mt. Albert Research Center.

RESULTS

CRITICAL-POINT DRIED SAMPLES were often too distorated to give good results; excessive folding of the samples could be seen by the naked eye. Attempts to restrain the critical point dried samples reduced visible folding but did not significantly reduce the distortion of muscle fibers and myocommata when viewed in the SEM. Freeze-dried samples remained similar in gross visual morphology to unprocessed samples and when viewed in the SEM showed little sign of folding or displacement of fibers or myocommata.

General description

The myomeres butt on to the myocomma (Fig. 2) to which they are attached by a fine network of collagenous processes that proceed from the myocomma to form the outer sheath on each myomere as noted by Love et al., (1969). The myomeres appear to fit into sockets on the myocomma and where the myomeres have been completely removed these sockets are evident (Fig. 3). The ends of

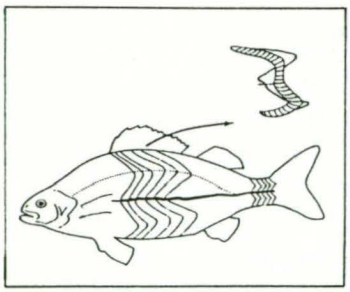

Fig. 1—Schematic diagram of the layout of fish muscle, showing the interlocking W-shaped myotomes separated by myocommata and a single myotome showing the complex double cone shape and the approximate orientation of the myomeres at the surface. Adapted from Ellis et al. (1978).

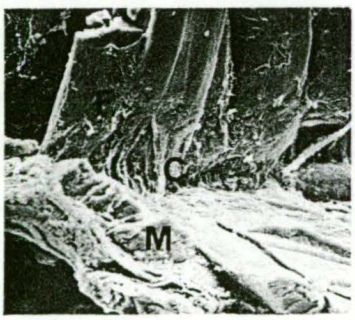

Fig. 2—Fine collagen fibrils C connect the myomeres F to the myocomma M. Fish L1 prerigor. Bar 0.1mm. Magnification: 269X.

Fig. 3—Sockets on the myocomma. Fish M. Bar 0.1 mm. Magnification: 263X.

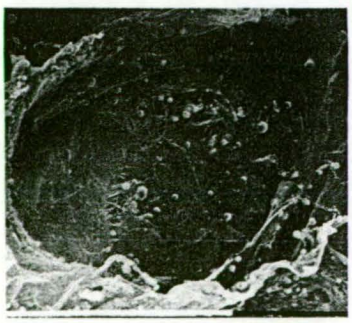

Fig. 4—Interior view of a socket on the myocomma. Spherical blobs may be remnants of former connections to the myomere. Fish M. Bar 0.1 mm. Magnification: 850X.

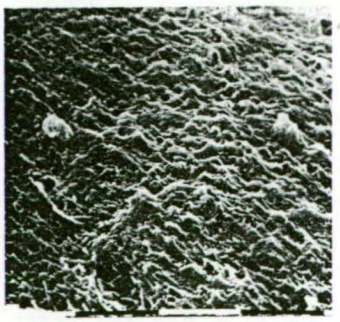

Fig. 5—The base of a fiber detached from the myocomma. Note raised points of former attachment. Fish N. Bar 0.01 mm. Magnification: 2880X.

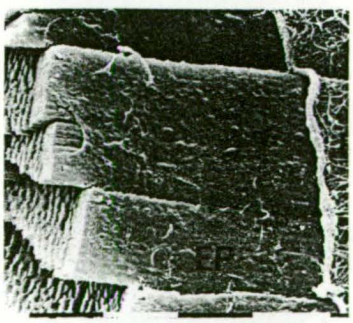

Fig. 6—Fractured fiber end with perimysium P peeled back and endomysium/plasmalemma EP intact. Fish L1. Bar 0.01 mm. Magnification: 2550X.

detached fibers (Fig. 5) and the inside of the sockets (Fig. 4) show either raised surfaces or small blobs of material that may previously have formed points of attachment.

Changes during rigor mortis

The structure of the pre-rigor muscle fiber with the typical elongated myofibrils near the periphery of the fiber and the more polygonal myofibrils in the interior is visible in Fig. 6. The connective tissue network of the perimysium has 'peeled' back from the fractured surface to reveal the plasmalemma and endomysium on the fiber surfaces. Viewed from different angles (not shown here) regular elevations running transversely across the fiber stood out in relief beneath the plasmalemma as observed in trout muscle by Schaller and Powrie (1971). A general view of typical fiber surface is shown (Fig. 7).

As the fish entered rigor the processes attaching the myomeres are still evident but in samples taken from fish in rigor, interfaces between the muscle fibers and myocommata are indistinct with the ends of fibers from adjacent myotomes jammed hard together (Fig. 8) and the transverse elevations are still evident (not shown here).

After 5 days storage in ice, when the fish are in the postrigor state the connections between fibers and connective tissue are less evident (Fig. 9) and at the fiber surface both the endomysium and plasmalemma layer beneath show signs of degradation (Fig. 10).

In samples taken after further storage in ice, muscle fibers free of connection to the myocommata occur (Fig. 11, 12) and the general area is often degraded and covered in debris (Fig. 12, 13, 14). The T system is still evident on the fibrils (not shown here) but the perimysium has generally deteriorated (Fig. 14, 15) although under the debris a fine reticular network is still evident.

Effects of solvent treatments

The nature and extent of dissolution or disruption by the solvents is fast and uncontrollable and some preparations are either badly disintegrated or amorphous or uninformative after treatment for only 1 hr.

The powerful action of urea causes nonspecific degradation noticeable in the myocommatal sheets from which 'tufts' of fibers readily break away (Fig.16). Treatment for

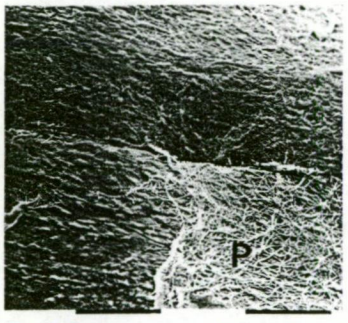

Fig. 7—Detail of muscle fiber surface of prerigor fish showing intact perimysium. Fish L1. Bar 0.01 mm. Magnification: 2550X.

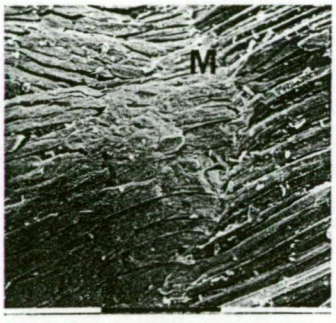

Fig. 8—Fish muscle in rigor. Interfacing myocomma M between adjacent myotomes runs from top to bottom. Fish L3. Bar 1 mm. Magnification: 41X.

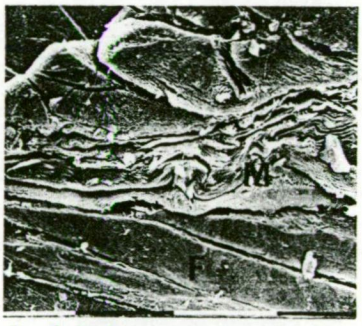

Fig. 9—Myofiber connective tissue interface in post rigor fish 5 days in ice after catch, no fibrous onnections between myofibers F which slant upwards from bottom right to myocomma M which runs across. The crumpled layers of myocommatal sheets are obvious. Fish L4. Bar 0.1 mm. Magnification: 348X.

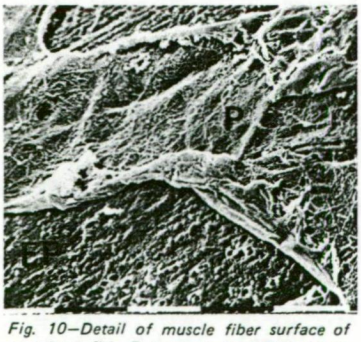

Fig. 10—Detail of muscle fiber surface of postrigor fish 5 days in ice after catch. Both perimysium P and endomysium/plasmalemma EP deteriorating. Fish L5. Bar 0.01 mm. Magnification: 2540X.

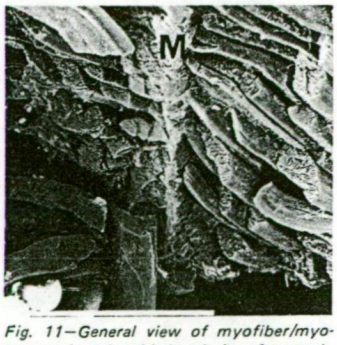

Fig. 11—General view of myofiber/myocomma junction 11 days in ice after catch. No connections between fibers F and myocomma M, which is situated vertically in centre. Fish L6. Bar 1 mm. Magnification: 79X.

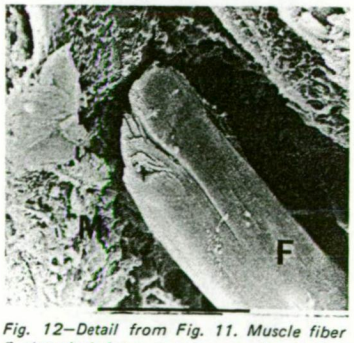

Fig. 12—Detail from Fig. 11. Muscle fiber F detached from myocomma M, no fine connections. Bar 0.1 mm. Magnification: 517X.

1 hr in a 1% SDS solution strips the outer layers of the myocommatal sheets leaving the larger, stronger fibers and the whole endomysium and plasmalemma, too, is attacked, apparently across the I band. Longer treatment with SDS (4 hr) accentuates this dissolution which runs transversely across the myofibers rather than between the fibrils (Fig. 17). It was also noted that after SDS treatment the samples did not turn yellow in the glutaraldehyde fixative. Yellowing normally occurs due to reaction of the glutaraldehyde with free amino groups.

Trypsin also causes general deterioration of the collagen fibrils and etches the collagen surfaces (Fig. 18). The overall degradation caused by 1M $CaCl_2$ gives no useful information. Muscle fibers dissolve readily in KCl but Fig. 19 shows the remnant of a fiber where it joins the myocomma. The fiber itself is swollen and hydrated and the perimysium is peeled back. With other preparations the endomysium and the plasmalemma disintegrates and apparent dissolution of the I band across the fibrils can be seen.

Exposure to water seemed also to result in disruption of the transverse system which collapsed below the surface of the myofibrils (Fig. 20). Sarcoplasmic proteins are water soluble and it is likely that they were extracted. Prolonged exposure to water for 6 hr caused more general disintegration in some fibers both across and between the myofibrils (Fig. 21).

DISCUSSION

FREEZE FRACTURING followed by freeze drying was the best of the methods tested for preparing samples to expose fiber/myocommata junctions. The use of solvents to preferentially remove some muscle components to expose underlying structures was hard to control.

The muscle fibers of blue grenadier bed into the myocommata and are attached to it by continuations of the collagenous fibrils (Fig. 2, 19) as reported for cod by Love et al. (1969). In terrestrial animals studies on the myotendonous junctions of the rectus abdominis muscle of the bull frog (Ajiri et al., 1978), the tongue of the guinea pig (Demmel et al., 1979), the mitral complex of the canine (Fenoglio et al. 1972), the papillary, tongue, diaphragm and gastrocnemius muscles of the guinea pig (Hanak and Böck, 1971) and the extensor carpi radialis longis and brevis muscles of the mouse (Trotter et al., 1981) have all shown the presence of small filaments, named microfibrils (Hanak and Böck, 1971), linking the muscle fibers to the connective tissue. Trotter et al., (1981) indicated that these microfibrils act as mechanical links at this junction. It seems clear from the present work that there are no connections along the course of the fiber and, in agreement with the observations reviewed by Gould (1973), the plas-

Fig. 13—A different area to that shown in Fig. 11, several fibers still show some attachment to the myocomma M and the area has degenerated. Fish L6, 11 days in ice after catch. Bar 0.1 mm. Magnification: 188X.

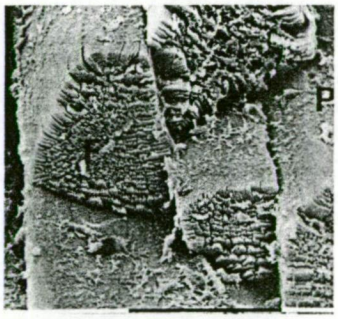

Fig. 14—General view of muscle fiber F ends at a fractured surface. Fish L6 stored 11 days in ice after catch. Note breakup of perimysium P cf. Fig. 6. Bar 0.1 mm. Magnification: 515X.

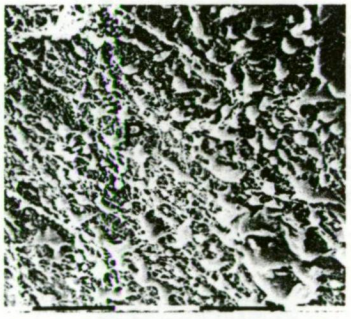

Fig. 15—Detail of perimysial P surface of a muscle fiber shown in Fig. 14 cf. Fig. 7. Bar 0.01 mm. Magnification: 2875X.

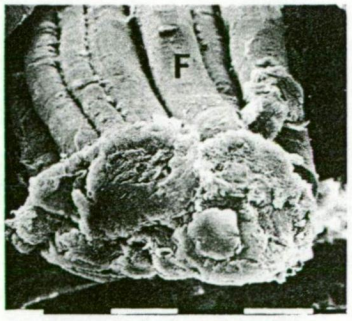

Fig. 16—Sample treated in 6M urea (1 hr). Tuft of fibres F loosened free of the myocomma. Fish O. Bar 0.1 mm. Magnification: 231X.

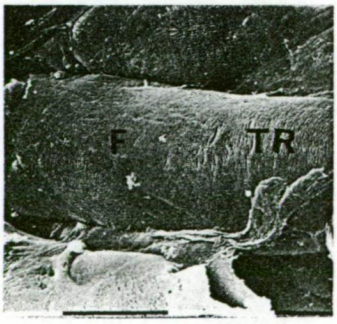

Fig. 17—Muscle fibers F treated 4 hr in 1% SDS. Note transverse fissures TR Fish O. Bar 0.1 m. Magnification: 359X.

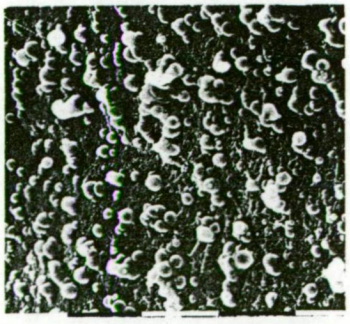

Fig. 18—Spherical blobs on the surface of myocomma from a sample treated with trypsin 1 mg/ml (1 hr). Fish O. Bar 0.01 mm. Magnification: 2620X.

malemma is independent of the perimysial (or reticuloendothelial) fibers (Fig. 6).

During chill storage the attachments between myomeres and the myocomma progressively deteriorate (Fig. 11, 12, 13) and indeed the whole sarcolemma deteriorates (Fig. 13, 14, 15). There are similarities to the degradative effects of cathepsins and collagenases on beef muscle reported by Eino and Stanley (1973).

The deterioration on the fibers (Fig. 5, 13) and the sockets (Fig. 4) is similar to that obtained with trypsin treatment (Fig. 18) and at times with KCl solutions (not shown). It is inferred from this that the progressive breakdown during storage is due to enzymic activity and that the enzyme(s) responsible must have powerful collagenolytic properties against the type of collagen of the perimysial fibrils. There are at least five genetically different types of collagen in connective tissues (Asghar and Henrickson, 1982; Eyre, 1980; Miller, 1982; Sims and Bailey, 1981) differentiated by the composition of the three helical chains which make up the molecule. Often more than one type of collagen is present in a tissue (von der Mark, 1981). Morphologically the collagen fibrils shown here resemble the perimysial felt work reported for rat, rabbit, cattle and sheep by Rowe (1981).

Almas (1982) isolated 'muscle cell envelopes' from cod muscle and concluded from the results of electrophoresis, chromatography and amino acid analysis that the collagen present was type I. This is in contrast to the results obtained on cow muscle by Bailey et al. (1979) who found that the perimysium was composed of a mixture of types I and III. The type III collagen appeared as a fine network. It seems obviously necessary to apply immunofluorescence or enzyme-linked immunoperoxidase techniques to fish muscle to ascertain the precise location and nature of the various collagen types present.

Different types of collagen have different susceptibilities to enzymic attack, for example types I and III are less resistant than type II (Harper, 1980). It is generally regarded that the initial attack on intact collagen is by specific collagenases and that once the initial cleavage of the helical polypeptide chains has been achieved other nonspecific proteinases can pursue the attack (Harper, 1980). Collagenases are ubiquitous and are closely associated with all types of collagen in a wide variety of tissues (Montfort and Perez-Tamayo, 1975) where they are probably bound to the collagen in the living animal (Pardo and Perez-Tamayo, 1975). Because of this association and their activity it is assumed that these collagenases function in the metabolism of the connective tissues. They are Zn^{2+} containing metalloenzymes that for full activity generally require Ca^{2+} (Cawston and Murphy, 1981); the collapse of the sarcoplasmic system would release the necessary calcium to stimulate the enzyme. Other collagenases and cathepsins with proteolytic activity are present in lysosomes in adjacent locations within the muscle fibers (Canonico and Bird, 1970; Reddi et al. 1972; Steiner et al., 1984). Fish muscle is known to have much greater catheptic activity than mammalian muscle (Siebert, 1958; Siebert et al., 1965).

Catheptic enzymes probably play a secondary role to the collagenases which are present as zymogens or proenzymes in the living tissue (Harper, 1980). Their activity is controlled by factors other than their levels alone (Montfort and Perez-Tamayo, 1975) and a number of inhibitory mechanisms have been found involving sarcoplasmic proteins (Hjelmeland and Raa, 1980), blood serum proteins and the plasma glycoprotein α_2-Macroglobulin (Starkey and Barrett 1977). Serum also contains inhibitors for cathepsins other than proteins; ATP also acts as an inactivator (Reddi et al., 1972). After death these control mechanisms would gradually cease to be effective.

The appearance of the myofibrils of blue grenadier is similar to that reported for trout (Schaller and Powrie, 1971) and cod (Almas, 1982). After 1 hr treatment with water (Fig. 20) they bear a striking resemblance to the dorsal muscle of rainbow trout stored 4 days postmortem at 3°C (Schaller and Powrie, 1971) where the transverse elements have collapsed below the surface of the fibrils. It is evident that water removes sarcoplasmic components (Fig. 20, 21) and thus some of the effects of the solvent systems used here may be due in part to the solvent effect of water alone. Water may have other effects such as removal of inhibitors and it is known that hypotonic solutions can rupture lysosomes (Dean and Barrett, 1976). This may have implications for the thawing in water of frozen fish and fillets of some species.

The T system and the I band seemed particularly susceptible to the action of SDS and breaks across the fibres rather than along their long axis occurred (Fig. 17). Similar transverse fissures have been noted in other preparations (not shown). These transverse fissures may be unique to the peripheral fibrils, with their elongated cross section, or they may indicate the presence of the connecting structures between myofibrils at the Z line level that are believed to be part of the organization of the overall muscle structure (Lazarides, 1980). It is not clear what to make of these observations but further research is warranted.

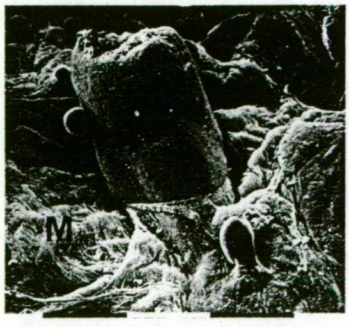

Fig. 19—Sample of muscle treated in 0.5M KCl (1 hr) showing remnants of a muscle fiber F attached to the myocomma M by a 'skirt' of perimysium P. Fish M. Bar 0.1 mm. Magnification: 289X.

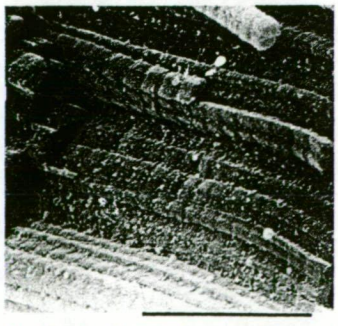

Fig. 20—Muscle fibrils from Fish O treated in water (1 hr). Note apparent collapse of T system below fibril surface. Bar 0.01 mm. Magnification: 5780X.

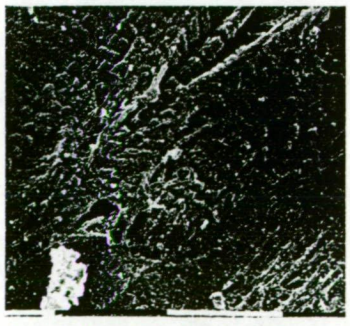

Fig. 21—Muscle fiber from Fish O treated in water (6 hr). Transverse and longitudinal disruption. Bar 0.01 mm. Magnification: 3890X.

Further work is also warranted on the detailed architecture of the myocomma and the myomere/myocommata junction using TEM and, on the types of collagen in fish tissue and on the collagenase(s) responsible for post mortem degradation.

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Degradation in Muscle Fibre–connective Tissue Junctions in the Spotted Trevalla (*Seriolella punctata*) Examined by Scanning Electron Microscopy

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The junctions between the muscle fibres and the connective tissues of the myocommata in the spotted trevalla (*Seriolella punctata* Forster) were investigated by scanning electron microscopy. In prerigor muscle, the fine collagen fibrils which arise from the myocommata to form the muscle cell envelope were evident. After the fish were stored several days in ice, progressive deterioration was observed in these fibrils. The structure and the degradation was similar in nature to that reported previously in blue grenadier.

Keywords: Collagen; collagenase; trevalla; myotendonous junction; fish muscle; muscle fibre; scanning electron microscopy.

1. Introduction

The structure of the junctions between the muscle fibres and the connective tissues of fish is of considerable interest to fish biologists and technologists. These junctions transmit the contractile forces of the muscles, and after death they are in part responsible for maintaining the integrity of the flesh.

Using light microscopy Love *et al.*[1] and Love[2] described fine collagenous processes arising from the myocommatal connective tissue planes which form sheaths surrounding each muscle fibre. Using scanning electron microscopy (s.e.m.) Bremner and Hallett[3] demonstrated the fine collagenous processes in the flesh of the blue grenadier (*Macruronus novaezelandiae* Hector) which confirmed these observations and illustrated the apparent socket-like nature of the invaginations on the myocomma into which the muscle fibres fit. Evidence was also presented indicating that in chill stored fish there was a progressive post-mortem breakdown of the fine collagenous fibrils that anchor the muscle fibres to the myocomma. This progressive breakdown was interpreted as being responsible for loss of structural integrity and resultant gaping of the flesh.

This present study extends these observations to a species with no close taxonomic relationship to the blue grenadier, spotted trevalla (*Seriolella punctata* Forster), also known as mackerel trevalla or silver warehou. This fish is a member of the family Centrolophidae and is of minor commercial importance. It is however closely related to the deepsea trevalla (*Hyperoglyphe antarctica* Carmichael) and warehou (*Seriolella brama* Gunther),[4] fishes of commercial importance in Australia and New Zealand. It is difficult to obtain prerigor samples from commercial fish such as these, which are caught by droplining and trawling respectively. Spotted trevalla was used in this study as live fish could conveniently be caught close to the laboratory.

2. Experimental

2.1. Fish

Juvenile samples of the fish (150 g, 23 cm long) were caught by line in the Derwent River estuary, Hobart, Tasmania, in June 1983 (Fish A) and in the nearby Huon River estuary in June 1984 (Fish B). They were given a sharp blow on the head and samples of flesh from the anterior dorsal muscle were taken and fixed in 3.2% glutaraldehyde in 0.16 M phosphate buffer containing 2% sodium chloride (wt:vol) and 0.5% sucrose at about 5°C.[3, 5] The time elapsed between the fish striking the hook and the sample being placed in fixative did not exceed 2 min. After the prerigor sampling, the fish were stored in ice. Further samples were taken and put in fixative when the fish were in rigor mortis (1 day after catching) and during postrigor storage on ice up to 14 days after catching. Two samples from each of three fish were taken at each time. The fixed samples were sent for microscopic examination to the DSIR Mt. Albert Research Centre, Auckland, NZ. Thus, samples were in fixative from between 7 to 20 days.

2.2. Preparation for s.e.m.

The samples were briefly washed in phosphate buffer, and frozen in dichlorodifluoromethane (−158°C) before transfer to liquid nitrogen (−196°C). They were then fractured under liquid nitrogen and freeze dried.[3]

After drying, the specimens were mounted on stubs and sputter coated with gold before examination with a Philips 505 scanning electron microscope.

3. Results

3.1. Prerigor samples

The detail at a fractured surface of a section of prerigor muscle (Figure 1) shows that the

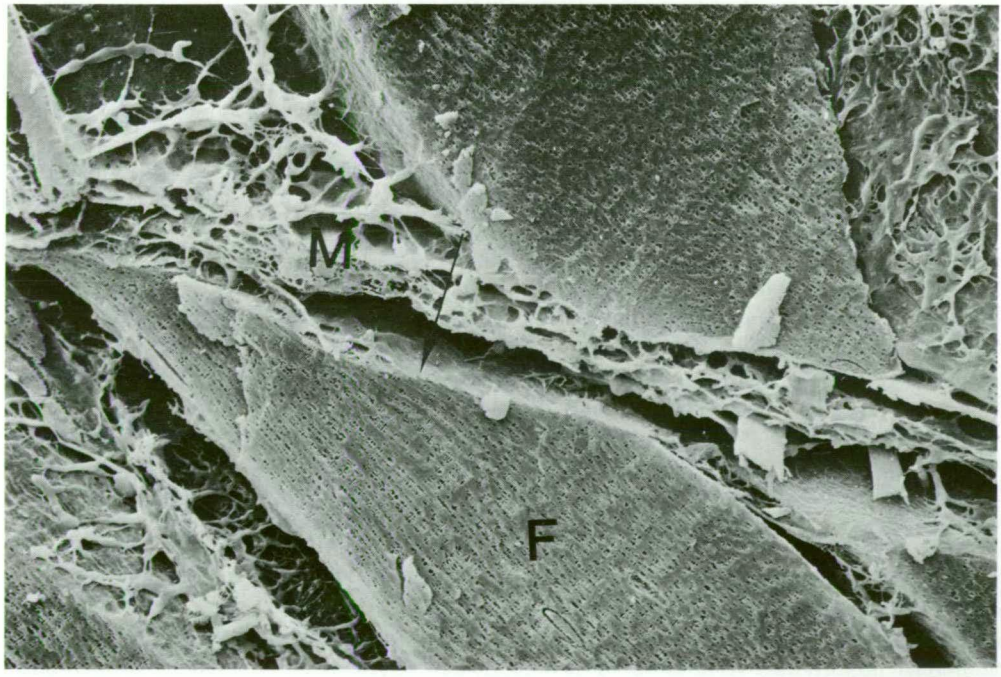

Figure 1. Fractured muscle fibre/myocommatal interface. Fine collagenous fibrils run from the muscle fibres to the myocomma which lies almost horizontally across the figure. The ends of the muscle fibre (F) are intimately connected to the surface layers (arrow) of the myocomma (M) which itself shows signs of being pulled apart. Prerigor (B1) fish caught June 1984. Magnification 1100.

myocomma and the muscle fibres have been forced slightly apart by the fracturing process. The muscle fibre ends are still in intimate contact with the outer surfaces of the myocomma. Between the muscle fibres the fine collagen fibres of the muscle cell envelope are evident. Similarly in Figure 2 the network of collagen fibrils that connects the muscle fibre to the myocomma is exposed.

The network of connective tissue fibrils of the muscle cell envelope can be seen in Figure 3 particularly on the muscle fibre in the centre of the field. Where the fibrils have been partly stripped off the adjacent (lower) fibre the striations are visible. At 10-times the magnification in a similar sample the series of holes of transverse tubules which open on to the extracellular space are evident (Figure 4). This figure bears a close resemblance to that published by Voyle[6] for meat (presumably beef muscle).

The longitudinal spacing of the holes is approximately 1.6μm, a value intermediate between the 1–2μm reported by Bertaud *et al.*[7] for the black mollie and in agreement with the range of values reported by Howgate[8] for the sarcomere lengths of cod.

3.2. Samples in rigor

Passive shortening was evident in samples taken from fish in rigor mortis (Figure 5).

3.3. Postrigor samples

In postrigor samples taken from fish held in ice for 4 days after catch there was evidence of breakdown and degradation of the fine connective tissue fibrils (Figure 6) at the myocommatal interface with some fibre ends only partially attached to the myocomma. With fish stored for 8 days in ice the muscle cell envelope degraded further and some muscle fibres were no longer connected to the myocomma (Figure 7). Breakdown is also evident along the muscle fibre

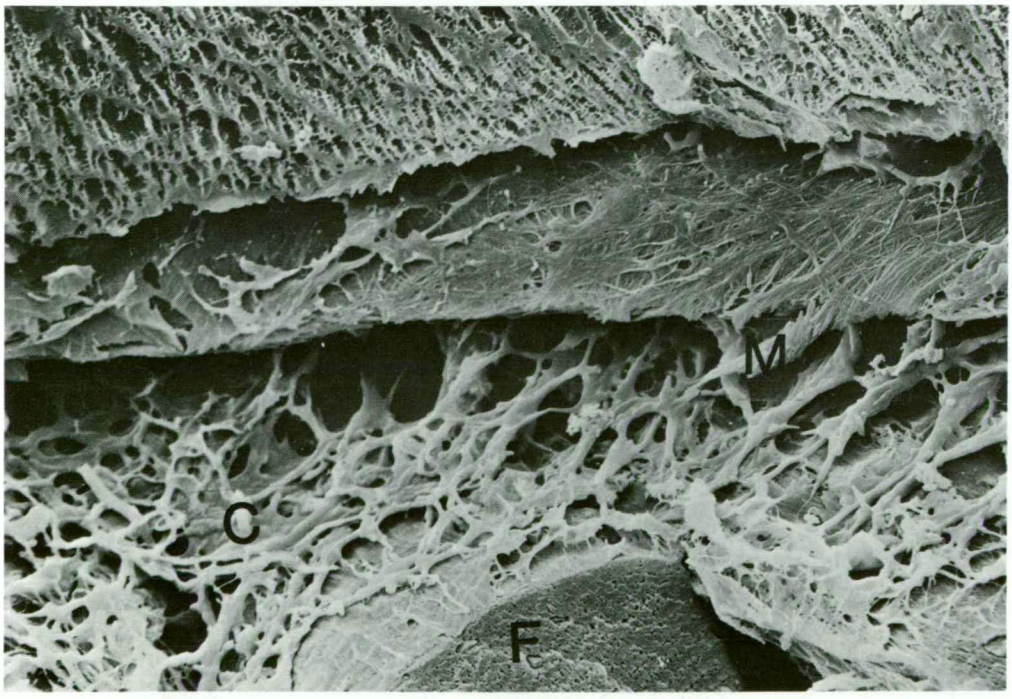

Figure 2. Freeze fracturing has exposed a thick network of collagenous fibrils (C) connecting the muscle fibres (F) (bottom of picture, centre) to the myocomma (M). Fish (B1). Magnification 1100.

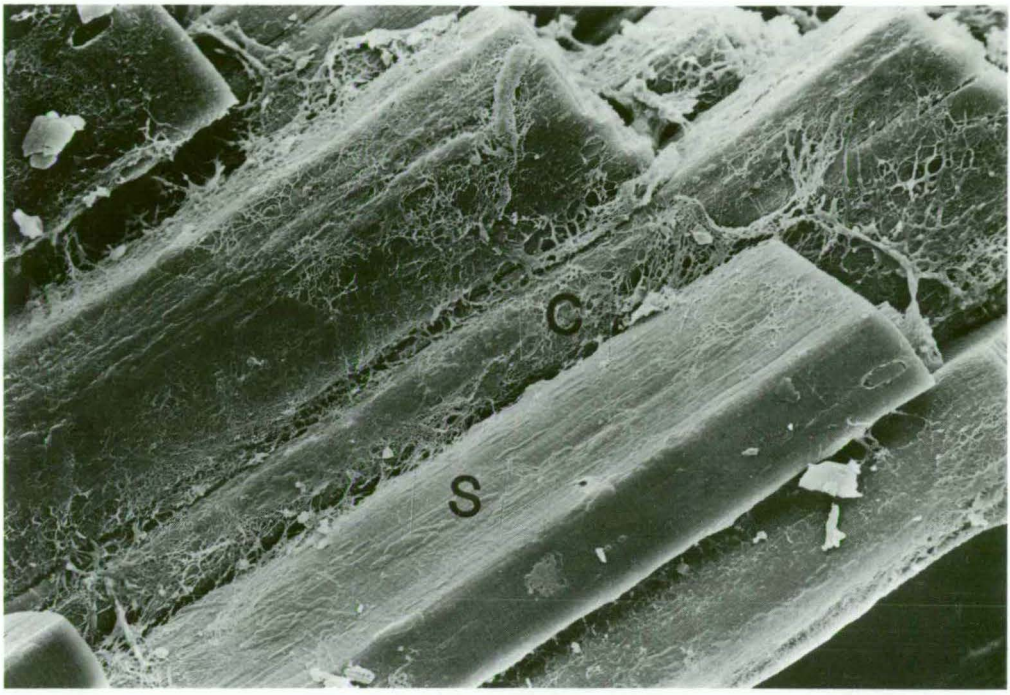

Figure 3. Muscle fibre surfaces near fractured face. Note network of collagen fibrils (C) and striations (S) on exposed muscle fibre. Fish (B1). Magnification 580.

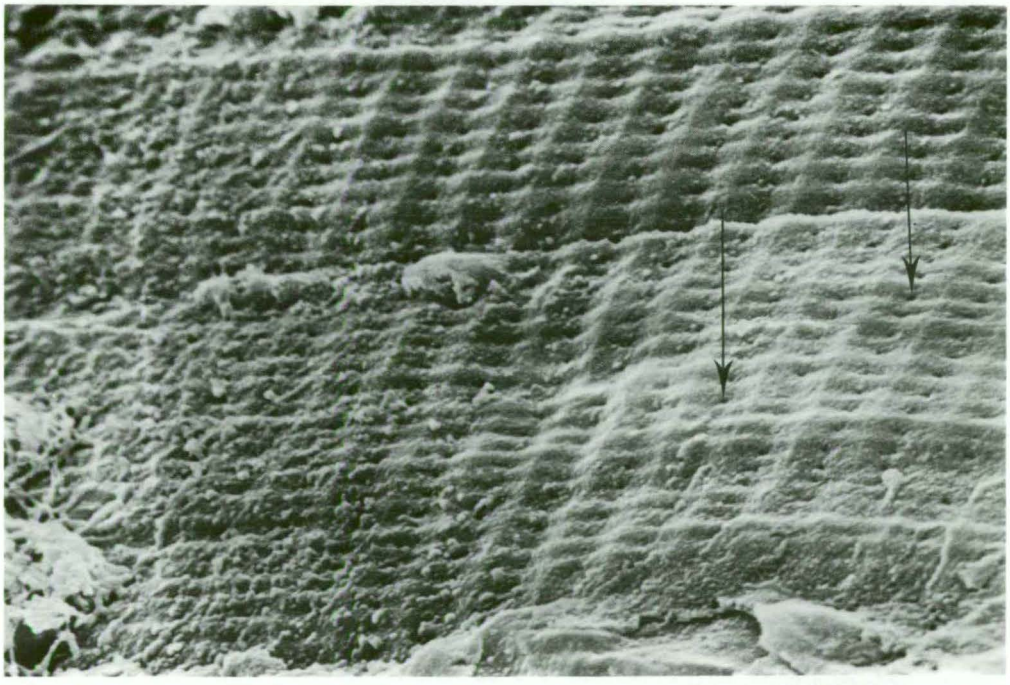

Figure 4. Exposed surface of a muscle fibre. Note openings of the transverse elements (arrows). Prerigor fish (B2). Magnification 5800.

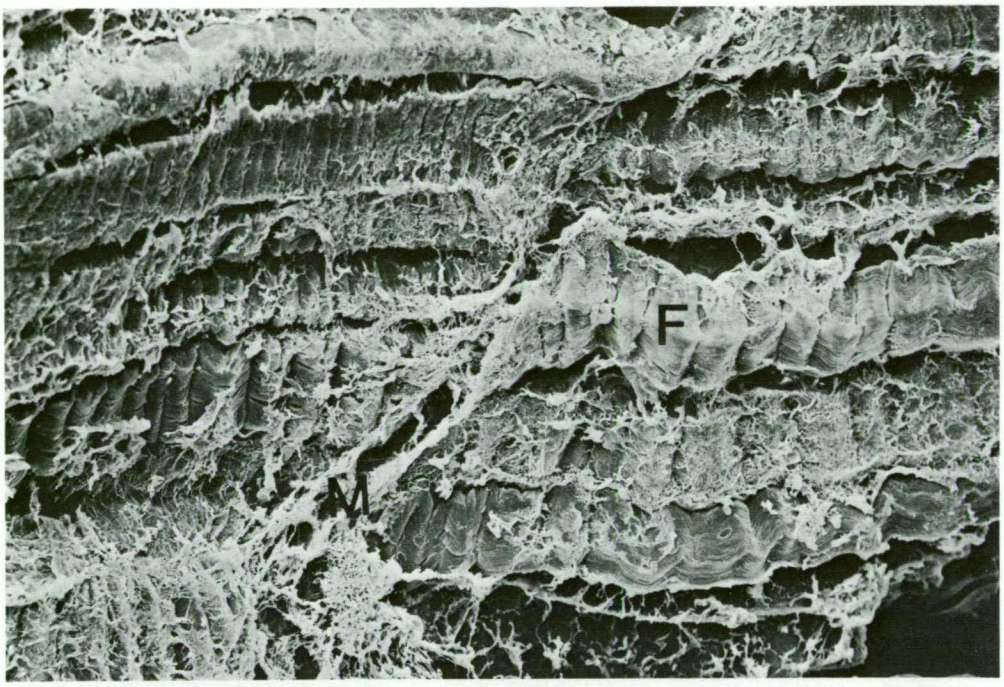

Figure 5. Muscle in rigor mortis. Note passively shortened crimped fibres (F) and myocomma (M). Fish (A1) June 1983. Magnification 205.

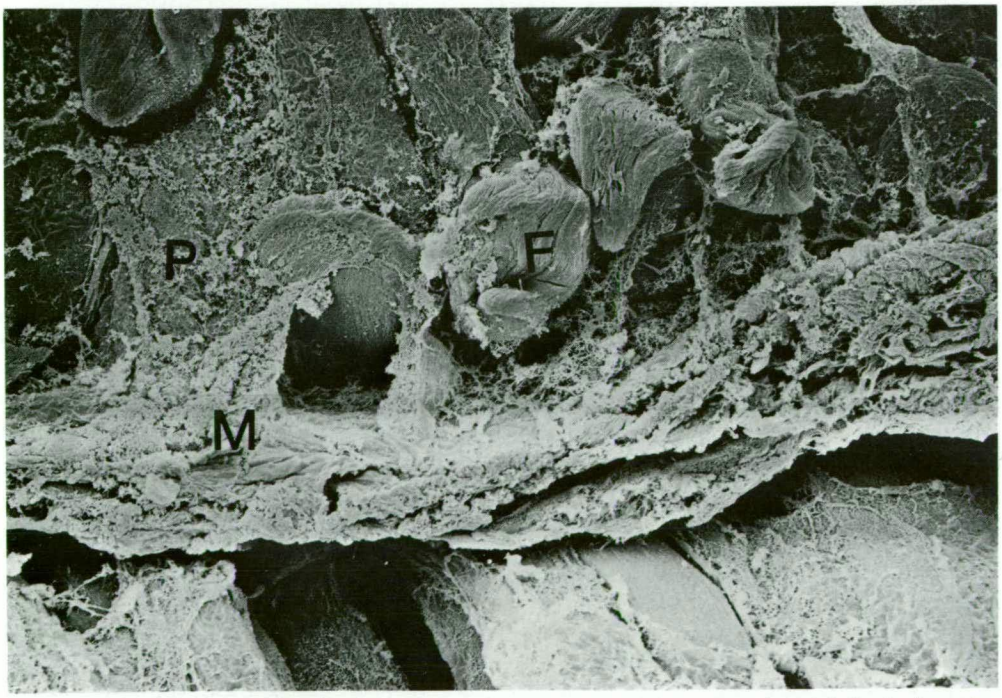

Figure 6. The fine collagenous fibrils of the perimysium (P) and myocomma (M) have been degraded allowing loosening of attachment of the muscle fibres (F). Fish (B2) stored 4 days in ice. Magnification 280.

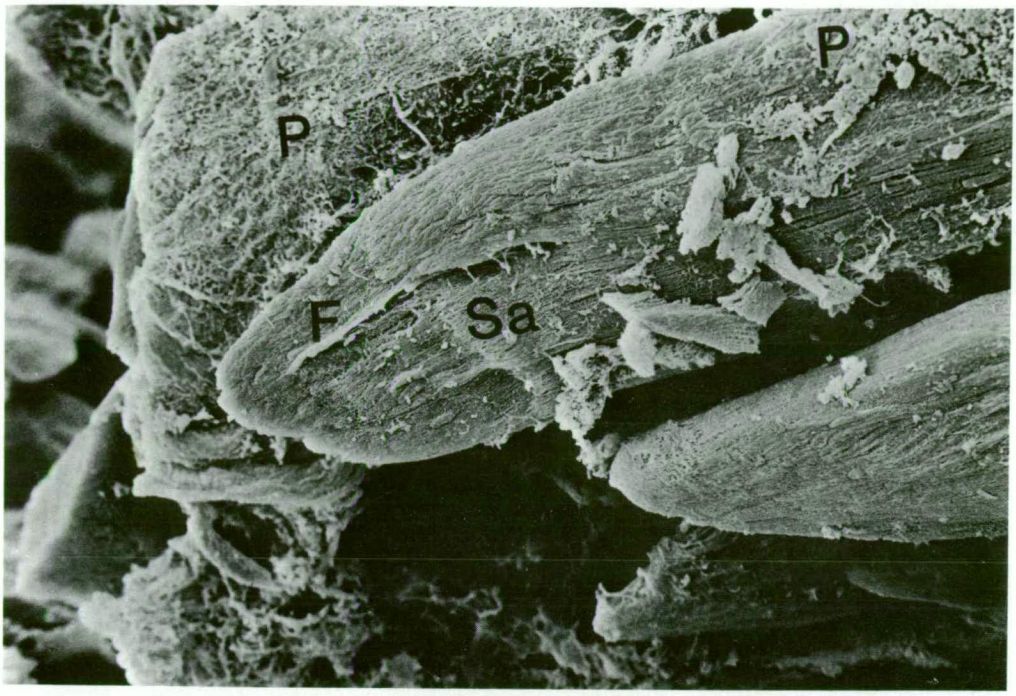

Figure 7. Muscle fibre ends (F) not attached to myocomma. General degradation of perimysium (P) and sarcolemma (Sa). Fish (B2) stored 8 days in ice. Magnification 570.

Figure 8. Degradation of the perimysium (P) and sarcolemma (Sa) on the surface of the muscle fibre. Fish (A1). Magnification 2270.

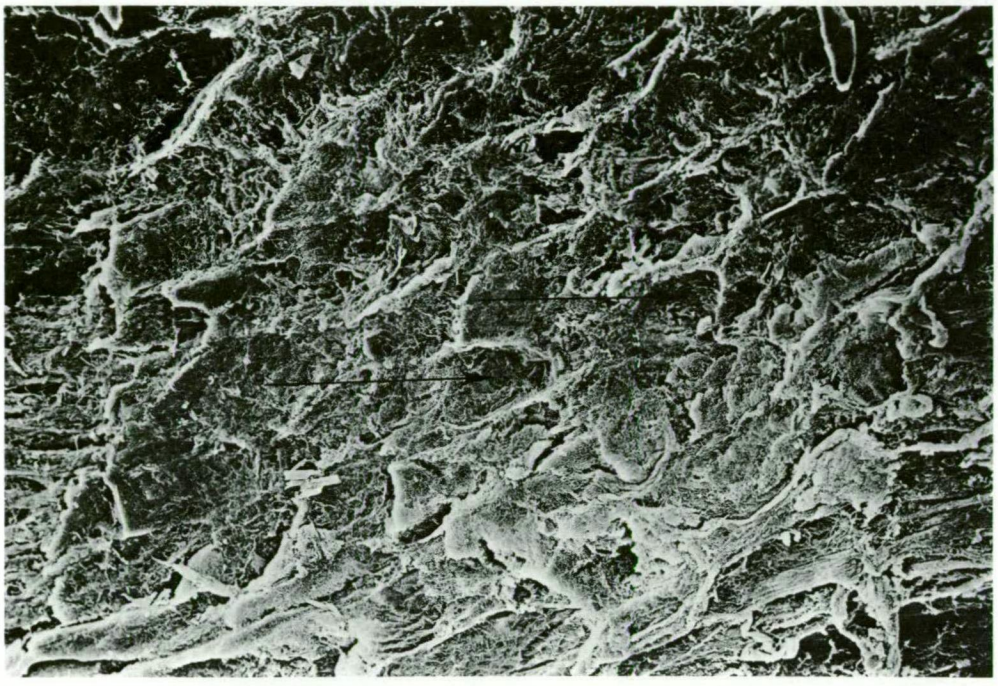

Figure 9. Surface view of myocomma showing evidence of sockets (arrow) formerly occupied by muscle fibre ends. Fish (A1) stored 4 days in ice. Magnification 220.

surface (Figure 8, compare with Figure 3). Where fibres are completely detached there was evidence of the remnants of the sockets into which they fitted (Figure 9).

4. Discussion

Unattached fibres (for example, Figure 6) were not observed in prerigor samples. The change from the clean appearance of the collagenous fibrillar networks seen in Figures 1, 2 and 3 to that in Figures 6, 7 and 8 where the collagen fibres were less well defined is considered to be due to enzymic degradation. There are similarities in the results of this work on spotted trevalla to that reported previously for blue grenadier.[3] The structure of the muscle fibre connective tissue junction and the appearance of the fine collagenous fibrils on the fibre surfaces and at the junctions was similar. The progressive breakdown of these fibrils appears to proceed by similar mechanisms that are consistent with enzymic attack by collagenases and/or other proteases.[3] It is the collagenous fibrils rather than the myofibrillar proteins that are attacked.

There were differences between blue grenadier and spotted trevalla. Examination of the two sets of prerigor samples (Fish A, June 1983 and Fish B June 1984) gave the subjective impression that the myocommata of spotted trevalla are thicker than those of blue grenadier. In the fixed tissue they were also more brittle and were more readily split along the axis of the myocommatal plane giving, at times, the appearance of a layered structure.

The spotted trevalla is less prone to gaping and the muscle fibres are not so readily detached from the myocommatal surface as in the blue grenadier. Nevertheless, during chilled storage, processes that degrade the fine collagenous fibrils of the myocomma and the perimysium are similarly at work. The inference is that this degradation is probably a widespread phenomenon that results in less cohesive flesh in chill stored fish. As such it acts independently but in concert with other degradative influences, such as increasing pH and the effects of spoilage bacteria, which lead to soft flesh.

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Fine Structure of the Myocommata–Muscle Fibre Junction in Hoki (*Macruronus novaezelandiae*)

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ABSTRACT

Transmission electron microscopic observations have been made of the interface between muscle fibres and myocommata of the fish, hoki (Macruronus novaezelandiae *Hector). The fine structure of the interface is similar to that recorded for other vertebrate muscle. Collagen fibres from the myocommata penetrate sarcolemma-lined invaginations into the base of the muscle fibre. A distinct basal lamina separates the sarcolemma from the collagen fibres. Microfilaments connect sarcolemma, basal lamina and collagen fibres. The myofibrils of the muscle fibre are connected to the inner sarcolemma surface by fine filaments from the final Z disc. During rigor mortis the structure of the interface remains unchanged. During post-rigor storage on ice there is progressive breakdown at the interface, particularly of the invaginating collagen fibres and the basal lamina, resulting in detachment of muscle fibres from the myocomma.*

Key words: Collagen, hoki, myotendinous junctions, fish muscle, muscle fibre, transmission electron microscopy, gaping.

myotomes posterior to the gill slit, dorsal of the main fat line. New Zealand caught fish were sampled immediately after catching, at the onset of rigor (24 h) and after storage on ice for 8 days. Tissue was fixed in 30 g litre^{-1} glutaraldehyde in 0·2 M sodium cacodylate buffer, pH 7·2. Samples were post-fixed in 10 g litre^{-1} osmium tetroxide in 0·2 M sodium cacodylate buffer, dehydrated in an ethanol series and embedded in Spurr's low-viscosity resin. Tasmanian caught fish were sampled at catching and after 11 days of storage on ice. Tissue samples were fixed in a mixture of 25 g litre^{-1} glutaraldehyde, 40 g litre^{-1} paraformaldehyde, 20 g litre^{-1} tannic acid, and 0·8 g litre^{-1} sucrose in 0·2 M phosphate buffer, pH 7·2. These samples were post-fixed in 10 g litre^{-1} osmium tetroxide in 0·2 M phosphate buffer, dehydrated in an ethanol series and embedded in epoxy resin. Ultrathin sections were cut using an LKB Ultrotome III and stained sequentially with a saturated solution of uranyl acetate in ethanol/water (1:1 v) and an aqueous solution of lead citrate. Sections were observed in a JEOL 100B transmission electron microscope.

3 RESULTS

3.1 Interface region in pre-rigor fish

Fish caught in New Zealand and Tasmanian waters gave comparable results. Invaginations into the muscle fibre base occurred at the interface of the fibre and the collagenous myocommatal sheet (Figs 1 and 2). These invaginations, which

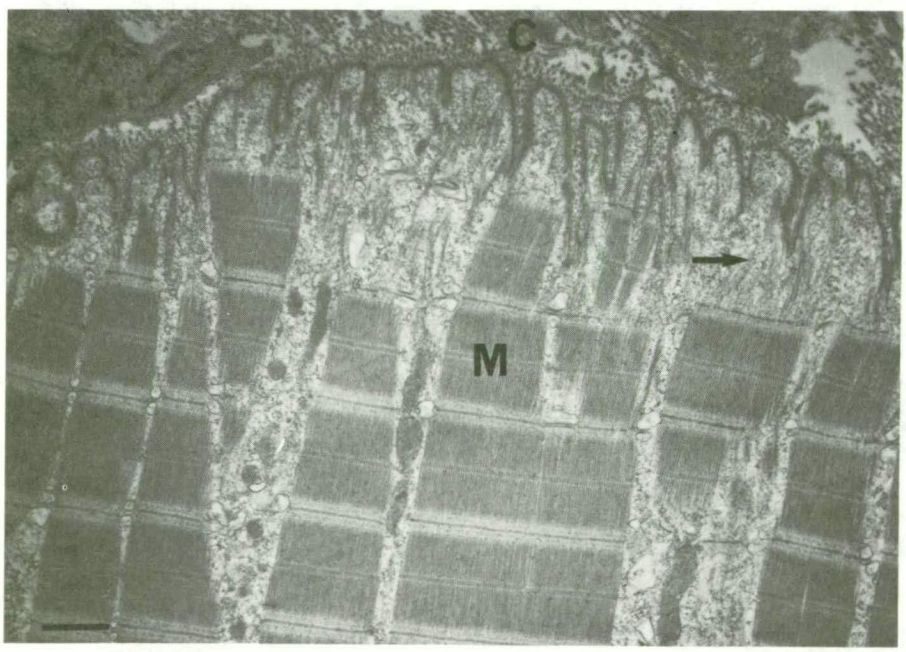

Fig 1. Muscle fibre base from a pre-rigor fish with groove-like, collagen-filled invaginations viewed in cross section. Thin filaments connecting the myofibrils to the sarcolemma are arrowed. M = muscle fibre, C = collagenous myocommata. Bar = 1 μm.

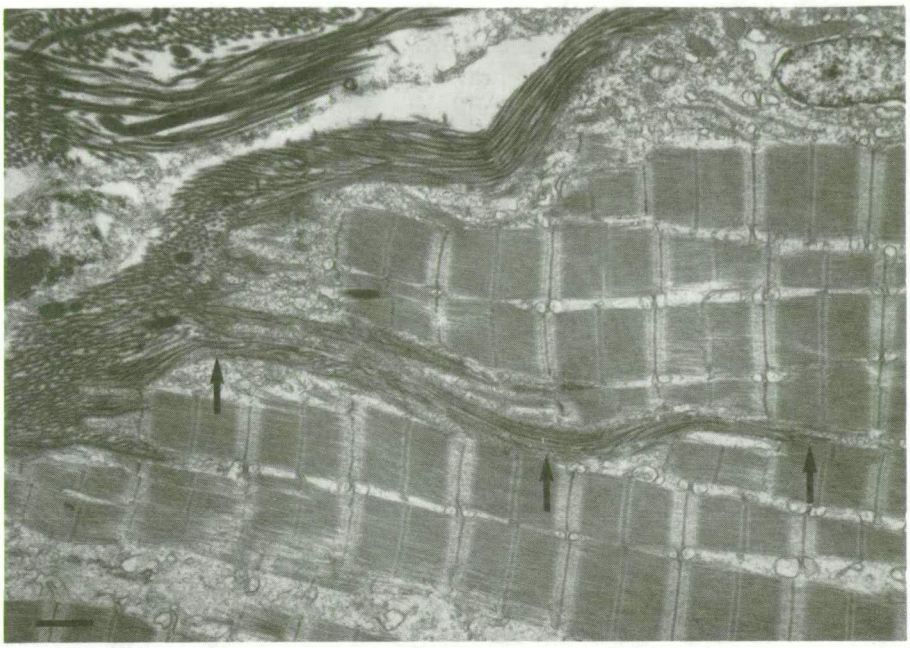

Fig 2. An elongate collagenous invagination from pre-rigor fish (arrowed) lying between myofibrils. Bar = 1 μm.

contained collagen fibres, extended for 1 to 10 μm into the fibre base. Shallow invaginations into the fibre base were common (Fig 1) and, frequently, groove-like (Fig 3). Short tubular invaginations were also present (Fig 4b). Deeper invaginations, though sometimes groove-like, were more often tubular or finger-like, sometimes twisting as they penetrated longitudinally up to 10 μm between muscle fibrils (Fig 2).

The invaginations of the fibre base were delimited by the sarcolemma, separated by an electron-lucent gap of 20–40 nm from the darkly staining amorphous basal lamina of width 30–40 nm (Fig 4). One or more collagen fibres (diameters ranging between 30 and 70 nm) were contained in the invaginations. The sarcolemma bounding the invaginations frequently showed further secondary invaginations into the sarcoplasm, not usually bounded by the basal lamina (Fig 6a). At the terminal end of an invagination the sarcolemma was often in close proximity to the bounding membrane of the sarcoplasmic reticulum (Fig 5). Often the basal lamina was absent from this region. Filamentous or spine-like bodies occurred between the

Fig 3. Diagrammatic reconstruction of the grooves in a fibre base as viewed end on to the fibre (compiled from a series of serial sections of which Fig 1 was a part). Dark represents muscle fibre base material and light the invaginations. The deepest groove was 2 μm. Invaginations of less than 0·3 μm are not recorded. Bar = 1 μm.

Fig 4. More detailed view of (a) a groove-like invagination, and (b) a short tubular invagination into the base of a pre-rigor muscle fibre. The sarcolemma (S), basal lamina (B), and collagen fibres (C) are clearly visible. Fibrillar processes (arrowed in (a)) connect the sarcolemma to the basal lamina. Bar = 1 μm.

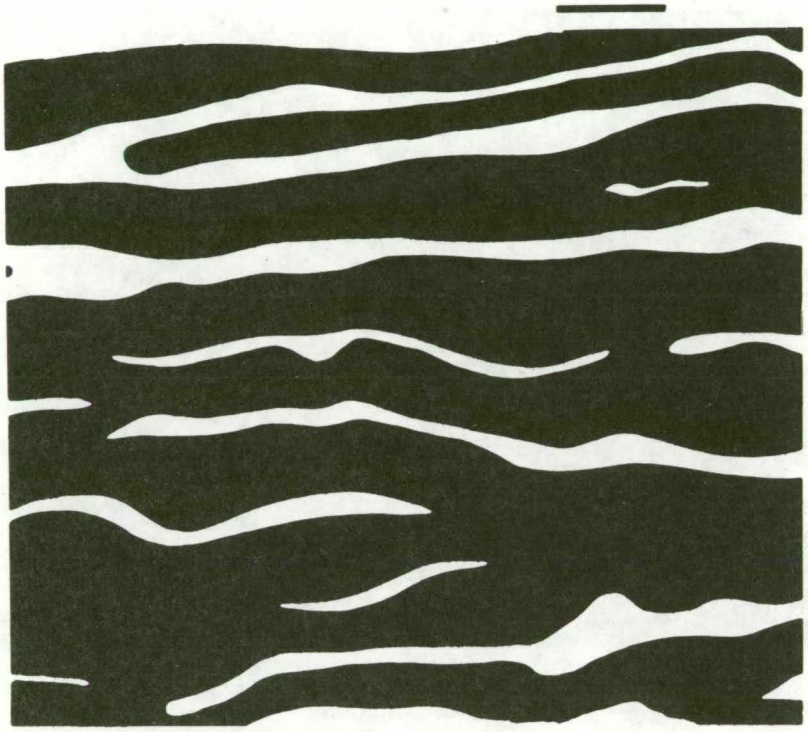

Fig 3

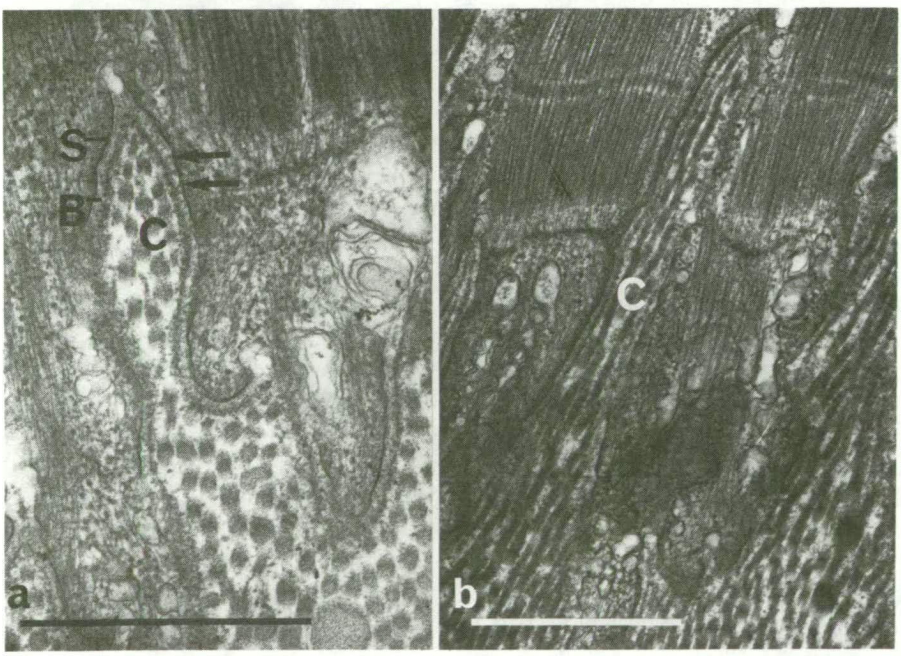

Fig 4

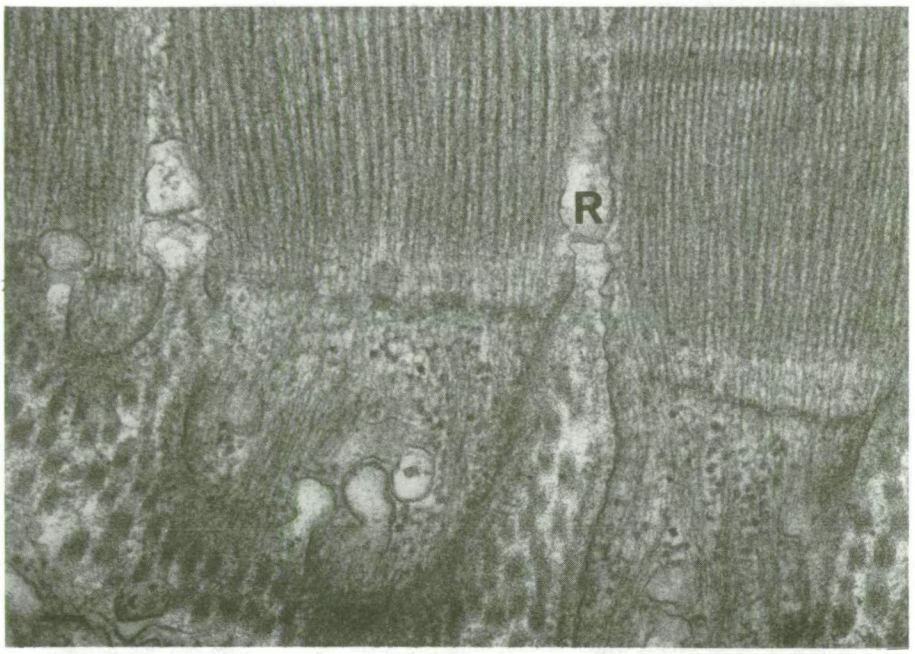

Fig 5. Terminal end of a groove-like invagination showing it in close apposition with a vesicle of the sarcoplasmic reticulum (R). Bar = 100 nm.

sarcolemma and the basal lamina, and between the lamina and the collagen fibres within the invagination (Figs 4a and 6). There was no sign of fusion between the collagen fibres of the invagination and either the basal lamina or the sarcolemma.

On the sarcoplasmic side of the muscle fibre base invididual muscle fibrils were connected to the sarcolemma by thin filaments similar in thickness to actin filaments (Figs 1, 4 and 7). These ran from the final Z disc. At the sarcolemma end of the thin filaments was a region of darker staining, amorphous material.

A diagrammatic representation of the invagination region is shown in Fig 8.

3.2 Interface region in rigor and post-rigor fish

In muscle from fish in rigor the overall structure of the interface region and the invaginations were similar to that found in pre-rigor muscle. However, there was a distinct increase in the amount of staining of the background matrix, resulting in a loss of contrast between this and the basal lamina and collagen fibres within the invaginations (Fig 9).

Fig 6. Details of an invagination showing fibres connecting through the basal lamina to the collagen fibres (solid arrows); (b) is a detail of (a). Secondary invaginations are indicated by open arrows. Bar = 100 nm.

Fig 7. Section of muscle fibre base, fine filaments (solid arrow) extend from the final Z disc (open arrow) of the myofibrils to the sarcolemma of the fibre base. Insert shows a detail of the base with a dark, amorphous region (arrowed) along the sarcolemma into which the filaments run. Bars (a) = 1 μm, (b) = 100 nm.

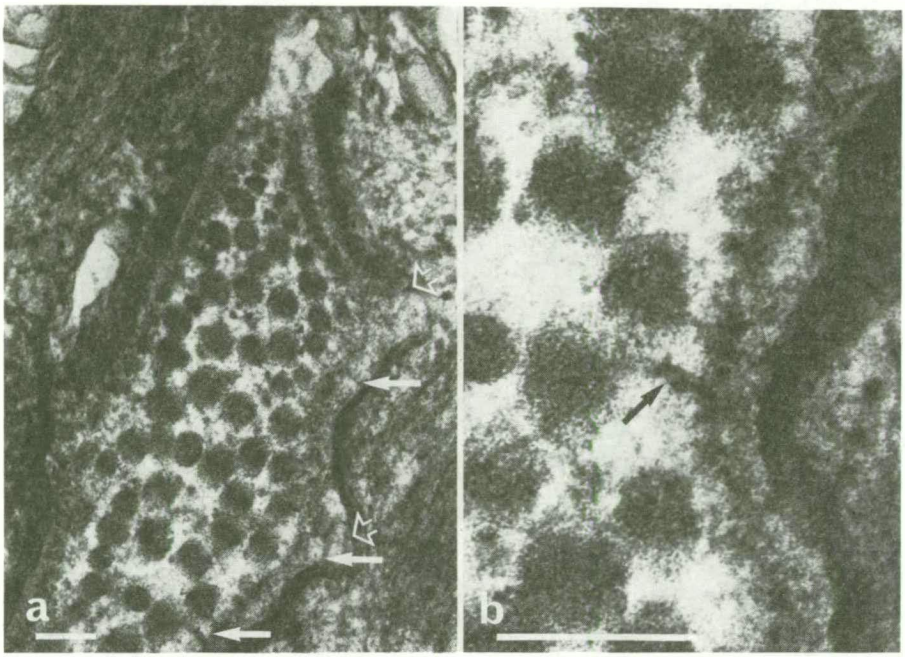

Fig 6

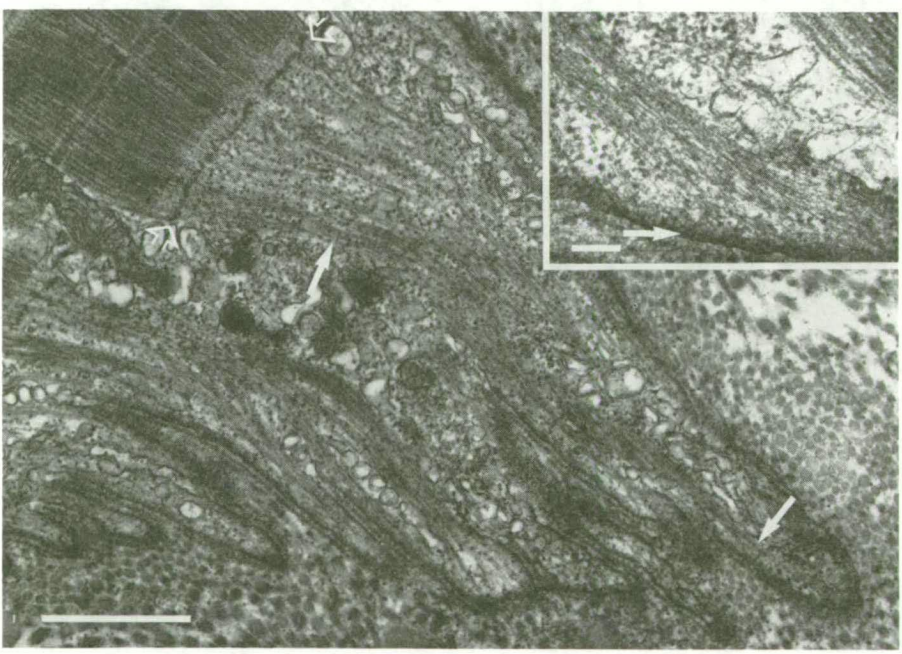

Fig 7

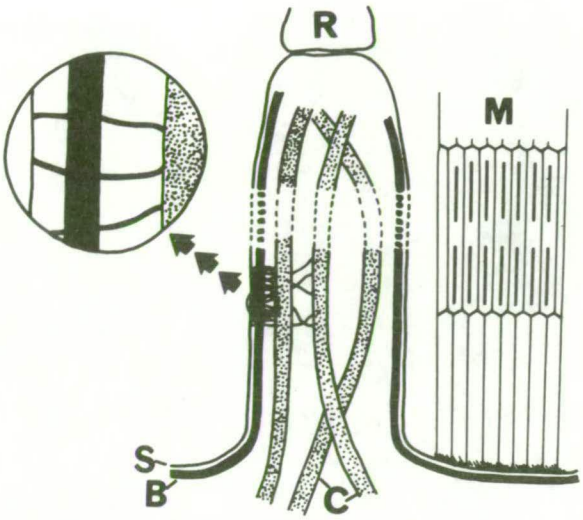

Fig 8. Diagrammatic view of the muscle fibre base region in pre-rigor fish. The invagination is lined by the sarcolemma and a basal lamina. Microfilaments connect the sarcolemma, basal lamina and collagen fibres (detailed in insert). Both basal lamina (B) and collagen fibres (C) terminate near the end of the invagination, which is closely appressed to the sarcoplasmic reticulum (R). Myofibrils (M) are connected to the sarcolemma of the muscle fibre base by fine filaments, which run into a darkly stained, amorphous region close to the sarcolemma (S).

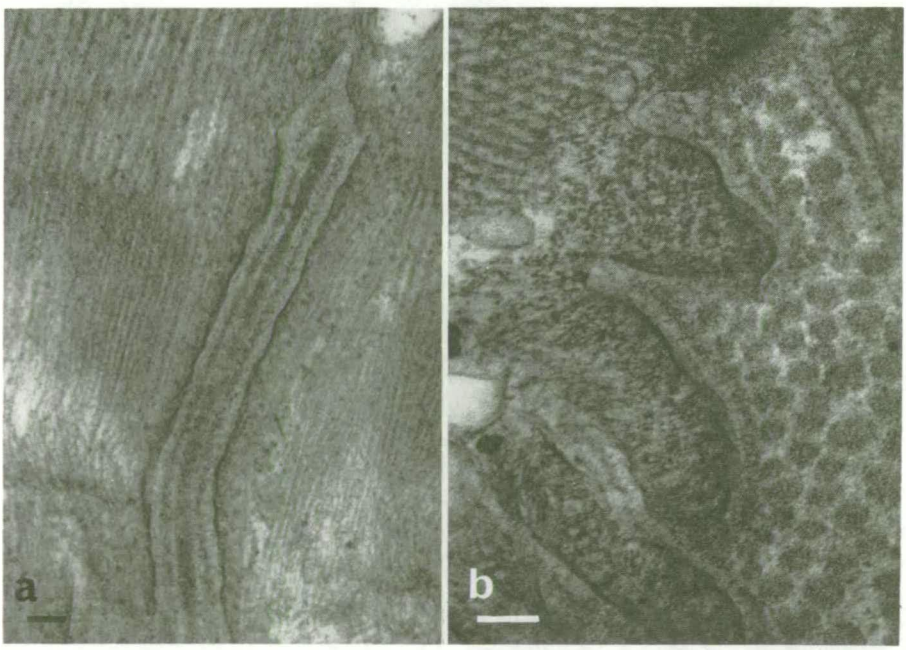

Fig 9. Tubular (a) and groove-like (b) invaginations into the muscle fibre base in fish muscle during rigor. The background matrix of the invagination stains more intensely than in pre-rigor fish. Although the definition of the collagen fibres and the basal lamina is thus less clear, the overall structure is the same as in pre-rigor fish. Bar = 100 nm.

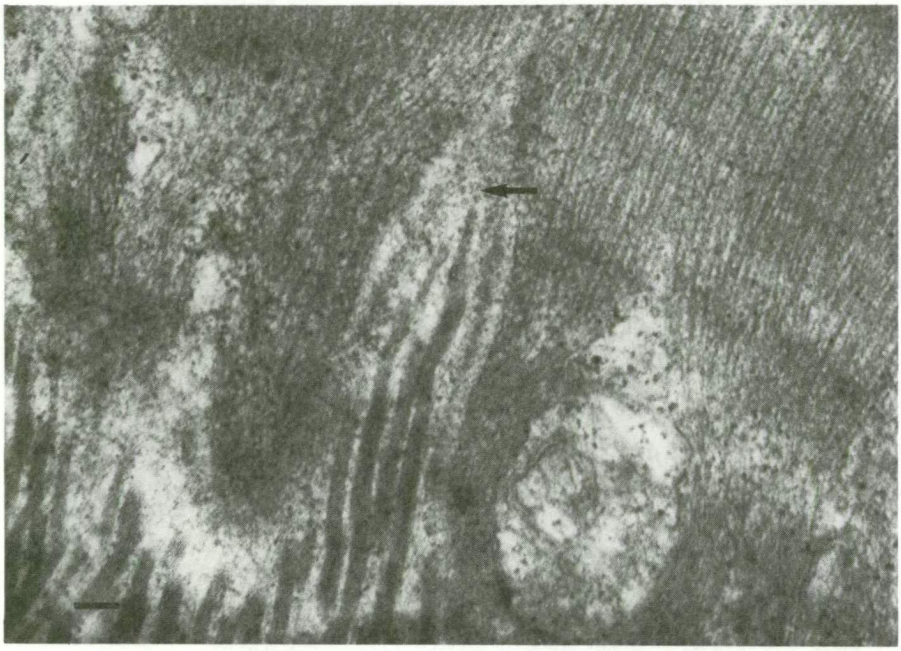

Fig 10. Invagination into the muscle fibre base in a post-rigor fish (stored 8 days). The basal lamina, although discernible, is indistinct and blurred. Small vesicles (arrowed) are visible in the terminal end of the invagination. There is considerable staining of the background matrix. Bar = 100 nm.

In muscle from post-rigor fish (8 days) the interface region showed considerable variation in structure, even within the same fibre. Some invaginations retained both collagen fibres and a discernible basal lamina (Fig 10). However, this latter was usually blurred. In some invaginations loss of the basal lamina and of collagen fibres had occurred to a greater or lesser extent. This appeared to start at the terminal region of the invagination (Fig 11). In many regions the fibre base and collagen sheet had parted and empty invaginations were apparent (Fig 12). Regions of empty and almost intact invaginations could be present close on the same fibre base; breakdown of the basal lamina could be seen in between. Invaginations of detached fibre bases contained neither the basal lamina nor collagen. However, the sarcolemma often remained intact at this stage (Fig 13).

In fish stored for 11 days the majority of fibres were more or less completely detached. Invaginations were not apparent at the muscle fibre base even when this was still in contact with the myocommatal sheets (Fig 14). In many instances massive vesicular deposits appeared between the fibre end and the collagenous sheets, and the sarcolemma had disappeared (Fig 15). However, despite this, the overall integrity of the muscle fibres, including the fine filaments to the fibre base region, remained intact (Figs 14 and 15).

In all samples the overall background matrix of the collagen appeared much more densely stained than that of pre-rigor fish.

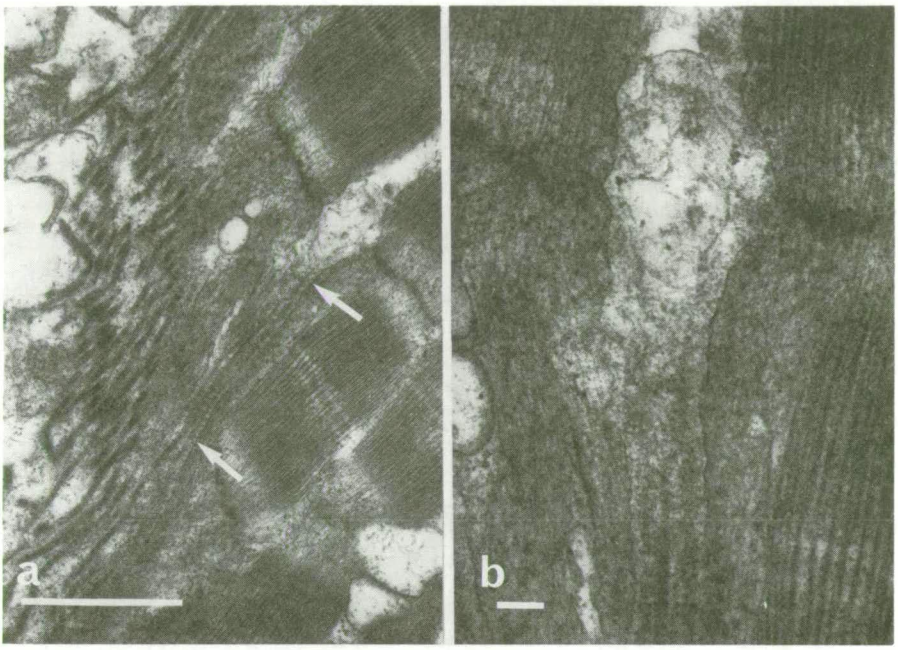

Fig 11. A tubular, but indistinct, invagination in a post-rigor fish (stored 8 days) is arrowed in (a). A detail of this (b) shows an apparent loss of collagen and the basal lamina from the terminal end. Bars (a) = 1 μm; (b) = 100 nm.

3.3 Pre- and post-rigor muscle fibre structure

Pre-rigor muscle fibres showed a compact fibril structure with obvious banding and a well developed sarcoplasmic reticulum (Fig 16a). At the fibre margin, successive layers of sarcolemma, basal lamina and collagen fibres made up the endomysium (Fig 16b). In post-rigor muscle there was a lack of cohesion between muscle fibrils and between the filaments composing the fibres, although the Z disc was still intact. Banding was indistinct (Fig 17a). The fibre margin retained the sarcolemma but lost its collagenous surround (Fig 17b).

4 DISCUSSION

The overall structure of the myocommata–muscle fibre interface is similar to that recorded in other vertebrates (Gelber *et al* 1960; Schwarzacher 1960; Muir 1961; Schippel and Reissig 1969; Hanak and Bock 1971; Korneliussen 1973;

Fig 12. Disconnected muscle fibre base/myocommata. Invaginations are empty of content. The region of the myocommata closest to the fibre base shows an area of amorphous stained material beneath which are collagen fibres. The basal lamina is no longer visible. Bar = 1 μm.

Fig 13. Detail of invaginations in the base of a disconnected muscle fibre. Apart from a few vesicles the invagination is empty. Only the sarcolemma (arrowed) lines the invagination wall. Bar = 1 μm.

Fig 12

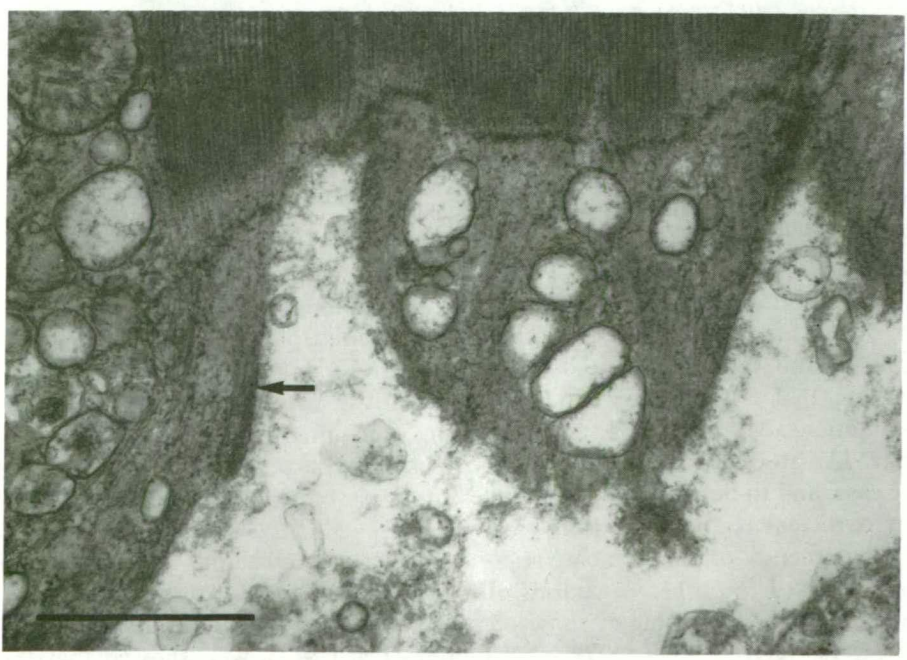

Fig 13

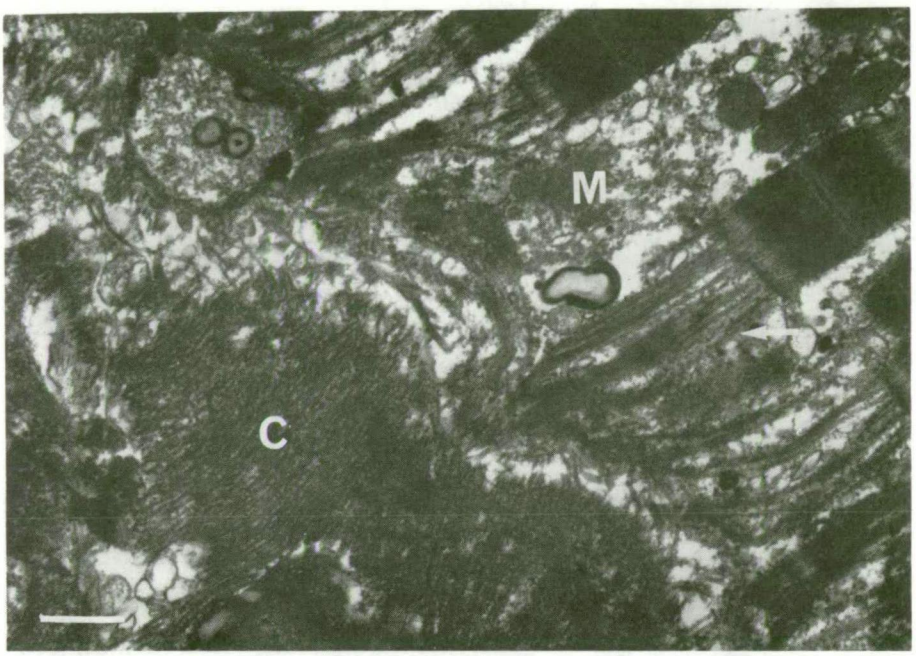

Fig 14. Fibre base region in an 11-day-stored post-rigor fish. Invaginations were not distinct, although the fibre base (M) appears to remain close to the myocommatal sheet (C). The fine filaments from the Z disc (arrowed) still extend to the basal region of the fibre. Bar = 1 μm.

Schattenberg 1973; Nakao 1975, 1976; Ajir *et al* 1978; Demmell *et al* 1979; Trotter *et al* 1985). Descriptions of the structure of the interface area in the seahorse (Schwarzacher 1960), pipefish (Schippel and Reissig 1969), hagfish (Korneliussen 1973) and lamprey (Nakao 1975) agree with our observations: ie invaginations of the sarcolemma containing collagen fibres from the myocommata. Characteristically the invaginations are finger-like, sometimes branched, and penetrate longitudinally between the muscle fibres. Depths of penetration reported are from 1–2 μm for lamprey (Nakao 1975) to 6–7 μm for guinea pig (Hanak and Bock 1971). Hoko exhibits invaginations covering the whole of this range. Additionally, large numbers of groove-like invaginations are present. These features are similar to those shown in the terminal ends of mouse plantaris muscle (Trotter *et al* 1985) and rat sternothyroid muscle (Ishikawa *et al* 1983). Serial sectioning indicates that most of this variation is real and not an artefact of different block face orientations. More detailed serial sectioning is required to discover whether the finger-like processes derive from the groove-like ones, as occasionally appears to be the case, and to determine whether different types of invagination are localised in any particular region of the fibre base.

The structure of the fibre base region at higher magnifications again resembles that reported for other organisms. The structures on the external side of the sarcolemma are the same as those observed by Hanak and Bock (1971), Nakao (1975, 1976), Ajiri *et al* (1978) and Trotter *et al* (1981), who showed that the sarcolemma, basal lamina and ingressing collagen fibres are apparently connected

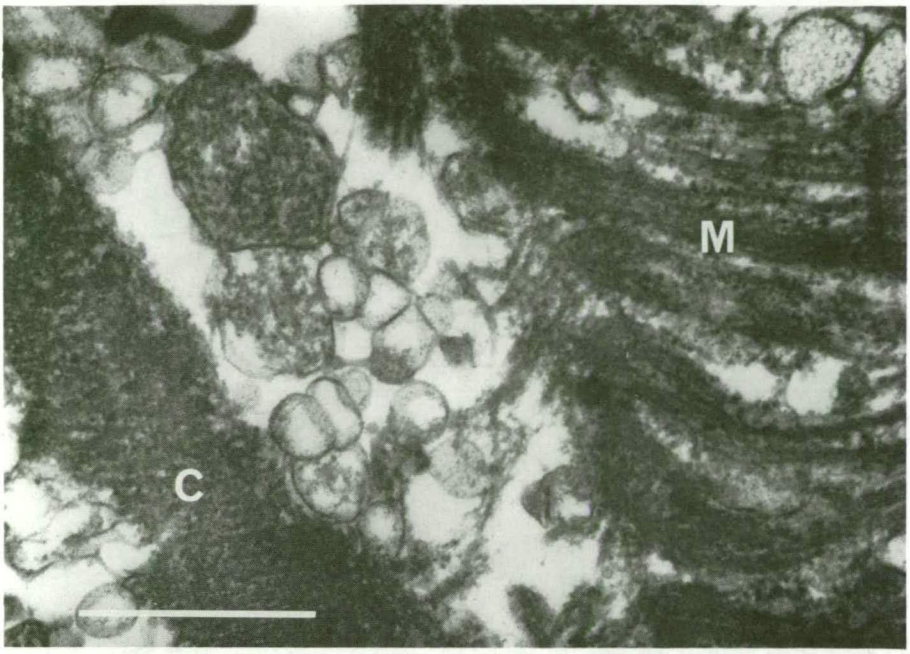

Fig 15. Similar fibre base region to Fig 14. The region between the fibre base (M) and the myocommata (C) contains large numbers of vesicles. There is no visible sarcolemma. Bar = 1 μm.

by microfilaments, which serve to attach the muscle to the connective tissue. Recent work indicates that the connections between the sarcolemma, the basal lamina and the collagen fibrils are exceptionally intricate (Trotter *et al* 1983; Keene *et al* 1987). An extended network of connecting plaques comprised of Type IV collagen and anchoring fibrils of collagen Type VII are normal features of the basement membrane zone of human skin and cornea (Keene *et al* 1987). Similar structures may exist in fish muscle but, to date, only collagen Types I, II and III have been isolated from fish (Kimura 1985), although collagen Type IV is a usual component of basal laminae.

Coupling of the sarcolemma of the invaginations and vesicles, presumably of the sarcoplasmic reticulum, was occasionally observed. This is consistent with the reports of Nakao (1975, 1976). Similarly the absence of the basal lamina from the terminal end of the finger-like processes is in accord with observations of lamprey and frog muscle (Nakao 1975, 1976). However, the basal lamina was not absent from the bases of the groove-like processes.

On the sarcoplasmic side of the fibre base the filaments that connect the final Z disc of the muscle fibril to the sarcolemma of the fibre base are presumably actin filaments (Schattenberg 1973; Maruyama and Shimada 1978; Trotter *et al* 1983). Other proteins of the cytoskeletal system such as vinculin (Geiger *et al* 1980), talin (Tidball *et al* 1986), zeugmatin (Maher *et al* 1985), and alpha-actinin (Goll *et al* 1969) have been found in myotendinous junctions and may also be present in fish as part of the electron-dense layer internal to the sarcolemma. Variability in the

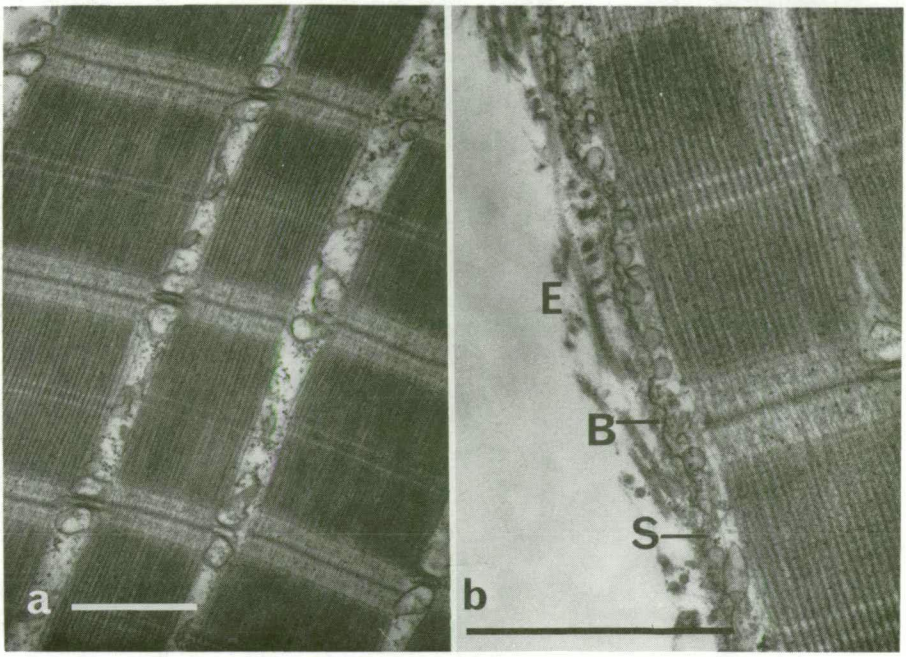

Fig 16. Muscle fibre of a pre-rigor fish: (a) myofibrils show distinct banding and well preserved sarcoplasmic reticulum; (b) margin has distinct sarcolemma (S), basal lamina (B), and endomysial collagen (E). Bar = 1 μm.

distance between the final Z disc and the fibre base is due in part to differences in section orientation and in part to actual differences in the fibril growth. (Fish muscle, unlike that of higher vertebrates, continues to grow throughout the life of the animal: Schattenberg 1973.) The sarcolemma region of attachment probably acts in a similar way to the membranous Z disc (Franzini-Armstrong and Porter 1964). It is from this region that a new Z disc must arise as the fibre extends. Thus, as in other vertebrates, the muscle fibrillar elements are firmly attached to the sarcolemma. In turn microfilaments connect the sarcolemma with the basal lamina and the collagen fibres derived from the myocommata. Trotter *et al* (1981, 1983) have shown that, even when the membranous nature of the sarcolemma is disrupted by lipid solvents, the connection and tension of these microfibrils remain unaltered. This suggests that they pass through the sarcolemma. Thus a firm linkage exists between the fibrils of the muscle fibre and the collagen fibres of the myocommata. Invaginations increase the surface area available for these connections and hence strengthen the interface. However, the magnitude of the stress at the junction is not the sole determinant of the degree of membrane folding (Tidball and Daniel 1986).

The disconnection of the muscle fibre end and the myocommata that results in gaping requires the breakage of at least one of the links between the muscle fibrils and the collagen fibres. The absence of any major structural difference between the fibre base region of hoki and that of other fish less prone to gaping (or indeed that of higher vertebrates), plus the absence of any major structural changes during rigor,

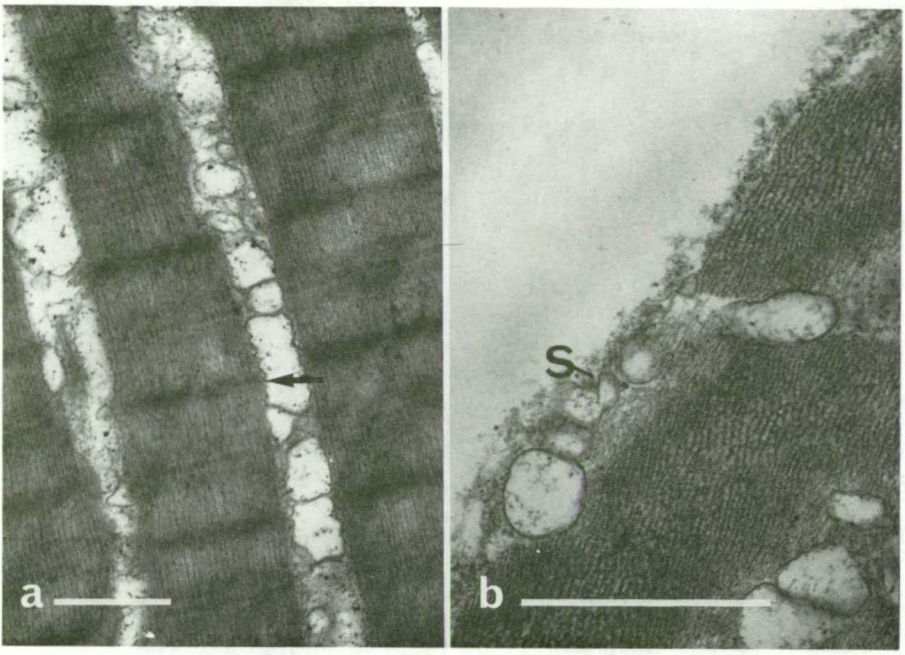

Fig 17. Muscle fibre of an 8-day-stored post-rigor fish: (a) myofibrils show little cohesion and banding is indistinct except at the Z disc (arrowed), and the sarcoplasmic reticulum (S) has degenerated into unconnected vesicles and membranous material, (b) margin has retained the sarcolemma but endomysial collagen and basal lamina are absent. Bar = 1 μm.

indicate that mechanical stress is unlikely to be the cause of post-rigor gaping. This, and observations on fish chill-stored for 8 and 11 days, support the hypothesis of Bremner and Hallett (1985) that gaping is caused by the high post-rigor activity of collagenases and other proteolytic enzymes at specific sites. This activity seems to be localised in the immediate vicinity of the basal membrane and within the invaginations. In most of the samples examined neither the bulk of the collagen of the myocommatal sheets nor the filamentous connections between the Z disc and sarcolemma showed major effects of enzyme activity. The present authors suggest that enzymic breakdown starts with the basal lamina and extends to ingressing collagen from the terminal end of the invagination, ie the sarcolemma itself is only disrupted and lost in the later stages. Although collagen within the invaginations is attacked, the dissolution of the basal lamina, and probably the microfilamentous connections to the sarcolemma, may be more significant in weakening the junction of the muscle fibre and the myocommata.

More work is required to identify the particular enzymes involved, their post-mortem activation and their precise site of activity. Why the changes in hoki should be so rapid remains a mystery. Similar enzymic processes almost certainly occur in other fish at different rates (Bremner and Hallett 1986). However, it is the rapidity of the process in hoki that causes a problem in the commercial fishery.

The results presented here suggest that, for this particular fish, changes in handling technique will not significantly improve the physical storage

characteristics of chill-stored fish, and that pre-rigor freezing will remain the most effective way of preserving the structure of the fish flesh.

ACKNOWLEDGEMENTS

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Fish Microstructure

H. ALLAN BREMNER[1] and IAN C. HALLETT[2]

The flesh of fish is comprised of interlocking blocks of muscle fibres joined at either end to collagenous sheets of connective tissue — the myocommata (Fig. 1). In the live fish, the forces of muscular contraction are transmitted through this junction, and in post mortem fish it is responsible for maintaining the integrity of the flesh during handling, storing and processing. In some species, progressive softening of the flesh and weakening of this myotendinous junction occurs and the muscle blocks (myotomes) separate and split. This effect is similar to the 'gaping' that can occur due to rigor contractions (Love, Lavety and Steel, 1969).

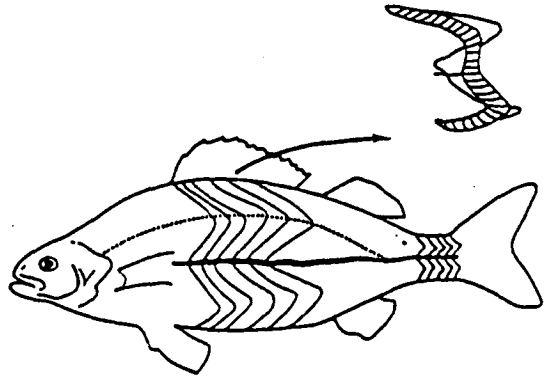

Fig. 1 — Schematic diagram of the layout of fish muscle, showing the interlocking W-shaped myotomes separated by myocommata and a single myotome. Adapted from Ellis et al. (1978).

The texture of raw fish depends on its structural integrity and this is of prime importance for the high-priced sashimi trade, as well as for normal sales and for processing. Soft and gaping fish are difficult to process; trimming and fillet losses occur and poorer products result. The study of the structure and changes to it are thus of considerable commercial and scientific interest.

Using light microscopy, Love (1970) and Love et al. (1969) showed that fine collagenous fibres from the myocomma appeared to surround each muscle fibre to form a sheath which extended the length of the fibre between neighbouring myocommata. This collagenous component of the muscle cell envelope was separated from isolated cod muscle cells using enzymatic treatments and shown to consist of a network of fine collagen fibres (Almas, 1982).

The detailed structure of the myotendinous junction has been studied in some unusual species of interest to science, such as the seahorse (Scwarzacher, 1960), pipefish (Schippel and Ressig, 1969), hagfish (Korneliussen, 1973), and lamprey (Nakao, 1975) using transmission electron microscopy (TEM). These workers all reported invaginations of the sarcolemma containing collagen fibres from the myocomma.

This paper reports on our recent investigations on commercially important species in which TEM and scanning electron microscopy (SEM) have been used to examine the architecture of the myotendinous junction in fish and its post mortem degradation in chilled storage (Bremner and Hallett, 1985, 1986; Hallett and Bremner, 1988).

SEM Studies

The three-dimensional view (Bremner and Hallett, 1985) of how the muscle fibres of blue grenadier (known in New Zealand as hoki; *Macruronus novaezelandiae* Hector) connect to the myocomma is seen in Fig. 2. When these muscle fibres are removed, the sockets on the myocomma into which they previously fitted can be seen (Fig. 3). Fine collagen fibres emerge from the myocomma to form part of the muscle cell envelope that runs the length of the fibre (Fig. 4), as noted by Love et al. (1969).

After chilled storage of the fish in ice for 5 days degradation of the cell envelope was evident (Fig. 5) with holes appearing in the cell membrane and obvious disruption of the fibrous collagen network. Progressive deterioration was seen and after 11 days in ice many of the muscle fibres were no longer attached to the myocomma, and degradation of the collagen was evident (Fig. 6, 7).

A strikingly similar result was seen in the unrelated species — the spotted trevalla (*Seriolella punctata*) (Bremner and Hallett, 1986). In the pre-rigor samples the muscle fibres are firmly attached to the myocomma (Fig. 8), whereas after only 4 days storage, deterioration of the

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Fig. 2 — Pre-rigor blue grenadier. Fine collagen fibres C connect the muscle fibres F to the myocomma M. Bar 0.1 mm.

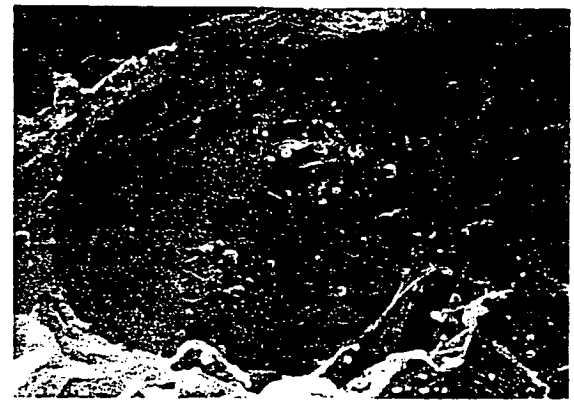

Fig. 3 — View of a socket on the myocomma into which a muscle fibre fitted. Bar 0.1 mm.

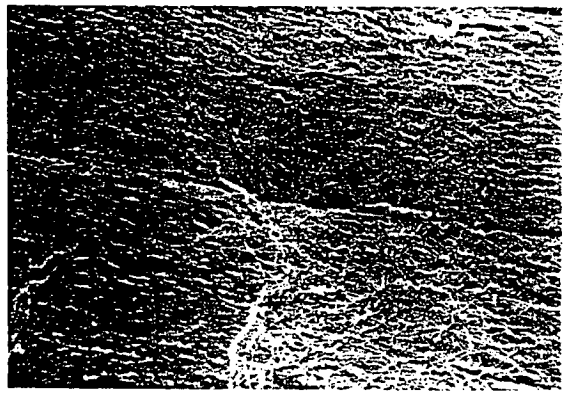

Fig. 4 — Pre-rigor fish. Detail of muscle fibre surface showing intact cell envelope and collagen fibres. Bar 0.01 mm.

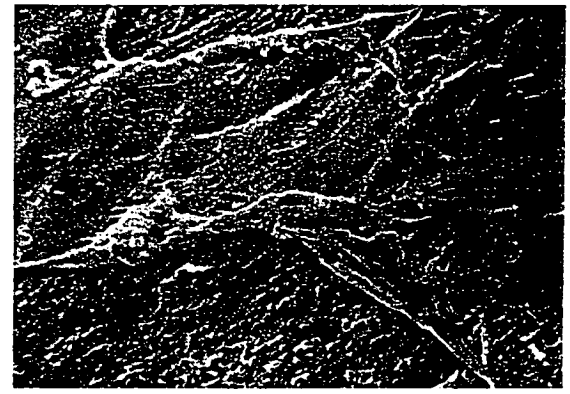

Fig. 5 — Post-rigor fish 5 days in ice after catch. Deterioration of sarcolemma and collagen. Bar 0.01 mm.

Fig. 6 — Fish stored in ice 11 days. General view of myotendinous junction. No connections remain between the muscle fibes F on the right and the myocomma M. Bar 1 mm.

Fig. 7 — Detail of fibre end from near centre of Fig. 6. The muscle fibre F completely detached from the myocomma M. Bar 0.1 mm.

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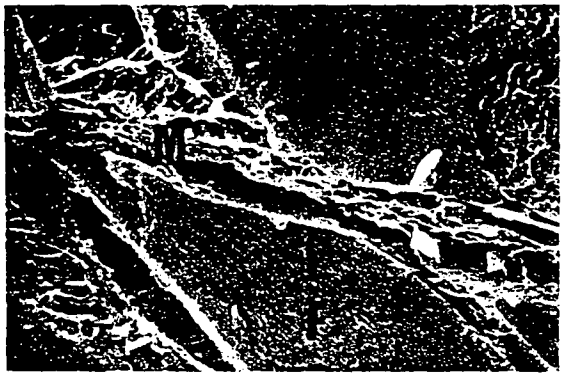

Fig. 8 — Pre-rigor spotted trevalla muscle at a freeze fractured face. Fine collagen fibrils attach the muscle fibres F to the surface layer of the myocomma M which lies almost horizontally across the picture. Bar 0.01 mm.

Fig. 9 — Trevalla stored 4 days in ice. Collagen of myocomma M and cell envelope has deteriorated allowing loosening of attachment of muscle fibres F. Bar 0.1 mm.

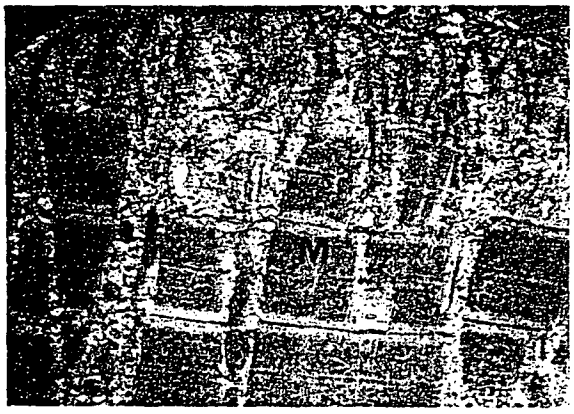

Fig. 10 — Muscle fibre base from pre-rigor blue grenadier with groove-like collagen-filled invaginations viewed in cross section. Thin filaments connecting the myofibrils to the sarcolemma are arrowed, myofibres M, collagenous myocommata C. Bar 1 μm.

Fig. 11 — Pre-rigor fish, a muscle fibre end featuring an elongate collagenous invagination (arrows) as it penetrates between the myofibres. Bar 1 μm.

Fig. 12 — More detailed view of a) a groove-like invagination and b) a short elongate invagination into the base of a pre-rigor muscle fibre. The sarcolemma S, basal lamina B, and collagen fibres C are clearly visible. Fibrillar processes (arrows) connect the sarcolemma to the basal lamina. Bar 1 μm.

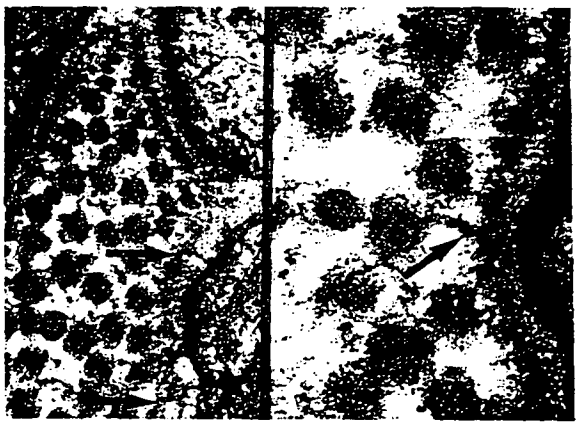

Fig. 13 — Detail of invagination showing fibres connecting through the basal lamina to the collagen fibres (arrows). Bar 100 nm.

Fig. 14 — Section of muscle fibre base, fine filaments (presumably actin; arrow) extend from the final Z-disc of the myofibrils to the sarcolemma of the fibre base. Insert shows detail of the base with electron dense region (arrow) into which the actin fibres run. Bar 1 μm, insert bar 100 nm.

Fig. 15 — Section from fish stored 8 days in ice showing a muscle fibre base disconnected from the myocomma. Invaginations are empty and there is an amorphous area near the fibre base under which collagen fibres are present. Bar 1 μm.

Fig. 16 — Detail of fibre base of a disconnected fibre. The invagination is empty and the basal lamina has disappeared but the sarcolemma is still present (arrow). Bar 1 μm.

Fig. 17 — Deteriorated fibre base from a fish stored 11 days in ice. There is extensive vesicle formation and disruption of the fibre base M, but the major strands of the collagen C of the myocomma are still obvious. Bar 1 μm.

collagen and detachment of the muscle fibres is obvious (Fig. 9).

Both these sets of observations indicated that deterioration of the fine collagen fibres of the myocomma and muscle cell envelope was mostly responsible for the loss of integrity in the flesh of the stored chilled fish and that gaping was not due to rigor contractions as can occur in frozen fish (Love et al. 1969). The nature of the deterioration points to enzymatic activity. Collagen, though, is normally regarded as being a fairly stable tissue so either there are some quite potent enzymes present with activity at 0°C, or the fish collagen is very susceptible to attack, or both situations occur. The similarity of the results obtained in two dissimilar species also indicates that the phenomenon is likely to be widespread, occurring to varying extents in most species.

TEM Studies

The fine structure of the myotendinous junction in pre-rigor blue grenadier muscle is complex and intricate (Hallett and Bremner, 1988). The muscle fibre ends are grooved (Fig. 10) and fine collagen fibres from the myocomma protrude into these grooves and invaginations for distances ranging from 1 to 10 μm (Fig. 11). The basal lamina external to the sarcolemma is well-defined (Figs. 10, 12, 13) and, in high

magnification, other fine connections can be seen linking the sarcolemma through the basal lamina to the collagen fibres in the invaginations (Fig. 13). Inside the muscle cell the well-defined banded myofibrillar structure is apparent (Figs. 10, 14) and fine fibres (presumed to be actin) stream from the last intact Z-line and are attached by an electron dense layer to the sarcolemma (Fig. 14).

During storage of the fish in ice, progressive degradation was seen to occur to the basal lamina, the fine connections and to the collagen fibres in the invaginations (Fig. 15). In some areas muscle fibres are seen to be free of the myocomma, the collagen in the invaginations has disappeared, only the sarcolemma remains in some places and in the fibre end itself vesicles have formed (Fig. 16). In other regions massive vesicular deposits between the muscle fibre and the myocomma are evident (Fig. 17).

Discussion

This more detailed evidence obtained by TEM corroborates the SEM work and the conclusions drawn from it. It is evident that there is considerable internal digestion of the collagen fibres and many of the fine structural features which anchor the muscle cell to the myocomma. The deterioration bears all the hallmarks of being due to enzymes but the location and exact nature of these enzymes is not known as yet. It is likely that they are intimately associated with the collagen itself (Pardo and Perez-Tamayo, 1975) as part of the mechanism that effects seasonal changes in the tissue during spawning.

The fine structure seen under the TEM is similar to that reported for myotendinous junctions of other vertebrate muscle (Hallett and Bremner, 1988), but there have been no similar reports of the lability of this structure. This may be because the conditions of chill storage of fish caught in cold and temperate waters are only about 10°C lower than their usual environment whereas for mammals this difference is over 30°C. This is too large a temperature difference for the enzymes to show significant activity. The collagen of fish flesh is well known to be more acid soluble than that of mammalian muscle due to a lower frequency of cross-links (Sikorski, Scott and Buisson, 1984). Kimura (1985, 1987) have reported the presence of a third alpha chain in the collagen molecule of the type I collagen of the skin of some fish. This has recently been confirmed for the collagen of the skin of the blue grenadier (Ramshaw, Werkmeister and Bremner, 1988). The collagen of the cell envelope and of the invaginations has not yet been studied but it is likely to have the same three chain structure in the molecule, although the degree of crosslinking may be less. It does seem to be more labile than the larger strands of collagen which comprise the main bulk of the myocomma.

These studies confirm the early suggestion of Banks (1962) that there is probably breakdown in the connective tissue in stored fish brought about by enzyme action. The breakdown occurs first in the very important fine connections and this leads to a loss of structural integrity of the flesh. These fine connections constitute only a minor proportion of the total structure but they anchor the major components and provide coherence to the flesh as a whole. Recently, Hatae, Tamari, Miyanaga and Matsumoto (1985) have concluded that softening of flesh during storage is more affected by changes in the overall structures than by changes in the major component proteins.

Conclusions

Softening in fish flesh is due to changes in the fine connections that anchor the three dimensional structure of the tissue. It is due to enzymes working with high activity at chill temperatures in the region of the basal lamina in the myotendinous junction. There is an obvious need to ascertain the nature of these enzymes and their location in the tissue before practical suggestions to overcome the problem can be attempted on anything other than an *ad hoc* basis. The enzymes may well have uses in food processing if they have suitably broad activity and can be obtained readily in sufficient quantity.

Further work on the nature of the fine connections between the basal lamina and the collagen and the sarcolemma is of prime importance in understanding the nature of this important and basic structure.

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Characterization of Type I Collagen from the Skin of Blue Grenadier (*Macruronus novaezelandiae*)

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The skin collagen of a fish, blue grenadier (*Macruronus novaezelandiae*), has been purified and characterized. The fish skin was readily soluble in dilute acetic acid, with no pepsin treatment needed. The collagen was purified by salt precipitation. Skin samples from fish of various ages showed that even in the oldest sample, more than 8 years of age, the collagen was still readily acid soluble. The purified collagen had a melting temperature of 22°C; the shrinkage temperature for the skin was 48°C. Its tissue distribution, examined by immunohistology, and its chemical properties indicated a close homology to mammalian type I collagen. However, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that three distinct α-chains were present. These were purified by ion-exchange chromatography on CM-cellulose and by gel permeation chromatography on Superose 6. The three purified α-chain fractions were examined by amino acid analysis and by SDS-PAGE of their cyanogen bromide fragments. These data indicated that the additional chain was genetically distinct, and most closely related to the α1-chain, from which it was poorly resolved on SDS-PAGE.

The collagen of fish tissues resembles the well-characterized mammalian and avian type I collagens, although in certain species it may have a distinct structure arising from the presence of an additional α-chain (1-3). When fish is used as a food source, the extent to which the collagen maintains the structural integrity of the tissue postmortem is important for product quality (4). In some species, for example, blue grenadier, the collagen noticeably deteriorates postmortem in the region of the myotendinous junction (5, 6), causing quality problems and economic loss. Characterization of the collagens in fish may assist in understanding the basis of this deterioration in quality.

MATERIALS AND METHODS

Isolation of collagen. Blue grenadier (*Macruronus novaezelandiae*, Hector, 1871) were obtained from the CSIRO research vessel, RV Soela. The age of specimens was estimated from length and weight measurements (7). Skin samples, cut from the dorsal region and dissected free of other adhering tissue, were diced and suspended in 0.1 M acetic acid, adjusted to pH 2.5 with HCl, for 20 h. After removal of insoluble material by centrifugation, 8000*g* for 1 h, the soluble collagen was purified by differential salt precipitation (8, 9).

SDS[2]-polyacrylamide gel electrophoresis. SDS-PAGE was performed according to the method of Laemmli (10), using 5% (w/v) polyacrylamide running gels for intact collagen chains and 12.5% polyacrylamide running gels for cyanogen bromide fragments, both with 3.5% (w/v) polyacrylamide stacking gels. After electrophoresis, gels were fixed and stained for 14 h in 0.1% Coomassie blue R-250 in methanol:acetic acid:water (5:1:5, v/v) and then destained in methanol:acetic acid:water (2:3:35, v/v).

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[2] Abbreviations used: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CNBr, cyanogen bromide; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

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Purification of collagen α-chains. Purified type I collagen was separated into its component α-chains by chromatography on CM-52 cellulose (Whatman CM-52) at 42°C, in 60 mM sodium acetate, pH 4.8, with elution by a linear gradient of 0–100 mM NaCl (11), followed by rechromatography of separated chain fractions under the same conditions. The α-chains were then separated from contaminating β-components by chromatography on Superose 6 (Pharmacia) in 50 mM sodium phosphate, pH 6.8, containing 150 mM NaCl and 2 M urea (12).

Chemical characterization of collagen α-chains. Purified α-chains were cleaved by 50 mg/ml CNBr in 70% formic acid for 4 h at room temperature, followed by lyophilization (13). Resulting fragments were analyzed by SDS-PAGE. For amino acid analysis, samples were hydrolyzed in 6 M HCl containing 0.01% phenol in evacuated tubes for 24 h at 108°C, and analyses were performed with a Waters HPLC amino acid analysis system using ninhydrin detection.

Collagen melting temperature determination. The melting temperature of collagen in intact skin was determined in 0.2 M NaCl, 10 mM sodium phosphate, pH 7.4, using a hydrothermal shrinkage apparatus as described by Bavinton (14), with a temperature increase of 1°C/min. The melting temperature of purified collagen in the same buffer was determined by measurement of circular dichroism at 221 nm (15), with the same rate of temperature increase.

Collagen solubility determination. Neutral-salt-soluble, acetic acid-soluble, and insoluble residue collagen fractions were prepared from powdered skin (16). The collagen content for each fraction was calculated as a percentage of the total collagen recovered in all three fractions. Collagen quantities were based on hydroxyproline values determined by amino acid analysis.

Antigenic characterization of collagen. Murine polyclonal antibodies to the purified collagen were produced in 12-week-old female, SJL/J mice that had been immunized intraperitoneally with 200 μg collagen emulsified in Freund's complete adjuvant. After 3 weeks they were further immunized intraperitoneally with 200 μg of the same antigen in Freund's incomplete adjuvant. Mice were bled 7 days after the last immunization and sera were tested for reactivity to the blue grenadier collagen by standard ELISA. Antibody specificity was further examined after separation of collagen chains by SDS-PAGE and transfer to nitrocellulose by electroblotting (17). Nitrocellulose sheets were then stained for protein with 0.1% amido black in methanol:acetic acid:water (5:1:5, v/v) for 3 min. For staining with antibodies, nitrocellulose membranes were blocked for 1 h in 5% Blotto (nonfat, dried milk powder) (18) and then reacted with antibody diluted 1:1000 in Blotto. Goat anti-mouse Ig (Bio-Rad), conjugated to horseradish peroxidase, was diluted 1:1000 in Blotto and used as the secondary antibody. Binding of antibodies was visualized using 0.3% 4-chloro-1-naphthol in 20 ml methanol containing 0.06% H_2O_2 added to 100 ml Tris-buffered saline, pH 7.4.

Immunohistology. Sections, 6 μm thick, were cut from frozen tissue using a freezing microtome. They were stained with the polyclonal antibody, diluted 1:100 in 0.15 M NaCl, 5 mM sodium phosphate, pH 7.4 (PBS), washed twice for 10 min in PBS, and then visualized with affinity-purified, fluorescein isothiocyanate-conjugated, sheep anti-mouse antibody (Silenus Laboratories, Melbourne) diluted 1:50 in PBS. After a further two washes for 10 min in PBS, sections were mounted in glycerol:water (9:1, v/v) containing 1 mM 1,4-phenylenediamine. Control slides were made either using preimmune serum instead of the mouse anti-collagen antibody or by omitting the mouse antibody.

RESULTS AND DISCUSSION

The fish skin collagen was readily soluble in dilute acetic acid, so pepsin treatment was not required. Examination of collagen solubilities indicated that the youngest samples had the greatest quantity of neutral-salt-soluble collagen, (Table I), while the amount of acid-insoluble collagen increased with age. The high solubility in acetic acid was present even in fish at least 8 years old (Table I), where most of the collagen, about 90%, was still acid-soluble.

High acid-solubility, which has been observed in skin collagen from other fish species (4), may reflect a slow rate of mature crosslink formation, with an accumulation of acid-labile, aldimine, crosslinks (19). If oxygen availability plays an important part in crosslink maturation (20), then the deficiency of mature crosslinks may result from a lower availability of oxygen in fish skin than in mammalian tissues (21), although other factors may also be involved. The tendency of blue grenadier to show food quality problems, due to lack of post-mortem structural integrity (5, 6), may result from the very low amount of mature crosslinks present in this species.

Acetic acid-soluble collagen was readily purified by fractional salt precipitation, and was precipitated at the same salt concentrations as those used for mammalian type I collagen purification (8, 9). There was no evidence of significant quantities of other collagens that precipitated at the

TABLE I

SOLUBILITY OF SKIN COLLAGEN FROM BLUE GRENADIER OF VARIOUS AGES

	Length (mm)	Weight (g)	Age (years)	Neutral salt soluble (%)	Acid soluble (%)	Insoluble (%)
Juvenile	310	106	1-1.2	8.2	87.8	4.0
Juvenile	315	106	1-1.2	10.0	87.7	3.3
Young adult	490	748	2	3.3	91.8	4.9
Young adult	610	798	3	3.0	91.5	5.5
Mature adult	960	3750	≥8	2.3	89.3	8.4
Mature adult	1020	4450	≥8	2.1	91.0	6.9

Note. The collagen in each fraction is given as a percentage of the total collagen of all three fractions. Collagen was taken from the same position in each fish, as determined from myotome number, analyzed in duplicate, and averaged. The age of fish was judged from fish length, using the data of Kenchington and Augustine (7).

salt concentrations used for fractionation of mammalian types III and V collagens.

The constituent α-chains of the collagen were separated by chromatography on CM-cellulose (Fig. 1); this gave a separation that was distinct from that obtained for mammalian type I collagens (11), and indicated the presence of an additional α-chain. This additional chain, designated the $\alpha 3$-chain, was hard to resolve from the first peak eluted, the $\alpha 1$-chain. Purification was achieved by repeated chromatography on CM-cellulose, followed by gel-permeation chromatography on Superose 6 to obtain samples free of β-components.

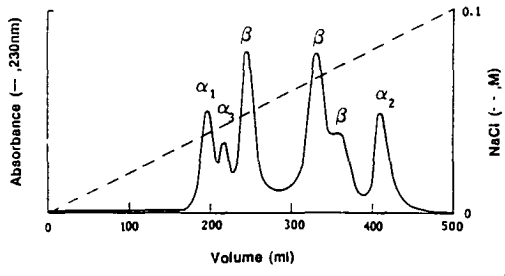

FIG. 1. Separation of the α-chains of blue grenadier skin collagen by chromatography on CM-cellulose (Whatman CM 52), 25 × 80 mm, in 60 mM sodium acetate, pH 4.8, with elution by a linear gradient of 0-100 mM NaCl, in a total elution volume of 500 ml. The peaks from which purified α-chains were prepared by further chromatography are indicated. The peaks labeled β each contained a range of β and higher polymer components.

SDS-PAGE demonstrated the three distinct α-chains in the collagen and in the purified fractions (Fig. 2), although the $\alpha 1$- and $\alpha 3$-chains were not well resolved. SDS-PAGE of fractions from the CM-cellulose column also indicated the presence of at least four distinct β-components (data not shown) in the three broad zones which contained the β-components and higher polymer forms (Fig. 1). Repeated chromatography was not sufficient to enable individual β-components to be prepared with sufficient purity to allow for accurate analysis of their chain compositions; this was probably due to the great similarity between the $\alpha 1$- and $\alpha 3$-chains.

The purified α-chains were examined by both amino acid analysis and CNBr diges-

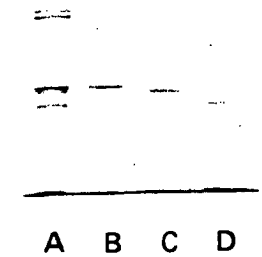

FIG. 2. SDS-PAGE of purified blue grenadier skin collagen (lane A) and the purified $\alpha 1$- (lane B), $\alpha 3$- (lane C) and $\alpha 2$-chains (lane D).

TABLE II

AMINO ACID ANALYSIS OF PURIFIED α-CHAINS

	α1	α2	α3
HO-Pro	69	60	60
Asp	47	52	49
Thr	26	26	24
Ser	46	53	49
Glu	82	65	88
Pro	96	99	94
Gly	345	347	347
Ala	135	121	138
Cys	0	0	0
Val	17	23	17
Met	15	13	10
Ile	8	11	10
Leu	14	23	14
Tyr	1	5	1
Phe	15	9	15
HO-Lys	4	7	4
His	3	11	12
Lys	29	22	26
Arg	48	53	42
Trp	nd	nd	nd

Note. Values are given as residues/1000. Tryptophan was not determined (nd).

tion. The amino acid analyses indicated (Table II) that each chain fraction had a distinct composition, with the α3-chain more closely resembling the α1- than the α2-chain. The CNBr fragment separations by SDS-PAGE also showed differences among all three chain fractions (Fig. 3). The α3-chain showed a very similar separation to the α1-chain, but a distinct, faster moving, band was present only in the α1-chain; both the α1- and the α3-chains showed patterns that were quite distinct from the α2-chain. Fewer CNBr fragments than might be expected from the amino acid composition were observed. This possibly indicates that several small fragments were formed which were not resolved by electrophoresis.

The melting temperature of intact skin was 48°C, and that of the purified collagen was 22°C, lower than observed for purified land mammal or avian collagens (36–41°C), and was consistent with the reduced imino acid content of the fish collagen (22). A correlation between melting temperature and approximate environmental temperature of an animal has been noted, originally with reference to fish collagens (23), but later found in other species as well (22, 24). Blue grenadier are found mostly in continental shelf waters, 400–700 m deep, where temperatures range from 7 to 12°C. However, at night they rise to feed to within 50–100 m of the surface, where temperatures in the summer may be near 20°C (J. S. Gunn, personal communication). The melting temperatures for the collagen and the skin of blue grenadier, which were higher than those for cold water fish, but lower than those for warm water fish (22), were consistent with this behavior pattern.

The tissue distribution of the collagen was examined by immunohistology using a murine polyclonal antibody. Since noncollagenous components of connective tissue can be highly antigenic when compared with collagens (25, 26), the antibody was shown to be highly specific for the collagen antigen by electroblotting. ELISA indicated that the antibody reacted with each of the purified α-chains. The immunohistology demonstrated a very broad distribution for the collagen, in skin, myocomma, vessels, intestine, and swim bladder (Fig. 4), consistent with the expected broad distribution of a type I collagen.

Previously, chromatography has shown that an α3-chain was present in carp skin (3, 27), but was absent in carp swim bladder (11). This suggested that multiple

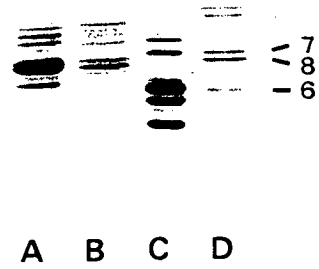

FIG. 3. Separation by SDS-PAGE of CNBr fragments from purified blue grenadier (A) α1-chain, (B) α3-chain, and (C) α2-chain, and (D) bovine α1(I)-chain.

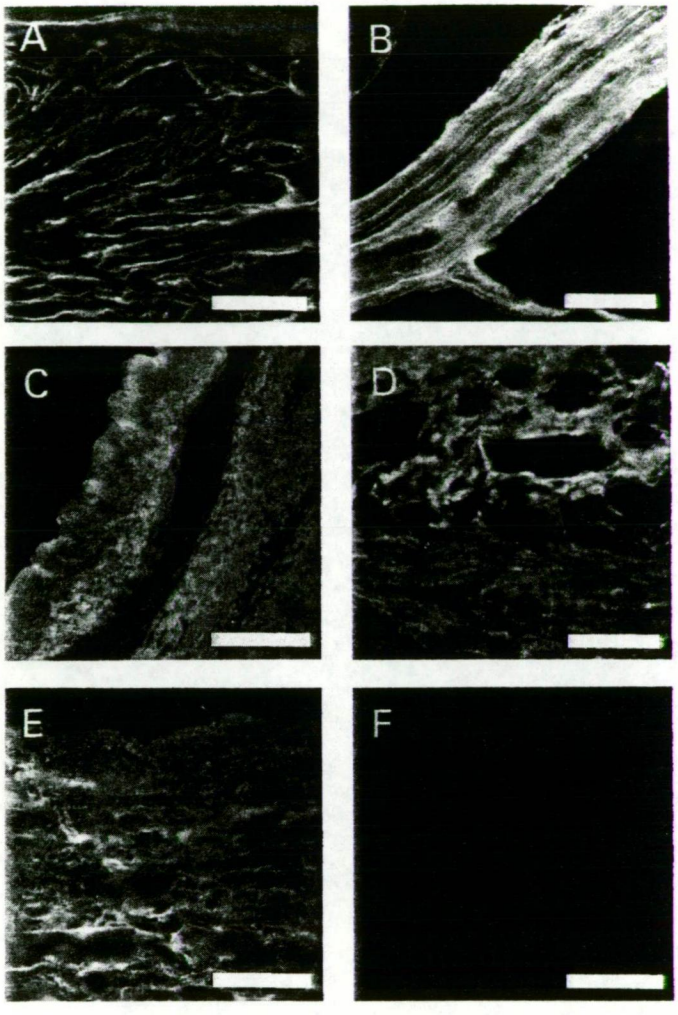

FIG. 4. Immunohistology of various tissues from blue grenadier using a murine polyclonal antibody against the purified skin collagen and fluorescein isothiocyanate-labeled, affinity-purified, sheep anti-mouse antibody. Tissues examined: (A) skin, (B) myocomma, (C) blood vessel, (D) intestine, and (E) swim bladder. Control sections, examined without the murine polyclonal antibody, indicated little nonspecific fluorescence, as shown for skin (F). Bars = 50 μm.

forms of type I collagen may be present in fish and have different tissue distributions. The α3-chain may be present as a separate homotrimer, although the number of observed β-components tends to suggest that it forms heterotrimers with the α1- and α2-chains.

The presence of an α3-chain in fish skin collagen was first reported for cod (1), and subsequently for other species (2, 3); these species, along with blue grenadier, are members of the subdivision Teleostei of the bony fish (class Osteichthyes). Since chromatographic studies on fish skin collagens from taxonomically different groups (11, 28) failed to show an α3-chain, this additional chain may be restricted to within one class of fish, and may be of taxonomic or phylogenetic value.

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