THE EFFECTS OF STARVATION AND REFEEDING ON THE MUSCULATURE OF THE MARINE TELEOST POLLACHIUS VIRENS L.

Charles Henry Beardall

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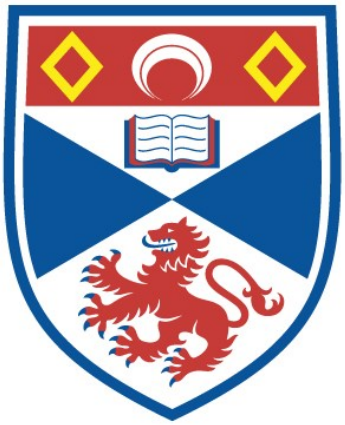
University of St Andrews



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The Effects of Starvation and Refeeding on the Musculature of the Marine Teleost *Pollachius virens* L

A Thesis submitted to the University of St.Andrews

for the degree of Doctor of Philosophy

by

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Summaries

Chapter 1

A brief introduction is given reviewing the physiology and biochemistry of fish myotomal muscle. Aspects of protein turnover and metabolism in muscle are discussed and related with previous studies on starvation and refeeding in both mammals and fish. In view of the ultrastructural similarities observed in muscle during myopathies in man, experimental myopathies in other animals, and some physiological stresses (for example starvation, denervation and disuse) a short review is given concerning the relevant literature.

Chapter 2

The marine teleost Pollachius virens L. undergoes a natural starvation during the winter months, and provides a reversible , non-pathological model for studying muscle wasting. In this study fish were kept without food under laboratory conditions for up to 12 weeks. The effects of starvation on muscle fibre cross-sectional area, volume fractions of mitochondria and myofibrils, and capillary supply were determined.

Starvation results in a preferential degradation of fast muscle myofibrillar proteins. For example, fibre cross-sectional area decreased from 1014 to 535 um^2 ($p<0.005$) and myofibrillar volume fraction from 79.0% to 56.4% ($p<0.001$) in fast fibres following 12 weeks starvation. In contrast there was little change in these parameters in slow muscle fibres.

Evidence is presented that M-line and thick filament breakdown occur as an initial stage of myofibrillar degradation. Sarcoplasmic reticulum in atrophied fibres often appeared swollen and multi-membraned lysosome-like vesicles were common.

The percentage of slow fibres (44 to 64% ; $p<0.025$) and fast fibres (51 to 86% ; $p<0.01$) without capillary contact increased and the percentage of fibre perimeter vascularised decreased during a 12 week starvation (6.3 to

3.3% in slow fibres and 2.8 to 1.1% in fast fibres). The volume fractions of mitochondria in slow fibres decreased in parallel to the decrease in capillary supply (from 34.6 to 18.6% ; $p<0.001$).

Mechanisms of myofibrillar degradation during muscle wasting are discussed.

Chapter 3

Starvation in the winter months is followed by a period of refeeding in the late spring, and the musculature degraded during the winter is regenerated. In the present study muscle fine structure has been investigated for fish kept without food, under laboratory conditions, for 74 days, and following 10, 20, and 54 days' refeeding. Muscle fibre cross-sectional area, capillary supply, and volume fractions of myofibrils, nuclei and mitochondria were determined from electron micrographs, using digital planimetry and stereological techniques.

In contrast to the starvation atrophy observed in chapter 2 the 74 day starvation reported in this chapter resulted in a decrease in the mean fibre cross-sectional area in both fast and slow muscle. However, in common with the previous experiment, a decrease in the fractional volume of myofibrils was only observed in the fast muscle (84.4% to 61.5%, $p<0.001$). Mitochondrial volume densities declined in both fast (5.2% to 1.5%, $p<0.001$) and slow muscle fibres (40.9% to 25.1%, $p<0.001$) after 74 days' starvation. In addition, intracellular glycogen stores were depleted and lipid droplets, present in slow fibres only, decreased from 7 4% to 0% of the fibre volume.

After 20 days refeeding, glycogen stores exceeded those observed in control fibres. Lipid droplets showed a transient increase after 10 days refeeding (5.3%), followed by a decrease at 20 days (0.9%), and a slow restoration to levels found in fed fish. Numerous ribosomes and polysomes were observed in the cytoplasm, mitochondria and nuclei of muscle fibres during refeeding. These were not evident in starved tissue. The number and

volume fraction of mitochondria gradually increased throughout the refeeding period.

In many starved fibres myofibrillar diameter is reduced, relative to controls, and the M-line and thick filaments are absent at the periphery of the myofibril. At 10 days (slow fibres) and 20 days (fast fibres) after refeeding the thick and thin filament lattice appears normal, although myofibrillar diameter is still less than in control fibres. In fibres from 10 day refed fish the formation of new sarcomeres appears to be initiated by the formation of the thin filaments, often attached to an electron dense area ("Z-body"). The Z body, invariably associated with a tubular membrane element, coalesce into a distinctive Z disc when the sarcomere nears a full complement of thick and thin filaments. Incomplete thick filaments form initially on one side of the sarcomere, within the thin filament lattice, and appear to assemble progressively towards the other. The possible mechanisms involved in the assembly of new sarcomeres are discussed.

Chapter 4

Experiments were performed using SDS/polyacrylamide gel electrophoresis, to consider the effects of starvation on the relative abundance of actin and myosin heavy chains. Both slow and fast muscle proteins were analysed from control and 66 day starved fish, using densitometric scans of comassie blue stained polyacrylamide gels. The results were expressed as the ratio of each protein relative to actin.

In fast muscle 66 days starvation resulted in a significant decrease in the relative abundance of myosin heavy chains ($p<0.01$) and a peak that may correspond to troponin T ($p<0.01$). In slow muscle, starvation did not cause significant changes in the relative abundances of any of the muscle proteins analysed. Starvation resulted in a general increase in the lower molecular weight proteins (< 30,000 daltons) in both muscle types.

The results are discussed with reference to the observations made in chapters 2 and 3.

Chapter 5

A wide range of biochemical parameters were determined to consider the metabolic effects of starvation and refeeding in saithe. Preliminary experiments involving a starvation for 84 days under laboratory conditions, resulted in significant decreases in both body condition indeces ($p<0.001$) and liver somatic indeces ($p<0.001$). Blood glucose remained elevated during the first 63 days of starvation but at 84 days had decreased to 30% of control values ($p<0.001$). Starvation for 84 days resulted in decreases of the glycogen stores in liver (91% decrease), fast muscle (73.2% decrease), and slow muscle (77.4% decrease).

The second experiment involved a starvation of 74 days after which there was a decline in the activities of both slow and fast muscle glycolytic enzymes (hexokinase and pyruvate kinase), but a fourfold increase in liver hexokinase activity. Aspartate amino transferase and glutamate dehydrogenase increased in most tissues, but only the liver had the potential to utilise alanine (high alanine aminotransferase activities). Carnitine palmitoyl transferase activity did not alter during starvation in slow or fast muscle, but significantly increased in the liver ($p<0.001$).

Upon refeeding (after 74 days starvation) the body condition index increased steadily, reaching that of control fish after only 28 days. In contrast muscle water contents did not return to control values even after 54 days of refeeding. An overcompensation of muscle glycogen stores, to values exceeding those found in control fish, was observed at 20 days of refeeding. Continued feeding resulted in the reduction of these energy stores to control values. Even though the apparent priority upon refeeding, was to replenish liver glycogen stores, the concentrations never significantly exceeded those

observed in control fish.

During the onset of refeeding muscle glycolytic potential remained depressed, whilst fatty acid metabolism increased. Liver metabolism (amino acid transferases, CPT, HK, and PK activities) gradually declined to that observed in control fish, with the exception of alanine aminotransferase activity which continued to increase upon refeeding, but declined steadily to control values in between 10-54 days. After 54 days of refeeding muscle glycolytic capacity (PK activities) and aspartate aminotransferase activities had increased to levels significantly elevated from those found in control fish. Carnitine palmitoyl transferase activities, however, steadily declined during the course of the refeeding.

The results are discussed with reference to previous studies on starvation and refeeding, both in mammals and fish.

Chapter 6

This chapter investigates the involvement of acid hydrolases and the lysosomal system in muscle atrophy and regeneration, during starvation and refeeding. Fish were kept without food for 66 days and 74 days in two separate experiments. After 74 days starvation they were refed for 54 days, during which samples were analysed at 10, 20 and 54 days of refeeding. A broad spectrum of acid hydrolases (acid proteinase, aryl sulphatase, β-glucuronidase, acid phosphatase, and acid ribonuclease) were monitored both during starvation and refeeding.

In control fish, acid hydrolase activities were higher in slow muscle (with the exception of acid phosphatase). A 66 day starvation resulted in an increase in all the lysosomal enzymes measured with the exception of slow muscle acid proteinase. There was a proportionally larger increase in the lysosomal enzyme activities in fast muscle. Upon refeeding, after a 74 day starvation, there was an immediate decrease in both slow and fast muscle acid

proteinase, and aryl sulphatase activities. Lysosomal enzyme activities after 10 days of refeeding were similar to those found in control fish.

Two lysosome rich fractions were obtained from the fast muscle of starved fish by differential centrifugation ; fraction II (10,000g pellet) and fraction III (22,500g pellet). At pH 5.0 both fractions were capable of degrading native actin and myosin heavy chains, but were relatively incapable of degrading tropomyosin. Both fractions were capable of degrading myosin heavy chains to a greater extent than actin. At pH 7.4, however, niether fraction was capable of degrading any myofibrillar protein.

The organelles present in fraction III from control fish were indistinguishable from those observed in fraction III from starved fish. Fraction II from starved fish, however, contained many multi-membraned vesicles that were absent in pellets from control fish. It was suggested that these vesicles were autophagic vacuoles (secondary lysosomes) and were atleast partially responsible for the increase in acid hydrolase activity of fraction II.

Cytochemical localisation of acid phosphatase activity in fast muscle from starved fish, revealed activity in the t-tubules and occasionally within the terminal cisternae of the sarcoplasmic reticulum.

The results suggest a role for lysosomal enzymes in the breakdown of myofibrillar proteins during starvation.

Chapter 7

The results are discussed with reference to the overall strategy of fish to survive starvation immediately followed by a period of rapid muscle repair and growth, during refeeding . The mechanisms involved in the disassembly and degradation of the myofibrillar proteins are discussed with specific reference to the involvment of the sarcoplasmic reticulum, t-tubules and proteinases. Suggestions are made for further work that may give an insight into some of the questions generated by this research.

Chapter 1

General Introduction

Introduction

During the natural life cycle of a fish there are many biochemical, physiological, and morphological changes that occur according to the season. These changes are ultimately the result of annual fluctuations in the environmental conditions, and are influenced by the nutritional and developmental status of the fish. The anual cycle of a fish may include (depending on the species) spawning migration, pre-spawning feeding, spawning, pre-migration feeding, wintering migration, wintering feeding. Ofcourse non-migratory species, for example many freshwater species, do not include so many phases in their general life cycles. Saithe (Pollachius virens L., previously Gadus virens) are a member of the gadoid family and form large shoals in the North West and East Atlantic. Saithe are the most active gadoid, containing the most red muscle, and are strongly migratory (Love, 1980). In the North East Atlantic large shoals are found off the northern coast of Norway, in the North sea, and in the waters around the Faroes and Iceland. At the end of the 1960's saithe fisheries expanded dramatically and now supply an important food resource in both the European and Russian fish markets. In the 1960's the maximum recorded catch from the North East Atlantic was 720,000+ tons per year, however since then the yearly catch has been in a steady decline and by 1979 had decreased to 400,000+ tons. Especially pronounced has been the decrease in the North Sea stocks which by 1979 had decreased from 36 % to 23 % of the total north atlantic catch (Reinsch, 1980). Of all the North East Atlantic fish stocks, the distribution, feeding and spawning of the North Sea saithe populations are least well studied. Generally it is considered that the northern regions above 59^{o} (latitude) are the areas of spawning, whilst the regions below 59^{o} are areas of feeding (Golubyatnikova & Malyshev, 1980). The size structure of the northern population varies from 33 to 110 cm

(average 65 cm) corresponding to variations in age from 2 to 13 years. In the southern range the size structure is smaller (33 to 60 cm) with the age ranging from 2 to 5 years. Spawning occurs offshore between January to April, tending to be later in the northern regions, at a depth of 100-200 metres (Wheeler, 1969). The eggs and larvae are pelagic in the top 30 metres, but by midsummer are found inshore. Young fish may spend 1-2 years inshore before moving to surface water offshore. South of 59° the average size of one year old fish was 32.8 cm (figure for 1973 - 1975). Immature fish remain in the surface waters for 1-2 years until moving to mid water regions where they spend the majority of their life. The diet of saithe is mainly Euphausiacae (especially for the size group 31-50 cm) and Trisopterus esmarkii N. (Norway pout). Other foods taken include haddock, blue whiting, young cod, sand eels, sprats, herring smelts, cope pods, and amphipods. Ninety percent of the diet of the larger fish (51-110 cm) consists of fish. In Britain the largest recorded saithe was caught off Lands End in 1921 and weighed 23.5 lbs.

Fish skeletal muscle

Fish display a wide variety of adaptations to a host of different niches supplied by the aquatic environment. In general , however, the design of fish trunk muscle is governed by the properties of the high density medium in which they live. Unlike terrestial locomotion, where the power required for motion usually increases linearly with velocity, to overcome the drag forces imposed by motion in water, the power required is proportional to the velocity cubed (Webb, 1975). These properties require that a disproportionately larger mass of muscle is needed with every additional increment in swimming speed. The buoyancy of muscle tissue in water partly offsets these limitations allowing fish to carry large masses of muscle specifically for fast swimming speeds, without the need for weight economy. Thus muscle may constitute between 40 -

75 % of the total body mass (depending on the species) of which up to 90 % may be fast contracting, white fibres, used during periods of fast swimming (Greer-Walker & Pull, 1975 ; Bone, 1978a). The metabolic limitations of supplying large masses of muscle with oxygen and nutrients are partially overcome by the reliance of fast fibres on anaerobic metabolism.

Fibre types

By using biochemical, histochemical, ultrastructural, and physiological criteria a number of fibre types have been defined in fish. The number of fibre types present varies between the species but is largely composed of two fundamental classes of fibre, red and white, originally classified according to their colour (Lorenzini, 1678, see Bone, 1978a). In the dogfish, Scyliorhinus canicula Bone (1966) has distinguished 5 fibre types in progressive layers from superficial red to deep white. In some teleosts, for example brook trout, Salvelinus fontinalis, and mackerel, Scomber scombrus, (Johnston & Moon, 1980b ; Bone, 1978b, respecively) only two fibre types exist, whereas in others three distinct fibres have been identified (e.g carp, Cyprinus carpio ; Johnston et al, 1977).

Slow muscle

In most fish, myotomal muscle contains a superficial, anatomically discrete, strip of muscle historically termed red muscle. More recently muscle types have been described by their myofibrillar ATPase activity and mechanical properties. Hence red muscle with a low ATPase activity and slow speed of shortening has been termed slow muscle (Barany, 1967). The quantity of slow muscle varies according to the activity of the species. For example active pelagic fish possess a larger proportion of slow muscle (29 % in anchovy, Engraulis encrasicholus), whereas demersal species can have as little as 0.5 % of the total musculature as slow fibre (Greer-Walker & Pull, 1975).

The quantity of slow muscle also varies along the trunk and with increasing body size (Magnuson, 1973). Slow muscle is characterised by large lipid and glycogen stores (Love, 1980), low myofibrillar ATPase activity (Johnston et al, 1974), high activities of tricarboxylic acid cycle enzymes (Johnston et al, 1977), high myoglobin concentrations (hence the red appearance of slow muscle), high capillarisation and slow maximum contraction velocities (around 20 - 25 % of those found in fast fibres)(Altringham & Johnston, 1982). Ultrastructurally fish slow muscle contains a volume density of mitochondria that is amongst the highest found in any striated muscle and, relative to other vertebrate slow muscle, a highly developed sarcoplasmic reticulum (s.r.) and tranverse tubule system (t-system). Indeed the s.r. and t-system of fish slow muscle even approaches that of some twitch fibres (Johnston, 1980a). Slow fibres are aerobic in nature and depend upon the oxidation of lipid as the major source of fuel for contraction (Bilinski, 1974 ; Driedzic & Hochachka, 1978).

Intermediate muscle

Little work has been done on the intermediate fibre types (sometimes referred to as pink fibres), but generally they are found to possess an aerobic capacity and volume fraction of mitochondria that is intermediate between slow and fast muscle (Johnston et al, 1977 ; Johnston & Maitland, 1980 ; Kryvi et al 1980). Intermediate fibres are present in many, but not all fish, and are situated in between the slow and fast fibre layers

Fast muscle

Fast fibres are characterised by a low aerobic capacity, high myosin ATPase activity, low capillary density, and fast maximal velocity of contraction (Bone, 1978 ; Love, 1980 ; Johnston, 1981a). Ultrastructurally fast fibres contain a low, but variable volume density of mitochondria (0.5 - 8%) and a high myofibrillar volume density (up to 90 %) (see Johnston, 1980b).

The myofibrils are elongated (ribbon shaped) at the periphery of the fibres. Fast fibres possess a more extensive s.r and t-system than slow muscle, even resembling that found in amphibian muscle (Peachey, 1965 ; Flitney, 1971). In fish muscle the prominent M-line is distinctively thicker in fast muscle than slow.

Whereas slow superficial fibres generally run parallel to the surface of the fish, deep fast fibres have a complex orientation which varies along the length of the body. In teleosts the myotomes form a W shape with the fibres arranged in a complex pattern. Their orientation is such that successive myotomes form a series of helices with their axis running parallel to the long axis of the body (Alexander, 1969). In general the fibres make an angle of up to 40^{o} with the lateral axis of the body (Alexander, 1969). This system requires that during swimming fast fibre contraction is virtually isometric, needing only a 2 - 3 % shortening of fibre length, irrespective of the position of the fibre along the body (Alexander, 1969).

In many fish smaller diameter fibres are found within the fast muscle. Even though these fibres possess some distinctive histochemical properties (for example SDH activity), is unclear as to whether they are a distinctive fibre type or, whether they represent the various stages of fast fibre growth (Johnston et al, 1975 ; Koreliussen et al, 1978).

Fibre innervation and recruitment

All fish slow fibres are multiply innervated with small diameter, myelinated axons, forming "en-grappe" type terminals along the fibre (Bone, 1966, 1970). Slow fibres are normally activated by junction potentials, even though it is thought that they may be capable of propagating action potentials (Stanfield, 1972). In hagfish, Eptatretus burgeri, the slow fibres are innervated by one axon from each myoseptum (Jansen et al, 1963), however in elasmobranchs and teleosts each fibre is innervated by at least two axons (Bone, 1966)

For fast fibres, two distinctive types of innervation exist.

Elasmobranchs, dipnoans, agnathans, chondrosteans, and holosteans possess focally innervated fast fibres, with "en-grappe" type terminations at one end of the fibre. A similar type of innervation is found in amphibians, reptiles, birds, and mammals. In dogfish, Scyliorhinus canicula, each fibre is innervated by two large diameter axons that fuse to form one end-plate (Bone, 1964,1972). These fast fibres show a propagating action potential that overshoots zero potential, similar to frog fast twitch fibres. However, among the majority of teleosts fast fibres are multi-terminally innervated. Each fibre recieves numerous nerve terminals from axons that run down the myosepta and then spread out across the surface of the myotome (Bone, 1964). In cod (Gadus morhua) fast muscle each fibre may recieve up to 23 motor terminations, depending on where the fibre is situated (Altringham & Johnston, 1981). Superficial fast fibres are found to have a greater density of end plates than deeper fibres (Altringham & Johnston, 1981). In experiments on Cottus scorpius (Shortspined cottus), Hudson (1969) reported two different electrical responses to stimulation of the spinal nerve ; junction potentials, leading to local, graded contractions, and spike potentials resulting in fast twitches. However it is unclear as to whether both these responses occur in vivo.

As swimming speed increases, the pattern of fibre recruitment is found to vary according to the innervation of the muscle. Focally innervated fibres, for example, are only recruited for burst swimming (Bone 1966). To exemplify this, at slow sustainable swimming speeds in the pacific herring, only slow fibres are active. As velocity increases above 5 lengths / second, fast fibres are recruited and fatigue occurs within 1 - 2 minutes swimming (Bone et al, 1978). For species with multiply innervated fast muscle, for example saithe (Johnston & Moon, 1980a), brook trout (Johnston & Moon,1980b), rainbow trout (Salmo gairdneri, Hudson, 1973), and common carp (Johnston et al 1977), the threshold speed for recruitment of the fast fibres

is much reduced (0.8-1.9 lengths / second in saithe, Johnston & Moon, 1980a), and therefore fast fibres are also active at slower cruising speeds. Studies on the recruitment of intermediate fibres in carp have shown the order with increasing speed to be red / intermediate / white (Johnston et al, 1977). It has also been reported that the recruitment of the fast fibres is dependant on the depth within the myotome, the superficial fibres being recruited before the deeper fibres (Johnston & Moon, 1980b).

Johnston and Moon (1980a) have reported a wide range of aerobic capacities in fast muscle from different species and have suggested that this correlates to the degree of involvement of fast muscle during sustained swimming. Other adaptations involving the fast muscle in cruise swimming and maximising swimming efficiency may entail a "flick and glide" locomotion (Videler, 1981). The drag on a continously swimming fish is 3.3 times greater than that on a gliding fish. A "flick and glide" locomotion may also allow a partial recovery of the muscle inbetween each "flick".

Metabolism

The basic pathways of intermediary metabolism are essentially the same in fish as in mammals. There are, however, differences in the relative physiological importance of the various pathways. A brief review of the intermediary metabolism in fish is given below. For more extensive reviews see Bilinski (1974), Driedzic and Hochachka (1978), and Walton and Cowey (1982).

Carbohydrate metabolism

In comparison with mammals carbohydrates are poorly utilised in fish. This assumption is based upon a poor control of blood glucose homeostasis, low activities of hexokinase, no detectable liver glucokinase activities, and in comparison with mammals, low levels of carbohydrate stores (Love, 1970, 1980 ;

Bilinski, 1974). For carnivorous fish, protein and lipid are the primary source of energy.

In both mammals and fish glycogen is the primary carbohydrate store and is found in all tissues, but mainly in the liver and the musculature. In mammals, hepatic glycogen stores are hydrolysed to maintain blood glucose levels, enabling the liver to function as an effective glucostat. During periods of stress hepatic glycogen stores are the primary source of energy, for instance during a starvation in the rat, hepatic glycogen stores are depleted within 24 hr (Fister et al,1970). Following glycogen depletion blood glucose levels are maintained by hepatic gluconeogenesis using amino acids from muscle proteolysis, lactate from anaerobic metabolism, or glycerol from triglyceride oxidation.

Many studies have reported that during steady swimming glycogen is utilised in both muscle fibre types (Pritchard et al, 1971 ; Johnston & Goldspink, 1973a). In slow muscle glycogen is metabolised aerobically whereas in fast muscle anaerobic metabolism predominates resulting in the production of lactate. In active fish the maximum scope for sustained aerobic activity is between 10 to 100 times less than that in small mammals and birds (Brett, 1972) and therefore the potential for anaerobic metabolism is relatively high. Indeed, for the goldfish (Carassius auratus) Smit et al (1971) have estimated that anaerobic metabolism may provide as much as 80 % of the total energy requirements during sustained swimming. Glycogen is the major source of energy for anaerobic metabolism and is found to be depleted rapidly in fast muscle, during burst swimming. In the rainbow trout, for instance, approximately 50 % of the fast muscle glycogen is depleted during a 15 second burst swim (Stevens & Black, 1966). There is, however, a larger concentration of glycogen in the slow muscle than the fast (see Bilinski, 1974), and Johnston and Goldspink (1973b) have reported that carp slow muscle may utilise glycogen at a rate two to three times greater than in fast muscle at a

swimming speed of 3 lengths / second.

Similar to mammals there are three main pathways for the metabolism of carbohydrates in fish ; glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle. The utilisation of glycogen is ultimately regulated at three points and is dependant on the activities of glycogen phosphorylase, phosphofructokinase, and pyruvate kinase (Driedzic & Hochachka, 1978). Glycogen phosphorylase, as in mammals, is found in two forms, an active form, phosphorylase a, and an inactive form (except in the presence of relatively high concentrations of 5'-AMP), phosphorylase b. The complex sequence of reactions resulting in the conversion of glycogen to glucose-1-phosphate is ultimately controlled by the conversion of phosphorylase into its active form (reviewed in Dreidzic & Hochachka, 1978). Phosphorylase activity can increase either as a result of hormonal action, for instance adrenaline, or via the influence of Ca^{++}. In slow muscle, which is well vascularised, hormonal control is likely to be more important, whereas in fast muscle Ca^{++} activation may be of more importance. As fish muscle hexokinase activity is relatively low, and phosphorylase activity relatively high (especially in fast muscle) glycogen is likely to be the only source of carbohydrate during periods of excessive demand (i.e.burst swimming) (Crabtree & Newsholme, 1972 ; Nagayama et al, 1972 ; Johnston et al, 1977).

Phosphofructokinase (PFK), catalysing the phosphorylation of fructose-6-phosphate (F6P) to fructose,1,6,bis-phosphate (FDP), is inhibited by ATP, citrate and phosphoenolpyruvate (PEP), and activated by AMP, FDP and ADP. Fish PFK is thought to be regulated in a similar manner as mammalian PFK. Some fish possess relatively high activities of PFK, even exceeding those found in some mammals (Crabtree & Newsholme, 1972). It has been suggested that, as in mammals, a substrate cycle between F6P and FDP is operating, and that high activities of PFK (and FDPase) create a system that is even more sensitive to fluctuations in AMP concentrations (Moon & Johnston,

1980).

Pyruvate kinase (PK) catalyses the hydrolysis of PEP to pyruvate. The activity of PK is closely linked with the gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (PEPCK) by way of a futile cycle. PEP is converted to pyruvate, and further to oxaloacetate, catalysed by pyruvate kinase and pyruvate carboxylase respectively. To complete the cycle oxaloacetate is converted back to PEP by PEPCK. In a similar manner as the PFK / FDPase substrate cycle, this futile cycle is thought to create a sensitive control of metabolic flux, where the simultaneous activation of one enzyme and inhibition of the other causes an amplified response to fluctuating substrate concentrations (Newsholme & Start, 1973).

The pentose phosphate pathway (PPP) provides an alternative route for carbohydrate metabolism from G6P. This pathway generates NADPH, for fatty acid synthesis, and pentose phosphates for nucleic acid synthesis. The PPP has been demonstrated in a variety of fish (Nagayama et al, 1972), but is considered to account for only a small proportion of the total glucose metabolism (Hochachka & Hayes, 1962). Although the highest activities of the PPP are found in the liver and kidney, PPP enzymes have also been reported in the musculature and especially in slow muscle (Hochachka & Hayes, 1962 ; Bilinski, 1974).

Under aerobic conditions pyruvate undergoes oxidative decarboxylation to acetyl CoA and entrance into the tricarboxylic acid cycle for complete oxidation. In fish the tricarboxylic acid cycle has been found to operate in a similar fashion to that in mammals (Gumbmann & Tappel, 1962a). However little is known about the regulation of the TCA in fish (see Driedzic & Hochachka, 1978).

Under anaerobic conditions pyruvate is converted to lactate, by NADH-linked lactate dehydrogenase (LDH). In many tissues, and especially fast muscle, this reaction is the terminal step of glycolysis. Lactate

dehydrogenase , in the presence of NAD^+, can also convert lactate back to pyruvate and is therefore the first enzyme in the gluconeogenetic pathway from lactate to glucose. Many isoenzymes of LDH exist made up from a combination of two distinct sub units, the M-type and H-type. This enzyme has been widely studied in fish and is found to have similar properties as that of mammalian LDH (see Tarr, 1972 and Coppes de Achaval, 1984 for reveiws).

Lipid metabolism

Lipid is the major energy reserve in fish, as in mammals. One gram of fat gives rise to 9.3 Kcal of energy in comparison with 3.7 Kcal for 1 gram of glycogen. Lipids therefore comprise a far more efficient energy storage medium. Fish have a variety of fat deposit sites. In elasmobranchs triglyceride (TG), the major lipid storage compound, is stored in the liver. In teleosts, storage deposits also occur as adipocytes throughout the musculature, and at specific sites in the viscera (Love, 1970). In general the lipid stores of the less active fish are mainly in the liver, whereas the active pelagic fish have adipose muscles with relatively small hepatic stores. During cruise swimming lipid is an important fuel for aerobic metabolism (Hochachka et al, 1977), especially for those fish that undergo spawning migrations (Bilinski, 1974). Slow muscle contains much larger lipid deposits than fast (Bone, 1966 ; Bilinski, 1974), and in salmonids is capable of oxidising long-chain saturated fatty acids at approximately 10 times the rate of that in fast muscle (Jonas & Bilinski, 1964 ; Bilinski, 1974). In addition, carnitine palmitoyl transferase activity, a measure of the fatty acid oxidation potential of a tissue (Crabtree & Newsholme, 1972) is generally much lower in fast than slow muscle, as are the enzymes involved in fatty acid oxidation (Bilinski, 1974). It is therefore evident that slow muscle is better adapted to utilise lipid than fast muscle. The same is true even in those species that recruit fast fibres during sustained swimming.

Triglyceride (TG) consists of a glycerol backbone with three fatty acid chains ranging from 12 to 22 carbons each. In mammals TG is hydrolysed to glycerol and non esterified fatty acids (NEFA) for transport in the blood via albumin complexes. Liver TG may also be transported in association with very low density lipoproteins (VLDL) or chylomicrons. The mechanisms of lipid transport and the uptake of lipids by extra-hepatic tissues is thought to be similar in fish as in mammals (Bilinski, 1974 ; Skinner & Rogie, 1978 ; Skinner et al, 1980). In mammals the oxidation of fatty acids results in the production of ketone bodies (acetoacetate and hydroxybutyrate), which are important during starvation as a fuel for kidney, heart, adrenals, and especially for the brain and nervous tissue. In fish, ketone bodies, and the enzymes associated with their metabolism, have only been found at any significant levels in elasmobranchs. In contrast free fatty acids are the product of lipid metabolism in teleosts and supply the main energy source for aerobic metabolism (Zammit & Newsholme, 1979).

Amino acid metabolism

Amino acids cannot be stored in the same manner as either carbohydrates or lipids. Excess amino acids or proteins are therefore deaminated and oxidised via the TCA cycle or converted into carbohydrates or lipids. Unlike mammals, the end product of nitrogen metabolism in fish is mainly ammonia, with uric acid, trimethylamine oxide, creatine and creatinine, excreted to a lesser extent. Ammonia production is less energetically expensive than the formation of urea and therefore the energy derived from protein catabolism is relatively higher. This adaptation may be of paramount importance, especially for those species of fish where dietary carbohydrate intake is low, and fat and protein intake is high

The degradation of amino acids proceeds by initial conversion into an intermediate of the TCA cycle, followed by either oxidation to CO_2 and H_2O, or

conversion to glucose or fatty acid. The initial step requires either deamination (via dehydrogenases or amino acid oxidases), transdeamination, or non-oxidative de-carboxylation. The enzymes utilised are invariably specific for a single amino acid. The major oxidative deamination enzyme found in fish is glutamate dehydrogenase, which is ultimately involved in the production of ammonia. Although D-amino acid oxidases have been reported, their precise function is unknown, as naturally occuring amino acids are in the L-isomer form (Fickeisen & Brown, 1977). Most species of fish studied possess amino transferases. As in other animals, alanine and aspartate aminotransferase are quantitatively the most important (Walton & Cowey, 1982). These enzymes appear to have similar properties as the respective mammalian enzymes (Cowey & Sargent, 1979). Decarboxylation is thought to be of lesser importance in the amino acid metabolism of fish, and has only been reported in a few species.

In fish muscle both glycine and histidine are found at high levels in the free amino acid pool and in some species may account for up to 50% of the total free amino acids (Driedzic & Hochachka, 1978). It is thought probable that fish muscle may be able to utilise histidine and indeed other amino acids, directly for energetic purposes even under routine metabolic conditions (Driedzic & Hochachka, 1978).

Seasonal starvation

Due to the seasonal fluctuations in food availability, many species of marine and freshwater fish have become exceptionally well adapted to survive prolonged periods of starvation. Under laboratory conditions for instance, both cod and plaice are able to survive without food for up to 6 months, whilst in one instance the european eel, Anguilla anguilla, has been reported to survive 4 years (Love, 1970). In wild populations the stress of this period (usually in the winter) is often accentuated by the need to build up the gonads and migrate to spawning grounds. As a source of fuel many fish are

adapted not only to utilise carbohydrate and lipid stores but also to mobilise large proportions of their fast muscle proteins. For instance in a 4 month starvation under laboratory conditions, plaice (Pleuronectes platessa) were found to mobilise up to 25 % of their fast muscle myofibrillar proteins (Johnston, 1981). The preference to degrade fast muscle is observed in many species that undergo severe starvations (Johnston & Goldspink, 1973a ; Patterson & Goldspink, 1973 ; Love, 1970, 1980), and is thought to allow fish to maintain slow muscle status, and hence cruise swimming capabilities, even when much depleted.

Even though many fish have adapted to survive prolonged starvation , the strategies employed vary amongst the species. For example the relative importance of maintaining certain energy stores at the expense of others, and the sequence and speed of mobilisation, varies considerably. In cod, hepatic carbohydrate stores are utilised prior to hepatic lipid. Once the hepatic reserves are depleted muscle glycogen and protein appear to be depleted simultaneously (Love, 1970 ; Black, 1983). In other species, for instance carp (Nagai & Ikeda, 1971), pike (Esox lucius, Ince & Thorpe, 1976b), and European eel (Larsson & Lewander, 1973), liver lipid is depleted prior to carbohydrate stores. In fact carp maintain hepatic glycogen stores at the expense of both liver lipid and muscle proteins, during the first weeks of starvation (Nagai & Ikeda, 1971). Despite the depletion of lipid stores from both liver and muscle , the lipid stores of the brain, heart, and gills do not decrease during starvation (Love, 1970).

Wide inter-specific variations are also evident in the concentrations of blood metabolites found during starvation. Blood glucose levels are either maintained by glycogenolysis or gluconeogenesis and the importance of each respective pathway varies amongst the species, as does the relative importance of different gluconeogenic precursors. In general, however there appears to be a decline in hepatic glycogenolysis (for example Moon & Johnston, 1980,

plaice) an increase in the levels of enzymes involved in hepatic gluconeogenesis and amino acid metabolism (alanine, aspartate, tyrosine, and leucine aminotransferase, GDH, PEPCK (Larsson & Lewander, 1973 ; Creac'h & Serfaty, 1974 ; Whiting & Wiggs, 1977 ; Zammit and Newsholme, 1979 ; Zebian & Creac'h, 1979 (cited by Walton & Cowey, 1982); Moon & Johnston, 1980 ; Morata et al, 1982)), and a decrease in the hepatic pentose phosphate pathway dehydrogenases (Yamachi et al, 1975 ; Lin et al, 1977).

In the musculature, starvation results in a decrease in the glycolytic capacity coupled with a general decrease in metabolic activity. In the laboratory severe starvation are often coupled with a lowering in oxygen consumption and a decrease in locomotory activity (Johnston, 1981b). During a 4 month starvation in plaice Johnston (1981b) reported extensive disorganisation of the fast musculature and water contents can reach as high as 90 %. In the same species slow muscle proteins were also degraded during the initial weeks of starvation, however these were thought to consist of non-structural, soluble proteins (Patterson,et al, 1974). With prolonged starvation, increased degradation was only observed in fast muscle (Johnston & Goldspink, 1973a).

Refeeding

There has only been a limited amount of research into the effects of refeeding after starvation, in fish. The effects of refeeding have long been known for the rat (Tepperman & Tepperman, 1958 ; MacDonald & Johnson, 1965). In these studies a transient increase in the activity of the pentose phosphate pathway and a massive increase ("overshoot") in hepatic glycogen (far exceeding those observed in control rats) was reported during the onset of refeeding. An overshoot phenomena has also been reported for phosphohexose isomerase, pyruvate kinase, L-glycerophosphate dehydrogenase, malic enzyme, dihydroxyacetone kinase, hexokinase , and glucokinase (Szepisi & Friedland,

1969). Additional work reported the glycogen overshoot to be paralleled by a rapid synthesis of RNA (Szepisi & Friedland, 1969 ; Pfeifer & Szepisi, 1974), which, it has been suggested, may be triggered by corticosteroids released from the adrenal cortex (Wurdeman et al, 1978).

Overshoots in either hepatic or muscle glycogen levels have not been observed in brook trout (Salvelinus fontinalis, Yamauchi et al, 1976) or rainbow trout (Jurss & Nicolai, 1976 (cited by Black,1983)) during refeeding. However in both cod (Black, 1983) and pike (Ince & Thorpe, 1976b) refeeding results in a glycogen overshoot in both tissues.

In refeeding experiments on cod, Black (1983) reported a dramatic rise in the RNA : DNA ratio. This same phenomenon, which suggests large increases in protein synthesis without cell division, has also been reported in carp, (Bouche et al, 1972) and bream (Abramis blicca, Bulow, 1969 (cited by Love, 1980)). Black also reported elevated levels of NEFA, lipogenesis, and pentose phosphate dehydrogenases, during refeeding in cod, and suggested their involvement in the replacement of membrane phospholipids.

Muscle protein turnover

Most proteins in animal cells are in a continuous state of turnover. The ultimate size of a muscle cell therefore depends upon a finely regulated balance between protein synthesis and degradation. Many studies have shown that this balance varies with changes in physiological conditions, for example, hormonal status, exercise, disease, dietary status, and following experimental manipulation, for example, stretch, denervation, and chronic electrical stimulation (Millward et al, 1978 ; Goldspink, 1978, 1980 ; Goldberg, 1980). It has now been demonstrated that many physiological agents are able to influence and regulate muscle protein synthesis independantly of any effect on protein degradation. The actions of the major regulatory

factors are considered below with specific reference (where studied) to fish.

Hormones

Insulin

As in mammals insulin is an important physiological factor regulating glucose homeostasis and protein balance specifically related to nutritional metabolism. Insulin is known to have a wide variety of metabolic effects. After feeding a rise in insulin increases net uptake of amino acids by the muscle cell and enhances their incorporation into muscle proteins. Insulin also independantly inhibits protein degradation possibly by directly suppressing the lysosomal system (Mortimore et al, 1978). The action of insulin on the lysosomal system is independant of glucose or amino acids and is thought to be separate from the stimulation of nutrient transport across membranes.

In lamprey injected insulin results in a reduction in plasma glucose, an activation of glycogen synthetase and an increased deposition of muscle glycogen (Bilinski, 1974). In sturgeon, salmon, plaice and lamprey, mobilisation of glycogen deposits, prior to spawning, are paralleled by a decrease in circulatory insulin. Ince and Thorpe (1975, 1976a, 1978) observed that insulin increased the incorporation of labelled glucose into liver and muscle lipid, increased the incorporation of labelled glucose and glycine into muscle proteins, decreased plasma free fatty acids and decreased the circulatory levels of 13 amino acids. It appears that in fish, as in mammals, amino acids and glucose trigger the release of insulin (Ince & Thorpe, 1975 ; Ince, 1979). However, catecholamines (fast acting hormones), and especially adrenaline, can inhibit the release of insulin, possibly to enhance the utilisation of energy reserves during periods of high activity (Ince & Thorpe, 1977).

Pituitary and thyroid hormones

It has long been known that the removal of the pituitary gland causes a reduction in protein synthesis and a concomitant reduction in protein degradation (Goldberg, 1980). In normal growing rats where synthesis exceeds degradation (hence net growth) removal of the pituitary causes a larger reduction in synthesis than in degradation, resulting in a state of equilibrium (no net growth). Treatment of hypophysectomised growing rats with growth hormone, returns protein synthesis to normal levels, but has no effect on protein degradation (Flaim et al, 1978 ; Goldberg, 1969a). In some fish, growth hormone plays an important role in survival during starvation. In carp, European eel, and salmon, Salmo salar, hypophysis activity has been reported to increase during starvation (see Love, 1980).

Thyroidectomy is also known to decrease protein breakdown. To reverse this response administration of triiodothyronine (T_3) or thyroxine (T_4) increases the rate of protein degradation back to normal. As with growth hormone, T_3 and T_4 have also been shown to stimulate protein synthesis (Goldberg, 1980). Hyperthyroidism leads to a general loss of body weight enhanced by severe muscle wasting, and is thought to be primarily due to an increase in proteolysis. It has been postulated that thyroid hormone directly influences the lysosomal system, causing either an increase in the production of lysosomes or an increase in the activities of lysosomal enzymes.

Glucocorticoids

Glucocorticoids promote the net breakdown of muscle proteins. They facilitate the mobilisation of amino acids from muscle, decrease DNA and protein synthesis (Goldberg, 1969b) and decrease the capacity for muscle to take up amino acids (Kostyo & Richmond, 1966).

Cortisol, a major glucocorticoid, has been found in a wide variety of fish (Chester-Jones et al, 1974) and is thought to have similar metabolic functions in fish, as in mammals. Cortisol is secreted by the adrenal cortex

under the influence of adrenocorticotrophic hormone (ACTH), which in turn is secreted from the pituitary. Elevated levels of circulating corticosteroids are generally associated with non-feeding periods in the life cycle of fish and are thought to be important in enhancing gluconeogenesis during migration, where muscle provides a substantial protein reserve for gluconeogenic precursors. For example during the upstream migration of the sockeye salmon, Oncorhynchus nerka Idler et al (1959) reported a six-fold increase in plasma corticosteroids paralleled by a 60 % fall in body protein. Similarly Dave et al (1975) reported an increase in plasma cortisol during starvation in the European eel, and the same has been found in plaice during the winter months (Love, 1980). Conversely in starvation experiments on cod, Black (1983) reported no significant increases in plasma cortisol, and also reportedthat gluconeogenesis was not enhanced. Administration of exogenous cortisol has been reported to increase blood glucose, serum amino acids and liver aspartate amino transferase activities in the Japanese eel, Anguilla japonica, indicating proteolysis and the enhancement of gluconeogenesis (Chan & Woo, 1978). Dave et al (1975) working on the European eel, reported an increase in blood glucose and liver glycogen, and a decrease in plasma and muscle triglycerides (TG). It has become apparent that glucocorticoids , and specifically cortisol, are therefore fundamentally important in the control of protein catabolism and the stimulation of gluconeogenesis during fasting, in atleast some species of fish .

Glucagon

Glucagon is secreted from the cells of the Islets of Langerhans in mammalsand generally causes the opposite effects to insulin (i.e. an increase in blood glucose via glycogenolysis). Glucagon also stimulates lipolysis and gluconeogenesis, and its action is thought to involve a second messenger (possibly cyclic AMP which can mimic the stimulatory effects of glucagon increasing gluconeogenesis from lactate in the Japanese eel (Haya hi &

Ooshiro, 1975)). Exogenous injection of glucagon has been reported to increase glucose release in the eel (Larsson & Lewander, 1972 ,European eel ; Chan & Woo, 1978b, Japanese eel ; Renaud & Moon, 1980a, Anguilla rostrata, American eel), increase plasma FFA in pike (Ince & Thorpe, 1975), and stimulate gluconeogenesis in carp and American eel (Murat et al, 1978 ; Renaud & Moon ,1980a).

Dietary regulation of protein turnover

In vitro studies have shown that plasma amino acids can influence both protein synthesis and degradation. Fulks et al (1975) reported that branch chain amino acids alone can increase protein synthesis and inhibit protein degradation. Whether branched chain amino acids are of such importance in vivo is unknown. However, intravenous injections of leucine, or other branched chain amino acids , has been reported to reduce protein catabolism in patients undergoing surgery (Goldberg, 1980), and is proving useful in the treatment of human diseases where protein breakdown is excessive (Goldberg, 1984).

Glucose also inhibits degradation, but is found to have no influence on the rate of protein synthesis (Fulks et al, 1975). Glucose in the presence of insulin, however, is found to have a synergistic effect on protein synthesis and an additive effect on muscle protein degradation. The mechanism of inhibiting protein degradation is unknown, but is thought not to be simply a case of glucose supplying a substrate for ATP production and hence reducing the need for muscle gluconeogenic precursors.

Given a protein-free diet, young rats reduce both protein synthesis and degradation (Millward et al, 1978). The decrease, however, is greater for synthesis, such that after 10 days there is a net decrease in muscle weight. Conversely, in fasting animals, degradation rates are substantially elevated whilst synthesis is much reduced, producing a negative net nitrogen balance. Surprisingly protein degradation rates can increase in some anabolic

circumstances (i.e. during muscle growth in young rats, where degradation rates are found to be relatively high) and decrease in some catabolic circumstances (i.e. protein free diet)(Millward et al, 1978). Generally it is the rate of synthesis that compensates for any changes in the rate of degradation, with a proportionally larger rate modification. Hence the rate of protein synthesis is thought to be the overall controller of muscle mass (Millward et al, 1976).

Passive stretch, denervation, and disuse

It has long been realised that by increasing work load muscle growth can be stimulated and that diminished activity by disuse or denervation, can result in retarded growth or muscle atrophy. Further investigation of these phenomena show that if a muscle is immobilised (via a plaster cast for instance) in a non stretch position, protein synthesis decreases whilst degradation increases, and hence there is a net loss in muscle weight (Goldspink, 1978). The extent of these changes varies with the muscle type and is generally greater in the slow than fast muscle, corresponding to their differing basal rates of protein turnover (Goldspink, 1980 ; Guba et al, 1980). Slow muscle therefore has a higher general metabolic activity, higher rates of protein turnover, and exhibits more pronounced changes in protein turnover during immobilisation. It is interesting to note that this is not the case in starved tissue where fast muscle exhibits the largest changes in protein turnover and the highest overall atrophy (for instance plaice, Johnston, 1981b).

A comparison of the changes in protein turnover rates observed during immobilisation (via plaster cast) and denervation, show that initially similar patterns of change are found in both protein synthesis and degradation rates (Goldspink, 1980). Overall wasting however, is lower in denervated tissue, due to a stimulation of protein synthesis after the initial decrease.

Alternatively if the denervated muscle is immobilised in a non stretch position (fully shortened) then greater atrophy is observed than in a muscle that is either singularly immobilised or denervated. Increased synthesis, after the initial phases of denervation, is thought to be due to the influence of passive stretch or spontaneous fibrillation. Stretch is found to induce growth even in the absence of any nutritional, innervatory, or hormonal support, and is therefore considered to be possibly the most important factor regulating muscle growth in vivo. The link between stretch and growth is not fully understood. It is possible that stretch may directly increase membrane transport (a phenomenon observed in stretched muscle) which may in turn induce some other mechanism that increases the anabolic processes. Stretching of embryonic muscle cells in culture causes an accumulation of amino acids within the cell, increased incorporation of amino acids into proteins, and activation of the Na pump (Vandenburgh & Kaufman, 1980). Vandenburgh and Kaufman (1980) postulated that the stretch induced activation of the Na pump may be the fundamental event stimulating growth in muscle cells. Stretch may also effect mitosis in satellite cells, which supply muscle cells with myonuclei during muscle growth.

Morphologically, the atrophies observed during denervation and immobilisation of muscle are very different. Denervation results in a decreased fibre diameter, whereas immobilisation tends to influence the sarcomere number and hence longitudinal growth (Goldspink et al, 1974 ; Williams & Goldspink, 1976). Goldspink (1980) also reported that inactive muscle (via immobilisation or denervation) became more sensitive to the hormones that increase catabolism (i.e. glucocorticoids) and less sensitive to the hormones that increase anabolic processes (i.e. insulin).

In the absence of any active or passive tension, Ca^{++} has been reported to increase muscle catabolism by the stimulation of both degradation and synthesis, but with the net balance towards increased degradation (Etlinger et

al, 1980). The changes are similar to those observed in denervated muscle and several muscular dystrophies (Kameyama & Etlinger, 1979). The presence of tension enhances the effects of Ca^{++} such that synthesis is further elevated whilst degradation is reduced, resulting in a nitrogen balance similar to that observed during muscle growth (i.e. hypertrophy). It has been proposed that the increase in protein synthesis caused by either electrical stimulation or stretch, may result from increased intracellular Ca^{++}. The mechanisms for Ca^{++} stimulation of proteolysis are unknown but many possibilities remain. Ca^{++} may modify proteins rendering them more susceptible to proteolysis or may stimulate proteolytic enzymes, for instance the Ca^{++} activated neutral protease found in mammalian muscle (Busch et al, 1972 ; Dayton et al, 1976, 1982). It has been suggested that this enzyme could even initiate myofibrillar breakdown. Ca^{++} activation of proteolysis may involve an increase in the number of lysosomes, increase in membrane permeability to lysosomal enzymes, or even an increased uptake of proteins into lysosomes.

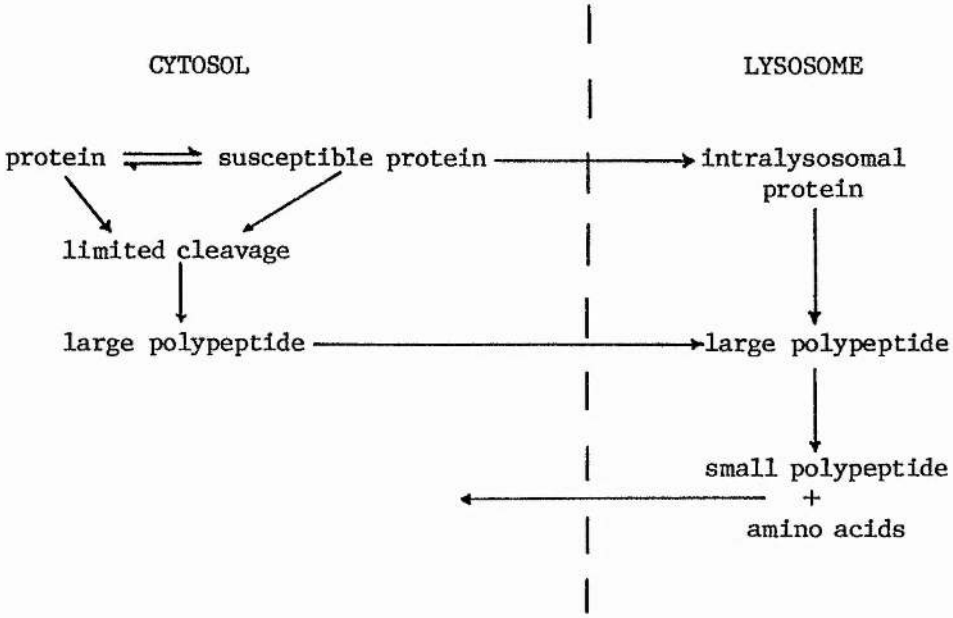
Kameyama and Etlinger (1979) investigated the possible involvement of Ca^{++} in muscle atrophy during human muscular dystrophies. Common features of dystrophies include an increased permeability of the plasma membrane to the high Ca^{++} concentrations normally present in the extracellular fluid, and a decreased ability of the s.r. to sequest Ca^{++}. By using the Ca^{++} ionophore A23187 to increase the transport of Ca^{++} across membranes, Kameyama and Etlinger demonstrated that by increasing the flux of Ca^{++} into normal muscle, changes occur that are similar to those observed in muscular dystrophies.

Mechanisms of protein Degradation

The rate of turnover of a protein can be directly correlated to a number of physical characteristics. Proteins with a high turnover rate are generally more negatively charged (Dice & Goldberg, 1976), more hydrophobic (hence absorbed to a greater extent by membranes) (Segal et al, 1978) and have a

larger sub-unit size (Naya et al, 1976). Under normal cellular conditions it is clear that protein conformations not only determine their cellular functional properties, but also influence their rate of turnover. In states of elevated proteolysis, for example starvation, it is interesting to note that these correlations are either much reduced or absent (Dice & Walker, 1978).

The possible translocations and transformations by which a protein may be degraded can be summarised by the following diagram :



The rate limiting step/s in protein degradation are unclear, as too are the intricacies of the mechanisms involved. There are, however, three possible sites for the gross regulation of protein degradation - transformation of a protein into a susceptible form, internalisation into the lysosomal system, or proteolysis (both in the cytosol or within the lysosome). Initially, a protein may be converted into a form that renders it more susceptible to proteolysis. This may depend on either the nature of the protein molecule itself or the presence and/or activity of proteolytic enzymes. If the former, then susceptibility may involve conformational changes, perhaps exposing susceptible peptide bonds to hydrolysis, or limited proteolysis into subunits. Indeed the correlation between turnover rate and

molecular size is closest to the size of the sub unit rather than the whole molecule (Dean, 1978). Alternatively degradation of some proteins may be triggered by the removal of a covalently bonded tag, as, for example, sialic acid is removed from circulating glycoproteins, rendering them susceptible to greater uptake and ultimately digestion, by the liver (Ashwell & Morrell, 1971).

An alternative site for the control of selective degradation may not rely on the nature of a protein, but may depend on the activity of the lysosomal system. This may involve increased synthesis of proteolytic enzymes, activation or inhibition of enzymes already present in the cell or plasma (possibly by de-activation of protease inhibitors or selective proteolysis of precursor molecules producing active enzymes), or the movement of enzymes into specific cellular compartments (i.e. lysosomes) where the environment is conducive to proteolytic activity.

The uptake of a protein into the lysosomal system may be a site of selectivity in turnover rates. Since the turnover rates of proteins are related to the hydrophobic characteristics of a protein (under normal cellular conditions), selective transport into the lysosome, may be important in regulating protein degradation rates (Dean, 1978). The interaction of macromolecules with the lysosomal membrane is thought not to be receptor-mediated and therefore gross selectivity on the basis of the physical properties of each protein could explain the correlation between turnover rates and hydrophobic characteristics (Dean, 1978).

Once within the lysosomal system, proteolysis proceeds by the action of endopeptidases (proteinases) cleaving the peptide bonds within the central region of the poplypeptide chain. Further degradation to amino acids is completed by the removal of C-terminal peptides or dipeptides, by exopeptidases (peptidases) (Barret, 1980). For further discussion on the function and properties of lysosomes and acid hydrolases see chapter 6.

Functions of protein turnover

Under normal nutritional conditions protein turnover is both continuous and extensive. In the adult rat, red oxidative muscle is thought to degrade and re-synthesise 8 - 10 % of the total mass, per day, whilst for fast glycolytic muscle 4 - 5 % is degraded and re-synthesised per day (Millward et al, 1978). Such an extensive turnover appears a seemingly wasteful process.

During periods of stress, for example starvation, it is fair to assume that the functions of increasing protein degradation and decreasing synthesis are to provide precursors for gluconeogenesis in the liver and kidney, substrates for oxidation in muscle, and substrates for the synthesis of new proteins essential for adaptation to the stress. As muscle tissue provides the largest protein reserve in the body, the mobilisation of muscle protein is an essential adaptation to survival.

Under basal conditions none of these functions would apply. Many theories have been postulated to explain the purpose of a continuous and extensive turnover system. One theoretical advantage of a basal turnover may be to regulate the concentration of proteins, for instance the non-equilibrium, regulatory enzymes. Indeed it does appear that many rate limiting enzymes have rapid turnover rates. This would imply that by fine control of the synthesis/degradation rates these enzymes would be able to respond rapidly to changing requirements. Within the same hypothesis it can be seen how a basal turnover will enable an organism to adapt to changing nutritional, environmental, or developmental conditions. For example in developing rabbit muscle there is a progressive alteration in the relative abundance of three myosin heavy chain isoforms (Whalen et al, 1981). There is some evidence to suggest that acclimation to altered environmental temperatures by poikilotherms, produces changes in the isoenzyme composition of L-lactate dehyrogenase, and possibly the relative abundance of troponin I isoforms, to suit the prevailing cellular conditions (reviewed by Johnston,

1983). However further evidence is required to correlate these changes to improved cellular operation in the new environmental regime.

Another requirement or possible function for basal protein turnover, relates to the synthetic errors that occur during the transfer of information from DNA to RNA. It has been estimated that up to 15% of all proteins contain at least one sequence error (see Schimke & Bradley, 1975). Proteins containing incorrect sequencing are often only partially functional or totally non-functional and therefore require removal before their accumulation causes cellular malfunction. Proteins containing amino acid analogues, which alter protein conformation in a similar way as synthetic errors, are found to be selectively degraded. In fact proteins containing errors may be degraded at a rate ten times the average of normal proteins. It is therefore suggested that the function of a basal protein turnover is to facilitate the rapid removal of synthetic errors simply by the selected acceleration of a system that is already in operation.

Muscle wasting diseases

There are a number of diseases in man that cause a wasting of the voluntary musculature. The most well known and common are probably the muscular dystrophies. Many studies of both biochemical and ultrastructural nature, have now been undertaken on congenital and experimental myopathies in other animals. It is evident that much of the information obtained from these studies is pertinent to the study of muscular diseases in man. It has also become clear that many of the degradative features observed in diseased muscle are common to those found in muscle subjected to such physiological stresses as starvation and disuse atrophy.

Ultrastructurally it appears that muscle can react to pathological injury (i.e.myopathies) with only a strictly limited number of responses and that these responses are rarely specific to any one condition. Due to the non-

specific response of muscle, especially in the later phases of wasting diseases, ultrastructural analysis has invariably failed to elucidate the importance of specific morphological changes, or elucidate whether a myopathy is a primary disorder of the muscle cell. However various characteristic ultrastructural abnormalities have been defined for a few human metabolic and congenital myopathies (Engel,A.G. 1973 ; Hudgson & Mastaglia, 1974).

The non-specific ultrastructural changes commonly observed during muscle wasting diseases, which are probably secondary responses and not of fundamental importance, include the susceptibility of the I band to degradation, disarray of the peripheral area of the myofibrils, increase in cytoplasmic material, fusion of myofibrils, increases in the number and size of vacuoles, invagination of the nuclear membrane and mitochondrial inclusions. Z disc degeneration has been reported in a number of myopathies (see Cullins & Fulthorpe, 1982) and in nemaline myopathy is thought to be the site of the fundamental pathological process producing the disorder. Selective degradation of the thick filaments, although uncommon, has been reported in a few myopathies, for instance hypotonia (Yarom & Shapira, 1977), childhood dermatomyosites and a case of acute quadriplegia (Yarom & Reches, 1980).

Muscular dystrophies

Muscular dystrophies are a group of disorders that are hereditary in nature and involve the atrophy of skeletal muscle. The primary causes of muscular dystrophy are unknown. It is assumed to be a primary disorder of muscle cells, however difficulties have arisen concerning the involvement of the muscular nerve and vascular supply. Alterations in the nerve and vascular supply to normal muscle cells, have both been reported to cause changes within the muscle that are similar to those found in dystrophies (Engel, W.K., 1973).

Duchenne muscular dystrophy (DMD) is the best known and most severe muscular dystrophy. The incidence at birth in males is 1 in 3,000 - 3,500

(Perry, 1984). Ultrastructurally little can be inferred about the pathogenetic mechanisms involved in DMD, as the morphological changes are non-specific. Initially there is a dilation of the s.r., with the presence of many vacuoles containing the products of cellular breakdown. Originally it was reported that the earliest ultrastructural abnormality was the selective degradation of the thick filaments (Pearce, 1964 (cited by Hudgson & Mastaglia, 1974)), however it is now accepted that the I band is more susceptible to degradation, as in other myopathies. In the later stages fibres undergo "coagulative" necrosis forming an amorphous mass in the centre of the cell. Engel,W.K. (1973) proposed that the muscle changes observed in DMD were primarily caused by abnormalities in the small blood vessels supplying muscle tissue, resulting in local ischemia. He reported that experimental ischemia in rabbits caused similar morphological events as seen in DMD. More recently, however, DMD has been considered to involve the occurence of membrane defects (see above Kameyama & Etlinger, 1979) and be a primary disorder of the muscle tissue. Unequivocal proof, however, has not yet been obtained.

In myotonic dystrophy it is thought that one morphological change of fundamental relevance is the abnormal communication of the tubular system with the extracellular space, and it has been suggested that the t-system is the origin of the myopathy (Schotland, 1970). This myopathy, along with murine dystrophy, may however, be of neurogenic and not myopathic origin (MaComen et al, 1971).

Hypo- and hyper-thyroidism are both clinically associated with muscle weakness and both the accumulation of glycogen granules and fibre necrosis (occassionally) have been observed. As with corticosteroid myopathy, the most striking ultrastructural changes occur in the mitochondria.

Denervation atrophy

In the absence or deterioration of a nerve supply to normal muscle,

changes are observed that are very similar to those found in the dystrophies (Hudgson & Mastaglia, 1974 ; Bird, 1975). Many studies have now reported a wide spectrum of metabolic and structural changes that occur within denervated muscle. In animal studies atrophy has been observed after the sectioning of entire motor neurone units, hence completely denervating a whole muscle simultaneously. In man, studies are restricted to partial denervation, occuring in such cases as spinal muscular atrophy and peripheral neuropathies. Despite these differences the changes observed in human and other animal atrophies are similar. Upon denervation of human muscle there is an initial hypertrophy, incorporating a proliferation of the s.r. and t-system, and an increase in mitochondrial numbers. Following this there is a decrease in myofibrillar diameter with degradative changes in the I band and Z disc. As in DMD selected fibres are degraded adjacent to apparently normal fibres. In denervation atrophy these fibres have been termed target fibres, and are characterised by the degraded myofibrils occuring in the centre of the fibre. Towards the periphery of the fibre the myofibrils appear progressively more normal. Target fibres are also observed in patients with hypo- and hyperkalemic periodic paralysis. Also present in human denervated muscle are targetoid fibres, essentially differing from target fibres by the degraded core being immediately surrounded by normal myofibrils (Hudgson & Mastaglia, 1974).

Parallel to the proliferation of the s.r , t-system and vacuoles, many studies have also reported increases in acid hydrolase activities in denervated muscle, and have implied an increased involvment of the lysosomal system and autophagic processes (Pollack & Bird, 1968 ; Bird, 1975).

Vacuolar myopathies

Vacuolar myopathies are defined as those myopathies in which fibre vacuolation is the typical or predominant pathological change (Engel,A.G.

1973). By this definition vacuolar myopathies include Periodic paralysis, chloroquine myopathy, several types of glycogenosis and lipid storage myopathy. Vacuolated fibres also occur in muscular dystrophies, polymyostis, paroxysomal myoglobinuria, and steroid induced myopathy. Muscle weakness and myofibrillar degradation is only associated with a few vacuolar myopathies. Engel,A.G. (1973) demonstrated the origin of some vacuoles to be the s.r. and t-tubules and reported that many were autophagic in nature, containing acid hydrolase activity.

As with other ultrastructural myopathic phenomena, no one type of vacuole is specific for any one disease. In addition to this the morphology of the vacuoles may change during the course of a disease.

In summary it becomes apparent that muscle responds to a wide variety of pathological injury with only a limited number of non-specific responses. It is also evident that many of the changes observed in myopathies can also be found in muscle undergoing controlled degradation during starvation. A study of the events occuring during the breakdown of large quantities of muscle, in separate fibre types, during a comparatively short period (albeit in fish), may therefore be pertinent to a further understanding of at least some of the secondary disorders observed in muscle wasting diseases.

In the present study the ultrastructural changes in muscle have been investigated during a cycle of starvation and refeeding in the saithe. In parallel with this a wide diversity of techniques have been employed to further understand the metabolic and proteolytic processes involved in the breakdown and regeneration of muscle.

Chapter 2

The effects of starvation on muscle ultrastructure.

Introduction

There have been a number of ultrastructural studies on muscle wasting both during starvation (Johnston, 1981b) and myopathies (Hudgson & Mastaglia, 1974). Ultrastructural changes in atrophied fibres, common to both diseased and starved muscle, include increased numbers of lysosomes, a reduction in myofibrillar diameter, and swollen terminal cisternae (Hoffstein et al, 1975 ; Johnston, 1981b ; Patterson & Goldspink, 1973).

Rats show approximately a 25% reduction in body and muscle weight following a 72 hour fast. Muscle degradation in 100 g rats is associated with an increase in protein degradation rates, a 50% decrease in protein synthesis rates and a substantial increase in cathepsin D activity, relative to fed controls (Li et al, 1979). Many marine fish from temperate and arctic regions undergo natural starvations that are much more severe than mammalian starvations. For example the European eel, Anguilla anguilla, has been reported to survive for over 4 years without food (Love, 1970), and many other species can withstand starvations lasting 3-6 months (Love, 1970 & 1980). Since these seasonal starvations are both reversible and non-pathological they provide an excellent model for studying the mechanisms involved in muscle wasting.

In this chapter the marine teleost Pollachius virens.L, was kept without food under laboratory conditions for upto 12 weeks. The effects of starvation on muscle fibre size, volume fractions of mitochondria and myofibrils, and capillary supply were determined.

Materials and methods

Experimental protocol

Saithe (Pollachius virens) of mean length 17.7 +/-1.0 cm were caught by line fishing from St.Andrews harbour during October-November (1980). The fish were kept in holding tanks at 10°C and starved for up to 3 months. Control fish, also caught in Oct-Nov 1980, were kept and fed for three days in the holding tanks prior to sampling. Two groups, comprising of six fish each, were starved for 5, and 12 weeks. Fish were sacrificed by a sharp blow to the head followed by transection of the spinal chord.

Ultrastructural studies

Muscle samples were dissected from myomeres 22 - 27 (numbered from the head) for fast muscle and myomeres 30 - 35 for slow muscle (fig.1). Bundles (3-5mm^2) of fast and slow fibres were pinned to cork, approximating resting length, and fixed in 3% Glutaraldehyde, 0.15M phosphate buffer, pH 7.2 (at 4°C) for 12hrs. The samples were dissected into smaller bundles (1-2mm^2), washed twice in buffer and postfixed in 1% Osmium tetraoxide, 0.15 M phosphate buffer, pH 7.2 (at 4°C), for 1hr. These were then rinsed in distilled water, dehydrated in a series of alcohols up to absolute, cleared in propylene oxide and embedded in Araldite CY212 resin (Ciba-Geigy). Ultrathin sections showing a gold interference pattern, were cut on a Reichert OMU2 ultramicrotome, placed on pyroxiline (2.5 % in amyl nitrate) coated grids (150 mesh). These were then double stained with 0.4% uranyl acetate in 50% alcohol (1 hr) and 0.04% lead citrate in 0.1 M carbonate free sodium hydroxide (10 min). Both stains were previously millipore filtered to avoid contamination. Sections were examined with a Phillips 301 electron microscope. For each of the six fish in the experimental groups, 12 blocks were made of each fibre type (6 T.S. and 6 L.S.). These were pooled together. From each starvation group of six fish there would therefore be 36 T S blocks

and 36 L.S. blocks. Twenty of each were randomly taken and cut, enabling between 45 to 73 fibres to be analysed (see tables 1 and 2).

Quantitative analysis

Due to the complex orientation of muscle fibres within fish myotomes, it is impossible to obtain a section with all the fibres in perfect transverse alignment. Estimates of component fractional volumes will be little affected by this, however, fibre areas and perimeters are highly dependant on the plane of sectioning. It is for this reason that only the fibres in true transverse section were selected for area and perimeter estimates. Fibre area, perimeter, and capillary contact length, were measured directly by tracing fibres (2% mean replication error) and capillaries from electron micrographs using a summagraphics digitiser in conjuction with a mini-computer (Walesby & Johnston, 1980).

Capillarisation

The following parameters were derived from these measurements to express vascular supply, as suggested by Totland, Kryvi, Bone and Flood (1980) ;

1. Percentage fibre perimeter vascularised (Capillary contact length per fibre / Mean fibre perimeter).

2. Mean perimeter served by one capillary (Mean fibre perimeter / Number of capillaries per fibre).

3. Capillary perimeter supplying 1 um^2 of fibre cross-sectional area (Capillary contact length per fibre / mean fibre area).

4. Capillary perimeter supplying 1 um^3 of mitochondria (Capillary perimeter supplying 1 um^2 of fibre cross-sectional area x 100 / Volume fraction of mitochondria).

5. Number of capillaries per mm^2 of fibre cross-sectional area (Number of capillaries (N_c) / Number of fibres analysed x mean fibre cross-sectional area (A_f) x 10^{-4}).

Muscle fibre ultrastructure

Quantitative analysis of fibre ultrastructure was carried out from electron micrographs, using a point count method (Weibel, 1973). A grid lattice spacing (d) was chosen so that d was > 1 and < 1.5 x the average diameter of the largest component (in this case myofibrillar diameter)(Weibel, 1973 ; Eggington & Johnston, 1982). As each cell contains components of varying sizes, there is an optimal grid lattice spacing for each component. For maximum accuracy it therefore follows that a grid should be used with multiple lattice spacings. However the error incorporated in using a single average value for d when the components are of similar size, is small in comparison to experimental error. The image of the electron micrographs were overlaid onto the test grid using a photographic enlarger. The magnification of the micrographs was such that the lattice spacing were equivalent to 2-5 μm. Component volume fractions were determined by Vv = Pl / Pt x 100 where Vv = fractional volume : Pl = number of points falling on a component : Pt = number of total points falling on the fibre. Each fibre analysed contained between 100 - 200 points. Note was taken as to whether the mitochondria were in the interfibrillar or subsarcolemmal fibre zones.

A student t-test (one-tailed) was performed on all results.

Results

Slow fibres from fed fish were characterised by a high volume fraction of mitochondria (figs.2,3) and a well developed sarcotubular system and capillary supply (table II). The mean size of slow fibre populations was not significantly different after 12 weeks of starvation (table I). However, frequency histograms of slow fibre cross-sectional areas reveal a decrease in fibres > 600 μm^2 and an increase in very small fibres (<50 μm^2) with starvation (fig.6b). This indicates that the mean cross-sectional area of

fibres is not a particularly sensitive indicator of fibre atrophy. Starvation results in a progressive decrease in the lipid content of slow fibres, until after 12 weeks starvation, no droplets are visible (figs.2,3,4 ; table III). In 5 week starved slow muscle, however, some fibres contained lipid droplets as well as degraded myofibrils. The volume fractions of mitochondria and capillary supply to slow fibres decreased following starvation (figs.2,4 ; tables I and III). Mitochondrial populations decreased to a greater extent in subsarcolemmal, than interfibrillar, zones (table III). The percentage of slow fibres without direct capillary contact was 44% in fed fish and 64% following 12 weeks starvation. There was also a two-fold decrease in the contact lengths between fibres and capillaries for the vascularised fibres (table I). The mean capillary area supplying 1 μm^3 of mitochondria was similar for fed and starved fish suggesting a parallel decrease in vascular supply and in the size of the mitochondrial compartment for these fibres (table I).

The decrease in volume fractions of mitochondria and myofibrils with starvation was accompanied by an increase in intracellular space.

Starvation resulted in a far greater decrease in fast muscle fibre cross-sectional area (figs.7,8,9,10), than in slow muscle. Large fast fibres (>1500 μm^2) were the most affected (fig.6a). For example, in fed individuals 9% of the fast fibres were >3000 μm^2 compared to only 1.3% after 12 weeks starvation (fig.6b). The breakdown of myofibrillar protein was also proportionally greater in fast than slow fibres (table IV). Around 60% of the total myofibrillar material is degraded by 12 weeks starvation ($p<0.001$)(table IV) resulting in a corresponding increase in intracellular space (table IV). The decrease in volume fraction of myofibrils with starvation varied little within the fast fibre population (fig.8,9,10). In contrast, whilst some slow fibres showed signs of atrophy others appeared little different from those of fed fish (fig.2,3,4). The mechanism for the decrease in myofibrillar volume

fraction appears to involve a uniform, rather than selective, decrease in myofibrillar cross-sectional area (fig.11). In fed fish myofibrillar growth appears to occur at the periphery of the fibres, as a result of the splitting of the large elongated myofibrils adjacent to the subsarcolemmal zone (fig.11). The products of these splits form the smaller internal myofibrils (fig.11). These sites also appear to be the areas where the myofibrils split during degradation (fig.20). The terminal cisternae of the sarcoplasmic reticulum appear to fuse and swell in degenerating fibres (figs.14,15), often invading the space inbetween the two halves of a splitting myofibril. A broad morphological spectrum of lysosome-like vesicles are present in the interfibrillar and subsarcolemmal space (figs.20,21,23-25). Some of these vesicles appear to contain electron dense material (figs.21,23-25).

Intracellular lipid droplets were absent from fast muscle fibres in both fed and starved fish. In fed fish the volume fraction of mitochondria in fast fibres was only 15% of that of the slow fibres (tables I and III). Fast fibres had relatively few mitochondria (4%) in the subsarcolemnal zone, compared to slow fibres (34%). Following 12 weeks starvation the volume fraction of mitochondria decreased to a greater extent (73% ; $p<0.001$) in fast fibres than in slow fibres (32%) (tables I and III). Starvation also resulted in a loss of mitochondrial cristae in the fast fibres, whereas slow fibre mitochondria retained most of their structural integrity.

The nuclei of starved fibres, in both muscle fibre types, were denude of euchromatin material (figs.5,14,16).

Discussion

Some species of fish are able to survive prolonged periods of starvation by utilising their muscle proteins (Love, 1970 ; Johnston & Goldspink, 1973a). For example, in the course of a 4 month fast, plaice degraded around 25% of

their myofibrillar protein, resulting in a muscle water content of 85-90% (Johnston, 1981b). Relatively little is known about amino-acid metabolism in fish. There is some evidence that certain amino-acids can be utilised directly for aerobic energy production even under normal nutritional conditions (Driedzic & Hochachka, 1978 ; Nagai & Ikeda, 1972). In addition, for species with limited lipid reserves, muscle proteins constitute the main reservoir of gluconeogenic precursors during starvation (Love, 1970 ; Moon & Johnston, 1980 ; Moon & Johnston ; 1981). In food deprived saithe, proteins are preferentially degraded from fast muscle fibres (tables I,II,III and IV). A relative sparing of slow fibres has also been reported for cod (Greer-Walker, 1971), crucian carp (Patterson & Goldspink, 1973), and plaice (Johnston, 1981b). In all these species slow fibres constitute less than 10% of the total musculature. In addition to the loss of myofibrillar material with starvation, there is also a general decline in the activities of glycolytic enzymes, particularly in the fast fibres (Moon & Johnston, 1980). This would be expected to reduce locomotory performance. However, a large proportion of the fast muscle (around 85%) is only required for peak swimming speeds, because these are energetically expensive relative to swimming at slow speeds. In general the cost of locomotion rises in proportion to the (swimming speed)^3 (Webb, 1975). Electromyographical studies have shown the order of recruitment of fibres with increasing swimming speeds, to be slow aerobic > fast aerobic > fast glycolytic (Johnston et al, 1977). The threshold speed for recruitment of slow fibres in 17cm saithe is 0.8-1.9 body lengths/sec. (Johnston & Moon, 1979). Thus both fast and slow fibres are recruited for moderate cruising speeds in this species. Burst swimming speeds involve the recruitment of the bulk of the fast fibres utilising both phosphoryl creatine and anerobic glycolysis from local glycogen stores essentially independant of the circulation (Johnston, 1981a).

It seems likely from consideration of the above factors, that utilisation

of fast fibres during starvation would result in a proportionally smaller loss of locomotory performance than utilisation of slow fibres.

In temperate latitudes natural starvation usually occur during the winter months, coinciding with the development of gonads for the spring spawning season (Love,1970). In many cases there is an active migration prior to spawning (Love, 1970). The requirement to maintain muscle contractility and hence swimming performance, would appear to conflict with the role of the skeletal muscle as a source of amino-acids for maintainence and gonad development.

In the present study M-line and myosin degeneration has been observed as the initial stage in myofibrillar dis. -assembly (figs.15-18). This causes the thin filaments (actin) to flare up into the interfibrillar zone until the Z-disc is degraded and hence myofibrillar diameter reduced (see figs.14-20,22). Degenerative changes in the M-line and Z-disc have been reported in 4 month starved plaice muscle (Johnston, 1981b). These observations are of particular interest in view of the Ca^{2+}activated neutral proteinases (CAP) found in mammalian and bird skeletal muscle (Busch et al, 1972 ; Azanza et al, 1979 ; Dayton et al, 1975 ; Dayton et al, 1976 ; Ishiura et al, 1980 ; Dayton et al, 1981 ; Reddi et al, 1983). Busch et al (1972) were the first to describe a Ca^{++} activated proteinase active in the neutral pH range, which is capable of removing Z-discs in vitro. Two forms of CAP have since been isolated with different Ca^{++} requirements and identical molecular weights (Dayton et al, 1981). In addition to releasing α-actinin from Z-discs, CAP also degrades troponin T, troponin I, tropomyosin, M-protein, and C protein in vitro. In vitro the enzyme is inhibited by a naturally occurring inhibitor of molecular weight 68,000 daltons (Takahashi-Nakamura et al, 1981).

Degeneration of the M-line and later the Z-disc at the periphery of myofibrils (figs.14,15,17,18) appears to result in an unravelling of the filament lattice and provides a mechanism whereby myofibrils can decrease in

diameter and yet still retain contractile activity. A characteristic of teleost muscle is the presence of elongated peripheral myofibrils in transverse section (figs.7,11) (Bone et al, 1978 ; Johnston, 1981a). In fast fibres from 12 week starved saithe these fibres can be seen to split where they become thin, possibly due to the mechanical forces exerted in the myofibril during contraction (fig.11c,d). Typically, fast fibres from 12 week starved fish contain many more myofibrils than comparable fibres from fed fish (fig.11c,d).

Lysosomes have been reported in a variety of vertebrate muscles and are known to contain a wide spectrum of cathepsins active at acid pHs (Bird et al, 1980). Cathepsins B, (EC 3.4.22.1) D, (EC 3.4.23.5) and L (E.C. 3.4.22.15) have been purified from rabbit skeletal muscle (Okitani et al, 1980 ; Schwartz & Bird, 1977), and are able to degrade myosin at acid pHs (3-4) in vitro (Bird et al, 1980 ; Okitani et al, 1980 ; Schwartz & Bird, 1977). A broad morphological spectrum of lysosome-like vesicles (including membrane whorls, myelin figures, autophagic vacuoles, and multi-vesicular bodies) are common in both fast and slow fibres of 5 and 12 week starved saithe (fig.17,18). In some cases electron dense material is evident within these organelles and this may represent fragmented myofibrillar material or residual material remaining after degradation, confirmation of either of these will require specific immunohistochemical studies.

A common feature of starved fish fibres is the swollen appearance of the sarcoplasmic reticulum (s.r.) often containing electron dense material (figs.15,22). There is evidence from mammalian muscle that acid hydrolases are normal constituents of s.r. and that vesicles of the s.r. give rise to lysosomes in atrophied muscle (Hoffstein et al, 1975). This would also appear to be the case for starved saithe muscle, but further evidence is required (see chapter 6).

It is evident that there is a major difference in the control of

myofibrillar breakdown between fast and slow fibres. For example, after 12 weeks' starvation a proportion of slow muscle fibres appeared little different from fed controls (fig.4), whilst others were severely atrophied (figs.4,5). In contrast, fast muscle myofibrillar proteins were degraded to a similar extent throughout the fibre population (fig.8,9,10). This same phenomena has also been reported to occur in some muscular diseases (Engel,A.G. 1974 ; Hudgson & Mastaglia, 1974). Factors affecting protein breakdown are so far known to include denervation, muscle length and activity, and the levels of certain hormones, particularly insulin and glucocorticoids (Goldberg, 1980 ; Goldberg et al, 1980 ; Goldspink, 1980). However, the factors responsible for a differential pattern of myofibrillar protein breakdown between fibre types is completely unknown.

Plaice respond to food deprivation with a 40% reduction in routine metabolism and a decrease in spontaneous locomotory activity (Johnston, 1981b). In the laboratory, individuals spend long periods motionless at the bottom of the tank. In contrast, food deprived saithe are active swimmers throughout starvation, with only a small observable decrease in burst swimming speeds (i.e. escape from capture). The mitochondria in slow muscle of plaice occupied a similar fraction of fibre volume (@ 24%) in both fed and starved fish. The cristae structure of the mitochondria was, however, less highly developed in starved individuals, consistent with a reduced aerobic capacity (Johnston, 1981b). In contrast the volume fractions of mitochondria in saithe slow muscle fibres dramatically decreased by around 50% following 12 weeks starvation (table III), with little change in the cristae structure. Degeneration of mitochondria was largely confined to the subsarcolemmal populations (table III), suggesting a functional difference between the two discrete mitochondrial populations.

There are many factors which affect tissue oxygenation in addition to the oxygen demand of the mitochondria. These include the number and surface area

of capillaries available for gas exchange, blood flow, perfusion distances, muscle myoglobin concentrations, and the various factors that influence the haemoglobin-oxygen equilibrium such as pH, PCO_2, and ATP concentrations (Prosser, 1973). Despite this there is a good correlation, for fish slow muscle, between the number of capillaries per unit volume of muscle fibre (NA(c,f)) and the volume fraction of mitochondria (Johnston, 1982). In contrast, for fast fibres, where other factors such as innervation and the removal of anaerobic waste products are important (Hoppeler et al, 1981), no such correlation is evident (Johnston, 1982). The capillary supply to fish muscle appears to be very adaptable, altering with both temperature and hypoxic acclimation (Johnston, 1982 ; Johnston & Bernard, 1982). It is significant that increases and decreases in mitochondrial volume fractions are closely matched by changes in the number of capillaries per unit volume of muscle fibre. In starved saithe the reduction in the mitochondrial volume for slow fibres is accompanied by a parallel decrease in capillary supply (tables I and II)

Table I

Effects of starvation on the capillary supply to slow muscle fibres.

Measured Parameters Mean ± S.E.	Symbol Calcn.	Nutritional Status Fed	5 week starved	12 week starved
Number of fibres analysed	A	55	51	73
Number of fibres without capillary contact	B	24	18	49
Number of capillaries associated with fibres analysed	C	59	33	29
Mean fibre cross sectional area (um^2)	D	238 ± 22	259 ± 26	274 ± 25
Mean fibre perimeter (um)	E	64 ± 3	63 ± 4	65 ± 3
Volume fraction of mitochondria	F	34.6 ± 1.9	23.7 ± 1.4	16.1 ± 0.9
Number of capillaries per fibre	G	1.1	0.7	0.4
Fibre perimeter in direct contact with capillaries	H_1 H_2	4.0 ± 0.7 7.1 ± 0.8	3.7 ± 0.7 8.2 ± 0.9	2.1 ± 0.4 5.8 ± 0.5
Calculated parameters				
Percentage of fibre perimeter in direct contact with capillaries	I_1 I_2	6.3 ± 0.9 11.1	4.7 ± 0.9 13.0	3.3 ± 0.7 8.9
Capillary perimeter per 1 um^2 of fibre area	J_1 J_2	0.018 0.03	0.014 0.032	0.008 0.021
Capillary area per 1 um^3 mitochondria	K_1 K_2	0.049 0.087	0.059 0.135	0.050 0.13
Number of capillaries per mm^2 of fibre cross-sectional area. N_A (c,f)	L	4507	2500	1450

1. Mean value for all fibres
2. Mean value calculated for vascularized fibres only

Table II

Effects of starvation on the capillary supply to fast muscle fibres.

Measured Parameters Mean ± S.E.	Symbol Calcn.	Nutritional Status Fed	5 week starved	12 week starved
Number of fibres analysed	A	55	45	62
Number of fibres without capillary contact	B	18	37	53
Number of capillaries associated with fibres analysed	C	31	8	9
Mean fibre cross sectional area (um^2)	D	1014 ± 161	881 ± 99	534 ± 87
Mean fibre perimeter (um)	E	113 ± 106	114 ± 8	80 ± 7
Volume fraction of mitochondria	F	5.1 ± 0.6	1.4 ± 0.3	1.4 ± 0.3
Number of capillaries per fibre	G	0.6	0.2	0.2
Fibre perimeter in direct contact with capillaries	H_1 H_2	3.1 ± 0.5 6.5 ± 0.5	1.2 ± 0.4 6.7 ± 0.8	1.3 ± 0.4 8.0 ± 1.7
Calculated parameters				
Percentage of fibre perimeter in contact with capillaries	I_1 I_2	2.8 ± 0.5 5.8	1.1 ± 0.5 5.9	1.1 ± 0.4 10
Capillary perimeter per lum^2 of fibre area	J_1 J_2	0.003 0.64	0.001 0.76	0.002 1.5
Capillary area per lum^3 of mitochondria	K_1 K_2	0.06 0.13	0.1 0.54	0.2 1.07
Number of capillaries per mm^2 of fibre cross-sectional area. N_A (c,f)	L	556	202	318

1. Mean values for all fibres.
2. Mean values calculated for vascularized fibres only

Table III

Effects of starvation on the ultrastructure of slow muscle fibres.

Component Volume fraction (Vv) % Mean ± S.E.	Nutritional status		
	Fed	5 week starved	12 week starved
Myofibrils	51.7 ± 1.3	47.4 ± 1.3	47.2 ± 1.0
Subsarcolemmal mitochondria	23.0 ± 1.6	14.1 ±1 0.9	7.7 ± 0.6
Interfibrillar mitochondria	11.9 ± 0.7	9.6 ± 0.7	10.6 ± 0.6
Lipid droplets	4.9 ± 1.0	6.7 ± 0.8	-
Intracellular space	8.3 ± 1.1	19.5 ± 1.8	33.2 ± 1.5

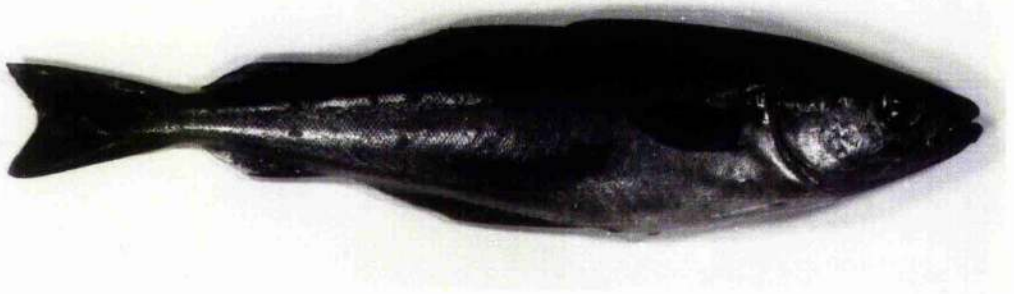
Table IV

Effects of starvation on the ultrastructure of fast muscle fibres.

Component Volume fraction (Vv) % Mean ± S.E.	Nutritional status		
	Fed	5 week starved	12 week starved
Myofibrils	79.0 ± 1.1	68.7 ± 1.2	56.4 ± 1.4
Subsarcolemmal mitochondria	4.9 ± 0.6	1.2 ± 0.3	1.2 ± 0.3
Interfibrillar mitochondria	0.2 ± 0.1	0.2 ± 0.04	0.3 ± 0.1
Lipid droplets	-	-	-
Intracellular space	13.0 ± 0.8	28.9 ± 1.2	41.1 ± 4.5

Fig. 1 Pollachius virens L. Lower diagram shows the distribution of slow and fast muscle in a transverse section through the musculature and the longitudinal orientation of the myotomes along the body. The areas of sampling for electron microscopic preparation are indicated for both slow and fast muscle.

1



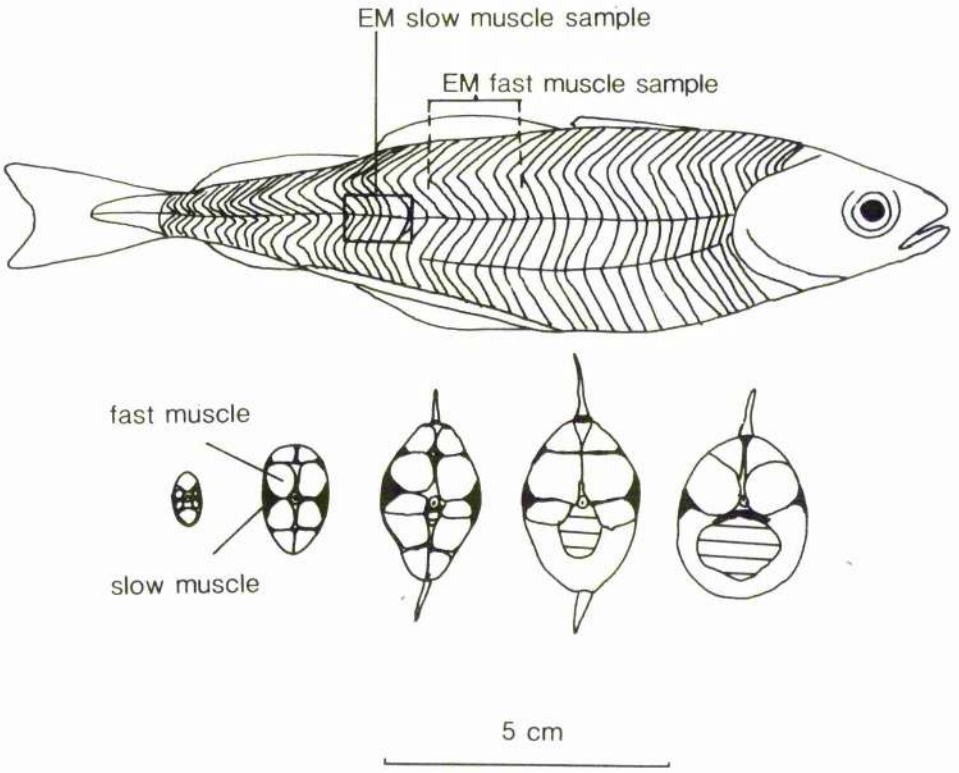

EM slow muscle sample
EM fast muscle sample
fast muscle
slow muscle
5 cm

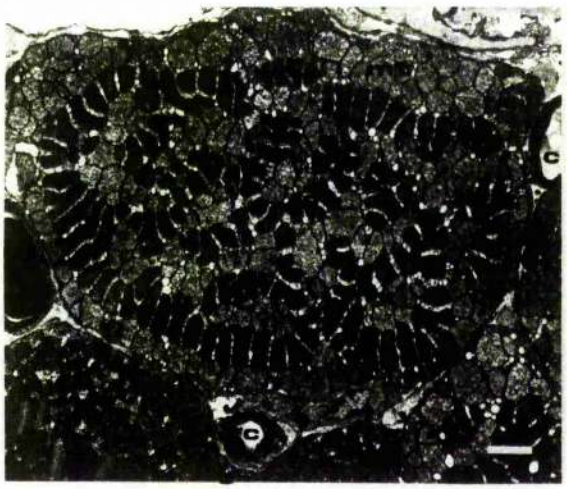
Fig. 2 Transverse section of slow muscle from fed saithe. Note high proportion of mitochondria (mt) surrounding myofibrils (m). Also note the extensive capillary supply (c) to the fibre. Bar 2 µm.

Fig. 3 Slow fibre from fed saithe. Note extensive lipid droplets (l). Bar 3 µm.

Fig. 4 Slow fibres from 12 week starved saithe. Note the differing extents of myofibrillar degradation in adjacent fibres. Bar 1 µm.

Fig. 5 Micrograph showing the right hand fibre from fig. 4 at a higher magnification. Note the degradation of the myofibrils (dm) and the apparently unaffected mitochondria (mt). Bar 1 µm.

2



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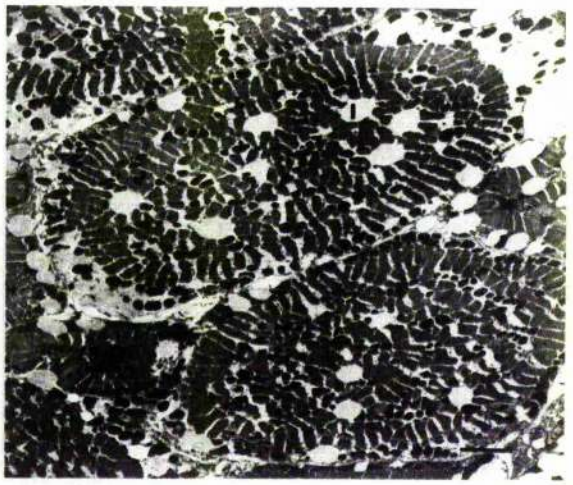

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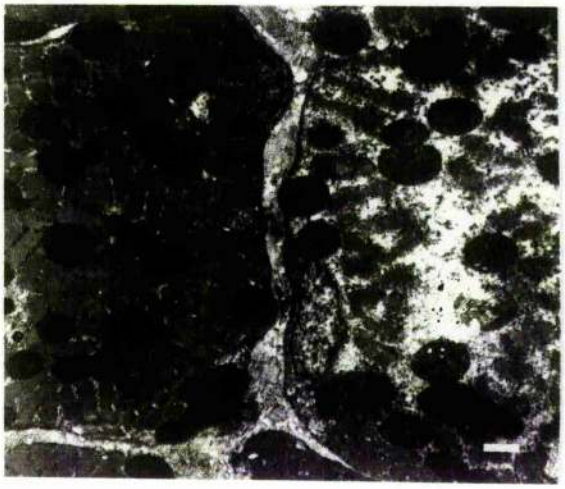

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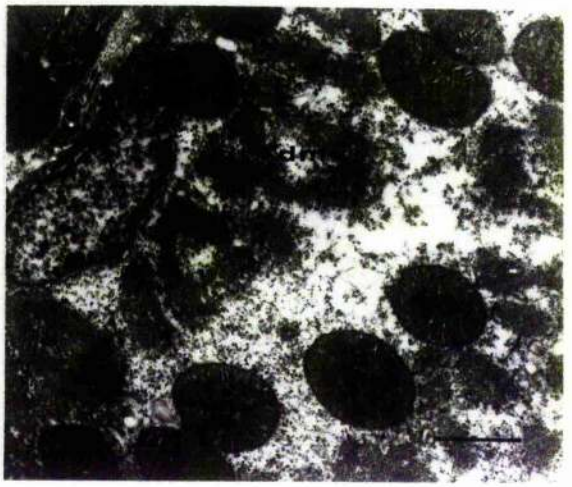
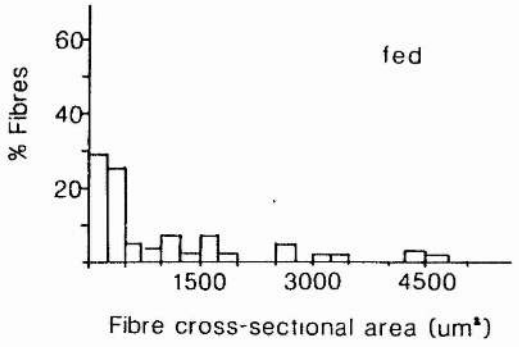
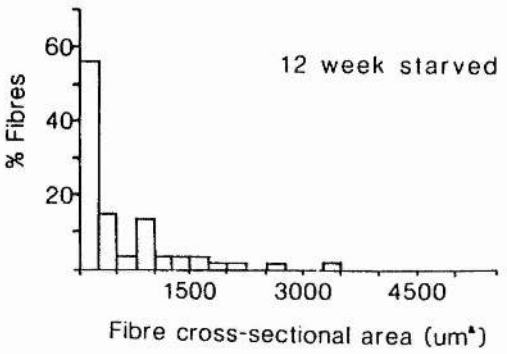


Fig. 6 a) Frequency histograms showing the distribution of fibre cross-sectional area for slow muscle in fed and starved fish.

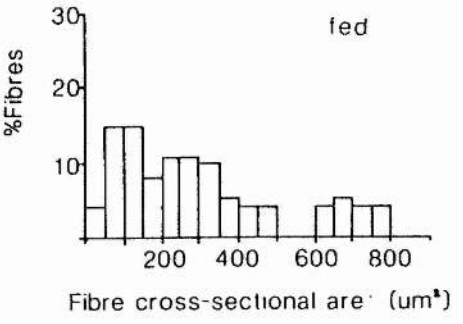
b) Frequency histograms showing the distribution of fibre cross-sectional area for fast muscle in fed and starved fish.

a. fast muscle





b. slow muscle



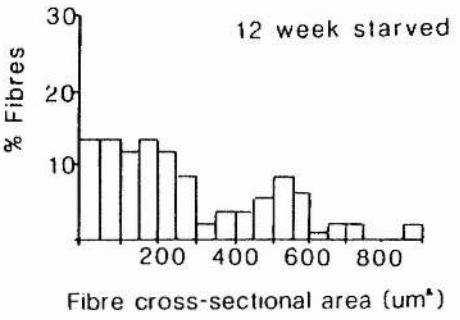
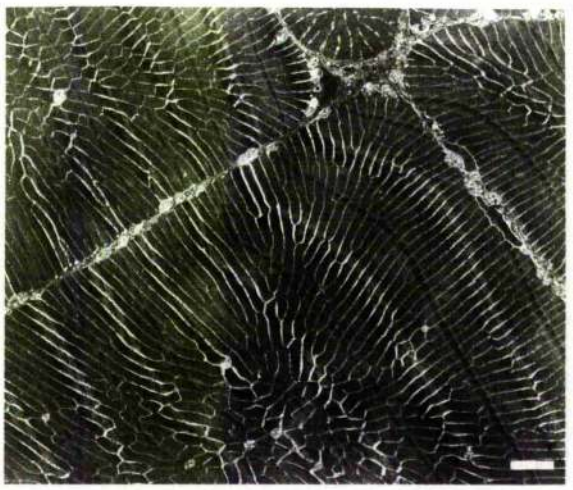


Fig. 7 Transverse section of a fast fibre from a fed saithe. Note the low density of mitochondria, espescially in the interfibrillar zone, and the high myofibrillar content. Bar 3 µm.

Figs. 8, 9, 10 Fast fibres from 12 week starved saithe. Note large interfibrillar space, reduced myofibrillar content, and depletion of mitochondria. 8) Bar 4 um. 9) Bar 5 um. 10) Bar 2 µm.

7



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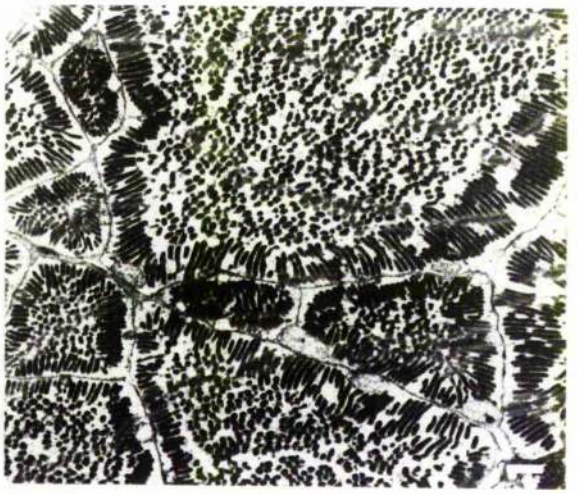

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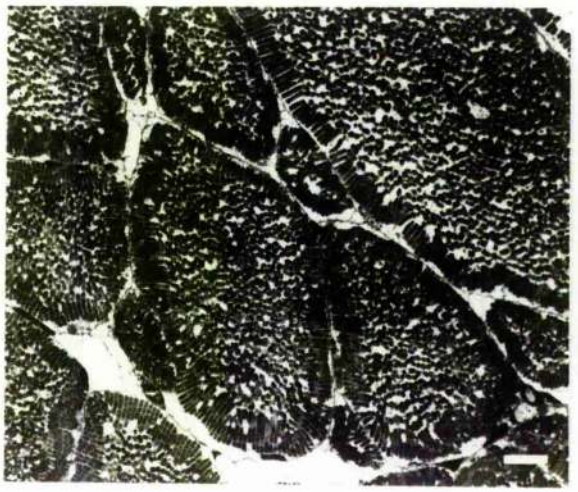

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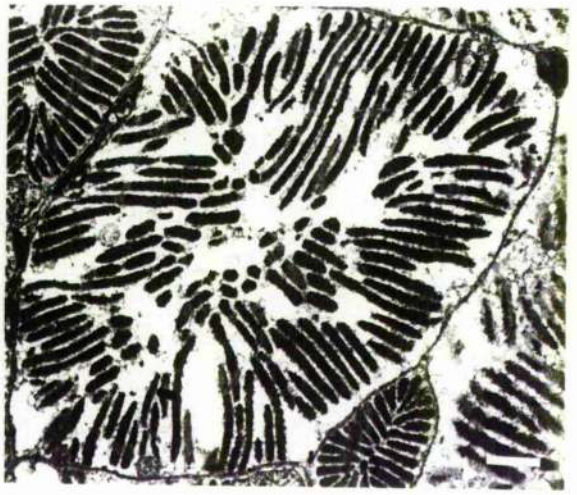
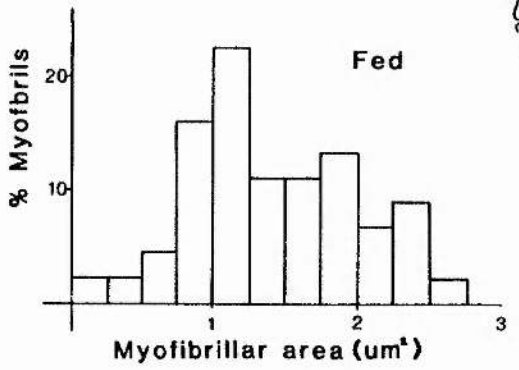


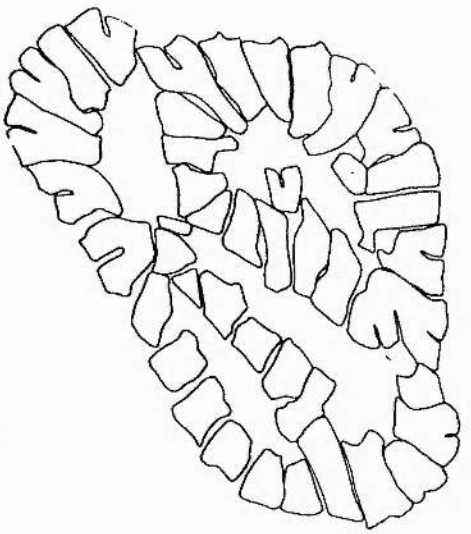
Fig. 11 Histograms showing the frequency distribution of myofibrillar cross-sectional area for fed and starved, slow and fast fibres. Each fibre shown has the mean volume fraction of myofibrils for its' respective group (see tables III & IV).

a) Frequency distribution of myofibrillar cross-sectional area for a single slow fibre form a fed fish.

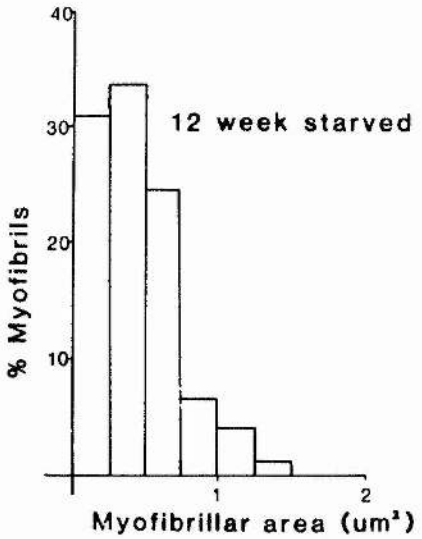
b) Frequency distribution of myofibrillar cross-sectional area for a single slow fibre from a 12 week starved fish.

a


Fed
% Myofbrils
20
10
1
2
3
Myofibrillar area (um²)



b


12 week starved
% Myofibrils
40
30
20
10
1
2
Myofibrillar area (um²)

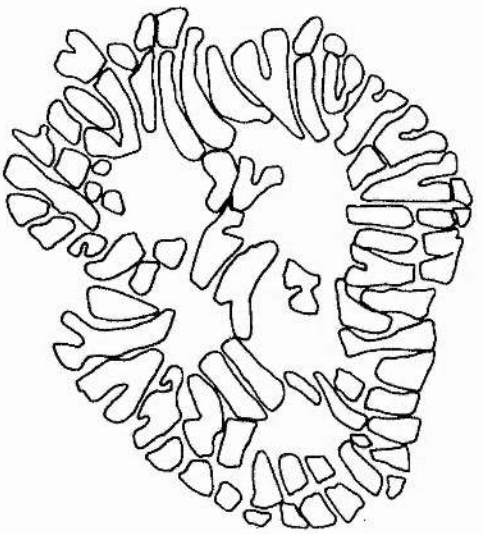
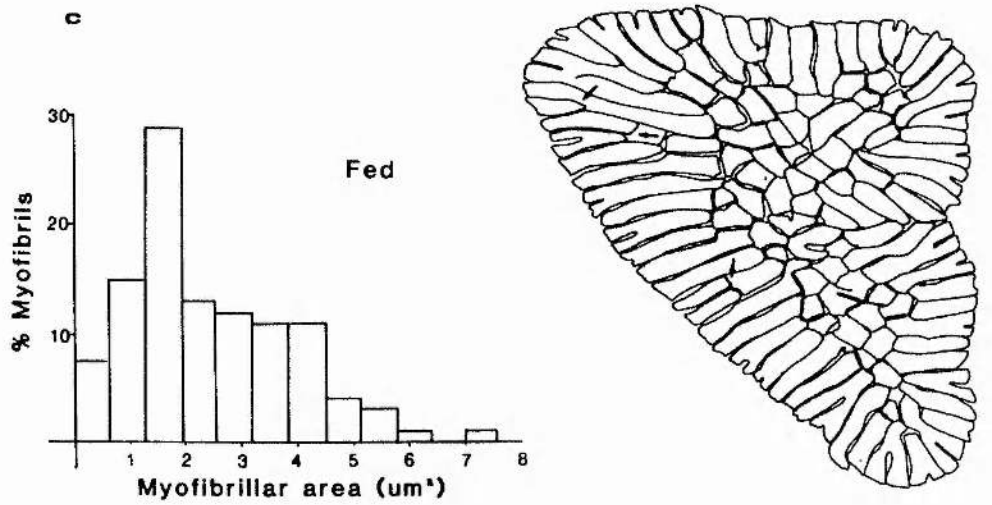


Fig. 11 (contd.)

c) Frequency distribution of myofibrillar cross-sectional area for a single fast fibre from a fed fish. The arrows indicate the possible sites where the elongated peripheral myofibrils have split forming internal myobrils.

d) Frequency distribution of myofibrillar cross-sectional area for a single fast fibre from a 12 week starved fish. Note the increase in the number of myofibrils and the uniform decrease in myofibrillar cross-sectional area.


c
Fed
% Myofibrils
30
20
10
1
2
3
4
5
6
7
8
Myofibrillar area (um²)

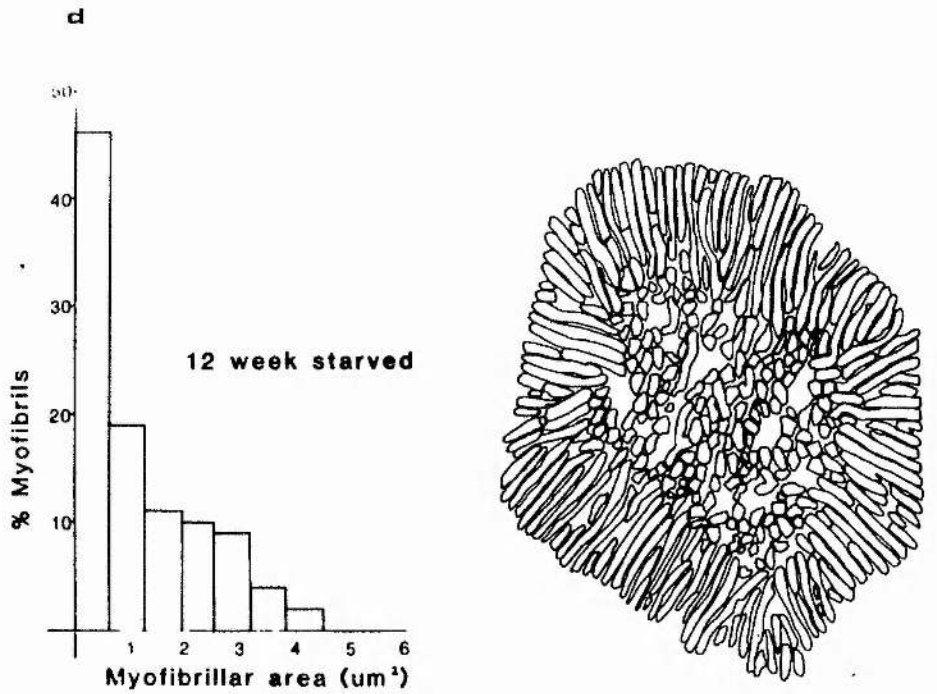

d
12 week starved
% Myofibrils
40
30
20
10
1
2
3
4
5
6
Myofibrillar area (um²)

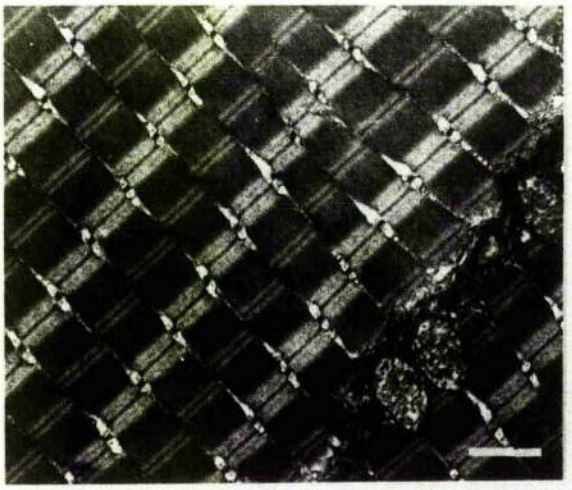
Fig. 12 Longitudinal section through fast muscle from a fed saithe. Bar 1 µm.

Fig. 13 Longitudinal section through fast muscle from a fed saithe. Bar 0.25 µm.

Fig. 14 Longitudinal section through fast muscle from a 12 week starved saithe. In comparison with figs.12 and 13 note the peripheral degradation of the myofibrils resulting in an increase in the interfibrillar space. Also note how the sarcoplasmic reticulum (s. r) can be seen to swell and occasionally fuse together. Bar 1 µm.

Fig. 15 Longitudinal section through fast muscle from a 12 week starved saithe. Note the peripheral degradation of the myofibrils and the breakdown of the peripheral section of the M line (arrows). A vesicle that appears to arise from the intermediate cisternae, can often be seen above the M line in degraded myofibrils. Bar 0.25 µm.

12



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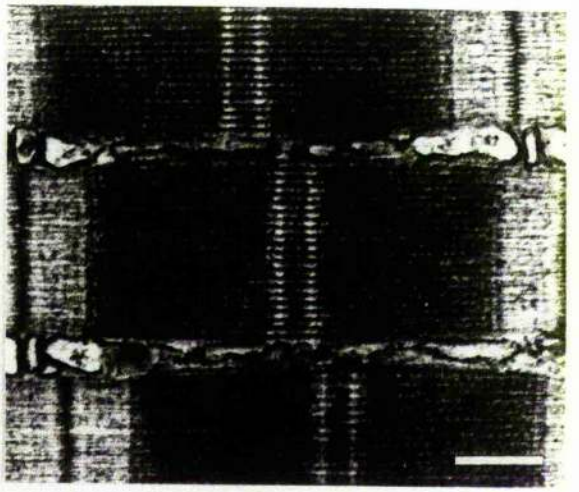

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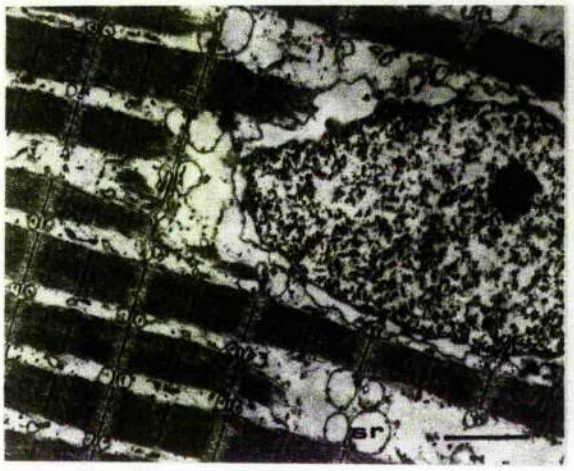

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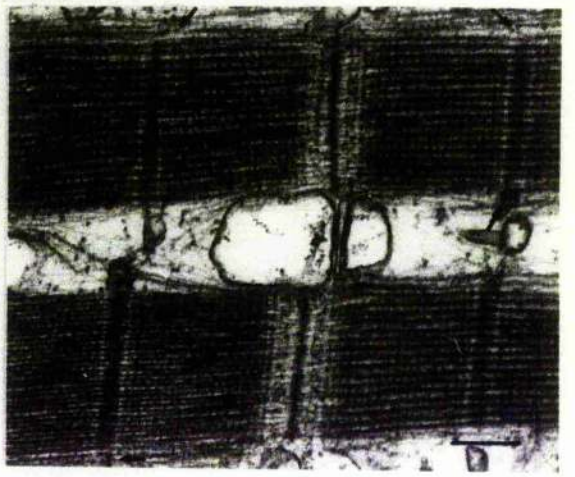
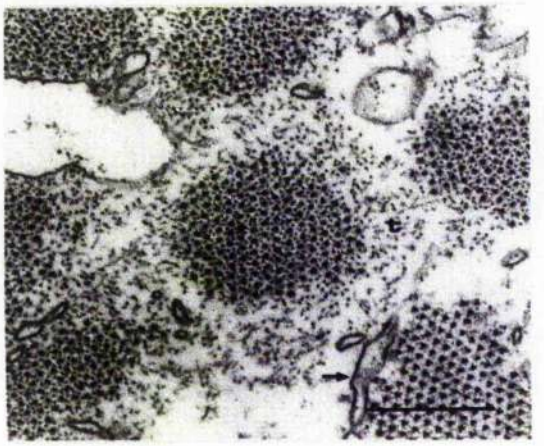


Figs. 16, 17, 18 Transverse sections demonstrating the preipheral degradation of the thick filaments. Fig. 16 shows undegraded myofibrils for comparison with figs.17 and 18. Note that in fig.17 the centre myofibril (sectioned inbetween the ends of the H and A zones) contains only thin filaments around the periphery (t). The bottom right myofibril is sectioned through the H or M zone, note the presence of the s.r. (intermediate cisternae) around the perimeter of the thick filaments (arrow). Bar 0.5 μm.

16

17



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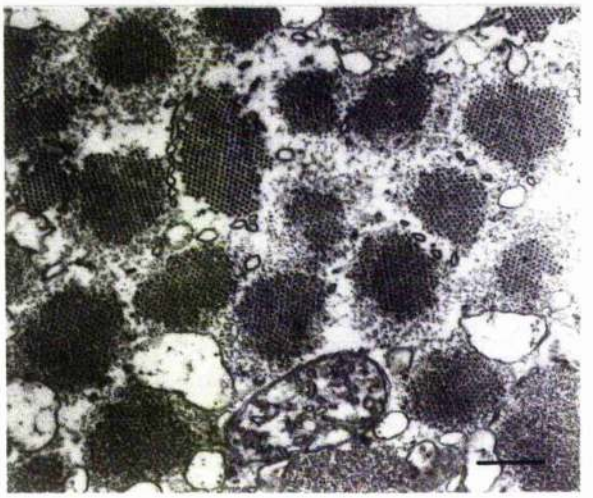


Fig. 19 Transverse section through fast muscle from a 12 week starved saithe. Note the peripheral degradation, vast interfibrillar space (compare with fig.7), and the lack of euchromatin within the nucleus. Bar 5μm.

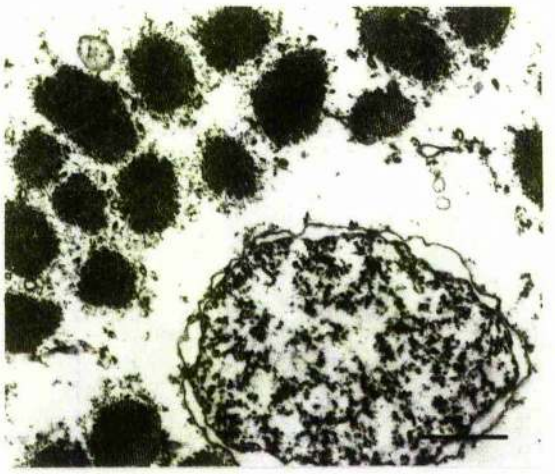
Fig. 20 Transverse section through fast muscle from a 12 week starved saithe showing lysosome like vesicle (ly). Arrows indicate the site of myofibrillar splitting, where peripheral degradation has caused the myofibril to become thin, resulting in the thick filaments no longer remaining in a straight line across the myofibril. Bar 0.5 μm.

Fig. 21 Lysosome like vesicles containing elecron dense material. Pictures taken from fast muscle from 12 week starved fish. Bar 0.25 μm.

Fig. 22 Myofibril from a 12 week starved fish showing the disassembly of the peripheal region of the myofibril. Note the electron dense material at the periphery of the fibril, in the interfibrillar space, and in the sarcoplasmic reticulum. Also note the thin filaments (t) flaring up into the interfibrillar zone and the disappearance of the thick filaments at the periphery of the myofibril. Bar 0.25 μm.

Figs.23,24,25 Double and multi-membraned vesicles observed in atrophied fibres. Note the electron dense material within the vesicles. Bar 0.25 μm.

19



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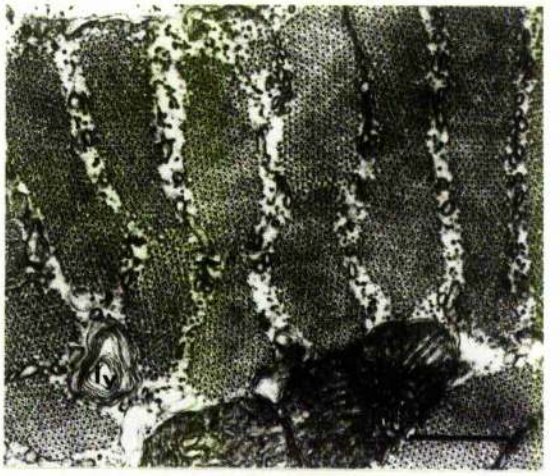

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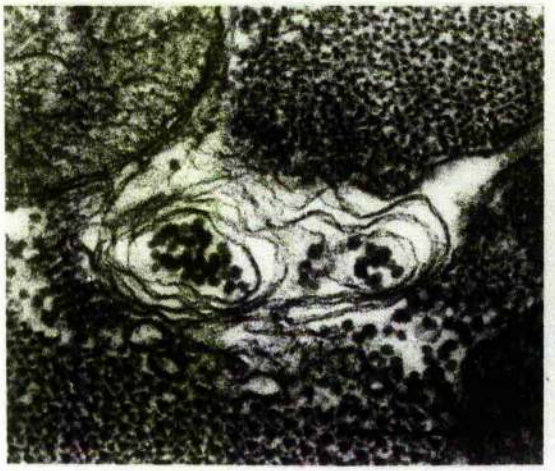

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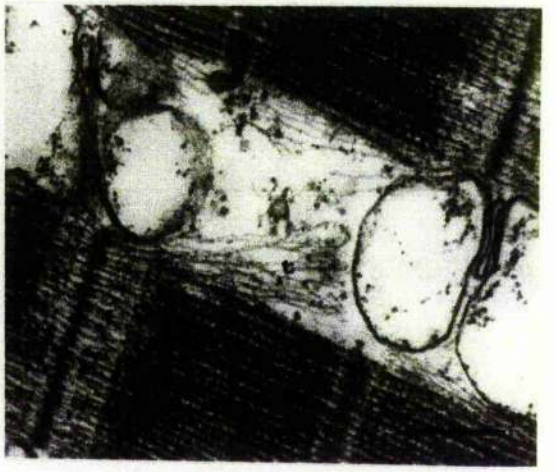

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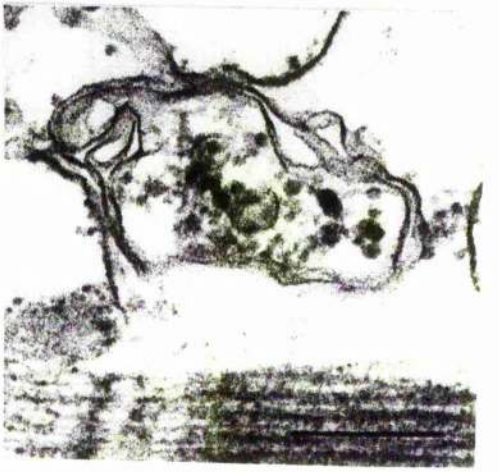

Chapter 3

The ultrastructure of muscle following starvation and refeeding.

Introduction

In many wild populations of marine fish, tissue water contents have been reported to increase during the winter months leading up to spawning in spring. For example the muscle water content in North Sea cod caught on the Aberdeen Bank, increases during December to March from 80.1% to 83.4% (Love, 1960), and in some cases reach 87% (Love, 1970). The most extreme natural depletion reported is in the American flounder, Hippoglossoides platessoides, where muscle water content has been found to reach as high as 96.2% (Templeman & Andrews, 1956). In chapter 2 it was reported that in saithe an experimental starvation of 12 weeks resulted in the preferential degradation of fast muscle protein, and this was paralleled by an increase in the water content from 79% to 86% (see chapter 5). During the same period the mean cross-sectional area of the fast fibres decreased by almost 50%, whilst the fractional volume of myofibrils decreased by 24%. These parameters indicate that during starvation a large percentage of the fast muscle contractile protein is degraded.

There have been a number of quantitative studies reporting the effects of starvation on fish muscle ultrastructure (Patterson & Goldspink, 1973 ; Johnston, 1981b), however the ultrastructural events that prevail during refeeding have not previously been investigated. In wild populations of North Sea cod muscle water contents are restored to normal in approximately 2 to 3 months following depletion in the winter / early spring period (Love 1980). In a biochemical study of refeeding following starvation in cod, Black (1983) reported a dramatic rise in tissue glycogen deposits paralleled by an increase in the RNA : DNA ratio, suggesting an increase in the net rate of protein synthesis. Black (1983) also reported increases in the activities of enzymes involved in lipogenesis and the pentose phosphate pathway and suggested that these may be involved in the replacement of membrane phospholipids. Upon refeeding there is therefore a rapid reversal of the processes involved in

muscle degradation and a period of elevated protein synthesis. Starvation and refeeding therefore provide a convenient model for studying the processes involved in repair and regeneration of muscle following severe atrophy.

In the present study saithe (Pollachius virens) were subjected to a starvation under laboratory conditions of 74 days and the effects on the muscle ultrastructure of 10, 20, and 52 days refeeding was investigated.

Materials and Methods

Experimental protocol

Saithe (Pollachius virens) of mean length 17.0 + 2.2cm were caught by line fishing from St.Andrews harbour during October and November (1982). The fish were kept in holding tanks at 10°C and starved for 74 days, during the same time of year as a natural depletion in the wild populations. After this starvation the fish were fed ad libitum once daily for 52 days, with a diet consisting of squid and herring. Groups of fish (consisting of 5 individuals each) were sampled after 74 days of starvation, and on days 10, 20, and 52 of refeeding. Control fish (also caught in Nov.1982) were kept and fed for three days in the holding tanks prior to sampling. Fish were sacrificed by a sharp blow to the head followed by transection of the spinal cord.

Electron Microscopy and quantitative analysis

Muscle samples were prepared and analysed as decribed in chapter 2. From each of the five fish in the 5 experimental groups 4 blocks were made and sampled (2 L.S. and 2 T.S.). Transverse sections were used for the quantitative analysis of organelle fractional volumes and fibre cross-sectional areas, whilst longitudinal sections were used for qualitative analysis only.

Results

Fibre cross-sectional area

Following 74 days starvation the mean cross-sectional area of slow fibres decreased by 60% ($p<0.001$). Fibre cross-sectional area increased progressively with refeeding (table I). Similar changes were observed for fast fibres (table II) where 74 days starvation resulted in a 50 % decrease in fibre cross-sectional area ($p<0.001$). Within the first 10 days of refeeding the average fast fibre cross-sectional area continued to decrease and thereafter increased progressively upon continued refeeding.

Energy stores

In fed saithe lipid stores occupy 7.4% of the slow fibre volume. These stores are totally depleted after 74 days of starvation (table III ; figs. 1, 2 & 3). Upon refeeding there is a rapid re-synthesis of lipid such that after 10 days lipid constitutes 5.3% of the slow fibre volume (table III ; fig.4). Inbetween 10-20 days refeeding the lipid stores decrease to only 0.9%, increasing to the levels found in fed fish after 52 days (table III ; figs.5 & 6). Upon refeeding there is a massive increase in glycogen deposits such that at 20 days of refeeding the subsarcolemmal space is occupied almost entirely of glycogen granules (fig.7). During 20-52 days of refeeding the subsarcolemmal space decreases from 16.9% to 0.7% of the fractional volume of the slow fibres. At 52 days refeeding most of the excess glycogen has been metabolised and the subsarcolemmal space is largely occupied by mitochondria and lipid droplets (fig.8).

Mitochondria

Mitochondria constitute 40.9 and 5.2% of the fibre volume in slow and

fast fibres respectively (Tables III & IV). Following starvation there is a decrease in both the number of mitochondria and the surface area of the inner cristae structure (figs.13 & 14). The fractional volume of mitochondria is reduced by 39% in slow ($p<0.001$) and 71% in fast fibres ($p<0.001$) after 74 days of starvation (tables III & IV). Starvation has a proportionally greater effect on the subsarcolemmal mitochondrial population. The fractional volume of mitochondria continues to decrease during the first days of refeeding and then increases gradually as refeeding continues (Tables III & IV). The subsarcolemmal populations appear to regenerate sooner than the interfibrillar mitochondria (tables III & IV). Polysomes were not observed in starved muscle mitochondria, but were common in both slow and fast muscle mitochondria after 10 days refeeding (fig.15). Figs.16 and 18 show the initial stages of what is assumed to be mitochondrial biogenesis. This appears to involve a clearing of cytoplasmic material prior to the synthesis of the outer mitochondrial membrane, in which polysomes can often be seen (fig.16). Presumptive mitochondria are often surrounded by glycogen particles. In 52 day refed fish the cristae structure of mitochondria is similar to that of control fish.

Myofibrillar degeneration following starvation

The fractional volume of myofibrils decreased by 23% in fast fibres following 74 days starvation ($p<0.001$, figs.9 & 10), but was unchanged in slow fibres (tables III & IV). However slow fibre cross-sectional area decreased by 60 % ($p<0.001$, table I) and hence there is also a significant degradation of contractile proteins within the slow fibre population. A feature of myofibrillar degradation was the breakdown of the M-line and thick filaments at the periphery of the myofibril (figs.19 & 20), resulting in the thin filaments flaring up into the interfibrillar space. In some fibres the myosin has been completely removed leaving adjacent Z-disc and thin filaments unconnected and yet still in alignment (figs.21 & 22). In most fibres however

the extent of degradation is restricted to the peripheral filaments causing a gradual decrease in myofibrillar diameter (fig. 19).

Myofibrillar regeneration during refeeding

Following starvation myofibrils remain in various stages of degeneration and can generally be classed into one of the following categories. 1. myofibrils with a normal appearance. 2. myofibrils with a decreased diameter and peripheral thick filaments absent (fig.19). 3. myofibrils with thick filaments totally absent but with the thin filaments and Z-discs still intact (figs.21 & 22). 4. myofibrils that are completely or largely disrupted and phagocytosed. A comparison of muscle from starved and refed fish indicates the following changes associated with refeeding. In fish sampled at 10 days of refeeding the peripheral integrity of the myofibrils appears to be restored by the replacement of thick filaments (fig.30), hence actin filaments are rarely observed intruding into the interfibrillar space. The second pattern of regeneration involves the production of new sarcomeres, especially at the ends of myofibrils where large areas of unfilled cytoplasm remain in starved muscle fibres. The production of new sarcomeres appears to progress in the following sequence. Initially electron dense areas can be seen, spatially orientated where the Z-disc and T-tubule will later be synthesised. Usually thin filaments are observed attached to the presumptive Z-disc (Z-body) where occasionally tubular membrane elements are apparent (figs.23-27). Thick filaments appear to form within the thin filament lattice, starting from the end of the myofibril and progressing into the newly forming sarcomere (figs.23-27). Z-bodies and tubular membrane elements were invariably associated with polysomes and were only observed after refeeding and not in fibres from starved fish. It therefore seems likely that these features are unique to regenerating sarcomeres and are not associated with continuing degradation.

Membrane systems

During starvation the terminal cisternae can be observed to swell and often fuse, creating large vacuoles frequently containing electron dense material. The intermediate cisternae can be seen to form a small vacuole (or vacuoles) above the M-line (fig.20). Many multi-membraned vesicles (probably secondary lysosomes) are present (figs.29). Also numerous vacuoles are present at the cell membranes. Upon refeeding there is a rapid decline in the number of free cytoplasmic vacuoles, especially between 10 and 20 days refeeding. The sarcoplasmic reticulum soon reduces in size and becomes more ordered. After 20 days of refeeding in slow muscle and inbetween 20-52 days in fast, the sarcoplasmic reticulum appears normal.

Nucleus

During starvation the nuclei and nucleolus become denude of euchromatic material. Within 10 days of refeeding however many ribosomes and polysomes are visible in both the nucleus and nucleolus (fig.31). As refeeding progresses the nucleolus becomes more electron dense and distinct (figs.32-34).

Capillary supply

In both slow and fast muscle there is a fall in the number of capillaries per fibre during starvation (tables I & II). During refeeding there is steady recovery in the capillary supply. However the number of capillaries per fibre only increases slowly and does not reach control values even after 52 days (tables I & II).

Discussion

During the starvation previously reported in chapter 2 neither the slow fibre mean cross-sectional area nor the volume fraction of myofibrils decreased significantly, whereas fast fibres showed substantial atrophy. These results are consistent with those for cod (Greer-Walker, 1971), Crucian carp (Patterson & Goldspink, 1973), and plaice (Johnston, 1981b). In contrast to this during the starvation reported in this chapter there was also a 60% decrease in the mean slow fibre cross-sectional area (table I). In common with the previous study the volume fraction of myofibrils remained unchanged in slow muscle, and decreased by around 27% in fast muscle. In studies on plaice Patterson et al (1974) reported that during a starvation of 1 month only fast fibre water contents increased significantly from controls. However, when the starvation was prolonged to 6 months the water contents of both fibre types increased significantly. It therefore seems likely that although the starvation in the present chapter was 10 days shorter than in the previous chapter, the fish may have been in a somewhat poorer condition prior to experimentation such that this starvation was in reality more severe. In support of this, another unique feature indicating the severity of the 74 day starvation, was the presence of fast fibres that were totally depleted of thick filaments.

Energy stores

In slow fibres there is a transitory accumulation of lipid droplets at 10 days of refeeding, but this is diminished again by 20 days (tables III & IV). This may reflect changes in the transport, storage sites and/or utilisation of lipid droplets during the course of refeeding.

Coinciding with the decrease in slow fibre lipid is a massive accumulation of subsarcolemmal glycogen. This also occurs in fast fibres, but only after a longer period of refeeding. Elevated muscle glycogen levels have

also been reported during the onset of refeeding in pike (Ince & Thorpe, 1976b), and cod (Black, 1983) and could provide another energy source for regenerating fibres.

Mitochondria

During 20-52 days of refeeding there is a large increase in the number and fractional volume of slow fibre mitochondria, particularly in the subsarcolemmal region (table III).

Mitochondria are known to be semi-autonomous, containing endogenous DNA capable of synthesising mitochondrial RNA, ribosomal RNA, transfer RNA, and a few proteins of the inner membrane. Two main hypotheses for mitochondrial biogenesis have been postulated (de Robertis & Bleichmar,1962). The first hypothesis suggests de novo synthesis of mitochondria from simple building blocks. During starvation in saithe there is a 15 % decrease in the total volume fraction occupied by mitochondria ($p<0.0001$, table III). Upon refeeding these populations are gradually restored. In 10 day refed fish many vesicles containing polysomes and occasionally tubular structures, were observed in slow muscle, especially in the subsarcolemmal region (figs.16 & 18). It is possible that these vesicles represent the initial stages of mitochondrial biogenesis. Evidence was also obtained for the repair of degenerate mitochondria, that have damaged cristae and/or a disrupted outer membrane, where a high density of polysomes and ribosomes were observed in association with the organelle (fig.17). The more rapid restoration of the subsarcolemmal, as opposed to interfibrillar mitochondrial volume fractions, again suggests a functional difference between these two mitochondria populations (table III).

Membrane systems

There is evidence from mammalian studies that acid hydrolases are normal

components of the sarcoplasmic reticulum (s.r.) and that the s.r. could be the source of many lysosomes in atrophied muscle (Hoffstein et al,1975, see chapter 6). In starved saithe the s.r. can be seen to swell and a broad morphological spectrum of vesicles are present in both the interfibrillar and subsarcolemmal space. Some of the vesicles are double or multi-membraned (membrane whorls, myelin figures, multi-vesicular bodies) and often contain electron-dense material (fig.29 ; see chapter 2). During refeeding the frequency and size of the s.r. and other vesicles rapidly decreases such that within 20 days of refeeding the s.r. of both fibre types appears similar to that of control fish. These events provide circumstantial evidence for the involvement of the s.r. and multi-membraned vesicles in myofibrillar degradation.

Myofibrillar degeneration during starvation

During starvation the myofibrillar volume fraction decreases by 25% in fast fibres, but remains unchanged in slow muscle (tables III & IV). Similar results have been reported in cod (Greer-Walker, 1971), carp (Patterson & Goldspink, 1973), plaice (Johnston, 1981b), and in chapter 2, for saithe. Filament disassembly by the initial degeneration of the M-line and myosin at the periphery of the myofibrils (figs.19 & 20) may provide a mechanism whereby myofibrils can be partially degraded and yet still maintain some contractility (see chapter 2). Recent studies on the cytoskeleton of skeletal muscle have demonstrated a range of proteins including vinculin, tubulin, spectrin, calmodulin, and cytoplasmic isoforms of actin, that are not observed using conventional uranyl and lead stains (Padro, Siliciano, & Craig, 1982 ; Wang, 1983 ; Pollard, 1982). Thin filaments, consisting mainly of actin, have been found to be relatively resistant to degradation during starvation. In atrophied muscle, some myofibrils, and indeed whole fibres, can be seen where all the thick filaments have been degraded and yet the sarcomeres (consisting

of thin filaments and Z-discs) still maintain alignment. This suggests that elements of the cytoskeleton may still remain intact even during severe myofibrillar degradation. The cytoskeleton may also have a role in the orientation and co-ordination of filament assembly during refeeding.

Myofibrillar regeneration following refeeding

Upon refeeding the repair and regeneration of the degraded contractile apparatus appears to involve two processes. The first process involves the replacement of the thick filament at the periphery of the myofibrils. This process is especially evident in 10 day refed slow muscle where, even though the myofibrillar diameter still appears much reduced, the peripheral lattice structure is restored to normal with a full complement of thick and thin filaments (fig.30). A similar phenomenon has been reported to occur during re-innervation of rat skeletal muscle following denervation atrophy (Jakubiec-Puka et al, 1982).

The second process involves the formation of new sarcomeres primarily at the myofibrillar ends and periphery. The temporal sequence for sarcomere formation appears to proceed with the synthesis of thin filaments, often attached to an electron dense area ("Z body"). Tubular membrane elements were frequently seen with, or prior to the formation of the Z body and may have a cytoskeletal function (figs.23,26 & 27). Z bodies appear to act as a focus of attachment for the thin filaments and may be responsible for co-ordinating the orientation of the thin filaments in the long axis of the myofibril. The gelating effects of α-actinin (a major component of the Z disc) on F actin (Maruyama & Ebashi, 1965) support the possible function of the Z body in binding and co-ordinating the assembly of the thin filaments. Z bodies initially appear as electron dense areas but later develope into distinctive Z discs. Coalescence of the Z body into a Z disc occurs when the sarcomere contains a high percentage of complete thick filaments and it has been

suggested (Kelly, 1969) that this process may occur when the sarcomere can generate sufficient tension to cause the alignment of the Z body into a distinctive Z disc.

From the present observations it is apparent that thick filament assemblage follows that of the thin filaments. This is in agreement with previous studies reporting on myofibrilogenesis in West coast newt, Taricha torosa (Kelly, 1968), and brown trout (Kilarski & Kozlowska, 1979), Xenopus cultured muscle cells (Peng et al, 1981), and rat soleus muscle regenerating after reinnervation (Jakubiec-Puka et al, 1982). In saithe muscle, thick filaments initially appear within the thin filament lattice closest to the completed end of the myofibril and extend progressively across the sarcomere. This observation is in contrast to the in vitro studies of Huxley (1963) who first reported that purified myosin can aggregate into filaments, similar in form and length to native myosin, depending on the pH, ionic strength, and MgATP of the external medium. Huxley (1963) proposed that the assemblage of the myosin filament commences from the middle region of the filament and recent work by Niederman & Peters (1982) has provided evidence that the bare zone area of myosin filaments can nucleate myosin filament assembly, in vitro. In the regenerating muscle of saithe the formation of thick filaments must presumably be influenced by other factors, as clearly myosin filament formation does not proceed from the central region (figs.23-27).

Filament synthesis and subsequent assembly into myofibrils has been the subject of many investigations and appears to vary between the species. In saithe muscle it appears that both filament types are synthesised in situ orientated within the myofibril possibly by binding to the Z body and/or by cross-bridge connections between the two filament types (Fischmann, 1967, 1970 ; Kilarski & Kozlowska, 1981). Filaments were not observed free in the cytoplasm unless in the vicinity of a potential Z body (figs.23-27). However,

it has been suggested (Fischmann, 1967) that free myofilaments may not be completly visualised when using the normal uranyl and lead stains. The observations in this study are consistent with those of Larson et al (1973) who also reported in situ synthesis of thick filaments assembled within the thin filament lattice, often in association with ribosomes and polysomes. Other studies have reported that thick filaments are polymerised to their final length in the cytoplasm, away from the thin filament and Z body complex, from where they are manipulated and inserted into the myofibril (Fischmann, 1967, 1970 ; Peng et al, 1981).

TABLE I Effects of starvation and refeeding on the capillary supply to slow muscle fibres (mean ± S.E.).

Measured Parameters	NUTRITIONAL STATUS				
Mean ± S.E.	Starved	10 days refed	20 days refed	52 days refed	Control
Number of fibres analysed	70	47	50	40	48
Number of fibres without capillary content	40	26	18	6	2
Number of capillaries associated with fibres analysed	22	16	27	36	53
Mean fibre cross sectional area (μm^2)	186±22	223±23	345±31	427±46	462±47
Mean fibre perimeter (μm)	57±3	66±5	75±4	85±6	92±6
Number of capillaries per fibre	0.3	0.3	0.5	0.9	1.1
Fibre perimeter in direct contact with capillaries [1]	1.3	1.8	3.3	4.4	4.8
[2]	4.3±0.3	5.4±0.3	5.2±0.3	5.2±0.3	5.0±0.3
Percentage of fibre perimeter in contact with capillaries [1]	2.3	2.8	4.4	5.2	5.2
[2]	7.4	8.2	6.9	6.1	5.5
Mean capillary cross-sectional area (μm^2)	11.6±1.7	9.7±1.6	12.9±1.1	14.8±1.0	12.9±2.9
Mean capillary perimeter (μm)	15.2±1.1	15.5±1.7	15.2±0.5	17.1±1.7	16.2±2.1

1. Mean values for all fibres.
2. Mean values calculated for vascularised fibres only

TABLE II Effects of starvation and refeeding on the capillary supply to fast muscle fibres (mean ± S.E.).

Measured Parameters	NUTRITIONAL STATUS				
Mean ± S.E.	Starved	10 days refed	20 days refed	52 days refed	Control
Number of fibres analysed	67	69	43	42	63
Number of fibres without capillary contact	58	55	30	26	44
Number of capillaries associated with fibres analysed	7	12	13	16	22
Mean fibre cross sectional area (μm^2)	653±87	587±77	763±76	1351±107	1306±186
Mean fibre perimeter (μm)	106±8	90±6	102±5	157±9	140±11
Number of capillaries per fibre	0.1	0.2	0.3	0.4	0.4
Fibre perimeter in direct contact with capillaries	1.0.6 1.5.5	1.0 5.5	1.9 6.2	2.6 6.7	1.5 5.1±0.8
Percentage of fibre perimeter in contact with capillaries	1.0.5 1.5.2	1.1 6.1	1.8 6.1	1.6 4.3	0.7 3.6
Mean capillary cross-sectional area (um^2)	9.9±1.7	7.5±1.5	16.1±3.0	17.1±2	11.5±2.6
Mean capillary perimeter (μm)	13.3±1.6	14.8±3.9	17.8±1.7	18.2±1.7	11.8±1.9

1. Mean values for all fibres

2. Mean values calculated for vascularised fibres only.

TABLE III Effects of starvation and refeeding on the ultrastructure of slow muscle fibres. The volume fraction (Vv) is given in % (mean ± S.E.).

Component Volume fraction (Vv) % Mean ± S.E.	NUTRITIONAL STATUS				
	Starved	10 days	20 days	52 days	Control
Myofibrils	48.9±1.9	49.5±1.3	58.9±1.8	53.7±1.1	47.0±1.0
Subsarcolemmal mitochondria	12.1±0.7	8.8±0.9	9.7±0.6	21.7±0.7	24.3±1.3
Interfibrillar mitochondria	13.0±0.8	7.9±0.9	5.5±0.4	9.9±0.8	16.6±0.9
Lipid droplets	0	5.3±1.4	0.9±0.3	8.9±0.9	7.4±0.7
Subsarcolemmal space	9.3±0.6	11.4±1.4	16.9±1.3	0.7±0.3	0
Interfibrillar space	14.9±1.3	14.0±1.6	7.2±0.8	3.5±0.4	3.4±0.4
Nuclei	1.8±0.4	1.7±0.5	1.3±0.3	1.1±0.3	0.8±0.3

TABLE IV Effects of starvation and refeeding on the ultrastructure of fast muscle fibres. The volume fraction (Vv) is given in % mean (mean ± S.E.).

Component Volume fraction (Vv) % Mean ± S.E.	NUTRITIONAL STATUS				
	Starved	10 day refed	20 day refed	52 day refed	Control
Myofibrils	61.5±2.8	64.2±1.7	74.4±0.4	78.6±1.1	84.4±0.7
Subsarcolemmal mitochondria	0.8±0.2	1.0±0.2	1.5±0.3	2.0±0.3	5.1±0.5
Interfibrillar mitochondria	0.7±0.2	0.4±0.2	0.3±0.1	1.1±0.3	0.1±0.1
Lipid droplets	0	0	0	0	0
Subsarcolemmal space	6.3±0.9	11.5±0.7	12.8±1.4	7.4±0.9	0.1±0.1
Interfibrillar space	25.9±0.9	19.1±0.7	13.7±1.2	11.3±0.6	10.1±0.4
Nuclei	0.8±0.2	1.0±0.2	1.5±0.3	2.0±0.1	0.5±0.1

Fig.1 Longitudinal section from a control fish slow muscle showing the density of mitochondria (mt), lipid (l), and glycogen (g). Bar - 1 μm.

Fig.2. Longitudinal section from starved fish slow muscle showing the total depletion of energy stores, partial degradation of myofibrils, and reduction in mitochondrial density. Bar - 1 μm.

Fig.3 Transverse section through slow muscle from a starved fish. Note the depletion of the mitochondrial populations and increase in interfibrillar space. Bar - 2 μm.

Fig.4 Transverse section through slow muscle from a 10 day refed fish showing lipid droplets (e.g. arrow) within the fibres. Bar - 5 μm.

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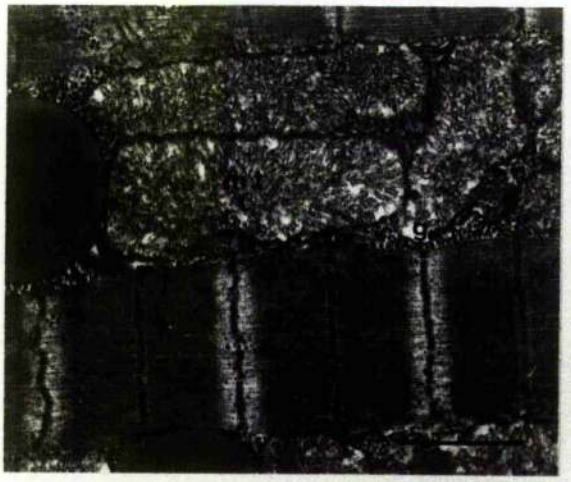

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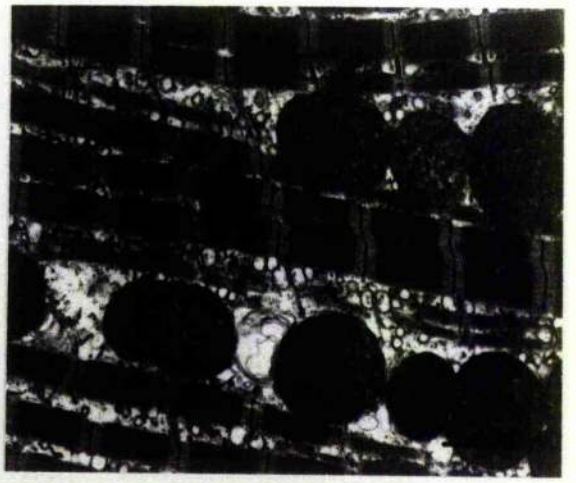

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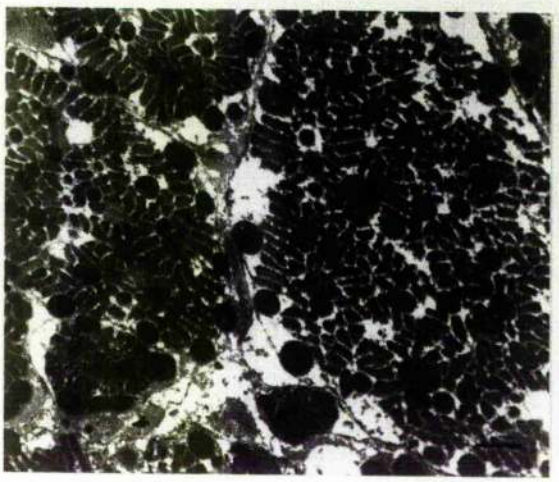

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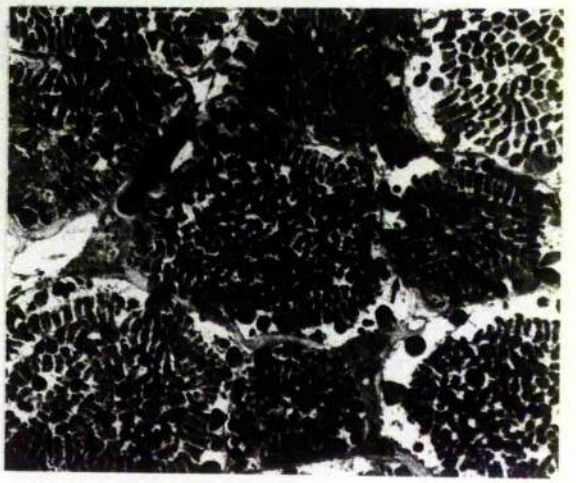

Fig.5 Transverse section through slow muscle from a 20 day refed fish. In comparison with fig.4 note the decrease in lipid droplets. The subsarcolemmal region contains massive quantities of glycogen but still few mitochonria. Bar - 5 μm.

Fig.6 Transverse section through slow muscle from a 52 day refed fish. The frequency of lipid droplets has again increased and the subsarcolemmal and interfibrillar regions are packed with mitochondria. Bar - 5 μm.

Fig 7 Longitudinal section through a slow fibre from a 20 day refed fish Note the high density of glycogen granules in the subsarcolemmal region Longitudinal section through a slow fibre from a 20 day refed fish. Also note that the fibre is sectioned at an oblique angle to the plane of the myofibrils and hence the myofibrillar ends are pointed. This fibre would therefore not be used to analyse the synthesis of new sarcomeres at the myofibrillar ends (see figs. 23-25). Bar - 1 μm.

Fig.8 Longitudinal section through a slow fibre from a 52 day refed fish. Note the restoration of the lipid droplets, mitochondria, and glycogen rossettes. Bar - 1 μm.

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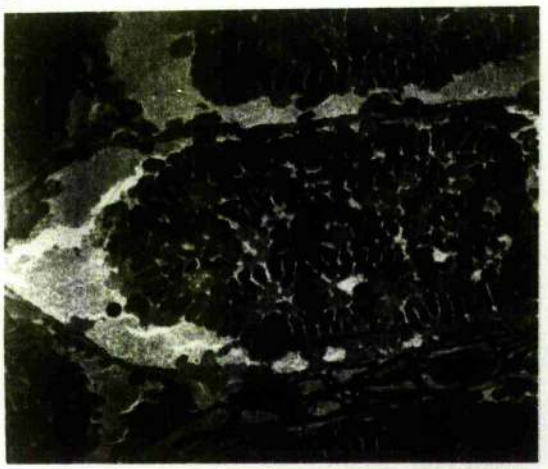

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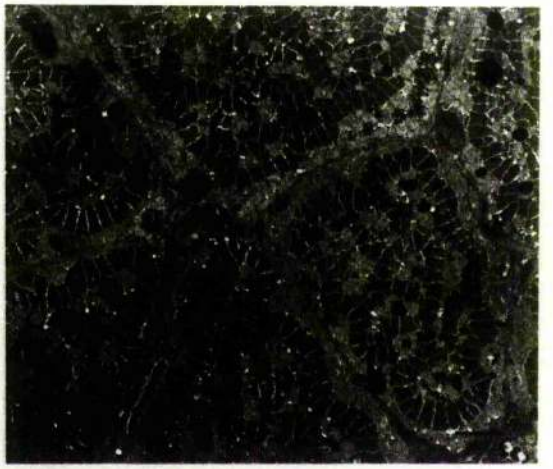

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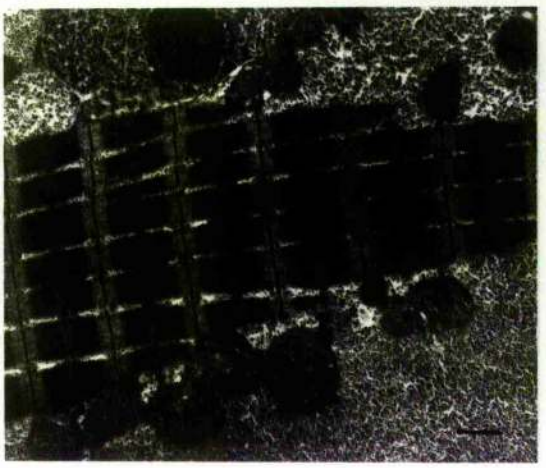

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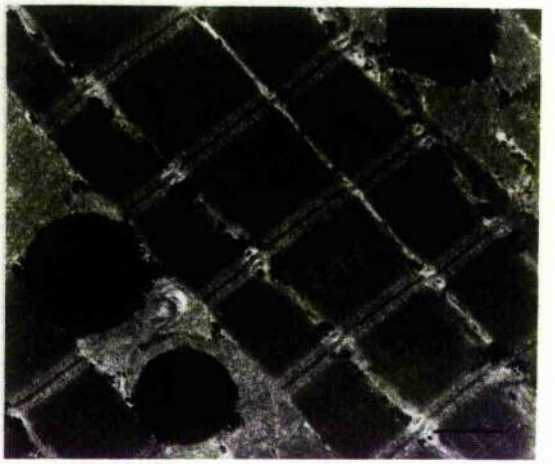

Figs.9-12 Transverse sections demonstrating the morphological changes in fast fibres during starvation and refeeding.

Fig.9 Fast fibres from a control fish. Note the high density of myofibrils, small interfibrillar space, and low density of mitochondria. Also note the characteristic elongation of the peripheralmyofibrils. Bar - 5.0 μm

Fig 10 Fast fibre from a 74 day starved fish. Note the dramatic decrease in myofibrillar cross-sectional area and the concomitant increase intermyofibrillar space. The nuclei are denude of euchromatic material. Bar - 5 0 μm

Fig.11 Fast fibres from a 10 day refed fish. The myofibrillar cross-sectional area and intermyofibrillar space appears similar to that in starved fibres. Bar - 5.0 μm.

Fig.12 Fast fibre from a 20 day refed fish. Note the increase in myofibrillar cross-sectional area and the presence of glycogen rossettes in the subsarcolemmal region. Bar - 5.0 μm.

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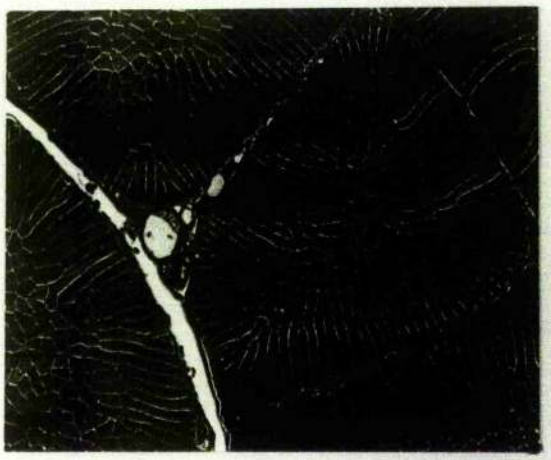

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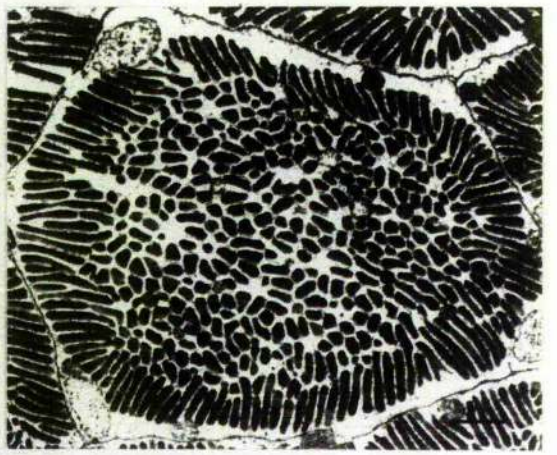

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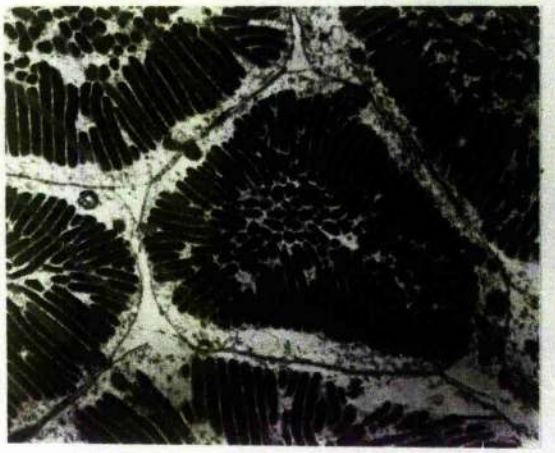

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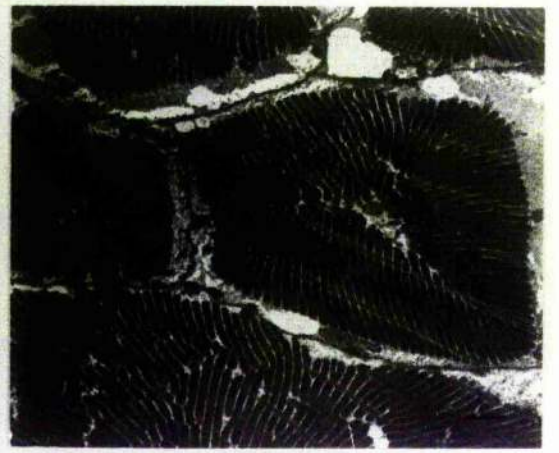


Fig.13 Mitochondria from control fish slow muscle. Note the high density of internal cristae structures. Bar - 1 μm.

Fig.14 Transverse section through fast muscle from a starved fish showing the degradation of the internal cristae structure. Bar - 1 μm.

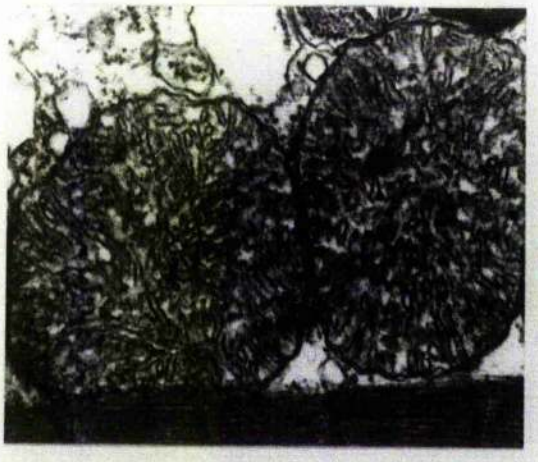
Fig.15 Regenerating mitochondria from a 10 day refed fish. Note the polysomes (arrow) within the outer membrane. Bar - 0.5 μm.

Fig.16 Longitudinal section through a slow muscle fibre from a 10 day refed fish. Note the clearing of cytoplasmic material and the presence of vesicles containing polysomes (arrows) in the subsarcolemmal region. Bar - 1 μm.

Fig.17 Partially atrophied mitochondria showing the abundance of polysomes both in association with the internal membranes and in the cytoplasm. Bar - 1 μm.

Fig.18 Subsarcolemmal vesicle from slow muscle in 10 day refed fish. Note the tubules within the outer membrane (t), which is occasionally double membraned, and the abundance of polysomes contained within the membrane/s. If these vesicles represent the de novo synthesis of mitochondria then the tubules may represent the synthesis of cristae structures. Bar - 0.5 μm.

13



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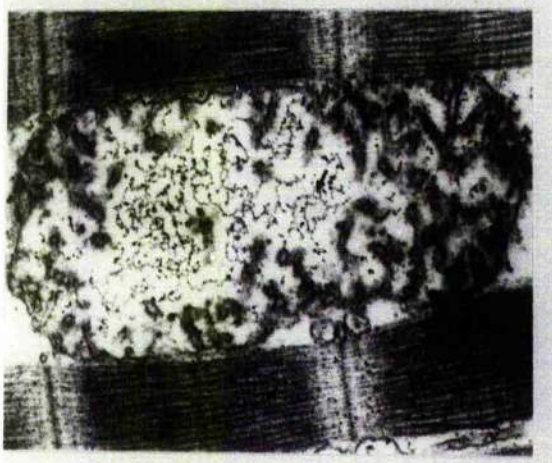

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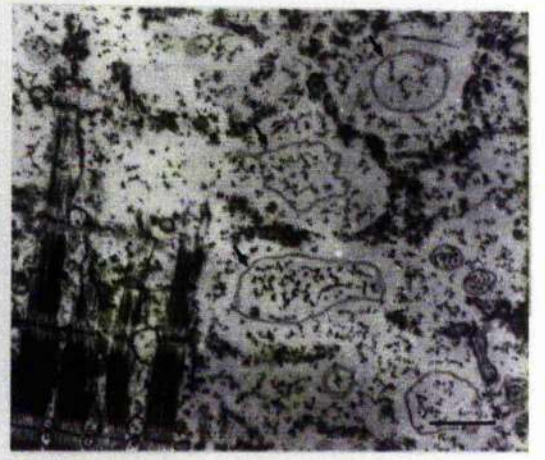

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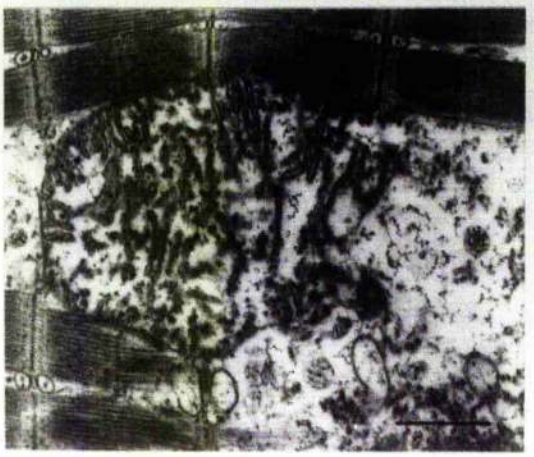

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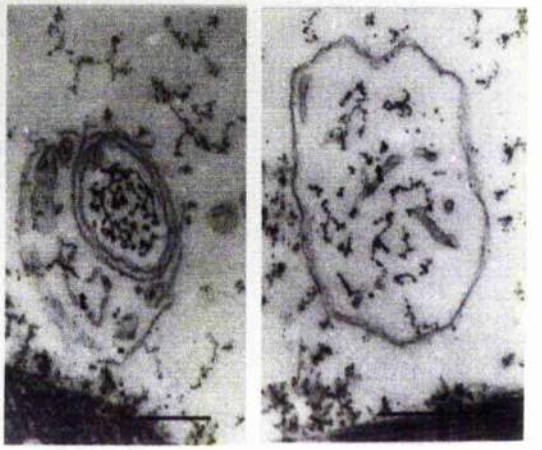


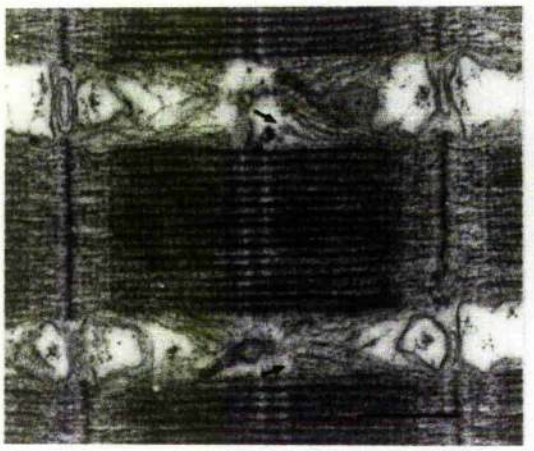
Fig.19 Longitudinal section through fast muscle from a starved fish. Note the degradation of the peripheral thick filaments and the thin filaments protruding into the interfibrillar space (arrows). Bar - 0.5 μm.

Fig.20 High powered micrograph again showing the peripheral degradation of the thick filaments. The severity of the degradation in this myofibril can be seen to be greater than that in figure 19. Note the vesicles above the M line (mv) which appear to originate from the intermediate cisternae. Also note that the peripheral thick filaments appear to be degraded from both the ends closest to the Z-lines (arrows) and from the central region. Bar - 0.5 μm.

Fig.21 Longitudinal section through fast muscle from a starved fish. In this fibre the thick filaments had been totally degraded. This phenomenon was only observed in a few fibres and was exclusively found in the fast musculature. Bar - 1 μm.

Fig.22 High powered micrograph showing one sarcomere from the fibre shown in fig.21. The arrow shows the degradation of the bare zone area of the one remaining thick filament in this sarcomere. Also note the periodicity along the thin filaments corresponding to the troponin complexes. Bar - 0.5 μm.

19



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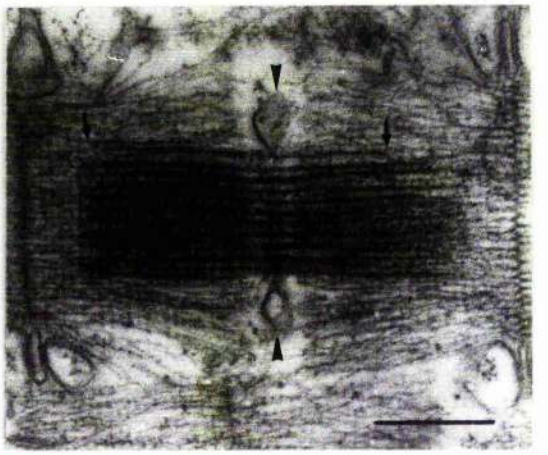

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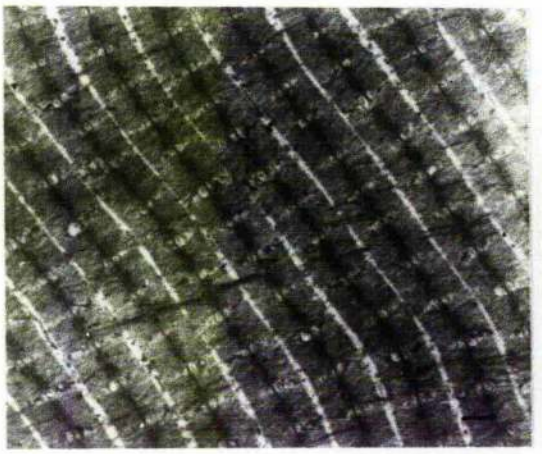

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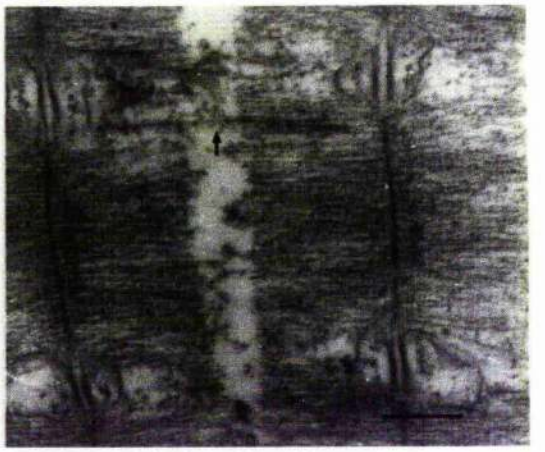
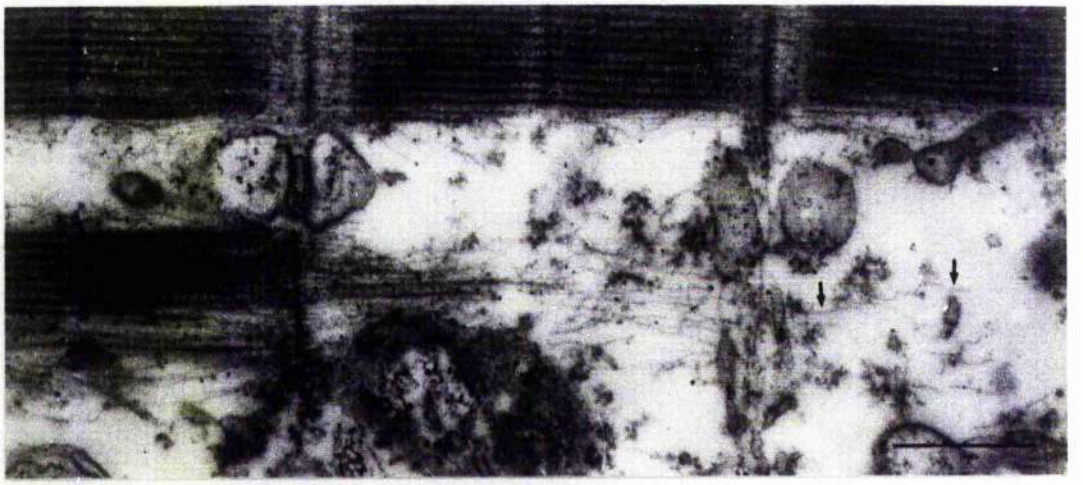


Fig.23 Longitudinal section from the slow muscle of a 10 day refed fish. Note the tubules (t) situated where the Z-disc will later form and the thin filaments apparently attached to this area (with the exception of some in the new sarcomere forming on the right (arrows)). Also note the formation of the thick filaments initially within the thin filament lattice closest to the myofibril and progressively extending across the sarcomere. Bar - 0.5 μm.

Fig.24 Longitudinal section from a 10 day refed fish (slow muscle). Note and compare the progressively increasing density of the Z-bodies (z) at the ends of the newly forming sarcomere. Thin filaments can be seen in the vicinity of the Z-bodies approximately spaced and orientated in the longitudinal axis of the myofibril. A thick filament (arrow) can be seen forming within the thin filament lattice. Bar - 0.5 μm.

Fig.25 Longitudinal section from slow muscle in a 10 day refed fish. This micrograph shows the formation of the electron dense Z-bodies (z) and tubular membrane elements before, or simultaneously with, the formation of the thin filaments. Note the abundance of polysomes (p) in the cytoplasm. Bar - 0.5 μm.

23



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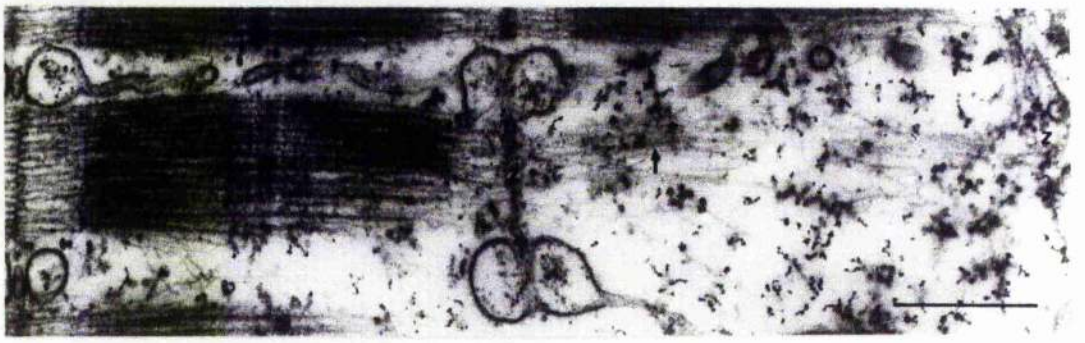

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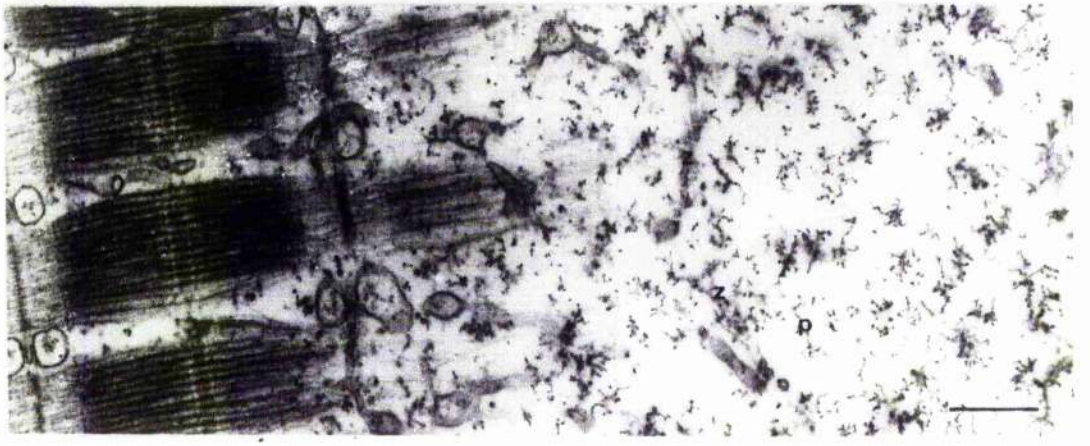

Fig.26 Sarcomere regeneration in a 10 day refed fish. Polysomes and ribosomes are evident in the cytosol and occasionally in association with the thick filaments (arrows). Tubule structures can be seen not only where the Z disc and t-tubule will later form (t), but also in the pre-M line region. Note the formation of the thick filaments initially within the thin filament lattice closest to the myofibril. The arrow heads point to the formation of thin filaments in the next sarcomere. Bar - 0 5 μm.

Fig 27 Longitudinal section through slow muscle from a 10 day refed fish. Thin filaments (arrow) can be seen forming in the end sarcomere whilst thick filaments are synthesised in the previous sarcomere. Note that these filaments have yet to be orientated in the long axis of the myofibril. This may occur when the filaments become attached to the Z-body. Again the thick filaments can be seen to form initially in the myofibril side of the new sarcomere. np - nuclear pore. Bar - 0.5 μm.

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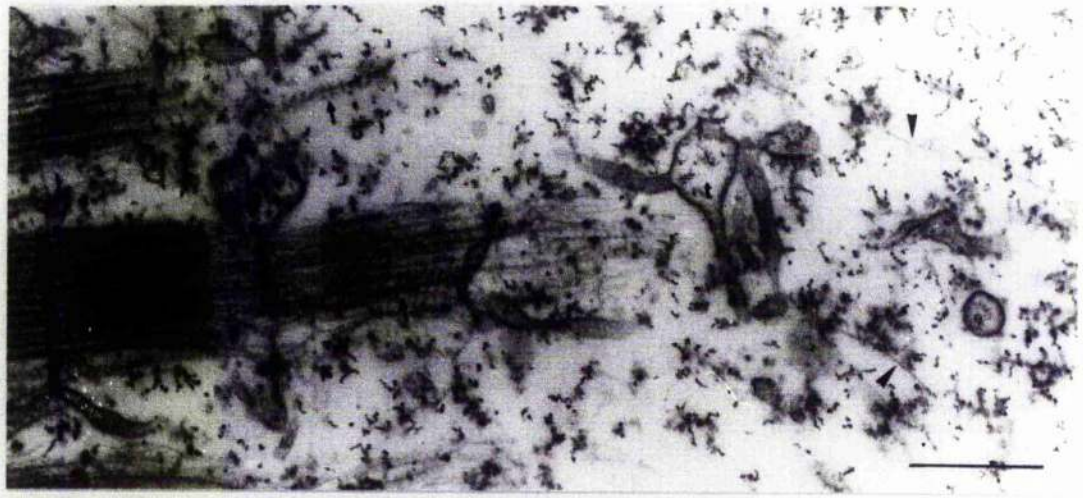

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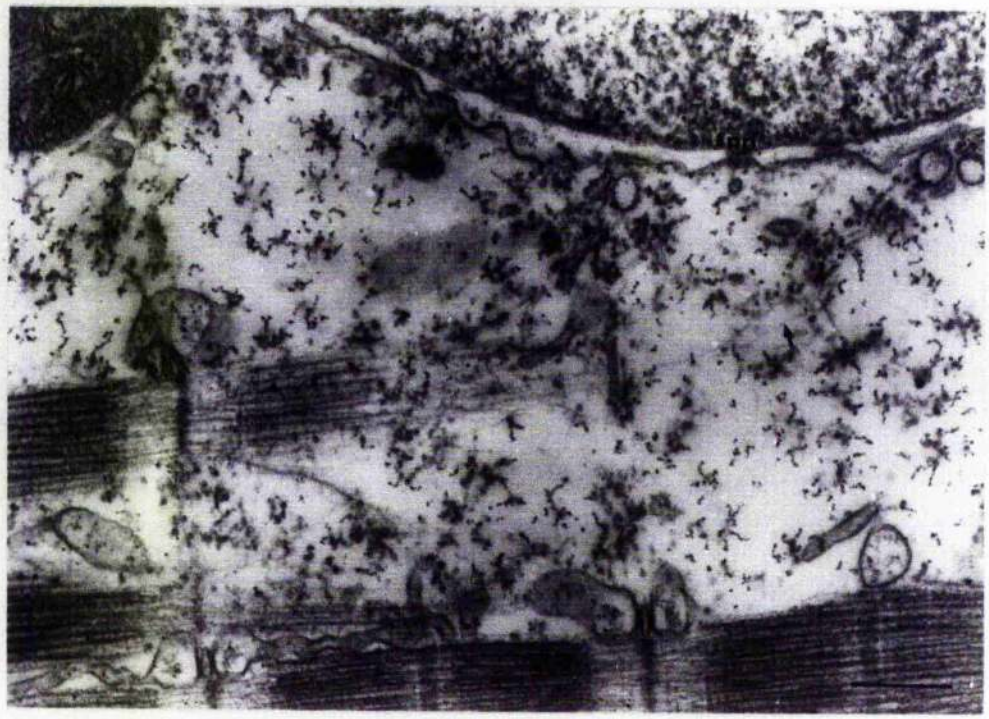
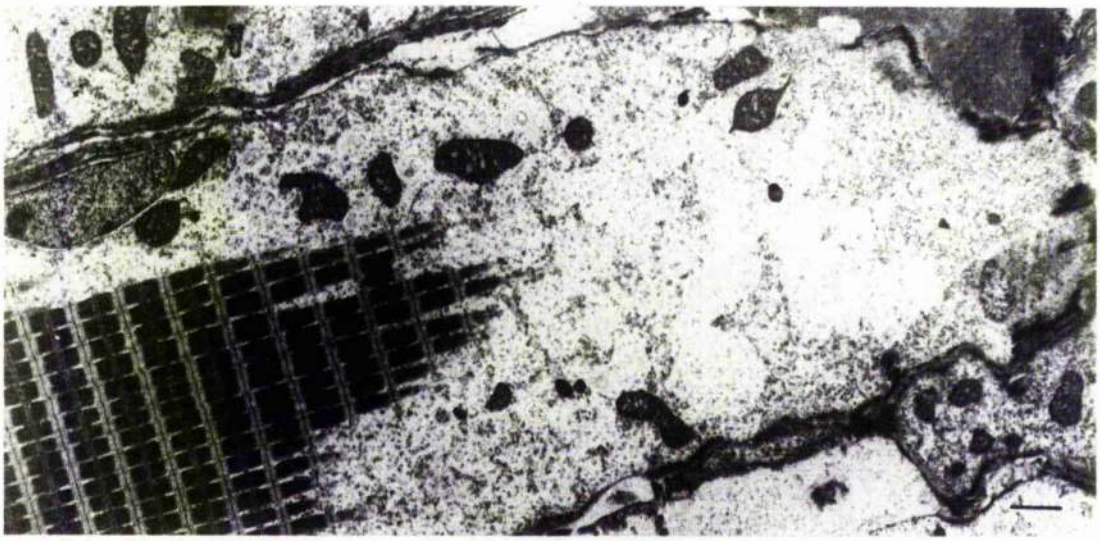


Fig.28 Longitudinal section through a slow fibre from a 10 day refed fish. This micrograph shows the large area of myofibrillar-free cytoplasm at the fibre ends and the clearing of cytoplasmic material prior to the biogenesis of the presumptive mitochondria. Bar - 2 μm.

Figs.29 Multi-membraned vesicles from a fast fibre of a starved fish. Note the electron dense material within the vesicles that probably represents residual material from the degradation of cellular proteins. Also note the swollen sarcoplasmic reticulum (s.r.). Bar - 0.1 μm.

Fig.30 Longitudinal section from 10 refed slow muscle. This micrograph demonstrates that whilst the interfibrillar space is still much elevated, in comparison with control fibres (see figs.1 & 8), the periphery of the myofibrils contain there full compliment of thick and thin filaments. Hence in contrast to starved myofibrils no thin filaments can be seen intruding into the interfibrillar space. Bar - 1 μm.

28



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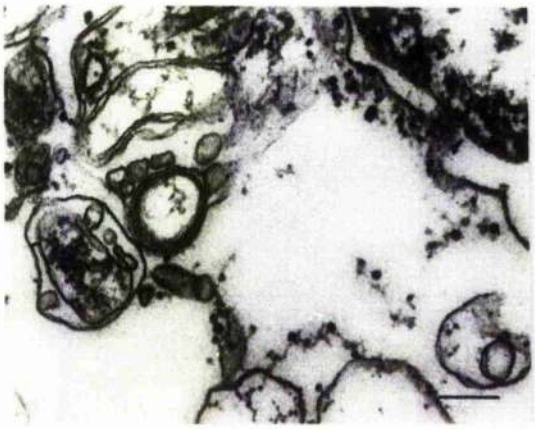

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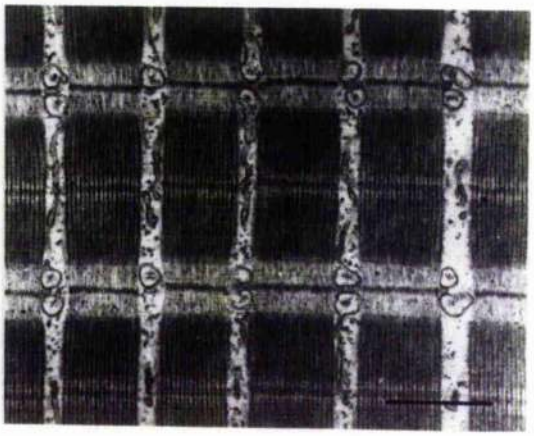

Fig.31 Nucleus from the fast muscle of a 10 day refed fish, showing the abundance of polysomes (arrow) within the nucleus and a nuclear pore (arrow head). Bar - 0.5 μm.

Fig.32 Nucleus from a 10 day refed fish showing the density of euchromatic material. Note the abundance of polysomes (p) in the nucleus, nuclear pore and surrounding cytoplasm. Bar - 1 μm.

Fig.33 Nucleus from the fast muscle of a 52 day refed fish showing the density of the nucleolus and surrounding euchromatic material. Bar - 0.5 μm.

Fig.34 Nucleus from a control fish slow fibre. Note the high density of mitochondria and the presence of lipid droplets. Bar - 1 μm.

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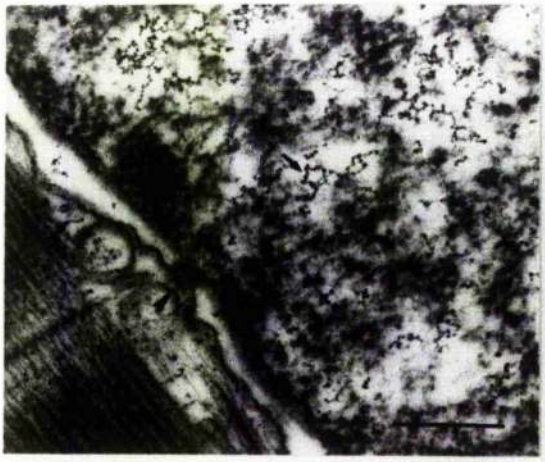

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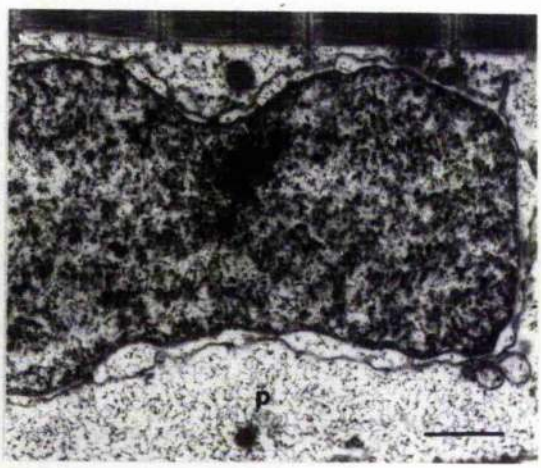

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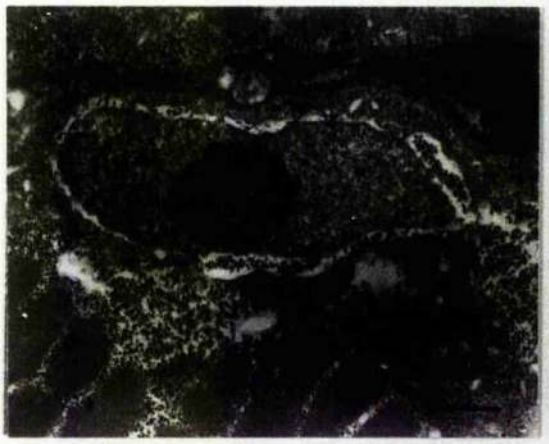

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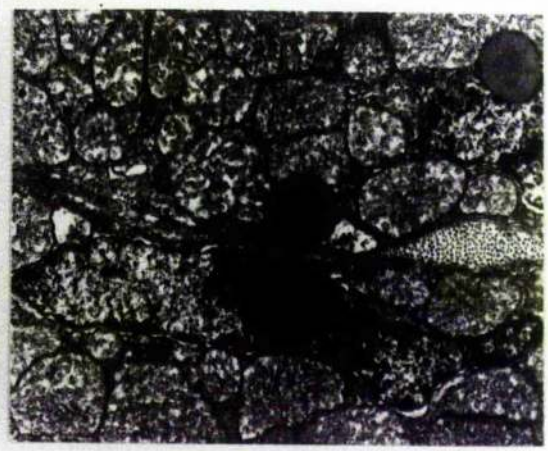


Chapter 4

The effects of starvation on the ratio of actin to myosin heavy chains

Introduction

During muscle atrophy there is a loss of myofibrillar protein. The ultrastructural observations made in chapters 2 and 3 suggest that the process of muscle atrophy involves a selective degradation of specific myofibrillar proteins (in particular the loss of peripheral thick filaments). The regulation and mechanisms involved in muscle atrophy are not fully understood, but clearly, for a net loss of protein, muscle degradation must exceed muscle synthesis. During experimental starvations in the rat it has been reported that muscle synthesis is decreased whilst degradative processes are enhanced (Millward, 1978 ; Goldberg, 1980). However it is unclear as to whether the processes involved in muscle atrophy during physiological stress, for instance starvation, result from an enhancement of the normal myofibrillar degradative processes or whether alternative pathways are involved.

Studies on the relative rates of protein turnover in mammals have shown the turnover rate of myosin to exceed that of actin (Koizumi, 1974). During muscle atrophy it might therefore be expected that myosin would degrade faster than actin, and indeed, during muscle atrophy following the denervation of rat skeletal muscle, Jakubiec-Puka et al (1982) reported that the ratio of myosin heavy chain (myosin H.C.) to actin decreased.

In this chapter the relative abundances of actin and myosin heavy chain have been studied by polyacrylamide-gel electrophoresis, in muscle from 66 day starved and control saithe.

Materials and Methods

Fish

Fish of mean length 33.9 ± 4.3 cm, were kept in holding tanks at 10°C and starved for 66 days. Control fish were kept and fed for one week prior to sampling. Each group, control and starved, consisted of four fish from which one sample of each muscle type was dissected and prepared for electrophoretic analysis.

SDS/polyacrylamide-gel electrophoresis

An adaptation of the method of Laemmeli (1970) was used for analysis of muscle fibre proteins. Slab gels (13 x 13 x 1.5 cm) were run in a Biorad Protan analytical apparatus at 5°. Two gels each containing 15 wells of 150 µl capacity, were run simultaneously at a constant current of 30 mA per gel. Gels of two different polyacrylamide concentrations were used, 7.5%, and 15%. The following stock solution were used and mixed in the quantities indicated to give the corresponding gels.

Solutions	7.5%	15%
1. 1.5 M tris-HCl (pH 8.8)	10 ml	10 ml
2. Acrylamide (29.2% acrylamide, 0 8% N'N'bis-methylene acrylamide)	10 ml	20 ml
3. Distilled water	19.5 ml	9.5 ml
4. N,N,N',N',tetramethylethylenediamine (TEMED)	0.02 ml	0.02 ml
5. sodium dodecyl sulphate (SDS) 10% (w/v)	0.4 ml	0.4 ml
6. ammonium persulphate 10% (w/v) (freshly prepared)	0.1 ml	0.1 ml

Solutions 1-4 were de-gased before the addition of 5 and 6.

Preparation and running of muscle samples

To 0.2 g of muscle tissue was added 2 ml of a treatment buffer consisting of 0.0625 M tris-HCl (pH 6.75), 2% SDS, 10% glycerol, 0.4% mercaptoethanol, and 0.001% bromophenol blue. The muscle tissue was partially crushed using a glass rod and the resulting homogenate boiled in a water bath for 3 min. A sample of 180 µg protein was found to give the optimum loading density .

Each gel was pre-run for 1 hr prior to samples being loaded. The pre-run conditions were identical to those of the run. The electrode buffer consisted of 0.25 M tris-HCl (pH 8.3), 0.192 M glycine, and 1% SDS. Gels were run for approximately 5-8 hr, depending on the acrylamide concentration, and were stopped when the tracking dye (bromophenol blue) was 1 cm from the gel buffer interface .

Gel staining and analysis

Prior to staining the gels were fixed in a solution of 40% methanol, 7% glacial acetic acid for atleast 12 hr. They were then stained for a minimum of 3 hr in a 2.5% solution of coomassiebrilliant blue, 40% methanol, 7% glacial acetic acid. Gels were destained by diffusion agianst several changes of the initial fixative. When destained adequately they were transfered to 7% glacial acetic acid for storage .

The gels were scanned using a Vitratron TLD 100 densitometric scanner at 570nm. The molecular weights of the proteins were determined by running standards of known molecular weight (66,000 to 14,200, Sigma Dalton marker VII-L) alongside the muscle samples .

Traces from the gel scans were analysed to measure the relative changes in identifiable peaks during starvation. A summagraphics digitiser was used to find the area under each protein peak. The results are expressed as the ratio of each protein to actin (i.e. area under respective peak : area under

actin peak).

Statistical analysis

A student t-test (one tailed) was performed on all results.

Results

On 7.5% polyacrylamide gels both myosin heavy chain and actin were present, with the intermediate peaks well separated (figs.1a & c). 15 % gels separated actin and the proteins of lower molecular weight (figs.1b & d).

During starvation there is a significant decrease in the fast muscle myosin H.C. : actin ratio ($p<0.01$) and the peak 1 : actin ratio ($p<0.02$) (table 1). In slow muscle there were no significant decreases, relative to actin, in any of the peaks measured (table 1).

There is a general increase in the lower molecular weight proteins (<30,000 daltons) in starved muscle from both fibre types (fig.1).

Discussion

In chapter 2 it was suggested that the initial stages of myofibrillar atrophy involve the peripheral degradation of the thick filaments. The occurence of this phenomena causes the thin filaments, still attached to the Z-disc, to protrude into the interfibrillar space. The results in this study clearly show that myosin heavy chain is degraded to a greater extent than actin in fast muscle during a 66 day starvation (table 1). Preferential thick filament degradation has also been reported in a few human muscle diseases (Yarom & Shapira, 1977 ; Yarom & Reches, 1980), experimental atrophies (Guba et al, 1977 ; Silverman et al, 1979 ; Jakubiec-Puka et al, 1981), and during metamorphosis in insect intersegmental muscle (Beaulaton & Lockshin,1977). The reasons for selective thick filament degradation are unclear. However the turnover rate of myosin has been reported to be greater than that for actin (Koizumi, 1974) and therefore a decrease in the rate of general protein

synthesis or increase in degradation could explain the relative loss of myosin observed during muscular atrophy. This hypothesis, however, may not explain the relative stability of the Z disc reported in many cases of muscular atrophy, as ∝-actinin, a component of the Z disc, has been reported to have a turnover rate that even exceeds that of myosin (Koizumi, 1974). It therefore appears that either selective changes in the individual protein turnover rates occur during atrophy or, alternatively , the removal of ∝-actinin, which accounts for less than 17 % of the Z disc protein (Robson et al, 1970), may not cause Z disc disassembly. However, investigation into the turnover rates of fish myofibrillar proteins are required to further elucidate the processes involved in this tissue during atrophy.

During starvation peak 1 was also found to decrease significantly (0.74:1 to 0.57:1, $p<0.02$) in fast muscle. Peak 2 was identified as tropomyosin by comparison with the migration of purified cod tropomyosin (courtesy of T.Crockford, prepared via the method of Smillie, 1982). It is therefore possible that peak 1, of molecular weight 40,700 daltons, is troponin T. In studies on the turnover rates of myofibrillar proteins in rabbit, it has been reported that troponin T possesses the highest turnover rate of all the myofibrillar proteins, with the exception of M-protein (Koizumi, 1974). During denervation atrophy, also in rabbit skeletal muscle, it is interesting to note that the relative abundance of troponin T decreases, as well as that of myosin H.C., tropomyosin, troponin I, and LC_3 (Guba et al, 1977). In contrast to this tropomyosin did not decrease in saithe fast or slow muscle during 66 days of starvation (peak 2). Guba et al (1977 & 1980) also reported characteristic differences in the ratios between the myofibrillar proteins depending on the cause of the muscular atrophy. It is therefore evident that the processes and mechanisms involved in myofibrillar degradation not only vary from species to species, but also the selectivity of the degradation for specific proteins, varies within a species depending on the initial cause of the atrophy.

Table I. Relative changes in the composition of proteins from fast and slow muscle during 66 days starvation in saithe. All figures are relative to actin. Data was obtained from densiometric scans of polyacrylamide gels using a summagraphics digitiser. The peak numbers correspond to those illustrated in fig. 1, Myo.H.C. - myosin heavy chain. Values given as mean ± S.E.

a) Fast muscle

Peak	mol.wt	control (n=4)	66 day starved (n=4)	
Myo H.C.	200,000	1.05 ±0.09	0.74 ±0.02	$p<0.01$
Actin	46,100	1	1	
1	40,700	0.74 ±0.04	0.57 ±0.03	$p<0.02$
2	37,400	0.56 ±0.04	0.56 ±0.03	
3	34,200	0 33 ±0.05	0.34 ±0.04	
4	17,600	0.90 ±0.07	0.81 ±0.09	
5	14,200	0.57 ±0.10	0.65 ±0.08	

b) Slow muscle

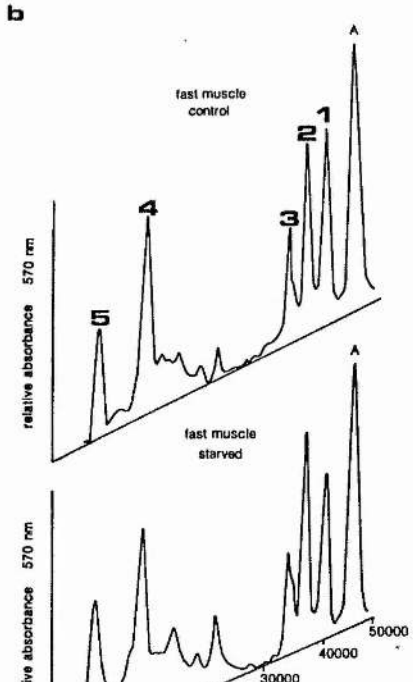
Myo H C	200,000	1.00 ±0.21	1.28 ±0.18	
Actin	46,100	1	1	
1	40,700	0 46 ±0.06	0.30 ±0.11	
2	38,000	0.29 ±0.08	0.45 ±0.21	
3	35,900	0.20 ±0.05	0.16 ±0.08	
4	18,000	0.73 ±0.18	0.71 ±0.13	
5	14,200	0.35 ±0.16	0.31 ±0.11	

Fig.1a Densiometric scans of 7.5 % polyacrylamide gel from control and starved fast muscle showing the proteins ranging from 40,000 to 200,000 +. MHC - myosin heavy chain, A - actin.

Fig.1b Densiometric scans of 15 % polyacrylamide gel from control and starved fast muscle showing the proteins ranging from 14,000 to 50,000. A - actin.

Fig.1c Densiometric scans of 7.5 % polyacrylamide gel from control and starved slow muscle showing the proteins ranging from 40,000 to 200,000 +. MHC - myosin heavy chain, A - actin.

Fig.1d Densiometric scans of 15 % polyacrylamide gel from control and starved slow muscle showing the proteins ranging from 14,000 to 50,000. A - actin.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fast muscle
control
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1
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fast muscle
starved
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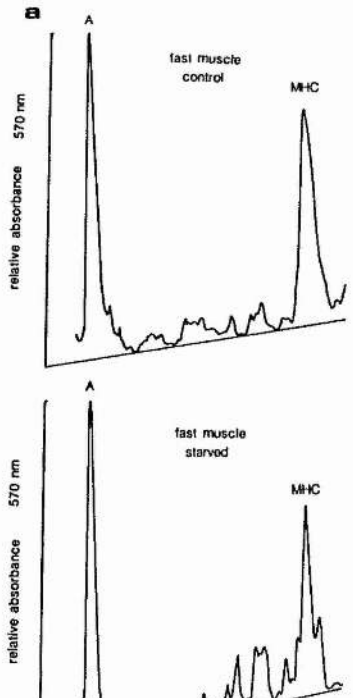
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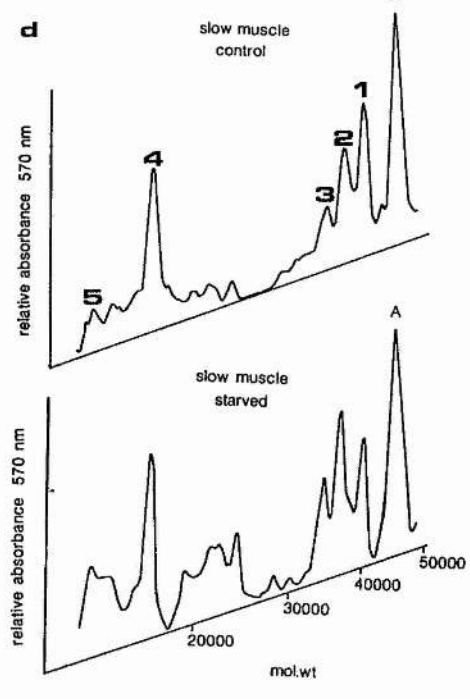
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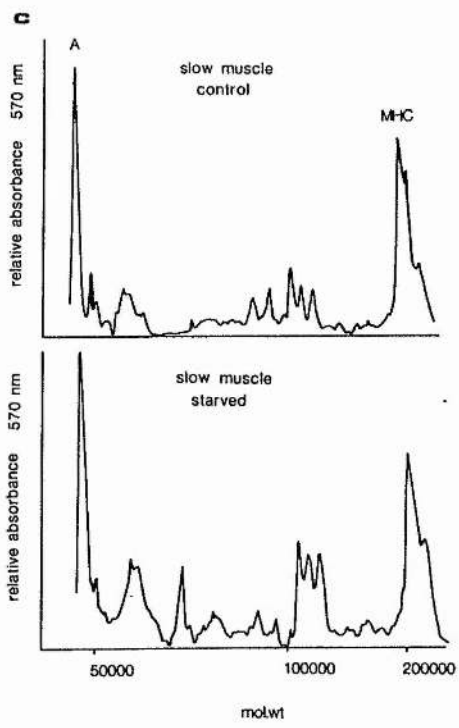
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Chapter 5

The metabolic effects of starvation followed by refeeding.

Introduction

Although there have been numerous biochemical studies concerning the effects of starvation on various species of fish, few people has studied the biochemical events occuring during refeeding. In mammalian studies, refeeding after starvation is characterised by an overshoot phenomenon, where energy stores and enzyme activities temporarily exceed those found in control animals (Tepperman & Tepperman, 1958 ; Pfeifer & Szepesi, 1974). In brook trout (Yamauchi et al, 1975), and rainbow trout (Jurss & Nicolai, 1976, cited by Black, 1983) no such phenomena were observed. However, in refeeding expeiments on cod (Black, 1983), pike (Ince & Thorpe, 1976b), and carp (Bouche et al, 1972) overshoots in the restoration of glycogen stores, liver lipid, and RNA levels were reported.

The primary sources of energy for carnivorous fish, such as saithe, are lipid and protein. Unlike mammals, carbohydrate is of lesser importance and this manifests in poor blood glucose homeostasis, no detectable glucokinase activity, low tissue hexokinase activities, and relatively low glycogen stores (Love, 1970, 1980 ; Bilinski, 1974). Lipid metabolism has been extensively studied in fish, (Bilinski, 1974 ; Driedzic & Hochachka, 1978) but there have been relatively few studies concerning protein metabolism. Increased transaminase activities have been linked to the mobilisation of muscle proteins during starvation, aswell as increased proteolytic enzyme activities (Creach & Serfaty, 1974). The activities of some key gluconeogenic enzymes (PEPCK and FDPase) have also been reported to increase in some fish (Moon & Johnston, 1980 ; Morata et al, 1982). However little is known of the processes involved in muscle protein-amino acid metabolism in fish.

In this chapter a wide range of biochemical parameters have been studied in parallel with the ultrastructural analysis of chapter 3. It is hoped that this will further elucidate the mobilisation of energy substrates during starvation and refeeding and give a better understanding of the mechanisms

involved in survival during these periods of excessive physiological stress.

Materials and methods

Two groups of fish were used in the following experiments. The first group of mean length 17.7 ± 1.0 cm were obtained from St.Andrews harbour in Nov.1980 and were the same group as used in chapter 2. The fish were kept in holding tanks at 10°C and starved for 84 days. Fish were sampled after 35 days, 63 days, and 84 days of starvation. These fish were used for preliminary experiments to determine tissue glycogen, blood glucose, tissue water, liver-somatic and condition indices during starvation.

Group II fish were used for measurements of enzyme activities, tissue glycogen, and tissue water content, and were of mean length 17.0 ± 2.2 cm. They were obtained from St.Andrews harbour in Nov.1982, and were the same group as used in chapters 3 and 6. Fish were kept in holding tanks at 10°C and starved for 74 days during the winter, the same period as a natural depletion in the wild populations. After this period of starvation the fish were fed once daily for 52 days (see chapter 3 for details). Fish were sampled after 74 days' starvation and on days 10, 20 and 54 of refeeding.

All control fish were kept and fed for three days in holding tanks, prior to experimentation.

Tissue preparation for enyzme assays

Fish were killed by a blow to the head followed by transection of the spinal cord. Samples of liver, fast and slow muscle were rapidly dissected, weighed, and kept on ice. Tissue was homogenised by 3 bursts of 20 seconds duration, with an Ultraturrex X 1010 homogeniser (4°C), in 8 vols (slow muscle ,liver) or 10 vols (fast muscle) of extraction medium. The extraction medium consisted of 50 mM Tris-HCl, 5 mM EDTA, 5mM $MgCl_2$, 1mM dithiothreitol, 75 mM sucrose, adjusted to pH 7.4 (5°C). Homogenates were centrifuged at 600

g for 10 min (5°C). Enzyme activities were measured in the supernatant.

Enzyme assays

All assays were performed at 15 °C in a Cecil CE 393 spectrophotometer, measuring the change in extinction of NADH, NADPH (340 nm), or DTNB (412 nm). Enzyme activities are expressed as μmols of substrate utilised per minute per gram dry weight of tissue. Dry weight was deteremined by dehydrating 100 mg of tissue to constant weight in a 60°C oven.

The assay conditions (given as final concentrations) were as follows;

Alanine amino transferase (Al.AT ; E.C.2.6.1.2)

Reaction:

$$\text{L-alanine} + \alpha\text{ketoglutarate} \xrightarrow{\text{Al.AT}} \text{pyruvate} + \text{glutamate}$$

$$\text{pyruvate} \xrightarrow[\text{NADH} \rightarrow \text{NAD}]{\text{lactate dehydrogenase}} \text{lactate}$$

Assay conditions:

Al.AT was assayed in a medium containing 100 mM Tris-HCl, pH 8.0 (15°C), 50 mM L-alanine, 10 mM α-ketoglutarate (αKG), 0.35 mM NADH, with excess glycerol stabilized lactate dehydrogenase (Boehringer-Mannheim Corp.), 1 ml final volume. Control assays contained the above medium with αKG omitted.

Aspartate amino transferase (Asp.AT ; E.C.2.6.1.1)

Reaction:

$$\text{L-aspartate} + \alpha\text{KG} \xrightarrow{\text{Asp.AT}} \text{oxaloacetate} + \text{L-glutamate}$$

$$\text{oxaloacetate} \xrightarrow[\text{NADH} \rightarrow \text{NAD}]{\text{malate dehydrogenase}} \text{malate}$$

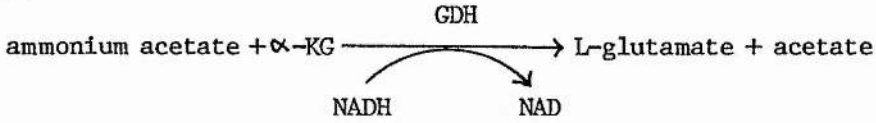
Assay conditions:

Asp.AT was assayed in a medium containing 100 mM Tris-HCl, pH 8.0 (15°C), 25 mM L-aspartate, 10 mM α-KG, 0.35 mM NADH, with excess glycerol stabilised

malate dehydrogenase, 1 ml final volume. Control assays contained the above medium with α-KG omitted

Glutamate dehydrogenase (GDH ; E.C.1.4.1.2)

Reaction:

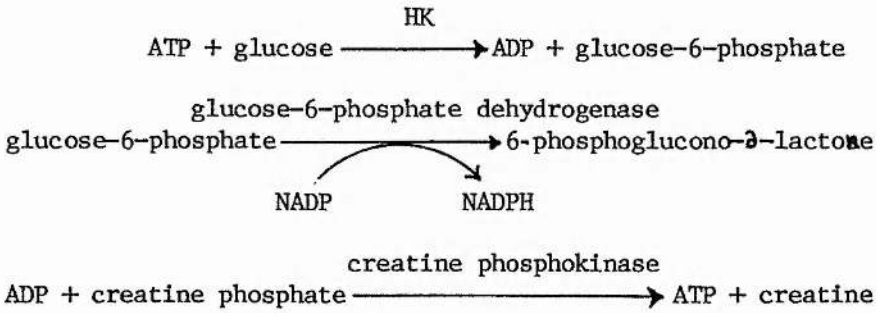


Assay conditions:

GDH was assayed in a medium containing 100 mM Tris-HCl, pH 8.0 (15°C), 10 mM α-KG, 30 mM ammonium acetate, 0.35 mM NADH, 1 ml final volume. Control assays contained the above medium with α-KG omitted.

Hexokinase (HK ; E.C.2.7.1.1)

Reaction: HK was assayed in an ATP regenerating system.

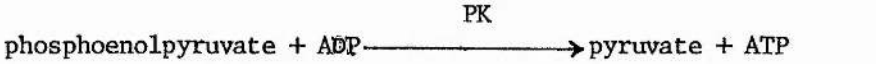


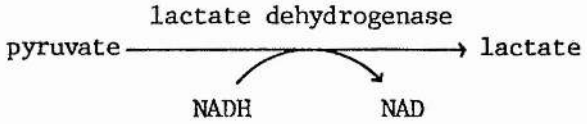
Assay conditions:

HK was assayed in a medium containing 85 mM Tris-HCl, pH 8.0 (15°C), 8 mM $MgCl_2$, 0.8 mM EDTA, 1 mM glucose, 2.5 mM ATP, 0.4 NADP,10 mM creatine phosphate, with excess creatine phosphokinase and glucose-6-phosphate dehydrogenase, 1ml final volume.Control assays contained the above medium with glucose omitted.

Pyruvate Kinase (PK ; E.C.2.7.1.40)

Reaction:



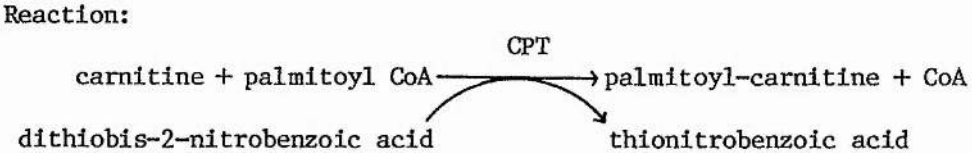


Assay conditions:

PK was assayed in a medium containing 50 mM Tris-HCl, pH 7.5 (15°C), 1.5 mM phosphoenolpyruvate (PEP), 2 mM ADP, 4 mM $MgCl_2$, 77 mM KCl, 0.32 mM NADH, and excess PK-free lactate dehydrogenase, 1 ml final volume. Control assay contained the above medium with the PEP omitted.

Carnitine palmitoyl transferase (CPT ; E.C.2.3.1.23)

Reaction:



Assay conditions:

CPT was assayed in a medium containing 60 mM Tris-HCl, pH 8.0 (15°C), 1.5 mM EDTA, 1.25 mM carnitine.0.25 mM 5-5'-dithiobis-2-nitrobenzoic acid (DTNB) in 40 mM phosphate, pH 8.0 (15°C), 50 uM palmitoyl CoA.

Control assays contained the above medium with the carnitine omitted.

Glycogen

Approximately 100 mg sample of liver, slow and fast muscle, were freeze-clamped in liquid nitrogen (-196°C). Frozen samples were digested in 1 ml 30% KOH (100°C) for 15 min and cooled. Ethanol (3.5 ml) was added and the solution re-boiled, cooled, and left overnight. The precipitate was collected and washed in 3 ml absolute ethanol. Ethanol was removed by heating in a water bath and the glycogen was hydrolysed to glucose by boiling for 2 hr in 2 ml 2N H_2SO_4 . The solution was neutralised using 2M K_2CO_3 and 100 ul aliquots removed for glucose determination. Glucose was assayed colorimetrically at 450 nm, via the following reactions, using Sigma kit no. 510 ;

$$\text{glucose} + H_2O + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconate} + H_2O_2$$

$$H_2O_2 + \text{o-dianisidine} \xrightarrow{\text{peroxidase}} \text{oxidised o-dianisidine (brown)}$$

The results are expressed as mg glycogen per 100 grams of tissue.

Blood glucose

Blood was taken from the heart immediately on death, using a heparinised hypodermic syringe. Care was taken to sample blood at the same time of day, from each fish. The glucose content of whole blood was analysed using sigma kit no.510 described above, and expressed as mg glucose per ml of whole blood.

Water content

Tissue water content was determined by dehydrating 100 mg of tissue to a constant weight in a 60°C oven.

Results

Body components

During starvation there is a significant decrease in both the condition index and liver-somatic index (table I). Almost 50% of the body weight was lost during the starvation and the condition index fell from 0.81 to 0.50 ($p<0.001$, table 1). Significant changes in the condition index did not occur until after 35 - 63 days starvation. Changes in liver texture and colour were also apparent. Upon starvation the liver changed from white / pink to dark red and became noticably less fatty. Tissue water contents increased in all tissues measured and especially in liver during the first 35 days of starvation (table I). The relatively low water content of liver in fed individuals probably represents the high content of fat in this tissue.

The body condition index increased within the first 10 days of refeeding, and after 54 days was significantly greater ($p<0.001$) than that of control fish (table II). After 20 days of refeeding the condition index was not significantly different from that observed in the control group. During refeeding tissue water contents decreased steadily. However, after 54 days, only liver had reached a value similar to that found in control fish. Both fast and slow muscle water content remained higher ($p<0.001$) than in control fish (table II). After 54 days of refeeding the fish had almost doubled in bodyweight relative to controls (table II).

Carbohydrate metabolism during starvation and refeeding

Starvation resulted in significant decreases of glycogen stores in liver (91 % decrease, $p<0.001$) and fast and slow muscle (73.2%, $p<0.01$ and 77.4 %, $p<0.001$, decrease respectively) (fig.1a). Liver glycogen decreased significantly during the first 35 days food deprivation and suffered the largest overall depletion.

Within the first 10 days of refeeding liver glycogen concentration increased, whilst fast and slow muscle stores remained unchanged or declined (fig.2). At 20 days of refeedng however, both fast and slow muscle glycogen was found to be elevated to concentrations significantly higher than those found in control fish ($p<0.001$). In fast muscle, glycogen concentrations were five times higher than those of control fish. Inbetween 20 - 54 days refeeding glycogen concentrations decreased towards control values (fig.2).

Blood glucose concentrations were maintained during the first 63 days of starvation. However, after 84 days they were found to be reduced to 30 % of control concentrations ($p<0.001$)(fig.1b).

Enzyme profiles during starvation

During starvation decreases in the regulatory enzymes of glycolysis, hexokinase and pyruvate kinase, were observed in both muscle tissues, but not

in liver (table III). Liver hexokinase increased four fold ($p<0.01$). Non-equilibrium enzymes give an indication of the maximum rates of flux through metabolic pathways. Hexokinase activity, catalysing the phosphorylation of glucose, gives a measurement of the maximum potential for aerobic glucose utilisation (Newsholme & Start, 1973). Fish liver PK (as opposed to mammalian liver PK) is not thought to be a regulatory enzyme as it displays hyperbolic kinetics with increased PEP concentrations. Therefore liver PK activity does not give a direct quantitative measure of the flux through this glycolytic step. Fish muscle pyruvate kinase, however is thought to be a non-equilibrium enzyme (Somero & Hochachka,1968 : Johnston,1975 ; Moon & Hulbert,1980), and represents the maximum flux through the anaerobic glycolytic pathway. Slow muscle maintains the highest activity of glycolytic enzymes in both control and starved tissue (table III).

Selected aminotransferase enzyme activities were measured to assess the potential of each tissue to utilise amino acids during starvation. Aspartate aminotransferase (Asp AT) was found to increase in both liver and fast and slow muscle, with the largest increase occuring in liver (table III). The highest activity of Asp AT was found in slow muscle from control and starved fish (table III). Alanine aminotransferase (Al AT) was found to increase nine fold in liver, but no increases were observed in either fast or slow muscle (table III). Glutamate dehydrogenase (GDH) activity increased in all tissues, however in liver and slow muscle the increase was not statistically significant.

Carnitine palmitoyl transferase (CPT) was measured as an indicator of the maximum rate of fatty acid oxidation (Crabtree & Newsholme, 1972b). Starvation results in an increase in liver CPT activity ($p<0.001$), whilst fast and slow muscle activities did not significantly change. Slow muscle possesses the highest potential for fatty acid oxidation in control fish. In starved fish liver activities exceed those of slow muscle

four fold

Enzyme profiles during refeeding

Muscle PK activities continued to decrease during the first 10 days of refeeding and then increased (table III). After 54 days, however, PK activities were significantly higher than those found in control fish muscle. Both slow muscle and liver HK activity decreased during the first 20 days of refeeding.

During the course of refeeding both fast and slow muscle Al AT activities were maintained at levels similar to those found in control fish (table III). Liver Al AT activity continued to increase during the first 10 days of refeeding, and then declined rapidly towards control values. Asp AT, elevated in all tissues during starvation, decreased during the first 20 days of refeeding, but only returned to control values after 54 days, in liver. Fast and slow muscle activities were found to increase again after 20 days, retaining an activity at 54 days of refeeding that was significntly elevated from that found in control fish.

The potential for fatty acid metabolism, as monitored by CPT activity, decreased in liver as refeeding progressed, until after 54 days activities were similar to control values (table III). Both fast and slow muscle CPT activity increased significantly during the first 20 days of refeeding. After this period muscle CPT activity declined.

Discussion

Carbohydrate metabolism

Fish respond to starvation with a variety of different metabolic strategies and the energy sources for survival vary among the species. "Fatty fish", such as Herring, have a high concentration of lipid in the musculature (upto 30 %) and do not break down protein even after several months of

starvation (Love,1970). In contrast, "non-fatty fish", with less extensive fat resources, rely on muscle protein catabolism to a much greater extent (Stimpson,1965 ; Moon & Johnston, 1980). In some non-fatty species it is apparent that muscle proteins are mobilised whilst carbohydrate reserves are maintained via gluconeogenesis (goldfish, Stimpson, 1965 ; carp, Wittenberger & Vitca, 1966 ; American eel, Renaud & Moon, 1980b). It has been suggested that this strategy may be employed as an adaptation to anoxia (carp can survive 2-3 months anoxia in ice-locked ponds with no detrimental effects), since carbohydrates are indispensible fuels for anaerobic metabolism (Hochachka et al, 1973). Other non-fatty species conserve muscle protein until lipid and glycogen stores are much depleted (Johnston & Goldspink, 1973 ; Malhorta & Sharma, 1981 ; Morata et al, 1982 ; Black, 1983).

During the initial 35 days of starvation in saithe, a non-fatty species, there is a large decline in the liver-somatic index, paralleled by a dramatic increase in the percentage water content of the liver (50 to 81%). Both parameters suggest a rapid depletion of liver lipid stores during the onset of starvation. It is also evident that saithe mobilise a large percentage of the hepatic glycogen store (70%) during the first days of starvation (fig.1a), as do cod (Black, 1983), trout (Morata et al, 1982), and pike (Ince & Thorpe, 1976). Just as hepatic glycogenolysis provides a temporary carbohydrate reserve for maintaining blood glucose during periods of starvation in mammals (Newsholme & Start, 1973), hepatic glycogenolysis may also be responsible for the maintenance of blood glucose during the first 63 days of starvation in saithe (fig.1b). Varying responses in blood glucose levels have been reported in fish during starvation. Blood glucose was found to decrease in European eel (Larsson & Lewander,1973), dogfish (Zammit & Newsholme,1979), trout (Morata et al, 1982), and pike (Ince & Thorpe, 1976), but remained unchanged in goldfish, even after 151 days of starvation (Chavin & Young,1970). Carp (Nagai & Ikeda, 1971), bass (Zammit & Newsholme, 1979),

and cod (Black,1983) were found to maintain blood glucose levels until the later phases of starvation where a significant drop was observed. A reduced level of blood glucose after prolonged starvation when glycogen stores have been much depleted, would suggest that gluconeogenesis is either not operating or only functioning at a low level. It also implies that during starvation fish tissue does not rely so heavily on glucose as a fuel source. It must be remembered, however, that during starvation there is a reduction in spontaneous activity aswell as a general decline in glycolytic metabolism (table III). Consequently the demand for glucose may also be expected to decrease. It has been suggested that during starvation in teleosts, fatty acids are the primary fuel supply (Larsson & Lewander, 1973 ; Zammit & Newsholme, 1979 ; Love, 1980 ; Morata et al,1982). Extensive work on blood metabolites in bass have reported increases in glycerol and non esterified fatty acids (NEFA), during starvation (Zammit & Newsholme, 1979). Black (1983), however, could find no corresponding increases in plasma fatty acid metabolites in cod during starvation. In contrast to mammals, where starvation results in a large and rapid increase in plasma free fatty acids, circulating lipids in fish are generally found to increase only after prolonged starvation and to a much lesser degree (Bilinski, 1974).

During refeeding an overcompensation in muscle glycogen concentration has also been reported in cod (Black,1983) and pike (Ince & Thorpe, 1976b). Interestingly no overcompensation was observed in the hepatic glycogen stores of either saithe (fig.2), or pike (Ince & Thorpe, 1976b). The reasons for the massive accumulation of glycogen within the muscle are not fully understood, but may result from the nutritional energy intake temporarily exceeding the energy demands of the regenerating tissue. The physiological function of the overcompensation may be an adaptation to the additional energy requirements needed for rebuilding tissue after depletion. The substrate source for muscle glycogenesis is not likely to arise from the endogenous gluconeogenesis of

dietary proteins as gluconeogenesis is not thought to occur in muscle tissue (see below). Blood glucose and/or glycerol have been suggested as the possible substrates. However, in cod, blood glucose was found to be depressed during the initial phases of refeeding and no glycerol was detected in the plasma (Black,1983). Glycerol could be derived from the hydrolysis of triglycerides which have been reported at elevated concentrations in blood from refed cod (Ross, 1978, cited by Black, 1983). Consistent with this it was reported in chapter 3 that in between 10 to 20 days refeeding, slow muscle lipid stores decreased whilst glycogen stores increased (see chapter 3). The utilisation of glycerol in the formation of glycogen, would require only one specific gluconeogenic enzyme, fructose 1,6 di-phosphatase (FDPase), which has been reported to increase in liver during refeeding in cod, but has not yet been measured in muscle during refeeding.

Enzyme profiles during starvation

Starvation in saithe was characterised by a general decline in muscle glycolytic potential whilst maintaining liver glycolyitic capacity. Maintenance of hepatic HK activity, whilst slow muscle declines to a value 70 % lower, allows the liver to posses s the highest competitive potential for blood glucose during starvation. Glucokinase, an isozyme of hexokinase not inhibited by glucose-6-phosphate, has not been found in fish. As all fish hexokinase isozymes are likely to be saturated with substrate, it appears that fish do not possessan enzyme control mechanism to deal with abnormal glucose loads. It has been suggested, however, that changes in glucose 6 phosphate and glucose 6 phosphatase could control liver glucose flux (O'Neil & Langslow, 1978 ; Moon & Johnston ; 1980). The decline in muscle glycolytic potential, coupled with the decrease in blood glucose levels during the later phases of starvation, suggests that the importance of glucose as a fuel is much diminished.

In some species of fish, and especially those that maintain carbohydrate

stores during starvation, glucose may arise from gluconeogenesis. Many studies have now reported an increase in liver gluconeogenesis during starvation (Zammit & Newsholme, 1979 ; Renaud & Moon, 1980b ; Moon & Johnston,1980 ; Morata et al, 1982). In mammals alanine and lactate are the major gluconeogenic precursors. Alanine constitutes 7-10 % of muscle amino acids, and yet accounts for 30-40 % of the amino acids released into the blood from muscle (Goldstein & Newsholme, 1976 ; Felig & Wahren, 1974). During starvation in rabbits, Odessey et al (1974) reported that alanine and glutamine released from muscle exceeded the amount that would be expected, whilst the release of the branched chain amino acids (BCAA), leucine, isoleucine, and valine, were less than expected. It was therefore proposed that an alanine - BCAA cycle exists where the synthesis of alanine, from pyruvate, involves the encorporation of the amino group released from BCAA oxidation. Branched chain amino acids are in turn degraded to glutamate and aminated to glutamine. This cycle primarily occurs in the muscle tissue and provides an important energy source for the muscle and substrates for hepatic gluconeogenesis. Amino acid metabolism in muscle involves the interconversion of many other amino acids (including aspartate and glutamate) via oxaloacetate, into alanine (Newsholme & Williams, 1978). Amino acid interconversion into alanine may require the presence of PEPCK to catalyse the conversion of oxaloacetate to pyruvate. This enzyme has been reported to increase during starvation in the slow muscle of plaice (Moon & Johnston, 1980), and therefore the same pathways may be involved in the synthesis of alanine in fish. In addition to this Moon and Johnston (unpubl. see Moon & Johnston, 1980) also reported an increase in plasma alanine during prolonged starvation in plaice. In fish glutamine is generally found to be at much lower plasma concentrations than alanine, and may not be of as much importance as in mammalian amino acid metabolism (Goldstein & Forster, 1970). Consistent with this Leech et al, (1979) reported that in spiny dogfish only alanine

(released from the muscle) was found to increase significantly during starvation (Leech et al, 1979). Studies on salmon during spawning migration, reported that glycine aswell as alanine increased in the white muscle amino acid pool, whilst aspartate, arginine, serine, and isoleucine decreased (Mommensen et al, 1980). It has also been suggested that the biosynthetic route for alanine production may differ from that in mammals, as the inhibition of PEPCK did not alter alanine production in dogfish (Leech et al,1979). Mommensen et al, 1980, suggested the importance of malic enzyme catalysing the conversion of malate to pyruvate and hence by-passing the need for PEPCK.

The role of gluconeogenesis in fish is not well understood, but is considered to be different from that in mammals. In fish, aerobic metabolism is much lower (approximately 30 times lower in plaice than in rat, Ross et al, 1967), whereas anaerobic metabolism is higher. Consequently the recycling of glucose from lactate (Cori cycle) is much more important in fish than in mammals (Renaud & Moon, 1980a). Renaud and Moon (1980b) reported that in American eel hepatocytes, the synthesis of glucose from lactate was enhanced during stavation, whilst alanine gluconeogenesis generally declined. However the involvement of amino acid gluconeogenesis was not studied during a prolonged starvation (i.e.in excess of 10 months, after which the carbohydrate stores are depleted). In other species, where carbohydrate reserves are reported to decline at the onset of starvation (for example plaice, Moon & Johnston, 1980), gluconeogenesis from muscle amino acids is thought to be of much more importance, especially during the later phases of starvation.

Interestingly in saithe, Al AT, Asp AT, and GDH all increased in liver during starvation (table III), and collectively suggest an increase in gluconeogenic potential. However it has to be understood that these enzymes may also be involved in reactions other than gluconeogenesis. The results from muscle activities infer that gluconeogenesis (atleast from alanine) does not

occur in this tissue during starvation, similar to plaice (Moon & Johnston, 1981), and cod (Black,1983). Gluconeogenesis is thought not to occur in fish or mammalian muscle due to low PEPCK and G6Pase activity, and no reported PC activity. Asp AT was found to increase in muscle aswell as liver. Increased muscle Asp AT activity may be involved in an increased flux of aspartate, via oxaloacetate, into either fatty acid metabolism, complete oxidation via Krebs cycle for aerobic energy production, or (as discussed above) interconversion into alanine. Similarly there was a small, but insignificant, increase in GDH activities in all three tissues. This may involve glutamate, via αKG, in the same processes. Increases in transaminase activities have also been reported to occur during starvation in European eel (Larsson & Lewander,1973), Japanese eel (Inui & Yokote,1974), trout (Jurss & Nicolai, 1976), carp (Zebian & Creach,1979, cited by Walton & Cowey, 1982), plaice (Moon & Johnston, 1981), and cod (Black,1983).

CPT activity was found to increase only in the liver during starvation in saithe, indicating an increased capacity for fatty acid metabolism and again exemplifying the importance of maintaining liver metabolism.

In summary saithe respond to starvation with the following general metabolic adaptations.

1. Muscle and liver energy reserves are initially depleted (as indicated by decreasing liver-somatic index and glycogen concentrations).

2. Skeletal muscle proteins are mobilised when energy stores are much depleted (indicated by the decrease in condition index, increase in muscle water content and ultrastructural events (see chapter 2)).

3. Skeletal muscle glycolytic capacity is decreased whilst liver glycolytic potential is maintained.

4. Amino acid metabolism is elevated in all tissues, but only the liver has the potential to oxidise alanine.

Enzyme profiles during refeeding

During the first 20 days of refeeding, slow muscle still maintains a reduced ability to utilise glucose (low HK activity, table III). Also during this period there is a transient accumulation of lipid within the slow muscle (chapter 3). It is therefore apparent that fatty acids are still the primary energy source during the onset of refeeding. In support of this, during the first 10 days of refeeding both fast and slow muscle glycolytic potential continues to decline (as indicated by PK activities, table III), whilst fatty acid metabolism increases (elevated CPT activities). By 54 days refeeding , however, muscle PK activities have returned to and exceeded control values, whilst CPT activity has declined to those found in control fish. These results imply that after 54 days of refeeding glucose has resumed its' function as an important fuel source for muscle tissue.

Consistent with the importance of fatty acids as the fuel source during the onset of refeeding, Black (1983) reported a dramatic increase in circulating NEFA in cod, and a similar phenomenon has been reported in rats when subjected to refeeding after a starvation (Tepperman & Tepperman, 1961).

The increase in activity of Al AT in liver continues during the initial 10 days of refeeding before dramatically declining towards control values (table III). Continuing Al AT activities may arise from the summation of alanine obtained from the break down of endogenous muscle during starvation, and the new dietary supplies. Increased hepatic gluconeogenesis (increased FDPase activity) was reported to occur in cod during refeeding (Black, 1983). Both muscle tissues in saithe remain relatively incapable of alanine deamination and their potential to utilise aspartate and glutamate declines during the first 20 days of refeeding. This later phenomena presumably indicates a decrease in endogenous aspartate and glutamate availability, created by the termination of muscle degradation, and/or a larger proportion of aspartate and glutamate, derived from dietary proteins, being used directly

to resynthesise muscle tissue. Also during this period other sources of energy are becoming available. Inbetween 20 - 52 days of refeeding Asp. AT activity again increases in muscle tissue, exceeding that found in fed fish. Cytosolic aspartate may either be incorporated into cellular proteins, interconverted into other amino acids, or injected into Krebs cycle for energy production or fatty acid synthesis. During this period (20 - 54 days) an increase in Asp AT may imply an increase in energy metabolism from aspartate. As the dietary supply of aspartate will presumably remain constant throughout refeeding this phenomenon may be caused by the completion of resynthesis of protein lost during starvation, and hence less demand on aspartate incorporation into proteins. Alternatively the elevated Asp AT activity found in 54 day refed fish may be a function of the refeeding diet.

In summary saithe respond to refeeding after starvation by the following general metabolic responses.

1. During the onset of refeeding muscle glycolytic potential remains suppressed, whilst fatty acid metabolism increases.
2. Carbohydrate stores are replenished with an overcompensation of muscle glycogen.
3. Liver metabolism (amino acid, fatty acid, and glycolytic) steadily declines towards control status, with the exception of alanine aminotransferase, which continues to increase during the onset of refeeding.
4. After 54 days of refeeding status quo has been resumed, except for an elevated condition index, increased muscle glycolytic capacity and high aspartate aminotransferase activities.

Table I. Changes in body index parameters during 84 days starvation in saithe. Mean ±SEM.

Parameter	Control (n=6)	35 day starved (n=5)	63 day starved (n=5)	84 day starved (n=5)
Body weight (g)	46.93 ±5.55	42.08 ±5.25	40.34 ±4.56	27.27** ±3.22
Body length (cm)	17.73 ±0.46	17.50 ±0.61	18.38 ±0.50	17.24 ±0.43
Tissue water (%)				
Slow muscle	76.7 ±2.2	79.7** ±1.8	82.5*** ±2.0	84.0*** ±0.9
Fast muscle	78.8 ±0.3	81.88*** ±0.8	84.5*** ±0.6	86.3*** ±0.3
Liver	50.5 ±3.4	75.0*** ±3.1	79.9*** ±1.2	81.3*** ±0.3
Condition index $\left(\frac{\text{body wt}}{\text{length}^3} \times 100\right)$	0.83 ±0.04	0.78 ±0.06	0 64** ±0.02	0 53*** ±0.02
Liver-somatic index $\left(\frac{\text{liver wt}}{\text{body wt}} \times 100\right)$	4.14 ±0.11	1.41*** ±0.08	1.23*** ±0.03	0.98*** ±0.02

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, significantly different from fed controls.

Table II. Changes in body index parameters during 74 days starvation followed by 54 days refeeding in saithe. Values given as mean ±SEM.

Parameter	Control (n=6)	starved (n=5)	10 day refed (n=5)	20 day refed (n=5)	54 day refed (n=5)
Body weight (g)	36.74 ±3.65	19.21 ±1.70	24.31 ±2.63	38.07 ±4.83	78.99 ±13.84
Body length (cm)	16.26 ±0.47	15.62 ±0.34	15.36 ±0.34	17.00 ±0.61	20.06 ±0.98
Tissue water (%)					
Slow muscle	77.9 ±0.3	84.0*** ±0.5	85.4*** ±1.0	78.2*** ±0.7	79.7** ±0.7
Fast muscle	79.4 ±0.3	86.1*** ±0.4	86.3*** ±0.4	81.3*** ±0.4	80.8** ±0.3
Liver	50.5 ±2.6	81.3*** ±0.7	79.0*** ±0.3	62.5** ±1.6	38.1 ±2.4
Condition index $\left(\frac{\text{body wt} \times 100}{\text{length}^3}\right)$	0.81 ±0.05	0 50*** ±0.02	0.66*** ±0.04	0.76*** ±0.05	0.94* ±0.05

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, significantly different from fed controls.

Table III. Activities (μmoles/min/g.dry wt) of selected enzymes during starvation followed by refeeding. Values given as mean ± SEM.

Enzyme	Tissue	Control (n=6)	Starved (n=5)	10 day refed (n=5)	20 day refed (n=5)	54 day refed (n=5)
Hexokinase						
	Slow muscle	2.53 ±0.27	0.78*** ±0.11	0.74** ±0.17	0.64*** ±0.15	/
	Fast muscle	0.18 ±0.07	0.01* ±0.01	0.11 ±0.06	0.14 ±0.04	/
	Liver	0.64 ±0.28	2.34** ±0.21	1.08* ±0.15	0.38 ±0.35	/
Pyruvate Kinase						
	Slow muscle	153.21 ±8.28	72.43*** ±4.27	48.22* ±17.96	89.1** ±6.32	202.71** ±24.02
	Fast muscle	101.07 ±7.10	55.75** ±2.4	21.40** ±9.94	54.75* ±5.54	186.28** ±59.56
	Liver	7.19 ±0.79	9.06* ±1.59	5.29 ±1.50	10.39* ±1.02	4.61 ±1.39
Carnitine palmitoyl transferase						
	Slow muscle	1.54 ±0.50	1.26 ±0.20	/	2.24** ±0.38	0.908 ±0.05
	Fast muscle	0.04 ±0.03	0.03 ±0.03	/	0.27** ±0.23	0.14** ±0.05
	Liver	0.64 ±0.10	4.26*** ±1.45	/	3.63*** ±0.59	0.51 ±0.15

table III (contd.)

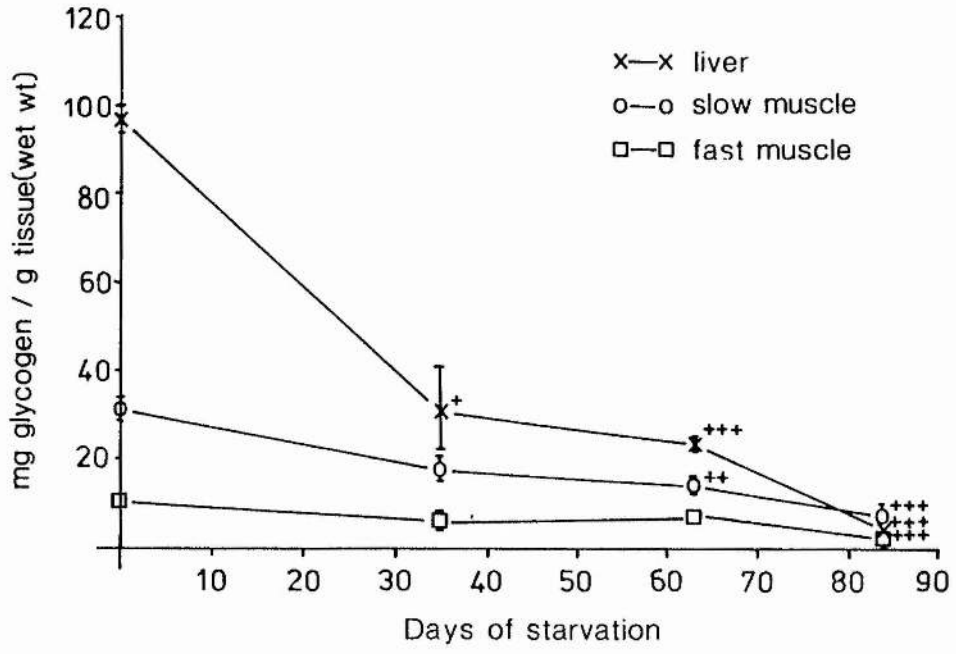
Enzyme	Tissue	Control (n=6)	Starved (n=5)	10 day refed (n=5)	20 day refed (n=5)	52 day refed (n=5)
Alanine amino-transferase						
	Slow muscle	6.23 ±1.16	4.37 ±1.02	3.66 ±1.53	2.67 ±0.78	4.87 ±1.18
	Fast muscle	4.36 ±0.08	4.30 ±0.29	4.18 ±0.68	2.45 ±0.29	2.53 ±1.26
	Liver	9.11 ±1.12	80.04*** ±3.35	97.13*** ±4.79	37.20*** ±0.89	12.11* ±1.00
Aspartate amino-transferase						
	Slow muscle	151.46 ±28.08	261.49** ±9.06	199.25* ±8.40	162.02 ±14.64	207.32* ±58.76
	Fast muscle	20.52 ±1.31	31.3*** ±3.09	30.11*** ±3.2	25.90* ±5.64	44.53*** ±3.38
	Liver	62.12 ±8.23	158.74*** ±9.28	151.46** ±16.7	87.47* ±4.79	32.19 ±2.38
Glutamate dehydrogenase						
	Slow muscle	0.72 ±0.54	2.18 ±0.414	N.D.	N.D.	N.D.
	Fast muscle	N.D.	0.06* ±0.05	0.10*** ±0.03	/	0.03 ±0.03
	Liver	0.06 ±0.02	2.72 ±0.67	1.88 ±0.14	/	0.46 ±0.09

* $p<0.05$
** $p<0.01$
*** $p<0.001$ Significantly different from fed controls.

Fig.1a Graph showing tissue glycogen concentrations during 84 days of starvation. Values given are mean ± S.E.M. + $p<0.05$, ++ $p<0.01$, +++ $p<0.001$

Fig.1b Graph showing blood glucose concentration during 84 days of starvation. Values given are mean ± S.E.M. +++ $p<0.001$.

a tissue glycogen



b blood glucose

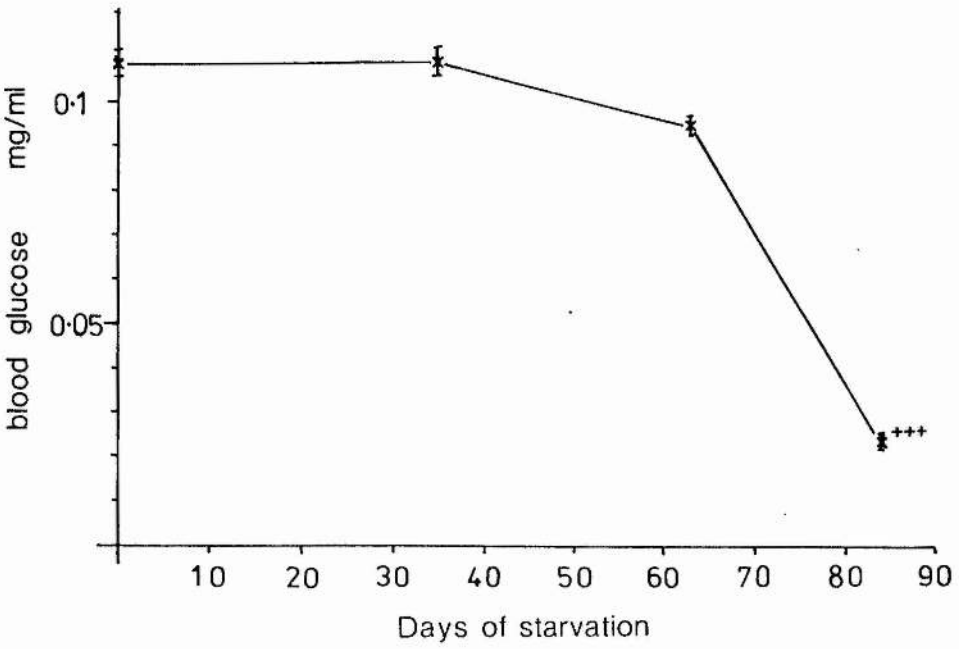
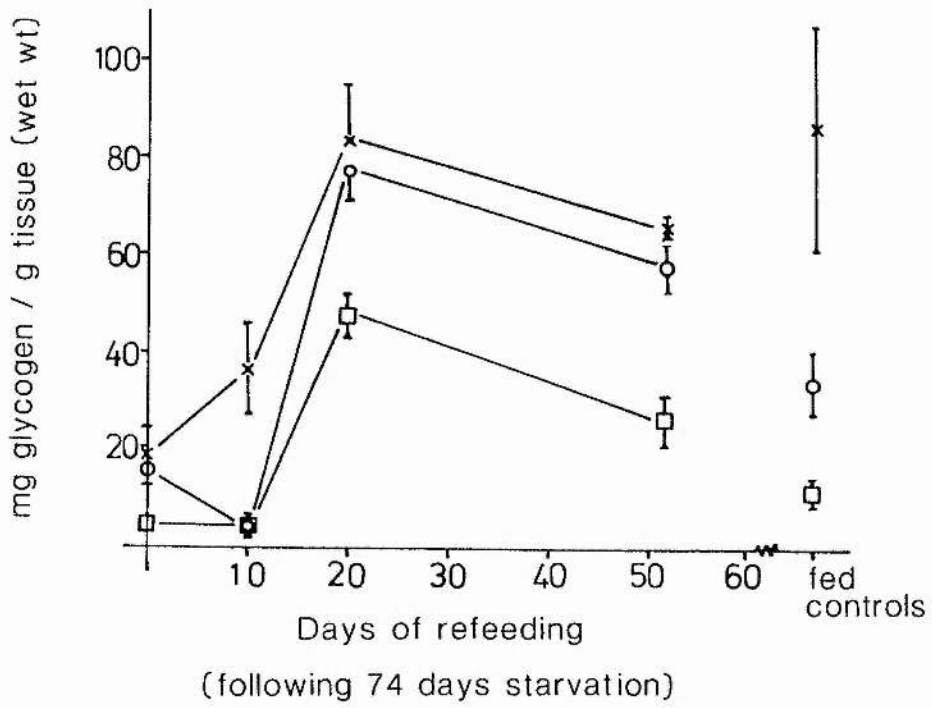


Fig.2 Graph showing tissue glycogen concentrations during 54 days refeeding following a 74 day starvation. Values given are Mean ± S.E.M.

tissue glycogen



×—× liver
o—o slow muscle
□—□ fast muscle

Chapter 6

Lysosomal enzyme activities in muscle following starvation and refeeding.

Introduction

This chapter is concerned with the processes involved in the breakdown of muscle cells and in particular the lysosome.

De Duve et al (1955) were the first to demonstrate the existence of a separate intracellular organelle, termed the lysosome, that contained a wide variety of acid hydrolases capable of degrading cellular substrates. Figure 1 summarises the hypothesised formation and dynamics of the lysosomal system in a normal eukaryotic cell. The synthesis and packaging of enzymes into primary lysosomes is not yet fully understood, but one major route is thought to be from the rough endoplasmic reticulum to the smooth endoplasmic reticulum and then containment into vesicles either in association with the golgi apparatus or in a specialised region of the smooth endoplasmic reticulum (Dean, 1978). In certain cell types some lysosomal enzymes may be of extracellular origin and have specific receptor-mediated sites for pinocytosis on the plasma membrane (Stahl et al 1978).

Much evidence suggests that the morphology of the lysosomal system is not the same for all cell types, and since muscle does not have much need for an absorptive, secretory or excretory function under normal conditions, it is thought that the requirement for a lysosomal system is much reduced. It is because of the relatively low activity of muscle lysosomal enzymes, that it was not until 1970 that Canonico & Bird first conclusively demonstrated the presence of an indigenous population of lysosomes in skeletal muscle tissue. They postulated that in muscle, lysosomes may exist as part of the sarcoplasmic reticulum (s.r.) and not as separate entities. Evidence for this was first based on zonal centrifuge studies (Stauber & Bird, 1974) and the observation that the sarcoplasmic reticulum appears to be analogous to smooth endoplasmic reticulum of other cell types. The involvement of the lysosomal system in normal muscle protein turnover is unclear. However, many studies

have reported massive increases in the lysosomal-vacuolar system, paralleled by increases in acid hydrolase activities, during such physiological and pathological conditions as hereditary muscular dystrophy, vitamin E deficiency (Zalkin et al, 1962 ; Li, 1980), vacuolar myopathies (Engel,A.G. 1973), denervation (Pollack & Bird, 1968 ; Schiaffino & Hanzlikova, 1972 ; Maskrey et al, 1977 ; Bass et al, 1984), protamine administration (Libelius & Lundquist, 1978), endurance exercise (Vihko et al, 1978), and starvation (Canonico & Bird, 1970 ; Li et al, 1979). Cytochemical studies have located many acid hydrolases within the sarcoplasmic reticulum (Fischmann, 1964 ; Schiffino & Hanzlikova, 1970 ; Topping & Travis, 1974 ; Hoffstein et al, 1975). Bird & Schwartz (1977) postulated that small quantities of acid hydrolase could be stored within the s.r. under normal cellular conditions and could be used in the formation of autophagic vacuoles during localised cellular degeneration. Engel,A.G.(1973) has suggested that the t-tubule system may also be involved in the formation of secondary lysosomes in some pathological conditions.

Previous studies on proteolytic enzymes found in muscle lysosomes, have so far found four proteinases that are capable of degrading myofibrillar proteins ; cathepsins B1, D, H and L (Schwartz & Bird, 1977, Bird et al, 1980 ; Okitani et al, 1980, 1981 ; Matsukura et al, 1984)). In addition to lysosomes, other muscle proteolytic systems may involve cytosolic neutral proteinases or alkaline proteinases. A Ca^{++}activated neutral proteinase capable of degrading myofibrillar proteins has been purified and characterised by Busch et al (1972) and Dayton et al (1976 & 1981). But although both neutral and acid proteinases are known to be capable of degrading myofibrillar protein, their mode of action in vivo is unclear. Many studies have also reported alkaline or "serine" proteinases capable of degrading native myosin, actin, troponin, and tropomyosin, however these are not thought to originate in muscle tissue and their involvement in muscular atrophy is unclear (see Bird et al, 1980 for review).

Only a limited amount of research has been dedicated to fish muscle proteinases and previous studies have identified and purified all three classes (acid, neutral, and alkaline) of proteinase. The majority of studies on fish muscle proteolytic enzymes have been concerned with acid proteinases, and in particular cathepsin D. Cathepsin D has been purified from carp (Makinodan et al, 1982a), winter flounder (Pseudopleuronectes americanus, Reddi et al, 1972), leather jacket (Tilapia mossambica, Doke et al, 1980), and cod (Seibert et al, 1965, see Makinodan et al, 1982a), and has a variable, but species specific, molecular weight ranging from 32,000 to 41,000 dalton. Carp cathepsin D has been reported to hydrolyse native myofibrils optimally at pH 3.2 - 4.0, but have no activity above pH 6.0 (Makinodan et al, 1982a). Other cathepsins (A, B, and C) have also been reported in carp muscle but their capacity to hydrolyse myofibrils has not been investigated (Makinodan & Ikeda, 1971).

There are only a few reports identifying neutral proteinases in fish muscle (Makinodan et al, 1979). Unlike the Ca^{++} activated neutral proteinase reported by Busch et al (1972), Makinodan et al (1979) found a neutral proteinase in carp muscle that was only slightly activated by Mg^{++}, and not influenced at all by Ca^{++}. Carp neutral proteinase was also reported by the same authors to have no activity against myofibrils or actomyosin and therefore its precise physiological function is unclear. Much work has been dedicated to alkaline proteinases in connection with food quality and texture (especially the himodori phenomenon during kamaboko production, see Iwata et al, 1979), however this proteinase, optimally active at around pH 8.0, has little activity below 55°C and it is therefore difficult to postulate any physiological role in vivo (Makinodan & Ikeda, 1977)

This chapter investigates the involvement of the lysosomal system in muscle degradation and regeneration, in saithe. The activities of a broad spectrum of lysosomal enzymes have been monitored both during starvation and

refeeding. Using differential centrifugation, lysosome-rich fractions were obtained from starved and control muscle. The proteolytic capabilities of the lysosome-rich fractions were investigated by incubations with native myofibrils, and electrophoretic analysis of the products. Fractions enriched in lysosomal activity were prepared by differential centrifugation and their ultrastructure investigated in an attempt to identify the organelles possessing acid hydrolase activity. In order to identify the organelles containing hydrolytic activity (lysosomes) in atrophic fibers, cytochemical techniques were employed to localise the activities of the lysosomal enzymes acid phosphatase and aryl sulphatase.

Methods

Fish

Two groups of fish were used for the experiments in this chapter. Group I were the same fish as used in chapter 3, obtained from St.Andrews harbour in Nov 1982 and of mean length 17.0 $\pm$ 2.2 cm. These fish were kept in holding tanks at 10^{o}C and starved for 74 days during the winter, the same period as a natural depletion in the wild populations. After this period of starvation the fish were fed once daily for 52 days (see chapter 3 for details). Fish were sampled after 74 days of starvation and on days 10, 20 and 52 of refeeding. Control fish were kept and fed for three days in holding tanks prior to experimentation.

The second group of fish (mean length 33.9 $\pm$ 4.3 cm), obtained from St.Andrews bay (Nov.1983), were kept in holding tanks at 10^{o}C, and starved for 66 days, again during the same period as a natural depletion in the wild populations. Fish were sampled after 66 days. Control fish were kept and fed for 1 week prior to experimentation.

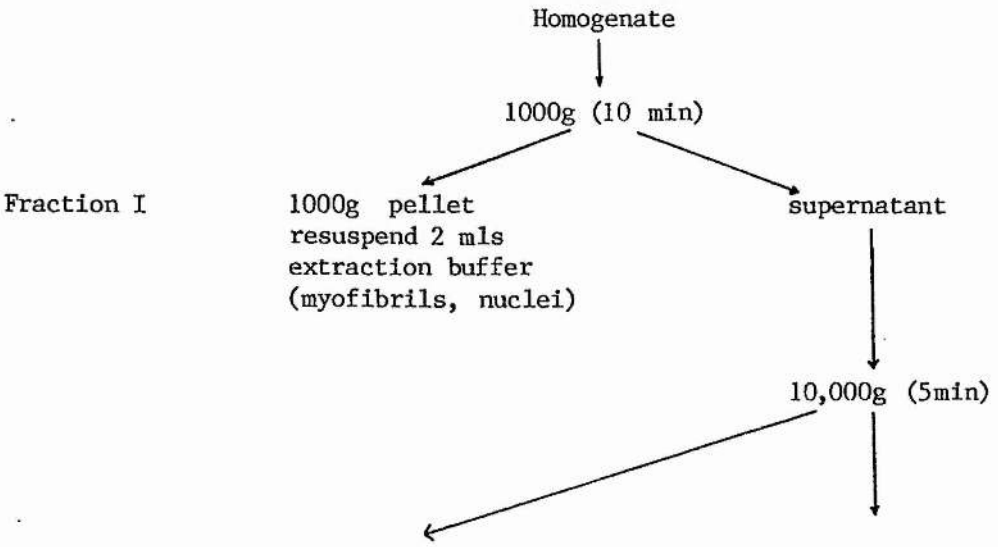
Tissue preparation

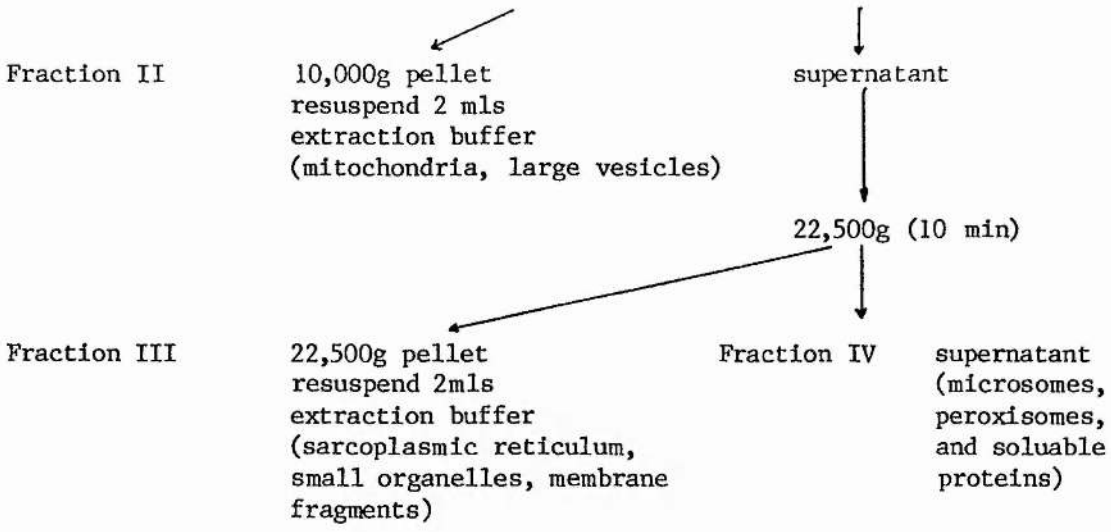
Total lysosomal enzyme activities

Both fast (approx.1.0 g dissected from myomeres 20 - 30) and slow muscle (approx. 0.3 g dissected from the posterior section of the lateral line) were homogenised in x 10 w/v of extraction buffer, consisting of 50 mM tris-HCl (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 5 mM EDTA, 1 mM dithiothreitol, 75 mM sucrose, 225 mM mannitol, pH 7.4 (5°C). Tissue was homogenised using an ultra-turrax X1020, at 3500 revs/min, with three bursts each of 20 seconds duration. Samples were kept on ice at all times. Homogenates were frozen and thawed twice, before centrifuging at 1000g for 10 min at 5°C (MSE Coolspin). Lysosomal enzyme activities were measured in the supernatant.

Subcellular fractionation of lysosomal enzyme activities.

Both fast (approx.1.0g) and slow tissues (approx.0.3g) were homogenised in x 7.5 w/v of an extraction buffer consisting of 10 mM tris-HCl (pH 7.4, 5°C), 20mM KCl, and 250mM sucrose. Tissue was homogenised by 2 x 2 sec. bursts with the ultra-turrax (3500 rpm), followed by two strokes at 1000 rpm with a glass\teflon homogeniser. The resulting homogenate was fractionated by differential centrifugation (5°C) as outlined below.





Each fraction was frozen and thawed twice prior to enzyme assays. Preliminary experiments were undertaken to ensure that two freeze-thaw routines were sufficient to break open the lysosomes. Triton X 100 was not used for this purpose due to interference with the protein assay.

Protein was determined according to Lowry (1951).

Enzyme assays

Activities of the lysosomal enzymes arylsulphatase, acid phosphatase, β glucuronidase, acid ribonuclease, and acid proteinase were measured during starvation and refeeding in fish from both group I and group II.

Preliminary experiments were undertaken to find the pH for optimal activity for each enzyme. All assays were subsequently performed at 25°C at their respective optimal pH in triplicate. Reaction media are given as final concentrations.

Acid proteinase (EC 3.4.23)

Acid proteinase (endopeptidase) activity was monitored by following the breakdown of bovine haemoglobin, measuring the resulting aromatic amino acids as reaction products, with Folins-Ciocalteu reagent (Canonico & Bird 1970).

Haemoglobin substrate was prepared by dialysing a solution of 2% haemoglobin in 0.05 M Na-acetate (pH 3.8, 5°C), against the same buffer for 48 hr. The resulting substrate was filtered before use.

The reaction medium consisted of 0.5 % haemoglobin substrate, 75 mM Na-acetate buffer (pH 3.8,25°C), and 125 µl enzyme supernatant (final volume 0.5 ml). Following a 24 hr incubation with substrate the reaction was stopped with 1 ml cold 5 % trichloroacetic acid (TCA). The tubes remained at 4°C for 30 min and were centrifuged at 1000g to remove the precipitated protein. Aromatic amino acids were measured in 0.5 ml of the supernatant using Folin-Ciocalteu reagent (Canonico & Bird, 1970). The optical density of the resulting solution was measured at 660 nm on a Cecil CE 393 spectrophotometer.

Standards of tyrosine were taken through the same procedure (excluding the incubation) and the results are expressed as nmoles of tyrosine equivalent per mg of protein per hour following the subtraction of appropriate blanks.

Acid Phosphatase (EC 3.1.3.2)

Acid Phosphatase, a hydrolase acting on phosphoric monoesters, was monitored by measuring the breakdown of p-nitrophenylphosphate, measuring nitrophenol as the reaction product.

The incubation medium consisted of 4 mM p-nitrophenylphosphate, 0.11 M Na acetate (pH 5.3, 25°C),and 125 µl enzyme supernatant (0.5 ml total volume). The reaction was initiated by the addition of substrate, incubated for 3/4 hr and stopped by the addition of 1.5 mls of 1.5 N NaOH. The optical density of the resulting solution was measured at 420 nm. Standards of p-nitrophenol were run through the experimental procedure (excluding the incubation) and the results expressed in µmoles p-nitrophenol equivalent per mg of protein per hour following the subtraction of appropriate blanks

Aryl Sulphatase (EC.3.1.6.1)

Aryl sulphatase, a hydrolase acting on sulphuric esters, was monitored by measuring the breakdown of nitrocatechol sulphate, measuring nitrocatechol as the reaction product.

The incubation medium consisted of 2.5 mM nitrocatechol sulphate, 80 mM

Na acetate (pH 5.1, 25°C), and 125 µl enzyme supernatant (0.5 ml final volume). The reaction was initiated by the addition of substrate, incubated for 24 hr and was stopped by the addition of 0.5 mls 5% TCA. The resulting solution was centrifuged at 1000g for 5 min to remove any precipitate. A 0.5 ml aliquot was added to 0.75 mls of 2.5 N NaOH and the optical density determined at 515 nm.

Standards of 4-nitrocatechol were run through the same experimental procedure (excluding the incubation) and the results expressed as µmoles 4-nitrocatechol equivalent per mg of protein per hour following the subtraction of appropriate blanks.

β-glucuronidase (EC 3.2.1.31)

β-glucuronidase, a hydrolase acting on o-glycosyl compounds, was monitored by measuring the breakdown of phenolphthalein β-glucuronide, measuring phenolphthalein as the reaction product (Gianetto and de Duve,1955).

The incubation medium consisted of 0.25 mM phenolphthalein β-glucuronide, 0.8 M Na acetate (pH 4.8,25°C)), and 125 µl enzyme supernatant (0.5 ml final volume). The reaction was initiated by the addition of substrate, incubated for 24 hr and was stopped by the addition of 1.5 mls of a solution containing 0.133 M glycine, 0.067 M NaCl, and 0.083 M Na_2CO_3 (pH 10.7,25°C). The resulting solution was centrifuged at 1000g for 5 min and the optical density determined at 540 nm.

Standards of phenolphthalein were run through the same procedure (except the incubation) and the results expressed as µmoles phenolphthalein per mg of protein per hour following the subtraction of the appropriate blanks.

Acid ribonuclease (EC 2.7.7.16)

Acid ribonuclease, a hydrolase acting on phosphoric diesters, was monitored by measuring the breakdown of RNA (type VI, from Torula yeast, Sigma chemicals).

The reaction medium consisted of 0.03 % RNA, 0.12 M Na acetate (pH 5.3, 25°C), and 125 µl enzyme supernatant (0.5 ml final volume). The reaction was initiated by the addition of substrate, incubated for 24 hr and was stopped by the addition of 0.5 ml of 10% perchloric acid, 0.25% uranyl acetate. A 0.1 ml aliquot was diluted with 1.0 ml distilled water and the optical density determined at 260 nm. The appropriate blanks were subtracted from the results.

Proteolytic capabilities of fractions II and III

Myofibrils were prepared from fast muscle of control fish, by the following technique. Muscle was extracted x 10 w/v in a buffer consisting of 10 mM tris-HCl (pH 7.4), 100 mM KCl, and 5 mM EDTA, and homogenised by three bursts of 20 sec duration with an ultra-turrax homogeniser (3500 rpm). Samples were kept on ice at all times. The resulting homogenate was centrifuged at 600 g for 10 min (5°C). The supernatant was discarded and the pellet resuspended in five times its volume of extraction buffer. The resulting solution was centrifuged at 2000g for 5min (5°C). The supernatant was discarded and the pellet again resuspended in five times its volume of extraction buffer. The resulting solution was centrifuged at 1250g for 5 min. The supernatant was discarded and the pellet resuspended in ten times its volume with extraction buffer. This was centrifuged at 400 g for 3 min and the top 2/3 taken as the myofibrillar fraction. Myofibrils were centrifuged and resuspended at 5 mg/ml in extraction buffer.

Myofibrils were incubated with fractions II and III prepared from the fast muscle of 66 day starved fish. Two sets of 6 incubation tubes were set up containing the following solutions;

1. 0.1 ml myofibrils + 0.3 ml extraction buffer (pH 7.4) (control).
2. 0.1 ml myofibrils + 0.1 ml extraction buffer + 0.2 ml 0.05 M Na acetate buffer (pH 5 0) (control).

3. 0.1 ml myofibrils + 0.1 ml fraction II enzyme supernatant (approx. 0.7 mg/ml protein) + 0.2 ml extraction buffer (pH 7.4).

4. 0.1 ml myofibrils + 0.1 ml fraction II enzyme supernatant + 0.2 ml 0.05 M Na acetate buffer (pH 5.0).

5. 0.1 ml myofibrils + 0.1 ml fraction III enzyme supernatant (approx. 0.9 mg/ml protein) + 0.2 ml extraction buffer (pH 7.4).

6. 0.1 ml myofibrils + 0.1 ml fraction III enzyme supernatant + 0.2 ml 0.05 M Na acetate buffer (pH 5.0).

The first group of 6 tubes were incubated for 8 hr, and the second for 24 hr, both at 25°C. At the end of the incubation 0.4 ml of polyacrylamide gel electrophoresis treatment buffer, containing 62.5 mM tris-HCl (pH 6.75, 25°C), 2 % SDS, 10 % glycerol, 0.4 % mercaptoethanol, and 0.001 % bromophenol blue, was added and the tubes boiled for 2 minutes. Each incubation was separately dialysed against fresh treatment buffer for 24 hr, to remove KCl, and analysed by SDS polyacrylamide gel electrophoresis, as described in chapter 4.

Molecular weight markers ranging from 66,000 to 14,000 daltons (Sigma Dalton marker VII-L) were run alongside the incubation tracks. Tropomyosin was identified by comparison with the migration of purified cod tropomyosin (courtesy of T.Crockford, prepared via the method used by Smillie, 1982).

Electron microscopic analysis of sub-cellular fractions II and III

Fraction II and III pellets, obtained by differential centrifugation, were prepared for electron microscopy as described in chapter 2. In between each dehydration and the embedding step, pellets were repacked using a Beckman microfuge (1 min spins).

Fraction II and III pellets were prepared from both fast and slow muscle, from control and 66 day starved fish (group II).

Cytochemical localisation of Acid phosphatase

Fast muscle from group two starved and control fish was prepared for cytochemical localisation of acid phosphatase by an adaptation of the methods by Miller & Palade (1964) and Hoffstein et al (1975). Small bundles (1-2 mm^2 cross-section) of fast muscle were fixed for 1 hr in a solution of 2% glutaraldehyde, 0.1 M cacodylate buffer (pH7.4, 5°C), and 0.1 M sucrose. They were then washed three times in 0.1 M cacodylate buffer (pH 7.4, 20°C), 0.15 M sucrose. The bundles were incubated for 1 hr at 35°C in incubation medium. The incubation medium was made up of two solutions. Solution A consisted of 3.78 g of β-glycerophosphate in 50 ml 0.05 M Na acetate (using 1 M acetic acid to pH to 5.3) and diluted to 100 ml. Solution B consisted of 0.12g $Pb(NO_3)_2$ in 84 ml 0.05 M Na acetate (using 0.05 M NaOH to pH to 5.3) and diluted to 100 ml. To 90 ml of solution B was added 10 ml of solution A (slowly, to avoid precipitation) and incubated for 1 hr at 55°C. The resulting solution was then filtered. To every 10 ml of the filtrate was added 0.68 g sucrose. To every 9ml of this solution was added 1 ml dimethylsulphoxide (DMSO). Control incubations consisted of the same solutions except that β-glycerophosphate was omitted from solution A.

After incubation, the fibre bundles were washed twice in buffer and prepared for electron microscopy as described in chapter 2.

Statistical analysis

A student t-test (one tailed) was performed on all results

Results

Lysosomal enzyme activities during starvation

Following the 66 day starvation of group II fish, there was an increase

in the total activities of all the lysosomal enzymes measured, with the exception of acid proteinase activity in slow muscle (figs.2a & b). Starvation resulted in a proportionally larger increase in the fast muscle lysosomal enzymes, than in the slow muscle enzymes (except for acid phosphatase).

Lysosomal enzyme activities during refeeding following starvation

Fig.3 shows the activities of acid proteinase and aryl sulphatase during the course of 54 days refeeding following a 74 day starvation. Upon refeeding the activity of both enzymes decreased within the first 10 days. After 54 days of refeeding acid proteinase activity in both fast and slow muscle is below that found in control tissue. The same is true for fast muscle aryl sulphatase activity.

Lysosomal activity of the sub-cellular fractions

The aryl sulphatase activities of the various cellular fractions are given in table I, and fig.4 correlates these activities with the percentage of the total protein in each fraction. Aryl sulphatase was selected as a marker for lysosomes for the following reasons. Firstly aryl sulphatase parallels the increases found in the other lysosomal enzymes during starvation (fig. 1). Secondly the increase in aryl sulpthatase activity during starvation, is relatively large . Thirdly there was less variability both within the assay itself and from fish to fish. The highest specific activities of aryl sulphatase occured in fractions II and III from both fibre types. During starvation there is an increase in activity in every fraction, but especially in fractions II and III. The relative increases were higher in fast than slow muscle.

Proteolytic capabilities of fractions II and III in degrading native myofibrils

Figs. 5 and 6 show the superimposed traces obtained from gel scans of the myofibrillar proteins ranging from 50,000 to 200,000 daltons. In the control incubations (with no added enzyme fractions) myosin heavy chain and actin (identified by their molecular weight and relative abundance) were only slightly degraded at pH 5.0, even after 24 hr incubation (fig.6). When the incubations included either fractions II or III then both myosin heavy chains and actin were almost completely degraded after 24 hr at pH 5.0. The capacity for fraction II to degrade myosin heavy chain appears to be greater than that of fraction III. Both fractions were capable of degrading myosin heavy chain to a greater extent than actin (figs.5 & 6). At pH 7.4 neither fraction II or III was capable of degrading actin or myosin heavy chain to any significant extent.

Figures 7 and 8 show the superimposed gel scans for the myofibrillar proteins below 50,000 daltons. Both fractions II and III appear to be relatively incapable of degrading tropomyosin. After 24 hr, however, tropomyosin appears to decrease in all the incubations (including control incubations) at both pH 7.4 and 5.0. Many of the peaks are masked by the increase of breakdown products during the incubations (shown in figs.7 and 8 where there is a transient increase in the peaks between 15,000 to 20,000 daltons). Figure 8b shows two gels of myofibrils incubated for 24 hours, from these gels the scans shown in figs.6 and 8 were obtained.

Electron microscopy of fractions II and III

Figs. 9 to 16 show electron micrographs of sections cut from fraction II and III pellets. There was no detectable differences between the pellets obtained from the slow or fast muscle, with the exception of lipid droplets found in fraction III from control slow muscle. The vesicles contained in

fraction III are shown in figs. 9 to 12. The type of vesicles and particulate matter do not appear to change during starvation and appear consist of mainly sarcoplasmic reticulum and disrupted mitochondria. Fraction II from control muscle contains mainly mitochondria and some smaller vesicles (fig.13). In starved muscle however many multi-membraned vesicles are also present (figs.14-16), some containing smaller vesicles and other electron dense material.

Cytochemical localisation of Acid phosphatase

Figs.17-20 show unstained sections from the fast muscle of starved fish, labelled for acid phosphatase activity. Reaction product was seen within the t-tubule system (figs.17-19), and occasionally in the terminal cisternae and other vesicles (fig.20). No acid phosphatase activity was found in any multi-membraned organelles. Reaction product was observed to accumulate in mitochondria and nuclei, however this also occured in control fibres, where the reaction substrate was absent from the incubation medium. No reaction product was visible in the t-tubule or other vesicles in control tissue.

Discussion

Haemoglobin is known to be exceptionally sensitive to breakdown by cathepsin D, a major carboxyl endopeptidase, at pH 2.5 - 5.0 (Barrett, 1978). Cathepsin B, a major thiol endopeptidase, is also capable of haemoglobin breakdown, but is optimally active at around pH 6.0, with little residual activity at lower pH's (Barrett,1980). In rat skeletal muscle and sockeye salmon fast muscle, acid proteinase activity (measured by the hydrolysis of haemoglobin) was completely inhibited by pepstatin, a pentapeptide inhibitor of carboxyl proteinases (Pennington,1977 ; Mommensen et al,1980, respectively)

It is therefore likely that by using haemoglobin as a substrate, a measure of cathepsin D activity is being obtained.

It is evident from the activities of the lysosomal enzymes, that control saithe slow muscle has a higher basal hydrolase activity (except for acid phosphatase) than fast muscle (fig.1). It is interesting to note that in studies on rat skeletal muscle the basal activities of acid cathepsin (using haemoglobin as the substrate) are higher in the extensor digitorum longus, a predominantly fast muscle, than in the soleus, a predominantly slow muscle (Goldspink et al, 1971). This trend however is reversed for RNase, acid alpha-glucosidase, and alkaline phosphatase which all exhibit higher activities in the soleus (Goldspink et al, 1971). Higher hydrolase activity in red oxidative muscle is consistent with this fibre type exhibiting higher turnover rates than fast glycolytic muscles, reported by Millward et al (1978).

It has long been known that fish muscle possesses relatively high activities of cathepsins and peptidases (Siebert et al, 1965), but few studies have reported the effects of starvation on muscle hydrolase activities. Creac'h (1972, cited by Love, 1980) reported that in carp muscle the activities of proteolytic enzymes were high but did not change to any significant extent during a prolonged starvation. In contrast Mommensen et al (1980), studying the spawning migration of sockeye salmon, reported a 70% decrease in white muscle protein paralleled by considerable increases in cathepsin D (six fold) and carboxypeptidase A (two fold increase). This group also reported that there was no decrease in red muscle protein concentration during the migration and that in conjunction with this cathepsin D and carboxypeptidase A activities remained unchanged. During a 66 day starvation in saithe, reported in this study, all the hydrolases measured increased with the exception of slow muscle cathepsin D (acid proteinase). The increases were proportionally larger in fast muscle (fig.1). These results therefore

suggest the direct involvement of acid hydrolases, and hence lysosomes, during muscle atrophy in saithe and are consistent with the elevated degradation observed in fast muscle during starvation (chapter 2).

There is conflicting evidence concerning the direct involvement of cathepsin D in myofibrillar protein degradation. Lockshin et al (1980), working on insect muscle, blocked the action of cathepsin D by administering pepstatin, and found that myofibrillar degradation continued as normal during metamorphosis. However, pepstatin has been found to inhibit the lysosomal degradation of rat myofilaments in vitro (Spanier et al, 1977), inhibit the degradation of muscle cells in culture (McGowan et al, 1976), and inhibit myofibrillar degradation in dystrophic chickens (Stracher et al, 1978). Different systems may therefore be involved in the catabolic processes of mammals and insects. No such inhibitory studies have yet been undertaken on fish muscle proteinases.

In this study there are three results giving supportive evidence for the involvement and importance of cathepsin D (acid proteinase) in muscle atrophy. Firstly there is a three-fold increase in fast muscle cathepsin D activity after 66 days starvation. During this period there is also a massive breakdown in fast muscle myofibrillar proteins (chapter 2). Secondly, in slow muscle where the utilisation of myofibrillar proteins is relatively small in comparison with fast muscle, there is no increase in cathepsin D activity. Any degradation of slow muscle myofibrillar proteins may be accounted for by decreases in synthesis rates (RNA concentrations have been reported to decrease three-fold during 30 weeks starvation in plaice ; Patterson et al, 1974). Thirdly, after the first 10 days of refeeding there is a dramatic decrease in cathepsin D activity (fig.3), to a value lower than that found in control fish. This is a period of net synthesis of a large mass of muscle fibre (chapter 3) and hence it would be advantageous to maintain a lower level of myofibrillar degradation than in control fish, where presumably the rate of

muscle growth is not so extensive. These results therefore infer that cathepsin D is directly involved in myofibrillar degradation. Furthermore it is clear that the lysosomal proteinases present in subcellular fractions II and III are capable of degrading myosin heavy chains and actin at pH 5.0 (figs.5-8).

Previous authors have suggested that the lysosomal system in muscle may be an integral part of the sarcoplasmic reticulum (Schiaffino & Hanzlikova, 1972 ; Stauber & Bird, 1974 ; Bird, 1975 ; Hoffstein et al, 1975), and indeed in an attempt to identify the subcellular organelles containing lysosomal enzyme activity, lysosomes were indistinguishable from the sarcoplasmic reticulum (figs.9-12). Only in subcellular fraction II were any morphological changes observed in the organelle populations that could account for the increase in aryl sulphatase activity seen in starved muscle (table I). As fraction II from starved fish muscle only contained mitochondria and multi-membraned vesicles (not observed in control muscle fractions)(figs.14 & 15), then these vesicles must be at least partially reponsible for the increased aryl sulphatase activity and may therefore be secondary lysosomes. In support of this, fraction II was also clearly capable of degrading native myosin heavy chain and actin, even to a greater extent than fraction III (figs.5 & 6).

Engel,A.G. (1973) demonstrated that in the myopathy acid maltase deficiency, the membranes of some secondary lysosomes originated from the t-tubule and that some of these lysosomes contained acid phosphatase activity. Many other authors have located acid phosphatase as well as cathepsins D and B, aryl sulphatase, and β glucuronidase within the sarcoplasmic reticulum (Hoffstein et al, 1975 ; Bird et al, 1978 ; Libelius & Lindquist, 1978 ; Schiaffino & Hanzlikova, 1972 ; Topping & Travis, 1974 ; Fischmann, 1964). In this study the cytochemical localisation of both acid phosphatase and aryl sulphatase were attempted. However the stain for aryl sulphatase, using the method of Hopsu-Havu et al (1966) as adapted by Hoffstein et al (1975), was

found to be unsuccessful due to the non-specificity of the precipitation Figures 17-20, taken of fast muscle from starved fish, show acid phosphatase activity within the t-tubule and occassionally within the terminal cisternae of the sarcoplasmic reticulum. In the light of other studies suggesting the involvement of the t-tubule in the formation of autophagic vacuoles (Engels, A G , 1973 ; Libelius & Lundquist, 1978 ; Jirmanova et al, 1977) it is interesting to note a similar involvement in fish muscle. Previous studies, however, have suggested that only the vacuolar membranes arise from the t-tubule and that acid phosphatase (and presumably other acid hydrolases) is acquired from the sarcoplasmic reticulum when forming secondary lysosomes. The findings in this study suggest that the origin of at least some acid phosphatase is extracellular, endocytosed into the muscle via the t-tubule Clearly further investigation is required. Lysosomal enzymes are, however, known to exist in plasma (Hickman & Neufeld, 1972 ; Goldstein et al, 1978) and much work has been done on the receptor-mediated pinocytosis of glycosidases (in particular β-glucuronidase) into fibroblasts and macrophages (Sly et al, 1978 ; Stahl et al, 1978 ; Diment & Dean, 1983). It is possible that some muscle hydrolases are of extracellular origin (Cannonico & Bird, 1970) and enter the muscle cell in a similar fashion as they enter fibroblasts and macrophages.

Table I. Specific Aryl Sulphatase activities in sub-cellular fractions I-IV (μmoles 4-nitrocatechol/mg protein/hr). Experiments undertaken on 66 day starved group II fish. Values are mean ± SEM.

Fraction	Slow control (n=4)	Slow starved (n=4)	Fast control (n=4)	Fast starved (n=5)
Fraction I	0.049 ±0.004	0.061 ±0.012	0.009 ±0.001	0.031* ±0.004
Fraction II	0.125 ±0.016	0.328* ±0.075	0.107 ±0.032	0.377* ±0.050
Fraction III	0.116 ±0.034	0.522** ±0.130	0.038 ±0.004	0.201** ±0.011
Fraction IV	0.052 ±0.171	0.343** ±0.043	0.009 ±0.001	0.073 ±0.027

* $p<0.01$
** $p<0.001$

Fig.1 Summary of the dynamics of the lysosomal system. Not all the transformations shown occur in every cell type

RE-rough endoplasmic reticulum
SE-smooth endoplasmic reticulum
GA-golgi apparatus
e-lysosomal enzyme
p-secretory protein
MI-mitochondria or other cellular organelle
s-soluable substrate for lysosomal digestion
AV-autophagic vacuole
PE-peroxisome
HL-hetero lysosome
MVB-multi-vesicular body
RB-residual body
Pi-pinocytosis
Ph-phagocytosis

(adapted from R.J.Dean,1978)

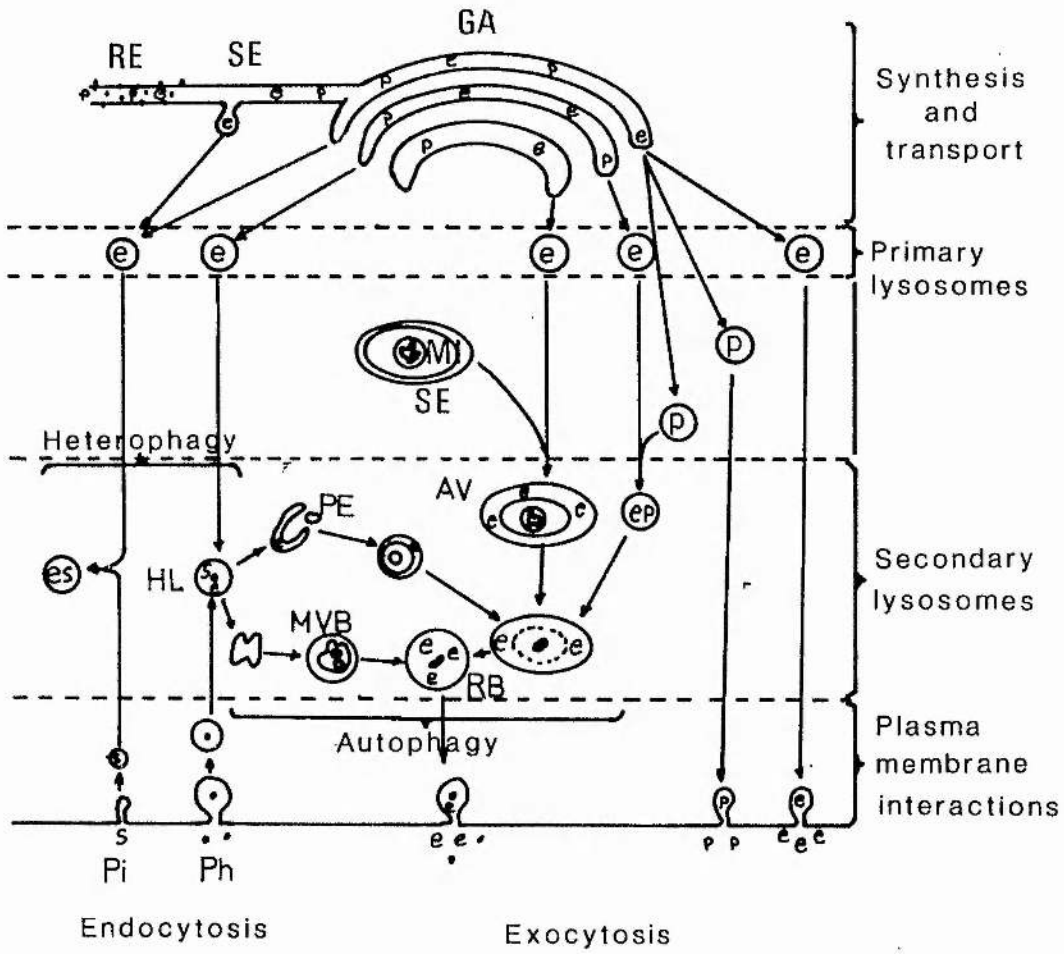

GA
RE
SE
Synthesis and transport
Primary lysosomes
MVB
SE
Heterophagy
AV
PE
HL
MVB
RB
Autophagy
Secondary lysosomes
Plasma membrane interactions
Pi
Ph
Endocytosis
Exocytosis

Fig.2a Histograms showing the activities of aryl sulphatase, β-glucuronidase and acid ribonuclease in muscle from control and 66 day starved saithe. Aryl sulphatase activity given in μmoles 4-nitrochatechol equivalent / mg protein / hour. β-glucuronidase activity given in μmoles phenolphthalein equivalent / mg protein / hour. RNase activity is given as the change in absorbance at 260 nm /mg protein /hour. Hatched bars - starved muscle. Clear bars - control muscle.

a

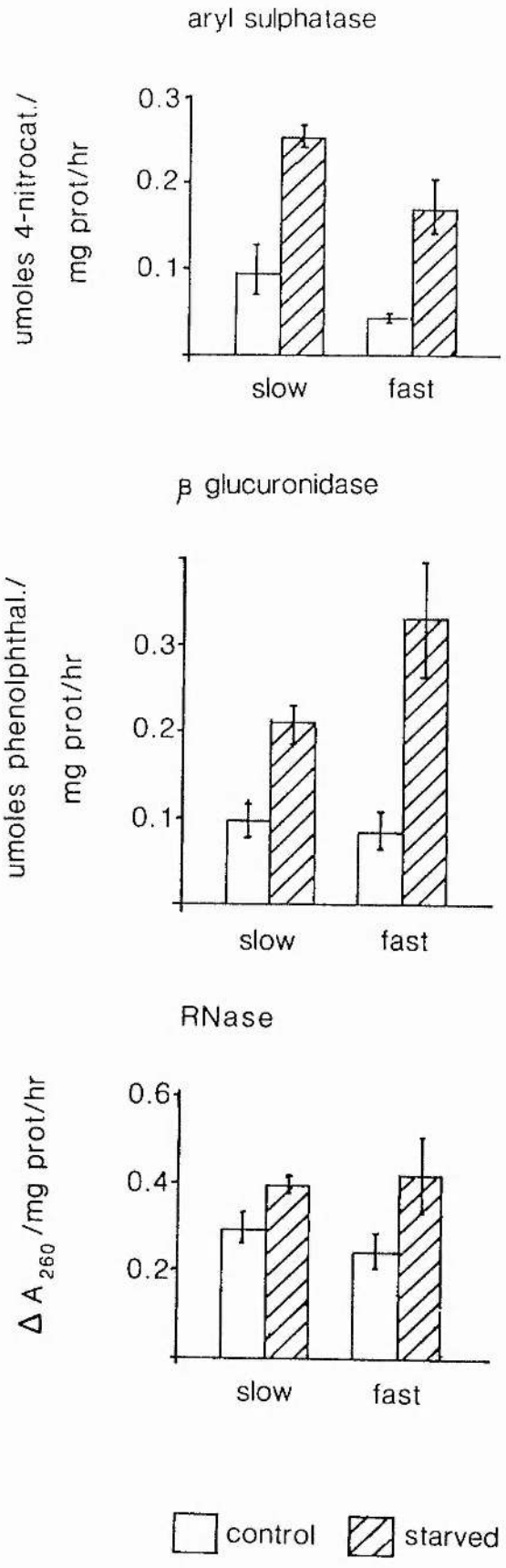

aryl sulphatase
umoles 4-nitrocat./
mg prot/hr
0.3
0.2
0.1
slow
fast
β glucuronidase
umoles phenolphthal./
mg prot/hr
0.3
0.2
0.1
slow
fast
RNase
Δ A 260 /mg prot/hr
0.6
0.4
0.2
slow
fast
control
starved

Fig.2b Histograms showing the activities of acid proteinase and acid phosphatase in muscle from control and 66 day starved saithe. Acid proteinase activity is given in nmoles of tyrosine equivalent per mg of protein per hour. Acid phosphatase activity given in μmoles of p-nitrophenol equivalent per mg of protein per hour. Hatched bars - starved muscle. Clear bars - control muscle.

b

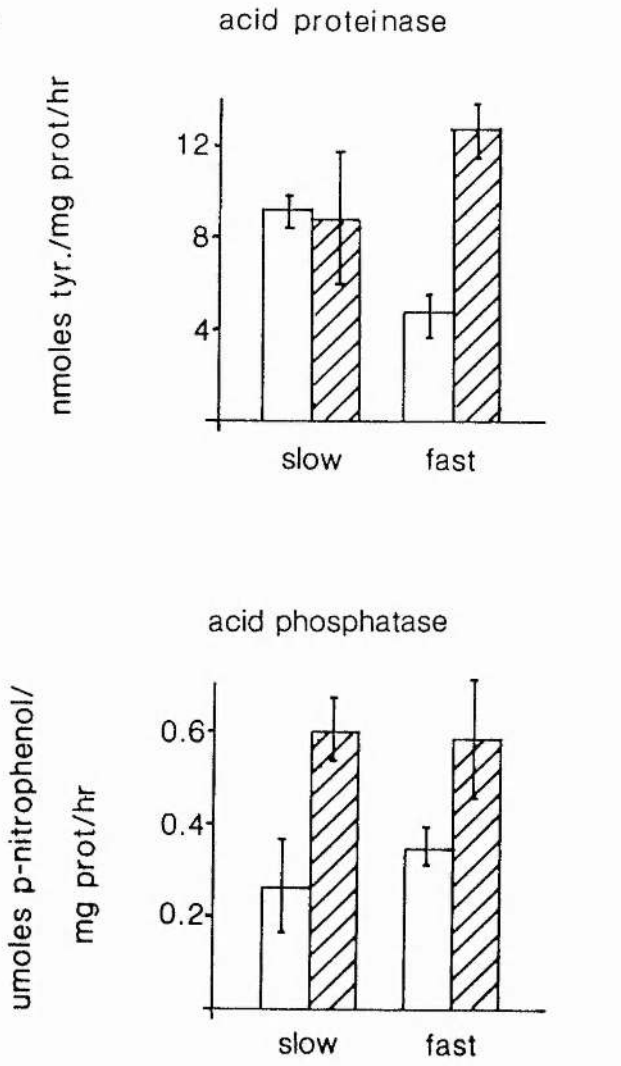
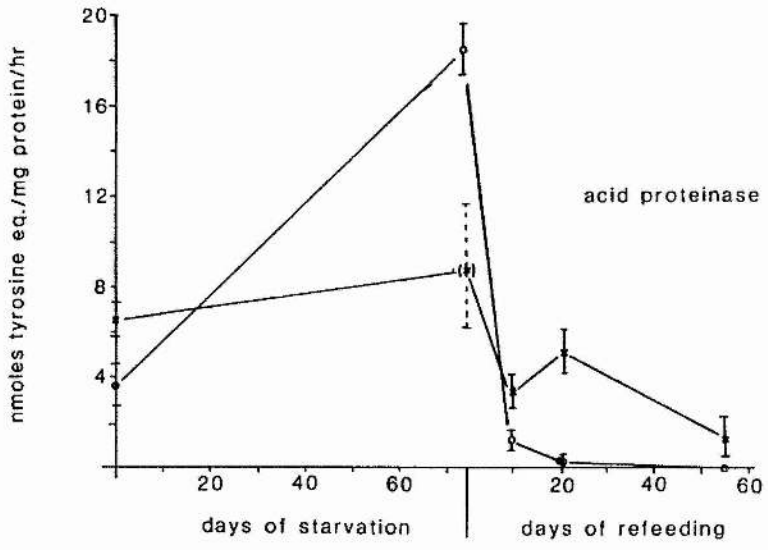
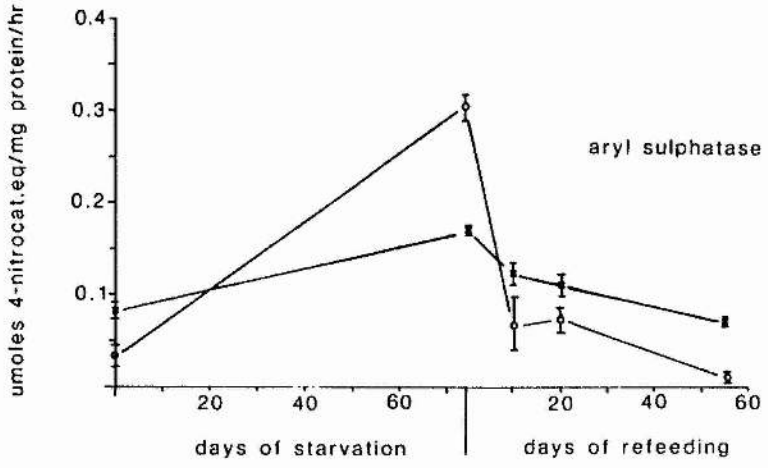

acid proteinase
nmoles tyr./mg prot/hr
12
8
4
slow
fast
acid phosphatase
umoles p-nitrophenol/
mg prot/hr
0.6
0.4
0.2
slow
fast
control
starved

Fig.3 Graphs showing the activities of acid proteinase and aryl sulphatase during a 74 day starvation followed by refeeding. Values given as mean ±SEM.

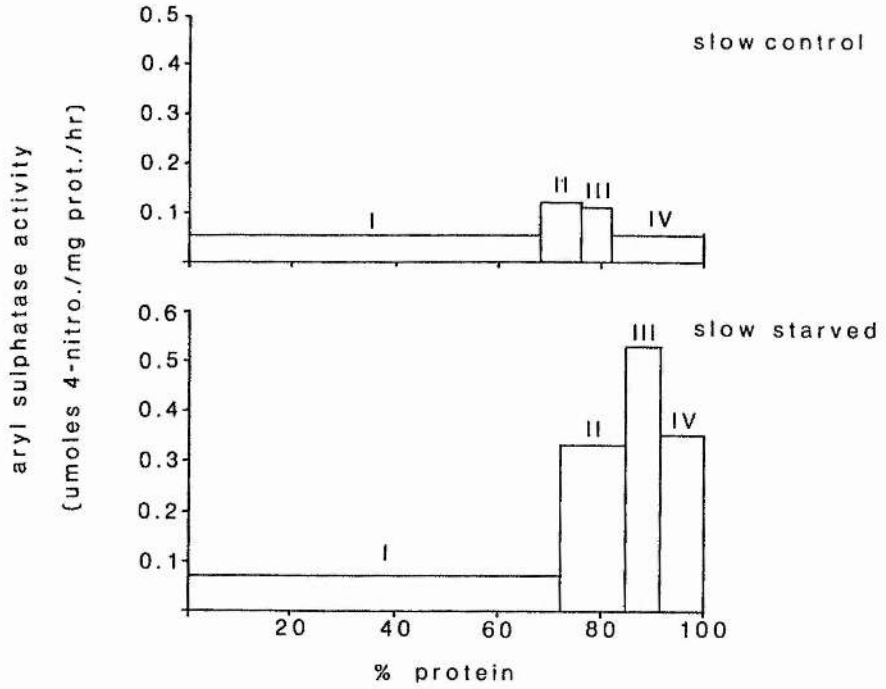




slow muscle

fast muscle

Fig.4 Histograms demonstrating the percentage of protein in each sub-cellular fraction, correlated with the specific aryl sulphatase acivity.


slow control
slow starved
aryl sulphatase activity
(umoles 4-nitro./mg prot./hr)
0.5
0.4
0.3
0.2
0.1
0.6
0.5
0.4
0.3
0.2
0.1
I
II
III
IV
20
40
60
80
100
% protein

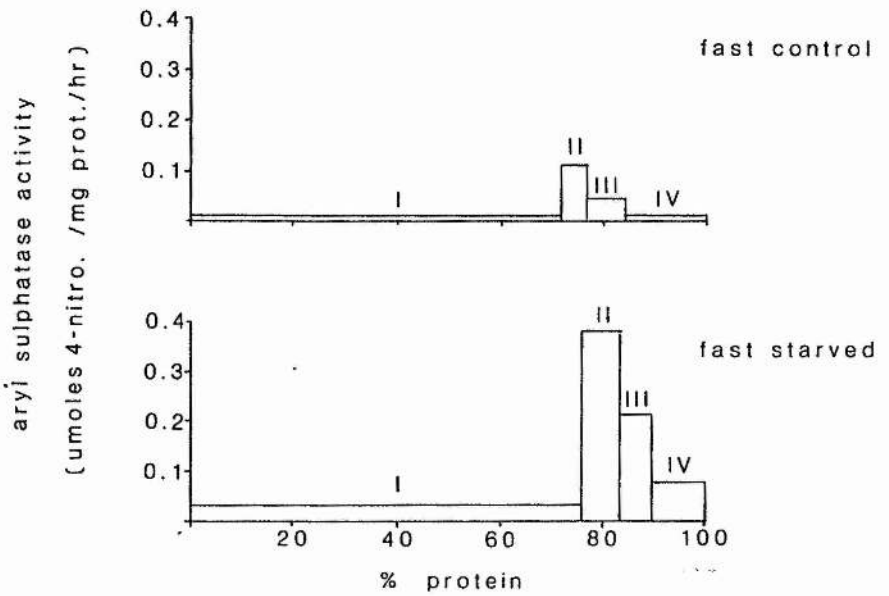

fast control
fast starved
aryl sulphatase activity
(umoles 4-nitro. /mg prot./hr)
0.4
0.3
0.2
0.1
I
II
III
IV
20
40
60
80
100
% protein

Fig.5 Traces from densometric scans of 7.5 % polyacrylamide gels. The traces show the reaction products from 8 hour incubations of lysosomal rich centrifuge fractions with myofibrils (prepared from control fish fast muscle). The top trace shows the 8 hour incubation of myofibrils alone at both pH 5.0 (dotted trace) and pH 7.4 (solid trace). When incubated with either fraction II or III there is a degradation of both myosin heavy chain (labelled myosin) and actin, at pH 5.0. Fraction II appears to possess the greatest potential for myosin heavy chain degradation and possibly actin degradation. Note the increase in proteins of molecular weight 100,000 to 200,000 presumably resulting from the breakdown of higher molecular weight proteins (for instance myosin heavy chain).

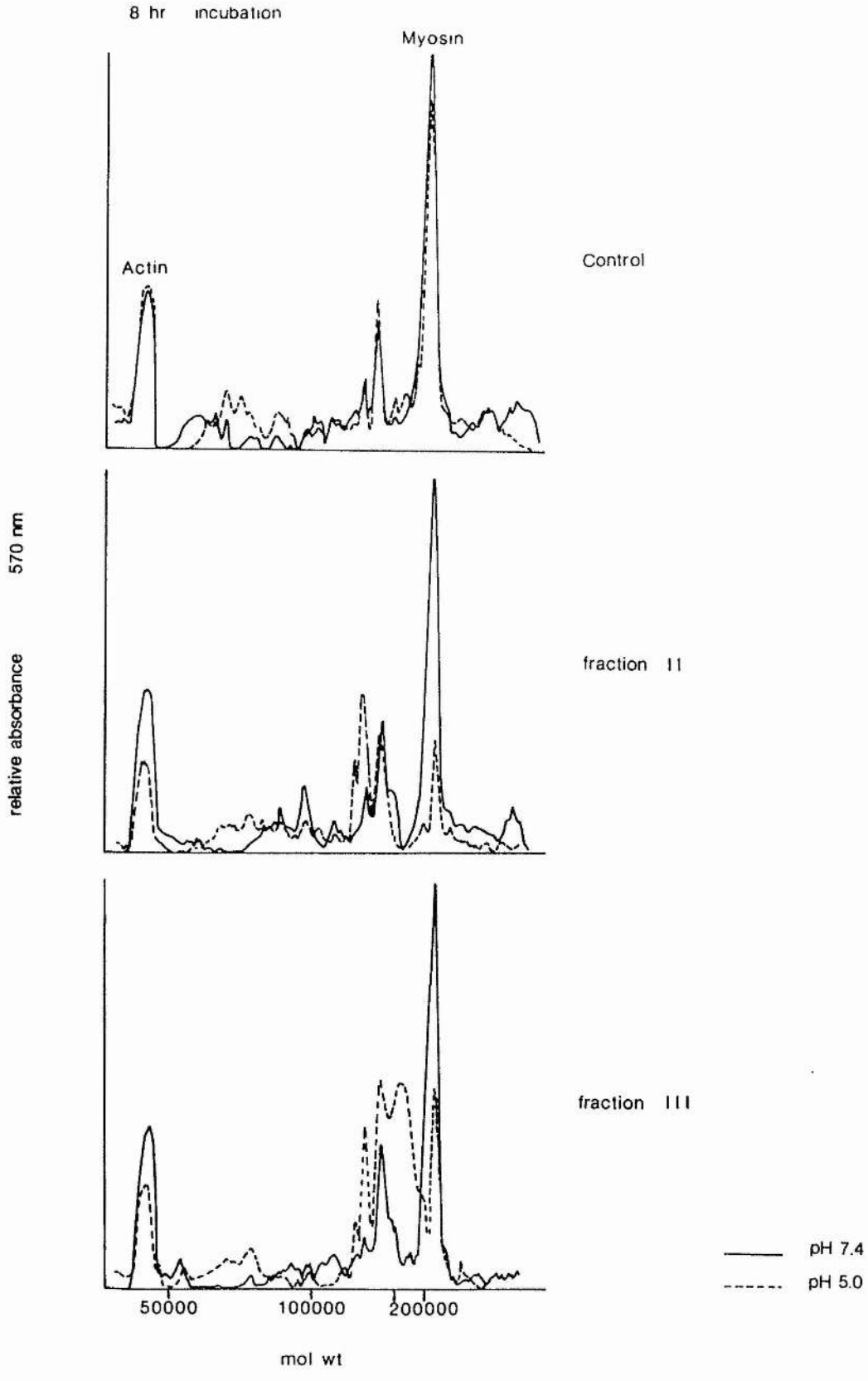

8 hr incubation
Myosin
Actin
Control
fraction II
fraction III
relative absorbance 570 nm
pH 7.4
pH 5.0
50000
100000
200000
mol wt

Fig.6 Traces from densometric scans of 7.5 % polyacrylamide gels. The traces show the reaction products from 24 hour incubations of lysosome rich centrifuge fractions with myofibrils (prepared from control fish fast muscle). Top trace shows the 24 hour incubation of myofibrils alone, at both pH 7.4 and pH 5.0. Note that at pH 5.0 (dotted line) there is a slight degradation of the myosin heavy chain (labelled myosin) and actin peaks. This shows the relative unstability of these molecules at pH 5.0 in comparison with pH 7.4. When myofibrils are incubated with either of the lysosome rich fractions then both myosin heavy chain and actin are much degraded. Fraction II appears to possess the greater potential for myosin heavy chain degradation.

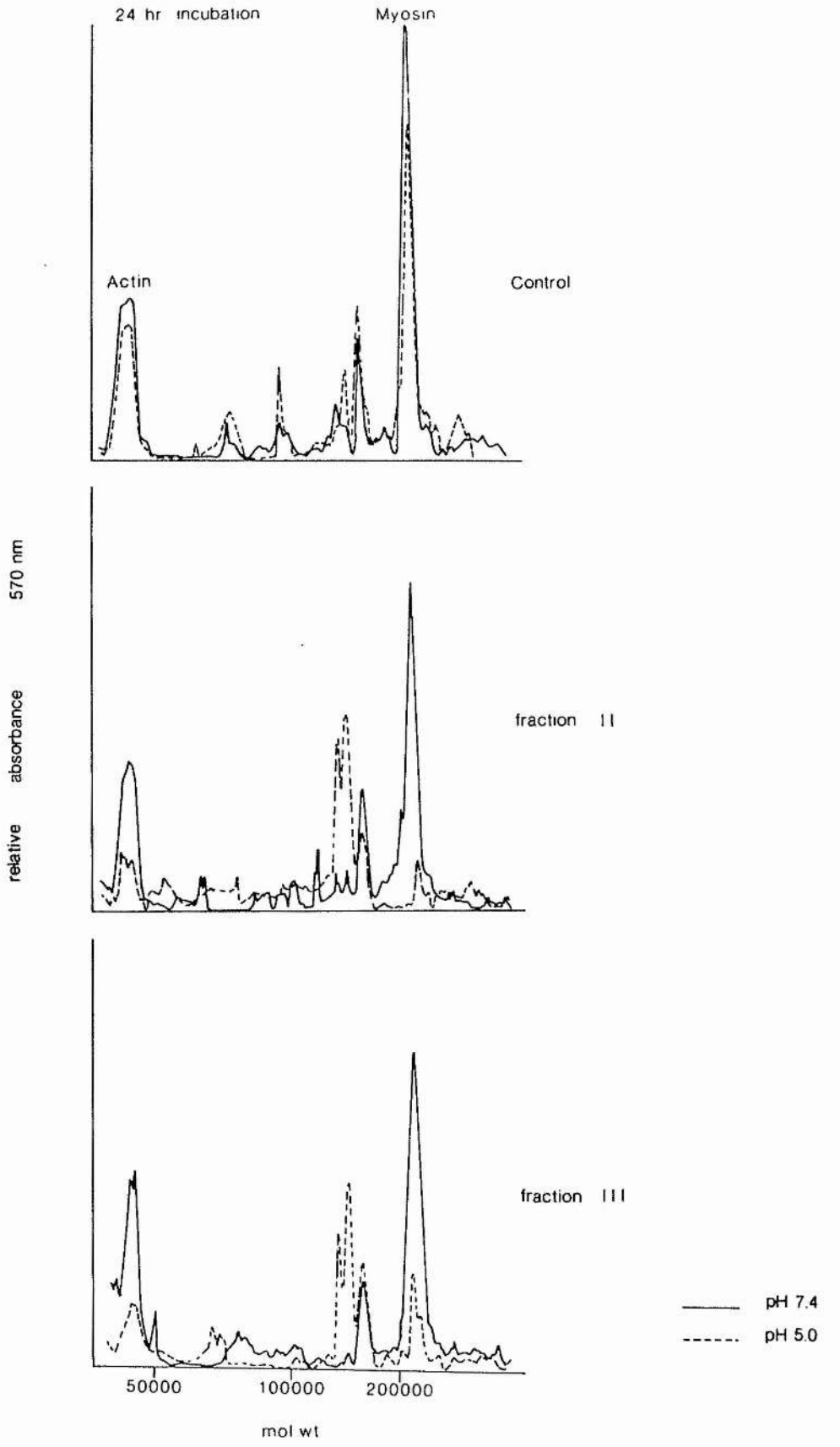

24 hr incubation
Myosin
Actin
Control
fraction II
fraction III
relative absorbance 570 nm
50000
100000
200000
mol wt
pH 7.4
pH 5.0

Fig.7 Traces from densometric scans of 15 % polyacrylamide gels. The traces show the reaction products from 8 hour incubations of lysosome rich centrifuge fractions with myofibrils. Note that relative to actin, tropomyosin is niether degraded at pH 5.0 nor at pH 7.4 even when incubated with the lysosome rich fractions. Below 30,000 daltons there is an increase in reaction products, even in control incubations, at pH 5.0. The build up of low molecular weight reaction products is especially prevalent in the incubation with fraction II at pH 5.0.

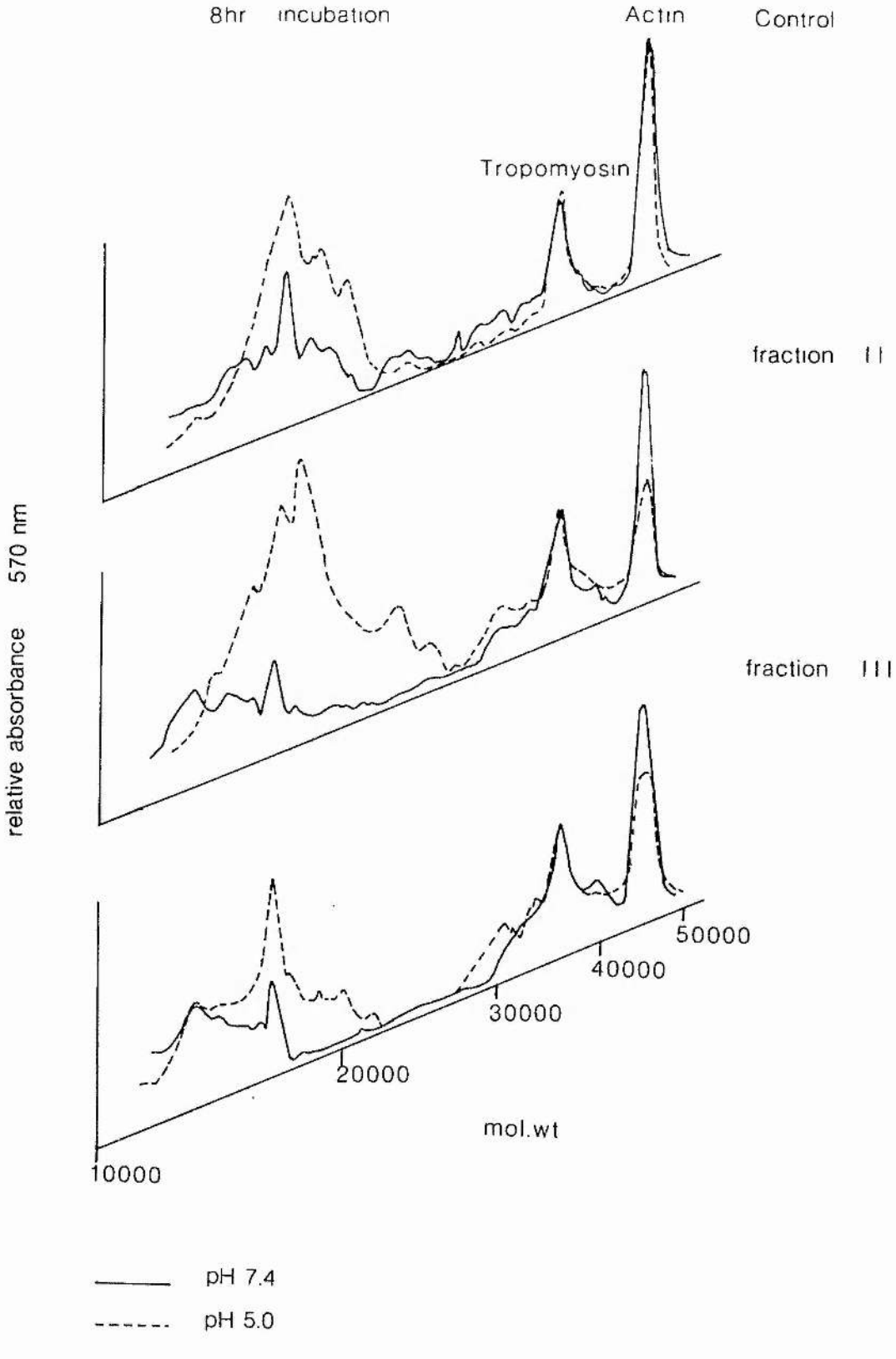

8hr incubation
Actin
Control
Tropomyosin
fraction II
fraction III
relative absorbance 570 nm
50000
40000
30000
20000
10000
mol.wt
pH 7.4
pH 5.0

Fig.8 Traces from densometric scans of 15 % polyacrylamide gels. The traces show the reaction products from 24 hour incubations of lysosome rich centrifuge fractions with myofibrils. Relative to actin the tropomyosin peak appears to be diminished in all incubations, at both pH 5.0 and pH 7.4. This suggests that tropomyosin is unstable after 24 hours of incubation at 25°C, and is subject to degradation without the involvement of lysosomal or indeed any other proteolytic enzyme. Also note the reaction products below 25,000 daltons are reduced in comparison with the 8 hour incubation. These proteins have presumably been degraded to molecules of below 15,000 daltons and possibly even to amino acids.

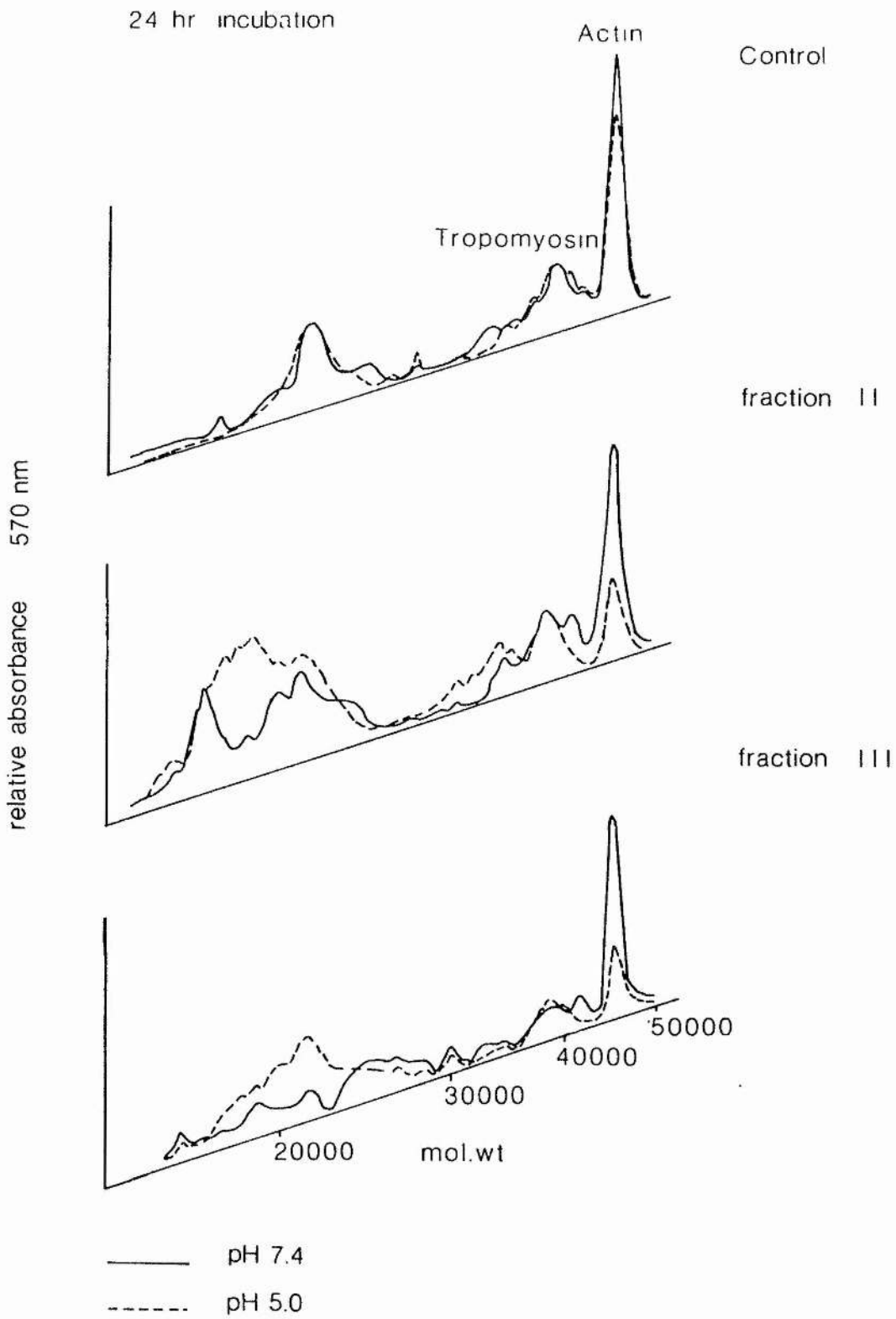

24 hr incubation
Actin
Control
Tropomyosin
fraction II
fraction III
relative absorbance 570 nm
50000
40000
30000
20000
mol.wt
pH 7.4
pH 5.0

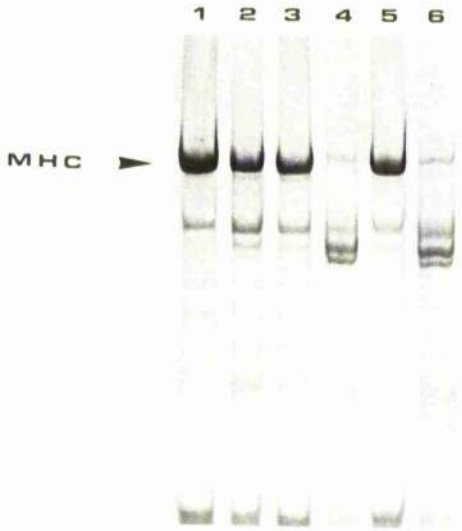
Fig.8b Photograph of 7.5 % and 15 % polyacrylamide gels showing myofibrils incubated for 24 hours (scans shown in figs. 6 and 8) with Fraction II and III. Well 1. Myofibrils incubated at pH 7.4 (control)

2. Myofibrils incubated at pH 5.0 (control)
3. Myofibrils incubated at pH 7.4 with fraction II
4. Myofibrils incubated at pH 5.0 with fraction II
5. Myofibrils incubated at pH 7.4 with fraction III
6. Myofibrils incubated at pH 5.0 with fraction III

MHC - myosin heavy chain, A - actin, TR - tropomyosin.

b

7.5 % gel



15 % gel

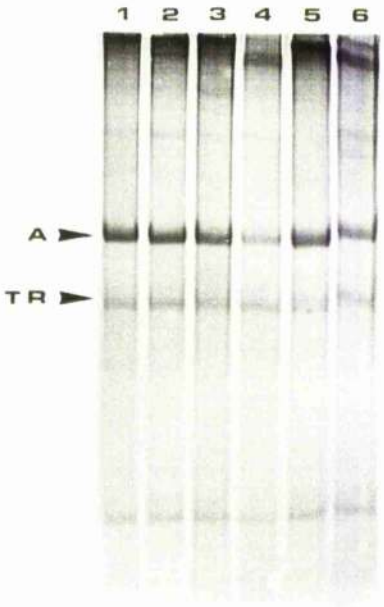


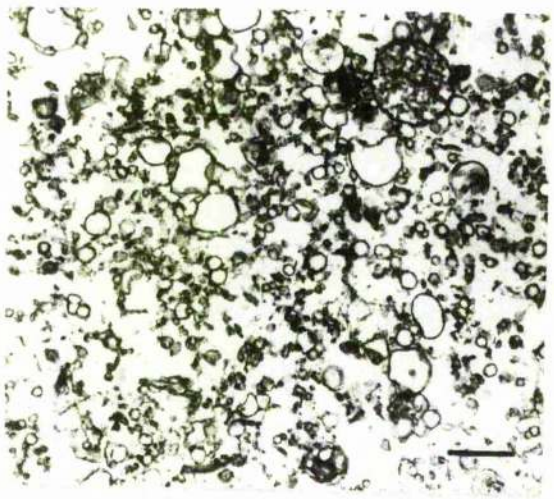
Fig.9 Electron micrograph of fraction III from the fast muscle of a control fish. Note the occasional mitochondria and the diversity of vesicles, presumably originating from disrupted s.r., or mitochondria. Bar - 1 μm.

Fig.10 Electron micrograph of fraction III from the fast muscle of a 66 day starved fish. The micrograph shows disrupted mitochondria (arrow) and the variety of vesicles. Note that multi - membraned vesicles are not present and that this fraction is indistinguishable from that in figure 9. Bar - 1 μm.

Fig.11 Fraction III from slow muscle of a control fish. Arrow again shows disrupted mitochondria and shows that some of the free vesicles arise from the mitochondrial inner membranes . Occasionally lipid droplets were also visible in this fraction. Bar - 1 μm.

Fig.12 Fraction III from the slow muscle of a 66 day starved fish, showing the morphology of the vesicles in greater detail. Bar - 1 μm.

9



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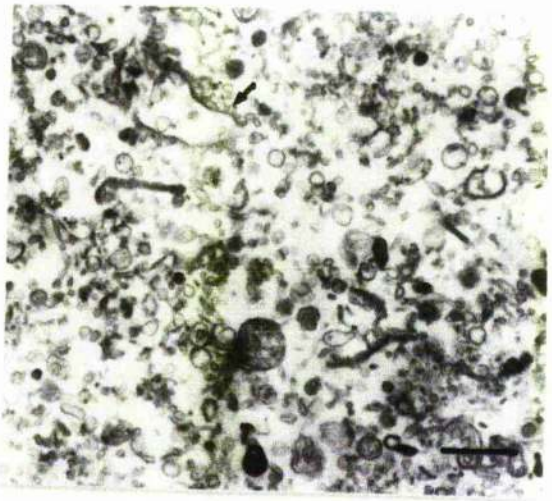

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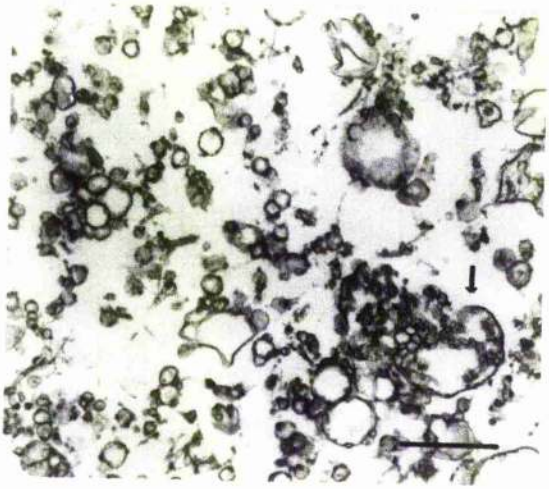

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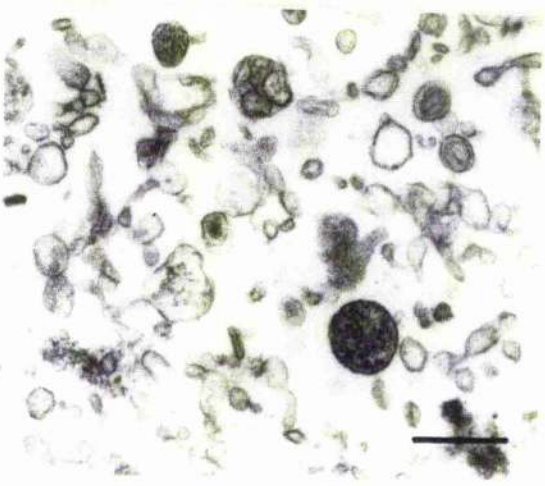
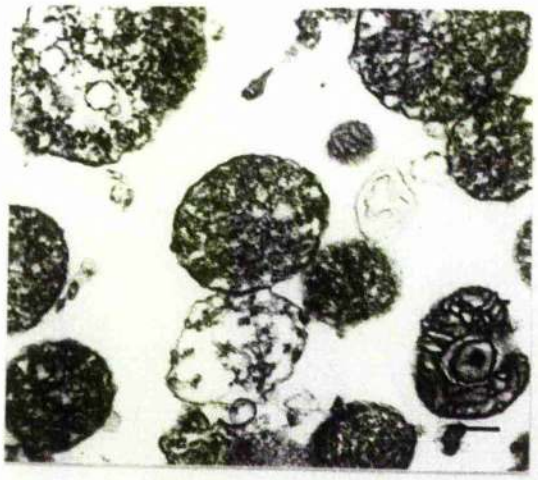


Fig.13 Fraction II from the fast muscle of a control fish, This fraction can be seen to contain mainly mitochondria and occasionally smaller vesicles Bar - 0.5 μm.

Figs.14 & 15 Fraction II from the fast muscle of starved fish, Micrographs show the multi-membraned vesicles, often containing electron dense material. Similar vesicles were not observed in control fractions. Bar - 0.5 μm.

Fig.16 Fraction II from the fast muscle of a starved fish, showing the diversity of the internal mitochondrial membranes. Note the double-membraned vesicles within the outer mitochondrial membrane (arrows). This phenomena was not observed in control mitochondria and may therefore represent autophagic processes involved in the degradation of the internal cristae structures. Bar - 0.5 μm.

13



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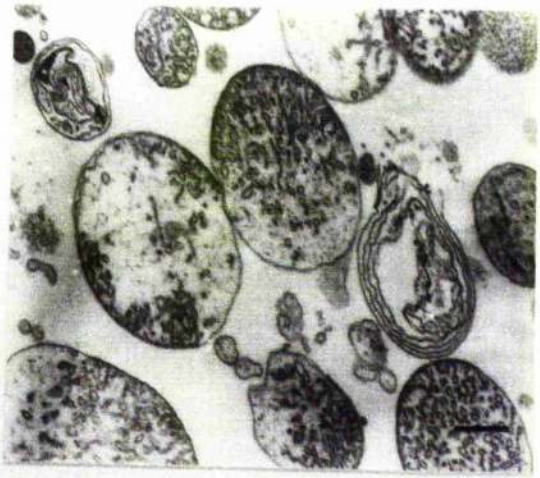
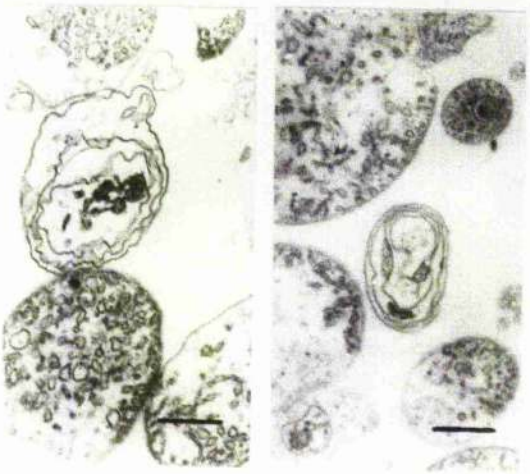


15



16

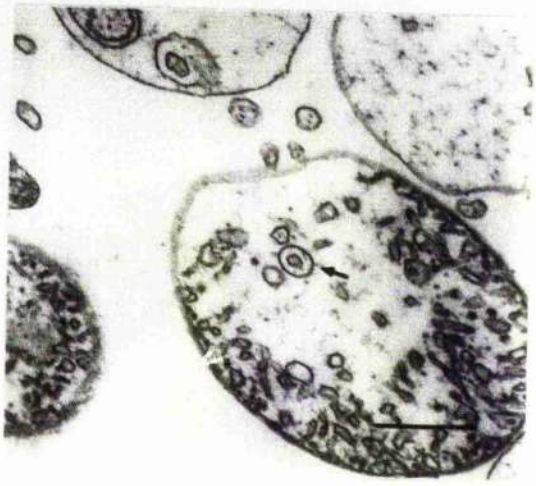


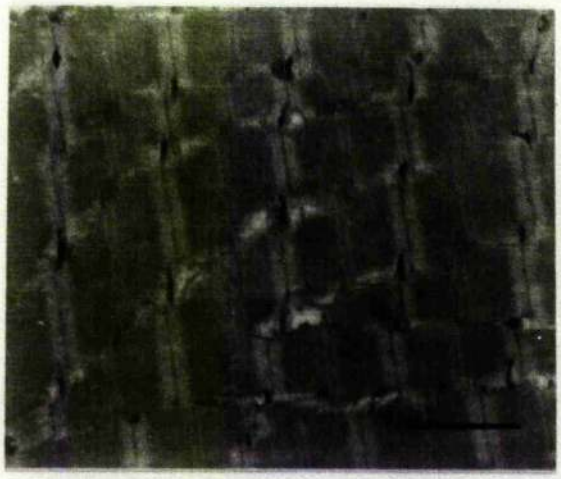
Fig.17 Unstained longitudinal section from starved fast muscle. The reaction product for acid phosphatase activity can be seen in the t-tubules. Bar - 1 μm.

Fig.18 Unstained longitudinal sections from starved fast muscle, again demonstrating the localisation of acid phosphatase in the t-tubule. Bar - 0.25 μm.

Fig.19 Detailed micrograph from starved fast muscle showing acid phosphatase within the t-tubule. Bar - 0.25 μm.

Fig.20 Unstained section from starved fast muscle, showing reaction product in the terminal cisternae of the sarcoplasmic reticulum. This phenomena was only occasionally observed. Bar - 0.1 μm.

17



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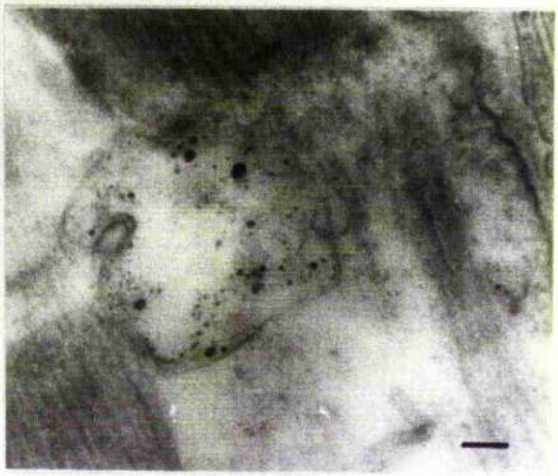

Chapter 7

General Discusion

General discussion

The response of fish to periods of starvation varies greatly from that of mammals, especially concerning the duration and extent of starvation that can be tolerated. Most healthy adult humans for instance , can tolerate a weight loss of 5-10 % with little functional disorganisation, however weight losses exceeding 40 % cannot be sustained (Levenson & Steifter, 1983). The differences between starvation in mammals and fish can be attributed mainly to the ectothermic nature of fish, allowing a basal metabolic rate that can be 5 to 10 fold lower than in a mammal of comparable size (Bennet & Ruben, 1979) In addition, due to the physical properties of water, fish require little energy to maintain posture and position and are able to carry a large mass of white muscle, solely for fast swimming and energy reserves, with little extra metabolic expense. It is interesting to note that during starvation in humans there is a preferential degradation of the red fibres. In contrast to this fish have adapted to maximise the advantages of carrying a large mass of muscle by the preferential degradation of the white muscle during starvation However, even amongst fish there is a large inter-specific variation in the strategies employed to survive periods of low food availability, and since only approximately 20 (out of 20,500) species have been studied in experiments on starvation, it is not possible to outline the complete range adaptations that have evolved. For saithe the sequence and sources of energy mobilisation have been reported (chapters 2 and 5) and are similar to those reported for other gadoid species (for example the cod) where much research has been dedicated to studying starvation in both the wild populations and under laboratory conditions (see Love, 1970, 1980 : Black, 1983).

There is an inverse relationship between the liver and white muscle glycogen concentrations and the post mortem pH of the respective tissue (Love, 1980). Post mortem pH has direct importance to the suitability of fish muscle

as a food since the lowering of the pH has been found to greatly affect the strength of the myocommata, causing a phenomenon called "gaping", where the muscle tends to fall apart. The lowering of tissue pH not only causes "gaping", hence rendering it unsuitable for processing, but also causes the muscle to be tough when cooked and unsuitable for freeze storage. The post mortem pH of cod has been reported to drop during the early summer and has been related to overshoots in the replacement of carbohydrate stores during refeeding in the wild populations. The experiments in this thesis have also reported overshoots in carbohydrate stores and although no studies (to the authors knowledge) have reported tissue glycogen or post mortem pH of white muscle in the wild saithe populations, it is likely that during the early summer saithe muscle may also be rendered unsuitable for processing for the same reasons as cod.

The precise mechanisms involved in dismantling the contractile apparatus during fibre atrophy remain unclear. However there are many observations in this thesis that may help to elucidate some of the processes involved and lead to suggestions for further experimentation. The involvement, or changes in the s.r.and t-system during fibre atrophy have been reported in the majority of studies concerned with muscle breakdown, and the presence of acid hydrolases within the s.r. and t-tubules has interesting implications. The formation of primary lysosomes, or membranes for primary lysosomes, from the t-tubule implies that there must be a time when the excitation-contraction coupling system becomes redundant (i.e. when the t-tubule becomes separated from the sarcolemma). At this point the sarcomere would no longer retain contractile properties and rapid degradation of the contractile proteins could proceed without further detrimental effects to the performance of the sarcomere. Indeed this scenario may explain the degradation of sarcomeres to differing extents along the same myofibril. Similarly can the s.r. maintain its normal functions during severe local atrophy ? As atrophy proceeds the s.

r. becomes progressively more dilate, the terminal cisternae of one sarcomere often fusing with the neighbouring terminal cisternae and possibly with the t-tubule. Further experimentation into the normal functions of the s.r. (i.e. Ca^{++} regulation) during the course of a severe atrophy (possibly by measuring the influx of $^{45}Ca^{++}$ into isolated s.r. (see McArdle & Johnston, 1980)), may help to clarify whether the s.r. maintains its Ca^{++} regulatory functions even when fragmented and distended. It is possible that different lysosomal vesicles and indeed sections of the s.r. may contain different complements of acid hydrolases (Topping & Travis, 1974), and therefore possibly maintain specialised local functions during fibre atrophy (for instance the vesicle observed above the M-line may be specifically involved in the degradation of the M-line and thick filaments). Further cytochemical or immunohistochemical localisation of other hydrolases may help to clarify not only the involvement of the s.r. in myofibrillar degradation, but also whether distinct populations of vesicles exist during muscular atrophy in saithe.

The internal pH of s.r. in normal muscle is approximately neutral (Martonosi, 1984). At this pH acid hydrolases would only be partially active. It is therefore evident that if the s.r. or t-tubule forms or becomes lysosomal in nature then the internal pH must become more acidic. The internal pH of a lysosome is thought to be maintained primarily via a Donnan equilibrium across the membrane. Because of the restricted permeability of the membrane to macromolecules, substantial amounts of negatively charged compounds (glycoproteins and phospolipids) will be contained within the vesicle, causing a passive accumulation of protons, and hence acidic internal pH (see Dean, 1978). In addition to this, intralysosomal hydrolysis may generate protons, and it has also been suggested that a proton pump may be in operation (Schneider & Cornell, 1978). It is therefore possible that during the formation of a vesicle from the s.r. or t system, an acidic internal pH may be created passively via a Donnan equilibruim and hence hydrolytic

activity immediately increased further reducing the pH towards that for optimal hydrolase activity.

Ca^{++} activatated neutral proteinase (CANP) and increases in cellular Ca^{++} have both been implied in the possible control and initiation of breakdown of myofibrillar proteins (Dayton et al, 1975, 1980 ; Goll et al, 1978). CANP is thought to be especially active in pathological and necrotic muscle where plasma membranes may be disrupted, allowing intracellular Ca^{++} to equilibriate with extracellular Ca^{++} (Varley & Dhalla, 1973 ; Cullen et al, 1979 ; Cullen & Fulthorpe, 1982). It has been suggested, though not proven, that CANP may be responsible for dismantling the myofibril and fragmenting myofibrillar proteins to monomers or lower molecular weight compounds. It is of interest to note that the CANP purified from mammalian and bird skeletal muscle (Busch et al, 1972: Dayton et al, 1976 ; Goll et al, 1978 ; Dayton et al, 1980) is capable of releasing α-actinin from the Z disc and degrading C protein, tropomyosin, troponin T and troponin I, but not myosin heavy chain , actin, or troponin C. Conversely the lysosomal enzymes reported in this thesis were capable of degrading myosin heavy chain and actin, but were relatively ineffective at degrading tropomyosin. The monomers of actin and myosin and the lower molecular weight fragments released from the dissembly of the myofibril, may therefore be degraded to amino acids within the lysosomal system (see, for instance, Dayton et al, 1975). Furthermore previous authors have suggested that the accumulation of degradation products created by the extralysosomal autolysis of the contractile proteins, may trigger the formation of lysosomes and autophagic vacuoles (Schiaffino & Hanzlikova, 1972 ; Cullen & Pluskal, 1977 ; Cullen et al, 1979). This hypothesis is consistent with the possible mechanisms involved in lowering the pH of the s.r. and t-tubule (suggested above), as when intralysosomal hydrolysis is initiated (perhaps by the presence of sub-units or myofibrillar fragments) then the protons generated will lower the pH and hence further enhance hydrolytic

activity. However, CANP has not been reported in fish and indeed the only neutral proteinase purified to date is not only unaffected by Ca^{++}, but also incapable of degrading any myofibrillar proteins (Makinodan,1979). Other cytosolic proteases may be present in fish muscle to substitute the action of mammalian CANP, perhaps even cathepsin L which is also capable of dismantling the Zand M-lines in intact myofibrils from rabbit (Matsukura et al, 1984). Clearly much ground work is yet to be done concerning fish muscle proteinases and whilst fish muscle atrophy exhibits many features incommon with mammalian and bird muscle, distinctive differences do appear to exist.

It is well documented that there is a large heterogeneity in the turnover rates of proteins, with half-lives ranging from minutes to days (Schmike, 1975). It is also well established that for most proteins studied, a correlation exists between the sub-unit size/s and the relative turnover rates (larger sub-units have faster turnover rates). This relationship, however, does not hold for all proteins, and is much reduced or even absent in muscle proteins during starvation in rat (Dice & Walker, 1978). During starvation Dice and Walker (1978) reported a selective enhancement in the turnover rate of the more stable proteins with smaller sub-units, and suggested that the degradative processes involved in muscle during starvation may be fundamentally different to those in normal muscle. They proposed that the proteins themselves may become more susceptible to degradation or the activity of a selected part of the degradative apparatus may increase. Both these factors may be regulated by the concentration of co-factors (for instance Ca^{++}) , membrane lipids, metabolic intermediates, ligands, and other proteins (whose concentrations will all change according to the metabolic condition of the cell) either affecting the conformational state of a protein (possibly exposing particularly labile peptide bonds) or the activity of proteolytic enzymes.

From the evidence reported in this thesis it is clear that during

starvation the changes in the relative abundance of myosin heavy chain (myosin H.C.) and actin are consistent with myosin H.C. having a higher turnover rate than actin, as reported in mammalian muscle (Koizumi, 1974). However the turnover rates of individual myofibrillar proteins have not been studied in fish. If the order of turnover rates for saithe myosin H.C. and actin are the same as in rabbit skeletal muscle, then clearly the changes in relative abundance between these two proteins could be attributed to a simple change in the normal rates of synthesis and/or degradation. Further investigation relating the turnover rates of the individual myofibrillar proteins in fish, combined with a more detailed study of the changes in the relative abundances of all the myofibrillar proteins during atrophy, would help elucidate the processes involved in myofibrillar degradation. The fundamental question yet to be answered is whether muscle atrophy during starvation in fish, simply results from a gross change in the normal rates of protein synthesis/degradation or whether selective processes are involved to enhance or inhibit the turnover rates of specific proteins.

The involvement of the cytoskeleton in muscle fibre regeneration and growth has recieved little attention. The research in this thesis has described the ultrastructural events and temporal sequences involved in myofibrillar assembly after degradation. Clearly from these observations the cytoskeleton may play an important role in co-ordinating the assembly of the myofibrils. There is therefore much scope for further investigation, possibly by immunohistochemical studies, into the functions of the cytoskeleton during both fibre dissembly and assembly.

In conclusion the research in this thesis has highlighted the benefits of employing a diversity of techniques to study starvation and refeeding and has generated many areas for continued investigation. The ultimate initiation and control of muscle breakdown, be it neuronal, nutritional, hormonal or otherwise has yet to be elucidated. During starvation in humans there is a

preferential loss of red fibres, so clearly there are differences in the control of muscle breakdown between fish and man. The general mechanisms involved in the degradation of muscle are similar both in fish and mammals, however there does appear to be subtle differences in the enzymes and mechanisms involved that warrant further investigation. Further research into the involvement of the lysosomal system under normal cellular conditions, perhaps by following the fate of radio-labelled proteins (similar to the study of Gerard and Schneider (1979)), may also be of value to clarify and compare the proteolytic mechanisms involved in basal protein turnover. Other areas that warrant further investigation (not mentioned above) may include a more detailed study of the intermediary metabolism (including plasma amino-acid levels) during both starvation and refeeding, monitoring plasma hormone levels (for instance insulin, glucagon, cortisol, and growth hormone), the purification and characterisation of lysosomal and neutral cytosolic proteinases, monitoring of the changes in pH of intracellular compartments during starvation (perhaps using flourescent pH sensitive dyes), and investigating the changes in gross protein turnover rates in the separate fibre types during starvation and refeeding. Finally an important aspect of the reseach in this thesis, of possible commercial importance, may be to relate the adaptations to starvation and refeeding reported under laboratory conditions to the seasonal adaptations found in the wild populations.

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