# THE EFFECTS OF TEMPERATURE ACCLIMATION ON THE EXPRESSION OF CONTRACTILE PROTEIN ISOFORMS IN THE SKELETAL MUSCLE OF THE COMMON CARP (CYPRINUS CARPIO)

Tony Crockford

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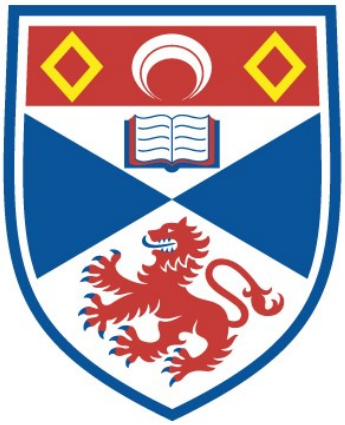
at the
University of St Andrews



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# THE EFFECTS OF TEMPERATURE ACCLIMATION ON THE EXPRESSION OF CONTRACTILE PROTEIN ISOFORMS IN THE SKELETAL MUSCLE OF THE COMMON CARP (Cyprinus carpio)

Submitted for the degree of PhD. at the University of St. Andrews by;

Tony Crockford

## DECLARATION

I hereby declare that the research reported in this thesis was carried out by me and that the thesis is my own composition. No part of this work has been previously submitted for a higher degree.

The research was conducted in the Department of Biology and Preclinical Medicine, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Prof. I. A. Johnston.

## CERTIFICATE

I hereby certify that Tony Crockford has spent eleven terms engaged in research work under my direction, and that he has fulfilled the conditions of General Ordinance No. 2 (Resolution of the University Court No. 1, 1967) and that he is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

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## SUMMARY.

Chapter 1.

Part A reviews the current knowledge of temperature acclimation in teleost fish,with particular emphasis on skeletal muscle. There appear to be two types of response to low temperatures, dormancy or a homeostatic response. The homeostatic response serves to compensate for the reduced reaction rate usually seen at lower temperatures. In some species both responses occur depending on the water temperature.

Part B reviews polymorphism in muscle proteins. All the myofibrillar proteins have been shown to exist as isoforms, which are differentially expressed in muscle types and with development. The isoforms expressed appear to be related to the contractile properties of the muscle.

Chapter 2.

The parvalbumin content, isoforms, and calcium binding characteristics were studied in the white muscle of 5° C and 25° C acclimated carp (Cyprinus carpio). The total parvalbumin concentration was 0.61–0.68mmols/kg wet weight. Two calcium binding sites per molecule and a dissociation constant of $2.1–2.4 \times 10^{-6}$ M were measured. No differences related to acclimation temperature were observed.

Chapter 3.

The myofibrillar ATPase from white muscle of 8° C and

20° C acclimated carp (Cyprinus carpio) was shown to increase in cold-acclimated fish at both high and low assay temperatures. Electrophoretic analysis of the myofibrillar proteins showed a unique myosin light chain isoform to be present in cold-acclimated fish, and a unique troponin I isoform to be present in warm acclimated fish. The presence of tropomyosin and troponin T isoforms in carp white muscle was also noted.

The (MLC3 + extra MLC):MLC1 ratio was found to be lower in cold- than in warm-acclimated fish

Chapter 4.

Myosin heavy chains and actin from the white muscle of carp (Cyprinus carpio) acclimated to 2° C, 5° C, 8° C, 11° C, 15° C, and 23° C were studied by peptide mapping. No differences were found between fish from any of the acclimation temperatures for either protein.

Chapter 5.

The major findings of the study are discussed, in relation to the mechanisms that produce protein isoforms, and with reference to further studies.

# CHAPTER 1.

## GENERAL INTRODUCTION

### A; TEMPERATURE ACCLIMATION IN TELEOST FISH.

The rate of a chemical reaction is usually directly related to temperature, and in fish a decrease in water temperature leads to a corresponding decrease in body temperature. In temperate fish, low water temperature leads to either dormancy or homeostatic responses which compensate for the change in temperature with a time course of less than a second to more than a month. In some species both responses may occur, depending on the water temperature.

#### Whole animal metabolism.

Oxygen consumption, may be used as an estimate of total energy production, providing there is no net input from anaerobic pathways, which is generally true for levels of activity that can be sustained for long periods. A number of measures of metabolic rate are commonly used (Fry, 1957; Doudoroff & Shumway, 1970). Standard or basal metabolic rate represents the oxygen uptake of animals in the post absorptive state and in the absence of activity. This provides an estimate of the maintenance energy requirement but is difficult to measure and interpret. Routine metabolic rate is more easily measured and is the basal metabolic rate

plus any contribution from spontaneous activity. The active metabolic rate is the oxgen consumption at maximum sustainable swimming speed. Sustainable swimming speeds are taken as those that can be maintained for 30-60mins (Brett, 1972). The aerobic scope for activity is the difference between the active and standard metabolic rate.

Locomotion in antarctic fish continues at temperatures below 0° C, but in most tropical species 15° C is the minimum temperature for locomotion. Species found in similar water temperatures, but with different activity patterns and body shape may exhibit variation in active metabolic rates. For example, the active metabolic rate of the sockeye salmon (Oncorhynchus nerka) (Brett, 1964) is 5-times that of the lemon sole (Microstomus kitt) (Duthie & Houlihan, 1982). These genetically fixed differences between species must be taken into account when the effects of temperature on metabolic rate are studied.

In the sockeye salmon (Oncorhynchus nerka) acclimation from 5 to 15° C produces a 4- fold increase in basal metabolic rate and a 2-fold increase in active metabolism, compared with a 15- fold difference between basal and active metabolic rates. Above 15° C the active rate declines and results in lower aerobic scope for activity (Brett, 1964). Largemouth bass (Micropterus salmoides) is a warmer water species and the aerobic scope increases with acclimation

temperatures between 15 and 30° C, but is lower at 35° C than at 30° C (Beamish, 1970). The temperature at which the aerobic scope starts to decrease is possibly the point at which excessive energy demands of ventilation and circulation restrict increased oxygen supply to the tissues (Jones, 1971).

Acclimatory responses are usually most marked in species that live in environments that exhibit a large seasonal variation in temperature. In the low water temperatures of winter, some species cease feeding and become relatively inactive, and are thought to enter a dormant state (Crawshaw, 1984). This is characterized by extremely low metabolic rates that spare food reserves until prey numbers and water temperature increase in the spring, as seen in the american eel Anguilla anguilla (Walsh et al. 1983). In other species, cold-acclimation, occuring over several weeks, results in improved swimming performance at low temperatures relative to that of fish acutely exposed to low temperatures. Both the above responses occur in acclimated largemouth bass (Micropterus salmoides), a dormant state is entered below 7° C while over the range 7-30° C a similar level of spontaneous activity is maintained (Lemons & Crawshaw, 1985). In the same species feeding was found to be inhibited at 10° C, which is higher than is required to suppress locomotion (Lemons & Crawshaw, 1985)

The standard and active metabolic rates of the goldfish (Carassius auratus) both show significant temperature compensation responses (Kanungo & Prosser, 1959; Beamish & Mookherjii, 1964; Fry & Hochachka, 1970). However even in fully acclimated individuals standard and active metabolic rate decrease with a $Q_{10}$ of 1.6–2.0 as water temperature is reduced (Fry & Hochachka, 1970).

Temperature acclimation in fish muscle.

Sustained swimming activity is largely supported by the recruitment of a relatively small volume of aerobic slow muscle fibres (Bone. 1978; Johnston, 1981). As speed increases there is a sequential activation of slow > fast oxidative > fast glycolytic motor units (Johnston, Davison & Goldspink, 1977). Reduction of water temperature from 20° C to 10° C decreases the recruitment threshold for fast glycolytic fibres from 2.6 to 1.4 bodylengths/s (Rome, Loughna & Goldspink, 1984). This suggests that at 10° C the power necessary to sustain swimming speeds above 1.4 bodylengths/s cannot be provided by the slow oxidative fibres alone. However following acclimation to 8° C the recruitment threshold speed for fast glycolytic fibres has been shown to increase (Rome, Loughna & Goldspink, 1985). Cold acclimated carp have been shown to be able to swim faster with their aerobic muscle and have higher sustained swimming speeds than acutely cooled fish (Rome et al. 1985).

Changes in swimming performance and central patterns of muscle fibre recruitment with cold-acclimation are associated with a major remodelling of the skeletal muscle. The fractional volume of aerobic fibre types is significantly higher in cold- than warm-acclimated striped bass (Jones & Sidell, 1982), and goldfish, 1700 fast oxidative glycolytic fibres/myotome for fish acclimated to 31° C compared withover 3000 for fish acclimated to 3° C (Johnston & Lucking, 1978).

The activity of aerobic enzymes, when assayed at an intermediate temperature, has been shown to be higher in cold- than warm-acclimated fish in a variety of species, including goldfish (Smit et al. 1974; Sidell, 1980), green sunfish (Shaklee et al. 1977), and common carp (Johnston, Sidell & Driedzic, 1985). This in part results from the higher density of mitochondria in the muscles of cold-acclimated fish. Mitochondria occupied 25% of slow fibre volume in crucian carp acclimated to 2° C, compared with only 14% for fish maintained at 28° C (Johnston & Maitland, 1980), and in the slow muscle of goldfish the mitochondrial diffusion path was found to be 23% shorter in cold- than in warm-acclimated fibres (Tyler & Sidell, 1984). Higher aerobic enzyme activities have been reported in muscle mitochondria isolated from cold- than warm-acclimated fish (Hazel, 1972; Wodtke, 1981). However, the presence of a

similar amount of cytochrome c oxidase/mg of protein in mitochondria isolated from the slow muscle of cold- and warm-acclimated carp (Wodtke, 1981), and stereological analysis of the surface density of the inner mitochondrial membrane in cold- and warm-acclimated goldfish (Tyler & Sidell, 1984), suggest that cold-acclimation does not change the density of enzymes on the inner mitochondrial membrane. Changes in mitochondrial enzyme activity could occur as a result of the changes in membrane phospholipid composition that accompany temperature acclimation (Hazel, 1972; Van den Thillart & Modderkolk, 1978).

In crucian carp, cold-acclimation has been shown to result in relatively higher surface and volume densities of muscle capillaries (Johnston, 1982b), and a higher mitochondrial volume density (Johnston & Maitland, 1980). These results taken together suggest an increase in the potential for aerobic ATP production in the muscle of low temperature acclimated fish relative to acutely exposed fish. Assayed at intermediate temperatures the rate of oxygen utilization of muscle fibres is higher for cold- than warm-acclimated fish. This is likely to result in a higher active metabolic rate and an increase in the range of swimming speeds that can be sustained at low temperatures (Fry & Hochachka, 1970; Rome et al. 1985).

The power to swim at maximum speed is mainly derived

from the recruitment of the fast muscle system (Johnston, 1981). Sprint activity is initially fueled by the hydrolysis of phosphocreatine stores, rapidly followed by the activation of glycogenolysis resulting in the accumulation of lactic acid. In contrast to the results for aerobic enzymes, the activities of glycolytic enzymes in muscle are generally unchanged by temperature acclimation (Shaklee et al. 1977; Sidell, 1980, Johnston et al. 1985).

The maximal enzyme activities in tissues from fish acclimated to 5 and 25° C in the laboratory have been compared with those from winter and summer acclimatized fish from the wild (Kleckner & Sidell, 1985). It was found that the general pattern of metabolic response to temperature was similar in acclimated and acclimatized fish, although the enzyme activity tended to be higher in the laboratory held rather than the wild fish. An exception was creatine phosphokinase activity which increased in the slow muscle of the winter acclimatized compared with summer acclimatized fish but did not change between acclimated fish. The opposite result was found for creatine phosphokinase in fast muscle, and it was suggested that the differences were related to different swimming behaviour between wild and laboratory fish.

The myofibrillar ATPase activity of fast muscle fibres in goldfish was found to increase at low temperatures

following cold-acclimation (Johnston, Davison & Goldspink, 1975). The ATPase isolated from cold-acclimated goldfish was more susceptible to thermal denaturation than the ATPase from warm-acclimated fish (Johnston et al. 1975). This was the first demonstration of re-modelling of myofibrillar proteins of fish following cold-acclimation, and similar changes have now been reported in slow fibres in goldfish (Sidell, 1980), and the fast muscles of common carp, tench, and roach (Heap et al. 1985).

The mechanical properties of single demembranated fast fibres from warm- and cold-acclimated common carp, assayed at 7° C, have shown that following cold-acclimation, fibres develop twice the tension and have twice the unloaded contraction velocity of fibres from warm-acclimated fish (Johnston et al. 1985). Starvation, leading to very low levels of protein synthesis, prevents this acclimatory response (Heap, Watt & Goldspink, 1986).

The thermal characteristics of the myofibrillar proteins in some eurythermal species do not appear to be altered by acclimation, brook trout (Walesby & Johnston, 1981), rainbow trout (Penney & Goldspink, 1981), and flounder (Johnston & Wokoma, 1986) are examples.

The energy stores in muscle are also affected by temperature acclimation, more than double the glycogen concentration being found in muscle fibres from 2° C than

28° C acclimated crucian carp (Johnston & Maitland, 1980). The volume density of lipid droplets in slow muscle of the striped bass was found to be 15-times higher in cold- than warm-acclimated fish (Egginton & Sidell, 1986).

B; POLYMORPHISM OF MYOFIBRILLAR PROTEINS.

Skeletal and cardiac muscle systems exhibit a very wide range of variation in their physiological properties, however the basic elements of contraction, the myosin thick filaments and actin thin filaments, are very similar in all muscles. The variation that exists is achieved by the expression of different isoforms of the myofibrillar proteins. Isoforms are defined as proteins that differ in their primary sequence, but their secondary, tertiary and quaternary structures are similar. Isoforms of a protein perform the same role, however their biological activities may vary.

Myosin.

Myosin exists as a polymeric protein of molecular weight about 460,000, consisting of two heavy chains (MHC) of molecular weight about 200,000, two P-light chains which may be phosphorylated (MPLC), and two alkali light chains (MLC).

Myosin heavy chain (MHC) isoenzymes.

The myosin heavy chains have two distinct functional regions, the C-terminal half of the molecule is the region that forms the central filament of the thick filaments, the N-terminal half of the molecule forms the head that protrudes from the thick filaments, and contains the site of ATPase activity and site of interaction between the myosin

and actin molecules. The general structure of the MHC has been found to highly conserved (Kavinsky et al. 1983), but numerous isoforms of the MHC have been shown to exist, see Swynghedauw (1986) for a complete review.

In skeletal muscle there appear to be four major MHC isoforms, two developmental (embryonic and neonatal) and two adult (fast and slow), (Whalen, 1985). Fast MHCs have been further divided into those found in white fast IIB fibres, MHCfB, and those found in red fast IIA fibres, MHCfA (Salviati, Betto & Betto, 1982). The two fast MHCs have been found to coexist in single fibres of the rabbit (Staron & Pette, 1987b). Slow MHCs have also been subdivided into MHCs1 and MHCs2, and in chickens the two types of slow MHC coexist, with the MHCs1 isoenzyme being predominant following hatching (Rushbrook & Stracher, 1979). Isoenzymes of MHC have been shown in cardiac muscle, a homodimer of alpha-MHC is found in the atrium, while the ventricle contains both alpha-MHC and beta-MHC (Hoh et al. 1978). Molecular cloning data (Lompre et al. 1984), indicate that ventricular beta-MHC is identical to slow muscle MHC, and is encoded by the same gene. In adult rat ventricle three myosin isoforms have been found, two homodimers of alpha-alpha-MHC and beta-beta-MHC, and a heterodimer of alpha-beta-MHC (Hoh et al. 1978). An additional MHC has been reported in the superfast-contracting cat jaw muscle, which

differs from both fast and slow MHC (Rowlerson et al. 1981).

It would appear that there are at least eight MHCs isoforms, and the evidence that rat ventricular myosin exists as a MHC heterodimer may require the re-examination of previous results. Where the MHC content of a single fibre was thought to be homogeneous, further isoforms of MHC may be found.

Myosin light chain (MLC) isoenzymes.

Two classes of myosin light chain are found in skeletal and cardiac muscle, the P-myosin light chains (MPLC) which can be removed from myosin by reaction with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) without loss of ATPase activity, and the alkali myosin light chains (MLC) that cannot be removed from the myosin molecule without the loss of ATPase activity (Perrie et al. 1973). The myosin light chains are identified by a number which corresponds to their migration on SDS polyacrylamide electrophoresis, the less mobile having the lower number.

Fast muscle contains contains three myosin light chains, MLC1f, MPLC2f, and MLC3f, of approximate molecular weights 25,000, 18,500, and 16,500 respectively. A fast myosin molecule will contain two MPLC2f, and either two MLC1f, two MLC3f or one MLC1f and one MLC3f. This allows three isoforms of fast myosin with the homodimer of fast MHC to exist, which may be seen by pyrophosphate gel

electrophoresis where fast muscle myosin runs as three distinct bands (Lannergren, 1987). Slow muscle also contains three myosin light chains, MLC1s, MLC2s, and MPLC3s, of approximate molecular weights 27,500, 26,500, and 17,500 respectively, which, as in fast muscle, allows three isoforms of slow myosin to exist due to variation in the light chain content. The MWs of MLC1s and MLC2s are very similar, 27,500 and 26,500 respectively, which leads to broad banding patterns on pyrophosphate gel electrophoresis, and, as yet, only two adult slow myosin isoforms have been detected in mammalian muscle (Staron & Pette, 1987b)

In cardiac muscle four light chains have been described, two in ventricular muscle MLC1v and MPLC2v, approximate molecular weights 27,500 and 19,000, and two in atrial muscle MLC1a and MPLC2a, approximate molecular weights 25,000 and 20,500 (Sartore et al. 1978; Hoh et al. 1978). In rat, MLC1v appears to be similar or identical to slow muscle MLC1s (Hoh et al. 1978).

During development, fetal ventricles and atria contain a specific light chain MLCemb that is similar if not identical to MLC1a and is probably a similar or identical light chain to that found in fetal skeletal muscle (Whalen et al. 1983). This light chain appears to be expressed in many tissues during development but only in the atrium in the adult.

Myosin isoenzymes.

Isoenzymes of the myosin molecule are called isomyosins. The existance in both fast and slow muscle of two MHCs and three MLCs (Staron & Pette, 1987a; Billeter et al. 1981), means that nine fast and nine slow isomyosins could exist. However, possibly due to the difficulty in resolving such similar molecules by electrophoresis under non-denaturing conditions, only four adult fast and two adult slow isomyosins have been detected (Hoh & Yeoh, 1979; Pinter et al, 1981; Lannergren, 1987). The study of single fibres by electrophoresis under denaturing conditions has demonstrated the coexistence of fast and slow myosin subunits in single fibres (Salviati et al. 1982, 1983; Reiser et al. 1985; Staron & Pette, 1987a, 1987b), and this suggests that at least 60 theoretical isomyosins may exist (Staron & Pette, 1987b).

Actin.

Actin is the most strongly conserved of all the myofibrillar proteins and is the only protein for which no isoforms have been found in fast and slow skeletal muscle (Vandekerckhove & Weber, 1979). Only 5 of the total 375 amino acids vary between rabbit slow and bovine cardiac muscle (Vandekerckhove & Weber, 1979). This relative homology in structure suggests that the role of actin is very similar in all muscle types and that alterations in the

biochemical properties of thin filaments are due to the presense of isoforms of the calcium regulatory proteins (Dhoot & Perry, 1979), and not isoforms of the actin molecule.

Tropomyosin (TM).

Tropomyosin molecules are dimers of two subunit each with a molecular weight of about 33,000. Two isoforms of TM were first described and named alpha-TM and beta-TM (Cummins & Perry, 1973) and were shown to differ at 39 amino acid sites (Mak et al. 1980) leading to a small variation in electrophoretic mobility on SDS PAGE, the beta-TM being the less mobile. In general, fast skeletal muscle, contains both alpha-TM and beta-TM, while slow skeletal muscle was thought to contains only beta-TM, until two additional iosforms of tropomyosin, gamma-TM and delta-TM were found to be present in the soleus muscle of rabbit (Heeley et al. 1983), the four isoforms of TM may be resolved by the use of two dimensional electrophoresis. It is not known if the TM dimer can exist as a heterodimer, as well as a homodimer, of any of the four isoforms. Tropomyosin is not required for the ATPase activity of purified actin and myosin (Lehman & Szent-Gyorgyi, 1972) but TM has been shown to be essential for the $Ca^{2+}$-sensitivity of $Mg^{2+}$ ATPase activity of actomyosin in the presense of the troponin complex. Little functional variation between the isoforms of TM has been

shown. Variation between tissues is possibly related to the interaction between TM, and the troponin complex expressed in the muscle type. There is evidence for differences in the binding of troponin-T to the alpha-TM and beta-TM isoforms (Perry, 1985). Phosphorylation of the TM isoforms is higher in foetal muscle than in adult muscle, suggesting a possible role for posphorylation of TM in development rather than in the regulation of contraction (Heeley et al. 1985).

Troponin complex.

Isoforms of all the components of the troponin complex are found. In most species a single isoform exists which is characteristic of either fast, slow, or cardiac muscle.

Troponin-C (TN-C).

Fast and slow isoforms of troponin-C, the $Ca^{2+}$-binding component of the troponin complex, are found in skeletal muscle. Cardiac TN-C has been shown to have the same amino acid sequence as slow muscle TN-C. Fast TN-C contains two $Ca^{2+}$ specific binding sites, while slow and cardiac TN-C contain only one $Ca^{2+}$ specific binding site (Potter & Johnson, 1982). The filling of the $Ca^{2+}$ specific binding sites is considered necessary to trigger contraction, thus fast muscle would require the uptake of two $Ca^{2+}$ per TN-C molecule to activate contraction rather than the one required by slow or cardiac muscle (Perry, 1985). Although cardiac and slow TN-C possess the same amino acid sequence,

they may exhibit different $Ca^{2+}$ binding characteristics *in situ* due to interactions with their respective troponin-I.

Troponin-I (TN-I).

Troponin-I is the inhibitory component of the troponin complex which inhibits the ATPase activity of desensitized actomyosin (Wilkinson *et al* 1972). Fast, slow and cardiac muscle each contain a specific TN-I isoenzyme, and sequence differences involving up to 40% of the amino acid sites have been shown between the three isoforms. However, the two sites involved in interactions with TN-C and actin have been shown to be virtually identical in all three isoforms in rabbit muscle (Wilkinson & Grand, 1978). Phosphorylation of fast and slow TN-I at two sites has been observed *in vitro*, however the extent of phosphorylation, and its possible role in modification of the action of TN-I *in vivo* is unknown (Perry *et al*. 1975). Cardiac TN-I however has an additional 26 amino acids at its N terminal. This region of cardiac TN-I contains a serine residue at site 20 which is rapidly phosphorylated by cAMP-dependant kinase (Cole & Perry, 1975), and when isolated immediatly after death, cardiac TN-I possesses much more phosphate per molecule than does skeletal TN-I (Perry & Cole, 1974). In normally beating rabbit heart the serine 20 region is about 30% phosphorylated and on beta-adrenergic stimulation this rises to approach 100%. Phosphorylation of the serine 20 site of

cardiac TN-I increases the $Ca^{2+}$ concentration required for 50% activation of actomyosin ATPase, and the phosphorylation is reversible and returns to normal following beta-adrenergic stimulation (Perry, 1983, England, 1983). This suggests that phosphorylation of TN-I may play an important role in the expressed activities of the various isoforms of TN-I, especially cardiac TN-I. Modification of the $Ca^{2+}$ binding characteristics of the troponin complex may be dependent on interactions between TN-I and TN-C isoforms and not just on the TN-C isoform present.

Troponin-T (TN-T).

Troponin-T is the tropomyosin binding component of the troponin complex, it also binds TN-C in the presense of calcium (Ebashi et al. 1972).

TN-T exists as isoforms that are specific for fast, slow and cardiac muscle. In chicken fast muscle, three isoforms of TN-T have been observed. One (MW 33,500) is present in breast muscle, two (MW 30,500 and 29,800) are present in the leg muscle (Wilkinson et al. 1984), and in rabbit skeletal muscle two fast and two slow isoforms of TN-T have been observed. The two fast TN-T isoforms (TN-T1f and TN-T2f) appear to be associated with different dimers of TM, TN-T1f with the alpha-beta-TM heterodimer and TN-T2f with the alpha-alpha-TM homodimer (Schachat et al. 1985).

Combinations of myofibrillar protein isoforms in muscle fibres.

From the study of single fibres, electrophoretically (Young & Davey, 1981, Pette & Schnez, 1977), histochemically and electrophoretically (Salviati et al. 1982, 1983, Staron & Pette, 1987a, 1987b), their contractile properties and electrophoretically (Reiser et al. 1985, Lannergren, 1987), there appear to be five basic fibre types. The nomenclature used varies, but the most commonly used is; Type I the red slow-twitch fibre, Type IIB the white fast-twitch fibre, Type IIA the red fast-twitch fibre, Type IIC the intermediate fast-twitch fibre, and Type IC the intermediate slow-twitch fibre.

The general myofibrillar content of the various fibre types in adult skeletal muscle based on their histochemical properties appears to be;

Type I fibres (ATPase staining after preincubation at pH 4.3 and 4.5 high, and at pH 9.6 low) contain slow MHC, MLC1s, MLC2s, MPLC3s, slow troponins I, C, and T, and predominently beta-tropomyosin.

Type IIB fibres (ATPase staining after preincubation at pH 4.3 and 4.5 low, and at pH 9.6 high, and have a low glycogen content) contain fast MHCfB, MLC1f, MPLC2f, and MLC3f, fast troponins I, C, and T, and a higher proportion of alpha-tropomyosin than the type I fibres.

Type IIA fibres (ATPase staining after preincubation similar to type IIB fibres, but with a high glycogen content) contain MHCfA, MLC1f, MPLC2f, MLC3f, and MLC2s, the troponin complex and the tropomyosin proportions are as found in type IIB fibres.

Type IC fibres (ATPase staining present after preincubation at pH 4.3, 4.5, and 9.6, but the pattern of staining resembles that of the type I fibres) contain combinations of all the myofibrillar proteins with a predominance of the slow isoforms.

Type IIC fibres (ATPase staining present after preincubation at pH 4.3, 4.5, and 9.6, but the pattern of staining resembles that of the type II fibres) contain combinations of all the myofibrillar proteins with a predominance of the fast isoforms.

The overall scheme appears to be that in slow muscle type I fibres predominate, and in fast white muscle type IIB fibres predominate. However in intermediate muscles, and in muscle types previously thought to be of purely type I or type IIB, a continuum of expression of the myofibrillar proteins is found (Salviati et al. 1982; Schachat et al. 1985; Lannergren, 1987; Staron & Pette, 1987a, 1987b; Greaser et al. 1988).

Developmental changes in myofibrillar protein isoforms.

During development different isomyosins are found in

skeletal muscle: embryonic and neonatal myosins (containing embryonic MHC, neonatal MHC, and embryonic MLC) were found to be different isomyosins to adult fast and slow myosins (Hoh & Yeoh, 1979; Rushbrook & Stracher, 1979; Whalen et al. 1979, 1981). In rat and human muscle the sequential appearence and disappearence of two neonatal isomyosins during development (Fitzsimons & Hoh, 1981, Bandman et al. 1982) has lead to the following sequence of isomyosin expression being proposed (Whalen, 1985). Soon after their formation muscle fibres contain an embryonic myosin, which is replaced by a neonatal myosin. Following birth the adult myosin forms become established as the predominant forms.

There is little evidence for developmental changes associated with myofibrillar proteins other than myosin. It has been shown that the beta-beta-TM homodimer may exist in early development (Dabrowska et al. 1977), and it is proposed that during development the proportion of alpha-TM increases until it reaches the ratio of alpha-TM to beta-TM found in the adult muscle (Amphlett et al. 1976). In the rat embryo presumtive fast muscle was found to stain with fast troponin antibodies only, while presumptive slow muscle stains with both fast and slow troponin antibodies. The staining of slow muscle by fast troponin antibobies progressivly decreases until no staining is observed 12 days after birth (Dhoot & Perry, 1980).

The presense of neonatal and adult myosins in the same muscle fibre of the rat (Butler-Brown et al. 1982) suggests that the mechanism for the transformation from neonatal to adult muscle involves the replacement of neonatal myosin with adult myosin within existing muscle fibres, rather than the degeneration of neonatal type muscle fibres and their replacement with adult fibres.

Transformations between fast and slow fibre types.

The innervation of a muscle is an important determinant of the isoforms of myofibrillar proteins expressed. Cross innervation of one fibre type by nerves from another type, or direct stimulation of the muscle at the appropriate frequency has been shown to induce full or partial transformation between fibre types. The myosin, troponin and tropomyosin isoforms present are those appropriate to the muscle type normally innervated by the nerve used. These transformations have been shown for myosin (Buller et al. 1969; Barany & Close, 1971; Weeds et al. 1974; Salmons & Streter, 1976), components of the troponin complex (Amphlett et al. 1975; Dhoot et al. 1981), and tropomyosin (Heeley et al. 1983).

The role of myofibrillar protein isoforms in muscular responses to exercise are not well understood. However in rat under an extensive exercise programme, a proportional increase of type I slow fibres, from 10% to 27%, in fast

type II muscles has been shown. Also, the number of type IIB fibres decreased, the number of type IIA fibres increased, and in the fast muscles an increased slow myosin light chain proportion was found (Green et al. 1984). Conversion of fibre type has also been demonstrated in young rats by subjecting them to different levels of activity (Watt et al. 1984). However as changes in isoforms of the myofibrillar proteins occur during development normally, it is uncertain whether the changes seen in young rats were due to replacement of the fibre type within the muscle, or a change in the innervation of the muscle, which led to an altered developmental sequence being expressed (Goldspink & Ward, 1979; Goldspink, 1985).

Genetics of isoforms.

Isoforms of a protein may be produced in a various ways. The isoforms may result from the transcription of different genes. The rat has seven HMC genes that have been cloned. These genes encode for embryonic, neonatal, extraocular, fast IIA, fast IIB, ventricular/atrial, and ventricular/slow MHC isoforms. The isoforms are produced by the selective RNA transcription of these genes, in different muscles, and at different development stages (see Emerson & Bernstein, 1987, for review).

The isoforms may also be produced by alternative transcription of the same gene. Nabeshima et al. (1984)

showed that chicken MLC1 and MLC3 could be produced from the same gene by the selective transcription of precursor RNA. The precursor RNA is then spliced, excluding certain portions of the RNA to produce mRNAs for either MLC1 or MLC3. The control of differential transcription is presently unknown.

Isoforms of MHC may be produced from the same mRNA (Bandman et al. 1982). It was shown that the mRNAs for MHC in the posterior latissimus dorsi and pectoralis major muscle produced identical MHCs in vitro, but in vivo peptide mapping showed the MHCs to be different. This suggests that post-translational modifications occur that give rise to the MHC isoforms.

# CHAPTER 2.

## THE PARVALBUMINS OF THE WHITE MUSCLE OF COLD- AND WARM-ACCLIMATED CARP (CYPRINUS CARPIO).

### INTRODUCTION.

Parvalbumins (PVAS) are a group of acidic proteins with an approximate molecular weight of 12,000. They are found in the white muscle of lower vertebrates at a high concentration of about 1mM (Pechere et al. 1973). Their amino acid sequence contains little or no tyrosine or tryptophan, which leads to little or no absorbance at 280nm, and a distinctive UV absorbance spectrum.

Parvalbumins bind two calcium ions per molecule with a high affinity (approx. $k_d = 10^{-7}M$), and the same sites have been found to bind $Mg^{2+}$ with a lower affinity (Haiech et al. 1979a). Parvalbumins have been found to have extensive sequence homologies with other calcium binding proteins, such as the myofibrillar $Ca^{2+}$ binding protein troponin C (Collins, 1976; Perry, 1979b). PVAs are thought to to play an important role in the relaxation of muscle (Gerday & Gillis, 1976; Pechere et al. 1977). They have a higher binding constant than troponin C, and are thought to bring about relaxation by the removal of $Ca^{2+}$ from troponin C (Benzonana, Capony & Pechere, 1972). At the low

intracellular concentrations of calcium present in the cell during relaxation the PVAs are thought to be fully bound with $Mg^{2+}$. The rise in intracellular $Ca^{2+}$ associated with contraction leads to the dissociation of the $Mg^{2+}$ and the binding of $Ca^{2+}$. It is thought to be the time necessary for the dissociation of the $Mg^{2+}$ that prevents the PVAs decreasing the intracellular $Ca^{2+}$ before contraction has taken place. This important role in the relaxation cycle is thought to be necessary to allow relaxation before all the $Ca^{2+}$ has been resequestered within the sarcoplasmic reticulum.

METHODS.

Common carp (Cyprinus carpio) of length range 150-200mm were acclimated over a period of 7-9 weeks. The water temperature was gradually altered over a period of 1 week to produce two populations of fish, at 5° C and 25° C. The fish were then held at this temperature for 6-8 weeks before sampling. The light regime used was 12hr light:12hr dark.

Preparation of parvalbumins.

The preparation of total parvalbumins from carp white muscle was adapted from the method of Pechere and Focant (1965). All stages of the preparation of parvalbumins were performed at 0-4° C. The fish were killed by a blow to the head followed by decapitation. 0.6g of deep white muscle was removed from the region between the dorsal fin and the lateral line, taking care to avoid any red or pink muscle. The muscle was homogenised in 8ml of extraction buffer, 0.1M phosphate buffer pH 7.0 at 0° C, 0.05M NaCl, 1mM mercaptoethanol, for 3 x 30s using at Polytron homogeniser at speed setting 5. The homogenate was then centrifuged, 30,000g for 10min, and the supernatant retained. The pellet was re-extracted twice more and the supernatants combined and centrifuged at 30,000g for 20min. The supernatant was drawn off and glycerol added to a final concentration of 10%.

Gel filtration was carried out using a LKB

chromatography column (2.6cm x 40cm) packed with Ultrogel ACA54 equilibrated with parvalbumin extraction buffer, at a flow rate of 10ml/hr, at 4° C. 7ml of the parvalbumin extract was layered onto the column, and 4ml fractions were collected. The fractions were scanned to obtain their UV spectrum at 240-280nm, using a Cecil automatic scanning spectrophotometer, and the calcium concentration of the fractions measured by flame spectrophotometery. The distinctive UV spectrum and the calcium peak were used to identify the parvalbumin peak. The protein content was measured using the method of Lowry et al. (1951) as described in chapter 3. The parvalbumin content of the original muscle sample was calculated, assuming a molecular weight of 12,000.

Electrophoresis of parvalbumins.

The molecular weight of the parvalbumin fractions was estimated by electrophoresis on polyacrylamide gels in the presence of SDS. The gels used were composed of upper stacking gels of 5% acrylamide, and lower separating gels of 15% acrylamide. Preparation and electrophoresis of SDS gels is fully described in chapter 3. The standard molecular weight marker proteins of the Sigma MW-SDS-70L with a molecular weight range of 14,200-66,000, and cytochrome C (MW 12,300) were used to estimate the molecular weight of the parvalbumins by the method of Weber & Osborn (1969) as

described in chapter 3.

The isoforms of parvalbumin were separated by non-denaturing alkali Tris-glycine polyacrylamide gel electrophoresis. Preparation of the gels and electrophoresis was performed as described in chapter 3 for alkali-urea gels, with the following exceptions (Blum et al. 1977). The pH of the gels was 8.6, and $Ca^{2+}$ and urea were not included.

SDS gels were stained with coomassie blue to estimate molecular weight, and then stained with the Bio-Rad silver stain kit (see chapter 3) to check if there were any other protein bands present. Alkali gels were stained with coomassie blue.

Samples for SDS electrophoresis were prepared by the addition to final concentrations of SDS 2%, glycerol 10%, 2-mercaptoethanol 1%, bromophenol blue 0.001%. The samples were heated to 100° C, for 3min, then cooled on ice. The samples were used immediately, or stored at -25° C.

Samples for Tris-glycine electrophoresis were prepared by the addition, to final concentration, of glycerol 10%, 2-mercaptoethanol 1%, and bromophenol blue 0.001%. The samples were then gently heated to 20° C to facilitate the mixing of the glycerol, once this was achieved the samples were cooled to 4° C, and were stored at -25° C, prior to use.

Densitometry of alkali gels.

Relative proportions of the parvalbumin isoforms were measured by scanning densitometry of the alkali gels using a Shimadzu CS-9000 densitometer. The gels were scanned at 550nm, and the peak areas calculated by the integral computer. The proportion of an parvalbumin isoform present was calculated as the area under the appropriate peak expressed as a percentage of the total area beneath all the peaks.

Calcium binding characteristics.

The dissociation constant ($k_{diss}$) and the number of calcium molecules bound per parvalbumin molecule, were calculated using a flow dialysis method adapted from Haiech et al. (1979a) based on Colowick & Womack (1969).

Ultrapure water (Milli-Q) was used throughout the calcium binding studies. To minimise calcium contamination, only plastic vessels were used and all equipment was washed in 10% nitric acid, then extensively rinsed with ultrapure water. Dialysis tubing was prepared by boiling in neutralised EGTA, then washed thoroughly with ultrapure water.

Calcium was removed from the parvalbumins by dialysis at 4° C against 50 volumes of 0.15M KCL, 25mM Pipes (pH 7.3 at 25° C), 10mM EGTA, for 2 days, followed by dialysis at 4° C against 10 changes of 200 volumes of 0.15M KCl, 25mM Pipes

(pH 7.3 at 25° C) that had been passed through an ion exchange column filled with Chelex 100 (Bio-Rad) to remove calcium ions (Haiech et al. 1979a). The calcium concentration of the parvalbumins after dialysis, measured by flame spectrophotometry was found to be less than 100nM.

The flow dialysis cell (Fig. 1) consisted of two chambers separated by a dialysis membrane. The volume of the upper chamber was 2.4ml, and the lower chamber 2ml. The cell was prepared by placing a small magnetic bar in the lower chamber, then positioning a square of dialysis membrane to cover the lower chamber. The two halves of the cell were fixed together by bolts that passed through both halves of the cell, and a small magnetic bar placed in the upper chamber. The lower chamber was filled by pumping buffer through the chamber whilst moving the cell to ensure all bubbles were removed. The cell was then placed in a water bath to equilibrate to the experimental temperature (5° C or 25° C).

The buffer used to flush the lower chamber was the same as that used to equilibrate the parvalbumins, and was held in a reservoir cooled by the same water bath as the chamber. The parvalbumins were diluted to 0.2mg/ml and $^{45}CaCl_2$ added to a final concentration of 10μM (2.5μCi/ml). A sample of 2ml was injected into the upper chamber and the stirrer and pump started at a flow rate of 10ml/min. The cell was left

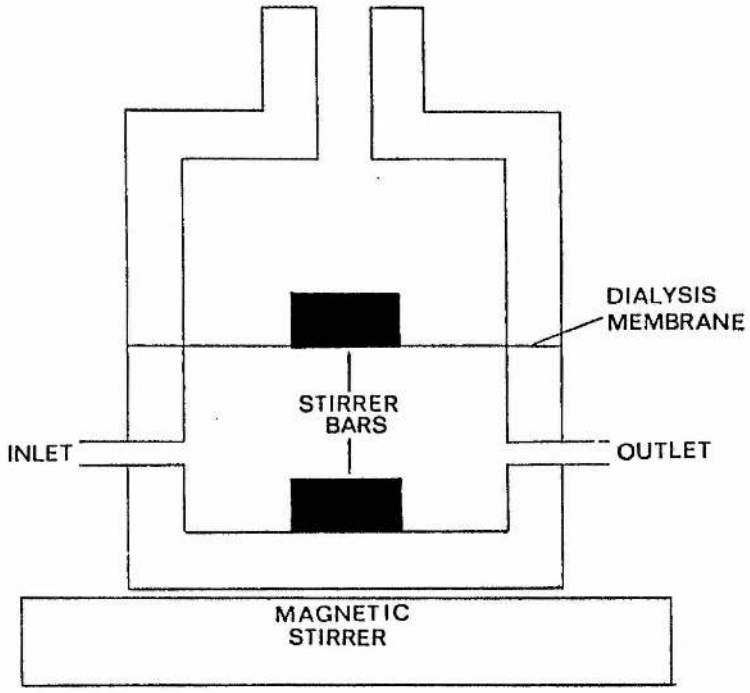



Figure 1. Flow dialysis cell used to measure the $Ca^{2+}$ binding characteristics of parvalbumins. The upper chamber contained the $^{45}Ca$-parvalbumin sample. Buffer was collected from the lower chamber and the $^{45}Ca$ concentration measured. The readings were used to estimate the dissociation constant and number of binding sites as described in the text.

to equilibrate for 1min, then fractions of 3ml were collected. Once six fractions had been collected, 10µl of 20mM $CaCl_2$ was added to the upper chamber, to give a final concentration of 20µM $Ca^{2+}$. The $Ca^{2+}$ addition was repeated every six fractions. Once the fractions from the parvalbumins with 60µM $Ca^{2+}$ had been collected, a final addition of 2µl of 1M $CaCl_2$ was made and a further 10 fractions collected (see Fig.7, page 46 for example). Colowick & Womack (1969) estimated that a steady state was set up once four times the volume of the lower chamber had been eluted. With a lower chamber volume of 2ml, and a flow rate of 10ml/min, a steady state should be achieved by the time 8ml have been collected. Fractions of 3ml were collected, so a steady state should have been reached after the third fraction. From each fraction 1ml was withdrawn and added to 9ml of scintillant, and counted for 5min using a Packard Tri-Carb scintillation counter.

Counts per minute were plotted against fraction number (Fig. 7). The steady state that is reached following the addition of non-radioactive $Ca^{2+}$ is only dependant on the concentration of free $^{45}Ca^{2+}$, and not on the total $Ca^{2+}$ in the upper chamber. The final addition of $Ca^{2+}$ leads to a steady state that is not changed by any further additions of $Ca^{2+}$. This indicates that the $Ca^{2+}$ bound to the parvalbumin was negligible, and that the rate of dialysis across the

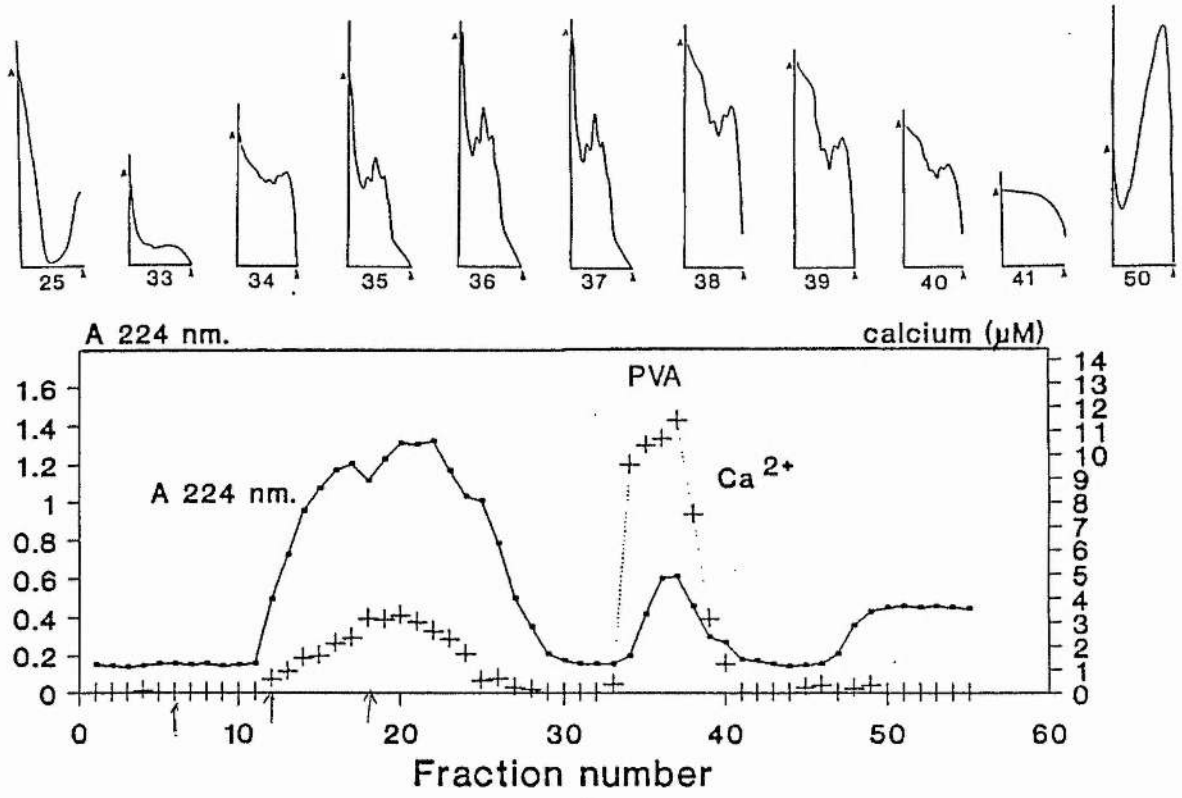



Figure 2. Various methods used to identify the fractions containing parvalbumins following gel filtration on Ultrogel ACA54. Absorbance measured at 224nm, the calcium concentration, and the UV spectra between 240 and 280nm. The numbers below the UV spectra correspond to the fraction number.

membrane was effectively the same as when all the $Ca^{2+}$ was unbound. Thus the counts per minute at the steady state following the final addition were taken to represent 100% of the $Ca^{2+}$ in the free state. By dividing any other observed value by this maximum value, gives the fraction of $Ca^{2+}$ free at that concentration. Once the fraction of $Ca^{2+}$ free at a give total concentration was known, then the bound $Ca^{2+}$ concentration could be calculated. The bound concentrations (B) were be plotted against bound/free (B/F) at a series of total concentrations (Fig. 8, page 47), (a Scatchard type plot). The slope of this plot gave the dissociation constant. The intercept on the bound axis gives the maximum molar concentration of calcium bound, which divided by the molar concentration of parvalbumin in the upper chamber gives the number of $Ca^{2+}$ molecules bound per parvalbumin molecule.

RESULTS.

A number of methods can be used to identify the PVA peak following gel filtration (Fig. 2). The PVAs appear as a peak when the absorbance at 224nm was measured, but not at 280nm. The calcium concentration of the same fractions was high, and the position of the calcium peak suggests that it is associated with a larger molecule. Free calcium would be expected to appear in later fractions. This together with the distinctive UV spectrum, makes the identification of the fractions containing PVAs less subjective than absorbance at 224nm alone. The fractions containing the PVAs were combined and used in subsequent assays.

The mean concentration of PVAs in warm-acclimated carp was lower than that of the cold-acclimated carp, however the wide range of values obtained for both groups prevented any significant difference being found (Table 1). Duplicate samples, prepared from separate muscle samples of the same animal gave consistant results, which showed that the variation in measured PVA concentration was not due to the methods used. Alkali electrophoretic analysis of the muscle extracts (Fig. 3), and purified parvalbumins (Fig. 4) showed the same banding pattern obtained by other workers (Pechere et al. 1973, Gerday et al. 1979b). SDS electrophoresis of the purified PVAs (Fig. 5) showed them to migrate as a single band with an apparent molecular weight of

| Acclimation temperature. (oC) | Parvalbumin concentration. (Mean ± S.E) | Range. |
|---|---|---|
| 5 | 0.684 ± 0.039 | 0.49–0.79 |
| 25 | 0.609 ± 0.045 | 0.39–0.78 |

Table 1. Total parvalbumin concentration in white muscle from 5° C and 25° C acclimated carp (mMol / Kg wet weight) (n = 12 throughout). The difference between 5° C and 25° C acclimated carp was not significant ($P \leq 0.05$, Mann-Whitney test)

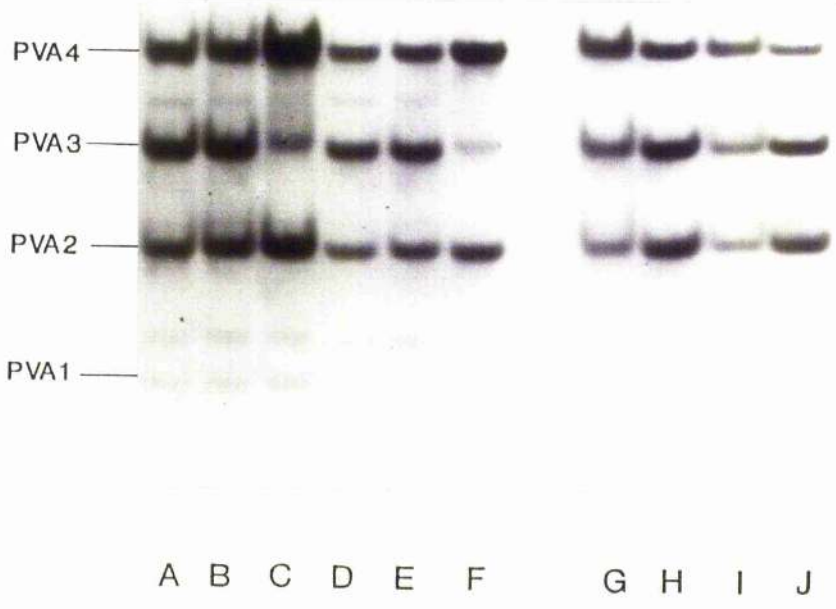



Figure 3. 10% Tris-glycine (pH 8.6) polyacrylamide gel of initial extraction of parvalbumins from white muscle of warm and cold acclimated carp. The lower portion of the gel containing the parvalbumins is shown. The gel was stained with coomassie blue. Conditions of electrophoresis were as described in text.

Key; A, C, E, G, I, cold acclimated carp. B, D, F, H, J, warm acclimated carp. PVA1, PVA2, PVA3, PVA4, bands corresponding parvalbumins 1, 2, 3, and 4.


Figure 4. 10% Tris-glycine (pH 8.6) polyacrylamide gel of parvalbumins, purified by gel filtration, from white muscle of warm and cold acclimated carp. Conditions of electrophoresis were as described in text.
Key; A, C, E, G, I, cold acclimated carp. B, D, F, H, J, warm acclimated carp. PVA1, PVA2, PVA3, PVA4, bands corresponding parvalbumins 1, 2, 3, and 4.

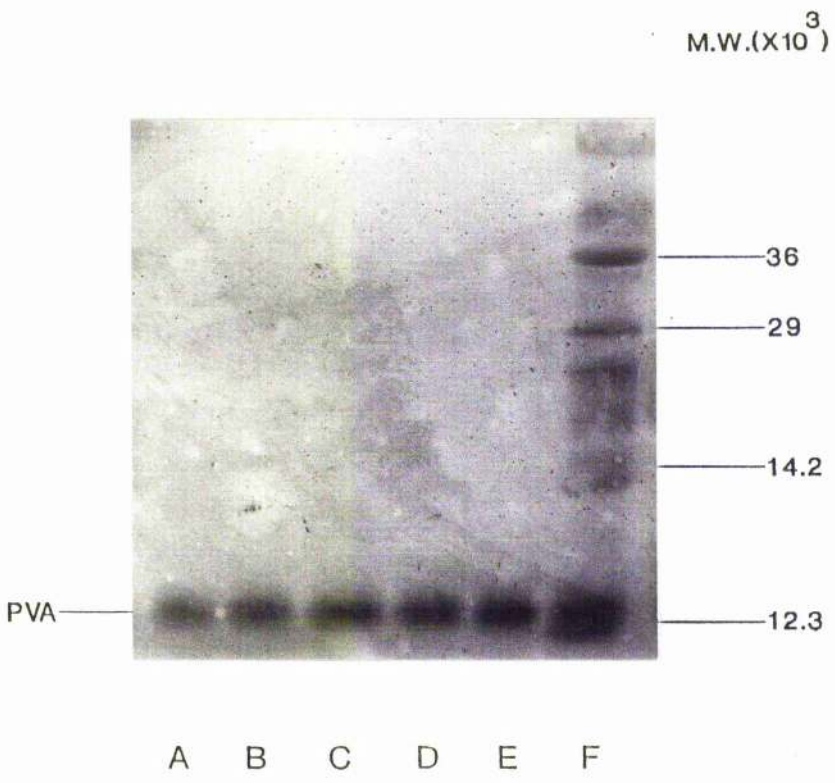



Figure 5. 15% SDS polyacrylamide gel of parvalbumins from the white muscle of cold and warm acclimated carp, purified by gel filtration. Stained by both coomassie blue and silver stain. Molecular weights were determined by running proteins of known molecular weight as markers. Conditions of electrophoresis were as described in text.
Key; A, C, E, purified parvalbumin samples from white muscle of cold acclimated carp. B, D, purified parvalbumin samples from white muscle of warm acclimated carp. F, Sigma MW-SDS-70L marker proteins and cytochrome C (MW 12,300). PVA, parvalbumin.

approximately 12,300. There was much variation between the relative proportions of PVA1, PVA2, PVA3, and PVA4 seen with alkali electrophoresis, which showed no relationship to acclimation temperature. This was clearly illustrated by the relative proportions measured by densitometric scanning (Table 2). As with the total parvalbumin concentrations, the mean proportions of each PVA were not significantly different between cold- and warm-acclimated carp. Variation in the proportions of the different PVAs was seen in both the muscle extracts and the purified PVAs, indicating that the differences are real and the relative proportions seen are present in the muscle, and are not an artifact brought about by the purification method. Densitometric scans (Fig. 6) show that acclimation temperature does not lead to a particular distribution of the four PVAs, the cold- and warm-acclimated fish can show identical patterns. Gerday et al. (1979b) reported that in experiments to isolate the individual PVAs from the lungfish, some specimens contained a low proportion of PVA3, and others a high proportion of PVA2. This indicates that variation of PVA components is not restricted to one sample or species, and is possibly a common occurence.

The plot of counts per minute against fractions eluted from the flow dialysis cell (Fig. 7) illustrates that a steady state was reached following each addition of calcium.

| Parvalbumin band. | Acclimation temperature. (°C) | Percentage. (Mean ± S.E.) | Range. |
|---|---|---|---|
| 4 | 5 | 53.17 ± 4.33 | 81.3–31.2 |
| | 25 | 51.08 ± 3.34 | 68.6–37.0 |
| 3 | 5 | 23.70 ± 4.02 | 0.0–40.7 |
| | 25 | 20.53.± 3.33 | 1.9–36.5 |
| 2 | 5 | 20.13 ± 0.82 | 14.0–24.1 |
| | 25 | 23.15 ± 1.38 | 14.8–31.2 |
| 1 | 5 | 1.71 ± 0.38 | 0.0–4.4 |
| | 25 | 2.35 ± 0.41 | 0.0–5.7 |

Table 2. Percentage distributon of parvalbumins 1, 2, 3, 4, from white muscle parvalbumin fractions from 5° C and 25° C acclimated carp (n = 12 throughout). Densitometric scans of Tris-glycine, pH 8.6 PAGE gels.

No significant ($P \leq 0.05$) differences were found between parvalbumins from 5° C and 25° C acclimated carp (Mann–Whitney test).

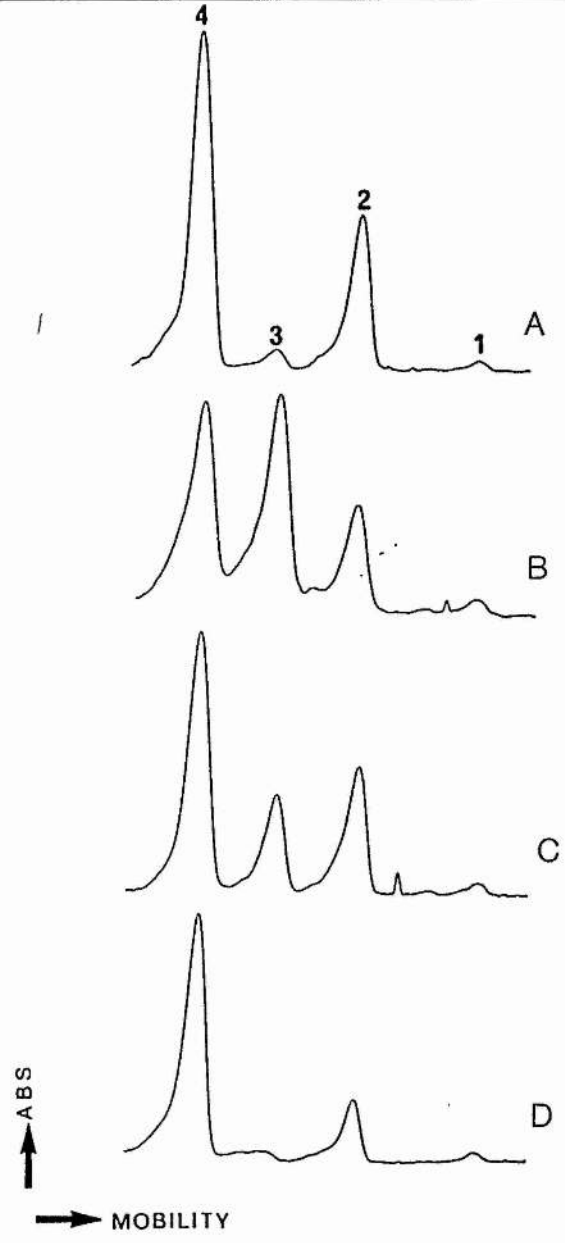



Figure 6. Densitometer traces of alkali-urea gels of parvalbumins from warm- and cold-acclimated carp. Key; 1, 2, 3, and 4 are peaks identified as PVA1, PVA2, PVA3, PVA4, respectively. A and B 5° C acclimated carp samples. C and D 25° C acclimated carp.

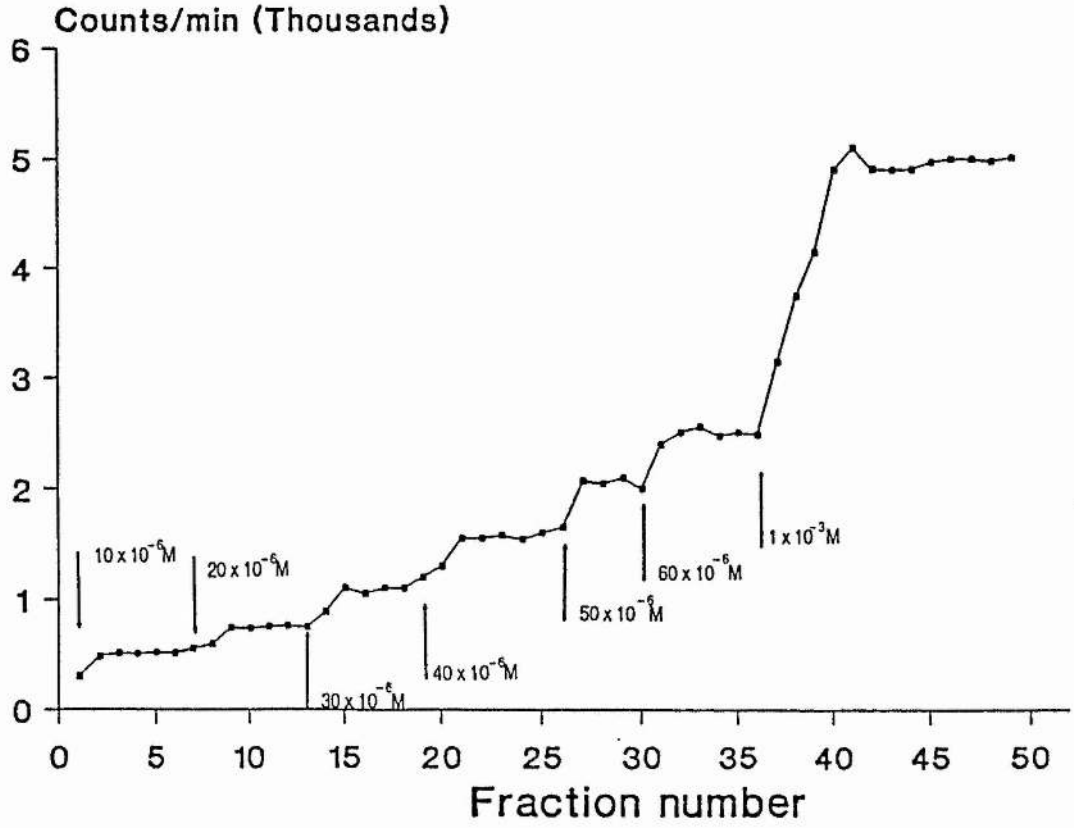



Figure 7. Plot of counts per minute against fraction from the analysis of the calcium binding characteristics of parvalbumins by flow dialysis. The ratio of bound to free calcium was calculated from the steady state reached following each $Ca^{2+}$ addition as described in the text. The inserted numbers show the total concentration of calcium following each addition.

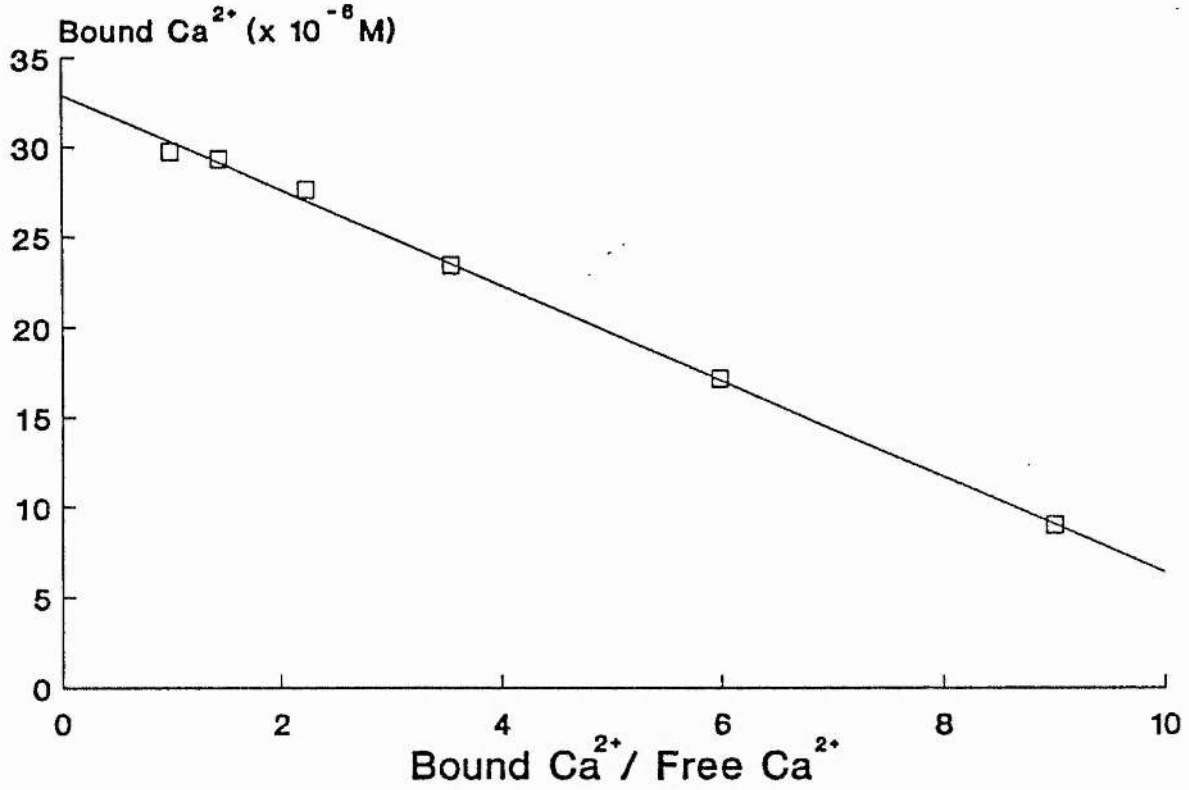



Figure 8. Scatchard plot obtained from the data in figure 7. The bound $Ca^{2+}$ and the bound/free $Ca^{2+}$ were calculated as described in the text. The dissociation constant was calculated from the gradient of the line. The intersect at the y axis is used to calculate the number of binding sites.

The Scatchard plot obtained (Fig. 8), showed very little deviation from a straight line, and the dissociation constants and numbers of binding sites measured were highly reproducable (Table 3).

The values for the dissociation constants 2.17-2.42μM are within the range generally found (4μM-0.1μM) and the binding of 2 molecules of calcium per molecule of PVA has been observed in many species (Pechere et al. 1971a, Benzonana et al. 1972).

The calcium binding characteristics of cold- and warm-acclimated carp (Table 3) show that there is little variation in either the dissociation constants, or the number of binding sites at either assay temperature.

| Acclimation temperature. (°C) | Assay temperature. (°C) | Dissociation constant (μm). (Mean ± S.E.) | $Ca^{2+}$-binding sites. (Mean ± S.E) |
|---|---|---|---|
| 5 | 5 | 2.42 ± 0.146 | 1.93 ± 0.069 |
| 5 | 25 | 2.22 ± 0.159 | 2.02 ± 0.087 |
| 25 | 5 | 2.32 ± 0.201 | 1.91 ± 0.067 |
| 25 | 25 | 2.17 ± 0.188 | 2.10 ± 0.207 |

Table 3. $Ca^{2+}$-binding characteristics of parvalbumin fractions from white muscle of 5° C and 25° C acclimated carp, assayed at 5° C and 25° C (n = 5 throughout). The Mann-Whitney test showed no significant ($P \leq 0.05$) differences with either acclimation or assay temperature.

DISCUSSION.

The total PVA concentration 0.6–0.7mM measured in the white muscle of cold- and warm-acclimated fish were similar to that measured by Pechere et al. (1977) 0.53mM and in the range of other fish species, lungfish 0.2mM (Gerday et al. 1979b), coelacanth 1mM (Hamoir et al. 1973), hake 1mM (Baron, Demaille & Dutruge, 1975). A correlation has been found between PVA concentration and speed of relaxation in different muscles of the guinea pig (Heizmann et al. 1982). The PVA concentration in the sartorious (a fast muscle with a high relaxation rate) was 0.25g/Kg, while that of the soleus (a slow muscle with a low relaxation rate) was <0.007g/Kg. In the muscle of different species the range of PVA concentrations is even larger. The mouse gastrocnemius, with a high relaxation rate contains 4.9g/Kg, whilst the horse deep gluteal muscle with a low relaxation rate contains <0.001g/Kg (Heizmann et al. 1982). The same correlation appears to exist in cardiac muscle, the PVA concentration of the rabbit heart is <0.01μmol/Kg (Baron, Demaille & Dutruge, 1975) whilst that of the heart muscle of the shrew, which beats at 1000 beats/min, is 19μmol/Kg (Le Peuch et al. 1978). This suggests that an increase in the rate of relaxation may be accompanied by an increase in the concentration of PVAs. PVA concentration can change under conditions that produce a lower relaxation rate, denervation

of the rat extensor digitorum longus increased the half-relaxation time from 9.4-19msec, and also resulted in a 20% decrease in the PVA concentration (Heizmann et al. 1982).

The rate of relaxation in the fin muscles of goldfish (Heap, Watt & Goldspink, 1987) and the myotomal white muscle of carp (Fleming, Altringham & Johnston, 1989) were shown to increase following cold- acclimation. If the rate of relaxation was dependant on the PVA concentration, then it would be expected to be higher in cold-acclimated fish. The results showed that there was no significant difference between the PVA concentrations of cold- and warm-acclimated carp white muscle. Therefore, although there may be a correlation between the PVA concentration and the relaxation rate when different tissues are compared, and in the same tissue following denervation, an increase in relaxation rate was not accompanied by an increase in PVA concentration following cold-acclimation.

The range of PVA concentrations found in both groups of fish, and the reproducability of the results, suggest that the total PVA concentration alone, may not be an important factor in the relaxation cycle.

In carp white muscle five isoforms of PVA were reported (Pechere, Demaille & Capony, 1971b), however others have only seperated the four components seen in this study

(Gosselin-Ray, Pironf & Gerday, 1978; Brandts et al. 1977; Iio & Hoshihara, 1984). In cod (Bhushana-Rao et al. 1969) and pike (Bhushana-Rao & Gerday, 1973) two isoforms were found, in hake three isoforms were found (Haiech et al. 1979b) and in the antarctic fish, Champsocephalus gunnari, five isoforms (Hamoir & Gerardin-Otthiers, 1979). The isoforms of carp white muscle PVA were found to be present as 46% PVA4, 16% PVA3, 32% PVA2, and 6% PVA1 by Le Peuch et al. (1978) which is different to the approximate 52% PVA4, 21% PVA3, 21% PVA2, 2% PVA1 found in this study, especially in the relative proportions of the PVA2 and PVA3 isoforms. This difference could be due to a number of factors, the assumption that the staining by coomassie blue is proportional to protein concentration in this study, or the assumption that $^{45}Ca$ binds equally to all the PVAs (Le Peuch et al. 1978) may not be valid. From the ranges of values obtained in this study it seems possible that the variation could be due to differences in the carp stocks used to measure the proportions of the PVA isoforms. There appeared to be no variation in the mean proportions of the four PVA isoforms between the cold- and warm-acclimated fish.

The isoforms of carp PVA have different calcium binding characteristics (Iio & Hoshihara, 1984). Their apparent calcium binding constants decreased in the order, PVA2 ($K_d = 2.8 \times 10^8 M^{-1}$) > PVA1 ($K_d = 1.25 \times 10^8 M^{-1}$) > PVA3 and PVA4

($K_d = 4 \times 10^7 M^{-1}$). The rate of response to a change in calcium concentration was also seen to vary between the isoforms, decreasing in the order PVA2 > PVA1 > PVA5 > PVA3 (Iio & Hoshihara, 1984). If the different characteristics of the PVA isoforms are an integral part of their function in the muscle, it would be expected that their relative proportions would be conserved. The individual variation observed in this study shows that the relative proportion of a PVA isoform is not fixed within a species. If all the PVAs isolated were in their active form, this would lead to the conclusion that the combined calcium binding characteristics of the total PVA fraction was not important in determining relaxation rates, otherwise large differences would exist between individuals.

The binding of 2 $Ca^{2+}$ per PVA, was in agreement with values found by other workers (Benzonana *et al*. 1972; Iio & Hoshihara, 1984). The calcium dissociation constants measured 2.17-2.42μM are in the range generally found for total PVAs 0.1-4μM (Pechere *et al*. 1971b; Benzonana *et al*. 1972). PVAs each contain a high and low affinity binding site, in carp the lowest affinity site is on PVA4 ($K_{diss} = 14\mu M$) and the highest on PVA1 ($K_{diss} = 0.5nM$) (Iio & Hoshihara, 1984). In this study only one affinity site was observed, and the inability to distinguish between the two sites by flow dialysis has been described previously (Haiech

et al. 1979a). It would appear that the measured dissociation constant was that of a combination of binding sites. It would appear that the calcium-binding characteristics of the total PVA fraction does not change with acclimation, when assayed at 5° C or 25° C.

The results suggest that the changes in relaxation rate seen in temperature acclimation are not accompanied by a change in total PVA concentration, expression of PVA isoforms, or in the calcium binding characteristics of the PVA fraction. These results would not appear to be consistent with the theory that relaxation rates are dependant on PVA concentration (Heizmann et al. 1982), and that changes in PVAs are not the mechanism for the acclimatory response of relaxation rates reported.

The variation in total PVA, and isoforms observed in individual fish has been seen previously. The absense of PVA3 in some carp but no variation in the other PVAs was seen on electrophoresis (Hamoir, Focant & Disteche, 1972). Considerable variation was reported in the proportions of all five PVAs that could be isolated from individual lungfish (Gerday et al. 1979b).

This suggests that both the total calcium binding capacity, and the proportions of the various affinity calcium-binding sites are not critical. Contraction is triggered by a rise in intracellular $Ca^{2+}$ to 10μM, the

estimated total $Ca^{2+}$ binding capacity of the PVAs is approximately 1.2mM (assuming two sites per PVA) thus allowing the binding of the $Ca^{2+}$ associated with 120 contractions. The excess calcium-binding capacity present in carp white muscle may be the reason that the PVA concentration does not need increase to allow higher relaxation rates. This could be the reason no difference is observed in any of the PVA characteristics measured following temperature acclimation.

# CHAPTER 3.

## ELECTROPHORETIC ANALYSIS OF WHITE MUSCLE MYOFIBRILLAR PROTEINS FROM COLD- AND WARM-ACCLIMATED CARP (CYPRINUS CARPIO.)

### INTRODUCTION.

The main division in fish myotomal muscle is between the red and white fibres which are arranged in anatomically distinct regions (see Bone, 1978). Red fibres contain more myoglobin, have a more extensive blood supply, a higher mitochondrial content, and smaller average diameters than white fibres (Bone, 1966). The ATPase activity of red fibres was found to be lower than that of white fibres in coalfish, cod and plaice (Johnston, Frearson & Goldspink, 1972), brooktrout (Johnston & Moon, 1980), tuna (Johnston & Tota, 1974) and carp (Johnston, Davison & Goldspink, 1977). Histochemically red fibre ATPase activity was labile at alkaline pH, whilst that of white fibres was less so (Johnston *et al*. 1974). The contractile properties of red and white fibres are also different, red fibres having a lower $V_{max}$ than white fibres in dogfish (Johnston, 1982a; Bone *et al*. 1986), tilapia (Flitney & Johnston, 1979), cod (Johnston, 1982a), and carp (Granzier *et al*. 1983). In carp, fibres of intermediate properties, between red and white

fibres, can be distinguished histochemically by the alkaline stability of ATPase activity (Johnston et al. 1974; Akster, 1983). These fibres have been described as pink fibres and have contractile properties between those of red and white fibres (Akster, 1985). Pink fibres appear to correspond to the type IIA, IIC and IC fibres of mammalian muscle (Salviati, Betto & Betto, 1982; Betto, Zerbato & Betto, 1986; Staron & Pette, 1987a, 1987b).

The red and white muscle of fish have been shown to contain different myosin light chains. Studies on carp, pike, dogfish, mackerel and angler fish, by electrophoretic techniques, have shown a general pattern of 2 myosin light chains specific to red muscle (MLC1s & MPLC2s), and 3 myosin light chains (MLC1f, MPLC2f & MLC3f) specific to white muscle (Focant, Huriaux & Johnston, 1976; Focant, Huriaux & Vandewalle, 1983). The pink muscles appeared to contain the myosin light chains of white muscle (Focant, Huriaux & Vandewalle, 1983). This pattern was basically the same a seen in other vertebrate muscle, however, two isoforms of MLC1s were found (Young & Davey, 1981; Salviati, Betto & Betto, 1982; Staron & Pette, 1987a, 1987b). The light chain pattern of the intermediate fibres has been been studied in mammals. Fibres with intermediate contractile properties contain various proportions of slow and fast muscle light chains (Young & Davey, 1981; Salviati, Betto & Betto, 1982;

Staron & Pette, 1987a, 1987b). There have been no previous studies on isoforms of the other myofibrillar proteins in fish. The presence of isoforms may be the mechanism for the acclimatory response to temperature seen in the contractile properties of carp white muscle (Altringham & Johnston, 1985; Fleming, Altringham & Johnston, 1989), and carp white muscle myofibrillar ATPase (Heap, Watt & Goldspink, 1985). The case for the molecular basis of acclimation being an alteration in the expressed isoforms of one or more proteins was supported by the lack of an acclimatory response in starved carp, in which protein synthesis is reduced to a very low level (Heap, Watt & Goldspink, 1986a).

The aim of this present study was to investigate the presense of different isoforms of the myofibrillar proteins by the use of electrophoretic techniques.

METHODS.

Common carp (Cyprinus carpio) were obtained from Humberside Fisheries (near Drifield, England) and held for several weeks in a re-circulated freshwater aquarium at 12-15° C prior to acclimation over a period of 7-9 weeks. The water temperature was gradually altered over a period of 1 week to produce two populations of fish, at 8° C and 20° C. The fish were then held at this temperature for 6-8 weeks before sampling. The light regime used was 12hr light:12hr dark. The fish were fed ad libitum three times a day with commercial trout pellets. The total length and weight of the fish used were (mean ± SD) 31 ± 2.5cm, and 735 ± 225g.

Preparation of myofibrils.

Fish were stunned by a blow to the head and then killed by decapitation. All the following procedures were carried out on ice. Fast muscle was removed from the dorsal epaxial region, care being taken to avoid superficial fast (pink) and slow oxidative (red) fibres. Red muscle was removed from the region below the dorsal line, taking care to avoid the fast (white) and superficial fast (pink) fibres.

Myofibrils were prepared from the muscle immediately following excision. The preparation of myofibrils was based on the method of Perry & Grey (1956). Muscle was minced with scissors and homogenized in 10 vol 100mM NaCl, 10mM Tris-HCl pH 7.2, 5mM EDTA, at 0° C , for 3 x 30s, the homogenate

placed on ice to cool between homogenizations. The homogenate was centrifuged at 6000g for 10min, and the pellet was washed and resuspended by a short homogenization at low speed, in 10 vol 100mM NaCl, 10mM Tris-HCl pH 7.2. The homogenate was centrifuged at 6000g for 10min, and the pellet washed a further 5 times, as described above. The pellet was resuspended in 20 vol of the wash buffer, and centrifuged at 400g for 2min. The myofibrils were contained in the top 1/2 of the supernatent, they were carefully removed with a pipette, and kept on ice.

An alternative method was used to check that all the soluble proteins were removed from the myofibrils, and that the banding patterns produced on electrophoresis were not due to contaminating proteins that had not been totally removed by the standard myofibril preparation technique. The method used a non-ionic detergent to dissolve the membranes (Solaro, Pang & Briggs, 1971). 1% Triton X-100 was included in the first two homogenization steps. The muscle was homogenized in 10vol 1% Triton X-100, 100mM NaCl, 10mM Tris-HCl pH 7.2, then left to stand for 30min before centrifugation at 6000g for 10min. The pellet was homogenized at low speed, in the same buffer and left to stand for 30min, then centrifuged at 6000g for 10min. The pellet was washed a further 5 times as described above.

Protein assay.

To obtain an accurate protein assay of myofibrils the Maddy and Spooner (1970) adaptation of the method of Lowry et al. (1951) was used, as it ensured complete dissolution of the myofibrils.

Stock solutions; 1. 2% Na deoxycholate,

2. 4% $Na_2CO_3$ ,

3. 0.1M NaOH,

4. 2% Na tartrate,

5. 1% $CuSO_4$ ,

6. 1 vol Phenol Folins reagent + 2 vol $H_2O$,

7. A solution, stable for 1 day, was prepared with 100ml stock 2 + 100ml stock 3 + 2ml stock 4 + 2ml stock 5.

50µl of sample, standard or appropriate blank was added to 0.45ml solution 1, and mixed well until the sample was fully solublised. 2.5ml of solution 7 was added, mixed well and left for 5min. 0.25ml of solution 6 was added, mixed rapidly and left for 60min, then read at 695nm. Bovine serum albumin was used to construct a calibration curve from 0.5 mg/ml to 3 mg/ml. Each assay was performed in triplicate.

Myofibril ATPase assay.

The ATPase activity was measured by the following method (White, 1982).

Solutions used;

A. Myofibril suspension, 0.5 mg/ml protein in 100mM NaCl, 10mM Tris-HCl pH 7.2

B. $Ca^{2+}$ buffer, 62.5mM Tris, 3.8mM $MgCl_2$, 0.2mM $CaCl_2$, pH 7.4 at 20° C.

C. EGTA buffer, 62.5mM Tris, 3.8mM $MgCl_2$, 0.2mM EGTA, pH 7.4 at 20° C.

D. 13.3% SDS, 0.12M EDTA, pH 7.0.

E. 0.5% (w/v) ferrous sulphate, 0.5% (w/v) ammonium molybdate, 0.5M $H_2SO_4$, made fresh daily.

F. 20mM Disodium ATP at pH 7.0.

ATPase assays were carried out in water baths at 8° C and 20° C.

As the myofibrils were in suspension, rather than solution, it was important that all incubations were continually agitated to prevent precipitation of the myofibrils. In a small beaker, 1ml solution A was pre-incubated, with 5.5ml of either solution B or C, for 5min. Before the ATPase reaction was started a 0.65ml sample was removed and placed in a test tube containing 0.25ml of solution D, which stops any ATPase activity, then 0.1ml of solution F was added. This was the zero time incubation. The

ATPase reaction was started by the addition of 0.9ml solution F and mixed well. Samples of 0.75ml were removed at intervals (1min for 20° C and, 2min for 8° C incubations) and placed in test tubes containing 0.25ml solution D. Colour development was initiated by the addition of 2.0ml solution E, was complete after 15min and stable for at least 60 min. The absorbance was read at 550nm.

A calibration curve was constructed between 25nmol and 600nmol phosphate, by using standard phosphate solutions in the place of the myofibril solution, and water in place of the ATP solution. The results were plotted as μmol Pi released/mg protein against time, the rate of Pi release was calculated from the slope of the initial straight line portion of the rate curve. The results were expressed in μmol Pi released/mg protein/min.

The rate of $Ca^{2+}$ activated ATPase was calculated as, the rate of $Ca^{2+}$ $Mg^{2+}$ ATPase minus that of EGTA $Mg^{2+}$ ATPase.

$$\text{Calcium sensitivity (\%)} = 1 - \left( \frac{\text{EGTA } Mg^{2+} \text{ ATPase}}{Ca^{2+} \; Mg^{2+} \text{ ATPase}} \right) \times 100$$

Electrophoretic techniques.

Alkali-urea gel electrophoresis was performed by modifications to the method of Focant & Huriaux (1976).

SDS gel electrophoresis techniques were based on the method of Laemmli (1970).

Isoelectric focusing was carried out using an adaptation of the method of Radola (1980).

Two-dimensional techniques were derived from the above methods, and also O'Farrell (1975), and Cleveland et al. (1977).

Preparation of samples for electrophoresis.

Alkali-urea gels: myofibril samples were dissolved in 3 vol (w/v) of buffer; glycine 20mM to pH 8.9 with iM Tris base, urea 12M, 2-mercaptoethanol 5% (v/v), BPB 0.001% (w/v), and either 5mM $CaCl_2$ or 10mM EGTA, to give a final protein concentration of 5mg/ml. The samples were homogenised in this buffer then left at 25° C for 2h before use. If not to be used immediately the samples were stored at -20° C.

SDS gels: myofibril samples were prepared by the addition of an equal volume of: Tris-HCl 120mM (pH 6.75), SDS 4%, glycerol 20%, 2-mercaptoethanol 2%, bromophenol blue 0.002%, to give a final protein concentration of 5mg/ml. The samples were then heated to 100° C,for 3min, then cooled on ice. The samples were used immediately, or stored at -25° C.

IEF gels: myofibril samples were prepared by the addition of solid urea to 8M, Noniodet P-40 to 2%, and 2-mercaptoethanol to 0.2%. The samples were then placed in a water bath at 20° C for 2 hours. Single fibre samples were prepared by placing the fibre in 8M urea, 2% Noniodet P-40, 0.2% 2-mercaptoethanol. The fibres were crushed against the side of the test tube and left at 25° C for 60min prior to use.

Electrophoretic equipment used.

Three basic gel forms were used, vertical slab gels, horizontal slab gels, and thin layer IEF gels.

Vertical slab gels were used for all SDS PAGE, and most alkali urea PAGE applications. The equipment used was a Protean Cell (Bio-Rad) which allowed gels of 160mm x 160mm to be cast, at a thickness of 0.75mm, 1.5mm, or 3mm. Well formers produce sample application wells that allow a maximum of 50µl, 100µl, or 150µl of sample to be applied.

Horizontal slab gels were used for alkali urea PAGE, and were cast using a gel casting kit for the LKB Multiphore II, which allowed the casting of 240mm x 120mm gels with a thickness of 2mm. The equipment incorporates an upper plate with projections into the space where the gel will be formed, creating either 5µl or 10µl sample wells.

Thin layer IEF gels were cast using the following equipment, which was devised from both the LKB ultrathin gel

casting equipment and Radola (1980). One base plate 180mm x 130mm x 4mm float glass, one gel plate 180mm x 120mm x 2mm float glass, two Teflon spacers 0.1mm x 180mm x 10mm, six large bulldog clamps, and a 1kg weight of area 100mm x 100mm. The Teflon spacers were cleaned as described below, then the lower surface that comes into contact with the base plate was smeared with a thin layer of silicone grease to ensure a good seal. The glass plates were also cleaned as described below, then the base plate was silanized to repel the gel, and the gel plate silanized to ensure adhesion of the gel.

Equipment cleaning.

All the equipment to be used in electrophoretic techniques was soaked in a solution of Decon overnight prior to use, and then cleaned using a paper towel soaked in Decon. The equipment was then extensively rinsed in tap water to remove the detergent, rinsed with Milli-Q water, then left to drain. The equipment was then dried using clean paper towels. The glass plates were degreased with acetone, firstly by wiping with a paper towel soaked in acetone, then rinsing with a stream of acetone, which also removes any dust that may have settled on the plates. The plates were finally left for the acetone to evaporate, and were ready to use.

Silanization of glass plates.

To prevent the adhesion of the gel to the plate used to form the gel it was pretreated thus; the plate was washed with detergent, thoroughly rinsed with tap water and finally with deionised water. The plate was dried, rinsed with acetone, and placed aside to dry. Approximately 1ml of Sigmacote was poured onto the plate, spread evenly over the surface using a clean tissue, and left 15 min to dry. The plate was rinsed with deionised water and wiped dry prior to use.

To promote the adhesion of the gel to the glass plate used to support the gel, the plate was cleaned as described above, and once dry after the acetone rinse, 2ml of 0.3% Silane A 174 in 50% ethanol poured onto the surface and spread evenly using another glass plate. Once the surface had been coated the excess was poured off and the plate left for 15min, after which the surface was rinsed with deionised water and dried with a clean tissue (Radola, 1980). The plates were used immediately or stored wrapped in tissue.

Gel preparation.

The same preparation methods were used for all vertical PAGE gels, the only difference being the reagents used.

The reagents other than acrylamide, BIS or BAC, and SDS, were weighed and made up to half the final volume and the required pH. Acrylamide, BIS or BAC, and SDS were added,

and water to the final volume, minus that of the TEMED and ammonium persulphate to be used. The solution was degassed in a large vessel connected to a vacuum pump for 5min. TEMED was added and the vessel rotated gently to mix well, but keep the introduction of oxygen to a minimum. Ammonium persulphate was added and mixed in a similar fashion.

The gel solution was placed in a tube fitted with a tap, and poured to the required depth in the prepared gel cassette. During filling, the cassette was gently rocked to prevent eddy formation. Gels that required no stacking gel were filled to the top and the well former placed in position. Polymerization occurred within 15min, but the gels were left for at least 2h before use.

Gels requiring a stacking gel were filled to the correct depth, and overlaid with isobutanol. Polymerization occured within 15min, and the gels were left for at least 30min before the stacking gel was added.

The stacking gel solution was prepared as for the separating gel, except that before the TEMED and ammonium persulphate were added, the isobutanol was removed from the separating gel. The surface of the gel was washed with water, and a small volume of the gel solution used to equilibrate the top of the separating gel to the stacking gel solution. The gel solution used for equilibration was poured off prior to the stacking gel being added. The well

former was placed in position, and polymerization occured within 15min. The gel was used within 30min of the stacking gel polymerizing, providing 2h has elapsed since polymerization of the separating gel.

The well former was left in place prior to use of the gel. When the gel was ready to be used, the well former was removed and the wells washed twice with electrode buffer, and then placed in the chamber ready for electrophoresis.

Horizontal gels were prepared as a separating gel described above with no stacking gel, and poured into the cassette and left to polymerize for 2hr before use. Prior to use the cassette was dismantled to leave the gel on the 1mm thick glass supporting plate. This was achieved by removing the clamps and the spacer, then cooling the gel and plates in a refridgerator for 5min, which allows the plates to be more easily removed. A spatula was placed between the upper and lower plates and slowly twisted to move the plates apart, at the same time a scalpel blade, dipped in electrode buffer, was placed between the gel and the well forming plate and moved along the surface of the gel. Twisting the spatula allowed the well former to be removed, leaving the gel in place with no air trapped beneath it. This must be carried out with care to prevent air becoming trapped between the gel and the supporting plate. This may be removed by gently pressing on the surface of the gel but can

lead to distortion of the gel and affect the gel pattern obtained.

Isoelectric focusing gels were prepared by assembling the plates in the following order. The two Teflon spacers were placed along the long edges of the base plate and firmly clamped in position. The base plate was put on a flat surface and the gel plate placed on top, supported on both sides by the spacers. The gel solution was prepared by combining all the reagents except the TEMED and ammonium persulphate, and dissolving them by warming with the hands. Heating to a high temperature to dissolve the urea leads to a loss of buffering capacity, by "forced ageing" of the Pharmalytes (Radola, 1980). The gel solution was gently degassed using a vacuum pump for 2min. If urea crystalised on the surface of the vessel, rotating it in the palms of the hands usually gave sufficient heat to redissolve it. The TEMED and ammonium persulphate were then added and gently mixed by rotating the vessel. The upper plate of the casting apparatus was slid back until half the lower plate was uncovered, and the gel solution poured onto the exposed surface. The upper plate was slowly slid back into position over the lower plate. The gel fills the space between the two glass plates by capillary action. Once the plates were flush, the weight was gently lowered onto the upper plate, and any gel solution expelled was removed. The gel

polymerized within 20min and was ready to use 1 hour after pouring. The gel was removed attached to the upper plate, by inserting a scalpel blade between the two glass plates and gently twisting. As soon as the gel begins to separate from the base plate it was removed by a single flowing movement to prevent bubbles forming on the surface of the gel, which appear to alter the banding patterns in that region of the gel. The gels were immediately placed on the cooling plate of an LKB Multiphor isoelectric focusing unit, set at 10° C. Any uneven edges on the gel should be carefully cut with a scalpel to give a straight edge, and excess gel removed. The gels were be run as described in the following section.

Alkali-urea PAGE gels.

The final concentrations used to make a 10% gel were; Glycine 100mM to pH 8.9 with 1M Tris base, urea 8M, $CaCl_2$ 1mM, 2-mercaptoethanol 0.1%, 9.67g acrylamide + 0.33g Bis/100ml. Polymerisation was initiated using; TEMED 100µl/100ml, ammonium persulphate 50mg/100ml.

Electrode buffer; Final concentrations were; Glycine 100mM to pH 8.9 with 1M Tris base, $CaCl_2$ 1mM, 2-mercaptoethanol 0.1%.

Running conditions; The gel apparatus was run at 5° C, at 50V initally, until the samples were seen to have entered the gel and then at 400V until the BPB front reaches the end of the gel.

SDS polyacrylamide gels.

Upper stacking gels; a final concentration of 0.125M Tris-HCl (pH 6.8 at 20° C), 0.1% 2-mercaptoethanol, and 0.1% SDS was used for all stacking gels. The final concentration of acrylamide + BIS was given by the %age gel required, usually 5%. BIS made up 3.3% of the total acrylamide in all cases. Polymerisation was initiated by the addition of 0.1ml TEMED per 100ml of gel, and 50mg ammonium persulphate per 100ml of gel.

Lower separating gels; a final concentration of 0.375M Tris-HCl (pH 8.8 at 20° C), 0.1% 2-mercaptoethanol, and 0.1% SDS was used for all separating gels. The final concentration of acrylamide + BIS was given by the %age gel required. BIS made up 3.3% of the total acrylamide in all cases. Polymerisation was initiated by the addition of 0.1ml TEMED per 100ml of gel, and 50mg ammonium persulphate per 100ml of gel.

Electrode buffer; 25mM Tris brought to pH 8.3 with glycine, 0.1% 2-mercaptoethanol, and 0.1% SDS.

Running conditions; samples were run into the gel at 30V. Once the samples had entered the gel the voltage was increased to 150V and run until the bromophenol blue front reached the end of the gel.

Isoelectric focusing gels

Gel composition; final concentrations used were; Pharmalyte

mixture 3%, urea 8M, Nonidet P-40 2%, 2-mercaptoethanol 0.1%, acrylamide 4.835g /100ml,BIS 0.165g /100ml. Polymerisation was initiated by the addition of, TEMED 30µl/100ml,ammonium persulphate 50mg/100ml.

The Pharmalyte mixture used depended on the pH range required. For linear gradients from pH 3 to pH 10, only Pharmalyte 3-10 was used, for extended gradients in the pH 2.5 to pH 5.5 range, 2.25% Pharmalyte 2.5-5.5 plus 0.75% Pharmalyte 3-10 was used.

Electrode buffers; cathodic buffer 2M ethylenediamine, anodic buffer 0.025M glutamic acid, 0.025M aspartic acid.

Running conditions; isoelectric focusing electrode wicks were soaked with their respective electrode buffers, then placed on the gel surface. The wicks should reach across the whole gel, but not extend beyond it. Small rectangles (10mm x 5mm) of Whatman No.3 filter paper were placed along the centre of the gel for the application of samples, one piece allowed a sample of 10µl to be applied, however samples of 60µl were applied by stacking five paper pieces on top of each other. Once the samples had been applied the electrodes were positioned and focusing started. The power output setting was 10mA, throughout focusing, but for the first 30min restricted to 200V maximum, the voltage was then set to a maximum of 1500V, which was usually reached within a further 20min. The sample application pieces were removed

following 60min of focusing, extensive streaking of the bands occurred if they were not removed. The gel was focused for a further 60min, then removed and placed in fixative immediately. The gels were stained with coomassie blue or silver stained as described below.

STAINING METHODS.

Coomassie blue.

Solutions used for all coomassie blue procedures; fixative/destaining solution; 400ml methanol, 70ml acetic acid, 530ml ultrapure water.

Staining reagent; 1.25g Coomassie Brilliant Blue G dissolved in 200ml methanol, made up to 500ml by addition of 35ml acetic acid and 265ml water. This reagent was filtered twice before use.

Normal procedure used for all gels except isoelectric focusing.

Gels were immersed in fixative solution for at least 2h, this leads to the fixing of the protein bands and also removes urea or SDS from the gel. The gels were transferred to staining reagent for at least 2h. Gels were destained in destaining solution until the background had cleared. The destaining solution was periodically replenished by passing it through a column of activated charcoal. The gels were stored in 5% acetic acid. Gels that had not stained or

destained evenly were stored in the destaining solution last used, which should still contain a small amount of coomassie blue, and therefore be light blue. Storage in this solution leads to an even clearing of the background and may increase the staining intensity of the bands. Sigma technical bulletin (MWS-877L) reports that this will lead to discolouration of some protein bands, however this was not observed in the case of myofibrillar proteins.

Rapid procedure.

The following procedure was used when bands were to be cut out and subsequently analysed by further electrophoresis. The gels were immersed in the staining reagent, and were not pre-fixed. The gels were stained for 10min and destained rapidly with replenishment of the destain as often as possible. The gels destained within 30min. The gels were then transferred to water, which was repeatedly changed, to wash out the methanol and acetic acid. The pH of the water was measured, to check when washing was complete. This rapid procedure was necessary if hydrolysis of the proteins was to be avoided, and was sensitive enough to identify the bands to be cut out. The water washes also remove SDS and urea, and if sufficient changes were used no interference from either was observed during subsequent procedures.

Coomassie blue staining of IEF gels.

The gels were firstly immersed in a 20% TCA solution, which both fixes the proteins and washes out the Pharmalyte, which stains the background if not removed. Gels were fixed for 5min, and became opaque during this stage. The solutions used to stain the gel from this point were the same as for the above method. The gels were transferred to fixative solution, and equilibrated for 10min. This stage equilibrates the gel to the correct pH and methanol concentration, and clears the background prior to staining, otherwise the stain intensity is greatly reduced. The gels were placed in staining reagent for 5min, then destained until the background had cleared. Gels were left to dry at room temperature, and stored with no further treatment.

Silver staining.

Two methods of silver staining were used, both obtained as kits, the Bio-Rad silver stain kit, derived from the method of Merril et al. (1981), and the Sigma silver stain kit, based on the method of Heukeshoven & Dernick (1985). Gels were stained as per instructions supplied with the kits with the following variations.

Bio-Rad

To obtain a clear background, two additional deionised water washes were performed. An additional wash after the

oxidizer step, and the deionised water wash following the silver reagent step was performed for the same total time indicated in the schedule, but using a change of water when half the wash period had elapsed. These additional washes appear to reduce the carry over of reagent, and reduce the precipitation of silver on the surface of the gel.

Sigma

The deionised water wash, between the fixing and silver equilibration steps, was performed for the same total time period but six changes of water were used. The rapid water wash prior to development of the gel was performed twice. This led to a slower development of the bands, but the contrast between the bands and background was improved.

The reducer step was not normally used as the additional wash before development reduced background staining and it was found unnecessary, except where staining was allowed to proceed past the best end point for background clarity to identify minor bands on overloaded gels. These bands could be visualised and the reducer used to obtain the best contrast between the bands to be visualised and the background.

Comments

Both methods worked well, and gave approximately a 25-fold increase in sensitivity over coomassie blue staining for the actin bands. However myosin, tropomyosin, and

troponin C did not silver stain well and coomassie blue was used when these bands were to be studied.

The Sigma stain kit was eventually used in preference to the Bio-Rad kit. The reason for this preference was that the timing and temperature of the Bio-Rad stain was critical, and agitation had to be constant to obtain the best results. The water washes following the oxidizer step were critical: too little, and the background stained heavily, too much and the protein bands did not stain to full intensity. The temperature sensitivity meant that a water bath had be used to obtain optimal results.

The Sigma stain was less time and temperature dependent, as the only time dependent step was the equilibration to the silver reagent, which appeared to be complete in the time given in the schedule, as increasing the equilibration time did not lead to any variation in the staining intensity observed in either the background or the bands. The decreased temperature sensitivity allowed the staining to be performed at room temperature without the use of a water bath. It was the ease of use and reproducibility which led to the choice of the Sigma kit over the Bio-Rad silver stain kit.

2-dimensional electrophoresis and related techniques.

Samples obtained by cutting out bands or tracts from one gel and analysing them on a different type of gel were

performed using the same basic method. The first dimensional gel was stained and destained by the rapid coomassie blue method. Once the band pattern could be observed the gel was placed in a large volume of ultrapure water at 4° C. The water is replaced every 15min until the pH of the water does not fall following its addition to the gel. The band patterns could still be seen, and the proteins appeared to be fixed by virtue of the very low ionic strength within the gel. This water wash/fixing method appears to have a number of advantages. It eliminates possible artifacts due to the presence of residual destain solution in the gel, and the swelling of the gel caused by the decreased ionic strength seems to increase the rate of equilibration to a new buffer. The bands of interest were cut out of the gel and added to an equal volume of double concentration sample buffer appropriate to the second dimension and left to equilibrate for 30min at 25° C. The equilibrated gel pieces may be stored at −25° C. Vertical slab SDS electrophoresis and isoelectric focusing were both used as the second dimension and the samples were applied as follows. The gel pieces for SDS were cut into pieces small enough to fit into the sample wells, and the wells were drained of all buffer. The gel pieces were positioned at the bottom of the wells with the aid of a small spatula. Once all the samples had been applied, electrode buffer was carefully overlaid, and the upper

electrode chamber filled. At this point any of the sample buffer remaining from the equilibration is added to the sample wells. The SDS gels used as a second dimension were made with a very long stacking gel, up to half the total length of the gel. This appeared to decrease the streaking of bands that tended to occur when a normal length stacking gel was used.

The gel pieces for isoelectric focusing as the second dimension were placed directly on the gel surface taking care that no bubbles were trapped beneath. A small volume of the sample buffer was applied to the edges of the gel slice to ensure a good electrical contact between the sample and the gel. For individual bands, sample buffer used to equilibrate the gel pieces was used, whereas for IEF of whole tracts from SDS gels , fresh sample buffer was used to avoid any artifacts due to leeching of proteins during equilibration The gel was then run exactly as described for isoelectric focusing in the section above.

Estimation of the relative proportions of tropomyosin and troponin-T isoforms.

The ratios of alpha-TM to beta-TM, and the two TN-T bands, were estimated from the relative areas under the peaks of densitometric scans of coomassie blue stained SDS gels. Densitometry was performed at 550nm as described in chapter 2. The best resolution was found to be in 13%

polyacrylamide gels.

The relevent portion of the gel was scanned at maximum resolution to reveal the double peaks, and the peak areas separated by dropping a line vertically from the lowest point between the two peaks. The area of each peak was then calculated using the computer software supplied with the densitometer. White muscle samples from 10 cold- and 10 warm-acclimated carp were analysed.

Estimation of the relative proportions of the myosin light chains.

The relative proportions of MLC1, MPLC2, and MLC3 were estimated from the relative areas under the peaks of densitometric scans of coomassie blue stained 13% polyacrylamide SDS gels. Densitometry was performed at 550nm as described in chapter 2. The relevent portion of the gel was scanned at maximum resolution and the area of each peak calculated using the computer software supplied with the densitometer.

## RESULTS

### Myofibrillar ATPase.

The ATPase activity of myofibrils was calculated from the straight line portion of the Pi production curve. This was found to be necessary, as preliminary results showed that the rate did not appear to stablise until 1 min after the addition of the ATP to start the reaction (Figure 9). The myofibrillar $Ca^{2+}Mg^{2+}$ ATPase activity of cold-acclimated carp showed a significant increase when assayed at both 8° C ($p < 0.005$) and 20° C ($p < 0.025$) (Table 4). The $Q_{10}$ of the ATPase activity was found to be 2.38 for 8° C acclimated carp and 3.15 for 20° C acclimated carp. This suggests that the optimum temperature for the cold-acclimated carp ATPase was lower than that of the warm-acclimated carp. The response of the ATPase of carp myofibrils appears to be a change in the ATPase enzyme system that leads to a higher activity over the temperature range 8-20° C, but possibly at the expense of thermal stability.

The EGTA $Mg^{2+}$ ATPase only showed a significant response to acclimation when assayed at 20° C ($p < 0.005$). The EGTA $Mg^{2+}Ca^{2+}$ ATPase activity at 8° C was low and difficult to measure accurately under the same conditions used for the $Ca^{2+}$ ATPase, this may be the reason for no significant difference being observed. This result tends to suggest that the changes in the ATPase activity, when measured at 20° C

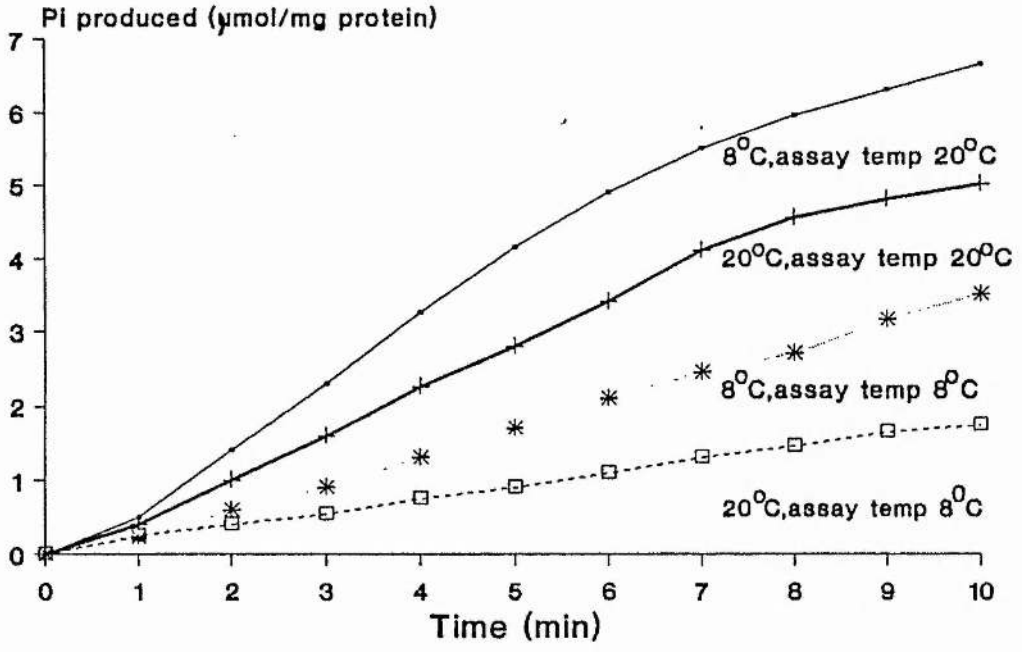



Figure 9. Rate of Pi production of cold- and warm-acclimated carp assayed at 8° C and 20° C. ATPase activity was calculated from the slope of the line over its linear phase. The 8° C acclimated carp can be seen to have a higher ATPase activity at both assay temperatures. The rate of Pi production was variable during the initial minute of the reaction.

| Assay temperature (°C) | Acclimation temperature (°C) | ATPase activity $Ca^{2+}$ | ATPase activity EGTA | Calcium sensitivity (%) |
|---|---|---|---|---|
| 8 | 8 | 0.362 (0.024) | 0.052 (0.012) | 85.6 |
| | 20 | 0.184 (0.014) | 0.032 (0.004) | 82.6 |
| 20 | 8 | 0.862 (0.049) | 0.158 (0.011) | 81.7 |
| | 20 | 0.580 (0.048) | 0.099 (0.009) | 82.9 |

Table 4. White muscle myofibrillar ATPase activities of cold-and warm-acclimated carp. The assays were carried out as described in the text. Units of ATPase activity are: $\mu$mols $P_i$ released/mg protein/min. Values are: Mean (S.E.).

are not due to alterations of the calcium regulatory proteins alone, as there was no significant loss of calcium sensitivity.

Electrophoretic analysis.

SDS electrophoresis of myofibrils from warm- and cold-acclimated carp (Fig. 10) showed no apparent differences. Plots of log MW of standard proteins against mobility relative to the bromophenol blue front (Fig. 11). Using both the 14,000-70,000 Sigma MW-SDS-70L kit, and the 30,000-200,000 Sigma MW-SDS-200 kit, enables estimates of the molecular weight of all the myofibrillar proteins. The bands were identified by procedures to be described later in the text. The apparent molecular weights were; myosin heavy chains (MHC) 205,000; actin 44,500; alpha-tropomyosin 37,000; beta-tropomyosin 36,000; troponin T 31,000; troponin Tx 32,000; myosin light chain 1 25,000; troponin I 21,000; myosin light chain 3 20,000; troponin C 18,700; myosin light chain 2 16,500. All the bands appeared to have molecular weights in the expected range for white muscle of vertebrates, except myosin light chain 3. The decreased mobility of carp MLC3 on SDS electrophoresis at alkaline pH was also seen by Huriaux & Focant (1978). The densitometric scanning of the tropomyosin/troponin T region of the gel (Fig. 10a) was used to estimate their relative proportions. The ratio of alpha-tropomyosin to beta-tropomyosin was 1:1,

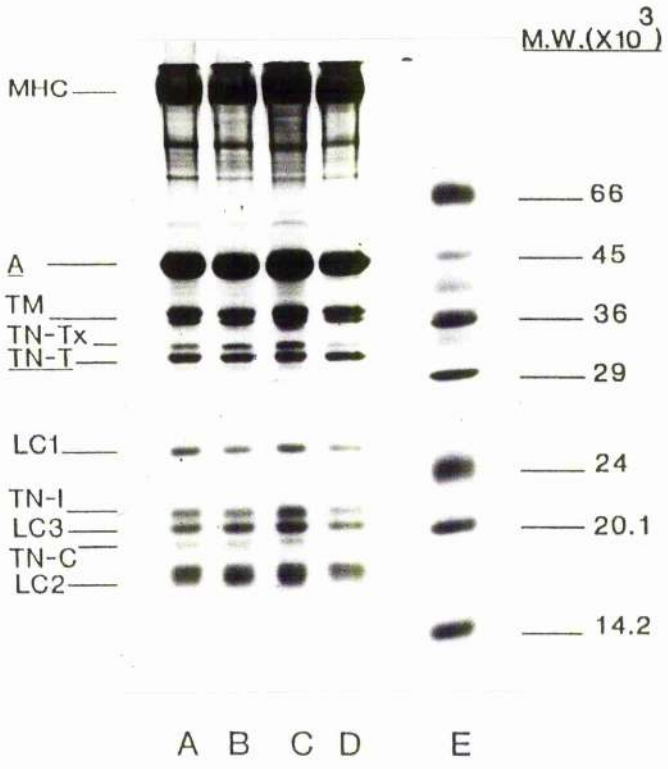



Figure 10. 13% SDS polyacrylamide gel of white muscle myofibrils from cold and warm acclimated carp. Stained by coomassie blue. Molecular weights were determined by running proteins of known molecular weight as markers. Conditions of electrophoresis were as described in text.
Key; A, C, cold acclimated carp. B, D, warm acclimated carp. E, molecular weight marker proteins. MHC, myosin heavy chain. A, actin. TM, $\alpha$-tropomyosin and $\beta$-tropomyosin. TN-x, possible troponin T isoform. TN-T, troponin T. LC1, myosin light chain 1, TN-I, troponin I. LC3, myosin light chain 3. TN-C, troponin C. LC2, myosin light chain 2.

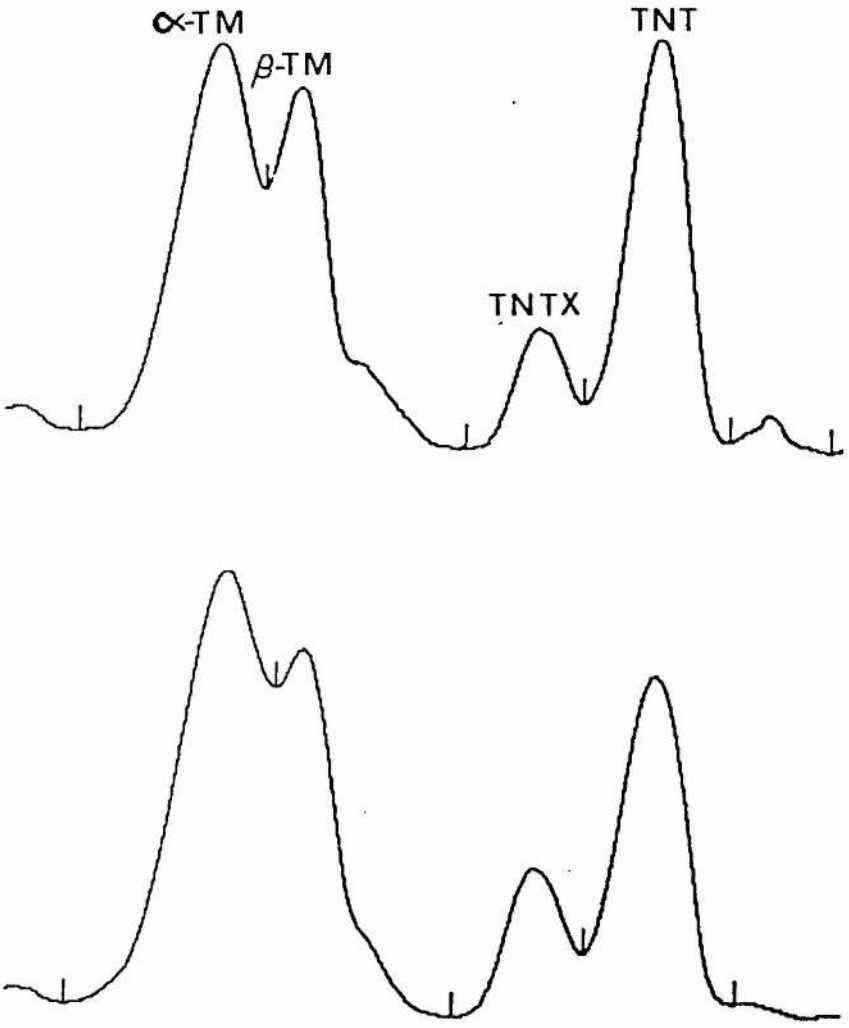



Figure 10a. Densitometric scan of the tropomyosin and troponin T region of a 13% SDS polyacrylamide gel stained with coomassie blue. The scan was used to estimate the relative proportions of the isoforms of each protein. The gel was scanned and analysed as is described in the text. Key; ∝-TM ∝-tropomyosin, β-TM β-tropomyosin, TNT troponin T, TNTX troponin T isoform described in the text.

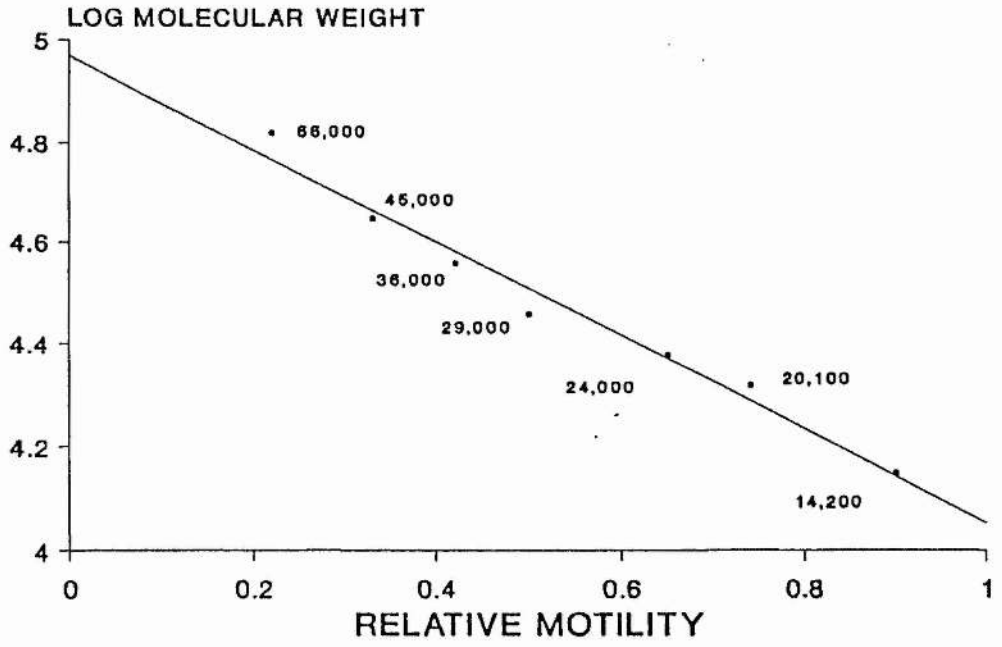



Figure 11. Plot of log molecular weight against relative motility for molecular weight standard proteins on a 13% SDS polyacrylamide gel. The plot was used to calculate the apparent molecular weight of unknown proteins. The conditions of electrophoresis were as described in the text. The inserted numbers indicate the known molecular weight of the standard proteins.

| Acclimation temperature (°C) | Percentage α-Tropomyosin (Mean ± S.E.) | Percentage β-Tropomyosin (Mean ± S.E.) | Ratio αTM:βTM |
|---|---|---|---|
| 8 | 50.1 ± 3.47 | 49.9 ± 3.47 | 1.0:1.0 |
| 20 | 50.0 ± 3.04 | 50.0 ± 3.04 | 1.0:1.0 |

**Table 5.** The relative proportions of α-Tropomyosin and β-Tropomyosin in myofibrils from the white muscle of cold-and warm-acclimated carp. The percentage of the total tropomyosin identified as each isoform was calculated as described in the text. No significant difference was shown between cold-and warm-acclimated samples by a Mann-Whitney test ($P \leq 0.05$).

and no significant difference was seen between cold- and warm-acclimated fish (Table 5). The ratio of troponin T to troponin Tx was 3.9:1 and 3.3:1 for cold- and warm-acclimated fish respectively (Table 6). The difference was not significant when tested by the Mann-Whitney test.

Alkali-urea electrophoresis of myofibrils (Fig. 12) was performed in the presence and absence of free calcium. The banding patterns of purified myosin on alkali-urea gels have been extensively studied to analyse differences between species and fibre types (Huriaux & Focant, 1974). Therefore the banding pattern on the alkali urea gel was expected to show the general pattern described by Huriaux & Focant (1974). The expected pattern would be in increasing mobility, light chain 1 and contaminating tropomyosin, light chain 2, and light chain 3. Troponin I, troponin T, actin, and MHCs do not enter alkali-urea gels. There was no apparent difference between the cold- and warm-acclimated fish. The ability of troponin I and troponin C to form a stable complex in the presence of 8M urea is well known (Syska, Perry & Trayer, 1974). Extra bands that changed their mobility depending on the presence or absence of free calcium indicated troponin C or troponin C + troponin I complex. The disappearance of band 4 in the absence of calcium and the appearance of band 6, suggests that band 4 was troponin I + troponin C complex and that band 6 was

| Acclimation Temperature (°C) | Percentage Troponin T (Mean ± S.E.) | Percentage Troponin $T_x$ (Mean ± S.E.) | Ratio TNT:$TNT_x$ |
|---|---|---|---|
| 8 | 79.6 ± 2.15 | 20.4 ± 2.13 | 3.9:1.0 |
| 20 | 76.8 ± 1.24 | 23.2 ± 1.23 | 3.3:1.0 |

Table 6. The relative proportion of Troponin T and Troponin $T_x$ in myofibrils from the white muscle of cold- and warm-acclimated carp. The percentage of the total Troponin T identified as each isoform was calculated as described in the text. No significant difference was shown between cold-and warm-acclimated samples by the Mann-Whitney test ($P \leq 0.05$)

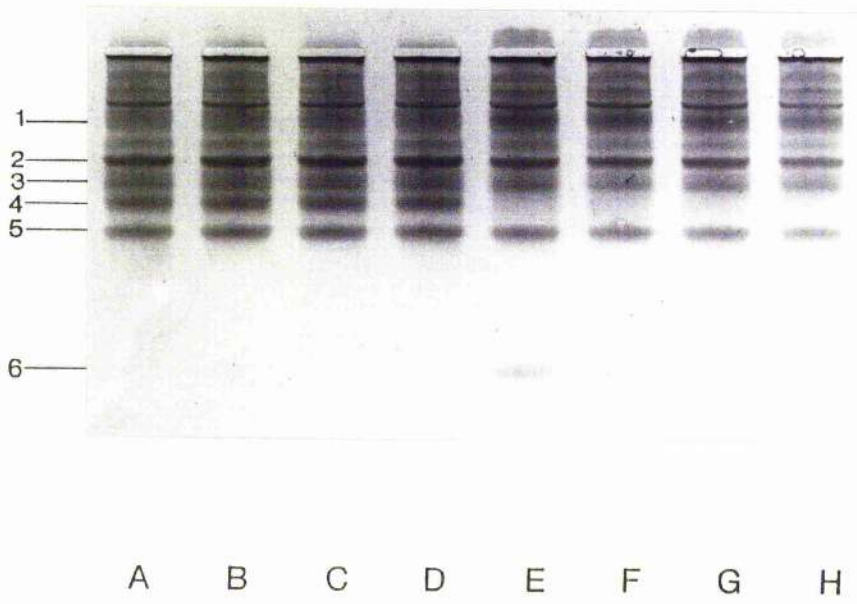



Figure 12. 10% Alkali-urea (pH 8.9) polyacrylamide gel of myofibrils from white muscle of warm and cold acclimated carp. Stained with coomassie blue. Samples containing 5mM calcium and 5mM calcium + 10mM EGTA were compared. Conditions of electrophoresis were as described in text. Key; A, C, cold acclimated carp samples containing 5mM calcium. B, D, warm acclimated carp samples containing 5 mM calcium. E, G, cold acclimated carp samples containing 5mM calcium + 10mM EGTA. F, H, warm acclimated carp samples containing 5mM calcium + 10mM EGTA. 1, tropomyosin. 2, light chain 1. 3, light chain 2. 4, troponin I + troponin C. 5, light chain 3. 6, troponin C. Bands were identified as described in text.

troponin C.

The remaining bands 1, 2, 3, and 5 were run on an SDS gel (Fig. 13). From the expected banding pattern on the alkali urea-gel and the apparent molecular weights, band 1 appeared to be mostly tropomyosin (TM); band 2, MLC1; band 3, TM, MPLC2 and MLC3; band 5, MLC3. The pattern seen in Fig. 13 suggests that the banding pattern on alkali-urea gels may not be as simple as expected. The presence of major TM bands 1 and 3 but not in band 2 indicate the presence TM with two possible charges, but the same molecular weight. A possible reason for this difference was the presence of phosphorylated and non-phosphorylated forms of TM. There appeared to be no difference between cold- and warm-acclimated samples in the banding pattern seen or the relative proportions.

The bands from alkali-urea gels, thought to be TN-I + TN-C, and TN-C, were run on an SDS gel (Fig. 14). The samples ran with molecular weights that would indicate that running alkali-urea gels in the presence and absence of calcium was a useful technique for studying TN-C, and TN-I + TN-C complex. It was also a simple technique for obtaining markers for the identification of banding patterns in myofibrils on SDS gels. No differences were observed between cold- and warm-acclimated samples.

To check that the pattern was not altered by the

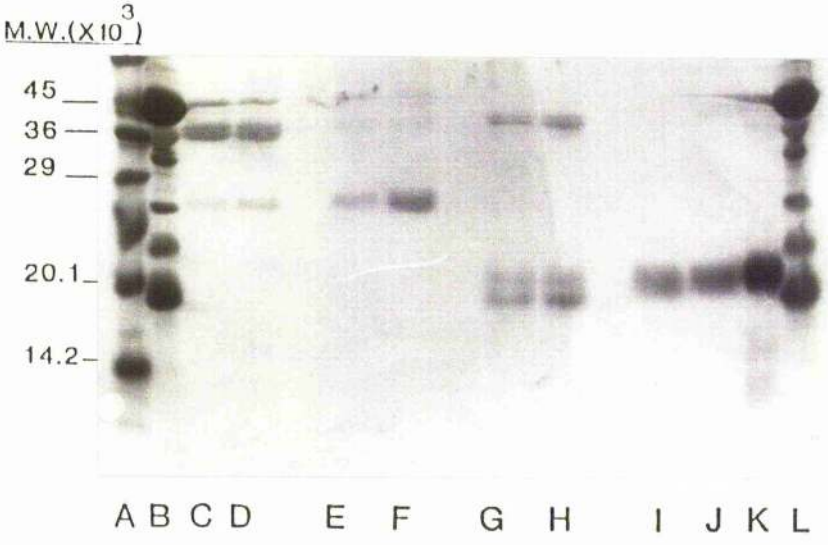



Figure 13. 13% SDS polyacrylamide gel of samples removed from a 10% alkali-urea (pH 8.9) polyacrylamide gel.The major bands, on the alkali-urea gel, other than those proposed to be TN-I and TN-C, were used . Samples were from white muscle of cold and warm acclimated carp. Stained with coomassie blue. The preparation of samples and the conditions of electrophoresis were as described in the text.
Key; A, MW standard proteins. B, myofibrils from cold acclimated carp. C, band 1 from figure 12, tropomyosin, from cold acclimated carp. D, band 1 from figure 12, tropomyosin, from warm acclimated carp. E, band 2 from figure 12, light chain 1, from cold acclimated carp. F, band 2 from figure 12, light chain 1, from warm acclimated carp. G, band 3 from figure 12, light chain 2, from cold acclimated carp. H, band 3 from figure 12, light chain 2, from warm acclimated carp. I, band 5 from figure 12, light chain 3, from cold acclimated carp. J, band 5 from figure 12, light chain 3, from warm acclimated carp. K, MW marker 20,100. L, myofibrils from warm acclimated carp.

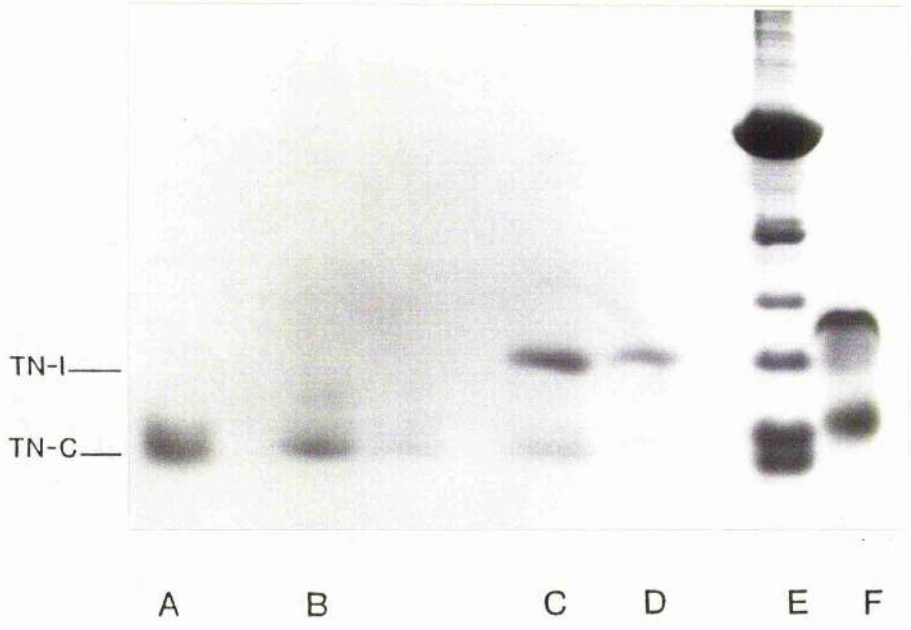



Figure 14. 13% SDS polyacrylamide gel of TN-I + TN-C, and TN-C samples removed from a 10% alkali-urea (pH 8.9) polyacrylamide gel. Samples were from white muscle of cold and warm acclimated carp. Stained with coomassie blue. The preparation of samples and the conditions of electrophoresis were as described in the text.

Key; A, band 6 from figure 12, troponin C, from cold acclimated carp. B, band 6 from figure 12, troponin C, from warm acclimated carp. C, band 4 from figure 12, troponin I, from cold acclimated carp. D, band 4 from figure 12, troponin I, from warm acclimated carp. E, myofibrils from cold acclimated carp. F, MW marker proteins, 20,100 and 24,000.

2-dimensional nature of the technique, a TN-I + TN-C band from an alkali-urea gel and a TN-I band from an SDS gel were run on an SDS gel(Fig. 15). The mobility of the TN-I was the same from both sources. The TN-C band, with a motility of approximatly 20,000, stained very weakly with coomassie blue: this is common and TN-C can be very hard to visualise (Fig. 10), since it does not stain well with silver

The TN-I + TN-C complex and the TN-C samples from alkali-urea gels were run on isoelectric focusing gels (IEF) and migrated to opposite ends of the gel (Fig. 16). They appeared to run as single bands, TN-I with an approximate pI of 9, TN-C with an approximate pI of 3.5. The stable complex formed in the presence of 8M urea that is calcium dependent, the apparent molecular weights and the extreme pIs all indicate that the bands identified on SDS electrophoresis, alkali-urea gels and IEF gels are indeed TN-I and TN-C. No difference was observed between cold- and warm-acclimated carp.

Myofibrillar samples were run on an IEF gel (Fig. 17). The greater resolution achieved on IEF gels resulted in a large increase in the number of bands observed. The acid anodic end of the gel containing the acidic proteins, the myosin light chains, tropomyosin, and troponin C, was characterized by a large number of bands. To achieve greater resolution in this area of the gel, an extended gradient

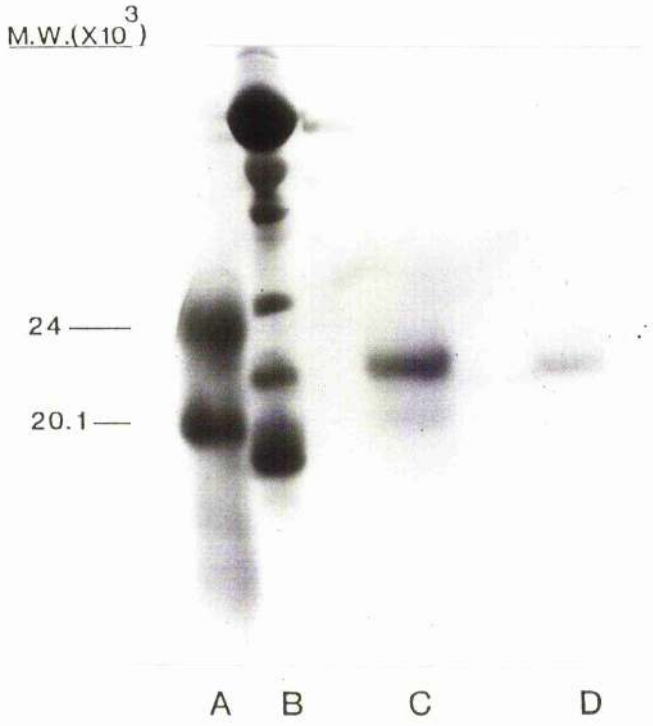



Figure 15. 13% SDS polyacrylamide gel of white muscle TN-I + TN-C removed from a 10% alkali-urea (pH 8.9) polyacrylamide gel, and TN-I removed from a 13% SDS polyacrylamide gel. Stained with coomassie blue. The preparation of samples and the conditions of electrophoresis were as described in the text.

Key; A, MW marker proteins 24,000 and 20,100. B, myofibrils from cold acclimated carp. C, troponin I + troponin C band from cold acclimated carp. D, troponin I band from cold acclimated carp.


Figure 16. Ultrathin polyacrylamide isoelectric focusing gel with linear gradient between pH 3 and pH 10, of white muscle TN-C, and TN-I + TN-C samples obtained from an alkali-urea gel. Samples were from cold and warm acclimated carp. Stained with coomassie blue. Conditions of sample preparation and isoelectric focusing were as described in the text.

Key; A, troponin C from cold acclimated carp. B, troponin C from warm acclimated carp. C, troponin I + troponin C from cold acclimated carp. D, troponin I + troponin C from warm acclimated carp.

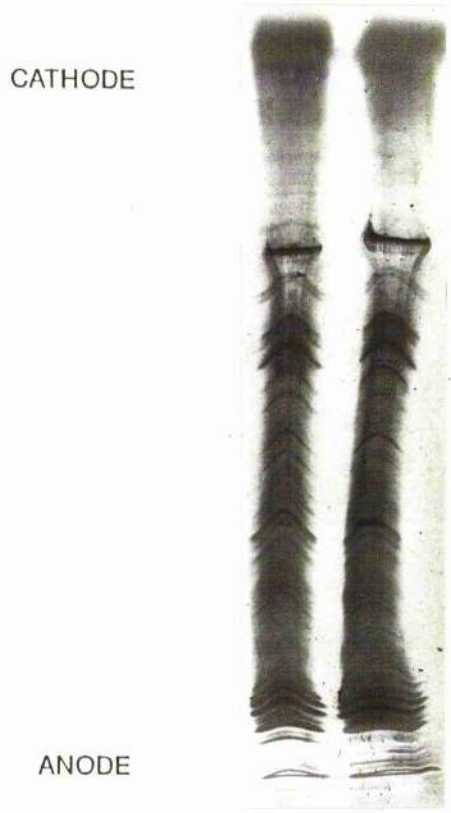



Figure 17. Ultrathin polyacrylamide isoelectric focusing gel with linear gradient between pH 3 and pH 10, of myofibrils. Samples were from the white muscle of cold and warm acclimated carp. Stained with Sigma silver staining kit. Conditions of sample preparation and isoelectric focusing were as described in the text.
Key; A, myofibrils from cold acclimated carp. B, myofibrils from warm acclimated carp.

over the pH 2.5-5.5 range was used. Gels with an extended gradient were used rather than gels of a narrow pH range as it allowed samples to be applied at the cathodic end of the gel, where any precipitation of proteins would not interfere with the final gel pattern. Also the distortion caused by the migration of proteins into the electrodes was minimised. IEF gels of cold- and warm-acclimated carp showed a difference in the banding pattern observed (Fig. 18). The band in the region marked X, was prominent in all cold-acclimated carp samples, and was reduced in intensity or absent in all warm-acclimated carp. The major bands seen in the pH 3.5-5.5 region of the IEF gel were numbered for subsequent analysis by SDS electrophoresis (Fig. 19).

SDS electrophoresis of the bands removed from the the IEF gel revealed that band 1 on the IEF gel appeared to be light chain 1, band 3 appeared to be mostly light chain 2, with a small proportion of tropomyosin, and band 5 appeared to be light chain 3 (Fig. 20). The other bands were not evident when run on the SDS, however band 2 from the IEF gel appears to be a combination of tropomyosin and light chain 2, and the position of band 6 on the IEF gel corresponds to that of troponin C obtained from alkali-urea gels. Band X, the band present in cold-acclimated samples could not be seen on these SDS gels.

Two-dimensional (2-D) electrophoresis, with an SDS gel

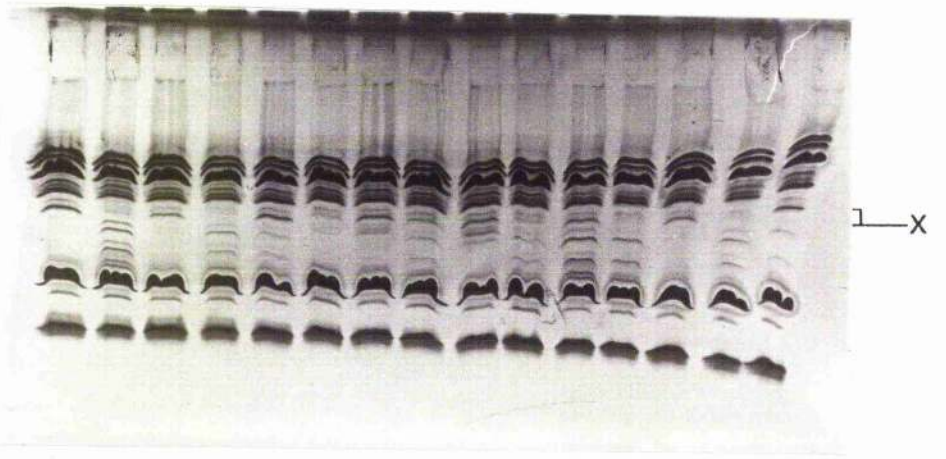



Figure 18. Ultrathin polyacrylamide isoelectric focusing gel with extended gradient between pH 2.5 and pH 5.5, of myofibrils. Samples were from the white muscle of cold and warm acclimated carp. Stained with coomassie blue. Conditions of sample preparation and isoelectric focusing were as described in the text.
Key; A, C, E, G, I, K, M, O, myofibrils from cold acclimated carp. B, D, F, H, J, L, N, myofibrils from warm acclimated carp. X, position of extra band in cold samples.

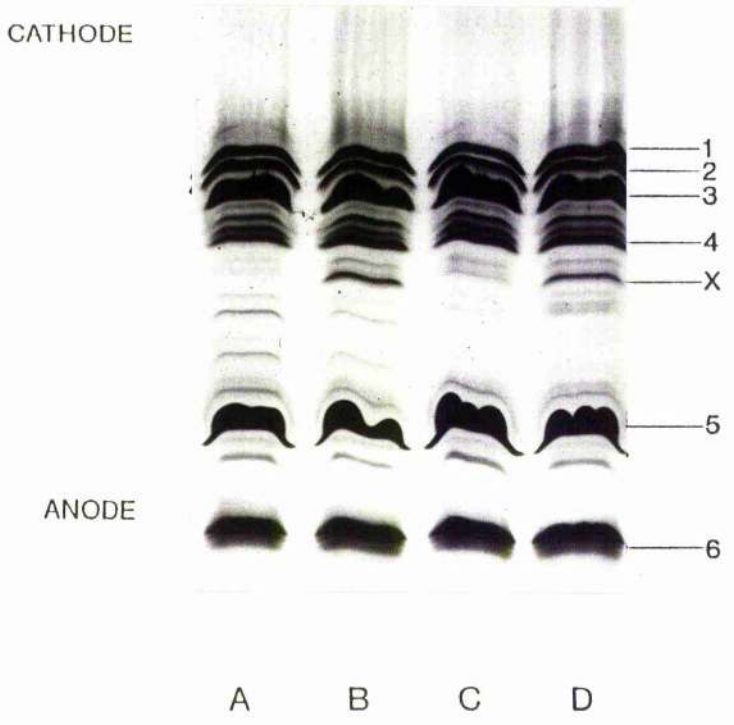



Figure 19. Ultrathin polyacrylamide isoelectric focusing gel with extended gradient between pH 2.5 and pH 5.5, of myofibrils. Samples were from the white muscle of cold and warm acclimated carp. Stained with coomassie blue. Conditions of sample preparation and isoelectric focusing were as described in the text.

Key; A, C, myofibrils from warm acclimated carp. B, D, myofibrils from cold acclimated carp. 1, light chain 1. 2, unidentified band. 3, light chain 2 and trace of tropomyosin. 4, unidentified band. 5, light chain 3. 6, troponin C. Bands were identified as described in the text.

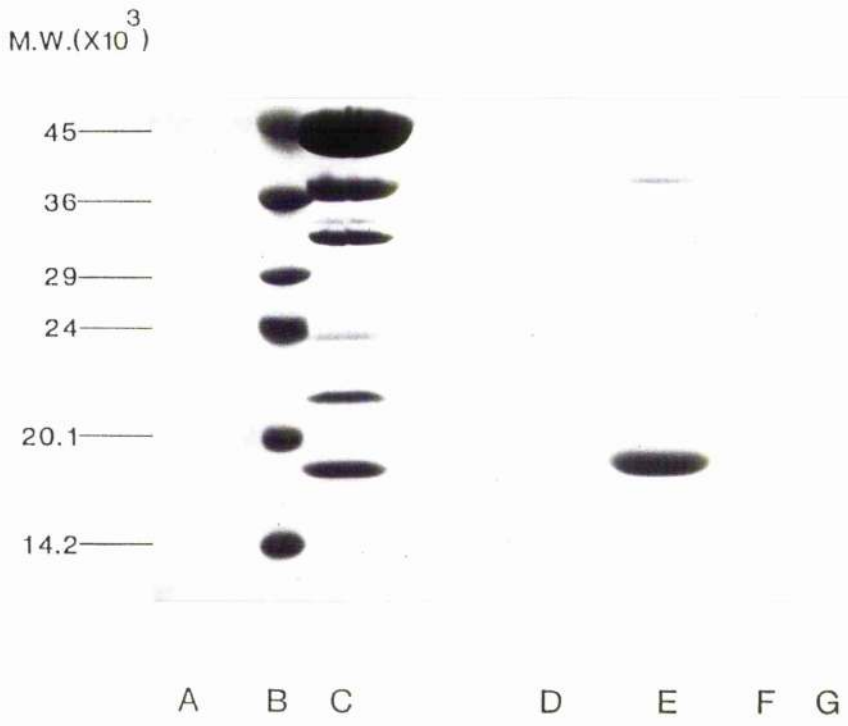



Figure 20. 13% SDS polyacrylamide gel of samples removed from an ultrathin isoelectric focusing polyacrylamide gel.The major bands as indicated in figure 19 were used . Samples were from white muscle of cold acclimated carp were used. Stained with coomassie blue. The preparation of samples and the conditions of electrophoresis were as described in the text.

Key; A, light chain 3, band 5 from fig. 19. B, MW marker proteins. C, myofibrils from cold acclimated carp. D, band 4 from fig. 19. E, light chain 2, band 3 from fig. 19. F, band 2 from fig. 19. G, light chain 1, band 1 from fig. 19.

as the first dimension, and IEF as the second dimension was used in preference to the usual method, of IEF as the first dimension. The main reason for the use of this method was that the banding pattern of myofibrillar proteins on SDS gels was well known, allowing the use of bands as markers in the second dimension. Myofibril samples from cold- and warm-acclimated carp were analysed by 2-D electrophoresis (Figs. 21 and 22). Troponin I and troponin T were well separated from the other proteins, both having pIs about 9. The troponin T spot had a tail which corresponded in position on the SDS dimension to the band labelled as troponin Tx in the normal SDS myofibril gel (Fig. 10). Troponin Tx, being a protein with very similar apparent molecular weight and pI as troponin T, would appear to be an isoform. For cold-acclimated carp an apparent extra band was seen in the same pH region as the extra band seen in IEF gels (Figs. 21 and 19). The position of this band suggested an apparent molecular weight between that of light chain 1 and troponin I.

Two-dimensional electrophoresis of myofibrils was performed, using an extended gradient between pH 2.5 and 5.5, and a first dimension of SDS in the presence of 8M urea (Figs. 23 and 24). The change in the apparent molecular weight of tropomyosin by the inclusion of 8M urea, is a well known method of identifying this protein (Sender, 1971). On

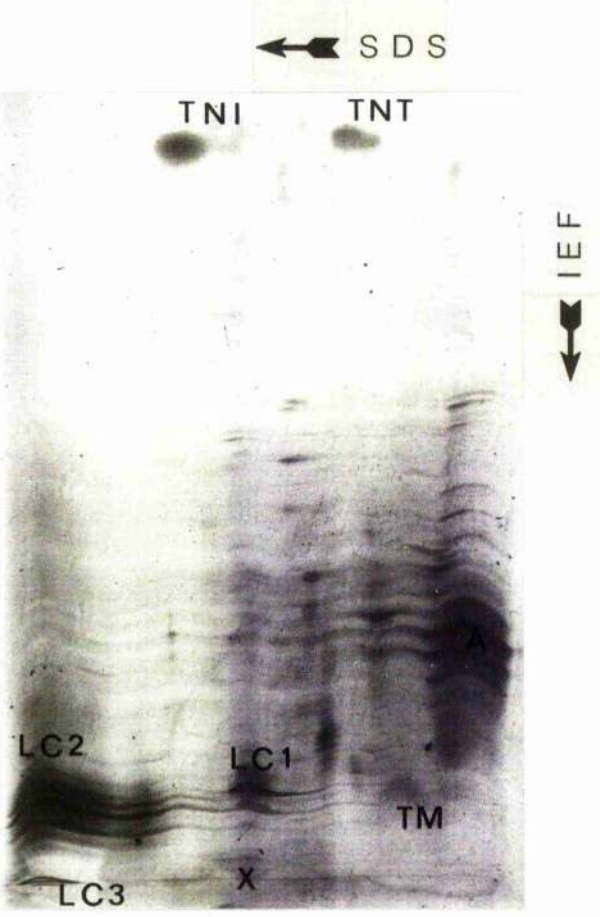



Figure 21. 2-dimensional electrophoresis of myofibrils from white muscle of cold acclimated carp. First dimension SDS electrophoresis on 13% polyacrylamide gel, second dimension on ultrathin polyacrylamide isoelectric focusing gel with linear gradient between pH 3 and pH 10. Stained with Sigma staining kit. Conditions of sample preparation and electrophoresis were as described in the text.
Key; A, actin. TM, tropomyosin. TNI, troponin I. TNT, troponin T. LC1, light chain 1. LC2, light chain 2. LC3, light chain 3. X, extra band seen in cold myofibrils.

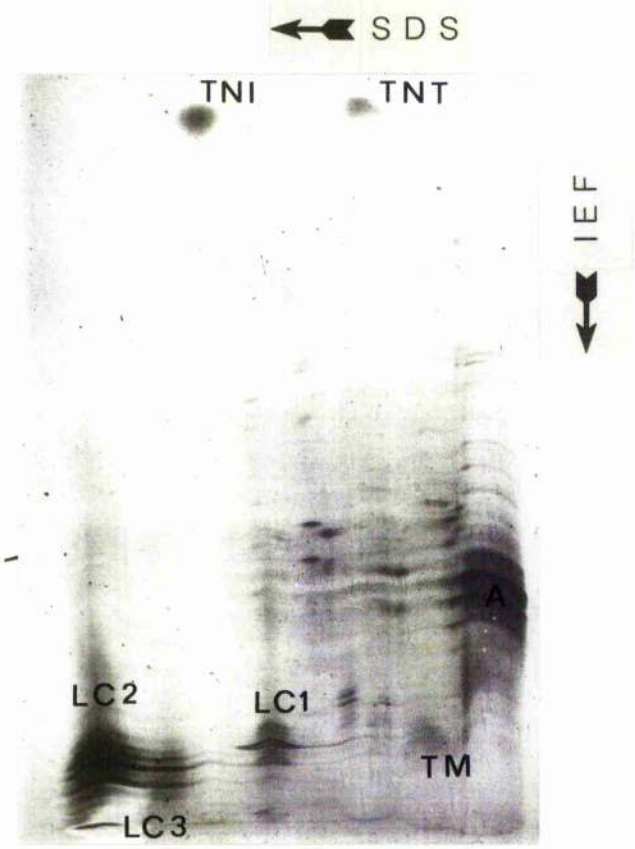



Figure 22. 2-dimensional electrophoresis of myofibrils from white muscle of warm acclimated carp. First dimension SDS electrophoresis on 13% polyacrylamide gel, second dimension on ultrathin polyacrylamide isoelectric focusing gel with linear gradient between pH 3 and pH 10. Stained with Sigma staining kit. Conditions of sample preparation and electrophoresis were as described in the text.
Key; A, actin. TM, tropomyosin. TNI, troponin I. TNT, troponin T. LC1, light chain 1. LC2, light chain 2. LC3, light chain 3.

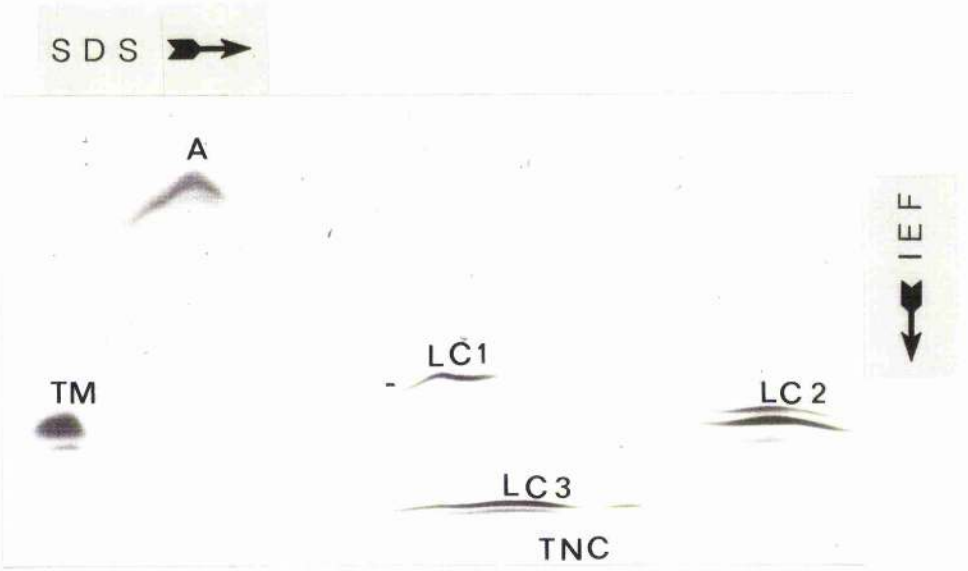



Figure 23. 2-dimensional electrophoresis of myofibrils from white muscle cold acclimated carp. First dimension SDS + 8M urea electrophoresis on 13% polyacrylamide gel, second dimension on ultrathin polyacrylamide isoelectric focusing gel with extended gradient between pH 2.5 and pH 5.5. Stained with coomassie blue. Conditions of sample preparation and electrophoresis were as described in the text.

Key; TM, tropomyosin. A, actin. LC1, light chain 1. LC2, light chain 2. LC3, light chain 3. TNC, troponin C.

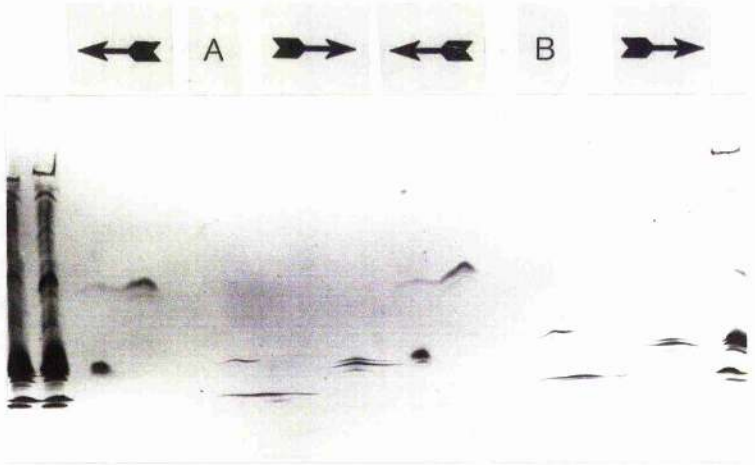
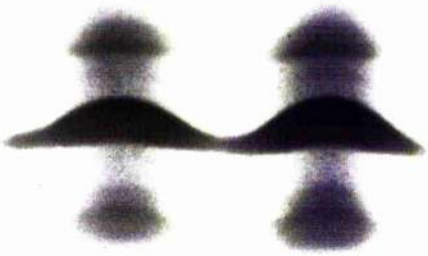



Figure 24. 2-dimensional electrophoresis of myofibrils from white muscle of cold-and warm-acclimated carp. First dimension SDS + 8M urea electrophoresis on 13% polyacrylamide gel, second dimension on ultrathin polyacrylamide isoelectric focusing gel with extended gradient between pH 2.5 and pH 5.5. Conditions of sample preparation and electrophoresis were as described in the text.

Key; A, myofibrils from cold-acclimated carp. B, myofibrils from warm-acclimated carp.

2-D electrophoresis differences in apparent pI of the light chains may sometimes be seen (Fig. 24). To look for possible variation in IEF mobility between the light chains of cold- and warm-acclimated carp, light chain bands identified from SDS and alkali-urea gels were cut out and run on IEF gels (Figs. 25, 26, 27 and 28). This analysis of the light chains revealed that there were no apparent differences in pIs between the bands identified as light chains, and that the variations seen on 2-D electrophoresis were artifacts. All the light chains gave multiple band patterns, however it was not possible to determine if they were due to different proteins or modified form of the same protein.

IEF of red and white myofibrils from cold- and warm-acclimated carp revealed that a double band was present in red myofibrils, in the same approximate position as the extra band in cold-acclimated carp (Fig. 29). This suggested that the extra band seen in cold-acclimated carp could be a red muscle protein. Both the band seen in red muscle samples and that of cold-acclimated white samples proved difficult to run on a second dimension SDS gel. Eventually by running large numbers of the same samples and combining the bands, SDS electrophoresis of the extra band was possible (Fig. 30). This revealed that the red muscle and the cold-acclimated white muscle both contain a band or bands with a similar pI. The pI is between that of white muscle



A B

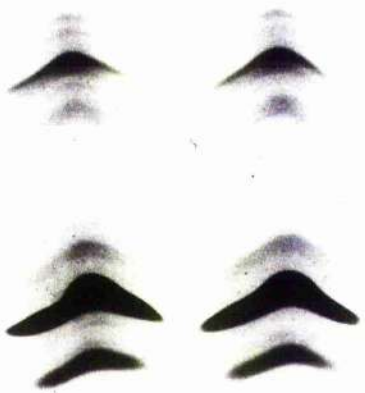
Figure 25. Ultrathin polyacrylamide isoelectric focusing gel with extended gradient between pH 2.5 and pH 5.5, of myosin light chain 1 isolated from electrophoresis on a SDS polyacrylamide gel. Samples were from white muscle of cold and warm acclimated carp. Stained with coomassie blue. Conditions of sample preparation and isoelectric focusing were as described in the text.
Key; A, light chain 1 band isolated from cold acclimated carp. B, light chain 1 band isolated from warm acclimated carp.

A B

Figure 26. Ultrathin polyacrylamide isoelectric focusing gel with extended gradient between pH 2.5 and pH 5.5, of myosin light chain 1 isolated from electrophoresis on an alkali-urea polyacrylamide gel. Samples were from white muscle of cold and warm acclimated carp. Stained with coomassie blue. Conditions of sample preparation and isoelectric focusing were as described in the text. Key; A, light chain 1 band isolated from cold acclimated carp. B, light chain 1 band isolated from warm acclimated carp.

A B

Figure 27. Ultrathin polyacrylamide isoelectric focusing gel with extended gradient between pH 2.5 and pH 5.5, of myosin light chain 2 isolated from electrophoresis on a SDS polyacrylamide gel. Samples were from white muscle of cold and warm acclimated carp. Stained with coomassie blue. Conditions of sample preparation and isoelectric focusing were as described in the text.
Key; A, light chain 2 band isolated from cold acclimated carp. B, light chain 2 band isolated from warm acclimated carp.



A B

Figure 28. Ultrathin polyacrylamide isoelectric focusing gel with extended gradient between pH 2.5 and pH 5.5, of myosin light chain 3 isolated from electrophoresis on a SDS polyacrylamide gel. Samples were from white muscle of cold- and warm-acclimated carp. Stained with coomassie blue. Conditions of sample preparation and isoelectric focusing were as described in the text.
Key; A, light chain 3 band isolated from cold-acclimated carp. B, light chain 3 band isolated from warm-acclimated carp.

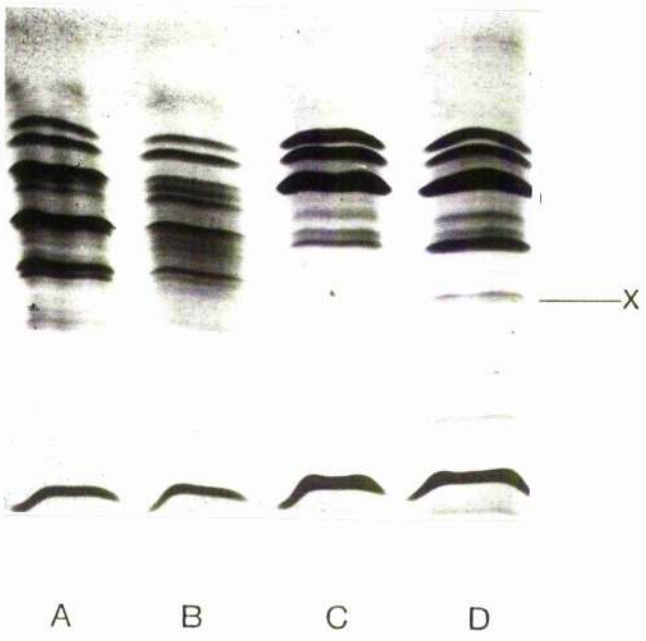



Figure 29. Ultrathin polyacrylamide isoelectric focusing gel with extended gradient between pH 2.5 and pH 5.5, of red and white myofibrils. Samples were from cold and warm acclimated carp. Stained with coomassie blue. Conditions of sample preparation and isoelectric focusing were as described in the text.

Key; A, myofibrils from red muscle of warm acclimated carp. B, myofibrils from red muscle of cold acclimated carp. C, myofibrils from white muscle of warm acclimated carp. D, myofibrils from white muscle of cold acclimated carp. X, extra band seen in cold acclimated carp.

light chains 2 and 3 (Fig. 19). The apparent molecular weight of the band was about 20,000 (Fig. 30). The pI, and the apparent molecular weight suggest that the extra protein found in the white muscle of cold-acclimated carp could be a red muscle myosin light chain.

To check that the extra band was not due to contamination of the white muscle samples by red fibres, single fibres from deep in the white muscle were dissected out and identified by their distinctive appearance. IEF of these single fibres revealed the presence of an extra band in cold-acclimated carp, in the same position as that seen in myofibril samples (Figs. 31 and 32) The small protein content of the single fibres has made it impossible as yet to run an SDS gel of the cut out band to estimate its molecular weight. As the single fibres contain the soluble muscle proteins as well as the myofibrillar proteins, it is not known if the extra band seen in the cold-acclimated myofibrils is the same as the extra band seen in the single fibres of cold-acclimated carp. Other variations in the banding patterns on IEF of single fibres from cold- and warm-acclimated carp were also seen, but the nature of these proteins remains obscure (Fig. 32).

The extra band found in the cold-acclimated white muscle with a molecular weight of 20,000 will co-migrate with LC3 on SDS gels. Therefore, the relative proportions of

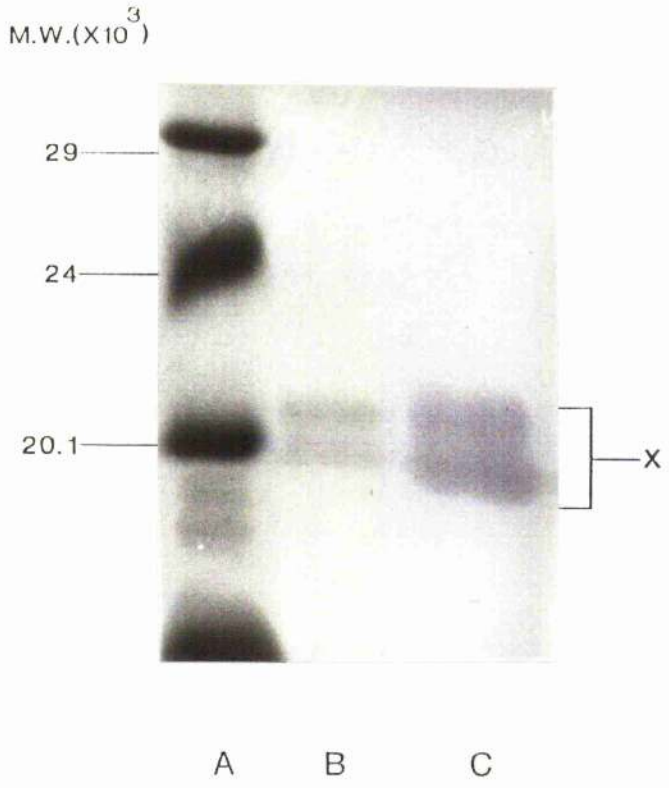



Figure 30. 13% SDS polyacrylamide gel of extra band present in isoelectric focusing. Samples from cold acclimated carp white muscle, and corresponding band from cold acclimated carp red muscle.Samples were removed from an ultrathin isoelectric focusing polyacrylamide gel. Stained with coomassie blue. The preparation of samples and the conditions of electrophoresis were as described in the text. Key; A, molecular weight marker proteins. B, sample from white muscle of cold acclimated carp. C, sample from red muscle of cold acclimated carp. X, double band pattern seen in both red and white muscle.

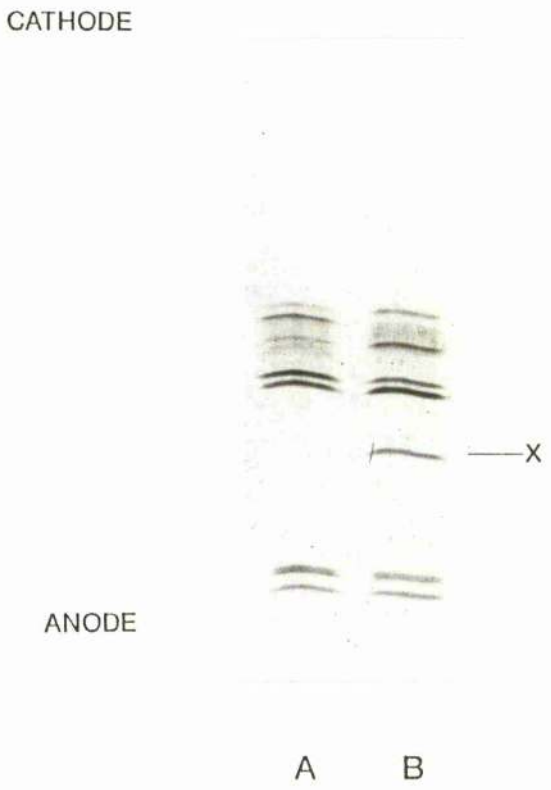



Figure 31. Ultrathin polyacrylamide isoelectric focusing gel with extended gradient between pH 2.5 and pH 5.5, of single white fibres. Samples were from cold and warm acclimated carp. Stained with coomassie blue. Conditions of sample preparation and isoelectric focusing were as described in the text.

Key; A, single fibre from warm acclimated carp. B, single fibre from cold acclimated carp. X, extra band, appears to be the same band present in white muscle samples from cold acclimated carp.

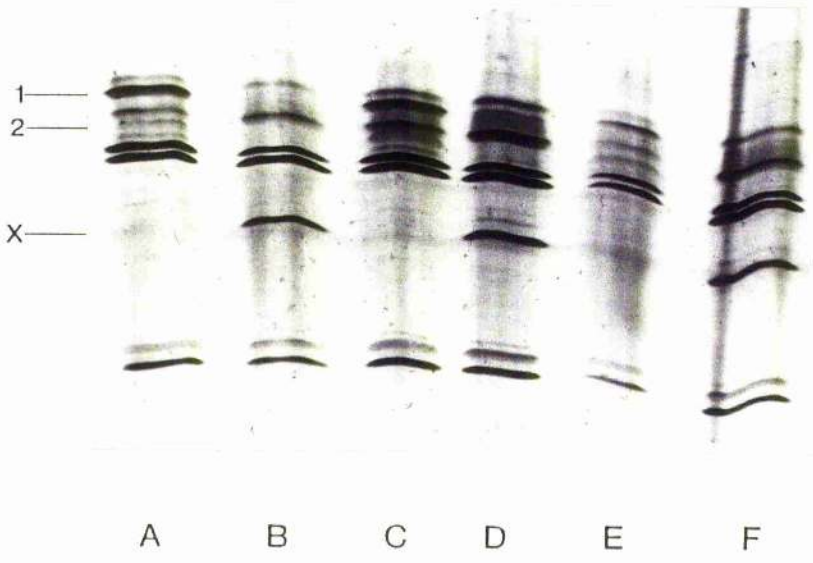



Figure 32. Ultrathin polyacrylamide isoelectric focusing gel with extended gradient between pH 2.5 and pH 5.5, of single white fibres. Samples were from cold and warm acclimated carp. Stained with Sigma silver stain kit. Conditions of sample preparation and isoelectric focusing were as described in the text.

Key; A, C, E, single fibres from warm acclimated carp. B, D, F, single fibres from cold acclimated carp. X, extra band present in cold acclimated carp. 1 and 2, regions of the gel where there appears to be a difference between warm and cold acclimated carp single fibres.

the myosin light chains calculated from densitometery of SDS gels must take this co-migration into account (Fig. 32a). The MPLC2:(MLC1 + MLC3 + extra band) ratio was approximately 1 in both cold- and warm-acclimated carp, which adds support to the suggestion that this extra band is a myosin alkali light chain (Table 7). The ratio (MLC3 + extra band):MLC1 was significantly lower in cold- (2.29:1) than warm-acclimated (2.87:1) carp (Table 7).

During the development of the techniques used for second dimensional SDS gels, an extra band was observed in myofibrils from warm-acclimated carp, with a mobility slightly greater than troponin I (Fig. 33). Densitometric scanning showed the extra band as a shoulder on the major troponin I band (Fig. 33a) IEF showed that it co-migrated with troponin I. The band appears very close to the troponin I band, and was difficult to see, however in 12 warm- and 12 cold-acclimated carp, the apparent extra band was observed in all the warm-acclimated carp but in none of the cold-acclimated samples.

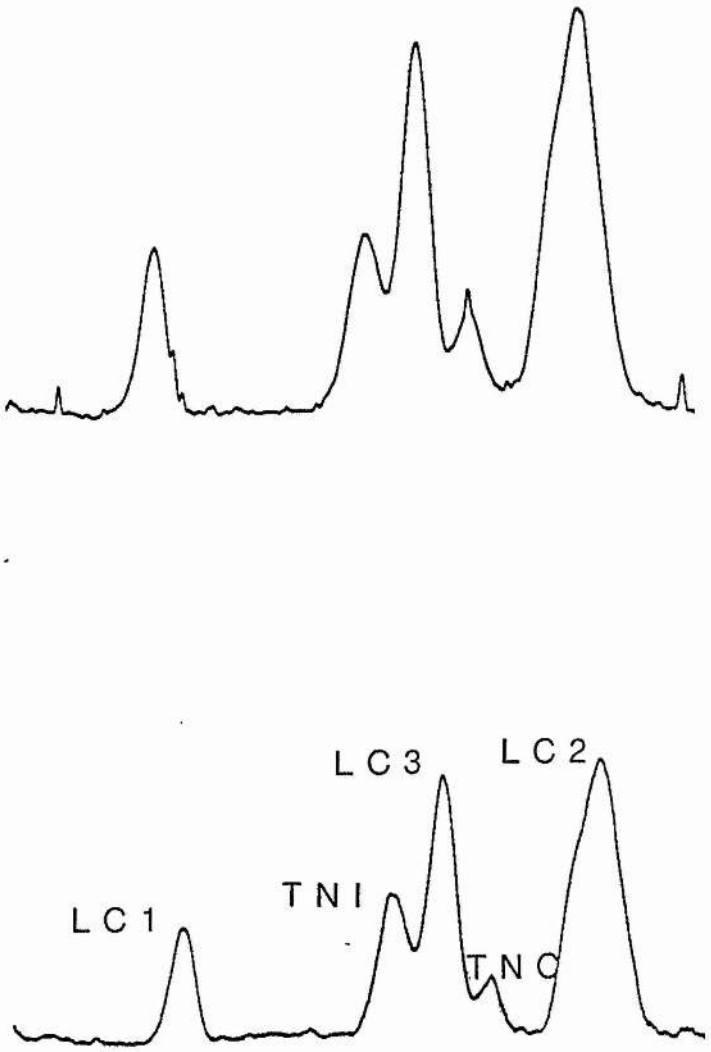



Figure 32a. Densitometric scans used to calculate myosin light chain ratios in cold- and warm-acclimated carp. 13% SDS polyacrylamide gels of myofibrillar samples were run as described in the text, and stained with coomassie blue. Key; LC1 myosin light chain 1, TNI troponin I, LC3 myosin light chain 3, TNC troponin C, LC2 myosin light chain 2.

| Acclimation temperature (°C) | MLC3 : MLC1 ratio (mean ± SD). | MPLC2 : (MLC1 + MLC3) ratio (mean ± SD). |
|---|---|---|
| 8 (n=19) | 2.29 ± 0.11 * | 1.07 ± 0.03 |
| 20 (n=19) | 2.87 ± 0.12 * | 1.09 ± 0.03 |

Table 7. The ratios of myosin light chains from the white muscle of cold- and warm-acclimated carp. The relative proportions of the myosin light chains were determined from densitometric scanning of 13% SDS PAGE gels stained with coomassie blue.

* The difference between the MLC3 : MLC1 ratio of 8° C- and 20° C-acclimated fish is significannt at the $P<0.002$ level (Mann-Whitney test).

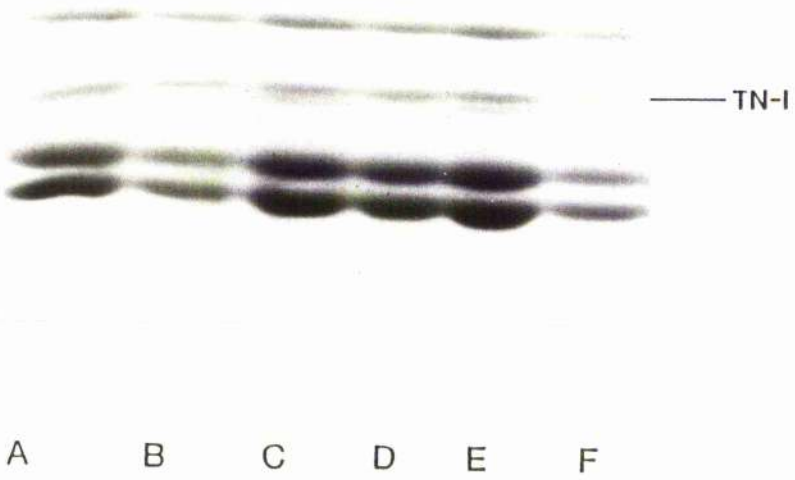



Figure 33. 13% SDS polyacrylamide gel, with an extended upper stacking gel, of white muscle myofibrils from cold- and warm-acclimated carp. Stained by coomassie blue. Conditions of electrophoresis were as described in text.
Key; A, C, E, warm-acclimated carp. B, D, F, cold-acclimated carp. TN-I, troponin I.

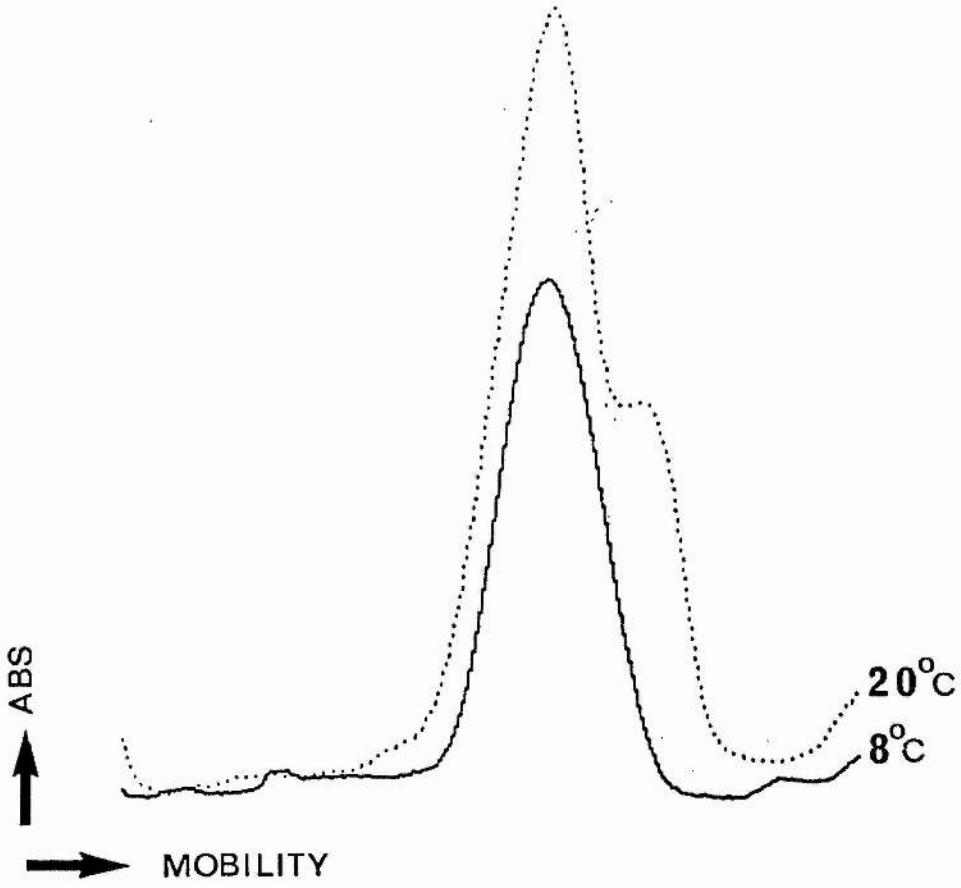



Figure 33a. Superimposed densitometric scans of the troponin I region of a 13% SDS polyacrylamide gel with an extra long stacking gel. The samples were from 20° C- and 8° C-acclimated carp. The shoulder was seen on the trace of all warm-acclimated fish and appeared to be a troponin I isoform. The conditions of electrophoresis were as described in the text.

## DISCUSSION.

### Myofibrillar ATPase activity.

Cold-acclimated carp had a significantly higher myofibrillar ATPase at both assay temperatures (Table 4), confirming the results reported by other workers (Heap et al. 1985). For example the fin muscle from cold-acclimated goldfish had a higher ATPase activity at a range of assay temperatures (0-30° C) (Heap et al. 1987), and the ATPase isolated from cold-acclimated goldfish was more suceptible to thermal denaturation than the enzyme from warm-acclimated fish (Johnston et al. 1975). In some other species myofibrillar ATPase activity appears to be fixed, and is not altered by temperature acclimation. This is the case for the brook trout (Walesby & Johnston, 1981), rainbow trout (Penny & Goldspink, 1981) and mummichog (Sidell et al. 1983). Some species, for example the mummichog, become relatively torpid at low temperatures (Sidell et al. 1983).

### Identification of proteins.

The main problem with the interpretation of the banding patterns obtained by SDS electrophoresis was identifying which band corresponded to which protein. It was not possible to label the SDS pattern by simply comparing the relative motility with results from other workers, unless the same species and the same methodology was used. An example of the possible errors that may occur due to direct

comparison has been shown with respect to the mobility of MLC3 of carp and goldfish muscle (Huriaux & Focant, 1978,1985). On SDS electrophoresis at alkaline pH, the apparent molecular weights of the light chains of most species decrease in the order MLC1 > MPLC2 > MLC3, however in carp and goldfish the order MLC1 > MLC3 > MPLC2 was seen. This anomaly was confirmed in the present study. Variations in mobility have also been observed in the thin filament proteins of different species of fish (Johnston & Walesby, 1979). This illustrates the problems in identifying the banding patterns of different species solely on the basis of molecular weight. The mobility of MLC3 varied with the pH of the electrophoresis buffer, whilst that of MLC1 and MPLC2 did not (Huriaux & Focant, 1978). Variations in the electrophoresis conditions can clearly lead to the misidentification of bands. It is therefore essential to identify the proteins on criteria other than just their molecular weight. The following criteria were used in the present study:

Myosin heavy chains and actin.

The bands labeled as MHC and actin were identified by their apparent molecular weights and the intense staining relative to the other bands, indicating their relative abundance.

Myosin light chains.

The myosin light chains were identified by their descending order of pIs, their apparent molecular weight, and by the unambiguous identification of troponin I and C bands.

Troponin I and troponin C.

Troponin I and C were identified as the proteins that form a stable complex in the presence of 8M urea, and when dissociated possessed highly basic and acidic pIs respectively. The electrophoretically purified troponin I and C were then used to identify the bands on SDS gels.

Tropomyosin.

Tropomyosin was identified by its apparent molecular weight on SDS electrophoresis in the presence and absence of 8M urea, and on its acidic pI. Alpha-tropomyosin and beta-tropomyosin were labelled according to apparent molecular weight.

Troponin T.

Troponin T was identified by its highly basic pI, and its apparent molecular weight which remained unchanged in both the presence and absence of 8M urea.

The pattern of protein banding found by SDS electrophoresis of carp white myofibrils was similar to that reported for carp white muscle actomyosin by other workers (Dabrowska & Szpacenko, 1977; Focant, Huriaux & Vandewalle,

1983).

Actin.

Actin is a highly conserved protein with a molecular weight in carp of 43,000 (Dabrowska & Szpacenko, 1977). In the present study the molecular weight appeared to be 44,500. No difference in pI or molecular weight was observed between cold- and warm-acclimated fish.

Myosin.

None of the electrophoretic techniques used were sensitive enough to differentiate MHC isoforms, but they were suitable for the investigation of the MLCs. The apparent molecular weights of the light chains obtained in this study were MLC1 25,000, MPLC2 16,500, MLC3 20,000. These are similar to those reported by Focant and Huriaux (1976), MLC1 25,000, MPLC2 17,500, MLC3 20,500. From urea gel electrophoresis and isoelectric focusing the pIs appeared to be similar to those of other workers (Dabrowska & Szpacenko, 1977, Focant et al. 1983). No changes in these identified myosin light chains were seen with acclimation.

Myofibrils from cold-acclimated fish contained an extra band on IEF gels which had a pI between that of MPLC2 and MLC3, and an apparent molecular weight of 20,000. On this basis it is likely to correspond to a myosin light chain, and its molecular weight showed that it was not a phosphorylated form of MPLC2. This band was found in the

same proportions in myofibrils prepared with and without the use of Triton X-100. This suggests that it is not a contaminant protein caused be the incomplete removal of the soluble proteins.

The ratios of MPLC2:(MLC1 + MLC3 + extra band) in both cold- and warm-acclimated fish from densitometric scanning of SDS gels were the same (Table 7). This suggests that the extra band that co-migrates with MLC3 was also an alkali light chain, replacing either MLC1 or MLC3 on an equimolar basis. The ratio of MLC3:MLC1 has been shown to vary considerably in fish fast muscle from a variety of species (Huriaux & Focant, 1985). The MLC3:MLC1 ratio varied from 9.1:1 in the goldfish to 1.1:1 in the gudgeon. In this study the MLC3:MLC1 ratios for cold- and warm-acclimated carp were 2.29:1 and 2.87:1 respectively, similar to the 3.3:1 reported by Huriaux and Focant (1985), however their results do not state the temperature at which the fish were kept. These results suggest that changes in the alkali light chain composition accompany temperature acclimation, with the proportion of MLC1 higher in cold- than warm-acclimated fish, and an additional alkali light chain present in cold-acclimated fish. Johnston, Sidell and Driedzic (1985) showed that carp acclimated to 7° C had twice the unloaded contraction velocity of 23° C fish. Greaser et al. (1988) have shown that in single rabbit muscles the $V_{max}$ increased

as the proportion of MLC3 increased. These results suggest that the remodeling of both the relative proportions and isoforms of the myosin light chains may be important acclimatory responses.

Preliminary data showed red muscle myofibrils contained a light chain band with a similar molecular weight and pI, which may be an identical protein to that seen in cold-acclimated carp. The band did not arise from contamination of the white muscle sample with red fibres since a similar banding pattern was found with single fibres.

There are two classes of light chain in fast muscle. The alkali light chains MLC1 and MLC3, which may be removed from myosin by treatment with guanidine-HCl or alkaline conditions, which results in the complete loss of ATPase activity (Weeds & Lowey, 1971). Cross-hybridisation experiments on rabbit myosin subfragment 1 have shown that the alkali light chains have a role in the regulation of ATPase activity (Wagner & Weeds, 1977). The use of mild dissociating conditions that produce myosin subfragment 1 (S1) essentially free of light chains, has shown that the light chain free S1 heavy chain retains an ATPase activity similar to native myosin (Wagner & Giniger, 1981). The use of light chains from different muscles show that around 66% of the difference in ATPase activity between different

myosins is due to the MHC isoforms (Wagner & Giniger, 1981). This suggests that the alkali light chains may not be essential to myosin ATPase activity, as was previously thought, and their role may be in the regulation of the ATPase activity. The other class of light chain may be removed from myosin by treatment with dithiobis-(nitrobenzoic acid) (DTNB). These light chains (MPLC2) may be phosphorylated and are not essential to the ATPase activity of myosin, but they may have a role in the $Ca^{2+}$-dependent modulation of actomyosin interactions (Pembrick, 1980). Thus it would appear that all the subunits of myosin may contribute to the final ATPase activity of a myosin isoform.

Calcium regulatory proteins.

In fish muscle tropomyosin and troponin isoforms have not been reported, although banding pattern differences on SDS and alkali-urea gels of red and white muscle myofibrils indicate that isoforms must be present (Focant, Huriaux & Johnston, 1976). Mammalian muscle contains isoforms of the troponin complex specific to slow and fast type IIB fibres, and an alpha-tropomyosin to beta-tropomyosin ratio that is fibre type specific. The fibre types IC, IIC and IIA, show coexistance of various proportions of tropomyosin and troponin isoforms of the slow and fast fibres (Young & Davey, 1980; Salviati, Betto & Betto, 1982; Staron & Pette,

1987a,1987b).

In this study the band labelled as tropomyosin ran as a double band on both SDS electrophoresis and on isoelectric focusing, and on 2-dimensional electrophoresis with urea present or absent in the first dimensional SDS gel. This suggests that both alpha-tropomyosin and beta-tropomyosin are present in carp white muscle. However Dabrowska and Szpacenko (1977) reported that only alpha-tropomyosin is present in carp actomyosin, and Focant et al. (1981), using a similar electrophoresis procedure (but with 15% acrylamide gels), showed actomyosin to produce a single tropomyosin band. The appearance of two tropomyosins could be due the use of myofibrils rather than actomyosin, although the loss of a particular tropomyosin during the preparation of actomyosin would seem unlikely. A more likely explanation would seem to be that the use of 13% gels achieved a better separation in this region of the gel, which allowed the tropomyosin isoforms to be visualised. However, Dabrowska and Szpanenko (1977) separated alpha-tropomyosin and beta-tropomyosin from rabbit, but only found one tropomyosin from carp. The following observations in the present study suggest that tropomyosin is present as two isoforms of slightly different molecular weight and pI. 1; the apparent molecular weight change on SDS electrophoresis in the presence of urea. 2; co-migration on alkali-urea gels. 3;

the small variation in pI. The results appear to indicate that the expression of tropomyosin is similar to that of other vertebrates. Further clarification of beta-tropomyosin in carp white muscle would require the purification and characterisation of the different isoforms.

Troponin T of carp actomyosin has been tentatively identified as a small band of apparent molecular weight 58,000 that remains in desensitised actomyosin samples by Dabrowska and Szpacenko (1977). However, its presence in desensitised actomyosin, its relatively large molecular weight, and the lack of information on its pI, suggest that it may not be troponin T. In this study troponin T has been labelled as the double band with an apparent molecular weight of 31,000-32,000. The reason for this identification is that this band appears to be absent in desensitised actomyosin (Dabrowska & Szpacenko, 1977), its apparent molecular weight is close to that found in other vertebrates, and its pI is basic, similar to that of troponin I, found in other vertebrates. Without functionally testing the 58,000 and 31,000-32,000 proteins it is not possible to know which is troponin T, however the 31,000-32,000 protein appears to have all the characteristics of troponin T from other species. The appearance of troponin T as a double band on both one and 2-dimensional electrophoresis, both bands having the same pI

but slightly different apparent molecular weights, suggest that they may be isoforms. Three fast troponin T isoforms were found on SDS electrophoresis of rabbit muscle by Greaser, Moss and Reiser (1988), and were expressed as a continuum within single fibres from fast muscle . The $V_{max}$ of the fibre was seen to correlate with the troponin T isoform contained, although the fibres could not be shown to be different histochemically (Greaser, Moss & Reiser, 1988). The absence of correlation between histochemical typing and the composition of the regulatory protein complex was also seen by Schachat, Bronson and McDonald (1985). The isoforms of the regulatory proteins appear to be important to the contractile properties measured in single fibres, and there may be more variations present in the histochemically typed fibres than was previously thought.

In vertebrates, troponin T isoforms were found to be correlated to the the $V_{max}$ of fast fibres (Greaser, Moss & Reiser, 1988), and tropomyosin isoforms correlated to the troponin T isoforms present (Schachat, Bronson & McDonald, 1985). In this study the bands that appear to be troponin T and tropomyosin did not change their relative proportions following temperature acclimation, suggesting that the acclimatory response did not involve a change in the troponin T or tropomyosin isoforms.

Troponin C had a molecular weight of 18,700 similar to

the 18,000 reported by Dabrowska and Szpacenko (1977). The highly acidic pI, and the complex formation with troponin I that was both stable in 8M urea and calcium dependent confirmed the identification. No evidence for troponin C isoforms was seen.

Troponin I had a molecular weight of 21,000, similar to the 21,400 reported by Dabrowska and Szpacenko (1977). The binding of troponin I to troponin C confirmed this identification. An extra band was also seen in myofibrils from warm-acclimated fish. On SDS electrophoresis using a long stacking gel the protein migrated with an apparent molecular weight slightly less than that of troponin I. Isoelectric focusing showed that its pI was the same as troponin I. This suggests that the protein is an isoform of troponin I. Studies on the troponin I/troponin C complex did not not reveal the presence of a troponin I isoform. Therefore this band was only tentatively identified as a troponin I isoform present in warm- but not cold-acclimated carp myofibrils.

The regulatory proteins have been show to play a role in temperature acclimation in fish (Johnston, 1979). The only difference observed in the regulatory proteins was the possible presence of a troponin I isoform in warm-acclimated fish. One possibility is that the major troponin I isoform may be responsible for the high ATPase activity measured at

low temperatures (Heap et al. 1985; Rome et al. 1985), and that the presence of the second troponin I isoform results in a lower ATPase activity.

# CHAPTER 4.

## PEPTIDE MAPPING OF MYOSIN HEAVY CHAINS AND ACTIN FROM CARP (CYPRINUS CARPIO) ACCLIMATED TO A RANGE OF TEMPERATURES.

### INTRODUCTION.

Different isoforms of myosin heavy chain have been demonstrated in single fibres, from adult skeletal muscle, that have been categorised histochemically (Young & Davey, 1981; Salviati, Betto & Betto, 1982; Staron & Pette, 1987a, 1987b), and by their contractile properties (Reiser *et al*. 1985a; Sweeney *et al*. 1986; Lannergren, 1987). Variations in the isoforms of MHCs have also been reported in developing muscle (Whalen *et al*. 1979; Dalla Libera, 1981; Reiser *et al*. 1985b), regenerating muscle (Sartore, Gorza & Schiaffino, 1982), and dystrophic muscle (Rushbrook & Stracher, 1979). The existence of MHC isoforms specific to certain fibre types suggests that they may have a direct role in determining the contractile properties of the fibre. Studies with *Xenopus* skeletal muscle fibres, have shown differences in the contractile properties of two fibre types with the same myosin light chain composition, but different MHCs (Lannergren, 1987). The unloaded contraction speed of rabbit muscle fibres ($V_{max}$) was found to correlate with the

type of MHC isoform present (Reiser et al. 1985a, 1985b). The MHC composition of rat gastrocnemius and diaphragm muscle was seen to change following chronic denervation and reinnervation showing that the MHC isoform present in a muscle may change under certain conditions (Carraro et al. 1981).

In carp (Cyprinus carpio) myofibrillar ATPase activity increases in the series slow < intermediate < fast muscle fibre types. The activity of fast muscle myofibrils was 4-times that of slow muscle myofibrils (Johnston, Davidson & Goldspink, 1977). Histochemical studies on the stability of myosin ATPase with respect to pH have shown five muscle fibre types in carp (Akster, 1983). The myosin light chain composition of red and white muscle myosins of various fish species appears similar to that of mammalian myosins (Focant, Huriaux & Johnston, 1976). This suggests that there may also be isoforms of the MHC present in fish muscle.

The contractile properties of goldfish fin muscles (Heap, Watt & Goldspink, 1987), and carp myotomal muscle fibres (Johnston, Sidell & Dreidzic, 1985) are know to be altered by several weeks temperature acclimation. Altringham and Johnston (1985) showed that at 7° C, fibres from cold-acclimated carp generated more force than those from warm-acclimated carp. In the same fibres the ATP turnover per myosin head was found to be lower in cold- than

warm-acclimated fish.

Cross-hybridisation of regulatory proteins from cold-acclimated fish to desensitised actomyosin from warm-acclimated fish alters the ATPase towards that of cold-acclimated natural actomyosin, and vice versa (Johnston, 1979). However the ATPase of the cross-hybrid actomyosin remained intermediate between the ATPase of the two host actomyosins. It therefore seems likely that part of the acclimatory response to temperature remains in the desensitised actomyosin, even though its expression may depend on the presence of the regulatory proteins. The apparent correlation between contractile properties and MHC isoform, and the ability of MHC composition to change under certain conditions, points to a change in the MHCs as a possible mechanism for the acclimatory response seen in fish muscle function.

The presence of isoforms of MHC have been investigated by many methods: peptide mapping (Dalla Libera, 1981; Bandman, Matsuda & Strohman, 1982; Sweeney *et al*. 1986), immunological analysis (Whalen *et al*. 1979; Sartore, Gorza & Schiaffino, 1982), non-denaturing electrophoresis (Hoh, McGrath & White, 1976; Lannergren, 1987), and SDS electrophoresis (Reiser *et al*. 1985a, 1985b; Staron & Pette, 1987a, 1987b).

To perform immunological analysis it is necessary to

obtain antibodies specific to one of the MHC isoforms and see if the MHC present in a muscle binds to it. The preparation of antibodies to carp white muscle MHC was beyond the scope of the present study. Analysis by non-denaturing electrophoresis can only be used to identify MHCs if the light chain composition of the myosin sample is known not to vary, otherwise different mobilities of myosins observed could be due to the light chains and not the MHCs. The apparent molecular weight of isoforms of MHC are very similar, making the detection of some MHC isoforms, by SDS electrophoresis, impossible. Peptide mapping allows differences in molecular weight of small portions of the MHCs to be analysed, and variation in the amino acid sequence of isoforms may be detected by this method. For these reasons peptide mapping is the most commonly used method to identify MHC isoforms, and the technique was adopted in this study.

Myosin ATPase activity has been found to correlate with speed of shortening (Barany, 1967). The response to acclimation seen in fish muscle, and the apparent role of MHC isoforms in mammals, suggests similar isoform expressions may contribute to temperature acclimation in fish. Isoforms of actin have not been shown. However the possibility of actin isoforms does not seem to have been investigated in response to temperature acclimation.

The aim of the present study was to determine if MHC and actin isoforms were present in carp white muscle, and if present, did they correlate with temperature acclimation.

METHODS.

Common carp (Cyprinus carpio) were acclimated over a period of 6-9 weeks. The water temperature was gradually altered over a period of 1 week to produce populations of fish, at 2° C, 5° C, 8° C, 11° C, 15° C and 23° C. The fish were then held at this temperature for 5-8 weeks before sampling. The light regime used was 12hr light:12hr dark. The total lengths and weights of the fish used were (mean ± SD), 2° C-acclimated, 23.2 ± 1.6cm, 391 ± 70g; 5° C-acclimated, 23.2 ± 1.6cm, 391 ± 70g; 8° C-acclimated, 22.3 ± 1.6cm, 354 ± 47g; 11° C-acclimated, 23.9 ± 1.6cm, 402 ± 96g; 15° C-acclimated, 23.6 ± 1.8cm, 400 ± 76g; 23° C-acclimated, 24.9 ± 1.1cm, 435.7 ± 68.5g.

Preparation of samples.

Well washed myofibrils in suspension were prepared as described in chapter 3, and mixed with an equal volume of sample buffer, Tris-HCl 125mM pH 6.75, 20% glycerol, 4% SDS, 1% mercaptoethanol, and 0.002% bromophenol blue, to give a final protein concentration of 10mg/ml. The sample was heated to 100° C for 3min, and cooled before use or storage at -25° C.

The sample buffer used to prepare samples for BAC gels contains a lower mercaptoethanol concentration than that normally used for SDS electrophoresis as higher concentrations lead to a breakdown of the interface between

the stacking and separating gels. Trial runs with a range of mercaptoethanol concentrations on BIS crosslinked gels showed that this did not affect the banding pattern that resulted.

BAC crosslinked gels.

3mm thick vertical slab gels were used for the preparation of both actin and MHC. The lower separating gels were BAC crosslinked gels of 8% acrylamide for MHC and 11% acrylamide for actin. The gels were prepared as described in chapter 3, for SDS PAGE, except that BAC was substituted for BIS on an equimolar basis, and different volumes of TEMED and ammonium persulphate were used to polymerise the gel (Hansen, 1976). As the MW of BAC is 260.14 and that of BIS is 154.17, this resulted in BAC being 5% of the total acrylamide when used in place of BIS. As BAC has a lower solubility than BIS it was necessary to heat gently to fully dissolve it.

The amount of TEMED and ammonium persulphate used were different to those used for BIS gels, as the BAC gel was not fully soluble due to non-specific crosslinking if the same polymerisation conditions were used. To ensure soluability BAC gels were polymerised with 3ml TEMED and 7mg ammonium persulphate per 100ml of gel solution. The gel was poured, 1cm of isobutanol layered on top, and the gel took at least

30min to polymerise and no more than 90min. If reagents or temperature varied and led to faster or slower polymerisation then the amount of ammonium persulphate used was adjusted. The gel was left for at least 3hr prior to pre-electrophoresis.

Pre-electrophoresis of the separating gel was necessary to remove excess TEMED and ammonium persulphate from the gel prior to electrophoresis. Preliminary experiments showed that their presence interfered with the banding patterns observed. A large band at the top of the gel with a mobility consistent with an apparent MW of 200,000-250,000 was found on re-electrophoresis to contain a large amount of actin, as well as MHC, and the apparent MW of tropomyosin was increased to 50,000, as was also observed in SDS PAGE in the presence of 8M urea (Sender, 1971). Pre-electrophoresis of the separating gel overnight at 30V, with 2l of an electrode buffer, Tris-HCl 0.372M pH 8.8, 1% SDS, resulted in BAC gels which gave the same banding pattern as obtained with BIS gels (Fig. 10).

A 3cm upper stacking gel, a BIS crosslinked gel with 5% acrylamide was used for both actin and MHC separation, and was prepared exactly as described in chapter 3.

100µl samples were loaded and the gel run as described for BIS gels in chapter 3.

Staining of BAC gels.

The gels were stained using either the rapid coomassie blue method (see chapter 3) or by the copper staining method based on that of Lee et al. (1987). This staining method has the advantage over the fast coomassie blue method in that it was rapid, and as an acid fixation step was not used, the proteins were less prone to hydrolysis during the staining procedure. Following electrophoresis, the gel was immersed in 0.19M Tris pH 8.8, 0.1% SDS for 10min, to fully equilibrate the gel to both SDS and Tris, which was necessary for even staining of the gel. The gel was rapidly washed with deionised water, and placed in a solution of 0.3M copper chloride for a period of 5min at room temperature. The gel was washed in several changes of deionised water, and could be stored indefinitely in deionised water. The stained gel was observed by placing the gel on a glass plate with a piece of matt black card beneath, and illuminating the gel from above. The proteins appeared as clear bands against an opaque background.

Electroelution of proteins.

Once the gel had been stained and destained with coomassie blue, the relevent bands were cut out using a scalpel, and placed in 100ml beakers.

Coomassie blue stained gel slices were extensively

washed with ultrapure water to remove the acetic acid and methanol. This was seen to have been achieved, when the pH of the water did not decrease on equilibration with the gel slices.

To remove the copper chloride from copper stained gel slices, they were washed in 20 volumes (w/v) with three changes (10min each) of 0.25M EDTA, 0.25M Tris-HCl, pH 9.0, the solution was agitated during the washes. The gel slices were then washed three times for 10min with 100ml of ultrapure water.

The gel slices were cut into small pieces, and solubilised by adding 1 volume (v/w) of buffer, Tris-HCl 50mM pH 7.4, 2% SDS, 20% mercaptoethanol, 0.001% bromophenol blue.

Electroelution chambers adapted from Allington et al. (1978) (Fig. 34) were prepared by soaking overnight in 1% Decon, then rinsing well with tap water and finally ultrapure water. Dialysis membrane squares (25mm x 25mm) were cut then boiled in two changes of ultrapure water, finally they were soaked in fresh ultrapure water. The chambers were assembled by holding the dialysis membrane across the end of the tube, and sliding the silicone tubing ring over until the membrane was securely held. The electrode baths were filled with buffer, Tris-HCl 50mM pH 7.4, 2% SDS, and the chambers fitted and filled with the

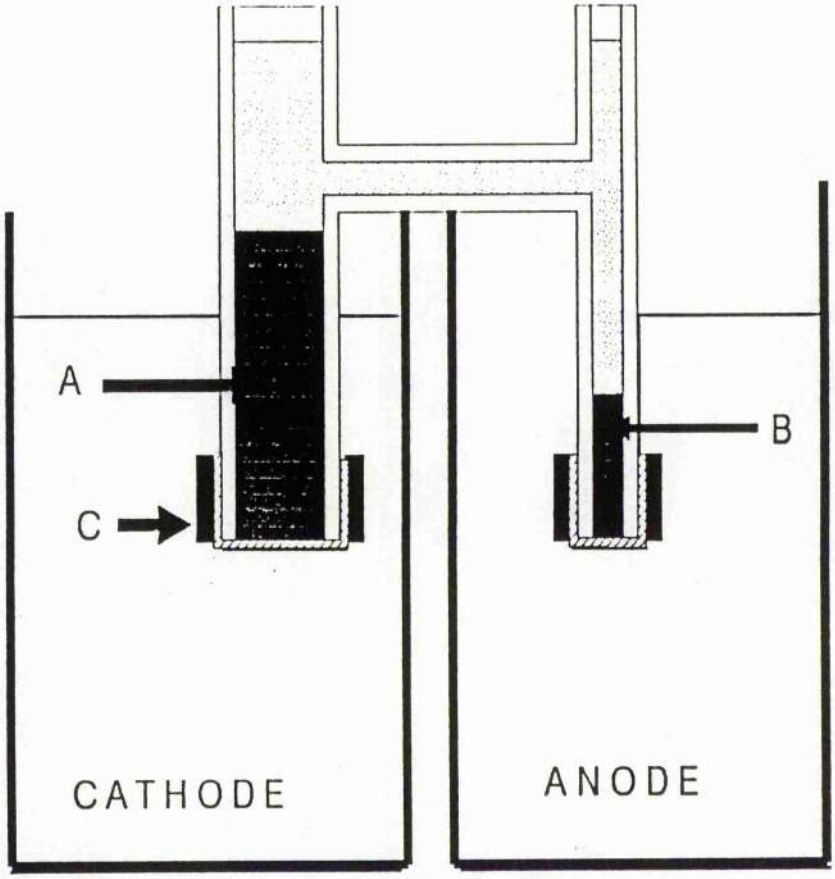



Figure 34. Electroelution chamber used for concentrating and buffer exchange in the preparation of myosin heavy chains and actin for peptide mapping. The methods and buffers used for electroelution are described in the text.
Key; A large bore arm of the chamber for original sample, B narrow bore arm of the chamber for collection of the sample following electroelution, C silicone rubber sleeve sealing the dialysis membrane in place.

same buffer, care being taken to ensure no bubbles were trapped within the chamber.

0.2ml of sample collection buffer, Tris-HCl 50mM pH 7.4, 2% SDS, NaCl 150mM, was carefully layered into the bottom of the small arm of the chamber, and the liquified gel sample layered into the bottom of the larger arm of the chamber.

The use of a sample collection buffer with a higher ionic strength than the electrode buffer decreased the rate of migration of the proteins in this part of the chamber, due to the SDS-protein complex contributing less to the overall charge movement. This allowed the concentration of the proteins, but prevented overconcentration at the membrane which led to precipitation and reduced recovery of the proteins.

Electroelution was performed at 4° C with 2.5mA/chamber, for 4hr or until the bromophenol blue showed a sharp interface, and the sample was sufficiently concentrated. The buffer was removed from above the sample, then a long form pasteur pipette used to remove the sample.

Proteolysis of the proteins.

Peptide mapping of actin and MHC was performed using two proteases. V8 protease from Staphylococcus aureus (Sigma) which cleaves proteins at the COOH site of aspartic acid and

glutamic acid, and Type 1-S chymotrypsin from bovine pancreas (Sigma) which cleaves proteins at the COOH site of tyrosine, phenylalanine and tryptophan.

Protease stock solutions were made by dissolving in Tris-HCl 50mM, pH 7.4, to give a solution of activity 100 units/ml, and aliquots stored at -25° C until required.

Proteolysis, at 25° C, was performed by preincubation of the protein sample and the protease for 5min, the reaction was started by the addition of 10µl of protease to 50µl of protein solution. The reaction was terminated by heating the sample to 100° C for 2min, then cooled and stored on ice prior to electrophoresis.

SDS electrophoresis of the samples was performed as outlined in chapter 3, using 0.75mm thick gels. A 3cm stacking gel of 5% acrylamide was used for both actin and MHC peptide maps, the separating gel for actin was 18% acrylamide, and that for MHC was 15% acrylamide. The sample size used was varied between 10µl and 30µl to allow analysis of the major and minor bands produced.

Silver staining of the peptide maps was carried out using the methods described in chapter 3.

RESULTS.

The results presented are representative of the final banding patterns produced by the peptide mapping of a total of 60 fish acclimated at either 2° C, 5° C, 8° C, 11° C, 15° C, or 23° C.

The methods used to obtain purified MHC and actin were found to be very effective. It was possible to obtain MHC and actin in a concentrated solution without the use of dialysis or freeze drying, both techniques that were used with little success, especially in the preparation of MHCs. The samples obtained produced single bands upon electrophoresis, with no evidence for proteolysis during their isolation, or contamination with other proteins (Fig. 35).

MHCs and actin from carp acclimated to a range of temperatures were peptide mapped with V8 protease (Fig. 37, 40). There were differences in the staining intensity of a number of the bands, but the same banding pattern was seen in all the samples. When MHC samples from the two extremes of acclimation temperature 2° C and 23° C were compared they gave banding patterns that were identical (Fig. 38).

Actin samples from 2° C and 23° C acclimated carp were also digested with V8 protease and run on the same gel with samples of actin that had not been digested, and V8 protease, 5-fold that used in the digestions (Fig. 39). This

A B C D E F G H I

Figure 35. 10% SDS polyacrylamide gel of of electrophoretically purified myosin heavy chains and actin. The samples were prepared from white muscle myofibrils from cold- and warm-acclimated carp. Stained by the Sigma silver stain kit. Conditions of electrophoresis were as described in text.

Key; A, myosin heavy chains from 2° C-acclimated carp. B, C, D, actin from 2° C-acclimated carp. E, myofibrils from 2° C-acclimated carp. F, myosin heavy chains from 23° C-acclimated carp. G, H, I, actin from 23° C-acclimated carp.

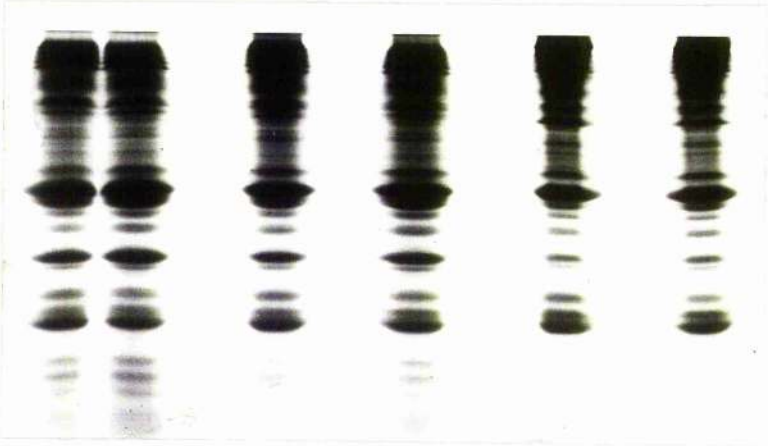



Figure 36. 12% SDS polyacrylamide peptide map of white muscle myofibrils from cold-and warm-acclimated carp, digested with V8 protease. Stained with coomassie blue. Conditions of electrophoresis and digestion were as described in text.

Key; The acclimation temperature of the carp, and the incubation period in V8 protease were as follows. A, 2° C, 2 hours. B, 23° C, 2 hours. C, 2° C, 1 hours. D, 23° C, 1 hours. E, 2° C, 0.5 hours. F, 23° C, 0.5 hours.

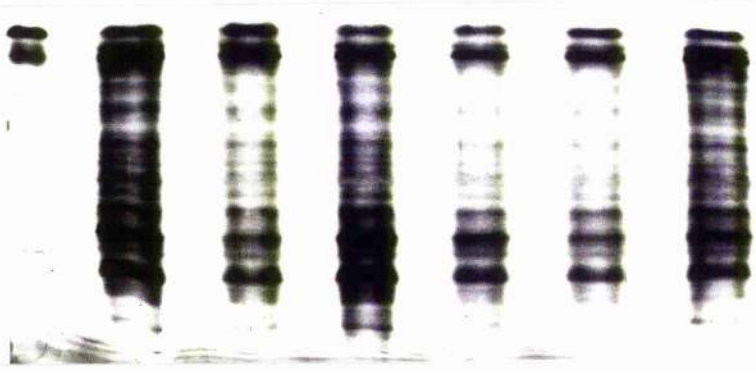
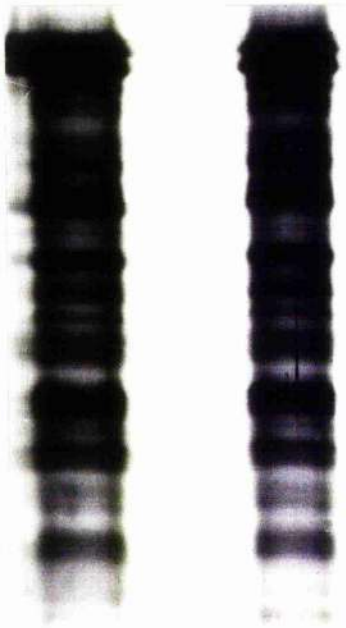



Figure 37. 15% SDS polyacrylamide peptide map of white muscle myosin heavy chains from carp acclimated to a range of temperatures, digested with V8 protease. Stained with Sigma silver stain kit. Conditions of electrophoresis and digestion were as described in text.
Key; A, Staphylococcus aureus V8 protease. The acclimation temperature of the carp were as follows. B, 2° C. C, 5° C. D. 8° C. E, 11° C. F, 15° C. G, 23° C.



A B

Figure 38. 15% SDS polyacrylamide peptide map of electrophoretically purified white muscle myosin heavy chains from cold-and warm-acclimated carp. Digested with Staphylococcus aureus V8 protease, and stained with Sigma silver stain kit. Conditions of electrophoresis and digestion were as described in text.
Key; A, myosin heavy chains from 2° C-acclimated carp. B, myosin heavy chains from 23° C-acclimated carp.

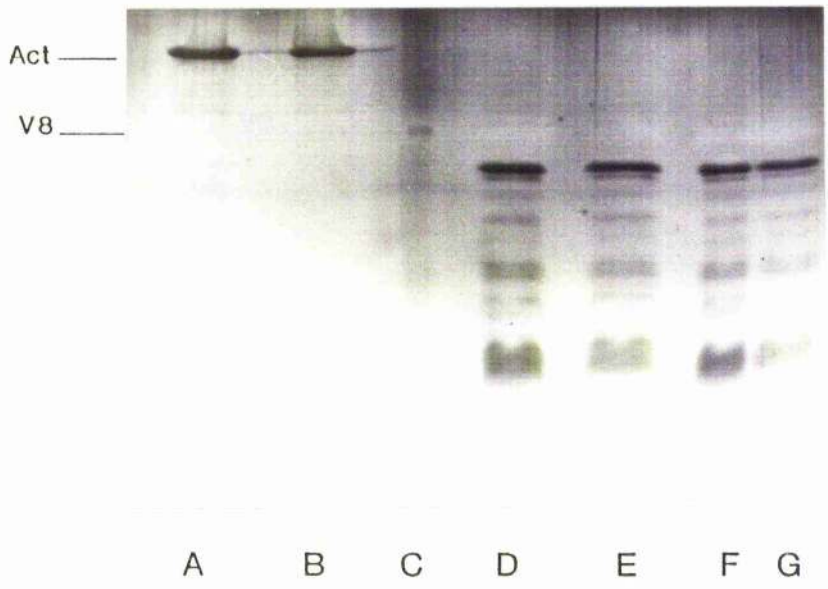



Figure 39. 18% SDS polyacrylamide peptide map of electrophoretically purified white muscle actin from cold and warm acclimated carp. Digested with Staphylococcus aureus V8 protease, and stained with Sigma silver stain kit. Conditions of electrophoresis and digestion were as described in text.

Key; A, non-digested actin sample from 2° C acclimated carp. B, non-digested actin sample from 23° C acclimated carp. C, myosin heavy chains from 2° C acclimated carp. B, myosin heavy chains from 23° C acclimated carp. C, Staphylococcus aureus V8 protease sample. D, F, actin samples from 2° C acclimated carp,digested with V8 protease. E, G, actin samples from 23° C acclimated carp,digested with V8 protease. Act, actin. V8, Staphylococcus aureus V8 protease.

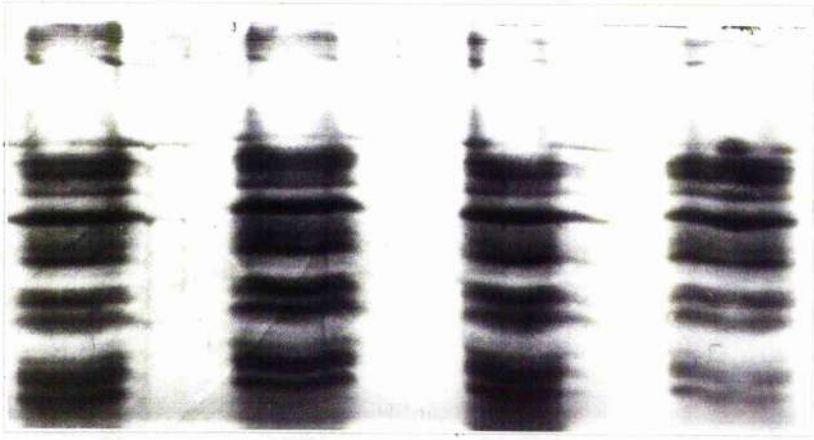



Figure 40. 18% SDS polyacrylamide peptide map of electrophoretically purified white muscle actin from acclimated carp. Digested with Staphylococcus aureus V8 protease, and stained with Sigma silver stain kit. Conditions of electrophoresis and digestion were as described in text.

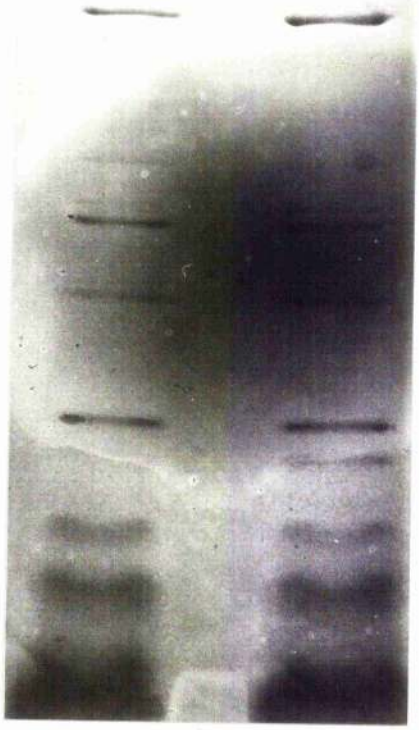
Key; A, sample from 2° C-acclimated carp. B, sample from 5° C-acclimated carp. C, from 11° C-acclimated carp. D, sample from 23° C-acclimated carp.

gel was run to check that the banding pattern found in the digested samples were not due to contaminants of either the actin sample or the V8. It was found to be necessary to run V8 samples every time a new batch was used as some supplies were found to produce multiple banding patterns.

MHC and actin from carp acclimated at 2° C, 5° C, 8° C, 11° C, 15° C, and 23° C were also peptide mapped with chymotrypsin (Figs. 41 and 42). The banding pattern obtained was different from that observed with V8 protease digestion, but did not vary with acclimation.

Peptide mapping of a large number of carp acclimated to a range of temperatures did not reveal any differences in either the MHCs or actin. The results presented are typical of the patterns that were found. In the initial experiments individual variations in banding patterns were observed, although they never appeared to be related to temperature of acclimation. As the techniques evolved into their final form, the same samples that had been stored at -25° C, or samples of the muscle from the same animal were re-analysed. The improved techniques showed no variation, and it appeared that with the technique of peptide mapping it was necessary to perform large numbers of experiments on the same samples before any conclusion could be drawn.

The analysis of peptide maps by scanning densitometry showed variation between samples, however the variation was



A B

Figure 41. 15% SDS polyacrylamide peptide map of electrophoretically purified white muscle myosin heavy chains from cold-and warm-acclimated carp. Digested with chymotrypsin , and stained with Sigma silver stain kit. Conditions of electrophoresis and digestion were as described in text.

Key; A, myosin heavy chains from 2° C-acclimated carp. B, myosin heavy chains from 23° C-acclimated carp.

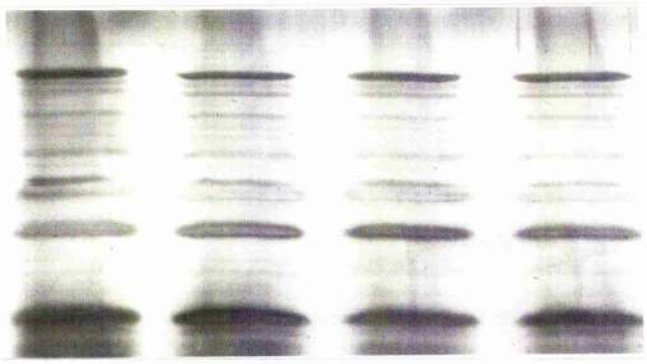



Figure 42. 18% SDS polyacrylamide peptide map of electrophoretically purified white muscle actin from acclimated carp. Digested with chymotrypsin, and stained with Sigma silver stain kit. Conditions of electrophoresis and digestion were as described in text.
Key; A, sample from 2° C-acclimated carp. B, sample from 5° C-acclimated carp. C, from 11° C-acclimated carp. D, sample from 23° C-acclimated carp.

small and was not detected by eye (Figs. 42-46). Replicate runs of samples showed that the variations appear to be due to differences between runs and were not consistently found in the same sample. This suggests that the analysis of the peptide maps by densitometric scanning was beyond the limit of reproducibility of digestion and separation in the techniques used.

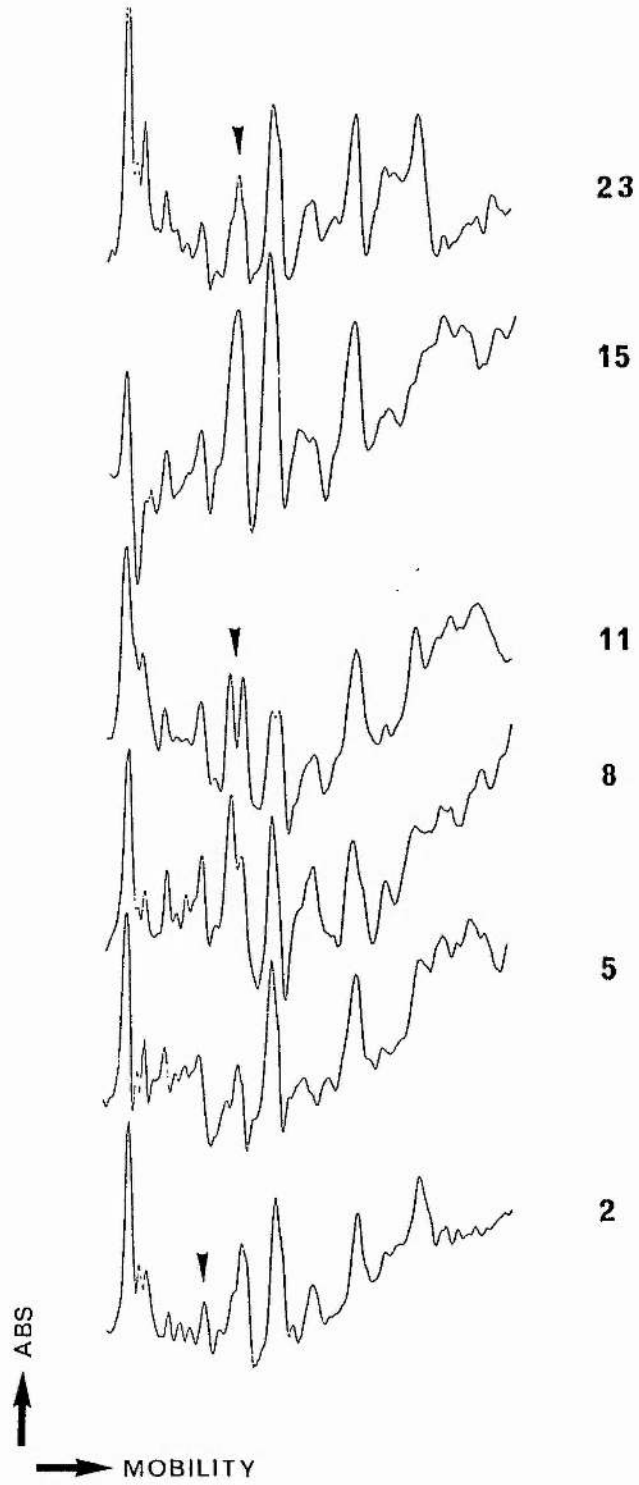



Figure 43. Densitometric scans of peptide maps of myosin heavy chains from carp white muscle using V8 protease. The scans were of peptide maps that appeared to be the same when examined with the naked eye. Densitometric scanning showed that there was variation in the peptide maps. The different scanning patterns did not appear to be related to acclimation temperature and were probably due to variations in digestion.

Key; The acclimation temperatures of the fish are given in °C. The arrows indicate regions of peak variation.

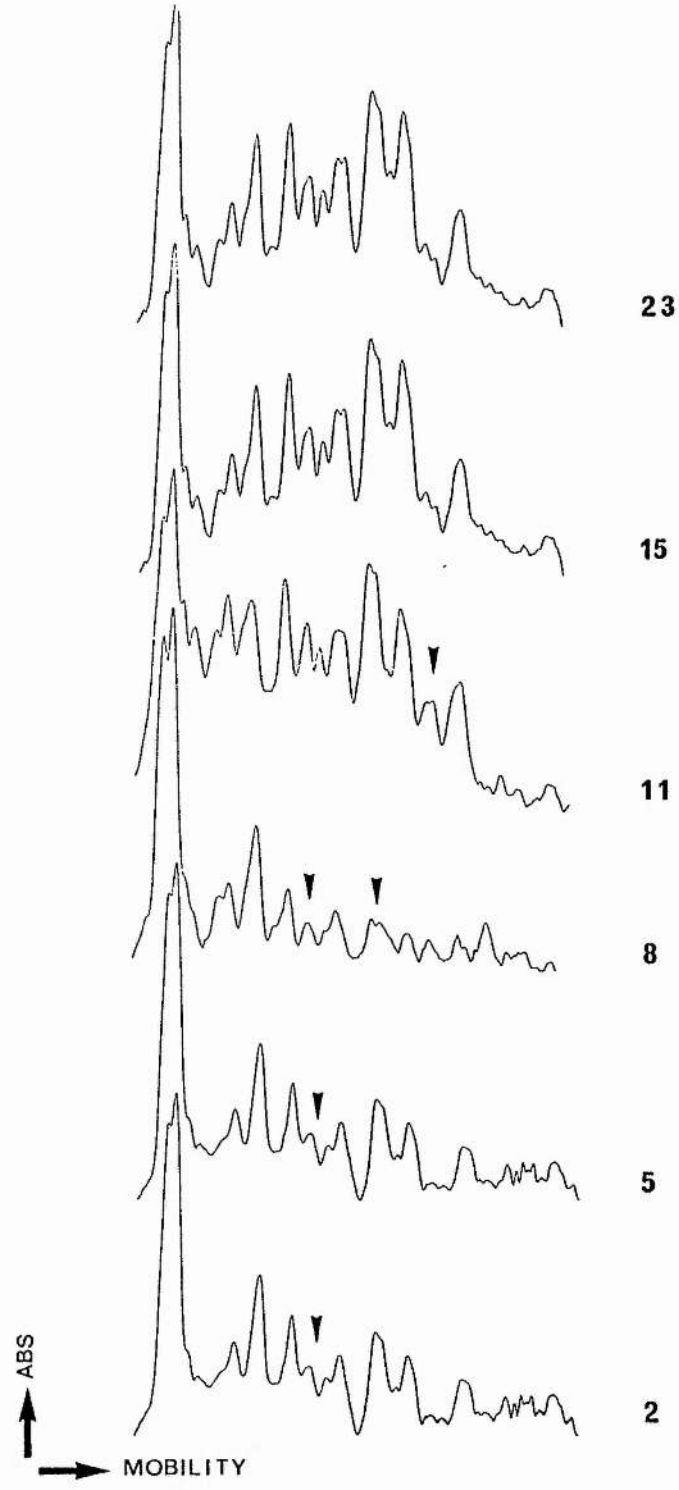



Figure 44. Densitometric scans of peptide maps of actin from carp white muscle using V8 protease. The scans were of peptide maps that appeared to be the same when examined with the naked eye. Densitometric scanning showed that there was variation in the peptide maps. The different scanning patterns did not appear to be related to acclimation temperature and were probably due to variations in digestion.

Key; The acclimation temperatures of the fish are given in °C. The arrows indicate regions of peak variation.

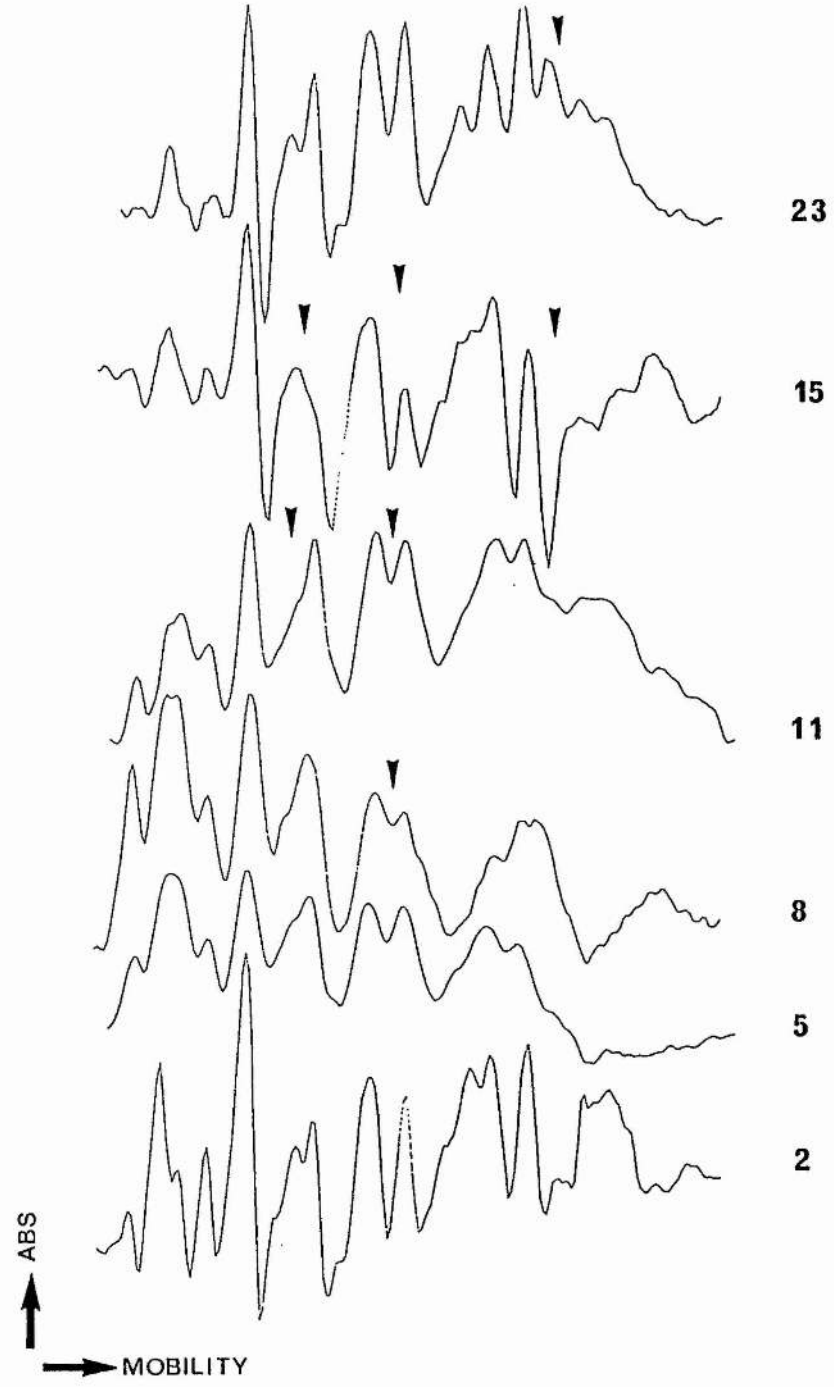



Figure 45. Densitometric scans of peptide maps of myosin heavy chains from carp white muscle using chymotrypsin. The scans were of peptide maps that appeared to be the same when examined with the naked eye. Densitometric scanning showed that there was variation in the peptide maps. The different scanning patterns did not appear to be related to acclimation temperature and were probably due to variations in digestion.

Key; The acclimation temperatures of the fish are given in °C. The arrows indicate regions of peak variation.

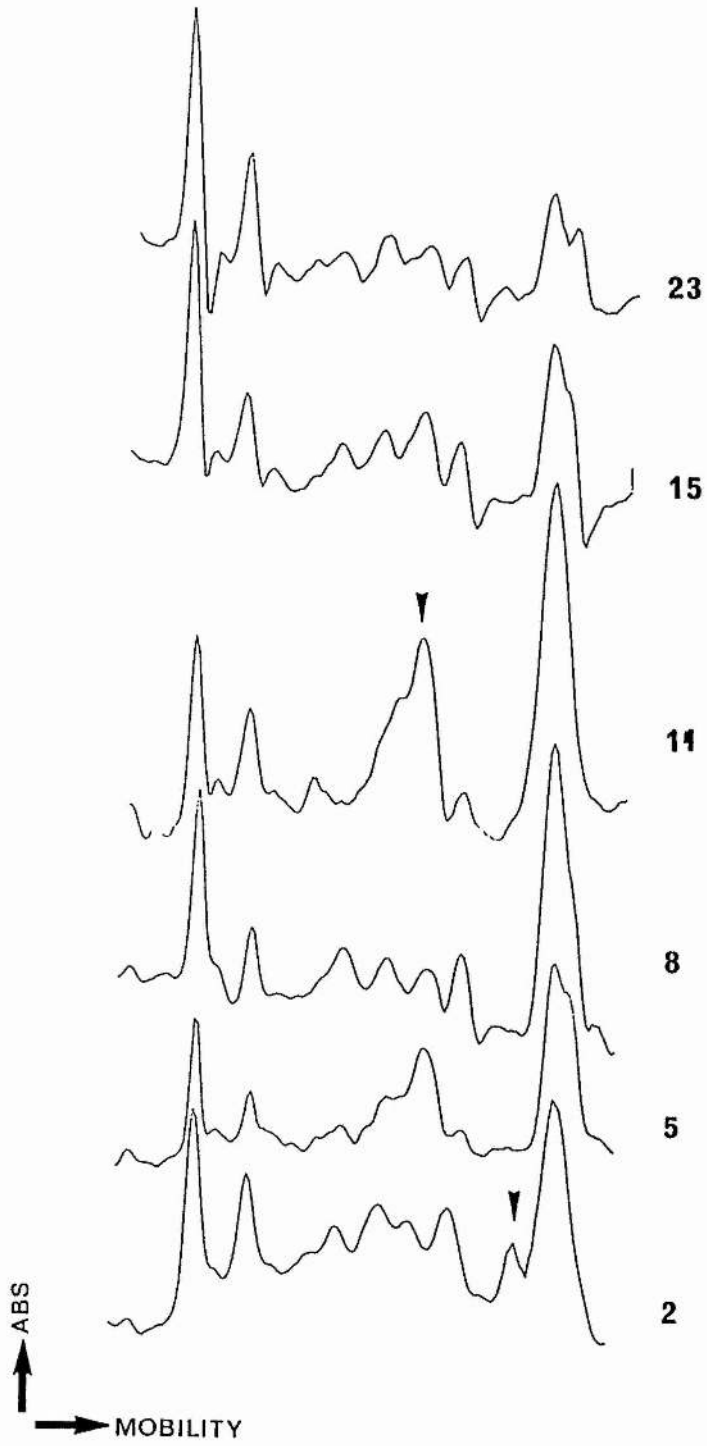



Figure 46. Densitometric scans of peptide maps of actin from carp white muscle using chymotrypsin. The scans were of peptide maps that appeared to be the same when examined with the naked eye. Densitometric scanning showed that there was variation in the peptide maps. The different scanning patterns did not appear to be related to acclimation temperature and were probably due to variations in digestion.

Key; The acclimation temperatures of the fish are given in °C. The arrows indicate regions of peak variation.

DISCUSSION.

The digestion of myofibrils by V8 protease over a range of time periods showed that care must be taken in the interpretation of gels obtained by partial proteolysis (Fig. 36). The relative proportions of a number of the bands changed with the various time periods used, however several of the bands seemed to be resistant to proteolysis by V8 protease. One of the problems encountered with the analysis of peptide maps was judging the degree of proteolysis that had occurred. The problem was decreased when coomassie blue was used to stain the gels as an increase in protein concentration leads to a higher staining intensity. When silver staining is used the response of a peptide may be quite variable. Most peptides stain more intensely as concentration increases, however some show negative staining whatever their concentration, and others increase in intensity until a certain concentration, or period of development is reached, and then begin to stain negatively. V8 protease with a sample loading that gave an intense band after a short period of development, on prolonged development led to negative staining, and if continued led to a clear band surrounded by a stained background. If the banding pattern of the bands that stain less intensely than others was to be analysed, then extended development times must be used. An increase in the concentration of the sample

applied, led to larger bands associated with the more intensely staining peptides, and decreased resolution of the less intensely stained bands. A balance was necessary between, a sufficiently large sample to allow bands to be observed, and small enough to allow adequate resolution. The balance may only be decided by trial and error, as the peptide pattern produced was unknown prior to electrophoresis.

The most commonly used method of peptide mapping of MHCs are adaptations of the method of Cleveland et al. (1977). The method involves the separation of the MHCs by cutting them out of SDS gels and running the MHCs out of the gel pieces into a second SDS gel. Once the MHCs are in the upper stacking gel, the protease is run into the gel until the bromophenol blue marker dye has nearly reached the interface between the upper and lower gel. The current is then switched off for 30min to allow digestion, then switched back on and the gel run as normal. This method has been used to investigate MHC isoforms in rabbit (Salviati, Betto & Betto, 1982), rat (Whalen et al. 1979; Carraro, Catani & Dalla Libera, 1981), chicken (Rushbrook & Stracher, 1979; Bandman, Matsuda & Strohnan, 1982) and chick embryo (Dalla Libera, 1981).

The method of Cleveland et al. (1977) is usually reported to give consistent results, however in preliminary

experiments in this study, variation in banding patterns was observed when different runs were carried out on the same sample. This inconsistency was also noted by Rushbrook and Stracher (1979), who noted shifts in the size range of peptides produced from repeated mapping of the same MHC sample. The suggested reason for lack of reproducibility was variation in the digestion time in the stacking gel. Peptide maps were compared from samples that appeared to produce equivalent digestion patterns (Rushbrook & Stracher, 1979). This approach did not appear sufficiently consistent to analyse possible differences in the less intensely staining bands, and a minor variation in isoform may have been overlooked. To overcome differences in the degree of digestion, soluble samples from BAC gels were digested prior to electrophoresis. This allowed control of, the amounts of protein and protease used, time period and temperature of digestion. The main problem using BAC gel samples was the high viscosity of the samples which led to uneven migration from the sample wells on electrophoresis, and distorted banding patterns. Electrophoretic elution of the MHCs from the BAC gel samples was to used reduce the viscosity of the samples, also concentrating the sample allowing smaller samples to be used resulting in better resolution between closely migrating bands. This also allowed bulk digests to be performed and analysed on different gels, allowing

multiple comparisons of the same samples.

Visual comparison of the peptide maps of MHC and actin using both V8 protease or chymotrypsin showed no evidence of isoforms being present. Densitometric scans of the gels showed differences in the banding pattern. The appearance of these peaks was not consistent on re-electrophoresis of the same samples, and did not apppear to be related to acclimation temperature. This suggests that the limit of resolution of peptide mapping was reached when differences in the banding pattern may only be seen by densitometric scanning and by studying minute variations in staining intensity. The care that must be used in selecting peptide maps with the same level of digestion, noted above, appears to be equally applicable to comparisons by densitometric scanning. No consistent difference in the peptide maps of MHCs or actin was seen on scanning. Peptide mapping may only be used to show that differences in amino acid sequence exist, it cannot be used to show that no differences exist. Thus the results only show that no isoforms of MHC and actin were observed.

# CHAPTER 5.

## GENERAL DISCUSSION

Major changes take place in the contractile properties of carp acclimated to different temperatures. The fast muscles from cold-acclimated fish develop twice the tension and have twice the unloaded contraction velocity of fibres from warm-acclimated fish (Johnston et al. 1985).

The striking feature of the present study was the relatively small variation in myofibrillar protein isoform found using electrophoretic techniques. The major finding was the presence of two proteins which were expressed over only one part of the thermal range. In white muscle from cold-acclimated fish an extra MLC was found. The MLCs are known from other studies to play an important role in the regulation of the myosin cross-bridge cycle. The significant variation in the proportions of MLC1 and MLC3 between the cold- and warm-acclimated fish further suggests that the myosin light chains may play an important role in the physiological responses observed in temperature acclimation. In addition white muscle from warm-acclimated fish had an extra troponin I. Calcium regulatory proteins have been shown to play an important part in the temperature acclimatory response of the actomyosin ATPase (Johnston,

1979).

The expression of isoforms associated with acclimation temperature seen in this study, do not necessarily provide the mechanism for acclimatory responses in contractile properties. These proteins are reported to have roles in the regulation of contractile properties of different fibre types that have no apparent variation in myosin heavy chain and actin isoforms (Staron & Pette, 1987a, 1987b). Forming only a small proportion of the total myofibrillar protein content of the muscle, they would provide a method of altering the physiological properties with minimal remodelling of the myofibrillar proteins (Greaser, Moss & Reiser, 1988).

Studies on the genetic basis of myofibrillar protein isoforms has revealed a multitude of possible production routes. Many isoforms of the myosin heavy chain have been found by peptide mapping (Dalla Libera, 1981; Sweeney et al. 1986), however, actin appears to have only two isoforms, one skeletal and one cardiac, when analysed by electrophoretic techniques. Both myosin heavy chains and actin belong to a multigene group, twenty genes coding for actin have been found in the mouse (Emerson & Bernstein, 1987), and in rat several sarcomeric MHC genes have been cloned, each appearing to encode for a specific MHC isoform (Emerson & Bernstein, 1987). It would appear that the presence of a

large number of multiple genes does not necessarily lead to the expression of an equal number of isoforms.

Post-transcriptional and post-translational changes have also been shown to produce isoforms. MLC1f and MLC3f from chicken, rat and mouse have the same gene, but variable splicing produces two specific mRNAs (Nabeshima et al. 1984; Daubas et al 1985). The same mechanism has been suggested for the troponin T isoforms seen in chicken fast muscle (Wilkinson et al. 1984). Bandman (1982b) showed that two MHCs which produced different peptide maps had the same mRNA, and the isoforms were produced by post translational changes.

Shotgun sequencing of cDNA has shown that as well as the known muscle-specific proteins, the sequence of new isoforms may be obtained before the isoform has been found expressed as the protein in vivo. Putney, Herlihy and Schimmel (1983) describe a new rabbit troponin T isoform, however its role and even if it is expressed in muscle is not known. The identification of protein isoforms by DNA sequencing may help to isolate expressed isoforms, and suggest improved methods of detection.

The links between physiological properties and the molecular mechanisms involved are only slowly beginning to be understood. The plasticity of fish muscle in response to temperature changes provides a good system for the study of

gene expression, under conditions that may occur naturally during the life cycle of certain fish species. The study of variations in the muscle proteins following temperature acclimation, and the associated physiological changes, may provide clues to the specific roles of these proteins.

Temperature acclimation in the carp, leads to changes in the properties of their muscles, which seems to involve the control of protein production by environmental factors. The study of isoforms of fish muscle protein expressed following acclimation, and the mRNAs involved would provide an insight into the link between the environment and the control of gene expression.

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