THE BIOCHEMISTRY OF THE SKIPJACK SWIMMING MUSCULATURE AND ITS APPLICATION TO METAECLIC CONTROL IN VERTEBRATE WHITE MUSCLE.

BY

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ABSTRACT

The tunas could be called the 'ultimate teleosts'. They have a high percentage of muscle and a high percentage of that muscle is red muscle, the muscles are kept at above ambient temperatures by a counter current heat exchanger and the respiratory capabilities of these fish are accordingly high. The behavioral culmination of these characteristics is manifested in swimming speeds, which can be extraordinarily high, on a sustained, or a burst basis. One of the hottest and fastest tunas, the skipjack, was used in a study to determine (1) when each muscle is active (2) when and where the muscle heat is produced and (3) what the advantage of the hot musculature is to the animal.

Evidence from E.M., histological, enzyme and metabolite studies suggest that the red muscle is qualitatively quite typical although its aerobic capacity is somewhat above that of other teleost red muscles. The white muscle has truely astounding anaerobic capabilities, but also displays an aerobic capacity not usually found in teleost white muscle.

Further examination of white muscle biochemical organization revealed a GP cycle which balances redox during the aerobic catabolism of glycogen and/or glucose. Both LDH (the terminal step in anaerobic glycolysis) and GPDH (the cytoplasmic arm of the GP cycle) are present in white muscle in high activities. Since these enzymes potentially compete for a common co-substrate, NADH, a tight control of these two enzymes seemed necessary to ensure mutual exclusive activity. Metabolite regulators of both enzymes were found which by

affecting the ability of each enzyme to compete for NADH, channel carbon and hydrogen to lactate and CO2 and H2O under anaerobic and aerobic conditions respectively.

The effect of temperature on metabolism was investigated and it is concluded that the stability rather than the absolute 'set point' of the body temperature is the more important feature.

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CHAPTER 1. GENERAL INTRODUCTION

The tunas (Scombridae) are represented by 13 species weighing frcm 1 to 500 Kg and inhabiting waters ranging from 5° C - 32°C. Some species are restricted to certain waters whereas cthers undergo extensive migrations. The tunas are mostly surface feeding fish whcse prey are fast swimming school fish and frequently squid. Tunas are of interest to the metabolic biochemist for three reasons, each of which I will discuss in turn.

Firstly, the tunas provide ideal material for the study of the roles of red and white muscle, a subject which has held the interest of physiologists and biochemists for at least 20 years. The tunas have a relatively large amount of muscle (Bcne, in prep.), a high proportion of this muscle is red, and red and white fibres are totally discreet allowing homogeneous sampling (Figure 1-1).

The relative positions of the red muscle masses in the array of scombrids encompass several alternatives. There is an increasing trend toward red muscle internalization from Scombercmerous (Seerfishes), to Sarda (bonito) and finally in Euthynnus pelamis (tuna), where the red muscle is contiguous with the vertebral column. E. pelamis is an example of relatively complete internalization, where little or no red muscle is found at the lateral surface of the swimming musculature. The Thunnus species have further developed the internalization phenomenon in several variations. The tropical cr warm water inhabitants (Thunnus tonggol, Thunnus atlanticus and Thunnus albacares) have a continuous, relatively internalized red muscle connecting the lateral surface with the

vertebral column and dorsal aorta - post cardinal vein complex. The bluefin species which inhabit broad ranges in the temperate oceans and polar seas have either a more intermediate red muscle placement (Thunnus maccoyii) or, as in the two Thunnus thynnus subspecies, the red muscle is completely internalized, with no lateral surface contiguity (Sharp, 1978). The red muscle in the tunas occupies about 20% of the total musculature, although the proportion of red muscle to total musculature varies along the length of the fish and varies between species (Sharp, 1978; Stevens et.al., 1974). This figure of 20% is a relatively high percentage when compared to other fish. The percentage of red muscle in the majority of fish "steaks" is between 1 and 10 although the percentage can rise to 36 at the posterior end of some species (Mosse and Hudson, 1977). The body of the tunas consists of 60-70% muscle, which is a relatively high proportion (Bone, in prep.).

Secondly, the tunas have warm muscles, which raises the questions: when, where and how is the heat produced and what effect does it have on the muscles' metabolism? The rate of heat transfer between blood and water across the gills of fish is approximately ten times that of oxygen transfer (in terms of half-time of equilibration) (Carey et.al., 1971). Thus it is evident that one "cost" of oxygenating the blood will be near complete equilibration of blood temperature to that of the ambient water. Fishes with normal circulation patterns therefore, are always perfusing the tissues with blood at ambient water temperature. The circulation in the tunas is such that heat is not lost from the muscle. Heat loss is prevented

by a counter-current exchange system, or rete. This rete is a network of arterioles and venules in close proximity. Heat coming out of the muscle in the venous blood, is lost to the arterial blood before it reaches the gills (Figure 1-1).

The rete in the tunas is organized in one of two ways. In some species, the central circulation is much reduced and a cutaneous vein-artery system runs along the side of the body at the level of the lateral line. The myotomal branches of these cutaneous vessels enter, and exit from the muscles, and form a lateral rete. The rete mainly serves the red muscle although the white muscle is supplied as well. There are two sets of cutaneous vessels in these tuna (bluefin, bigeye, albacore and yellowfin) and thus a rete on both the dorsal and ventral side of the red muscle mass (Carey et.al., 1971). In the rest of the tunas which have been examined (T. tongoll, T. atlanticus, E. pelamis, T. albacares and the black skipjack) a central rete is present (Carey et.al., 1971; Stevens et.al., 1974; Graham, 1973; Sharp, pers comm). The circulation is of the typical teleost type (except for the presence of only one post-cardinal vein) and in each segment, the dorsal aorta and the post-cardinal vein branch into small vessels and form a large three dimensional rete, just below the spinal cord. This rete serves both the red and the white muscles, but again the major mass of the structure is concerned with red muscle due simply to the lower vascularity of the white muscle. In the black skipjack and the skipjack, the dorsal aorta travels inside the postcardinal vein in the rete region and thus further maximises heat exchange before the gills (Graham, 1973; Stevens et.al.,

1974). These fish still retain the cutaneous rete, but its function has been superceded by the central rete; thus the former has atrophied. The outcome of these rete systems is warm skeletal muscles which can be up to 15°C above ambient temperature.

The majority of muscle temperature studies have invcolved the skipjack tuna (central rete) and bluefin tuna (lateral rete). The deep red muscle is always the hottest tissue reaching 12°C above ambient in the bluefin (Carey et.al., 1971; Carey and Lawson, 1973) and 9°C above ambient in the skipjack (Stevens and Fry, 1971). The white muscle temperature in these fish is also above ambient temperature. The temperature of the white muscle in both the skipjack and the bluefin is as high as the red at the junction of the red and white muscles and decreases with distance from the red muscle. Subcutaneous muscle temperatures (both red and white) are considerably lower than deep red or deep white muscle temperatures (Carey et.al., 1971; Hulbert and Brill, pers. comm.). A variety of other tunas have also been sampled and excess (of ambient) muscle temperatures range from 2°C - 13°C (Carey et.al., 1971).

All data on the bluefin tuna was collected in the field so accurate activity temperature relationships in this fish are not known. Data on skipjack tuna however (which can be held in captivity) suggest that:

1. the highest muscle temperatures are attained during the feeding frenzy which is high speed sustained swimming (Stevens and Fry, 1971).

2. excess temperature of the muscles of skipjack tuna

decreases after capture (Stevens and Fry, 1971).

3. activity during capture does raise the muscle temperature, but not to pre-capture levels (Stevens and Fry, 1971; Dizon and Brill, pers comm). Temperatures of other parts of the body such as the brain, eye and intestine are also above ambient temperature and have been shown to be served by retes (Carey et.al., 1971; Stevens and Fry, 1971). The heart of the tunas is obviously not served by a rete and is presumably at near-ambient temperature.

Thirdly, the tunas are unusually fast swimmers, which raises the question of the muscles' contribution to swimming speeds and the possible metabolic specialization for increased power output. The usual velocity term used with regards to fish swimming is lengths/second. Bainbridge (1958) has shown that the swimming speeds of different species of fish can be compared using this term. Fairly wide agreement exists that a cruising speed of 2 -3 L/second can be maintained for an hour or more by most fishes and values for burst swimming range from 6 - 20 L/second (Bainbridge, 1958; Wardle, 1975). The higher burst speeds (above) were measured over extremely short periods (0 - 5 seconds) and one was actually measured during a rush preceding a leap out of the water (Bainbridge, 1958).

It is difficult to measure the swimming speeds of tunas due to innumerable difficulties such as high oxygen requirements, extreme fragility of the fish and the difficulties of constructing an apparatus which will measure high swimming speeds of this relatively large fish. However, the evidence that is available suggests that both cruising and

burst speeds are comparably high. Skipjack tuna can swim at 8 knots for over and hour (Ccmmercial Fisheries Review, 1969), and a skipjack has been tracked at 6 L/second for over an hour (Dizon and Brill, 1978). Burst swimming speeds for tunas are high and are sustained for relatively long periods. Walters et.al., (1964) measured velocities of 21 L/second for yellowfin over 15 - 20 second periods, and skipjack have been repeatedly timed at 15-25 L/second and over (Dizon and Brill, pers. Comm; Yuen, 1966). The evidence for high swimming speeds is far from overwhelming, but simple observation was all that was necessary to convince me that at least skipjack tuna exhibit sustained and burst swimming speeds which are obviously in excess of that shown by other fishes.

With these three characteristics, the tunas offer the metabolic biochemist the opportunity to study an organism in which there are a variety of interrelated problems. The questions which are immediately obvious are:

1. At the purely descriptive level, how many fibre types is the skipjack musculature composed of and how do these types fit into the usual red-white muscle classification?

2. How is contribution to propulsion partitioned between the different muscle types i.e. at what swimming velocity does each muscle type contribute power and under what conditions is the metabolism of each muscle type aerobic and anaerobic?

3. Which muscle(s) produce(s) the heat, under what conditions is the heat produced in each muscle and what are the metabolic sources of heat?

4. Are there adaptations at the molecular level in the

muscles' metabolism which enable the tunas to swim at such high velocities for such long periods?

5. What is the advantage to the animal of the high temperature musculature?

I have tried to resolve these, and other questions which arose during my study, using the skipjack tuna (Euthynnus pelamis). The reasons for choosing the skipjack are a compromise between convenience and representation. The skipjack tuna are readily available, either as freshly frozen material, or as live animals. The frozen samples are available from Fisheries centers in San Diego and Honolulu. Live animals are kept in Honolulu (the only place in the world which keeps live tuna) where conditions allow a wide range of live animal experiments. The skipjack hardly falls short as a true representative of the tunas. It is not the hottest, nor the most thermoregulatory tuna, neither is it the biggest; but its muscle temperature excesses are only a few degrees below those of the bluefin, its red muscle is completely internalised and in terms of lengths/second, the swimming speeds of the skipjack are never matched. It is also, due to the holding facilities at Honolulu, a scientifically well known member of the tuna group and thus behavioral, anatomical and physiological data are available to support biochemical studies.

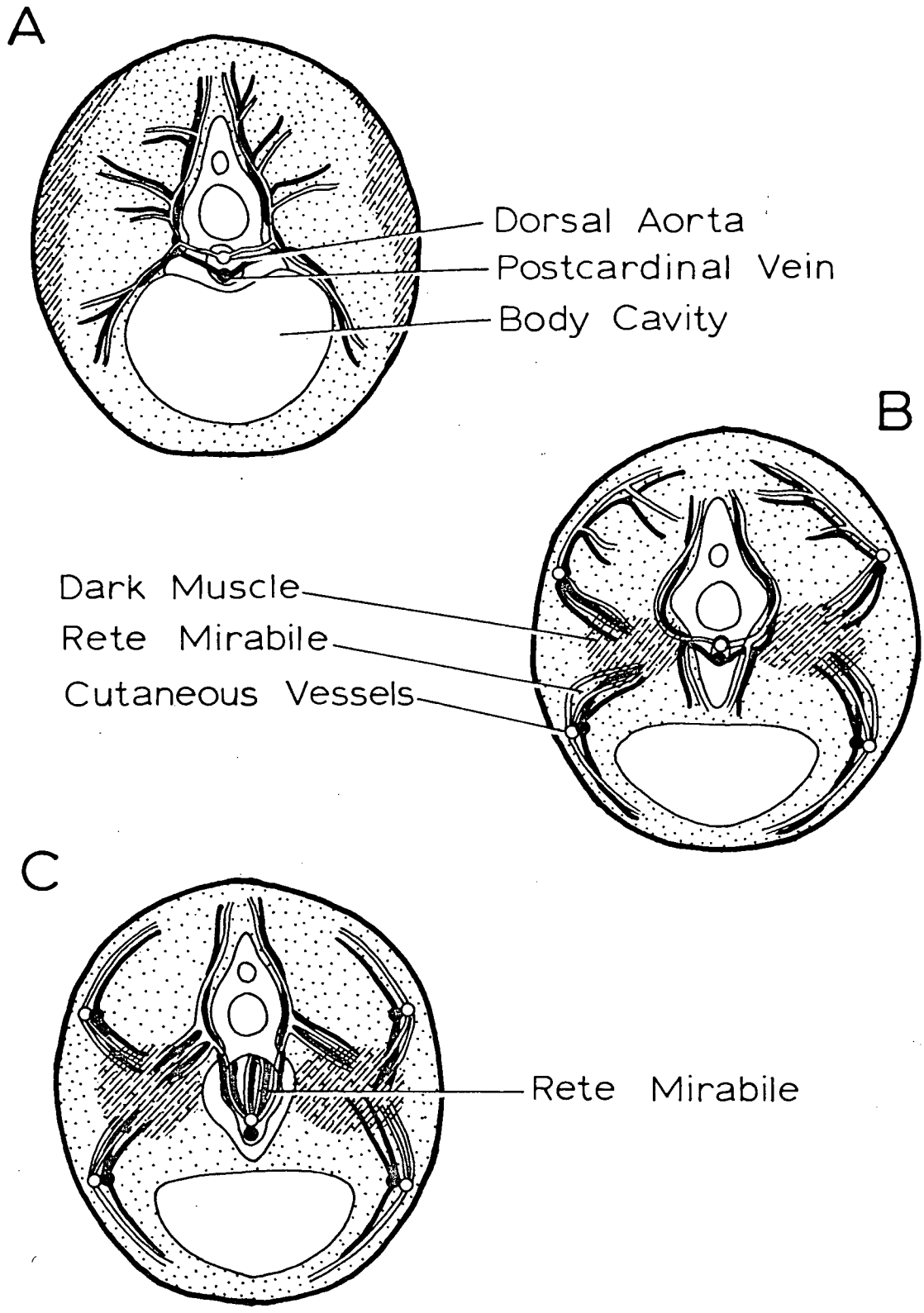
There are apparently three distinct populations of skipjack tuna. Young fish are found in food abundant shallow waters over the continental shelves. The adults migrate to the central pacific to breed and then presumably there is a migration of the young back to shallow waters. The fish found

off the coasts of the Hawaiian chain are a mixture of the three populations (Inter-American Tropical Tuna Commission bimonthly report, March-April, 1977; Sharp, pers, comm.). There is little known about their diurnal behavior. They do make forays into the depths all day, presumably to feed, and have been tracked down to 195 meters (Yuen, 1966). At night they hug the upper layer of water. Fishing activities indicate a major AM and PM cycle of "willingness to bite", but no more is known about feeding incidence.

The "hot spot" in the skipjack is next to the spinal chord under the leading edge of the main dorsal fin. The temperature decreases as one moves laterally, anteriorly and posteriorly away from this position (Graham, 1975; Dizon and Brill, pers. Comm.). The rete is about 97% efficient in exchanging heat (Neill et.al., 1976). Red muscle effects 27-84% of its heat exchange across the gills whereas this figure is 0-35% for the white muscle which loses most of its heat across the skin. (This is compared to 95% for all muscles in typical teleosts). Warming and cooling experiments yield no evidence for physiological thermoregulation in skipjack although skipjack tuna equilibrate with water temperature 60% as rapidly as typical teleosts (Neill et.al., 1976). Skipjack do not vary activity in a way detectably related to temperature (Neill et.al., 1976). Metabolism of free swimming skipjack is 3-5 times that for non-scombrid fishes of the same size (Neill et.al., 1976) and several features of the circulatory and respiratory physiology are consistent with the skipjack's high metabolic rate (Stevens, 1972).

Ultrastructural, histochemical and enzyme profile studies (Chapter 3) demonstrate that the skipjack musculature is composed of only two major (red and white) muscle types. Information on the white muscle shows it to be a tissue with a surprisingly high (even for white muscle) anaerobic potential. Equally surprising is the aerobic capability (based on carbohydrate fuel) which is demonstrated in the white muscle by these studies. Metabolite and temperature studies (Chapter 4) support the evidence presented in Chapter 3. The red muscle contributes aerobically to all swimming states, while the white muscle demonstrates an intense anaerobic metabolism during burst swimming and an aerobic contribution to steady state swimming. The white muscle has an active GP cycle (Appendix I) and thus an important aspect of metabolic control is the competition for NADH between GPDH and LDH. The ability of these two enzymes to compete for NADH can be perturbed by a variety of conditions (Chapter 5) allowing carbon flow to proceed along an aerobic or anaerobic route depending upon the tissue oxygen level.

Figure 1-1. Cross sections of fish . Cross sections of (a) a classical "cold" teleost; (b) the bluefin tuna, showing the lateral rete, and (c) the skipjack tuna, showing the central rete.


A
Dorsal Aorta
Postcardinal Vein
Body Cavity
B
Dark Muscle
Rete Mirabile
Cutaneous Vessels
C
Rete Mirabile

CHAPTER 2. MATERIALS AND METHODS

ANIMALS

Fresh

Fresh tissue samples of skipjack were obtained from fish being held in tanks at the National Marine Fisheries Center, Honolulu, Hawaii. Samples were only taken from fish which had been in captivity for less than three days. For certain comparative studies honey bees (*Apis melifera*) were collected from hives around Vancouver and white laboratory rats (*Rattus norvegicus*) and rainbow trout (*Salmo gairdneri*) were obtained from holding facilities at the University of British Columbia. Turtles (*Pseudomas scripta*) were purchased from NASCO Co Ltd, Fort Atkinson, Wisconsin and were kept at 10°C.

Frozen

Frozen tissue samples of skipjack tuna were obtained from the Inter American Tropical Tuna Commission in San Diego. The tuna were caught by oceanographic vessels, immediately frozen and transfered to the laboratory at dry ice temperatures. Again for comparative purposes, frozen tissue samples of Amazon fishes (*Osteoglossum bicirrhosum*, *Arapaima gigas*, *Hoplias malabaricas* and *Hoplerythrinus unitaeniatus*) were obtained from the Alpha Helix expedition to the Amazon River (Randall and Hochachka, 1978). These fish were frozen after capture and transfered to the laboratory on dry ice. Weddell seal heart was obtained from the Antarctic and was similarly frozen after

extraction and transferred to the laboratory on dry ice.

HISTOCHEMISTRY

Muscle pieces for histochemical analyses were excised from fresh skipjack, immersed for one minute in a bath of isopentane cooled with liquid nitrogen and then placed directly in liquid nitrogen. The tissue blocks were sectioned in a cryostat at -20°C. Sections (10 micron) were picked up on glass slides and allowed to dry 2-3 minutes before staining with haemotoxylin-eosin or processing specifically for either LDH, (see Abbreviations used) myosin ATPase or SDH. The slides were then mounted in 15% gelatin.

Succinate Dehydrogenase Staining

Slides were incubated at room temperature in 0.05M sodium phosphate buffer, pH 7.6, 0.05M sodium succinate and 1 mg/ml Nitro blue tetrazolium in a final volume of 50 mls. Slides were then washed in saline (4% NaCl), fixed in 10% formal saline for 10 minutes, washed in distilled water, taken through an ethanol series and mounted. The site of enzyme activity was evidenced by blue-purple diformazan deposits. Control slides were processed in the absence of succinate and showed no staining.

LDH Staining

Slides were incubated in a substrate solution containg 2g liquid DL-lactate, 0.22g NAD^+, 0.22g Nitro Blue Tetrazolium and 0.1 M Na cyanide in 50 ml of 0.06 M Na-phosphate buffer. Slides

were then rinsed in distilled water, taken through an ethanol series and mounted. Sites of enzyme activity were evidenced by the characteristic blue purple diformazan deposits. Control slides were incubated in the absence of DL-lactate and produced no staining.

General Staining

Slides were placed directly into haemotoxylin, rinsed in running tapwater and then placed in eosin then rinsed again in tapwater. Slides were then put through a graded ethanol series and mounted.

ELECTRON MICROSCOPY

Muscle tissue was collected from fresh fish and fixed in 3% glutaraldehyde and 100 mM sodium phosphate buffer, pH 7.4, containing 400 mM sucrose, for 1.5 hours followed by washing with sodium phosphate buffer and post-fixation in 1.5% osmium tetroxide. The muscle pieces were subsequently dehydrated in a graded ethanol series and embedded in Epon 812 according to Luft (1961). Thin sections were cut using glass knives fitted to a Porter Blum MT-1 ultramicrotome, negatively stained with Uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and viewed with a Zeiss EM-10.

ENZYME PREPARATION

Enzymes For Profiles Of Red And White Muscles

Tissues were excised from freshly killed fish and homogenised with a polytron PCU-2-110 in 19 volumes of 50 mM imidazole buffer, pH 7.0 containing 20 mM $MgSO_4$, 200 mM KCl and 1 mM EDTA. Mercapto ethanol (20 mM) was added for the preparation of hexokinase and PFK. The well stirred homogenates were spun at 12000g for 20 minutes and the supernatants used for the enzyme assays.

Enzymes For Kinetic Characterization

Skipjack white muscle GPDH was prepared from frozen muscle samples. Tissues were excised, blotted, weighed and homogenised in 10 mM Tris, 2 mM EDTA, 20mM mercapto-ethanol, pH 7.5, using a Sorval omnimixer for three 20 second periods. The homogenate was centrifuged at 12000g for 15 minutes. The supernatant was adjusted to 80% ammonium sulphate, spun at 12000g for 15 minutes and the pellet redissolved in a small amount of 50 mM Tris , 20 mM mercapto-ethanol, pH 7.4, and put on a 2.6 x 100 cm Sephadex G-100 column. The active fractions were pooled, concentrated by 80% ammonium sulphate and dialyzed for 45 minutes twice against 10 mM imidazole buffer, 20 mM mercapto-ethanol, pH 7.0, and loaded onto a 1.5 x 5 cm agarose-hexane-

nicotinamide adenine dinucleotide (NAD^+) column. The NAD^+ column was washed with buffer to remove any unbound enzyme and the enzyme was eluted with a 100 ml 0-300 mM KCl gradient. These simple steps led to highly purified enzyme preparations. Active fractions obtained from the affinity chromatography were pooled and stored in 80% ammonium sulphate, 20 mM mercapto-ethanol on ice in a 5°C cold room. The preparations were stable for at least one month.

GPDH from turtle white muscle and trout epaxial muscle (predominately white muscle) was prepared from fresh animals using the same procedure as described above for skipjack white muscle GPDH.

Skipjack white muscle LDH was prepared from frozen muscle samples. The muscle was cut into small pieces and homogenised in a Sorvall omnimixer for one minute in 20 volumes of 10 mM imidazole buffer, 20 mM mercapto-ethanol, and 1 mM EDTA, pH 6.5. The homogenate was spun at 12000g for 20 minutes and the supernatant was mixed with a batch of cellulose phosphate equilibrated with the homogenising buffer. All of the activity bound to the cellulose phosphate and was brought off between 250 and 400 mM KCl with a 2- to 3- fold purification. The enzyme was concentrated by bringing the solution to 80% saturation with ammonium sulphate, spinning at 12000g for 20 minutes and redissolving the pellet in homogenising buffer. The concentrated enzyme was then applied to an oxamate column. In the presence of 0.1 mM NADH, about 80% of the activity was bound to the affinity matrix. The column was washed several times with homogenising buffer and then the enzyme was eluted

with a small amount of homogenising buffer containing 500 mM KCl, pH 8.5. The resulting enzyme was a 15- to 20- fold purification and was stable in the elution buffer, plus 20 mM MgSO4, pH 7.0, for at least 2 weeks.

GPDH from honey bee flight muscle was prepared as for the GPDH from skipjack white muscle except that the NAD^+ column was deleted. The resulting preparation contained no measurable LDH.

LDH from turtle white muscle, rat brain and Amazon fish muscle was prepared by homogenizing the tissues in approximately 5 volumes of 50 mM imidazole buffer, pH 7.0, containing 2 mM EDTA. The homogenate was spun at 12000g for 20 minutes and the supernatant used for the assays of LDH. H4 and M4 LDH from rabbit muscle and beef heart respectively was purchased from Sigma Chemical Co; any contaminants were less than 0.01% of the LDH activity, there was no GPDH activity and the isozyme form was 99% homogeneous. GPDH from rabbit muscle was obtained from Sigma Chemical Co; any contaminants were less than 0.01% of the GPDH activity and there was no measurable LDH activity.

Enzymes For Gel Electrophoresis.

Enzyme preparations from frozen tissue were crude supernatants dissolved 1:4 in electrode buffer. Pure enzyme preparations in 80% ammonium sulphate were spun down and the pellet redizzolved in electrode buffer.

ENZYME ASSAYS

Enzyme activities were monitered in 1 ml cuvettes (1 cm light path) using a Unicam SP 1800 recording spectrophotometer. The reaction cuvettes were held in cell holders thermally equilibrated with a constant temperature bath and circulator. The rate was determined by the decrease in absorbance of NADH at 340 nm (in the case of citrate synthetase, the rate was determined by the increase in absorbance of DTNB at 412 nm).

Enzyme Assays For Muscle Profiles

All assays were done in 50 mM imidazole buffer, 10 mM MgCl2 and 100 mM KCl. NADH (0.15 mM) was used in all assays except phosphorylase, hexokinase, phosphoglucomutase, phosphoglucoseisomerase and creatine phosphokinase, in which 0.4 mM NADP was used.

Phosphorylase: pH 7.0, 2 mg glycogen/ml (omitted for control), 4uM glucose-1,6-diphosphate, 2 mM AMP and excess phosphoglucomutase and G6PDH.

Hexokinase: pH 7.5, glucose 1 mM, ATP 1 mM(omitted for control) and excess G6PDH.

Phosphoglucomutase: pH 7.5, 4.0 mM glucose-1-phosphate(omitted for control), 0.02 mM glucose-1,6-diphosphate, and excess G6PDH.

Phosphoglucoseisomerase: pH 7.5, 1.5 mM fructose-6-phosphate(omitted for control) and excess G6PDH.

Glucose-6-phosphate dehydrogenase: pH 7.6, 1 mM glucose-6-phosphate(omitted for control).

Phosphofructokinase: pH 8.0, 2 mM ATP, 5 mM fructose-6-

phosphate(omitted for control), excess GPDH, aldolase, and triosephosphate isomerase.

Aldolase: pH 7.5, 0.4 mM fructose-1,6-diphosphate(omitted for control), and an excess of GPDH and triosephosphate isomerase.

Triosephosphate isomerase: pH 7.5 6 mM glyceraldehyde phosphate (omitted for control), and excess GPDH.

Phosphoglycerate kinase: pH 7.5, 5 mM ATP, 10 mM glycerate-3-phosphate(omitted for control) and excess glyceraldehyde-3-phosphate dehydrogenase.

Enolase: 1 mM ADP, 1 mM 2-phosphoglyceric acid (omitted for control) and excess PK and LDH.

Pyruvate kinase: pH 6.5, 5 mM ADP, 5 mM phosphoenol pyruvate(omitted for control) and excess LDH.

Lactate dehydrogenase: pH 6.5, 10 mM pyruvate(omitted for control).

GPDH: pH 7.0, 2.0 mM dihydroxyacetone phosphate(omitted for control).

Citrate synthase: pH 8.0(Tris buffer), 0.1 mM DTNB, 0.3 mM acetyl CoA(omitted for control), 0.5 mM oxaloacetate.

Glutamate dehydrogenase: pH 7.3, 250 mM ammonium sulfate, 1 mM ADP, 10 mM a-ketoglutarate (omitted for control).

Malate dehydrogenase: pH 7.5, 0.5 mM oxaloacetate(omitted for control).

Glutamate/oxaloacetate transaminase: pH 7.5, 40 mM aspartate, 10 mM a-ketoglutarate (omitted for control), 0.1 mM pyridoxal-5-phosphate and excess malate dehydrogenase.

Glutamate/pyruvate transaminase: pH 7.5, 20 mM alanine, 10

mM a-ketoglutarate (omitted for control), 0.1 mM pyridoxal-5-phosphate and excess LDH.

Adenylate kinase: pH 7.5, 2 mM AMP(omitted for control), 5 mM ATP, 5 mM phosphoenolpyruvate, and excess PK and LDH.

Creatine phosphokinase: pH 7.5, 10 mM creatine phosphate, 1 mM ADP, 4 mM glucose, 2 mM AMP(to inhibit myokinase) and excess hexokinase and G6PDH.

Enzyme Assays For Kinetic Characterization

The assays used for kinetic characterization differed from those used for the muscle profiles (above) only in that a purified enzyme preparation was used and pH, temperature, substrate, product and modulator concentrations were varied. Values for the Michaelis-Menten constants(Km) were determined by double reciprocal plots of 1/velocity versus 1/substrate concentration. Inhibition constants (Ki) were obtained from Dixon plots of 1/velocity versus inhibitor concentration at varying substrate concentrations. Values obtained were reproducable to within +or -15%.

STARCH GELS

Starch gel electrophoresis was done on 13% starch gels at 5°C for 14 hours (LDH) and 9 hours (GPDH) with a current of 25 MA. The electrode buffer was 50 mM phosphate citrate buffer, pH 5.9 (LDH) or 6.3 (GPDH). The gel buffer was a 1:20 dilution of the electrode buffer. Both LDH and GPDH ran towards the cathode. Staining at 25°C was accomplished by overlaying the gels with filter paper soaked in 1 mM NAD^+, 0.1 mM phenazine

methosulphate, 1 mM Nitro Blue Tetrazolium and 50 mM GP or lactate in 50 mM Tris buffer, pH 8.0. Control gels were overlaid with filter paper soaked in the incubation medium minus GP or lactate and showed no activity.

METABOLITES

Muscle and blood samples were taken from fish that were performing at one of three different levels of activity, termed resting, burst swimming, or steady state swimming. Samples from resting tuna were obtained from animals swimming laps in circular pools at approximately 1-2 body lengths/second. For tuna, this is the only approximation to basal metabolism that exists, since, unlike many teleosts with swimbladders, the skipjack tuna lose hydrodynamic equilibrium and fall through the water column if their cruising speed decreases below about 1.2 lengths/second (Dizon, pers. Comm.). In terms of metabolite concentrations therefore, we are dealing with a working equilibrium system not directly comparable to "resting" mammalian muscles.

Burst swimming for periods of 7-10 minutes was obtained by attatching a line and hook to the lower mandible and releasing the tuna "on-line" into a circular tank, 5 meters in diameter, 1 meter deep. Under these conditions, bursts of up to 20 lengths/second can be achieved (Dizon, pers. Comm.).

Samples from tuna in high velocity, steady state swimming associated with feeding frenzies were obtained through the cocperation of local fishermen. These fish were hooked at sea at the stern of the boat and immediately hauled on board for

sampling. Sampling time was about 15 seconds longer than for the other two experimental groups (resting and burst swimming).

Preparation Of Tissue

Fish were netted and a 2 cm thick steak was quickly cut from the area immediately anterior to the leading edge of the dorsal fin. In the 1976 experiments (see Chapter 4), small pieces of red and white muscle (less than 1 gram) were excised from the steak and immediately frozen in liquid nitrogen; this procedure took less than 30 seconds. In the 1977 experiments (see chapter 5), the steak (which was less than 1 cm thick) was freeze clamped with Wollenburger tongs cooled in liquid nitrogen; this procedure also took about 20 seconds. Some of the frozen tissue was set aside for glycogen assays; the rest was powdered in a mortar and pestle, previously cooled by liquid nitrogen and then an aliquot of the powdered tissue was placed into a glass tube (cooled in a dry ice-ethanol bath). Immediately before and after the addition of the powder, the tube was weighed to the nearest milligram. Approximately 4 parts (by weight) of 8%(v/v) $HClO_4$ in 40% (v/v) ethanol was added to the cold powder, mixed quickly with a spatula and homogenized for 1 minute, at dry ice ethanol temperatures, with a polytron PCU-2-110. The resulting supernatant was centrifuged at 25000g for 10 minutes to produce supernatant "a"; the precipitate remaining in the homogenizing tube was re-homogenized in two volumes of 6% (v/v) $HClO_4$. This homogenate was added to supernatant "a" and spun at 25000g for 10 minutes. The "second" supernatant was adjusted to pH 6.0 by the slow

addition of 3M K_2CO_3 containing 0.5 M triethanolamine base, and then spun at 25000g for 10 minutes to remove the precipitated $KClO_4$. The final supernatant was measured to the nearest 0.1 ml and stored at -20°C.

Metabolite Assay Techniques

All metabolites were measured enzymatically and were based on the absorbance of the pyridine nucleotides at 340 nm. Assays were carried out on a Unicam SP 1800 dual-beam spectrophotometer connected to a strip-chart recorder.

ATP and glucose -6- phosphate were determined by the method of Lamprecht and Trautschold (1974); ADP, AMP, pyruvate, a-glycerophosphate, citrate and malate, by the method of Lowry and Passonneau (1972); fructose-6-phosphate and fructose diphosphate by the method of Racker (1974); lactate by the method of Sigma Bulletin #826; a-ketoglutarate by the method of Bergmeyer and Bernt (1974); creatine and creatine-phosphate by the methods of Bernt et al. (1974) and Lamprecht et al (1974); and glucose by the method of Bergmeyer et al (1974). Glyceraldehyde-3-phosphate and dihydroxyacetone phosphate were determined by the sequential addition of GPDH and triose phosphate isomerase. Glycogen was isolated and hydrolysed by the method of Osterberg, (1929) and assayed using the Sigma kit # 510 (Sigma Chemical Co. St. Louis). For amino acid determination, the perchloric acid extracts were filtered through millipore filters (0.45um) and applied to a Beckman 118C amino acid analyser. The samples were eluted with sodium citrate buffers (pH 3.25, 0.2N; pH 4.12, 0.4N; pH 6.4, 1.0N).

Preparation Of Blood

Blood drawn by cardiac puncture was added to an equal volume of 15% (v/v) $HClO_4$ and then spun at 25000g for 10 minutes. The supernatant from this spin was then treated as was the "second" supernatant above.

COMPETITION STUDIES

A. To determine the effect of GP upon different forms of GPDH, identical activities (as determined at 0.1 mM pyruvate or DHAP and 0.1 mM NADH, pH 7.0 at 25°C) of H4 LDH and either rabbit muscle or honey bee GPDH were put into a cuvette at 25°C containing 100 mM imidazole, pH 7.0, 0.1 mM pyruvate , 0.1 mM DHAP and 0.1 mM NADH; the incubation was done in the presence and absence of 2.0 mM GP. The NADH was monitered at 340 nm until the reaction was complete, at which time, 1 ml aliquots were added to 1 ml of 15% $HClO_4$. The resulting mixture was neutralised and centrifuged and the acid extract used for the determination of pyruvate and DHAP.

B. To determine the effect of creatine-phosphate upon defferent forms of LDH, rabbit muscle GPDH was incubated as above with either H4 or M4 LDH in the presence and absence of 20 mM creatine phosphate.

PROTEIN DETERMINATION

Protein concentrations were determined spectrophotometrically at 280 and 260 nm using the formula below (Layne, 1957):

mg protein/ml = 1.75(OD 280) - 0.74(OD 260).

TEMPERATURE MEASUREMENTS

Temperature measurements were taken with Yellowsprings Instrument (YSI) temperature probes (22 guage) which were linked to a YSI Telethermometer, accurate to 0.1°C. Temperature measurements with these probes were taken in deep red muscle (next to the spinal chord) and in deep white muscle (halfway between the red muscle and the dorsal edge of the fish) unless otherwise stated. Temperature measurements were taken at the level of the pectoral fins unless otherwise stated. Temperature tags came from Jim Rochelle and Charles Coutant, Oakridge National Laboratories and were pulse modulated temperature telemetary tags.

CHAPTER 3. HISTOCHEMISTRY, ULTRASTRUCTURE AND ENZYME PROFILES OF THE SWIMMING MUSCULATURE

INTRODUCTION

On the basis of colour, vertebrate striated muscle can be differentiated into at least two types, red and white. The red fibers owe their colour to the presence of a fine capillary net which surrounds each muscle, to the presence of myoglobin and to the presence of mitochondria and cytochromes (Love, 1970). The structural, metabolic and functional differences between these different fibers has been rigorously investigated in mammalian muscles and much of the terminology arises from these mammalian muscle studies. It is therefore expedient to firstly review the mammalian muscle literature.

MAMMALIAN MUSCLE

The nomenclature involved with the classification of mammalian muscles can become rather complicated. Romanul (1964), proposed a scheme including three major groups and a total of eight sub-groups. For the purposes of this thesis however, a discussion of the three major groups, red, white and intermediate, will suffice.

The characteristics of these three muscle types can be separated into three groups: ultrastructure, electrophysiology and enzymes and metabolism.

Ultrastructure

The white fibers have the largest diameter, being about twice the size of the red, and the intermediate fibres are intermediate in size. In general, mitochondrial content is

inversely related to the diameter of the fibers. The mitochondria in red fibers are present in interfibrillar rows and in pairs at the I band (filamentous mitochondria) and large spherical mitochondria aggregate at the periphery of the fiber. The mitochondria of white muscle are almost entirely filamentous pairs at the I band. Intermediate fibers have many of the red muscle characteristics, but peripheral and interfibrillar aggregations are less conspicuous. Z lines are thinnest in white fibers and thickest in red and there are clear ultrastructural differences between the three fiber types at their neuromuscular junctions (Gauthier, 1970; Hess, 1970). The development of the T system is related to the size of the fiber and to its speed of contraction . Thus the larger the fiber, and the faster the fiber (assuming the same size), the more extensive is the T system (Peachey, 1970).

Electrophysiology Studies

The contraction time of the white muscle is about 10 msec, that of the intermediate is 38 and that of the red is 18. Thus the red and white fibers in the mammals are classified as fast twitch and the intermediate is classified as slow twitch(Close, 1967; Edgerton and Simpson, 1969; Barnard et.al., 1971).

Enzymes And Metabolism

The time honoured method for distinguishing fiber types is histochemistry. This technique, coupled with actual enzyme assays clearly demonstrates the presence of at least three

fiber types in mammalian skeletal muscle. Using representative enzymes from glycolysis, B-oxidation and the Krebs cycle, the activities of myofibrillar ATPase, hexokinase activities, cytochrome concentrations, myoglobin concentrations, oxygen uptake, lipid stores and mitochondrial respiratory activity, the metabolic potential of the three fiber types appears to be as follows.

1. The red fiber has a high aerobic capacity, a moderate to high anaerobic capacity and high ATPase activity. (ATPase activity has been correlated with speed of contraction, (Barany, 1967)).

2. The intermediate fiber has a high aerobic, low anaerobic capacity and a low ATPase activity.

3. The white fiber has a low oxidative and high anaerobic capacity, and high ATPase activity. (Peter et.al., 1972; Barnard et.al., 1971; Khan, 1976; Edgerton and Simpson, 1969; Beatty and Bocek, 1970; George, 1962; Pande and Blanchaer, 1971; Pette and Dolken, 1975; Peter et.al., 1968). Unfortunately, there is one disturbing fact in the whole scheme, i.e. red muscle demonstrates as much or more glycogen than does white muscle (Baldwin et.al., 1973; Reitman et.al., 1973; Essen and Henriksson, 1974; Beatty and Bocek, 1970).

Results of in vivo and in vitro experiments are in keeping with these metabolic potentials. Numerous experiments have shown that the red muscle is an endurance muscle which uses predominately fat as a fuel for aerobic oxidation and that white muscle is a carbohydrate burning, more anaerobic muscle which is active during intense muscular activity(Terjung, 1976;

Baldwin et.al., 1973; Reitman et.al., 1973; Holloszy and Booth, 1976; Gollnick et.al., 1974). The intermediate fibers appear to behave in an intermediate fashion (Reitman et.al., 1973). There is presumed to be considerable overlapp between the two extreme fiber types as both types appear capable of aerobic and anaerobic metabolism using both lipids and carbohydrates as a fuel (Pande and Blanchaer, 1971; Reitman et.al., 1973; Baldwin et.al., 1973). Blanchaer(1964) suggests that white muscle may have the components of a GP cycle to facilitate redox balance during aerobic carbohydrate metabolism.

FISH MUSCLE

The morphology of fish striated muscle differs in one major respect to mammalian striated muscle. In many of the muscle fibers of fish, the fibrils are not even approximately cylindrical, but are flat or sheetlike (Nakajima, 1969). The peripheral fibrils tend to be more flattened than the central ones; thus the sarcoplasmic reticulum (SR) and T systems are constrained to a planar arrangement except near the center of the fiber (Peachey, 1970). Other ultrastructural diffences between fish and mammalian muscles include sarcomere length, innervation and the SR and T systems. The sarcomere length of the white muscles in the coalfish are slightly longer than that of the red and both are relatively short compared with those of the mouse (Patterson and Goldspink, 1972; Goldspink, 1968). Coalfish white muscle is multiply innervated (Patterson and Goldspink, 1972) which is in contrast to mammalian white muscle (Hess, 1970). Fish red muscle is distinguishable from other

vertebrate red muscle in having a highly organised SR and T system (Patterson and Goldspink, 1972; Kilanski, 1967; Peachey, 1970), in fact there is no significant difference in the % volume of the SR and T systems of the two fibers in the coalfish (Patterson and Goldspink, 1972). Apart from these differences, the pattern is similar to the mammalian one. The diameter of the white fibers is larger, the electrical activity of the two fiber types differs qualitatively, the innervation of the red fibers is multiple, and the width of the Z line is greater in the red than in the white. The red fibers are richer in capillaries, myoglobin concentration, lipid and glycogen deposits, oxidative enzymes (including lipase) and mitochondria, and the white fibers show more glycolytic enzyme activity and myofibrillar ATPase activity (Bone, 1966; Patterson and Goldspink, 1972; Johnston et.al., 1977; George, 1962; Takeuchi, 1959; Pritchard et.al., 1971; Patterson et.al., 1974; Mosse and Hudson, 1977; Lin et.al., 1974). Again red muscle is thought to be active during low to moderate aerobic swimming. Oxygen uptake of red muscle is greater than that of white, and electrophysiological studies show that the red muscle only is active at the lower swimming speeds (Bone, 1966; Johnston et.al., 1977; Lin et.al., 1974). White muscle appears to provide the propulsion at above sustained swimming speeds. Electrophysiological studies again support this (Bone, 1966; Johnston et.al., 1977) and glycogen depletion and lactate accumulation can be shown to occur in fish white muscle only at the higher swimming speeds (Pritchard et.al., 1971; Bone, 1966). As in the mammalian system, it is suggested that there

is considerable overlapping of function between the two major muscle types. Fish red muscle has the components of glycolysis, glycogen deposits and produces lactate at high swimming speeds. White muscle has active Krebs cycle components, lipid deposits and extracellular lipase (George, 1962; Johnston et.al., 1977; Pritchard et.al., 1971; Bone, 1966). The so-called intermediate fibers in fish myotomes have been reported in several studies and can occupy 10% of the myotome (Davidson et.al., 1976). Information on them however, is both sparse and contradictory. Bone (1966) classifies these fibers in the dogfish with the white fibers as they have the same pattern of innervation, the same scattered distribution of nuclei and low concentrations of lipid. These "pink" fibers however, are intermediate in size between the red and the white. In the carp the intermediate fibers are recruited at intermediate swimming speeds, have intermediate ATPase and oxidative enzyme activities, high PK and LDH activity and white muscle type myosin (Johnston et.al., 1977). Patterson et.al., (1975) find the intermediate fibers in five species of fish to be "intermediate" between the red and white fibers on the basis of glycogen and lipid stores and SDH, GPDH and phosphorylase activities. The fish intermediate fiber appears to be more like the white fiber than the red fiber, which is not the case in mammalian muscles.

RESULTS AND DISCUSSION: PART 1. ORGANIZATION OF THE SKIPJACK MUSCULATURE AT THE MYOTOMAL, CELLULAR AND SUBCELLULAR LEVEL

GENERAL OBSERVATIONS

In the skipjack, a small wedge of 'red' muscle occurs in the anterior half of the myotomal mass in a superficial lateral position. This wedge beccmes homogeneous with the white muscle in the posterior half of the fish (Figure 3-1). However, the bulk of the so-called deep-red muscle, is internalized and lies adjacent to the vertebral column (Chapter 1). Despite these complexities, myomeres consist cf both red and white muscle fibers. The boundary between the two is sometimes very sharp, but interdigitations are also evident (Figure 3-2).

HISTOCHEMISTRY

Two Basic Fiber Types

Although some subtle variations in fiber type can be distinguished at the E.M. level, these variations do not appear to be function- related, and by three histochemical tests, the skipjack myotome consists of cnly two basic fiber types.

Succinate dehydrogenase activity as an index of oxidative capacity, should be highest in fast-twitch red, intermediate in slow-twitch red and lowest in fast-twitch white (see

Introduction). When applied to tuna myotomal muscle, however, only two staining patterns are observed for SDH, with red muscle staining very darkly, white muscle staining lightly. By carefully monitoring the SDH reaction, it can be shown that red and white muscle display only one fiber type by this criterion; furthermore, in both muscles, the bulk of the SDH activity appears in peripheral rather than in myofibrillar positions (Figure 3-2). The lateral wedge stains as red muscle in the anterior half of the fish and as white muscle in the posterior half.

Although LDH activity normally may be taken as an index of anaerobic metabolism, the LDH-specific stain depends upon the back reaction (i.e. lactate oxidation to pyruvate); thus, like SDH, the LDH stain reaction can be used as an indication of oxidative capacity. When applied to the skipjack myotome, only two staining patterns are observed, red muscle staining very darkly, white muscle staining lightly (Figure 3-2). Again the wedge consists of red and white fibers in the anterior and posterior half of the fish respectively.

Lipid specific staining with sudan black again results in only two forms with the wedge characteristics being as they were for the SDH and LDH stains (Figure 3-2). The sudan black sections also demonstrate the interdigitations which can occur between red and white muscle and differential deposition or utilization of lipid in the red muscle (Figure 3-2). This latter observation does not represent different fiber types as (1) the pattern appears to be completely random, (2) it is not supported by differential SDH or LDH staining and (3) there are

no structural differences between fat loaded and fat depleted red fibers at the E.M. Level.

ELECTRON MICROSCOPY OF THE RED MUSCLE

The ultrastructure of the red and white muscle, although described in detail by Hulbert, Guppy and Hochachka (in press), is considered necessary for the logical development of this thesis, and so is described below in summary form.

Fiber Structure

In terms of size, skipjack red muscle sarcomeres are about 1.7um long. The fibers are substantially smaller than in white muscle, being between 12-53 um in diameter; an indication of the size variation is evident in Figure 3-1. The sarcoplasmic reticulum is fairly well developed, a feature common in teleost, but not mammalian red muscle (see Introduction).

Mitochondrial Abundance

Red fibers are extremely rich in mitochondria; up to 35% of the cross sectional area consists of mitochondria, a value that is higher than previously observed for other teleost red muscle (Hulbert and Moon, 1978; Patterson and Goldspink, 1972), but which correlates well with mitochondrial abundance in Scomber (Bone, 1978). Mitochondria are usually more abundant in peripheral regions adjacent to capillaries.

Capillarity

Like mammalian red muscle, skipjack red muscle is highly vascular. Each fiber is surrounded by 4-12 capillaries which is a high value compared to any other teleost(Mosse, 1978; Boddeke et.al., 1959).

Intracellular Fat

The abundance of mitochondria plus the numerous capillaries imply a highly oxygen dependent metabolism in skipjack red muscle. Not surprisingly, skipjack red muscle also contains large amounts of intracellular triglyceride. These triglyceride droplets may be myofibrillar in position, usually located near mitochondria, or they may be found peripherally. As already mentioned with reference to Figure 3-1, there is differential utilization (or deposition?) by different red muscle fibers; thus often there are fibers loaded with lipid droplets adjacent to fibers with the droplets totally depleted.

Exercised Red Muscle

No ultrastructural changes are observed in red muscle fine structure following severe exercise ('burst swimming'). What is notable, however, is a large depletion of glycogen granules and of intracellular lipid droplets.

ELECTRON MICROSCOPY OF WHITE MUSCLE

Fine Structure: Overview

The fine structure of skipjack white muscle differs in several aspects from the generalized concept of teleost white muscle. Firstly, skipjack white muscle contains more glycogen than does red muscle (this is quantified in Chapter 4). Secondly, glycogen granules are often sequestered away in membrane bound structures, termed glycogen bodies, and located primarily in interstitial regions. Thirdly, intracellular lipid drcplets are periodically observed. Fourthly, skipjack white muscle is well perfused, for capillary density is high compared to other teleosts. Finally, skipjack white muscle contains a substantial abundance of mitochondria.

General Fiber Structure

The basic 'contractile machinery' (i.e. the sarcomere fine structure) of skipjack white muscle is similar to that of other teleosts. In terms of the contractile protein composition, the ratio of actin/myosin is 6, as is typically observed elsewhere. In size, the skipjack white muscle sarcomere is 1.6-1.7 um long, well within the range of white muscle fibers in other teleosts (Hulbert and Moon, 1978). Compared to red fibers, white muscle fibers are large, and show a surprising range of

diameters (Figure 3-1). A highly complex sarcoplasmic reticulum is clearly evident.

Lipid, Mitochondria, And Capillarity

Skipjack white muscle contains small amounts of intracellular lipid. Even if such lipid droplets are rare (at least one/fiber) they are abundant compared to a fish such as the eel where lipid droplets may be found in every fifth fiber (Hulbert, pers. comm.). Their occurence in white muscle is supported by the histochemical studies (Figure 3-2). About 2.3% of the cross sectional area is occupied by mitochondria, compared to 0.1% in eel white muscle (Hulbert and Moon, 1978) and 1.1% in coalfish white muscle (Patterson and Goldspink, 1972). The mitochondria are at least 50% peripheral.

The occurence of unusually high amounts of mitochondria imply the need for an effective oxygen supply system. Capillaries in skipjack white muscle approach 1 capillary/fiber, again a value that is up to 10-fold higher than that of other teleosts (Boddeke et.al., 1959; Mosse, 1978). Although an effective oxygen delivery system is an absclute necessity if lipid is to be catabolized, E.M. Studies leave no doubt as to the far greater importance of glycogen as a carbon and energy source for skipjack white muscle.

Glycogen Storage

Glycogen depots in skipjack white muscle are unusual in two regards: in abundance and in storage mechanisms. Glycogen

is extremely abundant, far more so than in most teleost white muscle which typically stores only small amounts of glycogen (Walker and Johansen, 1977; Johnston and Goldspink, 1977). In fact, skipjack white muscle clearly stores higher concentrations of glycogen than does red muscle, in this feature resembling the mammalian, rather than the teleost, condition. White muscle glycogen is stored either as typical B-particles that are found both in myofibrillar and peripheral positions, or in distinct glycogen-membrane associations termed glycogen bodies (Hochachka and Hulbert, 1978).

Glycogen Depletion In Exercise

The participation of glycogen in white muscle metabolism can be conveniently demonstrated by sampling muscle following bursts of swimming. Thus, following such severe exercise, interfibrillar glycogen granules are almost completely utilized, exposing the highly intricate, and extensive sarcoplasmic reticulum that typifies white muscle. Similarly, peripheral and interstitial glycogen granules are strongly depleted. And perhaps most intriguing of all, even glycogen bodies sustain a potent mobilization of glycogen leaving glycogen bodies containing only the weakly absorbing matrix.

From all the above data, it appears that the skipjack myotome is formed from two main types of muscle fibers, one red and one white, presumably corresponding to slow-twitch oxidative and fast-twitch glycolytic fibers of other vertebrates. In other fishes, this arrangement has been

interpreted as a two-geared system (see Introduction). At low cruising speeds, only the red fibers are thought to be used, contracting at their relatively low optimal velocity; the fast fibers are activated only during burst swimming. The fast fibers have a higher optimal contraction velocity and therefore allow fishes to 'shift into higher gear' and propel themselves more rapidly without a great loss in thermodynamic efficiency (Goldspink, 1977). In this view, low gear function is aerobic; high gear function is anaerobic. Low gear function in skipjack red muscle could be primed by glycogen or fat catabolism, while 'high gear' function of white muscle would depend upon glycogen. Fine structure studies of red muscle indicate aerobic metabolic machinery surrounded by ample lipid and glycogen. White muscle, by contrast, has far fewer mitochondria, but is packed full of glycogen granules and moreover is well endowed with glycogen bodies; both sources of glycogen could be utilized for anaerobic metabolism. Thus, the two gear system clearly could work; but does it?

Obviously the white muscle of the skipjack has a high anaerobic potential, in terms of substrate (glycogen) for glycolysis. But it is also apparent that this muscle has aerobic capabilities in excess of any other teleost white muscle priviously studied. This paradox is readily overcome if it is assumed that in skipjack, the simple two-gear system is augmented by an overlapping of red and white muscle functions. The overlap is largely metabolic rather than mechanical and reguires that at least some (perhaps initial) white muscle work be supported by aerobic catabolism. If such an overlap occurred

it would explain the ultrastructural indications of a significant aerobic capacity in skipjack white muscle.

These initial studies provide only a hint of this possible overlapping function. However, as this thesis progresses, these ultrastructural studies are confirmed by more evidence, and it turns out that the white muscle in the skipjack tuna does indeed play a significant role in power output based on aerobic metabolism.

RESULTS AND DISCUSSION: PART 2. ENZYME PROFILES OF RED AND WHITE MUSCLE

To attatch any significance to measured *in vitro* enzyme assays, one must show that these measurements in some way reflect the *in vivo* metabolic organisation of the tissue in question. This reflection is one which is constantly assumed in metabolic studies but to my knowlege has never been discussed, although Newsholme and Start (1973), Crabtree and Newsholme (1972) and Sugden and Newsholme (1975), mention some of the problems which may be involved. I believe there can be a definite correlation between *in vivo* and *in vitro* values, but, mostly for my own peace of mind, I will briefly explain why there may be unseen dangers in always assuming that this is the case.

The rate of conversion of substrate to product in an enzyme reaction (assuming constant environment such as pH etc) is dependent upon the frequency of collisions between substrate molecules and the enzyme's active sites and thus on the concentrations of substrate and enzyme. The rate of conversion is also dependent upon the turnover number of the enzyme (only when the enzyme is the limiting factor in the reaction). Thus if a reaction is measured at saturating substrate levels (which it usually is in enzyme profile studies) the rate will be affected by the concentration of enzyme, and the turnover number. The latter characteristic may have no *in vivo* consequence if the enzyme is not the limiting factor. The concentration of enzyme, however will have an effect at all substrate levels. Maximal rates therefore, can imply

differences which would disappear under *in vivo*, non-saturating conditions.

The more obvious problems (such as physical denaturation during homogenisation) are mentioned by some authors involved in studies of this type (Pette and Dolken, 1975; Linzen and Gallowitz, 1975; Scrutton and Utter, 1968), but it is too often the case that the pitfalls are ignored and the conclusions drawn without hesitation. Conceivably much of the problem could be overcome by measuring activities under "physiological" conditions. Such conditions are however almost impossible to determine and do not take into account the many different metabolic states of the animal nor the difference in metabolic activity between and active and a sedentary animal. Thus maximum activities probably turn out to be the most meaningful of the two limited methodologies.

When a multi-enzyme pathway is considered the problem becomes even more complex. The maximum flux through a linear pathway depends upon the maximum potential of the rate limiting step in that pathway, which is usually a regulatory enzyme. It is often difficult to measure the potential of complex regulatory enzymes due to instability and the large numbers of potential enzyme effectors, and thus the activities of more active enzymes at a non-regulatory site, are usually taken as alternative indices. These latter enzymes are presumably present in such high activities to minimize or exclude the possibility that the step they catalyse ever becomes rate limiting. Therefore these activities may not be an accurate indication of the pathway's potential, but may represent an

"overkill" factor.

This is not to say that precise relationships between enzyme levels and some aspect of metabolism have not been demonstrated. Glycolytic rates have been correlated with the activities of phosphorylase, hexokinase and PFK (Crabtree and Newsholme, 1972). Simon and Robin (1971 and 1972) have demonstrated a good correlation between basal oxygen consumption and cytochrome oxidase activity and between PK activity and lactate production under anaerobioses. Salminen et.al., (1977) found that in mouse skeletal muscle, fatty acid oxidation capacity was highly correlated with cytochrome oxidase activity, as was fatty acid oxidation with MDH activity. Pande and Blanchaer,(1971) show a correlation between fatty acid oxidising capacity and the activities of the palmitate-activating enzyme and the carnitine palmitoyl-transferase in the mitochondria of rabbit red and white muscle. In addition many other correlations exist between enzyme activity, or simply enzyme existence, and the known metabolic strategy of the tissue (See Introduction).

Another type of correlation, one between the activities of 'sets' of enzymes in the same tissue is also found to be widespread and is a useful method of using more than one enzyme activity to determine the relative importance of metabolic pathways in a tissue. For example, Bass et al (1969) and Pette and Dolken (1975) find that groups of enzymes exist whose activities are found in comparable or even constant proportions in a variety of tissues or cells.

The activities of a variety of enzymes in skipjack red and

white muscle are given in Table 3-1. In terms of red and white muscle function, the picture is a typical one. All the glycolytic enzymes, with the exception of hexokinase are between 5 and 10-fold more active in the white muscle, and the activity of CS, the only real measure I have of Krebs cycle activity, is 7-fold higher in red muscle. In the skipjack, hexokinase is equally active in both tissues. Red muscle usually has more hexokinase activity than white (Peter et.al., 1968; Crabtree and Newsholme, 1972; Pette, 1966; Burleigh and Schimke, 1968). The rational behind this higher activity is that the substrate of hexokinase is glucose, a blood-born carbohydrate fuel which is burnt aerobically (when blood supply is adequate) by the more aerobic muscle type.

When compared to other fish red skeletal muscles (which on the whole do not differ significantly in terms of enzyme activities from corresponding mammalian skeletal muscles) the skipjack red muscle appears typical with perhaps the exception of LDH and GOT. The activities of these two enzymes are somewhat higher in skipjack red muscle than in other red muscles (Alp et.al., 1976; Crabtree and Newsholme, 1972; Beatty and Bocek, 1970).

The white muscle on the other hand is somewhat unique. Phosphorylase values are higher than other fish white muscle values and rank with the higher values which are found in some mammalian white muscles. (Most mammalian and fish muscle values are about 50 U/g, but the rat and the rabbit have higher values of around 100 U/g (Crabtree and Newsholme, 1972; Beatty and Bocek, 1970)). PFK values in the skipjack white muscle are

relatively low, but this is probably due to the extreme instability of the enzyme which showed signs of significant deterioration immediately after homogenisation. PK activities are about 3-fold higher in skipjack white muscle than in other fish white muscles (Hochachka et.al., 1978a; Beatty and Bocek, 1970) and the activity of LDH is the highest so far found in any tissue. The LDH activity in the skipjack white muscle is at least 5-fold higher, and usually 10-fold higher than any other value reported. CS activities in skipjack white muscle are not unusual, but the MDH and GOT values are about 1.5 - 2-fold higher than other white muscle values (Hochachka et.al., 1978a; Alp et.al., 1976). Srere (1969) has noted that CS is better extracted after freezing and thawing muscle tissue. Skipjack white muscle treated this way produces 7-8 U/g of CS, a value approaching that in some mammalian muscles. (Holloszy et.al., 1970). The activity of GPDH in the white muscle is unusually high. This enzyme, when in high activities usually functions as the cytoplasmic arm of the GP cycle, which balances redox during aerobic carbohydrate metabolism (Sacktor, 1976; Storey and Hochachka, 1975). There are usually higher activities of this enzyme in white muscle than red (Crabtree and Newsholme, 1972; Blanchaer, 1964) but a typical value in vertebrate white muscle is 5 - 25 U/g, with the highest values at 50 U/g (Hochachka et.al., 1978a; Beatty and Bocek, 1970; Crabtree and Newsholme, 1972).

The enzyme profiles both agree with and add to the conclusions drawn from the histochemical and ultrastructural data presented in Chapter 3. The red muscle is the more aerobic

of the two muscles as is demonstrated by its greater Krebs cycle activity and potential for amino acid metabolism. The low activity of the glycolytic enzymes in red muscle suggests that the majority of acetyl CoA for the Krebs cycle in red muscle is derived from lipid. Thus the skipjack red muscle appears to be a typical fish red muscle although the relatively high activities of GOT and LDH suggest that it may have an atypically high ability to metabolise carbohydrate aerobically (using a malate-aspartate shuttle to balance redox) and anaerobically, producing lactate. Also, the high activities of LDH in the red muscle (the red muscle LDH is kinetically H-type and thus has a higher affinity for lactate than does the white muscle LDH (see Figure 3-2 and Chapter 5)) could be for the oxidation of extracellular lactate. Red muscle mitochondria oxidize lactate at a faster rate than do white muscle mitochondria (Blanchaer, 1964), and Wittenberger et. al., (1975) argues that carp red muscle is the site of oxidation for white muscle lactate. With the quantities of lactate produced by the skipjack white muscle (Chapter 4), white muscle lactate could well be a major red muscle carbon source in the skipjack.

In the white muscle the glycolytic pathway is more than adequately represented and is terminated by a high activity of LDH. Thus there is ample opportunity for the anaerobic conversion of the high concentrations of glycogen (Chapter 3 and 4) to lactate. The unusual aerobic capacity suggested by the ultrastructure (Chapter 3), is supported by CS levels (in frozen and thawed tissue), levels of a-GPDH and hexokinase, and the high GOT levels could represent a capacity for augmenting

the Krebs cycle during aerobic metabolism (Dreidzic and Hochachka, 1976). The high levels of MDH may be correlated to Krebs cycle activity, but as there is also a cytosolic form of the enzyme (Siegal and Englard, 1962), the added activity could just as well be due to the cytoplasmic form. The white muscle thus would appear to be a somewhat abnormal tissue when compared to other vertebrate white muscles. It appears to have not only an extraordinary anaerobic potential, but a significant aerobic one as well.

The conclusions drawn in this chapter although consistent with the data cannot be taken as final as was pointed out in the introduction to this chapter. To begin with, there are no indicators of lipid metabolism and many of the values are activities of the near-equilibrium enzymes, rather than those which are rate limiting. As Atkinson (1977) points out, the solvent capacity of a cell is not infinite and thus the high activity of LDH and other enzymes could represent an increased turnover number rather than a higher concentration of enzyme. This data does not have to stand alone however. It is supported by data which precede and follow it and which are also consistent with the above metabolic arrangements.

Table 3-1. Enzyme profiles in red and white muscles. Enzyme activities (uM product/min/gram wet weight) at 25°C, and optimal substrate, cofactor and H^+ levels.

Enzyme	Red Muscle	White Muscle
Phosphorylase	22.0(0.64)*	106.2(11.14)
Hexokinase	1.2(0.78)	0.78(0.45)
Phosphoglucomutase	31.3(6.04)	152.8(46.48)
Phosphoglucose isomerase	84.4(14.00)	426.0(157.73)
Glucose-6-phosphate dehydrogenase	0	0
Phosphofructokinase	10.0(one value)	25.50(5.74)
Aldolase	35.5(10.05)	269.2(38.62)
Triose phosphate isomerase	1414.6(2 values)	9886.0(1763.23)
Phosphoglycerate kinase	371.1(34.8)	1982.7(433.01)
Enolase	77.7(9.23)	522.4(156.68)
Pyruvate kinase	195.2(37.0)	1294.9(249.5)
Lactate dehydrogenase	514.4(74.25)	5492.3(154.73)
α-Glycerophosphate dehydrogenase	21.7(2.77)	104.5(9.24)
Citrate synthase	20.6(0.00)	2.15(0.87)
Glutamate dehydrogenase	5.9(0.58)	3.0(1.15)
Malate dehydrogenase	723.4(30.95)	718.0(160.85)
Glutamate-oxaloacetate transaminase	101.9(6.18)	43.0(3.41)
Glutamate-pyruvate transaminase	7.7(2.25)	2.0(2.31)
Creatine phosphokinase	554.2(268.7)	516.4(101.21)
Myokinase	381.8(23.21)	946.7(102.02)

*n = 4 (±1 S.D.)

Figure 3-1. A plot of fiber diameters for red and white muscle versus frequency. Fibers were sampled from deep red muscle, deep white muscle, and from the lateral, posterior wedge, which in the skipjack tuna appears to be typical white muscle.

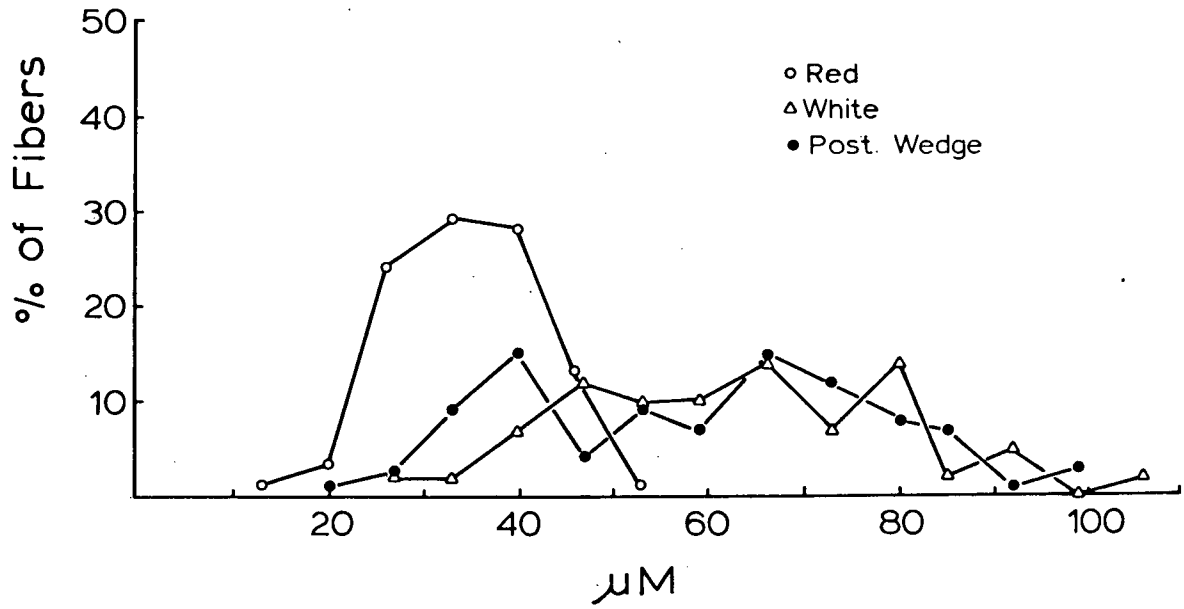

% of Fibers
50
40
30
20
10
20
40
60
80
100
μM
Red
White
Post. Wedge

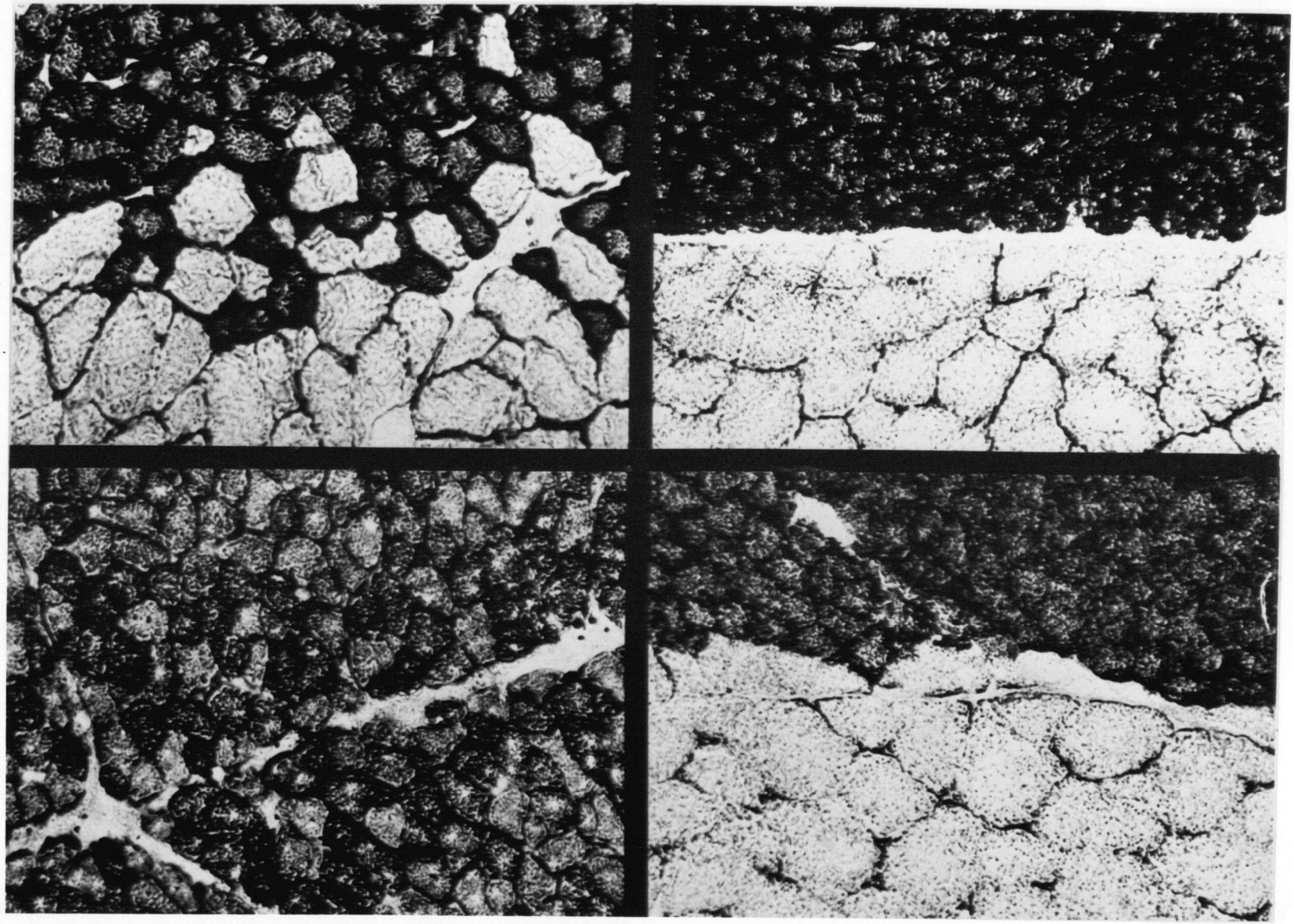
Figure 3-2. Histochemistry of the red and white muscle. In all cases except the lower right section, the red muscle is on the left and is stained more heavily; in the latter, the section is composed entirely of red muscle.

Upper left: specific stain for succinate dehydrogenase (X 160).

Upper right: specific stain for lactate dehydrogenase (X 160).

Lower left: sudan black staining for lipid (X 160).

Lower right: sudan black staining for lipid (X 160). Note the sharp demarkation between red and white muscle in the top two sections and the interdigitations in the lower left section. Also note the differential utilization or deposition of lipid in the lower right section.



CHAPTER 4. METABOLITE LEVELS AND TEMPERATURES IN THE RED AND WHITE MUSCLES DURING REST AND WORK

INTRODUCTION

Measurements of the non-protein components of metabolic pathways is a technique which is widely used to characterize metabolism in a tissue. The principle of the technique involves comparing metabolite levels before and after a situation which has in some way perturbed the tissue's metabolism. The more obvious metabolite level changes occur in glycolysis, the Krebs cycle and associated amino acid pathways and in the adenylate and creatine phosphate systems. These changes, and the conclusions that can be drawn from them are now well documented and are briefly summarized below.

During the initiation of flight in the blowfly (an example of intense aerobic glycolysis), trehalose, glycogen, abdominal polysaccharide and proline are depleted. During an hour of flight, ATP, ADP, arginine-phosphate and Pi levels remain constant. These observations are consistent with sustained aerobic work based on non-blood born carbohydrate with Krebs cycle augmentation being derived from proline (Sacktor and Wormser-Shavit, 1966). When aerobic glycolysis in the heart is stimulated, there are no changes in the levels of creatine-phosphate, ATP, ADP and glycogen (Neely et.al., 1972), and aspartate levels fall (Safer and Williamson, 1973). These observations are consistent with aerobic work based on blood-born glucose with aspartate providing the carbon for Krebs cycle augmentation. During acetate metabolism (Krebs cycle activation) in the heart, ATP levels remain constant and again aspartate levels decrease (Williamson, 1965), and when the work load on the heart is increased mechanically, long chain acyl

CoA levels decrease as flux through the B-oxidation pathway increases (Oram et al, 1973). With the onset of anaerobic work, the most obvious changes occur in the levels of end product(s), which in the majority of vertebrate tissues is lactate. Also during anaerobiosis the levels of pyruvate, ADP, AMP and creatine increase, and levels of creatine-phosphate and ATP decrease (Edington, 1973; Rovetto et.al., 1973; Lowry et.al., 1964; Neely et.al., 1975; Ford and Candy, 1972; Sacktor, 1970).

These kinds of changes which involve metabolites mainly at the beginning and ends of pathways, and those which are associated with the energy status of the cell, provide information on the fuel used for energy generation, the pathway used, the end-products accumulated and the energy charge of the cell during the work phase. Levels of metabolites throughout the pathway can occasionally increase or decrease, but usually, as a group, show no consistent correlation with flux. Specific metabolite levels within a pathway however, can be used very effectively firstly to locate regulatory enzymes and secondly to determine whether or not that enzyme, and thus the pathway involved, is activated or inhibited. Four enzymes in glycolysis have been found to be "far from equilibrium" and thus potential regulatory enzymes (see Newsholme and Start, 1973 and Rolleston, 1972, for a discussion of this point). These are hexokinase, PFK, GAPDH, and pyruvate kinase, whose mass action ratios in most tissues differ from their expected equilibrium by at least two orders of magnitude (Newshome and Start, 1973). Thus during the initiation of flight in the blowfly, the levels of FDP and pyruvate, products of two regulatory enzymes, rise

transiently as the pathway is activated (Sacktor and Wormser-Shavet, 1966). When aerobic glycolysis in the heart is stimulated, glucose and G-6-P levels fall as hexokinase and PFK are activated. When glycolysis in the heart is inhibited, metabolite level changes are consistent with inhibition at PFK and GAPDH (Williamson, 1965), and when fatty acid oxidation is inhibited, inhibition occurs before the Krebs cycle as evidenced by the build-up of long-chain acyl CoA derivatives and the depletion of acetyl CoA (Neely et.al., 1976). Metabolite studies on the Krebs cycle have suggested control at a variety of loci including citrate sythetase (LaNoue et.al., 1972), isocitrate dehydrogenase (Hiltunen and Hassinen, 1977) and a-ketoglutarate dehydrogenase (Safer and Williamson, 1973).

Non-regulatory enzymes, conversely, usually have higher activities and their mass action ratios are close to their expected equilibrium (Newsholme and Start, 1973; Rolleston, 1973; Williamson, 1965).

RESULTS

METABOLITES

Levels of various glycolytic and Krebs cycle intermediates, the adenylates, creatine, creatine-phosphate, amino acids and glycogen in tissues and blood are given in Tables 4-1 - 4-4. Table 4-1 and 4-2 are results from 1976 when tissues were simply dropped into liquid nitrogen. The data in Tables 4-3 and 4-4 are from 1977, when freeze-clamping with Wollenberger tongs was employed to quick-freeze the tissues in case the slightly longer freezing time in 1976 had led to artefacts (see Chapter 2). A comparison between Tables 4-1 and 4-3 demonstrates that there are differences which could be real differences, or possibly technique-based differences. The majority of the values however, are very similar, considering the difficulty of standardizing any work with skipjack. These differences will be mentioned in the following discussion. The glycogen data (Table 5-4) can be related to individual fish, which is a valuable asset and so has been presented in this form.

Glycogen

Glycogen levels in the skipjack white muscle are high compared to other teleost white muscle values (1000 vs 400 mg%) and are somewhat higher than skipjack red muscle values which is contrary to the situation seen in other teleosts (Walker and Johansen, 1977; Johnston et.al., 1977; Pritchard

et.al., 1971; Johnston and Goldspink, 1973). The skipjack values are in fact akin to mammalian values which tend to be around 1000 mg% with smaller differences between red and white fibers (Baldwin et.al., 1973; Ahlborg et.al., 1967). Barret and Conner (1964) found similar glycogen levels in white muscle of freshly caught skipjack.

Glycogen levels in the red muscle do not change between rest and sustained swimming, and the burst swimming glycogen levels are too variable for an average to be meaningful (Table 4-4). Values for the total glycogen and lactate pool in red muscle (Table 4-5), which are similar for all three states, simply demonstrate that glycogen carbon is either in glycogen or lactate and thus that there is no aerobic metabolism of glycogen i.e. there is no carbon lost as carbon dioxide. Glycogen levels in the white muscle are slightly lower during sustained swimming and drop markedly after burst swimming. Figures for total glycogen and lactate (Table 4-5) under burst conditions are as high or higher than those under resting conditions indicating that during burst swimming, glycogen is quantitatively converted to lactate. This relationship can most easily be seen in fish #3; glycogen values in the white muscle of fish #3 are the highest of the burst swim white muscle values whereas lactate values for the white muscle of fish #3 are the lowest of the burst swimming values (Table 4-4). However, the value for total glycogen and lactate under feeding conditions is lower (Table 4-5) indicating that aerobic carbohydrate metabolism is taking place in the white muscle during the feeding frenzy.

Glucose

Changes in glucose levels are complex. In the 1976 experiments, glucose levels in red muscle drop during feeding, but are unchanged during burst swimming; in white muscle, in contrast, glucose concentrations drop slightly during feeding, but rise dramatically during burst swimming. In the 1977 experiments, glucose concentrations in resting red muscle are lower than in the 1976 samples and show little change during feeding frenzies; however, on transition to burst swimming, glucose levels rise sharply. Not enough information is available on blood glucose levels to be certain of transient profiles; however, it does seem that red and white muscle glucose pools equilibrate with the blood during burst swimming, but not during steady state swimming.

Glucose-6-phosphate

G6P levels in the red muscle do not change in the 1976 experiments between rest and burst work and approximately double in the 1977 experiment. There is an increase in white muscle G6P levels with exercise in both experiments, again the increase is larger in the 1977 experiments. The difference here between the two experiments is the resting level of G6P which differs by a factor of 2.

Fructose-6-phosphate

Both red and white muscle levels of F6P increase with exercise, the trend being more pronounced in the white muscle.

Fructose Diphosphate

FDP levels in the red muscle do not vary with exercise in the 1977 experiment although there was a slight increase during burst swimming in the 1976 fish. The same is true for the white muscle; there was a 2-fold increase during burst swimming in the 1976 fish, but no changes with exercise in the 1977 fish.

Pyruvate

In both experiments pyruvate levels remain constant in the red muscle, but increase by a factor of 3 between rest and burst swimming in the white muscle. There is no change in pyruvate levels in the white muscle between resting and feeding fish in the 1976 experiment, whereas there is an increase (about 2-fold) in pyruvate levels between resting and feeding in the 1977 fish.

Lactate

Initial lactate levels are about 3-fold lower in both muscles in the 1977 experiments. Apart from this, the trend is the same. There is a slight increase in lactate levels in the red muscle during burst swimming and in the white muscle lactate levels increase by 3-6 fold during feeding and by a maximum of 20-fold during burst swimming. The 80-100 umol/g wet weight lactate concentrations produced in the white muscle after 10 minutes of burst work (Tables 4-1 and 4-3) are to my knowledge, the fastest rate of lactate production, reaching the highest levels ever recorded for muscular work. The jack

mackerel can produce 70 umol/g wet weight in 8 minutes, but control values were 40 umol/g wet weight (Pritchard et.al., 1971). In carp and coalfish exercised to fatigue, the lactate concentration increases to 12 and 20 umol/g wet weight respectively (Driedzic unpub.; Johnston and Goldspink, 1973) and it has been repeatedly shown that the level of lactate in salmonids can approach 30-50 umol/g wet weight (Black et.al., 1962; Stevens and Black, 1966; Hammond and Hickman, 1966; Bilinsky, 1974). A variety of mammals, exercised to exhaustion were found to accumulate 10-30 mM lactate in 10 minutes (Seeherman et.al., 1976). Blood lactate in the skipjack is not in equilibrium with the tissues under feeding or burst conditions, which at least in the latter situation is characterisic of teleosts (Black et.al., 1962). The lactate values in the blood of feeding fish however, as was the case for glucose, may not be comparable to the tissue values, although Barret and Conner (1964) published similar blood lactate levels with freshly caught skipjack tuna.

GP

GP levels approximately double during burst swimming in both tissues which is in accordance with other studies on fish, insects, and rats (Driedzic, Ph.D thesis; Edington et.al., 1973; Ford and Candy, 1972).

Citrate And Malate

Citrate and malate levels rise with exercise in the red

muscle in both 1976 and 1977 experiments. In the white muscle, citrate and malate levels do not change in the 1976 experiment and rise during burst work in the 1977 experiment.

Adenylates And Creatine-phosphate

There is some fluctuation in the levels of the adenylates in both tissues under the different exercise states. The most dramatic change is that of ATP levels in white muscle during burst swimming. The energy charge and adenylate pool size (Table 4-6) are higher in the white muscle. The energy charge is highest during feeding and lowest during burst swimming in both tissues. The pool size of the adenylates is highest during rest in both tissues and lowest during feeding in the red muscle, and during burst work in the white muscle. Creatine-phosphate levels in both experiments are at least 6-fold higher in the white muscle and drop (most markedly in the white muscle) in both tissues with exercise.

Amino Acids

The amino acid values in red and white muscle and blood during the three exercise states are given in Table 4-2. In the red muscle, aspartate and glutamate levels fall during burst swimming, and alanine, histidine and proline levels rise during feeding. In the white muscle, glycine and lysine levels fall during feeding and histidine levels, which are extremely high all the time, rise during feeding. Histidine, which is also present in high levels in carp muscle (Creach, 1966) may be in

the white muscle to buffer the large lactic acid fluctuations. The imidazole group on the histidine has a $PK^{\prime}$ of about 6.0, enabling it to act both as a proton donor and a proton acceptor at a pH near that of biological fluids. Also, histidine can be converted by histidine decarboxylase, to histamine, which is a potent vasodilator and thus could increase blood flow in the white muscle during burst work when histidine falls (Table 4-2).

Blood amino acid levels are very low and the data only represent one sample. There may be a rise in aspartate and glycine levels in the blood during burst swimming.

METABOLITE RATIOS

Various metabolite ratios are presented in Tables 4-7 and 4-8. As expected, hexokinase and PFK are far from equilibrium whereas PGI is very close to equilibrium (Table 4-7). Although glucose measurements do represent extra- and intracellular glucose pools, this does not change the conclusion that the hexokinase reaction is out of equilibrium. The mass action ratio is at least 4 orders of magnitude removed from equilibrium implying that glucose levels would have to be in error by at least 4 orders of magnitude in order that the hexckinase reaction be in equilibuium. The variation in the equilibrium of the hexokinase reaction (mentioned below) could however be negated by altering glucose concentrations by only one order of magnitude.

The degree to which the mass action ratio of regulatory enzymes differs from the expected equilibrium will vary,

depending upon the activation/inhibition state of the enzyme. Thus in the rat heart, PFK is farther from equilibrium under aerobic conditions (when glycolysis is inhibited) than under anaerobic conditions (when glycolysis is activated) (Williamson, 1965). In the skipjack muscles, there is no change in the mass action ratio of the PGI reaction as the activity state varies, nor in the mass action ratio of the hexokinase and PFK reactions between rest and burst swimming. However, upon the transition from rest to feeding, the hexokinase reaction mass action ratio becomes closer to equilibrium, indicating an activation of hexokinase, consistent with an activation of aerobic glycolysis. The mass action ratio for the PFK step however, moves further from equilibrium indicating either a PFK inhibition or simply that PFK has become the rate limiting step in glycclysis (Figure 4-1).

It is obvious from the work of Williamson (1965) and Neely et.al., (1975) that:

1. Meaningful changes in mass action ratios can only result from metabolite level changes which occur during the transition from one state to another. What my measurments probably represent, are steady-state metabolite levels after metabolism has adjusted to the new metabolic regime.

2. The locus in a pathway at which regulation is occuring varies as the transition is underway. Thus metabolite level-based evidence can easily present a picture which labels for instance one site as being regulatory, when in fact this is only correct for that instant in time.

I think what is important therefore in my data is the fact that

(a) the PFK and hexckinase reactions are out of equilibrium and (b) that the mass action ratios of these reactions change by up to an order of magnitude.

Lactate/pyruvate ratios (Table 4-8) are unfortunately not consistent between the two experiments. There is however, a definite increase in the white muscle lactate/pyruvate ratio upon burst swimming in the 1976 and 1977 fish. Assuming that LDH is a reaction which is close to equilibrium (Merrill and Guynn, 1976; Tischler et.al., 1977), an increase in this ratio signifies either a pH drop or that the NAD/NADH couple is beccming more reduced. Both occurences have been reported in working and/or anaerobic tissue (Macdonald and Jobsis, 1976; Steenbergen et.al., 1977).

CROSS OVER PLOTS

Crossover plots provide a method for localization of interaction or regulatory sites in complex enzyme systems (Williamson, 1970). The resting metabolite levels are set at 100% and the feeding, or burst swimming metabolite levels are plotted as relative values on the vertical axis (Figures 4-1 and 4-2). A cross-over point indicates that the changes in flux, and the changes in concentration of the reactants are experimentally inconsistent (i.e. some non-substrate agent must be affecting the rate of that reaction; thus the reaction is being regulated (Williamson, 1970)). Crossovers in glycclysis are typically seen at the hexokinase step, the PFK step, the GAPDH step and at the PK step (Williamson, 1970; Rovetto et.al., 1975; Williamson, 1965). My plots show three

crossover points (Figures 4-1 and 4-2), at hexokinase, PFK and LDH in both red and white muscles under at least some conditions. The crossover at the PFK step probably represents true sites of limiting function (Williamson, 1970). The crossover at hexokinase may be invalid as the glucose measurement includes extracellular as well as intracellular glucose and the one at LDH is probably due to the pH drop and the increased reduction of the NAD/NADH couple which cause the increase in the lactate/pyruvate ratio seen in Table 4-8. Since H^+ and NADH are substrates of the reaction this crossover does not imply that this is a regulatory step. A similar crossover at LDH is also shown in the data of Minikami and Yoshikawa (1966). Again one must remember that (a) my measurements do not represent the actual transition and (b) regulatory sites do not consistently show up on cross-over plots (Williamson, 1965; Rovetto et.al., 1975). The flucuations in glucose, G6P, F6P and FDP levels, however, clearly show hexokinase activation and PFK rate limitation during feeding and perhaps hexokinase inhibition during burst swimming.

TEMPERATURES OF THE RED AND WHITE MUSCLE

Ambient water temperatures, and the temperatures of the red and white muscle of individual fish under varying conditions, are given in Table 4-9. When the fish are resting, both muscles are about 1°C above ambient, the deep white muscle tends to be about 0.5-1.0°C lower than the deep red. The muscle temperatures are consistently highest during feeding when they are about 10°C above ambient in the red and 8-10°C above

ambient in the white. Muscle temperatures are also high after 10 minutes of "on line" swimming, but the maxima tend to be lower and the ranges higher in both muscles under these conditions.

DISCUSSION

This chapter has provided informaton on fuels which are being burnt under the different conditions, when end-products are accumulating and to what levels over what period of time. Also, it is now possible to say which enzymes have the potential of controlling flux through the glycolytic pathway and when and to what degree, both muscles' temperatures are increasing.

During burst swimming, the following metabolite concentration changes seem particularly important:

1. Creatine-phosphate levels in white muscle decrease by a factor of 10, in red muscle by about a half.

2. Particularly in white muscle, the adenylate pool size decreases.

3. Glycogen drops slightly in red muscle and markedly in white muscle.

4. Lactate accumulates in white muscle to levels 7-20 times higher than in the quiescent state. In red muscle, lactate levels increase, but modestly (1.5-fold).

These data, in the absence of any other, unequivocally identify anaerobic glycclysis as the predominant contribution to white muscle metabolism during this kind of swimming. At the same time, they indicate that red muscle metabolism is probably largely aerobic. That red muscle metabolism is activated under these conditions is indicated not only by the above data, particularly by the fall in ATP and creatine-phosphate levels, but also by the fact that there is a large rise in temperature, the hexose phosphates (G6P in 1977, and F6P) increase in

concentration and the pool sizes of citrate and malate increase with a concommitant drop in aspartate levels. The 10-fold increase in glucose concentrations in both tissues is curious and is confused by the fact that the measurement includes intra- and extracellular glucose. Glycogen may be preferentially mobilized during burst swimming with the concommittant inhibition of hexokinase, which is usually inhibited by the hexose-phosphates (Katzen and Soderman, 1975). This would cause glucose (extra- and intracellular) levels to rise as the intra cellular contribution (an unknown quantity) would rise. However, why are blood and tissue glucose levels in equilibrium when blood and tissue lactate levels are not (Table 4-1). Alternatively, perhaps glucose is mobilised from the liver, for the red muscle during burst swimming. But if blood flow to the white muscle is reduced during burst swimming (which is the case in other fish and which is also suggested by the non-equilibrium of the blood and muscle lactate pools) then glucose mobilization should not show up in white muscle. Unfortunately, it is clear that the glucose numbers are difficult to interpret and should not be relied upon.

In summary then, both red and white muscle metabolism contributes to burst swimming; red muscle work is sustained by an anaerobic and aerobic metabolism while white muscle work depends upon an anaerobic glycolysis more intense than any other thus far found in nature. This capability of the white muscle for anaerobic function is almost certainly the driving force behind the high burst speeds of which the skipjack is capable.

Does a similar division of metabolic function between red and white muscle occur during the steady state swimming associated with prey capture at sea? During such feeding frenzies at sea, when the highest sustained activity levels of skipjack tuna are thought to be reached, the relevant metabolite changes in red muscle can be summarized as follows:

1. Creatine-phosphate levels drop more dramatically, but ATP concentrations are sustained well within the normal range.

2. Red muscle glucose concentrations are low while hexose phosphate levels rise (F6P in the 1976 experiment, F6P and G6P in 1977) indicating a hexokinase activation Figure(5-1) ; at the same time, lactate levels remain in the normal range.

3. Citrate and malate levels rise.

These data are taken to mean (a) that red muscle is contributing to frenzy swimming, and (b) that the red muscle contribution is totally aerobic, powered at least in part by glucose oxidation.

By comparison, in white muscle:

1. Creatine-phosphate levels drop (as during "on line" swimming), but ATP concentrations, as in red muscle, are maintained in the normal range.

2. Glucose levels remain low, but G6P and F6P concentrations are high, consistent with a flow of glucose carbon into the glycolytic path; some of this carbon appears in lactate, which accumulates to levels 1/5-1/4 those observed in "on line" swimming.

These data are taken to mean that white muscle is contributing to frenzy swimming and that this contribution, as

in the case of red muscle, is largely aerobic, with only a minor anaerobic component. Plasma glucose seems preferred to glycogen as a carbon and energy source for the white muscle contribution to activated steady state swimming as glycogen levels remain high. Again, the unusually high cruising speeds which the skipjack demonstrates, are a consequence of the contribution by white muscle.

What of the heat generated. Where is it produced, and how is it produced? And what does the temperature of these muscles tell us about the muscles' metabolic strategies? This whole question is dependent unfortunately upon an unknown quantity, that is the amount of heat transfer between red and white muscle. There are three potential sources of heat available.

1. Anaerobic glycolysis, yeilding 32 Kcal/mole of glucose.
2. Glucose oxidation, yeilding 423 Kcal/mole of glucose.
3. Fat oxidation, yeilding 9.3 Kcal/gram of fat.

During burst swimming, the red muscle temperature increases by 10°C in 10 minutes. Basal oxygen uptake measurements by Neill et.al., (1976) of 2 mg oxygen/g/hr (assuming 1kg tissue = 1L of water), would raise red muscle temperature by 1.4°C/Kg/10 minutes if carbohydrate only were oxidised. A 7-fold increase in heat production would therefore be necessary to raise the red muscle temperature 10°C in 10 minutes. Considering that the above figure for oxygen uptake is for basal metabolism, and that it is for the whole body, not just the red muscle, and that lipid oxidation is not considered, it is well within the capabilities of the red muscle to produce enough metabolic heat, in 10 minutes, to raise its temperature by 10°C. The

white muscle on the other hand, also raises its temperature during burst swimming by about 10°C in some cases, and if totally anaerobic, oxidizes 50 mMoles/litre of glucose to lactate in 10 minutes. Again, on condition of the assumptions used above, 50 mMoles of glucose being oxidised to lactate in 10 minutes will only raise the temperature of the white muscle by 2.3°C. The conclusion is self-evident; either the white muscle is receiving heat from the red muscle, or aerobic metabolism is contributing to the temperature rise in the white muscle as a result of burst swimming. This latter explanation may explain the increase in citrate and malate levels in burst white muscle in 1977. The situation during feeding is a similar one. The red muscle is again the hot spot and quite capable of producing its own heat. The white muscle under these conditions heats up somewhat more than during burst swimming; average excess temperatures for white muscle under these conditions are 8-10°C (Table 4-9), only 0.3°C of which can be accounted for by lactate production (Table 4-4). If we assume that 60% of the glycogen depleted, about 10 umol glucose/g, during this kind of swimming is fully oxidised (Table 4-4), enough heat is generated to raise the white muscle temperature by 5-7°C. Aerobic glycogen metabolism in the white muscle during feeding can obviously produce the majority of the temperature increase without even considering glucose oxidation or conduction from the red muscle. The question of conduction (mentioned above) has not been resolved. As 50% of the heat must be lost across the body from the red muscle (Neill et.al., 1976), conduction between red and white muscle seems likely, but there is

evidence that the majority of this heat loss may occur over specific areas of the body (Dizon and Brill, in press).

Table 4-1. 1976 metabolite concentrations. Metabolite concentrations (umol/g wet weight wet weight or /ml) in red and white muscle and blood during three different exercise states.

	Rest		Feeding			Burst		
	Red	White	Red	White	Blood	Red	White	Blood
Glucose	1.83(0.39)	0.34(0.14)	0.18(0.09)	0.16(0.05)	1.25(0.57)	1.7(0.62)	2.35(0.51)	2.3
G6P	1.75(0.13)	1.57(0.26)	1.97(0.30)	2.7(1.02)		2.16(0.68)	3.87(1.06)	
F6P	0.25(0.14)	0.33(0.16)	0.58(0.12)	0.8(0.19)		0.46(0.07)	0.78(0.14)	
FDP	0.35(0.04)	0.35(0.1)	0.28(0.07)	0.35(0.14)		0.41(0.03)	0.69(0.11)	
DHAP	0.38(0.06)	0.32(0.05)	0.23(0.12)	0.12(0.05)		0.33(0.03)	0.3(0.08)	
G3P	0.22(0.04)	0.15(0.05)	0.11(0.03)	0.13(0.07)		0.18(0.05)	0.14(0.04)	
Pyruvate	0.14(0.03)	0.35(0.08)	0.18(0.10)	0.35(0.19)		0.19(0.04)	1.1(0.22)	
Lactate	12.3(2.1)	13.05(2.73)	10.47(1.82)	20.18(5.43)	4.9(1.82)	18.2(4.3)	84(10.4)	16.3
αGP	1.83(0.39)	2.0(0.54)	1.73(0.25)	1.65(0.24)		3.16(0.92)	0.37(0.34)	
Citrate	0.49(0.06)	0.25(0.09)	0.59(0.11)	0.25(0.03)		0.57(0.09)	0.20(0.04)	
αKG	0.14(0.02)	0.12(0.04)	0.14(0.06)	0.06(0.01)		0.12(0.04)	0.05(0.03)	
Malate	0.42(0.06)	0.19(0.22)	0.96(0.36)	0.25(0.045)		0.55(0.08)	0.31(0.06)	
Creatine	14.75(1.80)	17.35(2.80)				13.3(0.50)	23.5(1.76)	

	Rest		Feeding			Burst		
	Red	White	Red	White	Blood	Red	White	Blood
C-P	3.4(0.56)	14.25(2.70)	0.73(0.12)	1.65(0.59)		1.67(0.15)	1.35(0.48)	
ATP	4.2(0.39)	5.5(2.14)	3.55(0.44)	5.45(0.73)		3.33(0.32)	2.9(1.51)	
ADP	1.17(0.29)	0.72(0.10)	0.49(0.07)	0.46(0.03)		1.11(0.1)		
AMP	0.16(0.005)	0.09(0.03)	0.16(one value)				0.11(0.32)	

n = 5 (± S.D.)

Table 4-2. Amino acid concentrations. Concentration of amino acids (umol/g wet weight or /ml) in blood and red and white muscle during three different exercise states. Experiment done in 1976.

White Muscle

	Rest	Feeding	On Line
Aspartate	0.8*	0.4	0.55(0.5-0.6)
Glutamate	0.7	0.65(0.4-0.9)	0.6(0.5-0.7)
Glycine	1.8	0.6(0.4-0.8)	1.6(1.0-2.0)
Alanine	5.3(3.1-7.5)	1.3(1.2-1.4)	2.2(1.7-2.7)
Histidine	34.5(20-49)	58(50-66)	17.5(13-22)
Proline	0	0	0
Lysine	11.5	0	7.7

Red Muscle

	Rest	Feeding	On Line
Aspartate	1.1	1.75(1.5-2.0)	0.4(0.3-0.5)
Glutamate	2.8(2.4-3.1)	3.9(3.7-4.2)	0.65(0.6-0.7)
Glycine	3.1(2.0-4.2)	4.4(4.1-4.7)	2.8(1.9-3.6)
Alanine	3.2(2.8-3.6)	9.3(8.6-10.0)	2.2(1.7-2.7)
Histidine	0.9(0.3-1.4)	5.1(4.2-6.0)	2.5(2.1-2.9)
Proline	0	4.5(3.0-6.0)	0
Lysine			

Blood

	Rest	Feeding	On Line
Aspartate	-	0.13(0.1-0.17)	0.55(0.4-0.7)
Glutamate	-	0.3(0.2-0.4)	0.15(0.1-0.2)
Glycine	-	0.55(0.3-0.8)	1.1(1.0-1.2)
Alanine	-	1.4(0.7-2.0)	1.25(1.2-1.3)
Histidine	-	0.5(0.5-0.5)	0.35(0.3-0.4)
Proline	-	0	0
Lysine	-	0.2(0.2-0.2)	0.3(0.2-0.4)

*Values without accompanying ranges represent only one value. Other values are a mean of two.

Table 4-3. 1977 metabolite concentrations. Metabolite concentrations (umol/g wet weight wet weight) in red and white muscle during three different exercise states.

	Rest		Feeding		Burst	
	Red	White	Red	White	Red	White
Glucose	0.36(0.09)*	0.24(0.05)	0.52(0.20)	0.48(0.08)	2.03(0.43)	2.68(0.45)
G6P	0.82(0.13)	0.71(0.26)	1.48(0.16)	3.34(0.54)	2.5(0.41)	5.0(2.21)
F6P	0.14(0.05)	0.12(0.04)	0.27(0.09)	0.57(0.06)	0.38(0.09)	0.97(0.50)
FDP	0.19(0.05)	0.18(0.08)	0.11(0.05)	0.28(0.06)	0.20***	0.13(0.05)
Pyruvate	0.22(0.20)	0.2(0.07)	0.14(0.007)	0.69(0.22)	0.23(0.05)	1.47(0.46)
Lactate	3.1(1.17)	5.14(1.17)	5.82(2.26)	18.7(3.10)	7.2(1.86)	68.5(21.13)
Citrate	0.96(0.46)	0.30(0.17)	0.62(0.25)	0.1**	1.51(1.2)	1.15(0.49)
Malate	0.45(0.18)	0.12(0.05)	0.59(0.13)	0.18(0.06)	0.97(0.59)	0.47(0.31)
Creatine-P	4.76(0.31)	30.7(13.2)	1.31(0.47)	6.3(1.34)	2.86(1.06)	3.45(2.06)

*n = 5 (±S.D.)

**All other values were 0.0

***Average of two values

Table 4-4. Glycogen and lactate levels. Glycogen and lactate levels (umol/g wet weight wet weight) in red and white muscle during three different exercise states. Means of glyccgen values are expressed as umol/g (above) and as mg% (below).

	Red Muscle		White Muscle	
Fish No.	Lactate	Glycogen	Lactate	Glycogen
Rest:				
1	4.7	36.3	4.0	41.8
2	2.1	30.5	3.8	44.5
3	2.0	34.1	5.9	41.5
4	3.9	-	5.6	58.0
5	2.8	40.4	6.4	60.5
Mean ±S.D.	3.1(1.17)	35.3(4.1)	5.14(1.17)	49.2(9.2)
		635.0		885.6
Feeding:				
1	9.3	42.4	19.2	41.2
2	6.5	40.1	15.4	28.2
3	5.6	46.0	23.7	29.2
4	3.6	25.9	17.4	26.4
5	4.1	44.2	17.9	38.2
Mean ±S.D.	5.82(2.26)	39.7(8.0)	18.7(3.10)	32.6(6.6)
		714.6		586.8
Burst:				
1	9.6	6.9	84.5	21.6
2	8.1	18.3	80.6	19.1
3	6.0	43.6	32.0	59.05
4	7.5	50.1	75.6	9.6
5	4.8	49	69.6	22.6
Mean ±S.D.	7.2(1.86)	33.6(19.7)	68.5(21.13)	26.4(19.0)
		604.8		475.2

Table 4-5. Glucose equivalents. Total glucose equivalents (umol/g wet weight wet weight) from glycogen and lactate in red and white muscle during three exercise states.

	Rest	Feeding	Burst
Red Muscle	36.3 (4.2)	41.9 (12.6)	37.6 (17.6)
White Muscle	51.9 (9.5)	41.7 (7.0)	60.0 (11.7)

*Values ± S.D.

Table 4-6. Energy charge and adenylate pool size. Energy charge ((ATP + 1/2ADP)/ATP +ADP + AMP)and adenylate pool size (sum of the concentrations of ATP, ADP and AMP) in the red and white muscle during three exercise states using measured and calculated AMP values. Experiment done in 1976.

	Red Muscle			White Muscle		
	Rest	Feeding	Burst	Rest	Feeding	Burst
Measured AMP level µmol/g	0.16	0.16	0.17	0.09	0.16	0.11
Calculated AMP level µmol/g	0.55	0.28	0.66	0.26	0.17	0.43
E. C. measured	0.86	0.9	0.84	0.93	0.94	0.88
E. C. calculated	0.81	0.88	0.76	0.9	0.93	0.8
Adenylate pool measured µmol/g	5.57	4.2	4.57	6.42	6.12	3.64
Adenylate pool calculated µmol/g	5.92	4.32	5.09	6.48	6.08	3.96

Table 4-7. Metabolite ratios for enzyme reactions. Metabolite ratios for the hexokinase, phoshoglucoseisomerase and phosphofructokinase reactions during three different exercise states. Expected values from Newsholme and Start, 1973.

	Expected Equilibrium Constant	Rest	Feeding	Burst
Hexokinase				
76 Red	$3.9\text{-}5.5 \times 10^3$	0.29	1.5	0.43
76 White		0.59	1.4	0.36
PGI				
76 Red	0.36-0.47	0.14	0.29	0.21
77 Red		0.17	0.18	0.15
76 White		0.21	0.3	0.2
77 White		0.17	0.17	0.19
PFK				
76 Red	$0.9\text{-}1.2 \times 10^3$	0.4	0.06	0.31
76 White		0.13	0.04	0.19

Table 4-8. Lactate/pyruvate ratios. Lactate/pyruvate ratio during three exercise states.

	Rest	Feeding	Burst
Lac/Pyr			
Red 76	87.9	58.3	95.8
Red 77	22.1(14.78)	36.85	36.5(11.85)
White 76	34.6	58	77.4
White 77	27.64(8.19)	28.76(7.00)	45.55(5.76)

n = 5 (± S.D.)

Table 4-9. Muscle temperatures. Temperatures of deep red and deep white muscle (°C) during three exercise states.

	Fish No.	Temperature °C Red Muscle	H_2O	White Muscle
Rest:	1	25.0	23.0	25.0
	2	26.8	23.0	25.0
	3	25.0	23.0	24.0
	4	25.0	23.0	24.0
	5	25.0	23.0	24.0
	Mean ± S.D.	25.36(0.81)*		24.04(0.55)
Feeding:	1	34.3	24.4	31.9
	2	33.7	24.4	31.4
	3	33.6	24.4	32.5
	4	34.7	24.4	31.5
	5	35.2	24.4	32
	Mean ± S.D.	34.3(0.67)		31.90(0.44)
Burst:	1	34.6	23.0	31.0
	2	34.6	23.0	31.0
	3	32.0	23.0	27.6
	4	33.8	23.0	31.0
	5	32.0	23.0	27.0
	Mean ± S.D.	33.4(1.32)		29.52(2.04)

*±S.D.

Figure 4-1. Cross over plot; rest verses feeding. Cross over plot of the glycolytic pathway in red and white muscles. Resting values (100) verses feeding values. O, red muscle; x, white muscle.

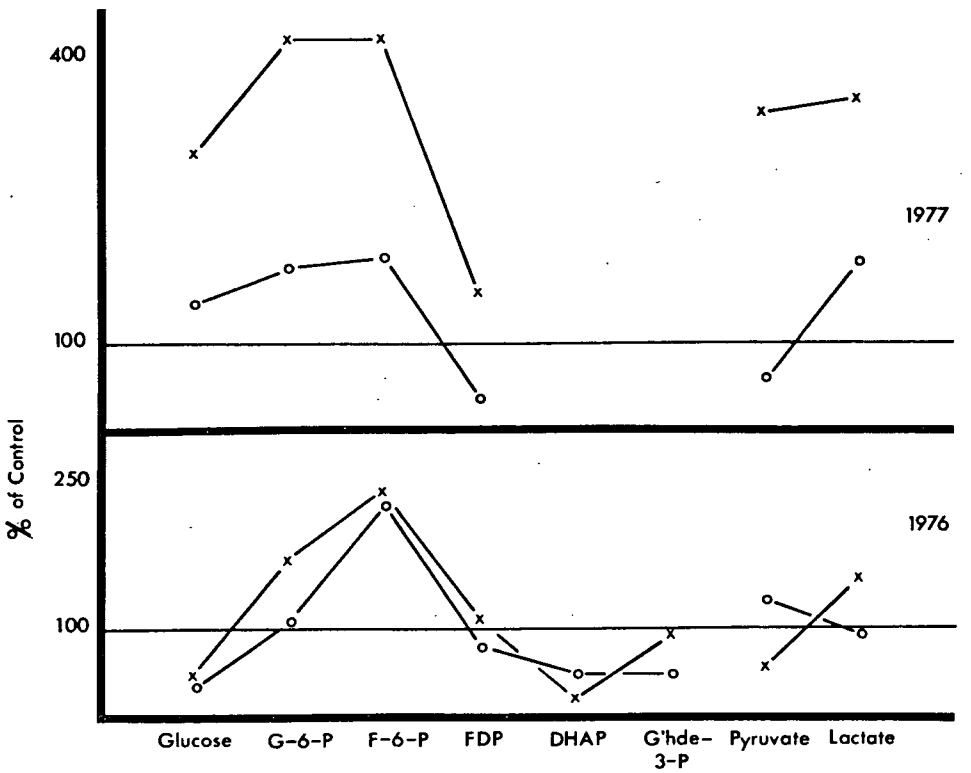
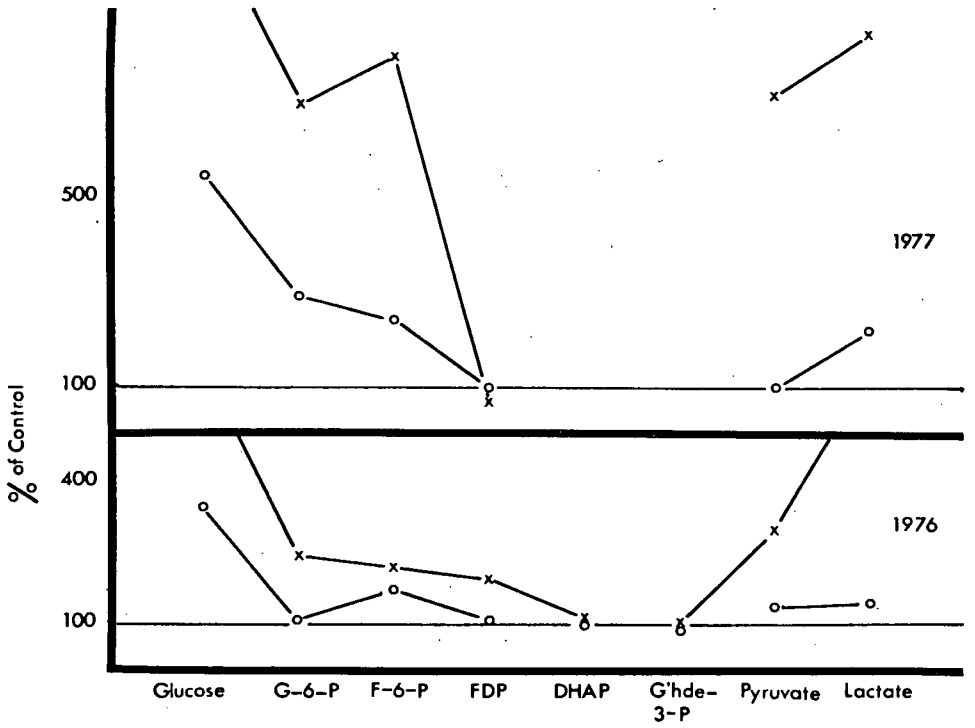

400
100
250
100
% of Control
1977
1976
Glucose
G-6-P
F-6-P
FDP
DHAP
G'hde-3-P
Pyruvate
Lactate

Figure 4-2. Cross over plot; rest verses burst. Cross over plot of the glycolytic pathway in red and white muscle. Resting values (100) verses burst values. O, red muscle; x, white muscle.


% of Control
500
100
400
100
1977
1976
Glucose
G-6-P
F-6-P
FDP
DHAP
G'hde-3-P
Pyruvate
Lactate

CHAPTER 5. ROLE OF DEHYDROGENASE COMPETITION IN METABOLIC REGULATION: THE CASE OF LACTATE AND A-GLYCEROPHOSPHATE DEHYDROGENASE

INTRODUCTION

Evidence presented in this thesis has so-far shown the skipjack to be a very active teleost (Chapter 1) with typical teleost red muscle and a somewhat unusual white muscle. The white muscle displays a high glycolytic potential (Chapters 3 and 4) which supplies most of the energy for burst swimming (Chapter 4), and an aerobic potential based on carbohydrate fuel (Chapter 3), primed by a GP cycle (Appendix I), which operates during high speed , steady state swimming and possibly during burst swimming (Chapter 4).

According to most current concepts, control of glycolysis in muscle and heart is achieved through metabolite regulation of key regulatory enzymes such as glycogen phosphorylase (Hers, 1976), hexokinase (Katzen and Soderman, 1975), and phosphofructokinase (Tsai et.al., 1975). In lower vertebrates, muscle pyruvate kinase also displays characteristics consistent with a regulatory role in glycolysis (Johnston, 1975; Randall and Anderson, 1975). However, in cases such as tuna white muscle, which displays an exceptional capacity for anaerobic as well as aerobic glycolysis, control of the above enzyme steps cannot account for the relatively exclusive function of either LDH (in anaerobic glycolysis) or GPDH (in aerobic glycolysis). The metabolism of skipjack white muscle thus presents a problem with regards to regulation.

The strategy in most tissues is to adopt carbohydrate metabolism as either an aerobic or an anaerobic mechanism of ATP production. Thus the renowned carbohydrate burners, the blowfly and the bumblebee, are obligate aerobes. Redox is

balanced with a GP cycle and LDH has been eliminated from their metabolic machinery. Thus the problem of competition between LDH and either PDH or the high levels of GPDH no longer exists (Sacktor, 1976; Crabtree and Newsholme, 1972). At the other end of the scale are the tissues with a high glycolytic potential, which have high activities of LDH and low activities of GPDH. Many vertebrate white muscles fit into this category; carbohydrate is used almost exclusively for anaerobic purposes and thus there is never a need for regulation of LDH and mainline glycolysis is regulated at the PFK, GAPDH, and PK steps (Crabtree and Newsholme, 1972). Thus, regulation of two potentially competitive dehydrogenases is essentially bypassed in most tissues at the genome level.

In the skipjack white muscle however, there is an active anaerobic metabolism terminated by high activities of LDH, and high activities of GPDH associated with a significant aerobic capacity. These two enzymes (GPDH and LDH) exist in the same compartment of the cell (Crabtree and Newsholme, 1972) and clearly compete for the same cytoplasmic supply of NADH. The consequences of such competition are potentially detrimental. Under aerobic conditions, significant LDH activity would reduce the flow of glucose-derived carbon and hydrogen to oxidative metabolic pathways, while under anaerobic conditions, significant GPDH function might drastically reduce the glycolytic ATP yield by channelling up to one-half of the glucose derived carbon and hydrogen into GP. Thus, when both enzymes occur in the same cell, minimizing sumultaneous function seems essential.

Such control is evidently achieved in the skipjack white muscle, as during anaerobic glycolysis, only lactate accumulates. Although GP levels do increase under anaerobic conditions, the overall change in concentration is insignificant compared to the total flow through glycolysis. During aerobic carbohydrate metabolism over long periods (as in feeding) lactate only accumulates to 1/10-1/5 of the anaerobic values (Chapter 4). Similar metabolite changes are also seen during aerobic and anaerobic carbohydrate metabolism in the brain (Lowry et.al., 1964), heart (Rovetto et.al., 1975) and in rat skeletal muscle (Edington et.al., 1973).

To try to resolve how this regulation is achieved, two types of studies were undertaken.

1. The GPDH and LDH from a tissue with an aerobic and an anaerobic carbohydrate based metabolism (skipjack white muscle) were characterised in order to ascertain what factors affected their activities.

2. Studies involving competition for NADH, between various LDH's and GPDH's were undertaken in order to determine whether the ability to compete for NADH was affected by (a) factors which perturb the activities of these enzymes, and (b) the isozyme sensitivity to these factors.

Temperature, pH, GP levels and creatine-phosphate levels affect the activities of skipjack white muscle GPDH and LDH. In two-enzyme experiments, the outcome of competition for limiting NADH depends upon both the kind of isozyme utilized and the concentration of the two regulatory metabolites, creatine-phosphate and GP.

PART 1. FACTORS AFFECTING THE ACTIVITY OF PURIFIED LDH AND GPDH FROM SKIPJACK WHITE MUSCLE.

RESULTS

Gel Electrophoresis

Starch gel electrophoresis shows one band of LDH in skipjack white muscle which migrates towards the cathode at pH 5.9. Skipjack red muscle has four bands of LDH; two migrate towards the cathode and two towards the anode at pH 5.9. The white muscle band does not co-electrophorese with any of the red muscle bands. The skipjack isozyme pattern is definitely non-mammalian although the kinetic comparison between the two tissues is much like an H-type (red muscle) and an M-type (white muscle) mammalian LDH comparison (Guppy, unpub.). LDH is a four subunit enzyme. In all mammalian tissues except sperm cells, there are two different subunit types which combine randomly to produce a maximum of five possible LDH isozymes (Markert, 1963). There is a unique homotetramer in mammalian sperm (Hawtrey et.al., 1975). In fishes, there is a third subunit type, and combination is not necessarily random so the pattern is more complicated (Horowitz and Whitt, 1972). There have been numerous LDH isozyme studies done on fish "muscle"; the number of LDH types in these muscles varies between 8 and 1 (Marquez, 1978; Wright et.al., 1975; Toledo and Ribeiro, 1978;

Horowitz and Whitt, 1972; Miller and Whitt, 1975; Gesser and Sundell, 1971). A more refined study of muscle LDH isozymes has been done on the goldfish by Wilson et. al. (1975); the red muscle has 4 bands and the white, 1-2 bands.

GPDH is a dimer. Trout muscles can have 1,2 or 3 bands (Utter and Hidgins, 1972) and various organs in birds and mammals can have 0-4 bands (White and Kaplan, 1969; Tsao, 1960). Skipjack white muscle has four bands of GPDH; the purified enzyme corresponds to the one major band typically found (Figure 5-1).

PH Profiles

The pH optima for the forward reaction of LDH is 6.2-6.5, for the forward reaction of GPDH is 7.0-7.4 (Figure 5-2). There is a sharp drop-off in activity as the pH departs from the optimum in both cases. At the pH optima of GPDH (pH 7.4), the activity of LDH is at about 1/3 Vmax, and vice versa. The pH optima of the reverse reaction is around 8.5-9.0 in both cases. These are typical pH responses of a dehydrogenase enzyme (Winer and Schwert, 1958).

Substrate Affinities

Both enzymes obey Michaelis-Menten kinetics, the substrate saturation curves being rectangular hyperbolas and double-reciprocal plots being linear. Michaelis constants (Km) are given in Table 5-1. The apparent affinities of the enzymes for NADH are very similar while the affinity of LDH for pyruvate is

5-fold lower than that of GPDH for DHAP. Due to pH effects, relative affinities for NAD^+ and NADH, and inhibition of the back reaction by NADH, activity in the forward direction is strongly favoured for both reactions.

A. Effects of co-substrate

Tuna white muscle LDH is unusually refractory to high pyruvate levels, its activity at 20 mM pyruvate being 92% of that at optimal pyruvate concentrations. The affinity of LDH for pyruvate is slightly dependent upon NADH levels and the Km drops from 0.33 to 0.25 mM as NADH levels drop from 0.1 to 0.02 mM (Figure 5-3a). NADH affinities double as pyruvate drops 10-fold, from 1.0 to 0.1 mM (Figure 5-3b). The affinity of GPDH for DHAP and NADH is not affected by the co-substrate.

B. Temperature effects

The affinity of LDH for pyruvate is strongly affected by temperature and rises as temperature rises, an effect previously observed for many LDH's (Hazel and Prosser, 1974). In contrast, the affinity for NADH is virtually unaffected by temperature (Figure 5-4a and 6-4b), a result that is rather unusual for ectothermic LDH's (Hazel and Prosser, 1975). High temperature decreases the affinity of GPDH for DHAP and NADH, more so for the latter; the Km values for both rise with temperature (Figures 5-4 c and d).

C. pH effects

Whereas the Km (NADH) is hardly influenced by pH, the affinity of LDH for pyruvate is strongly pH dependent (Figure 5-5 a and b). The Km(pyruvate) rises from 0.33 mM at pH 6.5 to 1.3 mM at pH 7.3. In the quiescent state, pyruvate occurs in

skipjack white muscle at about 0.3 umol/g wet weight (Chapter 4) and since the Km is important in setting the reaction velocity at low substrate concentrations, the observed effect of pH could serve to potently curb the LDH reaction under conditions of high pH.

ATP Inhibition

Both enzymes are affected by ATP; inhibition is 60% at 5 mM ATP (Figure 5-6). The inhibition is competitive with NADH , as is the usual case for dehydrogenases (Holbrock et.al., 1975), but is unaffected by temperature.

GP And Creatine-phosphate Effects

GP is an effective inhibitor of GPDH; the Ki is about 0.5 mM and this value is independent of temperature. Thus, the enzyme is 75% inhibited at 3 mM GP(Figure 5-7). GP has no effect on skipjack white muscle LDH.

LDH is inhibited by creatine-phosphate (Figure 5-8); the inhibition is mixed competitive, although the presence of creatine-phosphate does lower the Km(pyruvate) somewhat at all pH values tested(Figure 5-9). The Km(NADH) is not affected by creatine-phosphate. The inhibition by creatine-phosphate is strongly affected by pH and temperature: inhibition drops as pH and temperature rise (Figure 5-8). A similar creatine-phosphate inhibition of glyceraldehyde-3-phosphate dehydrogenase and of PK has been noted (Oguchi et.al., 1973; Storey and Hochachka, 1974a). The effect of creatine-phosphate however, is not a

general one for NAD-linked dehydrogenases since the compound does not affect GPDH activity at either low or high levels of substrate or coenzyme.

DISCUSSION

From our data, a complex *modus operandi* can be constructed of how, by an interaction of pH, creatine-phosphate, and perhaps temperature, the operation of the LDH reaction can be confined to certain situations, while under other circumstances, glycolysis can function aerobically with GPDH supplying the requisite NAD. When the muscle is not short of oxygen, the pH is relatively high (see Rahn, 1976 and Aickin and Thomas, 1977 for a discussion of intracellular pH), and thus GPDH is favoured because of the different pH optima of the two enzymes (Figure 5-2). Under these circumstances, pyruvate reduction is at a minimum because of the low affinity of LDH for pyruvate at neutral pH's (Figure 5-5a). Also, creatine-phosphate, which is at 15-30 mM in resting white muscle (Chapter 4), inhibits the LDH reaction (Figure 5-8). As soon as white muscle activation causes oxygen supply to be rate limiting, GP levels begin to rise (Chapter 4), and GPDH is inhibited (Figure 5-7). Meanwhile, creatine-phosphate levels drop drastically to around 3 mM (Chapter 4), and thus LDH is somewhat de-inhibited. Further de-inhibition is brought about as the pH, and thus the Km(pyruvate), decrease (Figure 5-5a). The decrease in pH could be due to the breakdown of creatine-phosphate (Macdonald and Jobsis, 1976) or perhaps to an increase in temperature in the working white muscle (Rahn *et.al.*, 1975). An increase in

temperature would also decrease the affinity of GPDH for NADH and DHAP. The affinity of LDH for NADH is not affected by temperature and although the Km(pyruvate) rises with temperature, so under these conditions does the pyruvate concentration. Hence, it is probable that a temperature increase would not offset the de-inhibition of LDH. When oxygen again becomes available as activity slows, GP is again oxidized in the mitochondria, GP concentrations return to normal, creatine-phosphate levels rise , pH rises, and LDH activity falls while GPDH activity rises. Both enzymes are affected by ATP levels (Figure 5-6), but ATP levels do not drop below 2 mM even in extreme bursts of swimming (Chapter 4) and therefore ATP levels alone probably are not involved in determining which of the two systems is working at any given time. The energy charge however, which decreases with exercise in white muscle (Table 4-6) could possibly have a differential effect on the two dehydrogenases. The effects of pH, ATP and creatine-phosphate on actual relative rates of the two enzymes are shown in Figure 5-10. This figure also shows that the seemingly unsurmountable 10-20-fold difference in the relative activities of the two enzymes can be reduced to a 2- to 3- fold difference by pH, ATP and creatine-phosphate.

Thus, looking at Figure 5-10, as oxygen levels drop, pH drops and creatine-phosphate levels drop, the situation on the left side of the graph is obtained, with LDH activity drastically in excess of GPDH activity. On return to steady-state swimming, oxygen rises, pH rises and creatine-phosphate rises; the situation on the right side of the graph is now

obtained, where the LDH excess is greatly reduced. In this way, a tissue obviously geared to an impressive anaerobic metabolism can allow for a low, but significant aerobic contribution using the same fuel source as it does during anaerobic bursts of swimming. The contribution to this regulatory scheme by temperature is unknown. Obviously temperature can have an effect, through pH changes (Rahn et.al., 1975), and through substrate affinities; but significant temperature changes only would occur between resting and either feeding and burst swimming, not between feeding and burst swimming (Chapter 4).

This is a convenient point to stress that I am using the words aerobic and anaerobic in relative terms. The white muscle is almost certainly never fully aerobic or anaerobic and thus both reactions will always be occuring simultaneously with a predominance of one over the other.

These data appeared at first to provide the solution to the problem of potentially competing dehydrogenases. However, the enzymes GPDH and LDH catalyse what are termed near-equilibrium reactions (Williamson et.al., 1967; Hohorst et.al., 1959). In vivo measurements of the substrates and products of "equilibrium" enzymes always show that the mass action ratio is approximately equal to the thermodynamic equilibrium constant (Chapter 4). It is usually assumed that since equilibrium enzymes always tend toward equilibrium, and usually display high activities, that their function is to simply transmit along a pathway, flux changes being generated elsewhere. As these enzymes are by definition always near or at equilibrium, they are considered unsuitable for regulatory loci and thus it

has become dogma that the "equilibrium" enzymes play secondary roles, if any, in metabolic regulation (Rolleston, 1972). However, if there is either competition for the substrate of an equilibrium enzyme, or if the substrate has an alternate way of being metabolised, alterations in the the rate at which the reaction comes to equilibuium could effectively re-route carbon flow. So if the various effectors discussed above altered the rate at which GPDH and LDH come to equilibrium, the path of carbon flow should depend upon the concentration (in the case of GP and creatine-phosphate) of these effectors, and the sensitivity of the enzymes to these effectors. This hypothesis was tested by firstly finding isozymes of the same enzyme which differ in sensitivity to specific metabolite modulators and secondly, by doing competition (competition for limiting NADH) experiments between LDH and GPDH using different isozymes and different modulator concentrations.

PART 2. COMPETITION FOR NADH BETWEEN GPDH AND LDH: THE EFFECTS OF IZOZYME FORM AND MODULATOR CONCENTRATION

RESULTS

LDH-creatine-phosphate Interactions

The LDH enzymes used were prepared from tissues intentionally chosen to represent a wide spectrum, from highly anaerobic muscles to the relatively aerobic metabolic organization of heart and brain. The kind of LDH present in such tissues usually correlates with its oxygen dependence. In mammals LDH occurs as a tetramer formed from random combinations of H and M subunits with the two homotetramers (H4 and M4) showing distinct kinetic characteristics (Holbrook et.al., 1975). Usually, M4 type LDH predominates in highly glycclytic tissues, while H type subunits are more abundant in aerobic tissues. However, kinetic features also can vary without corresponding alterations in electrophoretic properties (Hochachka and Storey, 1975). Moreover, in fishes, at least eight subunit types are now known (Markert et.al., 1975); kinetic specializations, although probable, have not yet been fully clarified.

Despite these complexities, creatine-phosphate was found to inhibit all the LDH's examined at least to some extent. Dixon plots of 1/velocity versus creatine-phosphate

concentration for LDH of tuna white muscle are consistent with creatine-phosphate inhibition being mixed-competitive with respect to either pyruvate or NADH (Figure 5-11 a and b).

The creatine phosphate sensitivity of the 12 preparations we studied appears to roughly correlate with the oxidative capacity of each tissue. The sources of LDH listed in Table 5-2, for example, are arranged approximately in order of increasing oxidative capacity (Randall and Hochachka, 1978; this thesis). This correlation is probably secondary and derives from the fact that the LDH isozyme function (Table 5-2) and content vary in these tissues (French and Hochachka, 1978). That is, creatine-phosphate sensitivity appears to depend upon the relative abundance of LDH subunits displaying M type versus H type properties. Not surprisingly, pure M4 LDH is one of the least creatine-phosphate sensitive preparations studies while pure H4 LDH is one of the most sensitive (Table 5-2).

H4 And M4 LDH Versus GPDH

The above experiments establish that large differences occur in LDH sensitivity to creatine-phosphate. Therefore, in the presence of creatine-phosphate, different LDH's should show differing capacities to compete with GPDH for a common source of NADH. Accordingly, appropriate competition experiments were set up to directly test this hypothesis.

Table 5-4a summarizes results of 2-enzyme competition experiments between rabbit muscle GPDH and either of two (H4 and M4) kinds of LDH enzymes in the presence and absence of creatine-phosphate. Egual initial activities of both

dehydrogenases lead to approximately equal contributions to total NADH oxidation in both cases. In the case of the M4 LDH, a creatine-phosphate resistant enzyme, creatine-phosphate had no measurable effect on the fraction of NADH oxidized by LDH. In contrast, fully three times more NADH was oxidized by GPDH compared to H4 LDH when 20 mM creatine-phosphate was included in the medium. Thus under conditions of limiting NADH, creatine-phosphate is an important modulator of LDH contribution to redox regulation.

GPDH Product Inhibition

My original interest in this problem arose from the finding that tuna white muscle contains GPDH at activity levels high enough to drain up to 1/2 the glucose-derived carbon into GP and thus to significantly reduce the already low energy yield of glycolysis (Chapter 3; Hochachka and Guppy, 1977). During glycolytic activation in this muscle, when LDH activity is favoured, GPDH activity needs to be and apparently is, dampened by GP product inhibition. Thus, in tuna white muscle, GPDH is unusually sensitive to product inhibition by GP, the Ki determined from Dixon plots being about 0.25 mM (Hochachka and Guppy, 1977). Table 5-3 indicates that a similar mechanism may operate in skeletal muscles of other vertebrates as well since all of the GPDH's examined show relatively low Ki values for GP. In tuna muscle (Chapter 4) and mammalian muscle (Rovetto et.al., 1975; Edington et.al., 1973) GP accumulates to values above the Ki range of GPDH. Thus, there is good correlation between enzyme data and tissue metabolite measurements,

implying that GP sensitivity may be of physiological significance.

Although sufficient information is not available on various vertebrate tissues to attempt to closely correlate GPDH sensitivity to GPDH isozyme type, two forms of the enzyme are known whose Ki values are far out of line with the typical vertebrate situation. These are the GPDH's from bee flight muscle and squid mantle muscle (Table 5-3). These two enzymes show the lowest sensitivity to product inhibition by GP of any GPDH's thus far known. Interestingly, they function in tissues that are extremely oxygen dependent and may never go anaerobic under normal physiological conditions; if made anoxic experimentally, these muscles sustain an accumulation of GP to much higher concentrations (up to 20 mM) than ever seen in vertebrate tissues (Hochachka et.al., 1975; Sacktor, 1976). Taken together, these data suggest that the GP resistant GPDH's should be more competitive with LDH under conditions of high GP levels than would be the typical vertebrate, GP sensitive, GPDH. If this is the case a physiological relevance of the low Ki for GP seen in vertebrate tissues is indicated.

GP Sensitive And Insensitive GPDH's Versus H Type LDH

The above hypothesis was directly tested by 2-enzyme competition experiments using one LDH form (H4) and two types of GPDH's (Table 5-4b). Rabbit muscle GPDH displays a high sensitivity to GP inhibition while the honey bee enzyme is strongly resistant to the reaction product (Table 5-3). When GP was absent from the incubation medium, the two forms of GPDH

competed with similar effectiveness for NADH. Their contributions to total NADH oxidation were not exactly equal (about 42% versus 50% of total NADH oxidation by the rabbit and bee GPDH's, respectively), possibly because of differences in their respective Km values for substrates and NADH (Storey and Hochachka, 1975). In sharp contrast large differences appeared in the behaviour of the two enzymes in the presence of 2 mM GP. Under these conditions, the fractional LDH-dependent NADH oxidation exceeded the oxidation due to rabbit muscle GPDH by nearly 10-fold while it exceeded the oxidation due to bee muscle GPDH by less than 2-fold (Table 5-4b). It is worth re-emphasizing that during anaerobic work in mammalian muscle, GP levels rise to about 3 umol/g wet weight (Rovetto et.al., 1975; Edington et.al., 1973), i.e. somewhat higher than the concentrations used in the above competition experiments. Thus, with both LDH and GPDH competing for the same limiting pool of NADH, a high GPDH sensitivity to reaction product strongly diminishes the amount of carbon and hydrogen that can be watefully channelled from "mainline" glycolysis into GP under conditicns of limiting oxygen.

Discussion

So with factors affecting the rate at which GPDH and LDH come to equilibrium, and with a common limiting substrate, one can see a role for "equilibrium" enzymes in the regulation of the aerobic to anaerobic transition in glycolysis. Under aerobic conditions creatine-phosphate levels are high and GP levels are low; LDH cannot compete for NADH under these

conditions, nor for pyruvate. The majority of carbohydrate carbon thus is oxidized in the mitochondrion and GPDH produces NAD^+ for continued glycclysis using catalytic amounts of DHAP. When oxygen becomes limiting, the creatine-phosphate pool is depleted and GP levels rise. The activity of GPDH consequently drops and competition for NADH now favours the deinhibited LDH. Pyruvate dehydrogenase (which I could not succeed in measuring in the skipjack) can no longer compete with the much higher activities of LDH and thus pyruvate is chanelled into lactate with no wastage of carbon at the triose phosphate level. Any other factors which vary with the anaerobic-aerobic transition, and which influence the rate of either reaction, such as pH, would also be part of this regulatory scheme. Competition is the key element here. Even if LDH and GPDH always tend towards thermodynamic equilibrium, in slowing down this tendency, the coenzyme of the reaction becomes unavailable as it is used by the competing dehydrogenase. The mass action ratio does not deviate from the equilibuium constant when the reduction reaction is slowed, since excess substrate can be chanelled off into glyceraldehyde-3-phosphate or acetyl CoA, respectively.

Central to this interpretation is the assumption that NADH at least under some conditions occurs at limiting concentrations, which is apparently assumed by Jomain-Baum et. al. (1978) who mention the concept of dehydrogenase competition in mitochondria. Until recently, reliable estimates of cytosolic NADH were not available. However, most previous measurements of NADH range between 0.03 and 0.15 umol/g wet weight; these values are for the whole cell and the NADH

concentration in the cytosol can only be less (Williamson et.al., 1971; Burch et.al., 1963; Kalkhoff et.al., 1966). NAD^+/NADH ratios in the cytosol of liver, brain, and fibroblasts vary from 7-2000(Merril and Guynn, 1976; Swartz and Johnson, 1976; Stubbs et.al., 1972). Assuming about a 1 mM pool size, NADH levels would be estmated at about 0.01 to 0.0005 mM. Such earlier estimates of cytosolic NADH concentration ranges have been closely checked using the technique of turbulent flow to rapidly lyse isolated hepatocytes (Tishler et.al., 1977). From these studies, the concentration of free NADH in the cytosol appears to be in the 0.06-1.5 uM range under differing metabolic conditions (starved versus fed nutritional states, with and without exogenous ammonia). Perhaps because the NADH binding site of dehydrogenases is conservative (Holbrook et.al., 1975),the affinity constants for NADH also are fairly constant, usually in the 0.01 to 0.02 mM range (Hochachka and Guppy, 1977; Holbrook et.al., 1975; Storey and Hochachka, 1975; Fields et.al., 1976). Thus it appears that the affinity constants are substantially higher than the lower limits of current estimates of NADH concentration in vivo. So it seems reasonable to assume, at least tentatively, that NADH would often, if not always, be limiting in the cytoplasm. At such times, creatine-phosphate and GP effects on LDH and GPDH respectively, would profoundly influence transitions between anaerobic and aerobic glycolysis in a tissue such as skipjack white muscle.

Table 5-1. Kinetic constants of LDH and GPDH from white muscle. LDH assays in the forward direction were done at pH 6.5; in the reverse direction at pH 8.5. GPDH assays in the forward direction were done at pH 7.0; in the reverse direction at pH 8.5. 50 mM imidazole was the buffer used, and all assays were at 25°C.

	NADH	Pyruvate	DHAP
LDH K_m	0.01-0.02 (depends on pyruvate con-concentration)	0.33 (slightly dependent upon NADH concentra-tion)	
αGPDH K_m	0.016		0.066

Table 5-2. Inhibition of LDH by 20 mM creatine-phosphate. Assay conditions: 0.1 mM pyruvate (0.3 mM pyruvate in the case of the Amazon fishes), 0.1 mM NADH, 25°C, pH 7.0.

Source of LDH	Type of LDH funtion	% Inhibition by 20 mM creatine phosphate
Hoplias white muscle	pyruvate reductase	25
Turtle white muscle	pyruvate reductase	26
M_4 from rabbit muscle	pyruvate reductase	29
Hoplerythrinus white muscle	pyruvate reductase	32
Skipjack white muscle	pyruvate reductase	38
Hoplias heart	bifunctional	39
Arapaima heart	bifunctional	39
Hoplerythrinus heart	bifunctional	41
Osteoglossum heart	bifunctional	47
H_4 from beef heart	lactate oxidase	71
Rat brain	lactate oxidase	71
Weddell seal heart	lactate oxidase	77

Table 5-3. The affinities for GP of GPDHs from various vertebrate and invertebrate muscles. Ki values were determined from Dixon plots at pH 7.0 (pH 7.4 for the turtle and the trout), 25°C.

Source of GPDH	$K_{i(GP)}$ (mM)
Tuna white muscle	0.25
Trout white muscle	1.1
Turtle white muscle	0.93
Rabbit mixed muscle	0.5
Honey bee flight muscle	5.0
Squid mantle muscle	15.0*

*Storey and Hochachka, 1975

Table 5-4. Competition for NADH oxidation between LDH and GPDH.

a. Competition between rabbit muscle GPDH and different LDH isozymes.

	0.0 mM C-P		20 mM C-P	
	% Total Oxidation by LDH	% Total Oxidation by GPDH	% Total Oxidation by LDH	% Total Oxidation by GPDH
H_4 LDH	52.4	47.7	25.6	74.3
M_4 LDH	49.5	50.6	48.3	51.7

b. Competition between H_4 LDH and different GPDH isozymes.

	0.0 mM GP		2.0 mM GP	
	% Total Oxidation by LDH	% Total Oxidation by GPDH	% Total Oxidation by LDH	% Total Oxidation by GPDH
Rabbit muscle GPDH	58.6	41.5	89.0	12.6
Honey bee	50.0	50.9	65.7	36.4

All values are a mean of 4 experiments.

Variation is ± 2%.

Figure 5-1. Starch gel electrophoresis of skipjack red and white muscle LDH and GPDH. For conditions of running and staining, see Chapter 2.

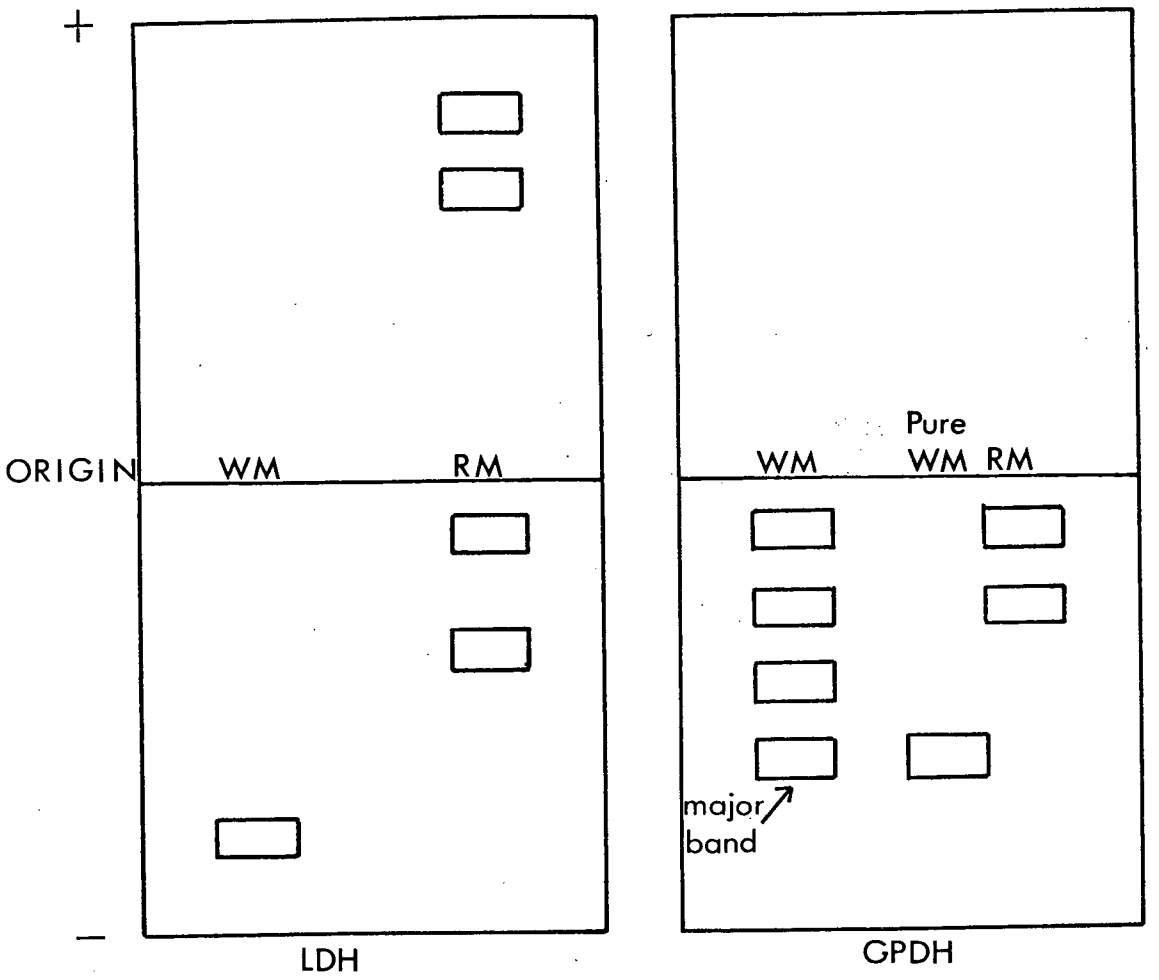

+
ORIGIN
WM
RM
−
LDH
Pure
WM
WM
RM
major
band
GPDH

Figure 5-2. Effect of pH on skipjack white muscle LDH and GPDH. 50 mM imidazole, 25°C. Assay conditions for LDH: 0.3 mM pyruvate, 0.1 mM NADH. Assay conditions for GPDH: 0.14 mM DHAP, 0.1 mM NADH.

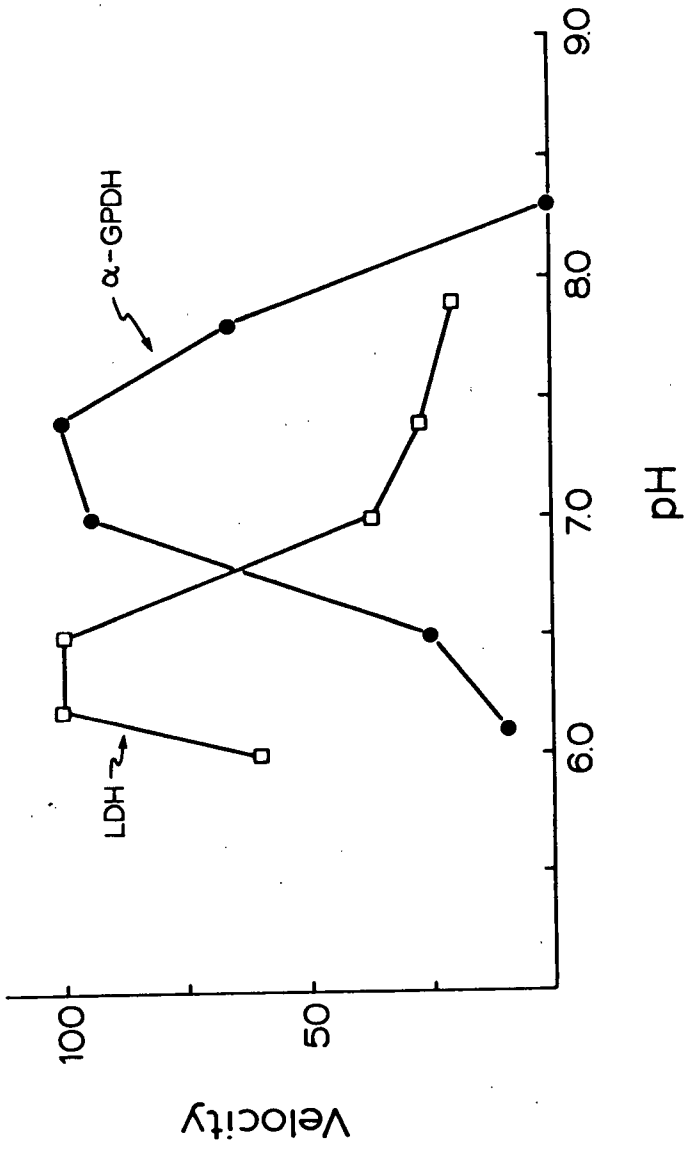

Velocity
100
50
6.0
7.0
8.0
9.0
pH
α-GPDH
LDH

Figure 5-3. Effect of the co-substrate on the Km of pyruvate and NADH of skipjack white muscle LDH.

A. The effects of NADH concentration on the Km of pyruvate of skipjack white muscle LDH. 25°C, 50 mM imidazole, pH 6.5.

B. The effects of pyruvate concentration on the Km of NADH of skipjack white muscle LDH. 25°C, 50 mM imidazole, pH 6.5.

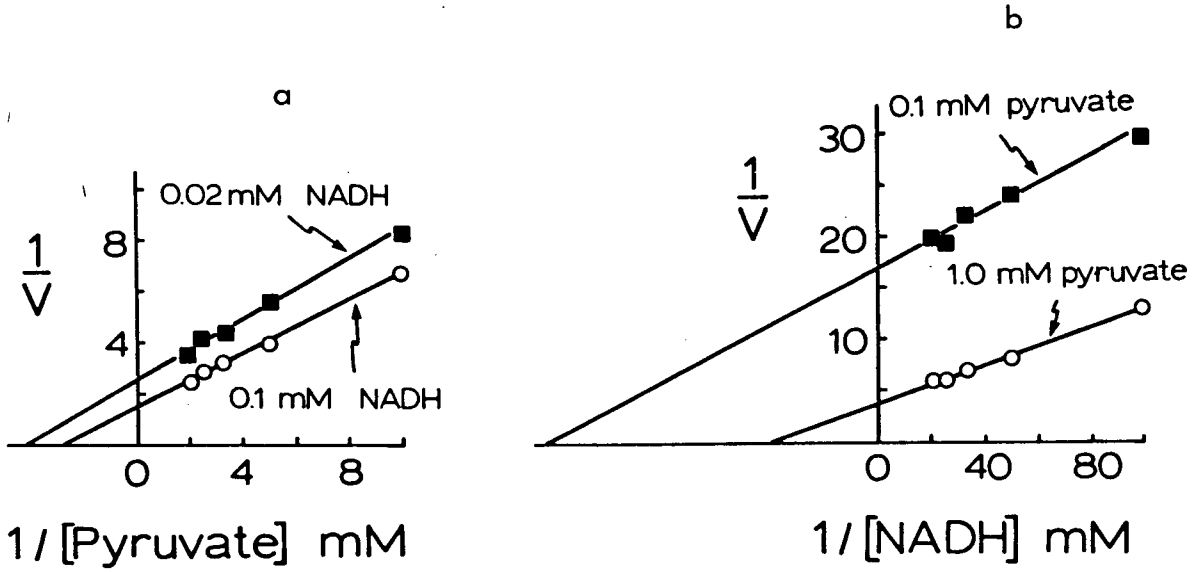

a
0.02 mM NADH
0.1 mM NADH
1/V
8
4
0
4
8
1/[Pyruvate] mM
b
0.1 mM pyruvate
1.0 mM pyruvate
1/V
30
20
10
0
40
80
1/[NADH] mM

Figure 5-4. The effect of temperature on the substrate affinity constants of skipjack white muscle LDH and GPDH.

A. The effect of temperature on the Km of pyruvate of skipjack white muscle LDH. 0.1 mM NADH, 50 mM imidazole, pH 6.5.

B. The effect of temperature on the Km of NADH of skipjack white muscle LDH. 0.5 mM pyruvate, 50 mM imidazole, pH 6.5.

C. The effect of temperature on the Km of DHAP of skipjack white muscle GPDH. 0.1 mM NADH, 50 mM imidazole, pH 6.5.

D. The effect of temperature on the Km of NADH of skipjack white muscle GPDH. 0.2 mM DHAP, 50 mM imidazole, pH 7.0.

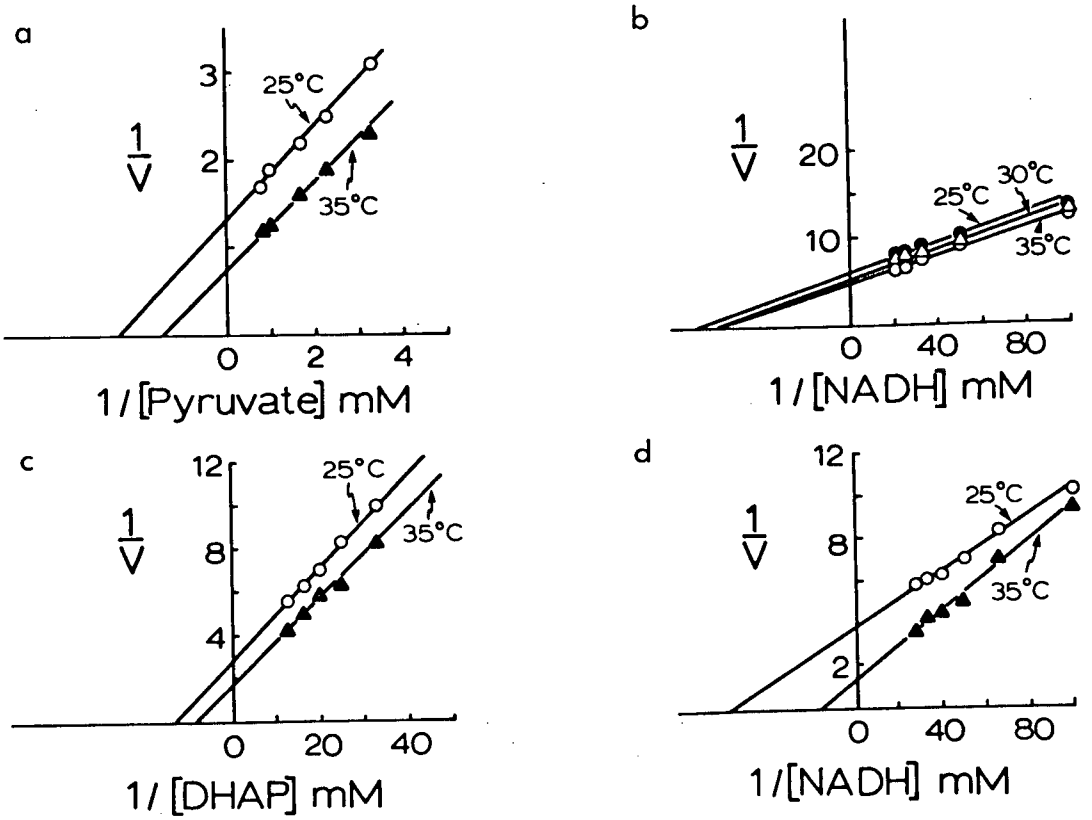
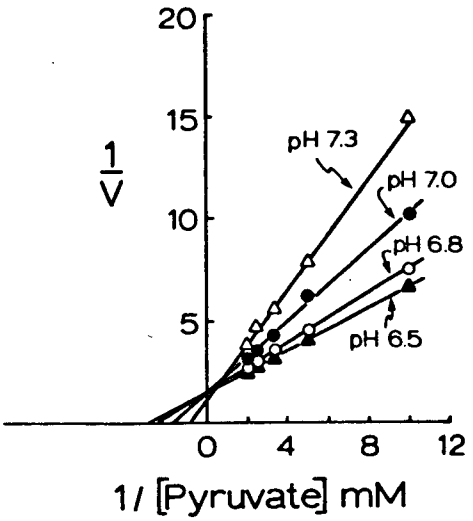

a
3
2
1/V
25°C
35°C
0
2
4
1/[Pyruvate] mM
b
20
10
1/V
25°C
30°C
35°C
0
40
80
1/[NADH] mM
c
12
8
4
1/V
25°C
35°C
0
20
40
1/[DHAP] mM
d
12
8
2
1/V
25°C
35°C
0
40
80
1/[NADH] mM

Figure 5-5. The effects of pH on the Km of pyruvate and NADH of skipjack white muscle LDH.

A. The effects of pH on the Km of pyruvate of skipjack white muscle LDH. 0.1 mM NADH, 25°C, 50 mM imidazole.

B. The effect of pH on the Km of NADH of skipjack white muscle LDH. 1.0 mM pyruvate, 25°C, 50 mM imidazole.


20
15
10
5
1/V
pH 7.3
pH 7.0
pH 6.8
pH 6.5
0
4
8
12
1/ [Pyruvate] mM

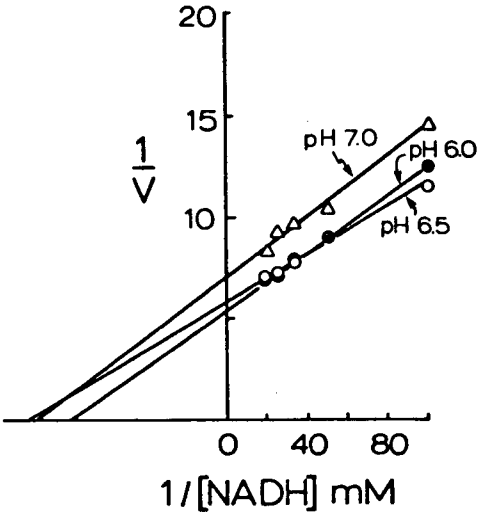

20
15
10
1/V
pH 7.0
pH 6.0
pH 6.5
0
40
80
1/[NADH] mM

Figure 5-6. The effect of ATP on the activities of LDH and GPDH from skipjack white muscle. Assay conditions for LDH: 0.3 mM pyruvate, 0.05 mM NADH, 50 mM imidazole, pH 6.5, 25°C. Assay conditions for GPDH: 0.1 mM DHAP, 0.05 mM NADH, 50 mM imidazole, pH 7.0, 25°C.

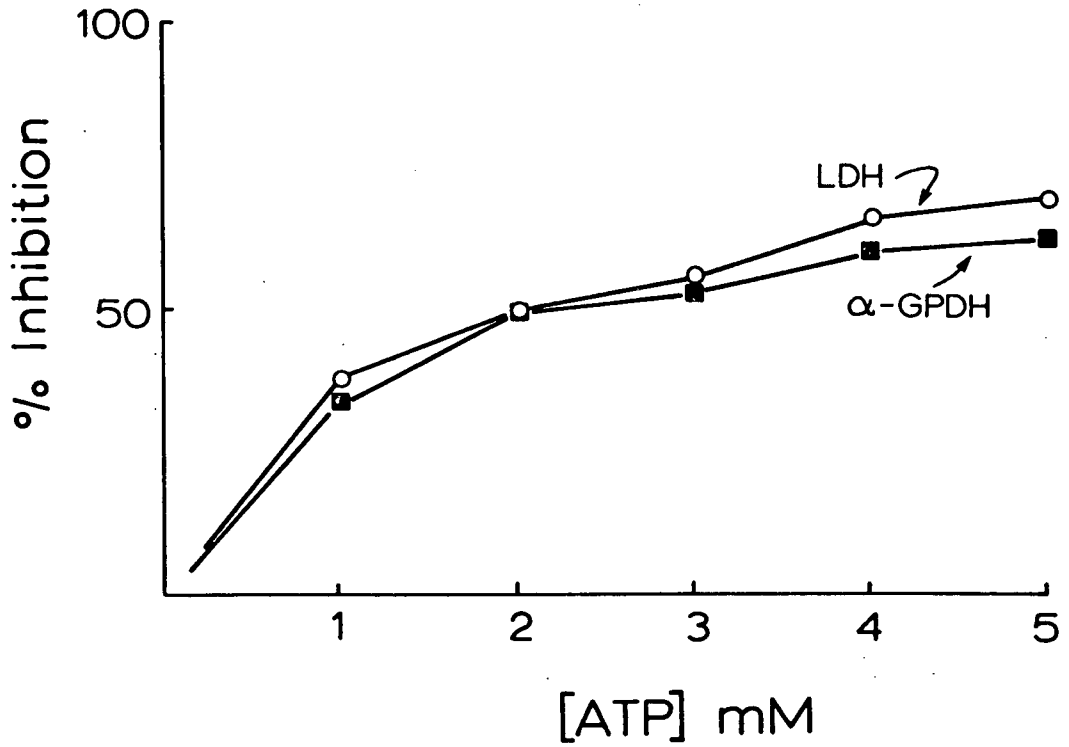

100
50
% Inhibition
LDH
α-GPDH
1
2
3
4
5
[ATP] mM

Figure 5-7. The effect of GP on the activity of GPDH from skipjack white muscle. 0.1 mM DHAP, 0.05 mM NADH, 50 mM imidazole, 25°C, pH 7.0.

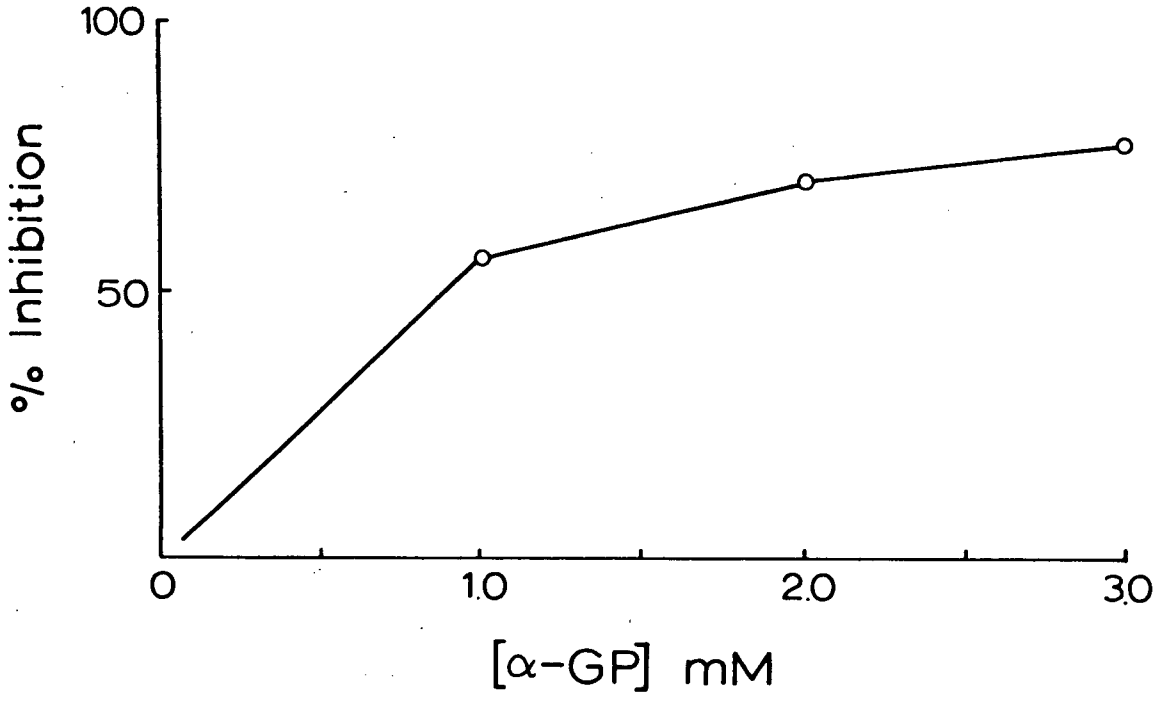

100
50
% Inhibition
0
1.0
2.0
3.0
[α-GP] mM

Figure 5-8. The effect of creatine-phosphate on the activity of LDH from skipjack white muscle. 0.5 mM pyruvate, 0.05 mM NADH, 25°C, 50 mM imidazole.

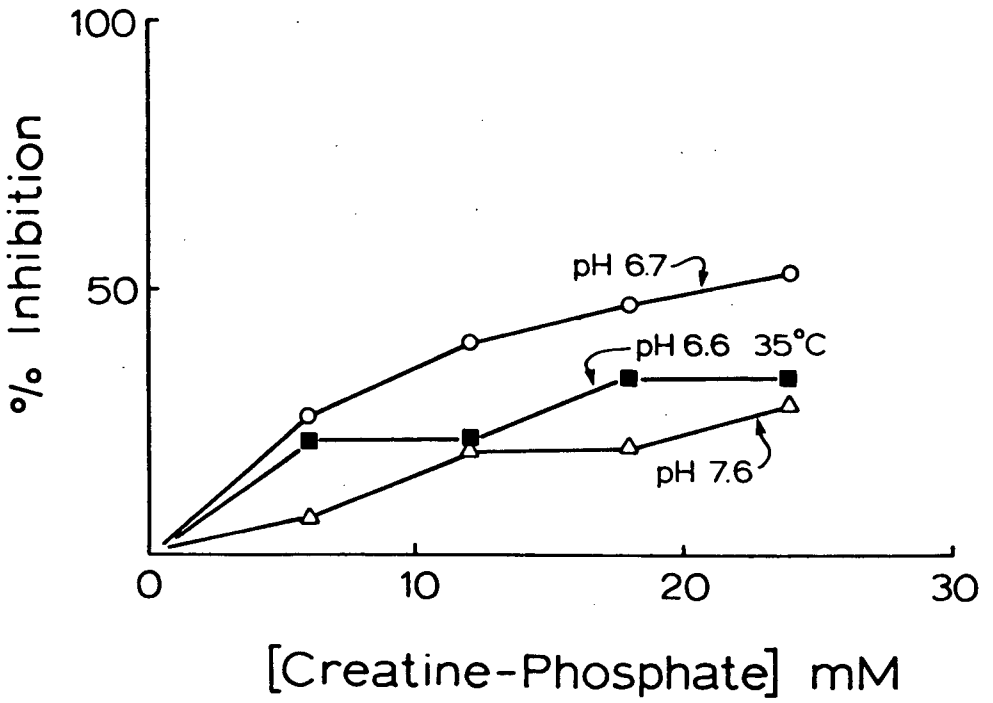

100
50
% Inhibition
pH 6.7
pH 6.6 35°C
pH 7.6
0
10
20
30
[Creatine-Phosphate] mM

Figure 5-9. The effects of pH and creatine-phosphate on the Km of pyruvate of skipjack white muscle LDH. 0.1 mM NADH, 20 mM creatine-phosphate, 25°C, 50 mM imidazole.

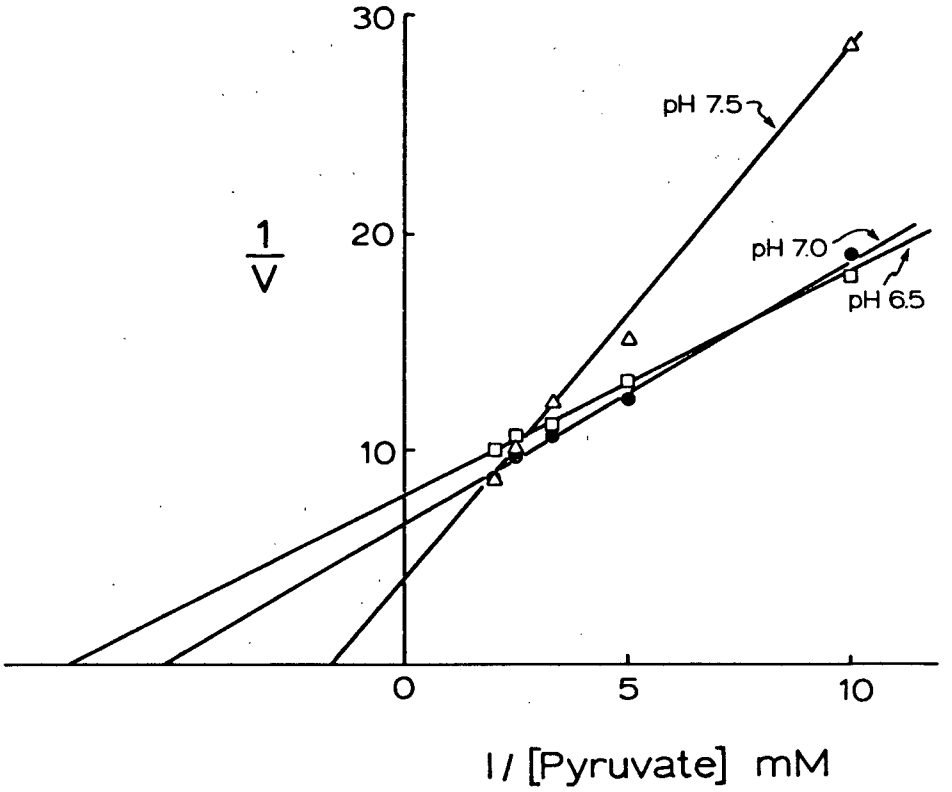

30
20
10
0
5
10
1/V
pH 7.5
pH 7.0
pH 6.5
1/ [Pyruvate] mM

Figure 5-10. Relative activities of LDH and GPDH in a crude 1:9 supernatant. 25°C, 50 mM imidazole. Closed circles: LDH, 0.1 mM pyruvate, 0.1 mM NADH; open squares: LDH, 0.1 mM pyruvate, 0.1 mM NADH, 30 mM creatine-phosphate, 6 mM ATP; closed triangles: GPDH, 0.1 mM DHAP, 0.1 mM NADH; open circles: GPDH, 0.1 mM DHAP, 0.1 mM NADH, 30 mM creatine-phosphate, 6 mM ATP.

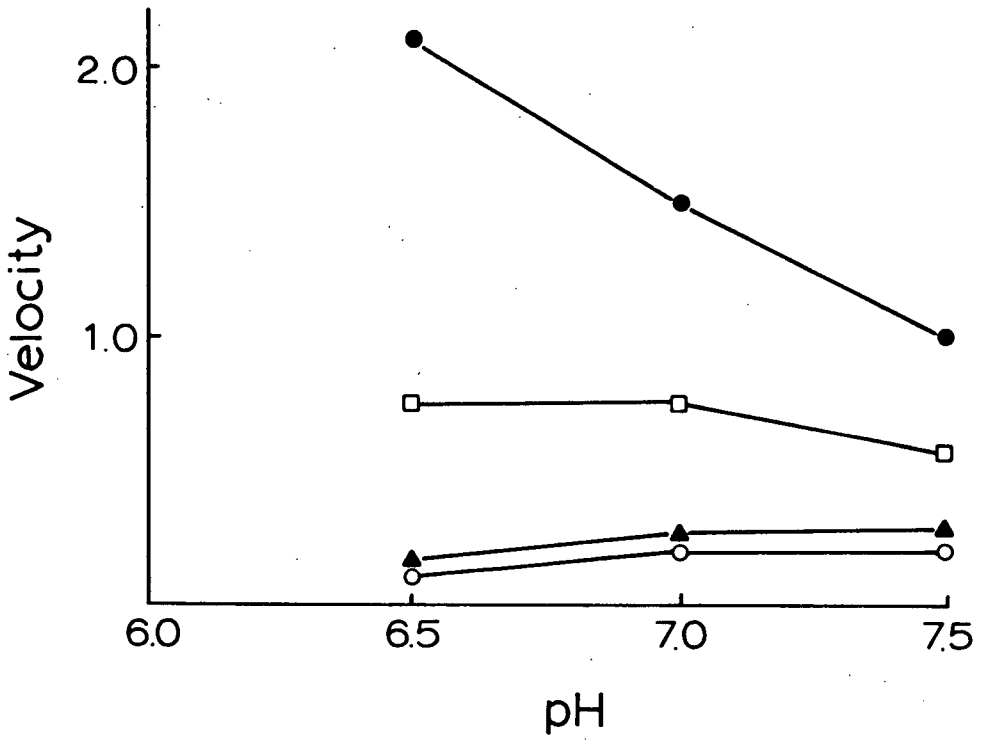
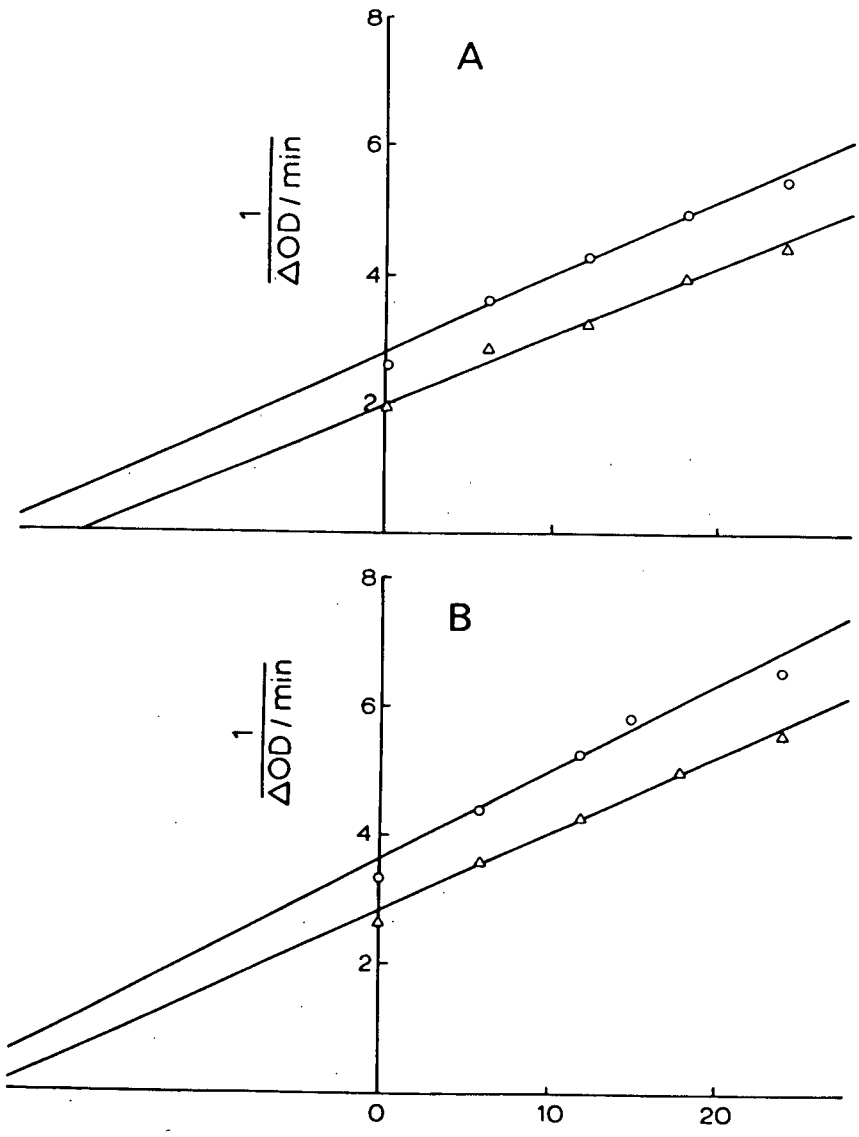

2.0
1.0
Velocity
6.0
6.5
7.0
7.5
pH

Figure 5-11. Kinetics of creatine-phosphate inhibition of skipjack white muscle LDH.

A. Dixon plot of 1/OD/min versus creatine-phosphate concentration. 0.05 mM NADH, 25°C, 50 mM imidazole buffer, pH 6.5. O, 0.5 mM pyruvate; X, 2.0 mM pyruvate.

B. Dixon plot of 1/OD/min versus creatine-phosphate concentration. 0.5 mM pyruvate, 25°C, 50 mM imidazole buffer, pH 6.5. X, 0.05 mM NADH; O, 0.02 mM NADH.


A
B
8
6
4
2
0
10
20
1/ΔOD/min
[Creatine phosphate] mM

CHAPTER 6. GENERAL DISCUSSION

When my study of the skipjack began, background information, which created the impetus for the study, consisted almost exclusively of behavioral, anatomical, and physiological data. The skipjack was known to swim at high speeds for long periods, its red muscle mass was relatively large, warm and totally discrete from the white muscle mass which was also warm; and its respiratory capabilities approached those of mammals (Chapter 1). The aim of my study was to add to the already available data information from the molecular level of organization which had been hitherto left virtually untouched.

Chronologically, my work began with a considerable bias towards temperature related studies. The bulk of this work is presented in Appendix III, although similar studies were also done on a cold water species, T. alulunga. The results of this work, although of interest to those working on pyruvate kinase, left little doubt in my mind that:

1. Temperature and metabolism were not as obviously interconnected in the skipjack as one might have thought.

2. More information on the skipjack, at different organizational levels, was needed before one could postulate on the role of temperature in the natural history of this fish.

I therefore embarked upon the study which represents the bulk of my thesis. Data from dead fish (ultrastructure, histochemistry and enzyme levels) provide an unambiguous picture of the different muscles' metabolic organisations, with the white muscle showing a surprising potential for diverse (aerobic and anaerobic) function. Studies with live fish support the former results and show the skipjack white muscle

to have impressive anaercbic capabilities as well as being a muscle capable of significant aerobic function using carbohydrate as a fuel source. These metabolite studies also considerably expand the information concerning heat production in the skipjack. When the heat is produced (in terms of swimming speed and fuel source) and in which muscle the heat is prcduced, are areas which are now considerably clearer although the question of heat conduction (mentioned in Chapter 4) still makes the exact assessment of white muscle heat production impossible. All of these results and their implications have already been discussed (Chapters 3 and 4).

From these results arose the question of metabolic ccntrol in the white muscle, a muscle capable of aerobic and anaerobic carbohydrate metabolism. So my interests descended to a lower level of organization with the end result being a novel metabolic control strategy whose implications are in no way restricted tc skipjack white muscle. Again, this latter part of my thesis, which includes Appendix I basically as results, and Appendix II as a relevant 'aside', has already been discussed (Chapter 5).

I initially set out with 5 questions to answer (Chapter 1). I have answered, or at least clarified, 4 out of 5 of these questions. The question which still remains unanswered is in fact the one to which I first addressed myself during my early studies on the skipjack, i. e. what is the advantage to the animal of either a high muscle temperature, or a circulatcry system which as an offshoot, produced high temperatures in the swimming musculature?

The function of the rete in the skipjack could be for the transfer of gas, metabolites, or heat, or for that matter, anything which differs in concentration between the venous and arterial flow in the rete. Heat exchange definitely occurs, metabolite exchange is a definite possibility, but has never been investigated, and Stevens et.al., (1974) have ruled out the possibility of gas exchange.

There is little one can say about metabolite, rather than heat exchange in the rete, as there is no evidence for or against this possibility. The rete could be a method of dumping for instance, the end-products of white muscle anaerobic metabolism into the red muscle for continued oxidation. But is the advantage of such a mechanism significant enough to justify the fact that skipjack tuna have a major heat loss problem. The magnitude of this problem becomes obvious when one considers that a significant aspect of skipjack behavior is a migration to cooler waters as they increase in size. This migration is related to the problem of larger fish needing cooler waters in order to thermoregulate satisfactorily (Dizon and Brill, 1978; Neill et.al., 1976; Sharp pers. Comm.).

If the function of the rete is to heat the muscles (a point on which most workers agree), how is its function regulated and what is the advantage of warm muscles. The first question, despite much work, remains unanswered. Nothing associated with the rete itself, nervous connections, or shunts, have ever been found (I have used L.M. and scanning electron microscopy) which would offer a reasonable mechanism for blood passing from the dorsal aorta, through the red

muscle, and to the cardinal vein without going through the rete. The lateral circulation in the skipjack, although much reduced (Kishinouye, 1923), could offer an alternate route for the blood, but whether this ever happens, and how much of the circulation could be accommodated this way, is completely unknown. As for the second quesion, there have been several suggestions, the majority of which are untenable.

1. If the P50 of skipjack haemoglobins exhibited mammalian-like temperature sensitivity, the amount of oxygen unloaded at the muscle would be large compared to most teleosts. However, such a system would also be vulnerable to water temperature at the gills, and it would seem that as the effect of temperature on most tuna haemoglobins is slight (Sharp, 1974), the skipjack haemoglobins would be no different.

2. Walters (1962) suggests that heat transfer from the muscle to the immediate boundry layer of water may be sufficient to alter the kinematic velocity of a very thin boundary layer of water; this would in turn energize the boundary layer and thus prolong laminar flow. However, the temperature near the skin is low; also one would think that in this case the white muscle should be the major target for heat conservation as it is contiguous with the skin almost all over the body.

3. Stevens (1974) suggests that since the spacial gradient between ambient and muscle temperature is sharpest in the region of the exchanger, red muscle temperature could be continuously compared to water temperature (arterial blood) in this area and consequently small changes in water temperature

perceived. However, tunas as a group are no more responsive to abrubt temperature changes than are other fishes (Dizon et.al., 1974).

Approaching the problem in more general terms, Neill et.al., (1976) postulate that the heat exchanger simply provides the muscles with thermal inertia and thus protects them from rapid changes in temperature and from short-term exposure to extreme temperature. Graham (1973) and Carey et.al., (1971) are both of the opinion that the high swimming speed of the tunas is a consequence of the higher muscle temperatures. Despite the wide range of possibilities covered by these latter suggestions, and the difficulty of testing them, they do point out that a similarity may exist between tunas and endotherms in general i.e. They both exhibit relatively constant and higher than ambient body temperatures.

In metabolic terms, the advantages of a constant body temperature are readily explained. Most biochemical processses and structures underlying metabolism are dependent for their integrity upon the sequential formation (or breakage) of weak, non-covalent bonding (Low and Somero, 1976; Hochachka and Somero, 1973; Fersht, 1978; Holbrook et.al., 1975). The important point to emphasize, however, is that such weak bonds are differentially affected by temperature, which is why it is more difficult for organisms to cope with thermal change than with a given absolute (high or low) temperature. Biochemical adaptations to low temperature are by definition maladaptive at high temperatures and vice versa (Hochachka, 1974). To be sure, many organisms can remain active over large thermal ranges, but

this is usually done at significant cost in terms of design of enzymes and probably metabolism. Thus, as pointed out by Heinrich (1977), a good strategy for highly active organisms is the tailoring of enzymes for specific temperatures and the regulation of body temperature. But why choose temperatures higher than ambient?

One possibility is to allow higher activity rates at high temperatures. This is an unlikely selective force for several reasons. For example, small dipterans weighing 1 mg or less, can contract the thoracic muscles at over 300 times/sec at 10°C while the small size of these insects makes endothermy of more than 1°C above ambient, impossible (Sotavalta, 1947). Also, Crompton et.al., (1978) point out that the speed of muscular contraction seems to be limited by factors other than temperature and is proportional to (body weight)$^{-0.125}$ for structural reasons.

The explanation for the high 'set point' in endotherms put forward by Heinrich (1977) and Crompton et.al., (1978) centers on the need for controlled heat loss. If the body temperature is above ambient, heat loss can readily occur by mechanisms (such as conduction and radiation) not involving significant water loss. But if the body temperature is not above ambient, evaporative heat loss is the only effective way of holding down the body temperature. Particularly for small terrestrial organisms, this process would necessitate detrimentally large losses of water. Thus the set point in endotherms appears to be a compromise between the disadvantage of the metabolic costs of heating up to well above ambient and the advantage of having a

high body temperature to facilitate heat loss to the environment. Set points are thus related to the upper range of environmental temperatures, which is why some primitive mammals, such as the hedgehog, are active at night (at lower environmental temperatures) and have lower set points (Crcmpton et.al., 1978).

I propose by analogy that the skipjack is an endothermic teleost somewhat akin to the hedgehog in terms of thermal regulation. Since the tuna is continuously active, muscle metabolism serves as an excellent heat source, while the sea serves as an excellent heat sink. Thus, the only barrier to endothermy is a way to dampen water temperature fluctuations which would be transmitted through the gills to the musculature in 'normal' teleosts, and that is the role of the rete. The rete mirable in the tuna cancels about 70% of the heat loss at the gills (Neill et.al., 1976), an admittedly drastic adaptational step, but without which there evidently is no way of avoiding complete thermal equilibration at the gills (Stevens et.al., 1974). As in the hedhehog, a primitive mammalian homeotherm with about a 30°C body temperature (Crompton et.al., 1978), the balance between heat production and heat loss is set to maintain a body temperature 5-10°C above ambient. If body temperature were set at ambient, temperature regulation by contrclled heat loss would be more difficult. If body temperature were set much higher, the metabolic costs would become prohibitive; even with the current arrangment, the metabolic rate of the tuna is many fold higher than that of other teleosts, approaching that of mammals of

comparable body size (Dizon et.al., 1978). Because only a short burst of swimming can warm up tuna muscles (Chapter 4) and because the tuna is probably more active at sea than in captivity, I assume that the resting temperatures observed in captivity are never found normally. That is, at sea the muscle temperature of the skipjack is probably as stable as in primitive mammals (+ or - 3°C). Indeed, Carey and Lawson, (1973) found the temperature of free swimming bluefin tuna to be remarkably constant. Thus, the key combination of a constant and higher-than-ambient body temperature, typical of other endotherms, seems also to be well expressed by the tuna.

If this interpretation is correct, and the set point in skipjack is related to environmental temperature, it follows that skipjack may not be able to lose enough heat to tolerate water temperatures much above 26°C. This prediction seems to have been verified, as mentioned earlier in the discussion, by field observations (Dizon and Brill, 1978; Neill et.al., 1976; Sharp, pers. comm.).

APPENDIX I. THE ALPHA-GLYCEROPHOSPHATE CYCLE IN SKIPJACK WHITE MUSCLE

INTRODUCTION

During the aerobic metabolism of glucose or glycogen in a muscle cell, the co-enzyme NAD^+ is reduced to NADH and pyruvate is formed in the cytsolic compartment. The continued production, and oxidation of pyruvate, requires an equivalent oxidation of cytosolic NADH by the mitochondrial electron transport chain, which regenerates NAD^+. However, direct transfer of the reduced co-enzyme NADH to the respiratory chain is prevented by a selective permeability barrier across the inner mitochondrial membrane to NADH as well as other metabolic intermediates (Lehninger, 1951; Purvis and Lowenstein, 1961; Greville, 1969). There thus arises a requirement for "hydrogen shuttles" for the transfer of reducing equilavents from the cytosolic to the mitochondrial compartment. The two most commonly accepted hydrogen shuttles are the malate-aspartate cycle and the GP cycle (Williamson et.al., 1973) (Figure I-1). In skipjack white muscle, the enzyme components of the malate-aspartate cycle are present. GOT activities however, although somewhat higher than those of other white muscles (Chapter 4) are low compared to GOT activities in tissues such as the mammalian heart (Scrutton and Utter, 1968) in which the malate-aspartate cycle is known to be the main reduced pyridine nucleotide transport system (Williamson et.al., 1973; Safer, 1975). This observaton, coupled with:

1. ultrastructural evidence suggesting unusual aerobic capabilities of skipjack white muscle (Chapter 3).

2. evidence from metabolite levels suggesting that white muscle is capable of aerobic work.

3. unusually high activities of GPDH (the cytosolic arm of the GP cycle) in skipjack white muscle (Chapter 4), raises the possibility that skipjack white muscle respiration is closely linked to the GP cycle

There is evidence that suggests an important contribution of the GP cycle in white muscles of vertebrates (Crabtree and Newsholme, 1972; Blanchaer, 1964), but at the time these experiments were initiated, direct demonstrations of functioning GP cycles in vertebrate muscles were not available.

MATERIALS AND METHODS

Experimental animals and enzyme assays have already been mentioned in Chapter 2.

Preparation Of Mitochondria

Samples of muscle were taken either from quiescent or fatigued tuna. In the first instance, slowly cruising tuna were caught with a sharp dip of a net and quickly sacrificed. Steaks about 3/4" in thickness were cut from the mid-portion of the body. About 8-10 gms of muscle were removed, blotted, weighed, then minced. To the mince was added Chappell-Perry medium (Chappell and Perry, 1954) or TES medium (Hansford and Johnson, 1975), in a 1:1 ratio (w/v). The Chappell-Perry medium contained 0.1 M KCL, 0.05 M Tris-HCL buffer, pH 7.4, 0.001 M ATP, 0.005 M MgSO4, and 0.001 M EGTA. The TES medium contained 0.12 M KCL, 20 mM TES (N-Tris-(hydroxymethyl)-methtyl-2-amino-ethane sulfonic acid), pH 7.2 and 10 mM KPi, pH 7.2. The mince

was ground in a precooled mortar and pestle. The resultant tissue slurry was diluted to a final 1:3 or 1:4 dilution using more homogenization medium; then homogenized in a glass homogenizer (teflon plunger). The homogenate was spun for 15 minutes at 600 x g, and the supernatant solution was then decanted and recentrifuged at 12000 x g for 20 minutes. The 12000g pellet obtained was washed, recentrifuged, then taken up in running medium(containing 0.12 M KCL, 20 mM TES, pH 7.2, and 10 mM KPi, pH 7.2) for respiratory studies. The same procedure was used in making preparations from exhausted muscle, but in this case the animal was first run for 5-10 minutes on a hook and line to partially deplete its glycogen reserves in white muscle (Chapters 3 and 4).

Respiratory Measurements

Oxygen uptake rate by mitochondria was measured using a Clark-type oxygen electrode attached to a Radiometer pO2 meter, with the radiometer output being followed on a mV Bausch and Lomb chart recorder. The incubation chamber was 2.5 ml in volume; its temperature was regulated with a water-jacket connected to a Lauda constant temperature bath and circulator (Brinkman Instruments, N.Y.). Hamilton syringes (10,50 and 100 uL) were used for the addition of metabolites, inhibitors, ions, and cofactors to the incubation medium without interruption of continuous monitoring of oxygen consumption.

Spectrophotometric Studies Of Mitochondria

Mitochondria, prepared as described above, were used in attempts to reconstruct the GP cycle. GP cycling in these experiments was monitered by following the change in OD (340) in a Unicam SP 1800 ultraviolet recording spectrophotometer at 25°C using 1 ml cuvettes and 1 cm light path.

RESULTS AND DISCUSSION

Isolated White Muscle Mitochondria

Skipjack white muscle mitochondria are rather difficult to study because of two possibly related problems. Firstly, using standard techniques for vertebrate muscle (Hansford and Johnson, 1975), it was not possible to get good coupling in skipjack muscle mitochondrial preparations. This difficulty presumably stemmed from some unknown metabolite, ion, or osmotic requirement. The second problem arose from the presence of rather massive glycogen reserves in skipjack white muscle (Chapters 3 and 4). In white muscle excised from quiescent animals, a large amount of glycogen was spun down during centrifugation and contaminated the mitochondrial pellet. In white muscle excised from animals that had swum to fatigue, much of the glycogen was depleted and hence its contamination of subsequent mitochondrial preparations was reduced. Of particular importance however, is that in such instances, the glycogen granules carry with them a number of enzymes

(Heilmeyer et.al., 1970), including, in the case of the skipjack white muscle, cytosolic GPDH and LDH. A close quantification of this effect was not attempted, but in a typical mitochondrial preparation from quiescent skipjack, a 1:10 dilution contained about 20 U/ml of GPDH and about 400 U/ml of LDH. In mitochondrial preparations made from muscle whose glycogen reserves had been at least partially depleted by strenuous swimming, the amounts of GPDH and LDH precipitating out with the glycogen were reduced to about 1/3 of the above values, due to the decreased amount of precipitating glycogen.

As is noted in Table I-1, GP by itself could serve as an acceptable substrate for skipjack white muscle mitochondria, increasing respiration over endogenous rates by about 6-fold. NADH alone causes a two-fold increase in oxygen uptake (demonstrating leakyness of the mitochondria), pyruvate and malate added together cause an oxygen uptake which is 2/3 that with GP as the substrate and the addition of succinate, which like GP donates its electrons to FAD, causes a 6-fold rise in oxygen uptake. These results demonstrate that at least one half of the GP cycle is operative in skipjack white muscle i.e. GP oxidase must be present in skipjack white muscle mitochondria and thus GP is oxidised, FADH is produced and respiration is stimulated. The relative levels of oxygen uptake with GP vs malate-pyruvate as a substrate are similar to those in mammalian white muscle mitochondria (Blanchaer, 1964) and insect flight muscle mitochondria (Sacktor, 1976).

Reconstruction Of The GP Cycle

Skipjack white muscle mitochondria were isolated as before and the pellet was taken up in a minimal volume of running medium, pH 7.2. A small volume (200 uL) of the mitochondrial preparation was added to 1 ml of running medium containing 0.1 mM NADH. In such preparations made from muscle of quiescent tuna, each cuvette would contain about 20 units of endogenous GPDH; in preparations from fatigued muscle, there would be about 7-10 units in each cuvette. Addition of GP to these mitochondria would then lead to NADH oxidation, which was followed at 340 nm. This technique was sensitive enough to allow a demonstration of the dependence of NADH oxidation on GP availability, to allow in effect the construction of GP saturation curves (Figure I-2) and the calculation of an apparent Michaelix constant for GP(about 0.4 mM). There is only one interpretation that can explain these data; it assumes that the added GP is oxidized by the mitochondria to DHAP, which then serves as the substrate for cytoplasmic GPDH, NADH being oxidized as a consequence. This experiment is thus a reconstruction of the GP cycle (Figure I-1). If this interpretation is correct, it leads to another important prediction: namely, that under such conditions, since the cycle only uses catalytic amounts of substrates, DHAP and GP should be at, or close to, some sort of steady state. On the other hand, NADH should be, and was, demonstrably depleted; once the change in E 340 was reduced to zero, addition of more NADH led to further oxidation and thus to reactivated GP cycling. In one experiment, 3-4 such rounds of NADH oxidation could be readily

demcnstrated, with no further addition of GP to the preparation.

The control properties of this reconstructed GP cycle were not fully characterized, but several significant observations were noted.

1. Addition of NAD^+ to the preparation caused a strong inhibition of respiration, presumably by a competitive block at the GPDH step.

2. ADP definitely activated the GP cycle at all levels of GP tested, saturating and non-saturating (Figure I-2), but as explanations of the results, these experiments could not distinguish between (a) a direct activation of GP oxidase, or (b) a simple coupling effect of ADP on phosphorylation and respiration.

3. The effects of GP concentration on the reconstructed GP cycle should be mentioned. Skipjack white muscle GPDH is unusually sensitive to GP (Chapter 6), thus high concentrations of GP should inhibit at least the cytosolic arm of the GP cycle. However, GP at up to 25 mM levels, did not inhibit NADH oxidation (Figure I-2). This suggests that the GP oxidase supplies the rate-limiting step in the aerobic process, although I am aware that this conclusion is not supported by (1.) above. A similar conclusion arises from measures of the relative activities of cytosolic GPDH and mitochondrial GP oxidase in various animals; the former activity is at least 3 times higher than the latter (Crabtree and Newsholme, 1972). The function of GP inhibition of cyosolic GPDH, therefore appears to take on importance only under anaerobic conditions,

when GP oxidase and electron transfer are blocked by an oxygen lack, and GPDH is inhibited so as to avoid a drain of carbon from mainline glycolysis to GP (Chapter 6).

From the above data, it is tentatively concluded that redox balance during white muscle respiration in skipjack is conserved by a GP cycle. This conclusion raises two important points:

1. Why would vertebrate white muscle, insect flight muscle and squid mantle muscle depend on a GP cycle instead of a malate-aspartate cycle as in mammalian heart (Williamson et.al., 1973) and vice versa?

2. Why are all the known NAD^+ regenerating systems (which include the malate-aspartate cycle, the GP cycle, the fatty acid cycle and the lactate pyruvate cycle) based on the same design i.e. a shuttle of hydrogen across the mitochondrial membrane with a resultant production of NAD^+ in the cytosol?

The second question is somewhat out of place here and is considered in detail in Appendix II. The first question needs clarification before it is considered. Is there a mutually exclusive separation of the malate-aspartate cycle and the GP cycle between the mammalian heart (for example) on one hand, and some vertebrate white muscles, insect flight muscle and squid mantle muscle on the other? Vertebrate white muscle displays higher activities of GPDH than GOT, whereas in mammalian heart, activities show the opposite trend (Opie and Newsholme, 1967; Crabtree and Newsholme, 1972; Scrutton and Utter, 1968). In Simplectoteuthis mantle, GPDH activities are 10-fold higher than GOT activities and in the blowfly flight

muscle and skipjack white muscle, oxygen uptake by mitochondria is greatest when GP is the substrate (Table I-1; Hochachka et.al., 1975; Sacktor, 1976). The evidence thus suggests that there is simply a predominance of the GP cycle system over the other in tissues which utilize carbohydrate as the primary carbon and energy source. However, tissues usually have the components of both systems and it has been shown that more than one cycle can be operative in one tissue (Williamson et.al., 1973; Cederbaum et.al., 1973).

What are the considerations when choosing one system over any other? In terms of energy yield, for each spin of the GP cycle (or, per mole of G6P converted to pyruvate), 7 moles of ATP are netted, 4 moles through the GP mediated transfer of reducing equivalents to oxygen, 3 moles through substrate phosphorylations in glycolysis. However, the overall aerobic yield is 36 moles ATP/mole G6P, a value slightly lower than the 38 moles obtained (by the mammalian heart for example) where the malate-aspartate cycle is used. Of these 36 moles of ATP, the formation of only 4 moles (i.e. Only 1/9th the energy yield) is obligatorily linked to the GP cycle. In these terms, therefore, the GP cycle does not seem to represent an unusually advantageous, or for that matter, disadvantageous, system. Another difference is that the mitochondrial component of the GP cycle uses FAD as its co-enzyme, rather than NAD as in the malate-aspartate cycle (Figure I-1; Appendix II). Thus if NAD were ever limiting in the mitochondria, selection for an GP cycle, rather than a malate-aspartate cycle, might ensue. Other considerations are that the malate-aspartate cycle may be more

readily reversable than the GP cycle simply because of the positive standard free energy change involved with reversing both GPDH and GP oxidase (Appendix II). Thus the malate-aspartate cycle may be useful in supplying cytoplasmic NADH for gluconeogenesis. The tissue location of the latter however, cerainly does not suggest this (the heart uses almost exclusively blood glucose as its carbohydrate energy source (Safer, 1975) and also Safer (1975) points out that the aspartate-glutamate exchanger in the malate-aspartate cycle is essentially unidirectional. The fact that the presence of an GP cycle correlates with a respiratory system based on carbohydrate, suggests that a functional link exists between the two, but its exact nature is as yet undefined.

Table I-1. Relative respiration rate of isolated white muscle mitochondra. 0.12 M KCL, TES running medium, pH 7.2, 30°C. Mitochondria isolated as described in Materials and Methods. A slow endogenous rate of O2 uptake is arbitrarily set at 100.

Conditions	Relative Respiration Rate
Endogenous	100
1 mM NADH	200
2 mM GP	610
1 mM pyruvate + 0.05 malate	400
10 mM succinate	600
2 mM GP + 1 mM NADH + 1 mM NAD^+	530

Figure I-1. Transport of reducing equivalents between cytosol and mitochondria: the GP cycle and the malate-aspartate cycle.

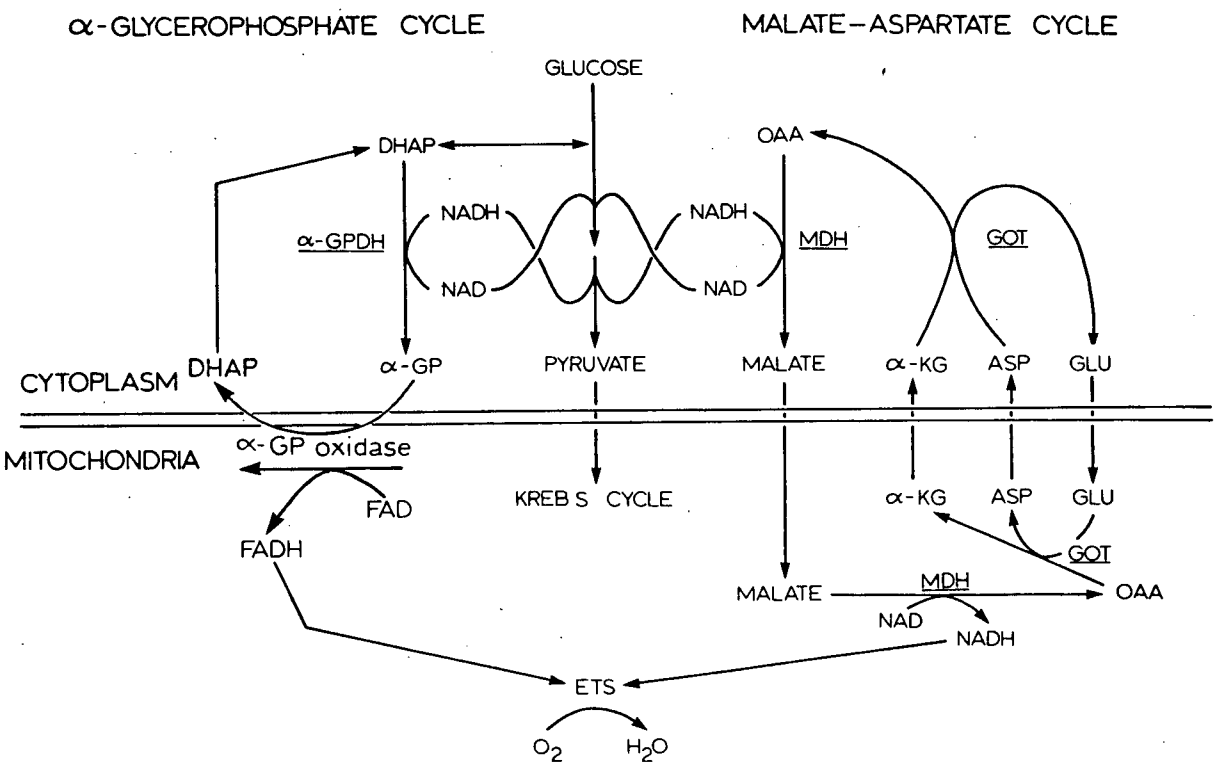
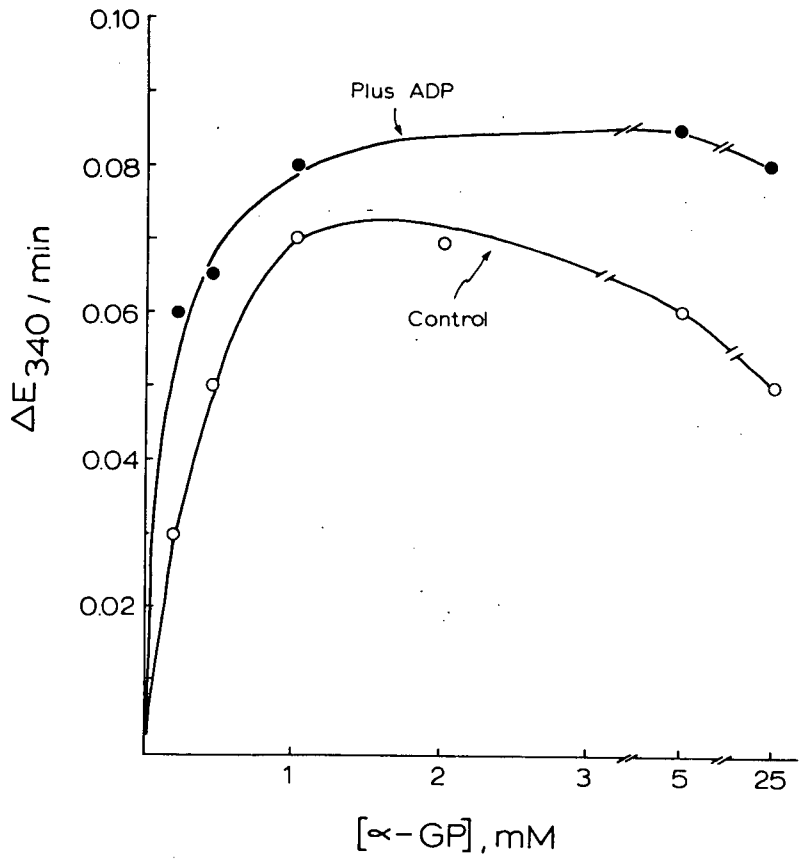

α-GLYCEROPHOSPHATE CYCLE
MALATE–ASPARTATE CYCLE
GLUCOSE
DHAP
OAA
α-GPDH
NADH
NAD
NADH
NAD
MDH
GOT
CYTOPLASM
DHAP
α-GP
PYRUVATE
MALATE
α-KG
ASP
GLU
MITOCHONDRIA
α-GP oxidase
FAD
FADH
KREB S CYCLE
α-KG
ASP
GLU
GOT
MALATE
MDH
OAA
NAD
NADH
ETS
O_2
H_2O

Figure I-2. Reconstructed GP cycle in isolated mitochondria from skipjack white muscle. In each experiment, 200 uL of freshly isolated white muscle mitochondria were added to a total volume of 1 ml of the running medium (see Materials and Methods), pH 7.2, 25°C, containing 0.1 mM NADH. The effect of increasing availability of GP was assessed in each experiment after subtracting a low "control" rate of endogenous NADH oxidation by the mitochondrial preparations. Control curve (open circles) refers to the effect of GP alone; the effect of including 1 mM MgADP is shown in the marked curve (dark circles).


0.10
0.08
0.06
0.04
0.02
ΔE 340 / min
Plus ADP
Control
1
2
3
5
25
[∝-GP], mM

APPENDIX II. THE IMPORTANCE OF WATER AND OXYGEN IN THE EVOLUTION OF HYDROGEN SHUTTLE MECHANISMS

INTRODUCTION

Primary metabolic pathways show little variation through the various biological kingdoms. Anaerobic energy production is accomplished by anaerobic glycolysis with lactate or ethanol as accumulating end-products (Rovetto et.al., ,1973; Edington et.al., 1973). In some cases, anaerobic energy production involves portions of the Krebs cycle and metabolites such as succinate accumulate (de Zwaan and Zandee, 1972). Aerobically, ATP can be produced in the mitochondria by the oxidation of fatty acids, glucose or glycogen, but lipid oxidation is the preferred aerobic pathway in most muscles which are called upon to provide a long term steady-state power output (Bilinski, 1974; Baldwin et.al., 1973). Whatever the pathway involved, energy production in the cell involves a partial or complete oxidation of substrate. Therefore, the design of an energy producing pathway must take into account a hydrogen acceptor which must (1) accumulate as long as substrate is being oxidized, and (2) always be available to accept electrons if substrate oxidation is to continue. This brings us to the role of hydrogen shuttles in the aerobic metabolism of carbohydrate.

Carbohydrate metabolism can assume a major role in aerobic ATP production. It is the only source of energy in some insect flight muscles (Sacktor, 1976), and it assumes prime importance in aerobic metabolism in squid mantle muscle (Storey and Hochachka, 1975). Mammalian brain, an obligately aerobic tissue which can account for up to 20% of the total oxygen used in the body, is strictly a glucose-burner (Dunn and Bondy, 1974), and

glucose is metabolised aerobically in the rat heart (Safer and Williamson, 1973). In the glycolytic sequence, glyceraldehyde-3-phosphate dehydrogenase reduces NAD. Under anaerobic conditions, NAD is regenerated by the LDH reaction, redox balance is maintained and lactate accumulates. Under aerobic corditions, pyruvate is no longer reduced to lactate since it enters the mitochondrion for further oxidation. Regeneration of NAD, however, is still necessary for the continuation of glycolysis. Since the inner mitochondrial membrane is generally considered to be impermeable to pyridine nucleotides (Greville, 1969, Lehninger, 1951; Lowenstein, 1961) cytoplasmic NADH cannot be oxidized directly by the mitochondrial electron transport system. Thus, with respect to redox balance, aerobic carbohydrate metabolism would seem to present somewhat of a problem.

THE SHUTTLES

Four mechanisms have been demonstrated by which the required NAD can be produced under aerobic conditions; these are described in detail below. The four mechanisms are the GP cycle, the malate aspartate cycle, the fatty acid cycle and the lactate pyruvate cycle (Williamson et.al., ,1973; Sacktor, 1976; Klingenberg, 1970; Grunnet, 1970; Storey and Kayne, 1977). These four cycles are similar in that in all cases reducing equivalents from the NADH produced by GAPDH are donated to a hydrogen carrier which then passes these equivalents to an intra-mitochondrial carrier which is finally oxidised by the electron transport system (Figures II-1,2,3).

The cytosolic hydrogen carriers of the malate-aspartate, fatty acid and lactate pyruvate shuttles actually cross the inner mitochondrial membrane (Safer, 1975; Whereat et.al., ,1969; Cederbaum et.al., ,1973; Storey and Kayne, 1977). GP however, the cytosolic hydrogen carrier in the GP cycle, cannot cross the inner mitochondrial membrane and GP oxidase is located on the outside of the inner membrane and transfers reducing equivalents from GP, across the inner membrane, to FAD (Klingenberg, 1970).

The NAD/NADH system is more reduced in the mitochondria (Williamson et.al., 1971) necessitating the NAD-linked shuttles to work against a gradient. So far only in one of the shuttles, has an energy driven step been found which could account for the "uphill" nature of these shuttles. This is the glutamate - aspartate exchanger in the malate-aspartate cycle, which is located in the inner membrane. This exchanger apparently takes advantage of the inward proton gradient (Safer, 1975) and thus could be the energy input into the malate aspartate shuttle . In the other shuttle mechanisms, no such energy driven step has been described, although the gradient will not be as inhibitory in the case of the GP cycle, as a flavin is the intramitochondrial hydrogen carrier rather than a pyridine nucleotide.

The malate aspartate cycle (considering the cycle as a reaction) exhibits identical standard free energy changes for the forward and reverse directions, as the cytosolic and mitochondrial enzyme components are identical. Thus this system has the potential of running backwards and possibly supplying

reducing equivalents for cytoplasmic gluconeogenesis, although the glutamate-aspartate exchange mechanism is said to be difficult to reverse (Safer, 1975). The GP and fatty acid cycles are more energetically favourable in the forward direction because of the mitochondrial flavin involvement (the GP oxidase reaction has a standard free energy change of -8 Kcal/mole in the oxidizing direction) and thus would need impossibly large concentrations of substrates in order to force the cycle backwards. The lactate-pyruvate shuttle is not really a cycle at all as the pyruvate formed in the mitochondria is probably oxidized in the mitochondria instead of being recycled. This shuttle, however, if disconnected from the Krebs cycle, could possibly run backwards and supply reducing equivalents to the cytoplasm (Figure II-3).

The operation of the GP cycle itself, produces 4 moles of ATP/mole G6P, the lactate pyruvate and malate aspartate cycles produce 6 and the fatty acid cycle produces 10.

DISCUSSION

Having described these shuttle mechanisms in detail, it is clear that they accomplish the second of the two design features mentioned in the introduction (i. e. they provide a continued supply of electron acceptor). However, far simpler mechanisms, which do not involve the mitochondria, but which accomplish the same end, come to mind.

1. Cytoplasmic NADH oxidase (Figure II-4). This system, although energetically unfavourable, could produce NAD for glycolysis and would have no mitochondrial components.

Membrane-bound NADH oxidase has been found in procaryotes (Junks and Matz, 1976) and NADH oxidation outside the permeability barrier of the mitochondria has been demonstrated in pidgeon heart (Rassmussen, 1969). There is no evidence that these oxidations involve cytochromes, oxygen or water, and the question of physiological significance is as yet unclear.

2. A cytoplasmic dehydrogenase system (Figure II-5 a and b). Such a system would also accomplish the goal of a complicated hydrogen shuttle system i.e. supplying NAD for glycolysis. However, again, such a system has never been described for an aerobic system and presumably does not exist. Why is it that such complicated systems have been selected over relatively simple ones which could accomplish the same end?

This is where the incorporation of the first design feature (see introduction) is realized. Aerobic work (carbohydrate or fat based) is a steady-state, long term process, which completely oxidizes the substrate (in this case, carbohydrate), to CO2 and H2O. It is terminated by a reaction which disposes of hydrogen, the last remaining element of the substrate. The substrate pool for this terminal reaction must be at least as large as the initial substrate pool (i.e. glucose or glycogen) if it is not to limit the whole process. Correspondingly, the final concentration of the end-product of the terminal reaction will at least equal the decrease in the concentration of the initial carbohydrate pool which occurs during the work phase. It is only because of cytochrome oxidase that aerobic metabolism is not considerably burdened by the substrates and products of its own terminal reaction. The co-

substrate (electrons and protons are the other) for cytochrome oxidase is oxygen, an ideal electron acceptor, which is available during steady-state aerobic metabolism in quantities which are inexhaustible. Oxygen is dissolved in the cell fluids, but the real oxygen pool is the atmosphere, an infinitely large pool, which is in equilibrium with the cell fluids during aerobic metabolism. So the problem of substrate pool size does not exist. The end-product of the reaction is water, the only "metabolite" which can accumulate without significantly affecting the already 40 M concentration in the cell. The qualities of the aerobic terminal reaction are perhaps more obvious if one examines the corresponding anaerobic reaction, LDH. This enzyme reaction (somewhat akin to the system proposed in Figure II-4) accumulates lactate, a potentially noxious metabolite, and anaerobic glycolysis is characteristically a short-term process which is probably limited by lactate build-up (Hulten et.al., 1975).

With these stratigies in mind, the disadvantages of the cytoplasmic aerobic, NAD regeneration mechanisms are very clear. Both processes would result in deleterious end-product accumulation; hydrogen gas in the case of NADH oxidase (Figure II-4) and metabolite "b" or "d" in the case of the cytplasmic dehydrogenase system. Also in the case of the latter, pool quantities of substrate "a" or "c" would be neccessary (Figure II-5 a and b). Hydrogen gas and the substrates and products of the cytoplamic dehydrogenase system could not be as innocuous as oxygen and water. So, these cytoplasmic mechanisms would balance redox, but would limit and detract from the long-term

nature of mainline aerobic metabolism. Their absence in nature is therefore hardly surprising.

The cytoplasmic redox balancing mechanisms have been discarded for what are now apparent reasons, and the nature of the aerobic metabolic process has been conserved. Nature has characteristically taken advantage of the suitability of oxygen and water as substrate and product of the terminal step of a process involved in long-term, steady state aerobic work. The result is that NAD is regenerated during aerobic carbohydrate metabolism by a mechanism with a design which ensures redox balance and yet places no burden on the efficiency of the aerobic process. Since a regeneration system involving a mitochondrial component produces extra reduced coenzyme in the mitochondria (Figures II-1,2 and 3), another "advantage" of these processes is the increased amount of ATP produced per mole of carbohydrate. This added ATP amounts to between 11 and 20% per mole of glycogen depending on the shuttle mechanism involved. The relative importance of this additional ATP is impossible to assess; it could definitely be an advantage, but a terminal reaction with an innocuous end-product and a limitless supply of substrate is a necessity.

Figure II-1. Transport of reducing equivalents between cytosol and mitochondria: the GP cycle and malate-aspartate cycle.

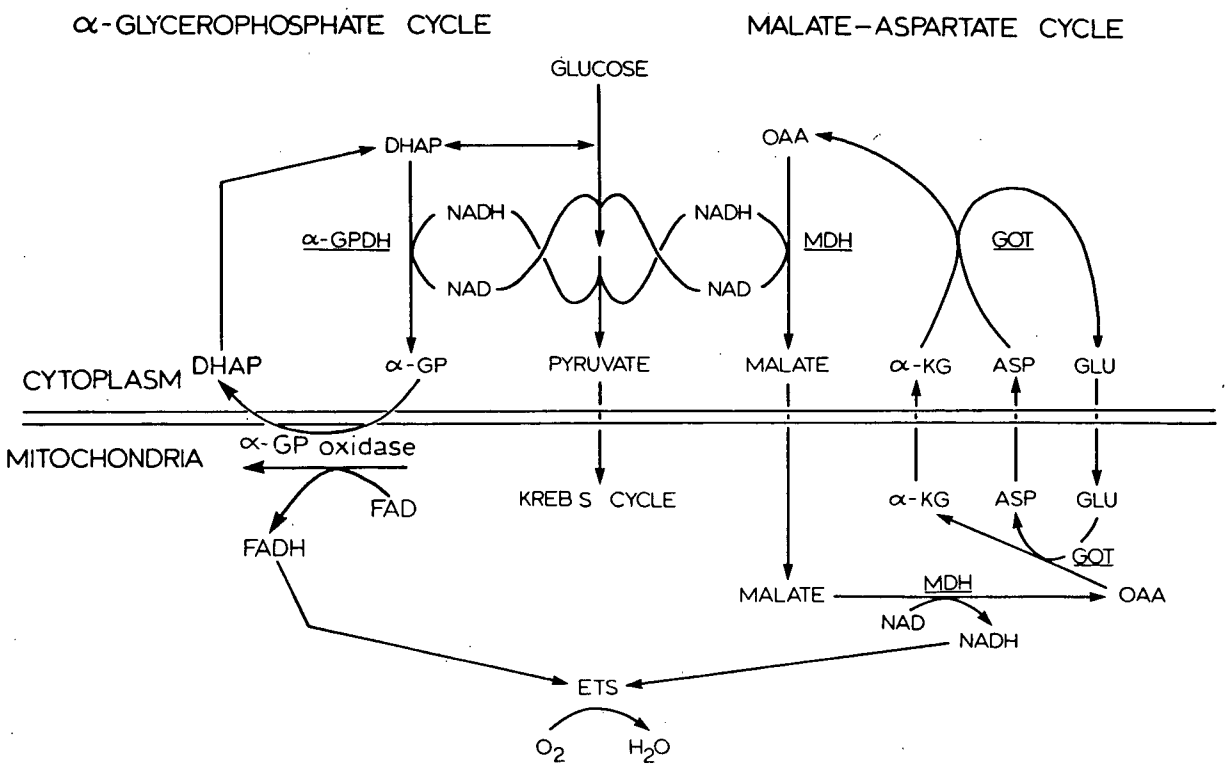

α-GLYCEROPHOSPHATE CYCLE
MALATE–ASPARTATE CYCLE
GLUCOSE
DHAP
OAA
α-GPDH
NADH
NAD
NADH
NAD
MDH
GOT
CYTOPLASM
DHAP
α-GP
PYRUVATE
MALATE
α-KG
ASP
GLU
MITOCHONDRIA
α-GP oxidase
FAD
FADH
KREB S CYCLE
α-KG
ASP
GLU
GOT
MALATE
MDH
OAA
NAD
NADH
ETS
O_2
H_2O

Figure II-2. Transport of reducing equivalents between cytosol and mitochondria: the fatty acid shuttle

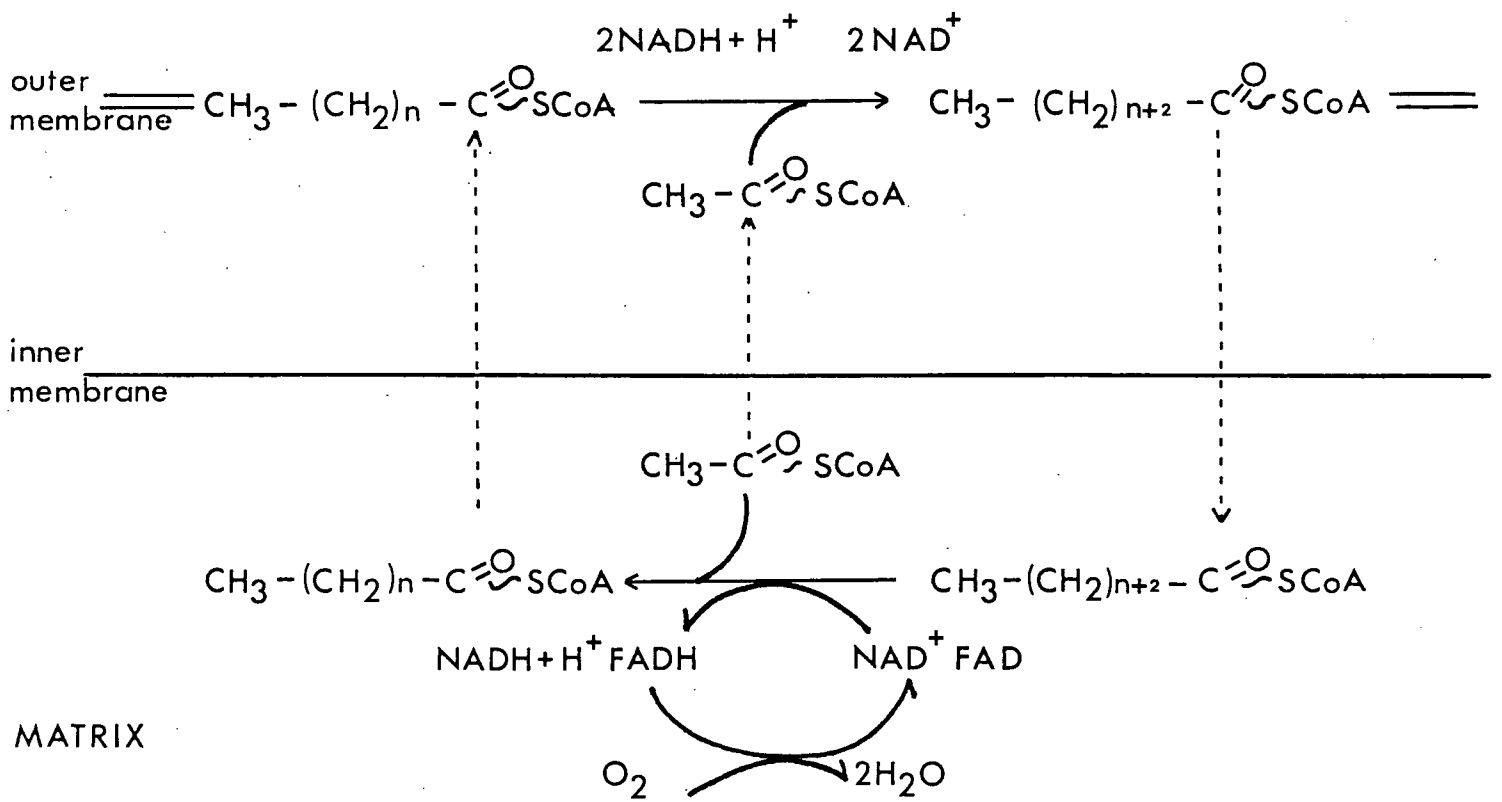

CYTOPLASM
2NADH + H+
2NAD+
outer membrane
CH3 - (CH2)n - C(=O)SCoA
CH3 - (CH2)n+2 - C(=O)SCoA
CH3 - C(=O)SCoA
inner membrane
CH3 - C(=O)SCoA
CH3 - (CH2)n - C(=O)SCoA
CH3 - (CH2)n+2 - C(=O)SCoA
NADH + H+ FADH
NAD+ FAD
MATRIX
O2
2H2O

Figure II-3. Transport of reducing equivalents between cytosol and mitochondria: the lactate-pyruvate cycle.

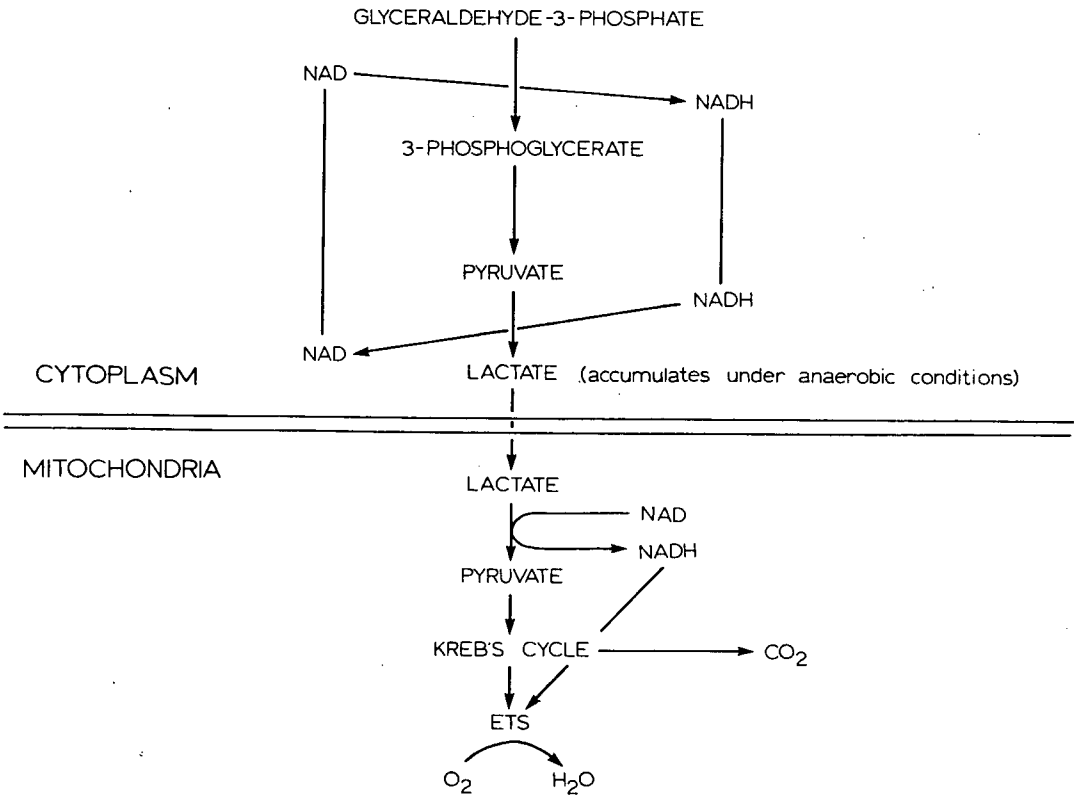

GLYCERALDEHYDE-3-PHOSPHATE
NAD
NADH
3-PHOSPHOGLYCERATE
PYRUVATE
NADH
NAD
CYTOPLASM
LACTATE (accumulates under anaerobic conditions)
MITOCHONDRIA
LACTATE
NAD
NADH
PYRUVATE
KREB'S CYCLE
CO_2
ETS
O_2
H_2O

Figure II-4. Mechanisms for the generation of NAD^+ from NADH in the cytoplasm: cytoplasmic NADH oxidase.

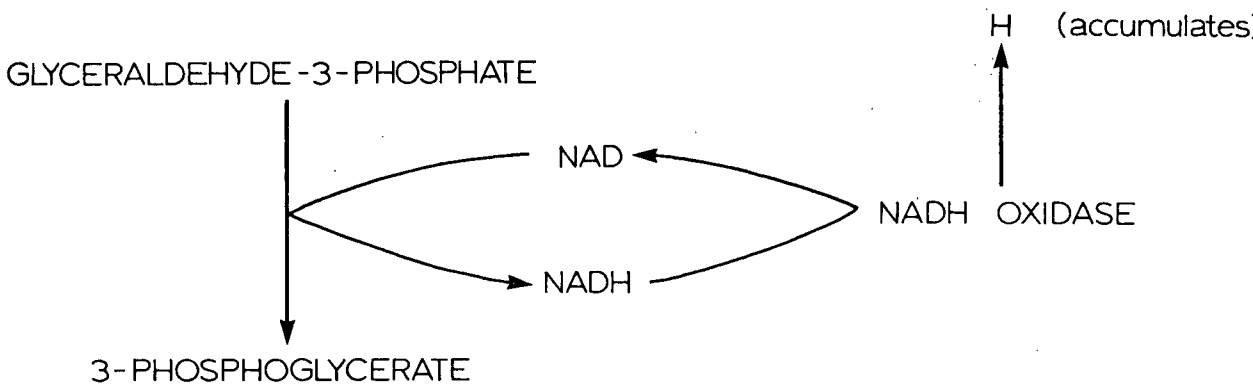
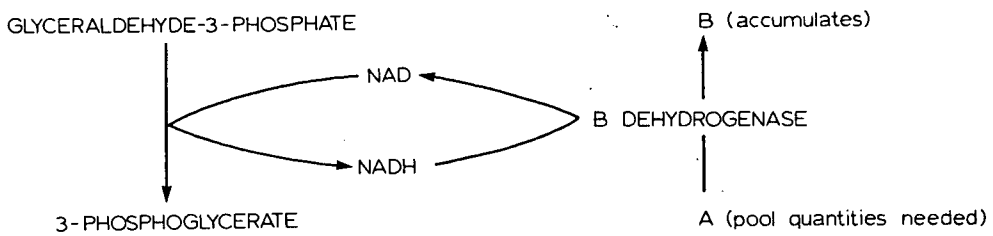
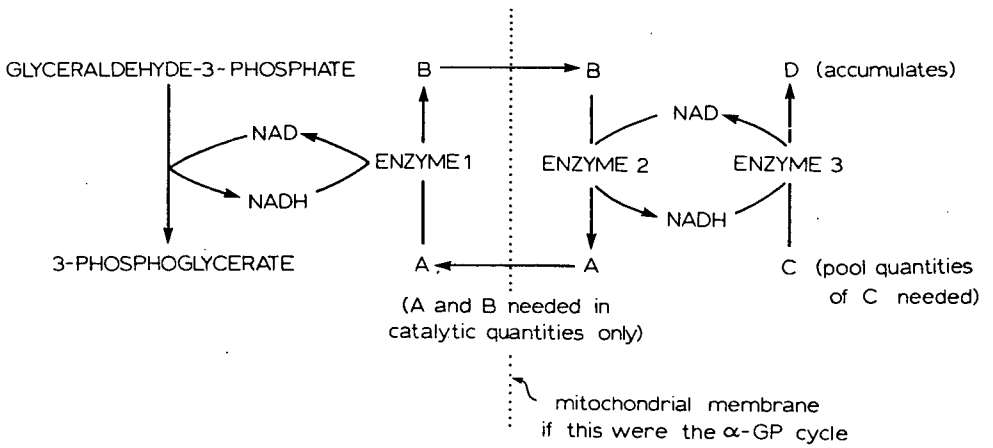

H (accumulates)
GLYCERALDEHYDE-3-PHOSPHATE
NAD
NADH OXIDASE
NADH
3-PHOSPHOGLYCERATE

Figure II-5, a and b. Mechanisms for the generation of NAD^+ from NADH in the cytoplasm: a cytoplasmic dehydrogenase.


GLYCERALDEHYDE-3-PHOSPHATE
NAD
NADH
3-PHOSPHOGLYCERATE
B (accumulates)
B DEHYDROGENASE
A (pool quantities needed)


GLYCERALDEHYDE-3-PHOSPHATE
NAD
NADH
3-PHOSPHOGLYCERATE
B
ENZYME 1
A
B
ENZYME 2
A
NAD
NADH
D (accumulates)
ENZYME 3
C (pool quantities of C needed)
(A and B needed in catalytic quantities only)
mitochondrial membrane if this were the α-GP cycle

APPENDIX III PYRUVATE KINASE FUNCTIONS IN HOT AND COLD ORGANS OF TUNA

INTRODUCTION

Because different major tissues function at significantly different temperatures in the skipjack (Chapter 1), this animal offers the unique opportunity for studying within a single organism, the influence of differential temperatures on controlled enzyme function. I chose to examine in this context, the catalytic and regulatory properties of pyruvate kinase in red muscle (highest cell temperatures) and in the heart (ambient cell temperatures).

Pyruvate kinase (E.C. 2.7.1.40) is an enzyme whose physiological functions are well understood. It occurs in mammals in two primary (L and M) forms. The L-type, or regulatory pyruvate kinase, has a low affinity for PEP, is strongly activated by FDP, and is under inhibitory control by ATP, alanine, and phenylalanine; PEP saturation curves are strongly sigmoidal. The M-type, or non-regulatory enzyme, has a high affinity for PEP and is largely refractory to modulation by ATP, alanine, phenylalanine, and FDP. Moreover, substrate saturation curves are Michaelis-Menten hyperbolas (Tanaka et.al., 1967; Leveille, 1968; Van Berkel, 1974; Seubert and Schonen, 1971).

In fishes, the situation seems more complicated. In the majority of cases, evidence suggests that fish muscle pyruvate kinase, although L-type in some respects, displays hyperbolic PEP saturation curves. These pyruvate kinases are sensitive to modulation by ATP and FDP, but the degree of sensitivity is low. Rat liver pyruvate kinase can be activated 10-fold by FDP and inhibited 90% by 5 mM ATP (Van Berkel et.al., 1972; Tanaka

et.al., 1967) whereas the respective figures for fish pyruvate kinases are 1.5-2.5-fold and 20-50% (Somero and Hochachka, 1968; Mustafa et.al., 1971; Johnston, 1975). The Km (PEP) of the fish enzyme is in the range of 0.1-0.4 mM which is intermediate between the PEP affinities shown by the L- and M-type mammalian pyruvate kinases, and the affinities for ADP appear to be slightly lower in fish pyruvate kinases than in the mammalian forms (Van Berkel, 1974; Johnston, 1975; Mustafa et.al., 1971).

Temperature studies on pyruvate kinase have been almost totally concerned with enzyme-PEP interactions in fish and invertebrate enzymes although there are some data on activation energies for the reaction (Low and Somero, 1976). In the organisms thus far studied, the Km(PEP) responds in one of three ways to temperature. Firstly, and most usually, it rises with temperature, but it also can be temperature independent and in one case actually falls with temperature (Somero, 1975; Hoffman, 1976). For the pyruvate kinase reaction, structural volumes of activation appear to be correlated with the adaptation temperature of the protein (Low and Somero, 1975) and catalytic efficiencies are related to cell temperatures (Low and Somero, 1976). There is no relatioinship, however, between the activation enthalpy and the physiological temperature range for a variety of pyruvate kinases from vertebrates and invertebrates (Low and Somero, 1976; Hoffman, 1976). Different pyruvate kinases are sensitive to metabolite effects at a variety of physiological temperatures, but no studies have been done on the effect of different temperatures

on the metabolite sensitivity of a single pyruvate kinase.

In the skipjack tuna, the heart and muscle pyruvate kinases occur in tissue-specific isozymic forms. The two enzymes differ in terms of the response of the Km(PEP) to temperature, but are similar in that both are more sensitive to metabolite regulation as temperature increases. Both enzymes are relatively insensitive to modulators which is a characteristic typical of fish muscle pyruvate kinases.

MATERIALS AND METHODS

Fish Samples

Fresh and frozen fish samples were obtained as is described in the main text (Chapter 2).

Preparation Of Pyruvate Kinase

Preparation of pyruvate kinase for specific activities has already been described (Chapter 2).

For electrophoretic purposes, frozen tissue samples were (a) homogenized in a minimal amount of 10 mM sodium phosphate buffer, 2 mM EDTA, pH 7.0, centrifuged at 12,000g for 20 minutes, (b) then concentrated by precipitation at 80% ammonium sulphate, and (c) resuspended in buffer. Before use, the muscle ammonium sulphate preparations were dialysed for one hour, with one change, against 1L of 50 mM sodium phosphate buffer, pH 5.7. The heart ammonium sulphate preparations were dialysed as above against Tris buffer (5.8g NaCl/L) 0.5 M, pH 9.0.

For kinetic studies the tissues were homogenized in 5 volumes of 10 mM sodium phosphate buffer, 2 mM EDTA, pH 7.0 (pH 6.5 for the heart enzyme), and then concentrated with 80% ammonium sulphate. The muscle fraction was dialysed twice against 1L of 50 mM sodium phosphate buffer, 50 mM KCl, pH 6.5, and then applied to a 2.5 x 40 cm cellulose phosphate column equilibrated with the dialysis buffer. A 50-400 mM KCl gradient

was then run through the column and the peak fractions collected and again concentrated with 80% ammonium sulphate. The precipitate was resuspended, then dialysed twice against 1L of 50 mM sodium phosphate buffer, 50 mM KCl, pH 6.0, and applied to a 2.4 x 40 cm SE Sephadex column equilibrated to the dialysis buffer. A 50-400 mM KCl gradient was then run through the column, the peak fractions were collected, concentrated with ammonium sulphate and stored at 0°C. Purification was between 40- and 50-fold. The procedure for the heart enzyme was essentially the same. The first column was a SE Sephadex equilibrated with 20 mM sodium phosphate buffer, pH 6.5, and the gradient was 20-600 mM potassium phosphate. The second column was cellulose phosphate equilibrated with 50 mM sodium phosphate buffer, 50 mM KCl, pH 6.0, and the gradient was 50-400 mM KCl. The purification factor for the heart enzyme was about 90-fold. By two criteria (total activity and Km(PEP)), the purified enzymes were stable at 0°C for at least one month. Before use, aliquots were dialysed, with one change, for one hour, against one litre of the assay buffer.

Assays

Pyruvate kinase activity was monitored in 1 ml cuvettes (1 cm light path) using a Unicam SP 1800 recording spectrophotometer. The reaction cuvettes were held in cell holders thermally equilibrated with a constant temperature bath and circulator. The pyruvate kinase reaction was linked to the LDH reaction and the rate was determined by the decrease in absorbance of NADH at 340 nm.

The assays for specific activites have already been described in Chapter 2. The assays for the kinetic data were done in 50 mM imidazole buffer using 0.15 mM NADH, 15 mM MgSO4; 100 mM KCl. All other concentrations are given in the text. Values for the Michaelis-Menten constants (Km) were determined by double-reciprocal plots of 1/velocity versus 1/substrate concentration. Inhibition constants (Ki) for ATP were obtained from Dixon plots of 1/velocity versus inhibitor concentration at varying substrate concentrations. Values obtained were reproducible within 15%.

Electrophoresis

Electrophoresis was done on 13% starch gels. The electrode buffer used was the dialysis buffer (see preparation of pyruvate kinase); the gel buffer was a 1:20 dilution of the electrode buffer. The gels were run for three hours at 50 mA and 400 V. To detect activity, the gels were overlaid with filter paper sturated with 0.15 mM NADH, 20 mM MgSO4, 100 mM KCl, 0.2 mM PEP, 0.5 mM ADP, 0.5 I.U. LDH, and 50 mM imidazole buffer, pH 6.5. Glucose (20 mM) and 5 I.U. Hexokinase were added to control ATP levels and to regenerate ADP. The presence of activity was determined under U.V. light; NADH absorbs at 340 nm and appears yellow, NAD^+ transmits the light and appears purple (Susor and Rutter, 1971).

RESULTS

Electrophoresis

Starch gel electrophoresis of muscle extracts displays one band of pyruvate kinase which migrates toward the cathode at pH 5.7. There are two bands, one major and one minor, in the heart of the skipjack; both migrate toward the anode at pH 9.0 and are clearly separable from the muscle form of pyruvate kinase. It was not possible to separate the two heart forms by column chromatography.

Specific Activities

The activities of pyruvate kinase (uM substrate converted/min/gm wet weight) in red muscle and heart are 195.2 (160.8-241.1; three values) and 126.6 (107.1-150.0; four values), respectively.

Effect Of PH

Both enzymes showed a broad pH optima around pH 6.5 at 20°C. The peak becomes sharper and slightly more acidic at 40°C (Figure III-1).

Affinities For PEP And ADP

Both forms exhibited hyperbolic saturation curves with respect to PEP and ADP, at 10°C through 40°C. The Km(PEP) of

the heart enzyme rises very slightly as temperature increases from 10°C to 30°C and then takes a four-fold jump to 0.55 mM at 40°C (Figure III-2). The Km(ADP) of the heart enzyme rises very gradually from 10°C to 40°C. The Km(PEP) of the muscle enzyme is always between 1.5- to 2-fold higher than that of the heart enzyme; it begins to rise at a significant rate at 20°C and rises constantly between 20°C and 35°C. The Km(ADP) of the muscle enzyme is between 2 and 3 fold higher than that of the heart enzyme and is unaffected by temperature (Figure III-2).

Effect Of ATP

At Km concentrations of PEP and ADP, both enzymes are weakly inhibited by ATP (Figure III-3). Inhibition of the two enzymes is similar (40% at 5 mM ATP) at 20°C. At 40°C the inhibition of the muscle enzyme is as it was at 20°C, but the effect on the heart enzyme is more pronounced, being 62% at 5 mM ATP (Figure III-3). ATP is competitive with PEP and non-competitive or uncompetitive with ADP as determined from Dixon plots at 10, 20, 30 and 40°C. The Ki values for ATP are high, reflecting the lack of inhibition and do not show any consistent variation with temperature. The Ki values for the heart enzyme are slightly lower than for the muscle enzyme (Table III-1).

Effect Of FDP

FDP activates the heart enzyme at 20°C and at 40°C by increasing the affinity of the enzyme for PEP; in contrast, the

Km for ADP is not affected by FDP. FDP activates the muscle enzyme only at 40°C and the activation is greater than that seen in the heart enzyme at 40°C. Again, the mechanism of activation involves a decrease in the Km(PEP), but no change in the Km(ADP) (Table III-2).

Effect Of Alanine

Alanine has a slight inhibitory effect at 20°C on both enzymes (25% inhibition at 5 mM alanine). At 40°C the inhibition is more potent and the muscle enzyme is affected to a greater extent, 50 and 75% inhibition of the heart and muscle enzymes, respectively, occurring at 5 mM alanine (Figure III-4). This increasing inhibition is graded over the temperature range. The mechanism of inhibition appears to be rather complex. Both the Vmax and Km(PEP) are affected by alanine at all temperatures.

Combined Effects Of FDP, ATP And Alanine

In both tissues, 0.1 mM FDP partially overcomes the effects of 5 mM ATP at 20°C; at 40°C the inhibition is totally reversed. Inhibition by 2 mM alanine is fully overcome by FDP at 20°C and 40°C in both tissues (Table III-3).

Q10 Values

Q10 values, when determined at Km values of substrate do not differ significantly between the two enzymes. The values are slightly lower than expected for a non-catalyzed reaction

between 10 and 20°C and substantially lower between 20°C and 30°C. The value between 30°C and 40°C is negative for both enzymes due to the large rise in the Km(PEP) at these temperatures (Table III-4).

DISCUSSION

There are two major electrophoretic types of pyruvate kinase found in adult mammals. Skeletal muscles tend to have a single form whereas the liver, kidney, brain and heart can have two or more forms. Some of the forms are tetrameric hybrids of the major parental forms (Cardenas et.al., 1973 a and b; Osterman and Fritz, 1973). The pyruvate kinase isozymes in Rana pipiens appear to be derived from the same parental types as in the mammals. Multiple forms exist in the liver and there is one tissue specific form in the cardiac and skeletal muscle (Schloen et.al., 1974). The only electrophoretic work on fish involves salmon which have five bands in the heart (probably tetramers formed from two subunit types), and one in the muscle (Guderley, pers. Comm.). The skipjack conforms to the trend in having only one form in the muscle and multiple forms in the heart. In mammals and salmon, however, one form is common to the heart and skeletal muscle, whereas this is not the case in the skipjack.

The specific activities of pyruvate kinase in the red muscle and heart of the skipjack are similar to that found in four species of Amazon fishes (Hochachka et.al., 1978 a and b). Pyruvate kinase activity in carp red muscle is about half that found in skipjack red muscle (Van den Thillart, 1977). Most values for mammalian muscles are somewhat higher and vary from 200 to 700 units/gram wet weight (Osterman and Fritz, 1973). These values however, are for mixed (red and white) muscles and are therefore not representative. The only value for a "red" mammalian muscle is 280 units/gram wet weight (Prewitt and

Salafsky, 1967). The muscle used for this determination was the soleus muscle of the cat, which may be composed of mammalian intermediate fibers (Edgerton and Simpson, 1969) and therefore again is not representative. I feel that specific activities are probably not significantly different between comparable muscles of mammals and teleosts.

A distinction between fish and mammals however, becomes obvious when the kinetics of pyruvate kinase and the effects of pH are considered. Pyruvate kinase from mammals, generally has an alkaline pH optimum, somewhere between 7.5 and 8.5 (Randall and Anderson, 1975; Borgmann and Moon, 1976), whereas the pH optima for most teleost pyruvate kinases are acidic (Johnston, 1975; Mustafa et.al., 1971). The pH profiles of the pyruvate kinases from skipjack red muscle and heart are thus typically teleost. The kinetics of the two enzymes over their physiological temperature ranges (18-25°C for the heart (Sharp, pers. Comm.); 20-34°C for the red muscle (Chapter 5) are typical of neither the M nor L form found in mammalian tissues. At these temperatures the Km(PEP) of the heart enzyme is 0.1 mM and that of the muscle enzyme varies between 0.14 and 0.5 mM. The Km(ADP) of the heart enzyme is 0.3 mM and the Km(ADP) of the muscle enzyme is 0.7 mM, which is unusually high. ATP inhibition and FDP activation are relatively modest, and alanine inhibition is negligable at temperatures below 30°C. These two enzymes are thus typically teleost; they are partially regulated enzymes exhibiting characteristics in between those of the two major forms found in adult mammals.

The effect of temperature on enzyme reactions has been the

object of prolonged investigation. It is now well established what should be the effects of temperature on enzyme reactions and how a protein can evolve to either diminish or heighten this effect (Somero, 1975). Much of this work has centered around the pyruvate kinases of fishes , resulting in a large data base fcr ccmparison. The binding of the highly charged PEP molecule to the enzyme must be highly dependent upon ionic interactions and thus should be adversely affected by temperature change (Somero, 1975). Conversely, the binding of the ADP molecule probably involves hydrophobic as well as icnic interactions and should not be as temperature dependent (Hochachka, 1974). Information to date shows that in the majority of pyruvate kinases, the Km(PEP) increases as temperature rises through the physiological range (Somero, 1975; Hoffman, 1976). In the one study on the influence of temperature on the Km(ADP), it was found that the affinity of trout pyruvate kinase for ADP was temperature independent (Scmero and Hochachka, 1968). The skipjack muscle enzyme shows a typical two-fold rise in the Km(PEP) over 10°C, whereas the Km(ADP) is unaffected by temperature. Both of the affinity constants of the heart enzyme however, are virtually temperature independent over the physiological temperature range. The muscle enzyme is thus a typical fish muscle enzyme (Somero, 1975) whereas the heart enzyme shares characteristics in common with some invertebrate enzymes whose affinity constants do not vary with temperature (Hoffman, 1976).

An important feature of skipjack red muscle is that its temperature is dependent upon muscle activity. Thus, a direct

Km-temperature relationship could prevent saturation of red muscle pyruvate kinase if PEP levels were to rise with activity. However, although PEP levels in skipjack red muscle are too low to be measured accurately, they definitely do not rise to levels which could present saturaticn problems. The Km(PEP)-temperature relationship therefore probably serves to render the pyruvate kinase reaction temperature independent in an active tissue subject to sudden, significant temperature variations. This latter conclusion is supported by the low Q10 values for the red muscle pyruvate kinase reaction (Table III-4). The significance of the flat Km(PEP)-temperature relationship of the heart enzyme is not clear. This shape of curve appears to be characteristic of eurythermic organisms which "opt" for the maintenance of enzyme-substrate affinities within certain optimal values, rather than temperature independence (Hoffman, 1976). Interestingly, however, in contrast to the enzymes of eurytherms, the heart pyruvate kinase shows relative temperature independence. How the lcw Q10 for the heart enzyme (Table III-4) is achieved is still unknown.

ATP levels in the red muscle do not vary significantly under the three exercise conditions (Chapter 5) and ATP effects are not temperature sensitive; the role of ATP in regulation at this site would thus seem to be minor. This is not to say that the energy charge, which may flucuate significantly (Table 5-6) does not play a significant role in regulation of red muscle pyruvate kinase.

The effect of alanine, however is strongly temperature

dependent (Figure III-4). The temperature of the red muscle is 1°C above ambient when the fish is at "rest", it is consistently high during feeding frenzies (i.e. during very fast sustained swimming), and may also be raised during burst swimming (Chapter 5). Alanine concentrations in the red muscle rise from 4 umol/g wet weight wet weight under resting conditions to 9 umol/g wet weight wet weight during feeding conditions (Chapter 4). Thus, at the high feeding temperatures, alanine would be inhibitory (Figure III-4).

FDP levels in red muscle fluctuate slightly between the various activity states, but never drop below 0.25 umol/g wet weight wet weight which is almost certainly enough to modulate the effects of both ATP and alanine at any temperature. This is a common paradox associated with pyruvate kinase research and there are many observations showing minimal FDP concentrations to completely overcome the regulatory effects of other metabolites (Koster et.al., 1972; Mustafa et.al., , 1971; Guderley et.al., 1976; Storey and Hochachka, 1974b).

PEP availability is obviously important, but unfortunately PEP levels are too low to be measured accurately. PEP may become a key element in control when levels drop due to inhibition of regulatory sites in glycolysis above pyruvate kinase.

Metabolite levels in the heart of the skipjack are not known, but blood levels of glucose and alanine hardly change between rested and active fish (Chapter 4). In the heart, temperature bears no relation to activity and thus the significance of the temperature induced pyruvate kinase

sensitivity to metabolites is as yet obscure.

Table III-1. ATP inhibition constants for the red muscle and heart pyruvate kinases from the skipjack. Dixon plots were done at three concentrations of PEP and at 0.2 mM ADP, pH 6.5.

Temperature °C	$K_{i(ATP)}$ mM	
	Muscle	Heart
10	0.5	1.7
20	3.5	2.5
30	2.0	2.4
40	4.0	1.5

Table III-2. The effect of FDP on pyruvate kinase from the heart and red muscle of skipjack. Heart assays done at 0.5 mM ADP and 0.1 mM PEP; muscle assays done at 0.2 mM ADP and 0.1 mM PEP. All assays done at pH 6.5.

	% Activation by 0.5 mM FDP	
	20°C	40°C
Muscle	0	50
Heart	14	30

Table III-3. The effect of 0.1 mM FDP on the activity of the heart and red muscle pyruvate kinases in the presence of 5 mM ATP or 2 mM alanine. 2.0 mM PEP, 2.0 mM ADP, pH 6.5.

	Activity (% of control)			
	FDP + ATP		FDP + Alanine	
	20°C	40°C	20°C	40°C
Red muscle	73	100	100	180
Heart	73	113	130	244

Table III-4. Q10 values of skipjack red muscle and heart pyruvate kinases. 0.04 mM PEP and 0.5 mM ADP.

Temperature range °C	Q_{10} Red muscle	Heart
10–20	2.3	1.9
20–30	1.57	1.3
30–40	.66	.66

Figure III-1. Relative activity of pyruvate kinase versus pH at 20°C and 40°C.

A. Relative activity versus pH at 20°C. ., muscle; x, heart; 0.2 mM PEP; 0.5 mM ADP.

B. Relative activity versus pH at 40°C. ., muscle; x, heart. 0.2 mM PEP; 0.5 mM ADP.

Relative Activity
100
50
20° C
40° C
6
7
8
pH

Figure III-2. Km(mM) versus T°C: skipjack red muscle and heart pyruvate kinases. ., muscle ADP; squares, muscle PEP; o, heart ADP; x, heart PEP. 0.2 mM PEP; 0.5 mM ADP; pH 6.5.

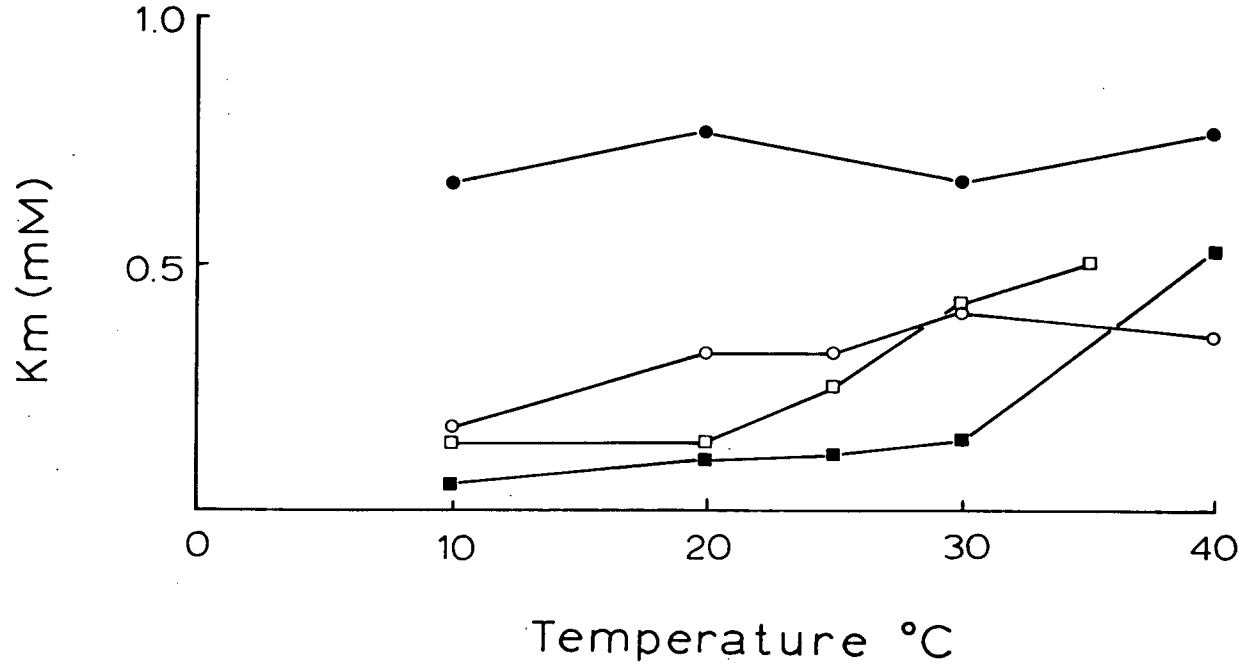

1.0
0.5
Km (mM)
0
10
20
30
40
Temperature °C

Figure III-3. % of control activity versus ATP concentration. Assays done at pH 6.5, 0.2 mM PEP and 0.2 mM ADP. ., muscle, 20°C; squares, muscle (40°C); o, heart (20°C); triangles, heart (40°C).

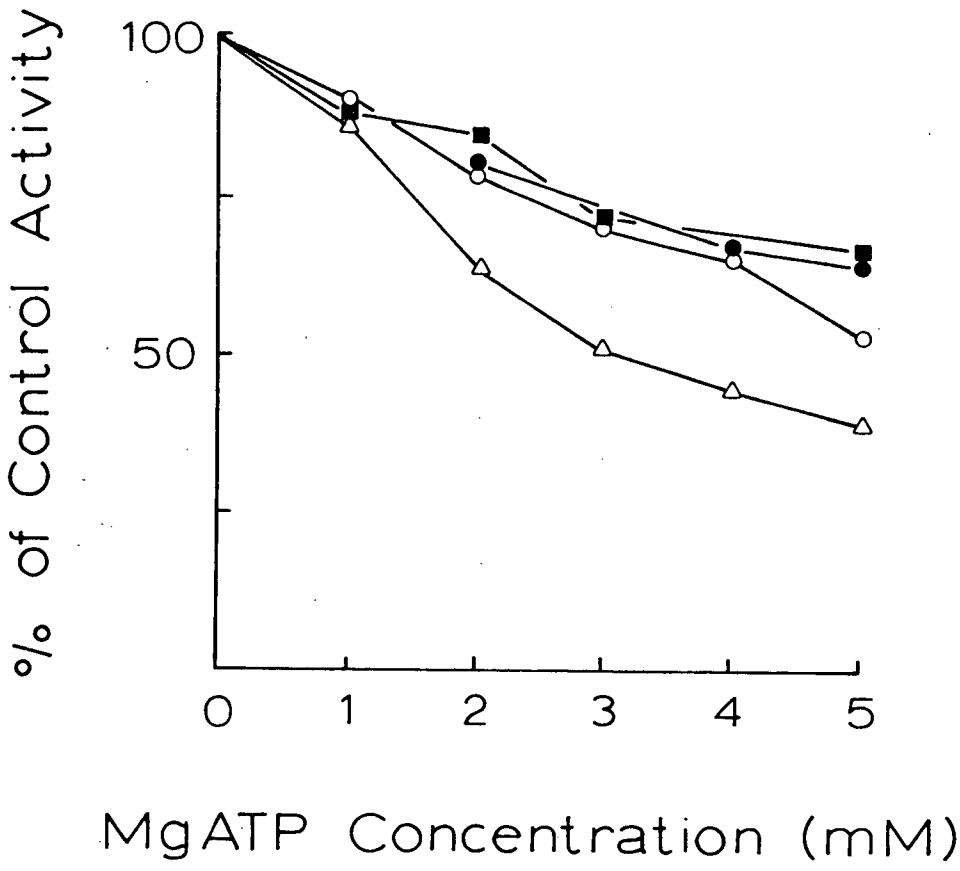
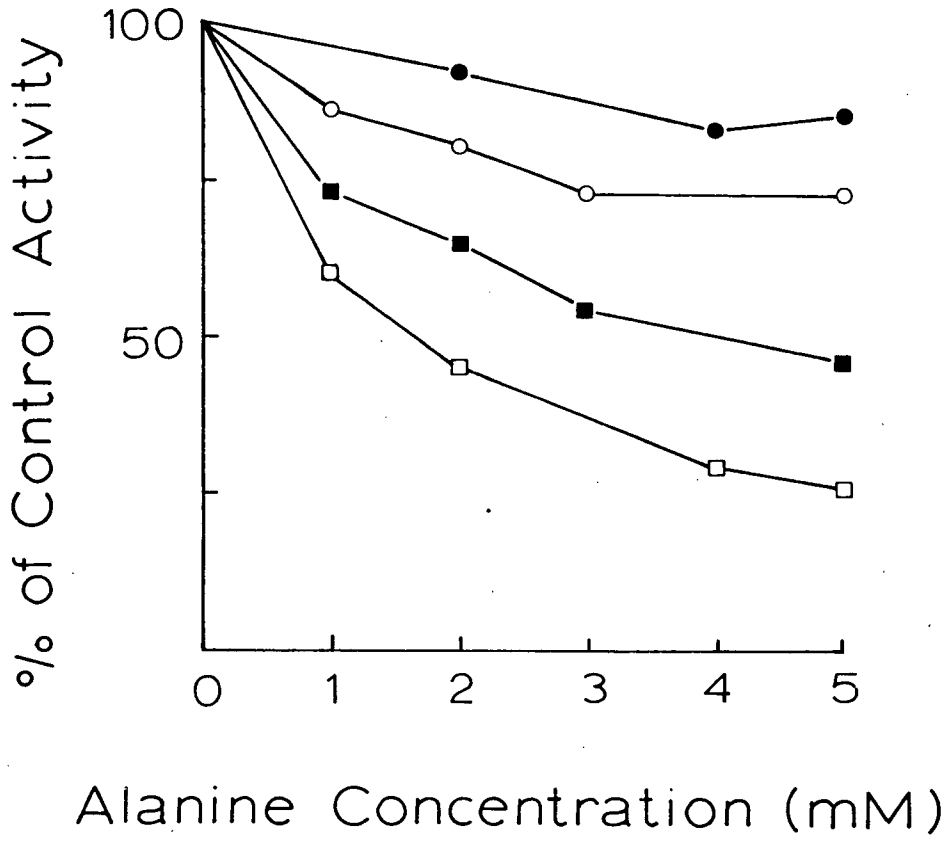

% of Control Activity
100
50
0
1
2
3
4
5
MgATP Concentration (mM)

Figure III-4. % of control activity versus alanine concentration. Muscle assays at 0.2 mM PEP and 0.2 mM ADP; heart assays at 0.1 mM PEP and 0.2 mM ADP. All assays at pH 6.5. ., muscle (20°C); squares, muscle (40°C); o, heart (20°C); triangles, heart (40°C).


100
50
% of Control Activity
0
1
2
3
4
5
Alanine Concentration (mM)

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ABBREVIATIONS USED

ADP: adenosine diphosphate

AMP: adenosine monophosphate

ATP: adenosine triphosphate

CS: citrate synthetase

DHAP: dihydroxyacetone phosphate

DTNB: 5, 5' dithiobis-(2-nitrobenzoic acid)

EDTA: ethylene diamine tetraacetic acid

EGTA: ethyleneglycol-bis-(-amino-ethyl ether) N, N'-tetra acetic acid

FAD: flavin adenine dinucleotide (oxidized)

FADH: flavin adenine dinucleotide (reduced)

FDP: fructose diphosphate

F6P: fructose-6-phosphate

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GOT: glutamate oxaloacetate transaminase

GP: α-glycerophosphate

GPDH: α-glycerophosphate dehydrogenase

G6PDH: glucose 6-phosphate dehydrogenase

G6P: glucose-6-phosphate

LDH: lactate dehydrogenase

MDH: malate dehydrogenase

NAD^+: nicotinamide adenine dinucleotide (oxidized)

NADH: nicotinamide adenine dinucleotide (reduced)

NADP: nicotinamide adenine dinucleotide phosphate (oxidized)

PDH: pyruvate dehydrogenase

PEP: phosphoenol pyruvate

PFK: phosphofructokinase

PK: pyruvate kinase

SDH: succinate dehydrogenase

SR: sarcoplasmic reticulum

T-: tranverse tubule

U: units of enzyme activity (uM product produced/minute)

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