ASPECTS OF ADAPTATION TO THE ENVIRONMENT IN THE WHELK BULLIA

With special reference to physiological effects of fluctuating temperatures in *Bullia digitalis* (Dillwyn)

by

Fernanda Maria Palma Ribeiro da Silva Tatley

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The work contained in this thesis is original, except where otherwise indicated either in the text or in the list of acknowledgements. In the papers submitted in the appendix, the sequence of authors is a reflection of the amount of input provided by each.

Signed

Fernanda Maria Palma Ribeiro da Silva

September 1985

BULLIA DIGITALIS (DILLWYN)

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SUMMARY

This thesis comprises an investigation of some metabolic compensations to temperature fluctuation in the whelk Bullia digitalis (Dillwyn).

Bullia digitalis is a most unusual and highly active prosobranch which inhabits sandy beaches on the west coast of southern Africa. This habitat is characterised by sudden and unpredictable thermal changes and erratic food availability. Measurements of oxygen consumption of intact whelks, indicated their metabolism to be relatively temperature independent, at all levels of activity, as evidenced by a flat rate-temperature curve over the thermal range commonly encountered in the field (10 to 22°C). This control is absent in whole body homogenate. This was interpreted as an energy conserving metabolic adaptation related to the unpredictable nature of the whelk's environment. Possible mechanisms contributing to this phenomenon include the presence of isozymes at key branch points, resulting in shunting of substrate flux through selected pathways as a function of optimal temperature. This mechanism can be particularly effective if distinct and specific control of enzyme substrate properties occurs simultaneously. Selected aspects of metabolism with reference to this phenomenon were thus examined.

A survey of population genetic structure of several Bullia species revealed a low level of polymorphism indicative of genetic homogeneity. Structural, physico-chemical and kinetic properties of D(-)LDH and D(+)ODH were examined, since both enzymes operate at the pyruvate branch point, the latter being highly important in the aerobic/anaerobic transition during muscular contraction. D(-)LDH is expressed as 5 isozymes in foot, whereas it is represented by only 3 in the mantle. In the foot the major isozymes (I and V) exhibit distinct temperature responses. D(+)ODH on the other hand is represented by only one enzyme form, except for a variant in the digestive gland. It is temperature sensitive, displaying distinct thermal optima of forward and reverse reaction. In addition, its optimal thermal range is desynchronous with that of D(-)LDH, such that the branch point is operative irrespective of temperature. Work of a preliminary nature on the chemical composition of B.digtalis suggested a carbohydrate dependent metabolism. This was further confirmed by determination of glycolytic flux, where glycogen is a primary source of energy. Additional supportive evidence is provided by the abundance of glycogen granules in foot muscle of the whelk. Changes which occur in metabolite ratio and adenylate energy charge in the course of a period of activity, are small, suggesting an ability to conserve energy. This was further supported by ^{31}P-NMR studies of cell free cytosol, showing that slight changes occur in the relative porpotions of metabolite peaks.

Although only a limited study was undertaken, results suggest that the flat rate:temperature curve depends on the summation effect of several physiological processes.

Finally analysis of electron micrographs of foot muscle of B.rhodostoma (a closely related species) indicates the presence of two distinct muscle fibres. One is found predominantly in the small anterior portion of the foot, the propodium, being a form of striated muscle; the other is confined to the larger remaining part of the foot (metapodium) and is more typical of molluscan smooth muscle. This may be of adaptive value considering the functional diversity of the foot.

An appendix presents papers published and in press, relevant to the above study.

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Since CHAPTERS 3, 4, 5, 6, and 8 are intended for publication, their format is different to that of INTRODUCTION, REVIEW, CHAPTER 7 and DISCUSSION AND CONCLUSION.

"THE BIOLOGICALLY SIGNIFICANT CAPACITIES OF AN ENTIRE ORGANISM ARE EXPLAINED BY ANALYSING THE ORGANISM INTO A NUMBER OF 'SYSTEMS' - THE CIRCULATORY SYSTEM, THE DIGESTIVE SYSTEM, THE NERVOUS SYSTEM, THE RESPIRATORY SYSTEM, etc.- EACH OF WHICH HAS ITS CHARACTERISTIC CAPACITIES. THESE CAPACITIES ARE IN TURN ANALYSED INTO CAPACITIES OF COMPONENT ORGANS AND STRUCTURES. IDEALLY, THIS STRATEGY IS PRESSED UNTIL PURE PHYSIOLOGY TAKES OVER...".

Cummings, 1977.

"... LAWS ESTABLISHING THE CAUSAL RELATIONSHIPS BETWEEN THE TRAIT AND ITS FUNCTIONAL EFFECT, AND LAWS ESTABLISHING THE CAUSAL RELATIONSHIP BETWEEN FUNCTIONAL EFFECT AND THE EXISTENCE OF THE TRAIT IN THE SYSTEM. THE FIRST SET OF LAWS MAY BE LAWS OF CHEMISTRY, PHYSICS, etc., (eg., THE LAWS WHICH ESTABLISH THAT CHLOROPHYL CATALYSES PHOTOSYNTHESIS)... THE SECOND SET OF LAWS ARE THE LAWS OF NATURAL SELECTION. THE CONDITIONS LIKEWISE FALL INTO TWO SETS: THE CONDITIONS NECESSARY FOR THE TRAIT TO HAVE ITS FUNCTIONAL EFFECT, AND THE CONDITIONS NECESSARY FOR THE TRAIT TO SPREAD THROUGH A POPULATION UNDER THE INFLUENCE OF NATURAL SELECTION."

Williams, 1976.

CHAPTER 1

INTRODUCTION: THE LIFE OF THE WHELK *BULLIA DIGITALIS* (DILLWYN) IN A FLUCTUATING TEMPERATURE REGIME

1.1. Some ecological considerations of the genus *Bullia*

The genus *Bullia* (Gray 1834) falls within the gastropod family Nassariidae, which belongs to the superfamily Buccinacea, the most advanced superfamily of the Neogastropoda. Our knowledge of the genus, excepting only its taxonomy, has beem summarised by Brown (1982). Members of the genus typically inhabit shallow and intertidal sands in temperate and warm latitudes around southern shores. There are at least thirteen species in southern Africa. These may conveniently be divided, on behavioural grounds, into two groups: those which are predominantly intertidal and use their broad, muscular feet to surf up and down the beach, and those which are mainly subtidal and do not surf. Around the Cape Peninsula of South Africa, surfing species include *B.digitalis* (Dillwyn), *B.rhodostoma* Reeve and *B.pura* Melvill, while *B.laevissima* (Gmelin), *B.diluta* (Krauss), *B.tenuis* Reeve, *B.annulata* (Lamarck) and *B.callosa* (Wood) represent the non-surfing, sub-tidal group (Brown 1971).

Bullia digitalis is the common intertidal species of South Africa's west coast, while east of Cape Point it is joined by *B.rhodostoma* and *B.pura*, all three species extending along the south coast. The species are thus sympatric over much of their geographical range, the latter tending to occur either higher up

the shore or in distinct patches (Brown 1982). This segregation appears to be due to differences in surfing ability (McGwynne 1980) and to sorting by water currents (Brown 1971). While an occasional individual of B.rhodostoma may be found on the west coast, it is extremely rare except for an isolated colony at Saldanha Bay (Brown 1977).

The present distribution of Bullia species in the south-west Cape, as well as some of their physio-ecological adaptations, may be viewed in the light of the geological history of the area. Until fairly recent times, the Cape Peninsula was an island, what is now the Cape Flats being under water (Taylor 1978). This stretch of water, known as the Cape Straits, thus joined Table Bay and False Bay. Eddies from the warm Agulhas Current, flowing down the east coast of South Africa may well have entered the Cape Straits to flow northwards up the west coast. Temperatures may thus have been different from those prevailing today and may have influenced the later evolutionary development of the species in the area (Fig. 1.1).

Present conditions on the two sides of the Cape Peninsula are rather different, False Bay shores sloping more gently and being less exposed to wave action than west coast shores. However, it is with regard to the different temperature regimes of the two coasts that different physiological adaptations are likely to be manifested (Brown & Jarman 1978; Brown & da Silva 1984). Firstly, False Bay waters tend to be warmer than those of the west coast, due not only to the warm

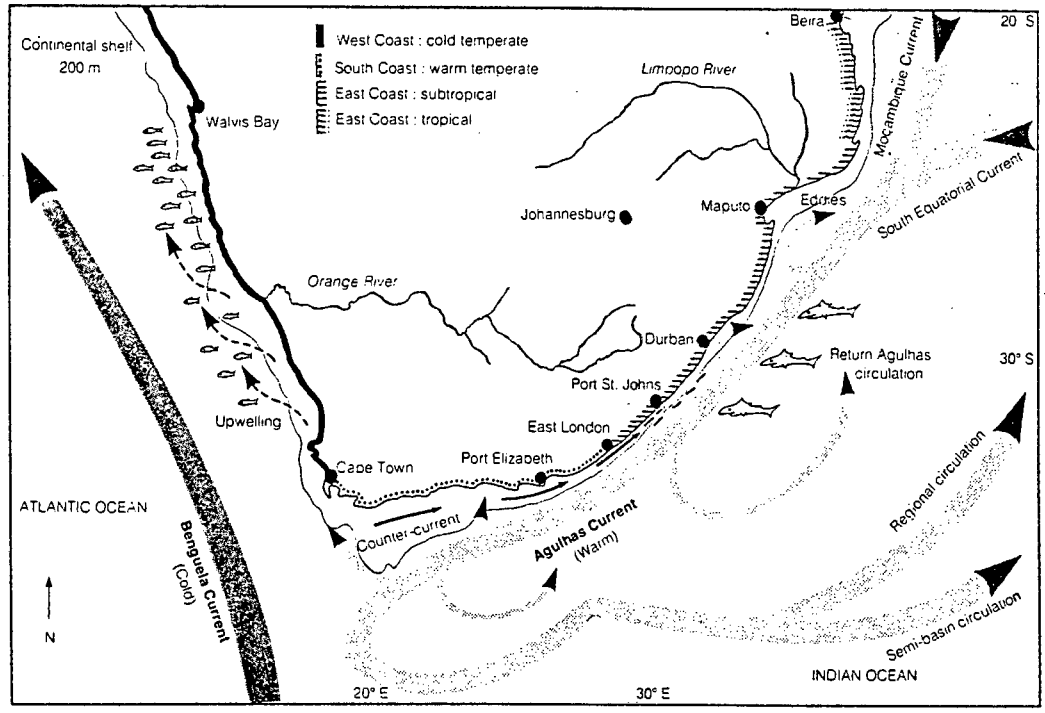


Figure 1.1. Map of southern Africa showing the major ocean currents (from Branch & Branch 1981).

Agulhas Current, which contributes a large proportion of the Bay's water, but also because, being shallow and having a long residence time, is heated by the sun (Day 1970). Temperatures range from 12 to 25°C in summer and from 8 to 20°C in winter, changes being relatively gradual, especially along the northern shores of the Bay. In contrast, temperatures of the water on the west coast may be up to 10°C lower than those of False Bay and the average difference is about 5°C. Of greater eco-physiological impact, however, is the extreme and rapid fluctuation in temperature encountered on the west coast, particular in summer. The water temperature may change from 17 to 8°C in a few hours (Branch & Branch 1981). This is due to coastal upwelling (Andrews 1974), warm water being blown away from the shore by the south-easterly wind in summer, to be replaced by cold, upwelling water. There can be little doubt that these wind-induced fluctuations in temperature have a far greater significance for the biota of the west coast than do average winter or summer temperatures (Brown & da Silva 1984).

Physiological and biochemical adaptations are also indicated by the highly erratic food supply to which the whelks are subjected. They are essentially carnivorous, feeding on carrion washed up onto the beach, and will accept virtually any animal material (Brown 1964, 1971). Observations over many years (Brown 1982), as well more restricted but quantative assessments (McGwynne 1980) show that carrion may be virtually absent from a beach for a whole season of the year, occurring erratically and largely unpredictably at other times. It is thus hardly

surprising that the whelk can consume the equivalent of a third of its tissue weight of food in a single meal (Brown 1961) and will live for months in the laboratory without feeding. Recent work has shown that Bullia's carvinorous diet may be supplemented by algae cropped from its own shell (da Silva & Brown 1984, Harris et al. 1985) and possibly by the uptake of dissolved organic matter (Colclough & Brown 1984). However, these alternative sources of nutrition can do nothing more than partially alleviate a desperate situation. Maximum utilisation of food is thus of great importance in the lives of the animals, not only with respect to the amount that can be ingested and the efficiency of assimilation, but also with regard to energy conservation (Brown 1981).

It may be noted that the staple diet of Bullia east of Cape Point consist of Cnidaria, notably Scyphozoa and siphonophores such as Physalia. This is due to the abundance of this carrion and not to choice on the part of the animals, which prefer carrion of higher calorific value, such as bivalve molluscs and the tunicate Pyura, when these are available (Brown 1964, 1981). However, washed up Cnidaria are far less common on the west coast than along the south coast, including the beaches of False Bay, so that Bullia digitalis on the west coast faces more severe food shortages than do Bullia populations east of Cape Point (Brown & da Silva 1984). Energy conservation is thus of even greater importance to them.

1.2. Temperature independence of metabolic rate in *Bullia digitalis*

The rate of metabolism in some species of *Bullia* varies with temperature in the classic manner expected of an ectotherm. This is demonstrated by rates of oxygen uptake in unfed, female individuals of *B.rhodostoma* from False Bay, these rates being temperature dependent at all levels of activity, although the rate-temperature curves tend to be linear rather than logarithmic (Brown & da Silva 1984) (Fig. 1.2.). This species also shows significant, though not extreme, seasonal temperature acclimation.

In contrast, *B.digitalis* from the west coast displays a largely temperature-independent rate of oxygen consumption, the Q_{10} of the routine rate, within the temperature range experienced by the animal in the field, being only 1.1 (Brown & da Silva 1979). Moreover, this flat rate-temperature curve is in evidence at all levels of activity (Brown & da Silva 1983) (Fig. 1.3). This has been interpreted as an adaptation favouring energy conservation and homeostasis/enantiostasis in a regime of widely fluctuating temperatures (Brown & da Silva 1984). In view of these flat rate-temperature curves and the fact that winter average temperatures on the west coast tend to be slightly higher than those of summer, it hardly surprising that *B.digitalis* does not acclimate (Brown *et al.* 1978, Brown & da Silva, 1979).

While the explanation of the temperature-dependent metabolic rates of *B.rhodostoma* is clear in terms of biochemical

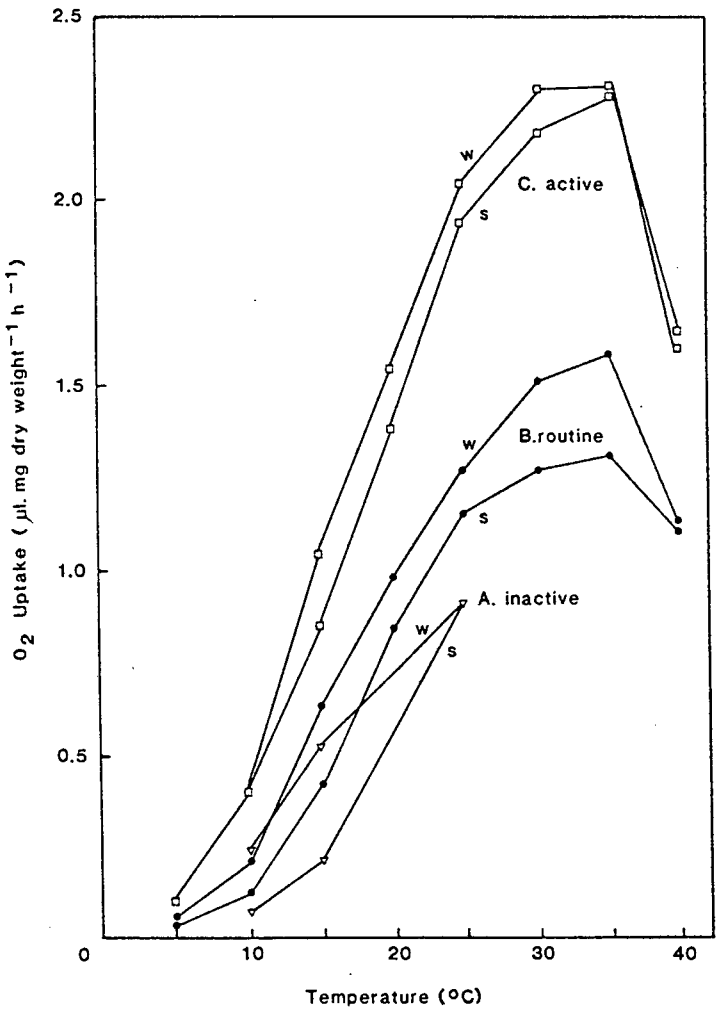


Figure 1.2. Acute rate-temperature curves for Bullia rhodostoma : A, inactive (after Dye & McGwynne 1980); B, routine rate in a constant water current; C, active rate; s, summer; w, winter (from Brown & da Silva 1984).

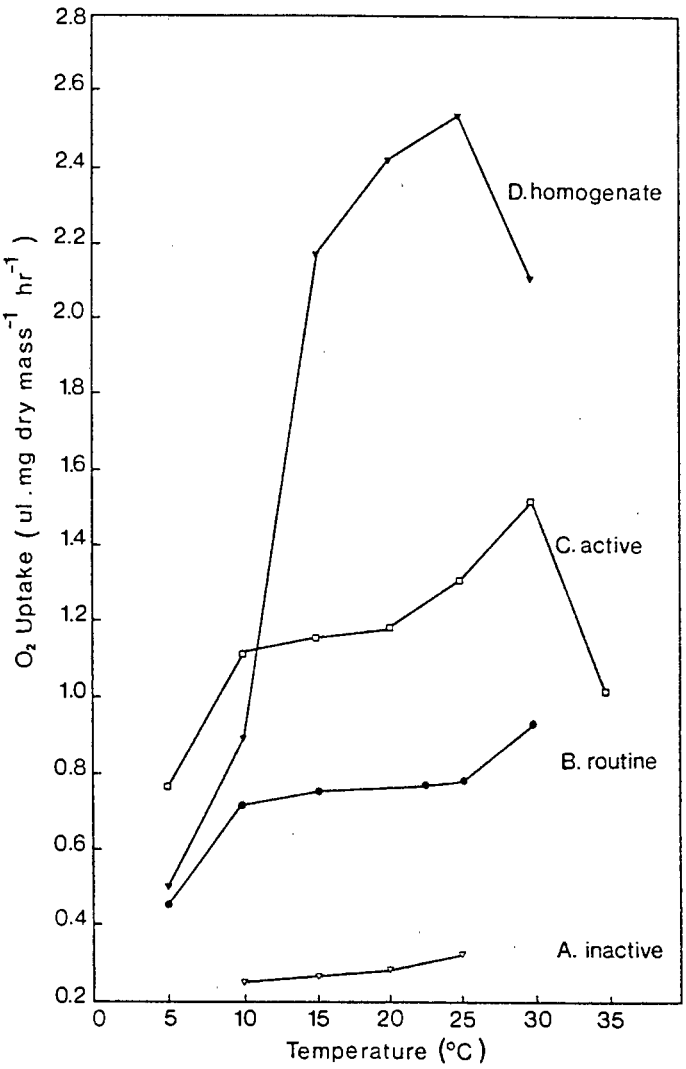


Figure 1.3. Acute rate:temperature curves for the whelk Bullia digitalis: A, inactive animals (after Dye & McGwynne 1980); B, routine rate for animals in a constant current (after Brown & da Silva 1979); C, active animals in a constantly changing current; D, whole tissue homogenates (Brown & da Silva 1983).

thermodynamics, the flat curves of B.digitalis require further investigations at cellular, sub-cellular and biochemical levels. As a first step towards the discovery of how the animal manages to control its metabolism in the face of fluctuating temperatures, the rate of oxygen consumption of whole body homogenates of B.digitalis was measured at various temperatures, covering the same thermal range as previously employed for intact animals. In marked contrast to the living animals, homogenates of B.digitalis were shown to have a fully temperature-dependent rate of oxygen uptake (Brown & da Silva 1983) (Fig. 1.3). This was construed as a direct consequence of the disruption of cellular function during homogenisation. Since protease inhibitors and reducing agents had been added to the homogenate during preparation, it is unlikely that differences between intact animals and homogenates were influenced by proteolytic degradation. It should also be noted that no substrate was added to the homogenate. Thus it is probable that drastic alterations in cellular compartmentation, pH gradients, endogenous substrate levels and pathway organisation were responsible for the loss of metabolic control.

It was thus decided to investigate further the nature of this metabolic control, for which a number of mechanisms have been proposed:

(a) The synthesis of a metabolic hormone, acting directly on the protein synthesising machinery or via cAMP, with consequent feed-back regulation.

(b) The presence of variants of certain enzymes, whose temperature sensitivity and kinetic properties differ, allowing each enzyme variant (allozyme or isozyme) to develop distinct temperature optima so that irrespective of temperature, the pathways in which the enzyme variants are involved are always operative.

(c) Enzyme-substrate interactions and consequent enzyme substrate affinities could change as a result of conformational or kinetic property alterations due to thermal variation. This could affect adenylate ratios and thus shift the cellular energy balance.

(d) In analogy to vertebrate metabolism regulation via cAMP, protein kinase dependent phosphorylation-dephosphorylation reactions, a similar mechanism could be available whereby selective modulation of an enzyme form regulates metabolic flux, contributing to metabolic independence of temperature. This system could operate as an enzyme cascade.

(e) No one single adaptation, but rather the summation effect of several aspects of metabolism could be responding, interacting with each other and contributing to the overall physiological behaviour described by the flat rate-temperature curve.

As will become evident, the complexity of the actual mechanisms involved rendered their systematic investigation beyond the scope of this analysis. Instead it was attempted to

investigate certain aspects of metabolism in B.digitalis which demonstrate some adaptive value (utilising some of the mechanisms proposed above), as it was suspected that the flat rate-temperature curve is an adaptive mechanism to compensate for environmental thermal variations.

CHAPTER 2

2.1. TEMPERATURE ADAPTATION IN ECTOTHERMS: A PHYSIOLOGICAL REVIEW

2.1.1. The paradigm of adaptation

The concept of adaptation refers to the modification of certain features typical of organisms, allowing for the augmentation of the ability with which an animal masters a particular environment (Hochachka & Somero, 1984). Adaptation comprises a myriad of interactions and responses ranging from the morphological, physiological, genetic, mechanical, behavioural, ecological to more fundamental biochemical ones. Thus, it appears that adaptation is the vastest paradigm of biology and that despite a conserted effort (Prosser 1973, Hazel & Prosser 1974, Newell 1976, Newell 1979, Hochachka & Somero 1984) towards understanding its nature, relationships, mechanisms, advantages and/or disavantages, we are barely touching the surface, in view of the multiple strategies employed by different organisms to attain the same end result. This is best summarised in the words of Wimsatt (1972), on the current understanding of biology: " In biology, at least, the picture is further complicated by another factor - that different theoretical perspectives are not nearly as well individuated as in the physical sciences. Thus, anatomical, physiological, developmental and biochemical criteria, not to mention paleontological information and inferences of phylogenetic relations and homologies, all interact with criteria of evolutionary significance in the analysis of organisms into functional systems and subsystems. This borrowing

of criteria for individuation of parts from different and diverse theoretical perspectives is one of the factors which make functional organisation in general and in biology such a conceptual morass at times."

Inspite of the diversity of responses to environmental conditions, so as to increase their adaptive value, some common principles transpire. These relate to the fundamental biochemical processes characteristic of all living organisms and comprise the following functions:

1. The preservation of structural cellular integrity.
2. The synthesis, mobilisation and utilisation of energy (ATP) so that metabolism is not disrupted.
3. Maintenance of overall control of physiological reactions to external and internal stimuli.

These are primordial requirements for the correct adjustment of an organism to varying environmental conditions and are thus the central dogma of adaptation.

2.2. ADAPTATION TO TEMPERATURE: FUNDAMENTAL TEMPERATURE EFFECTS

Temperature is the most important factor contributing to the distribution pattern of organisms on the intertidal shore (Stephenson & Stephenson 1972, Somero & Hochachka 1976).

The effects of temperature variation on marine organisms is dependent on the magnitude of the temperature change and on the suddenness with which it occurs. Biochemically and

physiologically rapid temperature changes are fundamental in determining what adaptive mechanism is to be favoured, when little time is available to compensate for environmental change by biochemical means (Somero & Hochachka 1976, Hochachka & Somero 1984). Therefore the larger the period over which a temperature change develops, the greater the possibility that the organism will evolve adequate cellular strategies to adjust to new conditions and enhance both adaptational success and survival rate. Because of the high price biochemical and physiological changes demand, these are only employed when behavioural adaptations have failed.

An understanding of the effects of temperature on living organisms goes hand in hand with the cognisance of the physical factors determining temperature, a measure of the intensity of the heat energy present in the system. But to appreciate the effects of temperature on organisms, one should be acquainted with the effects of temperature on reaction rates and equilibria.

2.2.1. The effects of temperature on reaction rates

It is generally assumed that reaction rates increase as a function of temperature, commonly expressed in terms of Q_{10}. The Q_{10} is defined as the ratio of reaction velocities at a temperature (T^{o}) ten degrees C apart and expressed as follows:

$$Q_{10} = \frac{\text{Velocity}\,(T^{o}+10^{o})}{\text{Velocity}\,(T^{o})}$$

The assumption that a doubling of reaction velocity for a 10°C rise in temperature (Q_{10} = 2) is associated with large relative changes in temperature is not always valid, as in many physiological and biochemical processes a Q_{10} = 2 is equivalent to a mere 3% absolute increase in temperature (10/(273+25)), at ambient temperature (Hochacka & Somero 1984). This apparent paradox was explained by Svante Arrhenius in the 1880's, by emphasising the importance of a fraction of the molecular population which is reactive at any temperature. Changes in temperature induce alterations in the size of this reactive population. These reactive molecules are characterised by having energy equal to or greater than the minimal ACTIVATION ENERGY required for the reaction. Activation energy in this context is equivalent to activation enthalpy ($\Delta H^{\ddagger}$) expressed in Kcal/mol. Temperature dependence of reaction rates is defined as follows:

$$\frac{d \ln k}{d T} = \frac{\Delta H^{\ddagger}}{RT}$$

where K is the rate constant, T is the absolute temperature in kelvin and R the universal gas constant. It is the relationship among reaction velocities, temperature change and $H^{\ddagger}$ that is fundamental to biochemical temperature adaptation.

2.2.2. The effect of temperature on reaction equilibria

In addition to effects on reaction velocity, temperature has marked effects on the equilibrium constants of biochemical processes, especially those involved in the reversible formation of non-covalent ("weak") chemical bonds.

These bonds are responsible for a wide range of of biologically structured functions and are easily affected by minimal thermal variation. Covalent bonds on the other hand, are relatively strong and require catalytic hydrolysis for their rupture; therefore they are much less susceptible to thermal changes (Hochachka & Somero 1984).

All of the biochemical structures stabilised by weak bonds have a feature in common: they are malleable rather than rigid and change in the course of functional activity. This was designated the "semistability" property by Alexandrov (1977). It is this maleability that is so important in the maintenance of higher degrees of structure and function.

2.2.3. The overall effects of temperature

Since the maintenance of a dynamic status quo is pivotal to living organisms, the reliance on weak chemical bonds imposes a sharp temperature dependence of macromolecular structure. In consequence this dependence causes the metabolic apparatus and its regulation to be extremely sensitive to temperature alterations. The rate effect of temperature will affect the velocities of metabolic fluxes and thus, on an evolutionary scale, there has been a concerted effort to compensate for temperature changes, though in temporary situations short term mechanisms will prevail (Hochachka & Somero 1976).

2.3. MOLECULAR MECHANISMS OF TEMPERATURE COMPENSATION IN ECTOTHERMS: Fundamental strategies

Metabolic regulatory responses are continuously used to maintain the normal functioning of an organism (Atkinson 1977). If environmental changes exceed the intrinsic capacity of the compensatory strategies, there can be severe consequences both to the organism and environment (Stebbing 1981).

A hypothetical time related sequence of events of the effects of the environment on an organism, as suggested by Blackstock (1984), is given in Fig. 2.1. It is apparent from this figure that despite the emphasis on adaptation, this is only the last resort and that considerable efforts are made by animals to minimise or avoid environmental stress. This is especially true of mobile species, though inapplicable to sessile forms.

The control of metabolic flux is achieved through different molecular levels, on variable time courses. Non-equilibrium enzymes have been associated with the regulatory function of pathways (Newsholme & Start 1973) though some exceptions have been observed (Groen et al. 1982). The responses of non-equilibrium enzymes are always fairly rapid and contrast with those of effectors and modulators, which may alter the utilisation and availability of substrates to enzymes. The forces of natural selection which contributed towards the descrimination of certain molecular forms responsible for enhanced survival of a species, may be considered a mechanism whereby alternative responses occur.

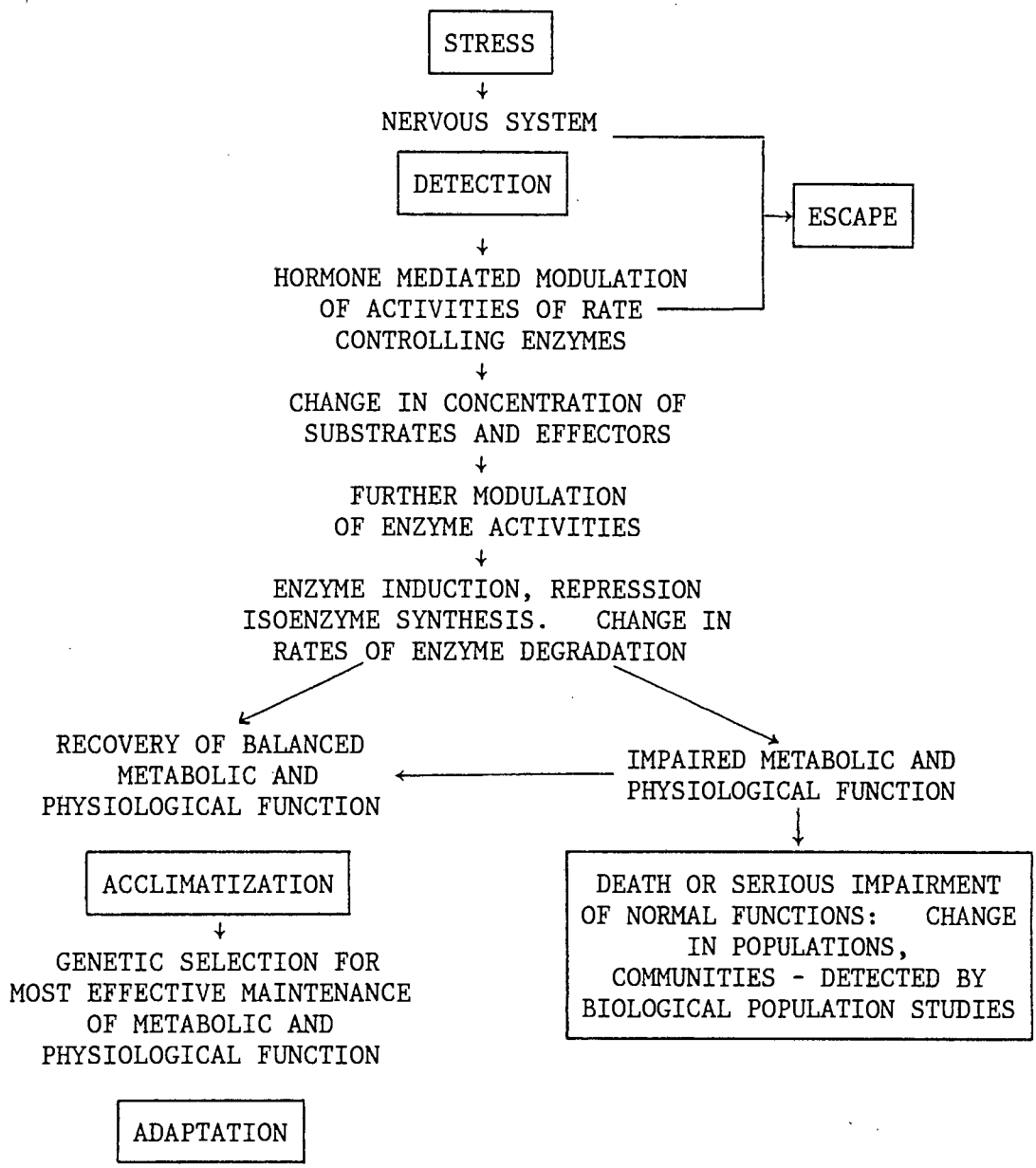


Figure 2.1 Time related sequence of effects of environmental stress on marine fauna (after Blackstock 1984).

This is a favoured field of study (especially with regard to intermediary metabolism), due to the many possibilities offered by the system (Hochachka et al. 1983, Storey & Storey 1983, de Zwaan 1983, Livingstone & de Zwaan 1983). Intermediary (carbohydrate) metabolism is normally closely associated with hormonal regulation, via varying levels of feed back regulation within the myriad of reactions comprising these pathways. In invertebrates the situation is complicated by the inter-relationships between glycolysis, Krebs cycle, fatty acid synthesis and pathways leading to the production of amino-, imino-acids and propionate, during bursts of activity which can cause a state of anaerobiosis. These often give rise to unusual end-products, used in the maintenance of redox balance, and are a consequence of the utilisation of pyruvate (rather than oxygen) as a terminal electron acceptor (Hochachka & Somero 1984). Phylogenetically the pathway of glycolysis is old, often leading to the erroneous belief that it is highly conserved and unchanged. In fact several functional reaction branches have undergone modification, namely the phosphoenol pyruvate and the triose phosphate branch points, as stressed by Hochachka & Somero (1973) and de Zwaan (1977), whereby metabolite levels are involved in the control of pathway flux.

In some marine invertebrates the modulatory effect of phosphagen levels has been associated with rate-controlling enzymes (Storey 1976), as is the case with arginine phosphate, a strong allosteric modulator (via feed back mechanism) which

inhibits PFK in the adductor muscle of the oyster Crassostrea virginica. However, this is generally not the rule in invertebrates and it appears that phosphagen modulation of glycolytic enzymes occurs via the forward activation of fructose biphosphate, the hexose phosphate product of the reaction catalysed by phospho-fructo kinase (de Zwaan 1977, Zammit et al. 1978, Zammit & Newsholme 1978, Ebberink & de Zwaan 1980).

A summary of carbohydrate metabolism is given in Fig. 2.2. indicating the complexity of reactions and interactions that can result in consequence of modulations of these pathways. There are inter-relations with energy producing mechanisms and one cannot over stress the dynamic status quo of the cell, whose disruption at one site may have marked overall effects.

Most ectotherms are unable to regulate their body temperature effectively by behavioural or circulatory-metabolic means, with the result that their body temperature tends towards equilibrium with that of the external environment (Hazel & Prosser 1974).

Despite the similarity between ambient and body temperature, many eurythermal ectotherms can compensate for variations in the thermal range of the environement by altering their physiology and/or biochemistry. These temperature compensations may occur over 3 distinct time courses, as suggested by Somero (1969 a), Somero & Hochachka (1969) and Hochachka & Somero (1973):

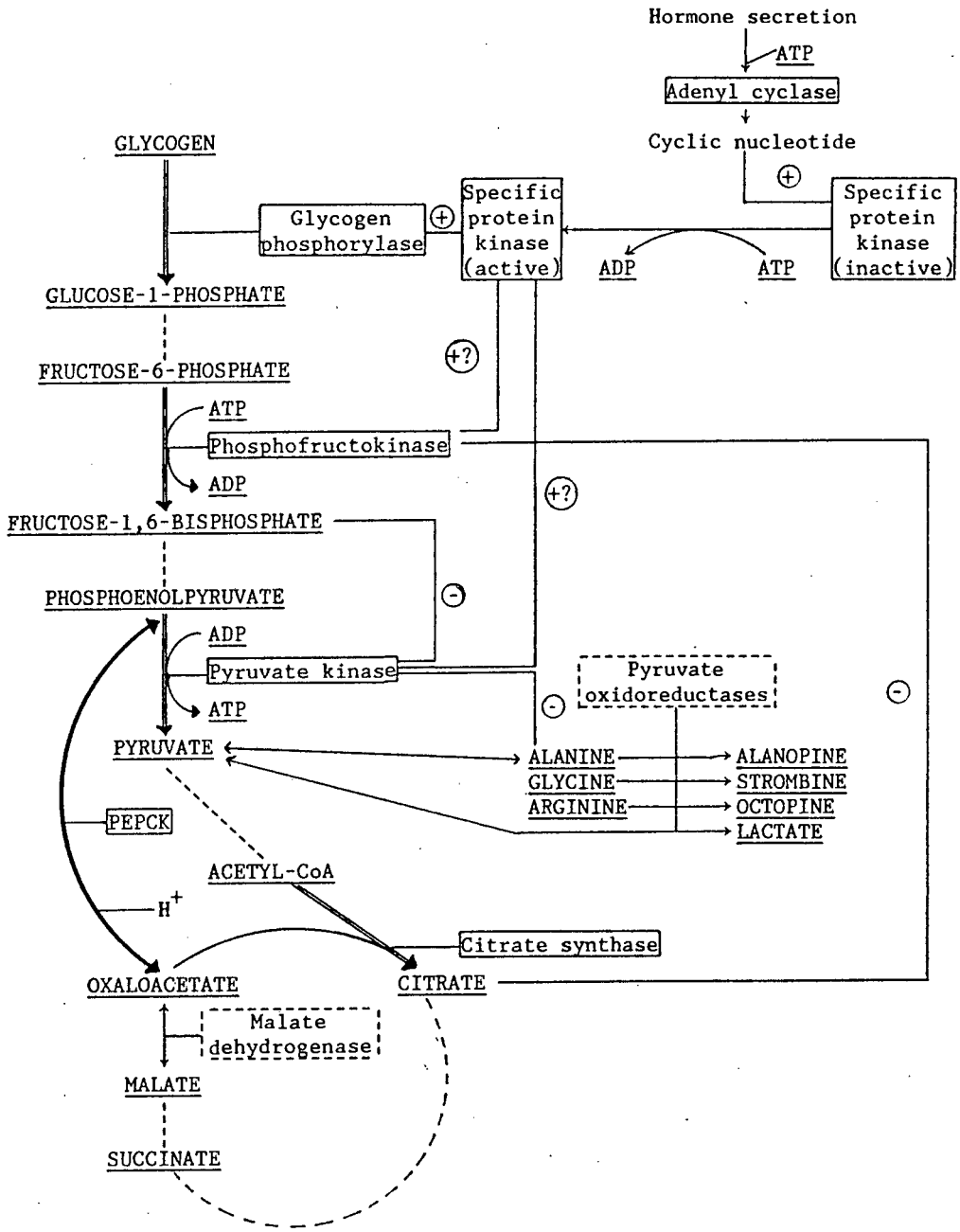


Figure 2.2

A generalised reaction scheme outlining some of the major regulatory influences on potential rate-controlling reactions of carbohydrate catabolism in marine invertebrates. Potential rate-controlling reactions are indicated by heavy arrows and the enzymes catalysing these reactions are named in the adjacent rectangles. Regulatory influences are indicated by the circles containing +, for activation effects or -, for inhibitory effects. Only one mechanism for activation of phosphorylase is included. The pyruvate oxidoreductases vary between species (see de Zwaan & Zurburg, 1981; Livingstone, 1983 for discussions of the rôles and phylogenetic distributions of these enzymes). The pyruvate oxidoreductase and malate dehydrogenase reactions are included to provide an indication of the metabolic routes for formation of end-products of anaerobic carbohydrate catabolism. For clarity the contributions of amino acids to these pathways and the involvement of coenzymes in reactions have been excluded. The reaction catalysed by phosphoenolpyruvate carboxykinase (PEPCK) is considered to have regulatory importance in view of the competition between this enzyme and pyruvate kinase for their common substrate phosphoenolpyruvate (see de Zwaan, 1977 for discussion of these mechanisms).

1. For some poikilotherms, mainly intertidal invertebrates, temperature compensation may be instaneous.

2. The majority of aquatic poikilotherms can acclimate, a process which occurs over several weeks.

3. Thermal compensations may result from exposure to a different thermal regime over many generations and thus develop in the course of evolutionary time span.

2.3.1. Instantaneous temperature compensations

Ectothermic organisms which are unable to control their body temperature often exhibit considerable degrees of temperature independence in their metabolic rates. Whereas one would predict that each 10°C change in body temperature would lead to an approximately 2 fold change in metabolic rate, this is not often observed (Prosser 1973). Some organisms are known to exhibit complete, or nearly complete, temperature-independence in certain processes. Analysis of standard and active rates of oxygen consumption of several intertidal species (sea anemone Acatina equina, whelk Littorina littorea, cockle Cardium edule, barnacle Balanus balanoides and the bivalve Mytilus edulis) disclosed an ability to maintain metabolism at a constant level, over the temperature range encountered in the field (Newell & Northcroft 1967, Newell 1969, Newell & Pye 1970 a,b). This pattern of immediate or instantaneous temperature compensation seems most characteristic of ectotherms which experience rapid and large changes in cell temperatures, e.g. intertidal invertebrates which may be exposed to air during low tide (Newell 1969), but

may also occur in other instances, such as organisms exposed to drastic temperature fluctuations, e.g. the amphipod Gammarus oceanus (Hallcrow & Boyd 1967), the oyster drill Urosalpinx cinerea (Shick 1972), the crayfish Orconectes (Wiens & Armitage 1961) and the land snail Helix aspersa (Newell 1966, 1967), the latter exhibiting marked temperature independence over part of its environmental temperature range. It has been suggested that temperature independence of whole-animal metabolism may result from properties of mitochondria, whereby the Q_{10} of succinate and pyruvate oxidation are lowered (Newell 1967). A reduction of respiratory rates implies economy of metabolic reserves during stress and a low temperature coefficient of metabolism minimises further depletion of reserves at high environmental temperatures (Newell 1973).

Instantaneous temperature compensations are generally achieved by the followimg mechanisms:

(a) Enzyme-substrate interactions

It appears that the most common means of reducing temperature sensitivity of an enzyme catalysed reaction relies on enzyme-substrate (E-S) interactions. A large variety of enzymes of ectotherms are characterised by a direct relationship between assay temperature and the K_m of substrates, such that at reduced assay temperatures less substrate is required to saturate the enzyme (Hazel & Prosser 1974).

The use of K_m as a dissociation or binding constant ($1/K_m$) for the formation of E-S complexes is only valid if reactions are adequately described by the 3 rate constant model of Michaelis-Menten equilibrium i.e. $E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P$, where $K_m = K_2 + K_3/K_1$, and only in some cases when $K_2 >> K_3$ does K_m approximate the dissociation constant for the E-S complex. As long as K_3 is not negligible relative to K_2 or there are discrete chemical intermediates in the pathway from the E-S complex to the product, the kineticly determined K_m is thus the reflection of a complex ratio of individual rate constants that invalidates any simple conceptual interpretation of binding affinities (Berhnard 1968, Harbisson & Fischer 1974, Ferdinand 1976).

Temperature induced changes in K_m may account for the low Q_{10} values characteristic of the standard metabolism of intertidal animals; this is indicated by extension of the range of temperature-independent metabolism observed with homogenates of the winkle Littorina littorea at physiological substrate concentrations (Newell & Pye 1971 a,b). However, Brown & da Silva (1983) found that even at physiological substrate concentrations, oxygen consumption of foot muscle homogenate of the whelk Bullia digitalis was temperature dependent, in contrast to the thermal independence of whole animal metabolism.

The understanding of the effects of temperature on the kinetic properties of ectotherm enzymes, relies on the distinction between two fundamentally different types of molecular bonding forces involved in maintenance of enzyme stability during catalysis (as mentioned above). For example a

specific reaction catalysed by a given enzyme may require the formation and cleavage of covalent bonds, normally strong and stable at physiological temperatures (Barrow 1961). Hence for a covalent bond to turnover at an appreciable rate, in a biological system, a catalyst is generally required. Under saturating levels of substrate the rate controlling step in a reaction mechanism will generally be the rupture of a covalent bond and therefore the Vmax of the reaction will depend on the amount of thermal energy present in the environment. Conversely the establishment of E-S and E-metabolite complexes, in addition to higher levels of biochemical structure including the tertiary and quaternary structure of proteins is stabilised primarily by weak-bonded forces (Hochachka & Somero 1972), and may contribute to temperature-induced changes in K_m. Due to low energy of the weak bonding forces, the bonds themselves are relatively temperature insensitive and the Q_{10} for rupturing such bonds is probably small. Thus, even though a change in temperature may not have a large effect on equilibrium distribution between formed and unbroken weak bond, it may cause alterations in enzyme catalytic function (Hazel & Prosser 1974). It follows that even if temperature induced modifications in protein structure are relatively minor, the shift in functional properties may be considerable. In consequence temperature changes may influence enzyme metabolism in the following manner:

1. Temperature induced modifications of enzyme structure may favour or impede the stable formation of E-metabolite

complex, due to low energy values of bonds involved in reactions, thus easily affected by environmental thermal energies.

2. Temperature may also alter the structure of the enzyme reflected by varying enzyme metabolite affinities as a result of changes in topography of the binding or active site (Hochachka & Somero 1973). Under subsaturating substrate concentrations, velocities are controlled to a large extent by K_m and the effects of temperature on K_m may regulate reaction rates at physiological concentrations. The positive thermal modulatory response of organisms that experience rapid predictable changes in habitat temperature, and in poikilotherms during instantaneous compensation, may result from natural selective pressures to modify the primary enzyme structure (Hazel & Prosser 1974).

Temperature may also affect E-S interactions with respect to substrate specificity. Such effects appear to be particularly important when binding forces between enzyme and substrate are predominantly hydrophobic in nature, as observed in the synthesis of phospholipids of the bacterium Escherichia coli (Sinensky 1971).

(b) Enzyme and branch point modulatory responses

Another factor is the effect of temperature on the interactions of enzymes with a variety of modulatory metabolites (Hazel & Prosser 1974). A positive thermal modulation has been

exhibited by some molluscan enzymes with respect to the effects of temperature on K_m. This was illustrated by the temperature dependence of adenosine deaminases from clams and scallops (Harbison & Fischer 1973), by the linear variation of the apparent K_m for D-isocitrate of NADP-dependent isocitrate dehydrogenase from Mytilus edulis between 5 and 23°C (Head & Gabbot 1980) and by the temperature dependence in Helix pomatia of D(-)lactate dehydrogenase affinity for pyruvate, despite the temperature independence of K_m of the same enzyme for D(-)lactate (Wieser & Wright 1978). In addition the K_m of arginine kinase (of the latter species) for L-arginine increased threefold between 10 and 25°C, though that of pyruvate kinase remained temperature independent, irrespective of temperature. Similar findings have been reported by Hoffmann (1976) on pyruvate kinase of Helix fulgens, Buccinum undatum, Littorina littorea and Mytilus edulis. A detailed study of the thermodynamic and kinetic parameters of octopine dehydrogenase of Pecten maximus disclosed some temperature independent properties, except for the K_m of L-arginine and pyruvate which increased directly with temperature (Luisi et al. 1975). It has been proposed that due to the frequent utilisation of adenylates as common substrates for many enzymes and their involvement in the regulaton of many others, effective metabolic regulation over the range of temperatures encountered by ectotherms would require adenylate interaction to be temperature independent (Hochachka & Somero 1968). Evidence in favour of this is provided by glugoneogenesis in the scallop Placopecten magellanicus, where as a result of cold stress , AMP becomes an inhibitory effector of the diphosphatase function in

addition to an increase in sensitivity of phosphenolpyruvate carboxykinase to AMP activation following minimal thermal change (O'Doherty & Feltham 1971). In squid muscle, the inhibition of malic enzyme by NADPH is enhanced by low temperatures (Storey et al. 1975), though the significance is unknown.

Metabolites have also been observed to exhibit temperature dependent responses. A classic example is that of temperature induced pyruvate inhibition of mudsucker LDH, below 25°C, whereby carbon flow is directed to the Krebs cycle, whereas above 25°C it is channeled into lactate (Somero 1973).

The regulation of metabolism via hormonal processes has grown in recent years, yet lacks a unitarian understanding of the mechanisms involved (Newsholme & Start 1973, Cohen 1978, Schole 1982). Currently the role of cAMP dependent protein kinases in the regulation of enzyme activity by phosphorylation-dephosphorylation reactions is considered to be the major mechanism by which metabolic fluxes in vertebrates are controlled in response to external stimuli (Cohen 1978).

In invertebrates, understanding of the hormonal control of metabolism with respect to environmental effects is in its infancy. Though extensive work has been carried out on the bivalve Mytilus edulis by Ebberink & Salimans (1982), it failed to demonstrate the activation of glycogen metabolism in the adductor muscle via cAMP interconversions of phosphorylases as is known in vertebrates (see Drumond et al. 1969, Mahler & Cordes 1971). Instead, it appears that the active monomer AMP-

phosphorylase complex production and activation is pH dependent. The regulation of PFK by a cAMP directed mechanism has not been established in invertebrates either, due to the high concentrations of nucleotide needed *in vitro* to induce changes, in contrast to the minimal concentrations present *in vivo* (Blackstock 1984). Evidence for cAMP effects on PFK kinetics has been gathered by Storey (1976) on the oyster *Crassostrea virginica*, though the effects were small. Benett & Nakada (1968) also reported some effect of this cyclic nucleotide on PFK of the bivalve *Mytilus californianus* and the abalone *Haliotis rufescens*. A similar effect was observed on the polychaete *Glycera alba* (Blackstock 1984). Investigations of PK in invertebrates has not revealed the typical cAMP mediated phosphorylation-dephosphorylation mechanism observed in vertebrates (Engstrom 1978, Hall *et al.*1979), but it appears that in invertebrates PK is regulated by allosteric activity of adenylates, phosphagens and glycolytic intermediates (Munday *et al.* 1980).

Our poor understanding of hormonal responses to environmental stimuli is due to the dearth of information of the processes involved. This has obviously added to the inability to make correlations between external stimuli and internal responses.

Instantaneous effects of temperature have also been observed at metabolic branch points, whereby the effectiveness of two or more pathways vary with temperature. In a complex multi-enzyme system, at branch point there may be competition for one

common substrate; temperature changes may affect the ability of the various pathways to compete for that substrate differentially, resulting in distinct catalytic rates (Hazel & Prosser 1974).

It is apparent that enzyme-temperature relationships are complex and intrinsically related to each other. Temperature not only affects Michaelis-Menten constants but also strongly influences pH levels (Somero 1978, 1981). Irrespective of the validity of a direct temperature-K_m relationship in certain instances, this may not be general. Natural selection may have allowed the development of enzyme species that are able to maintain ligand-binding properties at relatively constant levels despite environmental changes (Somero & Low 1977). These mechanisms have been discussed by Somero (1978) and Somero & Yancey (1978).

(c) Conformational changes

A different mechanism of immediate compensation has been reported for pyruvate Km of the Alaskan King Crab. This is an example of temperature dependent conformational change which results in the interconversion of 2 forms of the enzyme, each represented by a distinct set of kinetic properties (Somero 1969 b), above and below 10°C. Consequently above and/or below a certain critical temperature, a specific protein could display distinctly different functional properties. Such a mechanism for the instantaneous development of enzyme variants would give the organism two fundamentally distinct populations of enzyme

catalysts, each functioning optimally within a narrow independent regime, avoiding the disadvantageous long periods involved in the synthesis of a new enzyme (Hochachka & Somero 1973). However, no such mechanism have been reported in molluscs.

The maintenance of complex isozyme systems (simultaneous occurrence of enzymes catalysing the same reaction, but with differing kinetic properties) may extend the thermal range over which favourable modulatory interactions of the various isozymes can occur, resulting in instantaneous compensations.

2.3.2. Mechanism of thermal acclimation

When ectotherms are exposed to fairly lengthy periods of thermal stress, adaptive strategies are developed which allow the organism to exhibit similar rates of physiological activity despite widely different environmental temperatures. This process is acclimation (Prosser 1973).

Acclimatory responses demand a relatively long time span to become operative and are characterised by marked changes in the level of general protein synthesis (replication enzymes included) (Haselkorn & Rothman-Denes 1973). This may indicate that physiological acclimation involves feed back to the genome following the relays to the protein synthesising machinery. Such compensation thus reflects environmentally induced phenotypic variation, intrinsically related to the genetic make-up, which

cannot be altered by environmental stimuli (Prosser 1964). Therefore the phenotype is dynamic rather than static and may be markedly influenced by environmental physical factors (within limits established by the genome). In consequence warm and cold acclimated populations of a species would differ directly in the specific part of the genotype being expressed (Hazel & Prosser 1974). But acclimated populations may also differ in the rate at which a gene is being read or in the rate of degradation of the gene product (Vesell & Yielding 1966), such that the steady-state level of that gene product varies with the state of acclimation.

Seasonal acclimation in the pond snail Lymnaea stagnalis has been demonstrated for the heart rate (Harrison 1977), as winter animals show higher rates than summer ones, between 15 and 25°C; similar results were reported for the limpet Acmaea limatula (Segal 1956). Mechanistic implications of the relationship between function and temperature have been discussed by Gersch (1969), Lagerspetz (1968, 1974) and Vislobokov (1975). The effect of energy intake on temperature has been examined by Calow (1975) in the fresh water gastropods Ancylus fluviatilis and Planorbis contortus, whose absorption effeciencies were found to be temperature independent and the respective Q_{10} suggested an acclimatory response. In contrast, Widdows & Bayne (1971) reported a reverse temperature dependence in absorption effeciency of Mytilus edulis. Contrary to the findings in Littorina and Mytilus, which show seasonal changes in metabolism, Polinices duplicatus (an intertidal prosobranch) is characterised by temperature independent metabolism (Huebner 1973). In the

terrestrial snail Arianta arbustorium acclimation to different temperatures has negligible effects on oxygen consumption, both of intact animal and isolated tissues (Wieser et al. 1970) and a similar effect was observed in Donax vittatus (Ansell & Sivadas 1973).

In the field the organism reacts simultaneously to a multitude of factors, which for obvious reasons are difficult to simulate in the laboratory. Attempts to bridge the gap between laboratory and environmental conditions have been made by Bass (1977), by studying in conjunction the effect of temperature and salinity on respiration of the oyster Crassostrea virginica, results being more complicated than expected. Furthermore, when warm and cold acclimated Mya arenaria were subjected to varying temperatures and salinities, a change in the usual resposes occurred, suggesting that the compound effect of these two factors may significantly alter responses to one factor only (Dupaul & Webb 1970).

Changes in metabolic responses to temperature can be induced by starvation or hypoxia level (Davis 1967, Newell & Bayne 1973, Newell et al. 1978). These oxygen responses are often reflected by mitochondrial function and respiration, over the same thermal range (Newell & Pye 1971 b). Cell free homogenate of Littorina litorea acclimated at different temperatures in the laboratory, revealed the same capacity to become temperature independent coinciding with the temperature of acclimation in vivo (Newell 1973, Pye & Newell 1973). Evidence in favour of

changes in enzyme-substrate affinity accounting for temperature independence of metabolism of cell free and mitochondrial preparations has been gathered by Newell & Pye (1971b).

Widdows & Bayne (1971) have developed an "energy balance" equation as an index of the animal's responses to environmental change. The integration of the various physiological functions into an index of energy balance is useful as an indication of whole animal metabolism.

Mytilus edulis possesses compensatory mechanisms that allow adjustment to constant as well as cyclic temperatures (Widdows 1976). These refer especially to filtering rates.

Despite the rich literature on acclimation mechanisms, there is little information on molluscan adjustments to new temperatures (Hoffmann 1983). Changes have been observed in pyruvate kinase of Helix pomatia (Hoffmann 1976) and on succinated dehydrogenase (Precht et al 1973), lactate dydrogenase (Wieser & Wright 1979) and on some proteases (Mews 1957) of the same species, as well as on pyruvate kinase of Mytilus edulis (Camaselle et al. 1980), on malate dehydrogenase of Acmaea limatula (Markel 1976) and on alkaline phosphatase of Pila globosa (Brahmanandam 1976). A number of observations have also been made with respect to seasonal acclimation and reproductive cycles (see Livingstone 1981, Gabbot & Head 1980).

(a) Regulation of gene expression

Enzyme induction involves synthesis of an m-RNA precursor (Schimke and Doyle 1970) through gene regulation. This regulation can operate at 2 levels, one more immediate at the level of enzyme induction, the other involving inheritance of modified enzymes (through alteration of the coding genome). Both processes may be affected by post-translational modifications (Kacser & Burns 1981).

Only in recent years has the complexity of gene expression been appreciated (Hunt 1980, Caskey 1980). The possibility of silent genes has been raised by Currie & White (1981), as genes whose induction is purely a function of the type of stimulus received. These genes can be expressed fairly fast (1 to 1.5 hours) and have been found to be induced by heat shock (Hammond et al. 1982).

Protein modifications have often been associated with environmental temperature changes and have been extensively studied (Hochachka & Somero 1968,1973; Hazel and Prosser 1970, 1974; Somero 1973). These investigations suggest that enzymes and isozymes can be induced in the course of acclimation in such a way that different properties are selected and exhibited depending on the external conditions. It has been suggested that time spans may be too short to allow enzyme compensatory mechanisms to develop and that post-translational modifications have been underplayed (Somero 1973).

Enzyme regulation by quantity

Thermal acclimation may be mediated by temperature induced alterations in specific enzyme concentration. A large number of enzymes exhibit compensatory changes in maximal velocity in response to temperature stress, though none have been reported in molluscs. However, despite a wealth of data involving catalytic activity studies, few clearly indicate quantitative enzyme changes. One involves the fatty acid desaturase of Bacillus megaterium (Fulco 1972) and the other immunological studies of cytochrome oxidase following cold acclimation of gold fish muscles (Wilson et al. 1973). This effect was demonstrated by: (i) accelerated rate of protein synthesis in the cold; (ii) a zero-order decay of protein synthesising system when exposed to temperatures above 20°C; (iii) an irreversible first order inactivation of the enzyme system at higher temperatures.

Regulation of enzyme types - isozymes

Instead of producing more copies of the same enzyme at low temperatures, an organism can generate qualitatively different molecules, with better catalytical properties than the equivalent warm acclimated enzymes. This demands that the acclimatory process be characterised by a major biochemical restructuring of the organism, such that more adequate molecules are produced to ensure biological function in the force of changing environmental conditions (Hochachka & Somero 1973, Somero 1972). Synthesis of specific isozymes with well defined thermal limits has been reported for acetyl cholinesterase of trout brain (Baldwyn & Hochachka 1970), isocitrate dehydrogenase

from trout liver (Moon & Hochachka 1971, Whitmore & Goldberg 1972) and glucose-6-P-dehydrogenase from the lungfish (Hochachka & Hochachka 1973). Though it has been suggested that some variation may result from genetic polymorphisms, it is not clear the extent to which polymorphic variation contributes to thermal acclimation (Hazel & Prosser 1974).

In the examples given, cold and warm isozymes were considered ideally suited to function at their acclimation temperature because each enzyme variant exhibited a minimum in the K_m-temperature response at assay temperature corresponding to the acclimatory temperature at which that isozyme prevailed. Most enzymes for which kinetic data are available are characterised by a U-shaped K_m-temperature response. At assay temperatures above the temperature of minimum K_m, there is a direct relationship between ambient temperature and K_m. Because the temperature of minimum K_m (range over which positive thermal modulation occurs) depends on isozyme type, the environmental temperature range over which temperature independent catalysis occurs may change as a function of acclimation, since isozyme presence depends upon acclimation temperature (Somero 1972). Hence different isozymes, with different K_m-temperature responses, may account for the effect of acclimation that results in regions of temperature independent metabolism coinciding with the prevailing ambient temperature. In contrast, at temperatures below the temperature of minimal K_m, there is generally marked increase of this parameter (decrease in affinity), which together with the reduced kinetic energy at low assay temperature results in a strong

negative modulatory effect of enzyme activity. An increase in K_m of each enzyme on either side of the region of minimal K_m, implies rather restricted thermal working range, being essentialy inactive outside of this range by virtue of an extremely diminished substrate affinity (Somero 1972).

If minimal K_m coincides with average ambient temperature, a single isozyme cannot function optimally over a wide temperature range. K_m values of warm and cold acclimated enzymes would be so high on either side of the optimal temperature, that the rates of catalysis would be relatively insensitive to changes in substrate concentration, rendering the enzyme incapable of varying its catalytic rate as well as responding to modulatory substrate levels changes, a great dis vantage to the organism (Somero 1972).

It was stressed by Hazel (1972 a,b) that minimal K_m values and U-shaped K_m-temperature responses appear to be confined to isozymes, whereas other enzymes such as succinate dehydrogenase (Hazel 1972 a,b), PFK (Freed 1971), glyceraldehyde-3-P dehydrogenase (Cowey 1967, Greene & Feeney 1970) and choline acetyl transferases (Hebb et al. 1969) are characterised by a linear dependence of assay temperature over a wide temperature range. Moreover, the slope of the K_m-temperature response for these enzymes is not as steep as that reported for many isozymes (Somero & Hochachka 1969). Therefore, the slope of K_m-temperature curves for enzymes with a single molecular form is shallow enough to permit the enzyme to function throughout the entire

temperature range and yet steep enough to impart a certain degree of temperature independence. These differences in K_m-temperature characteristics of enzymes, probably reflect fundamental differences in primary structure and hence are genetic in origin, perhaps reflecting separate trends in natural selection of primary structures which result in distinct temperature independent catalytic function.

From the above work it was concluded that acclimation could involve the induction of enzymes, isozymes and modulation of enzyme properties. Hochachka & Somero (1973) have suggested that enzyme induction may be too slow to compensate adequately for the undesirable effects of radical temperature change and that in the short term non-genetic modifications may be more relevant, their role having been underestimated. This view is supported by some recent investigations of Thébault *et al.* (1980), who showed that substrate affinity of LDH of the tail of the shrimp *Paleamon serratus* (acclimated at 12°C) could change as a function of the period of time to which it was subjected to a higher temperature (20°C). Since the time required for acclimation was approximatelly 3 weeks, these authors suggested that enzyme induction was involved in the acclimation process and that more rapid diurnal changes in temperature may be compensated by conformational changes in enzyme structure.

Evidence collected from heat shock experiments on the other hand, suggest a more rapid genetic response. For instance, when the polychaeta *Scolelepsis fuliginosa* was subjected to a temperature increase of 7°C (in 10 minutes), synthesis of

additional esterase isozymes, distinct from those previously existing, occurred within one hour (Guerin & Kerambrun 1979). A similar effect has also been observed in esterase isozymes of Paelemon serratus larvae following heat shock (Trél lu et al. 1977 a, b, 1980).

(b) Secondary consequences of altered gene expression

So far only primary responses to environmental change have been considered in physiological adaptation, but other modifications could also occur. Alteration in protein and catalytic properties may give rise to certain modifications within their local micro-environment, such that associated processes are also affected, even though not in direct response to environmental change. These could include changes in the composition and type of membrane lipids and would be termed secondary effects of differential gene expression.

In order for proteins to change shape or assemble correctly, a "semistable state" is functionally important. Likewise, lipid based systems are characterised by a semistable structure. Many lipid systems are described by a"liquid-crystaline" state, a state which appears to have been conserved throughout evolution. These fundamental lipid properties and their interactions have been termed "homeoviscosity" by Sinensky (1974).

The degree of lipid unsaturation appears to increase with decreasing environmental temperature, as reflected by

changes in the physicochemical properties of membrane function. The most obvious of these effects is on membrane permeability. In consquence, the nature of the lipid milieu in the microenvironment of a membrane bound enzyme may markedly influence its catalytic activity. It is tempting to speculate that environmentally induced alterations in membrane structure not only maintain an appropriate membrane fluidity , but in addition may also modulate the activity of membrane bound enzymes during thermal acclimation (Smith & Ellory 1971).

Examination of succinic dehydrogenase from the inner mitochondrial membrane of gold-fish muscle revealed it to be more unstable in its lipid free form. When the total mitochondrial lipid extract from cold acclimated fish was added, the enzyme stability increased significantly in comparison to that which was reconstituted with warm acclimated lipids. This was interpreted as an indication that the specific activity of the enzyme may be partly or entirely explained by fundamental changes in membrane composition, rather than in quantity or quality of enzyme (Hazel 1972 a,b). It was further proposed that the fluidity of fatty acyl chains of membrane phospholipid may confer motional freedom, which allows enzymes of these membranes to undergo conformational changes and movements associated with their activity (Kimelberg & Paphadjoupoula 1972).

Apart from the main biochemical restructuring of an organism as reflected in qualitative and quantitative differences of catalytic proteins and major changes in membrane structure associated with thermal acclimation, additional changes in the

intracellular environment of a secondary nature may influence a variety of catalytic and cellular functions. One such factor is intracellular pH. The intracellular pH of poikilotherms may increase following cold acclimation, and enzymes that exhibit pH dependencies within the critical pH range (6.5-8.0) would be subjected to pH regulation or modulation (Hochachka & Somero 1973). Examples reported include activation of succinate dehydrogenase at low temperatures by pH fluctuations, (Hazel 1972 a,b), enhanced substrate affinity of citrate synthesase (Hochachka & Lewis 1970) and the extreme sensitivity of PFK of gold-fish muscle to pH changes of the order of 0.1 pH unit (Freed 1971).

A further possibility involves temperature induced alterations in inorganic cation concentrations (which are cofactors to many enzymes (de Zwann & de Bont 1975, de Zwann et al. 1975), as these may have modulatory enzyme functions. This appears to be true of divalent cations (Mg^{2+}, Ca^{2+}) which are more temperature sensitive than the monovalent counterparts (Houston & Madden 1968, Houston et al. 1968, Umminger 1969 a,b). Furthermore it is possible that temperature induces changes in metabolite and co-factor concentrations, that may also be involved in regulation of catalytic function either via allosteric interaction or directly by altering the extent of enzyme saturation (Freed 1971, Baslow 1967).

Though most thermal acclimation data have been concerned with qualitative description of the type of

physiological adaptation inducible by thermal stress, the nature of the primary inducing stimulus remains unclear (Blackstock 1984). It is possible that the thermal environment of the organism may be sensed by the central nervous system and that peripheral temperature adaptation subsequently affected by the release of relevant neurotransmitters or trophic agents, may in turn function as inducers or repressors of gene expression (Hazel & Prosser 1974). Nicaise & Amsellem (1983) described in detail the nature and types of molluscan muscular neurotransmitters but no attention was paid to stimulation by external environmental signals.

Lastly the possibility cannot be excluded that certain acclimation effects may result from a manifestation of prolonged direct effect of temperature without necessarily involving feedback regulation of the synthetic machinery. Non-genetic temperature induced synthesis of specific isozymes in molluscs has been reported for: pyruvate kinase of Mytilus edulis (Livingstone & Bayne 1974), malate dehydrogenase of Acmea limatula (Markel 1976), lactate dehydrogenase of Cepaea nemoralis (Gill 1978) and of Helix pomatia (Wieser & Wright 1978) and α-amylase of Choromytilus meridionalis (Seiderer & Newell 1979).

This involves accomodation for changes in the environment so as to maintain survival capacity. Considerable information exists on acclimation with respect to changes in the rate of controlling enzymes associated with energy yielding metabolism of marine invertebrates. Little information is however available on the evolutionary consequences of metabolic regulated

acclimated response, though such knowledge is fundamental to the understanding of adaptation of marine organisms to their environment (Blackstock 1984). This is not to understate the complexity of the problem involved in such studies together with the theoretical and technical difficulties of distinguishing between molecular and genetic regulatory responses of an organism to a changing environment.

2.3.3. Compensations to temperature variation on an evolutionary time scale

Adaptive regulatory responses to environmental changes arise over evolutionary rather than ecological periods and are considered to be achieved by genetic selection in generations following those exposed to long term environmental changes (Blackstock 1984).

Electrophoretic analysis of separable enzyme variants have been used as genetic markers in the assessment of genetic structure of marine invertebrates, the Mollusca having received considerable attention (Kohen & Mitton 1972, Berger 1973, Snyder & Gooch 1973, Gaines et al. 1974, Murdock et al. 1975, Campbell 1978, Lassen & Turano 1978, Levinton & Suchanek 1978, Buroker et al. 1979, Goshling & Willkins 1981). Other invertebrate groups, such as crustaceans (Schopf 1974, Tracy et al. 1975, Nelson & Hedgcock 1980, Bulnheim & Scholl 1981), echinoderms (Marcus 1977) and coelenterates (Walsh & Somero 1981) have also received attention.

Despite its frequent use, electrophoretic analysis is not without its problems. Interpretation of data depends on allele frequency and population size (Boyer 1974, Chaisson et al. 1976), associated with seasonal changes as well as natural biorhythms (Flowerdew & Crisp 1976, Kohen & Immerman 1981). In addition ambiguities have resulted from different schools of thought on heterozygosity. For instance Kimura (1979) suggested that heterozygosity reflects the neutrality of variants, an argument disputed by Koehn et al. (1981). However, the adaptive significance of electrophoretic differences is still little understood (Milkman 1978, Burton 1983) and the correlation between observed changes and phenotype survival or environmental exploitation demand further investigation (Blackstock 1984).

Electrophoretic results are further complicated by the method and buffer employed, due to variations in: pH, molecular sieving properties, molarity, molecular charge, factors which interfere directly with the protein separation and variant expression (see Brewer (1970) for more complete appraisal of problems). Contrary to expectations, protein changes in amino-acid composition may not alter the net charge on the enzyme (Ferguson 1980). Thus, in order for a significant effect to be noticed on the charge of the protein, post-translational modifications may be necessary. These are often a function of a cellular "steady-state" in response to external stimuli and may not be detected eletrophoretically (Moss 1979).

(a) Thermal compensation in metabolism and oxygen comsumption

The metabolism-temperature curves (oxygen versus assay temperature) for some Artic fish and crustaceans have been shown to be displaced towards lower temperature, compared with those for tropical ectotherms; extrapolation of curves, for tropical fish, below lethal temperature, revealed that the latter were typically below those of Arctic species, suggesting a clear elevation of the metabolic rate of Arctic fish at lower temperatures (Scholander et al. 1953). These compensations did not manifest a general capacity of the "cold" species to acclimate to cold, as it became apparent that markedly stenothermal species have lost the ability for acclimatory compensation (Somero et al. 1968)). Thus organisms exposed to a constant cold environment appear to be highly adapted to function at these temperatures, but have apparently lost thermal acclimation ability (Hazel & Prosser 1974).

(b) Thermostability

A wealth of evidence in support of the concept of evolutionary adaption to specific thermal environments has been obtained from studies of tissue thermostability.

Molluscs have been extensively studied in this respect (Hoffmann 1983) and as early as 1936 studies on the oxygen consumption of bivalves revealed that species endemic to northern

Europe comsumed more oxygen (irrespective of temperature) than the equivalent in the Mediterranean zone (Spärck, 1936). Evidence in support of evolutionary adaptation to specific thermal environments was reviewed by Skoog (1976), with special reference to thermal tolerance and thermostability studies. Wallis (1975) found an inverse relationship between the temperature of 50% incapacitation and geographical latitude in Mytilus edulis of eastern Australia, concluding that tolerance declines with increasing latitude. Similar relationships have been observed by Schlieper et al. (1967) for the same bivalve. Furthermore, it appears that thermostability in molluscs can vary appreciably without corresponding changes in heat stability of tissue cell or proteins (Arronet 1959, Konev & Burtseva 1970). In contrast interspecific comparisons of cell thermostability of homologous tissues from allied species always correlate well with environmental conditions (Ushakov 1964). Cells and macromolecules of ectothermes that inhabit warm environments tend to exhibit greater heat resistance than comparable cells of organisms inhabiting cooler environments (Evans 1948). Theede & Lassig (1967) tested the cellular resistance of four euryhaline bivalves (Cardium edule, Mya arenaria, Macoma baltica and Mytilus edule) from the North Sea, the Belt Sea and the Gulf of Finland, each of which have different salinities and temperatures. Bivalves from the Gulf of Finland (lower salinity) had lost their ability to survive freezing, though this was preserved by truly marine species. Rigby & Mason (1967) reported good correlations between heat and stability of collagen fibres of several marine and terrestrial gastropods, at temperatures encountered by the

species in the field. Generally proteins from organisms that inhabit warm environments also have a greater resistance to heat than those from organisms in cold environments (Hoffmann 1976). The basis for this variation is obviously genetic because long periods of temperature stress in the laboratory do not change the interspecific differences in protein thermostability (Oliver et al. 1971).

Heat stability or conformational flexibility of enzyme molecules depend not only on temperature, but also on surrounding interacting factors (Alexandrov 1977). Moreover it not possible to predict temperature responses of tissue specific isozyme, nor their significance. In the pulmonate mollusc Biomphalaria glabrata, for instance, the malate dehydrogenase variant present in albumen gland is the most thermolabile MDH of all organs (Narang & Narang 1974), though the significance of this is unknown.

Comparisons of muscle thermostability from populations of the same species occupying different thermal habitats showed no compensatory adjustments, suggesting that cell thermostability in ectotherms is a conservative species-specific characteristic. On the other hand, comparisons of cell thermostability of homologous tissues from allied species displayed a positive correlation with environmental conditions (Ushakov 1964). Thus, tissues of poikilotherms that inhabit warm environments are characterised by greater heat resistance than

the comparable cells of organisms inhabiting cooler environments. This may be an illustration of thermal adaptation on an evolutionary time scale.

Correlations between heat resistance of protein and the cells from which they were obtained, suggest that heat damage and cell death may be a consequence of the denaturation of protein complexes. Hence heat resistance of a cell is limited by the thermostability of its least heat resistant protein system (Ushakov 1964). Thermostability of the mitochondrial enzyme succinate oxidase, MDH, NADH dehydrogenase, MAO and cytochrome oxydase did not correlate with the phylogenetic history of the species, but rather with the thermal environment to which the species was adapted (Smith 1973 a, b, c). However it has been proposed that positive correlations between protein thermostability and the thermal environment do not necessarily validate a cause and effect relationship. Moreover, at least in bivalves, the possession of heat stable proteins appears to be correlated with ability to survive anaerobic conditions rather than environmental temperature alone (Read 1967). Furthermore it appears that proteins and their thermostabilities are species-specific characteristics, contrasting with whole animal stability, in that it is seldom that these properties can be modified by thermal acclimation (Ushakov 1964).

(c) Mechanisms of evolutionary adaptation to temperature

Enzymatic compensations

To date there is no systematic investigation of the specific activities of various enzymes from species of varying thermal environments. Attention has focused rather on the specific catalytic properties of enzymes that may be better adapted for catalysis under specific temperature regimes.

A balance must exist between structural stability of an enzyme and its catalytic efficiency in order for positive temperature modulation of enzyme-substrate affinity and reduction of free enthalpy of activation to be considered as a process of catalytic control (Hochachka & Somero 1973). Values of $H^{\ddagger}$ (enthalpy of activation) for homologous muscle pyruvate kinase of Haliotis fulgens, Buccinum undatum, Littorina littorea, Helix pomatia and Mytilus edulis correlated well with the habitat temperature of these species. In addition a direct relationship was found between the ratio of $H^{\ddagger}$ (10°C) to $H^{\ddagger}$ (30°C) and the variability of body temperature by these species (Hoffmann 1976). Evolutionary adaptation of enzymes to temperature has also led to the selection of minimal K_m values at temperatures that coincide closely with that of habitat temperature (Carrión et al. 1978). The minimum clearly defines a U-shaped or J-shaped K_m temperature plot in stenotherms (H.fulgens) and broadens to form a plateau in eurythermal organisms (L. littorea, B.undatum, M.edulis and H.pomatia). Variability in protein primary structure is supposed

to provide the avenue for natural selection of adaptive molecular properties (Hoffmann 1983).

Somero (1969a) suggested that in cold adapted ectotherms, natural selection would favour enzymes capable of lowering the activation energy for a given reaction; therefore in systems where only limited thermal energy is available , enzymes that are highly effective in reducing such energy barriers would seem advantageous. For some enzyme systems there is a direct relationship between habitat temperature and activation energy including: pyruvate kinase from tuna, trout, shrimp, king crab and Trematomus (Somero 1969a); fructose diphosphatase from lungfish and rainbow trout (Behrisch & Hochachka 1963a, b) and glyceraldehyde 3-phosphate dehydrogenase from rabbit and Antarctic fish (Green & Feeney 1970). In accordance with this hypothesis, glycolytic and pentose shunt enzymes (LDH, triose phosphate dehydrogenase, glucose-6-phodphate dehydrognase and 6-phosphogluconate dehydrogenase) of the yellow fin sole and tanner crab from the Bearing sea were characterised by low Ea values (Behrisch 1972). However care should be exercised with these results since although such a correlation exists for certain enzymes, it is by no means universal (Hochachka & Somero 1971). The disadvantage of this hypothesis is that it measures activity enthalpy ($\Delta H^{\ddagger}$) rather than the free energy of activation ($\Delta G^{\ddagger}$), neglecting changes in the activation entropy that may contribute significantly to $\Delta G^{\ddagger}$, since this is the determinant of reaction velocities. A more reliable criterion for comparing enzyme efficiency should be substrate turnover numbers, which are a more

direct measure of catalytic efficiency (Somero 1972). Comparisons of substrate turnover number for glyceraldehyde 3-phosphate dehydrogenase from rabbit, lobster and cod, for phosphorylase b from rabbit and lobster, and for LDH fom rabbit, chick, tuna and halibut have indicated that enzymes from ectothermes are considerably more efficient than homologous enzymes from mammals (Low et al. 1973), though such measurements are not available for the majority of ectotherms. It was suggested that natural selection may favour minimal activation energy (maximal turn over number) to coincide with temperature of normal catalytic function (Hochachka & Somero 1984) .

Evolutionary adaptation of enzymes to temperature may have also allowed selection of minimal K_m values at temperatures that coincide closely with those of habitat temperature. Hence, in the case of LDH from Trematomus the minimal K_m for pyruvate occured at 0°C (Antarctic fish), whereas the minimum Km for the lung fish was between 30 and 34°C (habitat temperature 30°C), while trout exhibited a minimum K_m value at intermediate temperatures (Somero 1969a).

Evolutionary adaptation has however failed to maintain a consistent effect on the absolute value of K_m. Low K_m values are not always predominant in the cold, as demonstrated by the absolute K_m values for Trematomus LDH which were consistently higher than those of lungfish enzymes, regardless of the fact that the former exhibited a minimal K_m-temperature response at lower assay temperatures. It was therefore concluded that

absolute K_m values may be selected in such a way as to allow the rate of catalysis to vary optimally in response to changes in physiological substrate concentrations rather than to establish thermal compensatory rates of activity (Hochachka & Somero 1973, Somero 1969a).

Alterations in the primary structure of proteins

Structural and catalytic properties of protein, such as optimal temperature, temperature of thermal denaturation, substate specificity, catalytic efficiency and temperature dependence of kinetic constants are determined by the genetic information encoded in the primary structure of all proteins (Kacser & Burns 1981). In terms of thermostability organisms that inhabit a cold environment for generations may have experienced mutations causing some of their proteins to become relatively heat labile; such mutations would not be lethal unless the organism were transfered to a much warmer environment. It is therefore not surprising that variations in protein functions associated with adaptation to a particular thermal environment may not be correlated with genetically determined differences in amino-acid composition and sequence.

Examination of protein variants from thermophilic microorganisms (optimal temperature for growth occurs above 45°C) indicates that hydrophobic interactions (maximally stable at 60°C) may contribute to the thermal stability of proteins. In several cases, proteins from thermophiles contained higher levels

of hydrophobic amino acids than homologous proteins of mesophiles (optimal temperature for growth is between 20 and 45°C) and many thermophilic enzymes underwent a conformational change in the region from 45 to 55°C without loss of activity. This conformational change (at temperatures in the vicinity of maximal stability of hydrophobic bonds) may be due to a "melting" of the hydrophobic cluster, thus enhancing protein stability without causing a gross change of the properties of the active site (Singleton & Ameluxen 1973). Differences in the thermostability of aldolase from thermophilic bateria and glyceraldehyde-3-P dehydrogenase from Antarctic fish and the corresponding rabbit muscle enzyme, were not detected in total amino-acid composition, though structural differences could be related more to changes in amino-acid sequence than composition (Hazel & Prosser 1974), which determines the folding characteristics of a protein.

2.4. CONCLUDING REMARKS

It appears that immediate temperature compensation in response to rapid thermal changes is a function of the direct effect of temperature on the physical and kinetic properties of the macromolecules, including enzyme interactions with substrate, effectors and modulators, as well as induction of conformational changes of proteins involved in branch points which may control metabolic flux.

Acclimation on the other hand is attained by the development of compensating adjustments in the physiology and

biochemistry of the organism concerned, while maintaining a steady-state of structure and function. This process can be achieved by three mechanisms, namely qualitative and quantitative alterations of enzymes and interactions of either with the surrounding environment. Acclimation is a semi-long term process requiring a few weeks to become established, through the involvement of alterations of the protein synthesising machinery.

Evolutionary adaptation is a process which occurs through various generations of an organism continuously exposed to a constant thermal regime. This may have given rise to natural selection of certain proteins, whose physical and catalytic properties allow an enzyme to function optimally at extreme thermal regimes, leaving no capacity to adapt to other thermal environments. Variation in protein primary structure is the vehicle of these adaptive mechanisms which are intrinsically dependent on genetic expression. Thus alterations in protein features can occur only via genome reorganisation.

CHAPTER 3

THE GENETIC PROFILE OF THE SANDY BEACH WHELK *BULLIA DIGITALIS* THE SIGNIFICANCE OF ENZYME VARIATION

INTRODUCTION

The genetic structure of gastropod populations is highly dependent on life history characteristics, which bear directly on the mode of larval dispersal. Gastropods such as *Littorina littorea* (Berger 1973) and *Nassarius obsoletus* (Gooch *et al*. 1972), which spawn into the sea, developing a pelagic larva, show little geographic genetic differentiation. In contrast, intertidal gastropods such as *Littorina saxatilis* (Snyder & Gooch 1973) and *Nucella (Thais) lamellosa* (Campbell 1978; Grant & Utter, unpublished), lack a pelagic larval stage and show greater genetic differences among populations. However, the dominant influence of larval dispersal on genetic population structure can be modified by other life history features. For instance, Johnson & Black (1982) have shown that patterns of recruitment in the intertidal pulmonate, *Siphonaria*, (which has a pelagic larva), result in genetic differences over small distances in the absence of large-scale geographic differentiation among regions.

The whelk *Bullia digitalis* is remarkably well adapted to its unpredictable environment (da Silva *et al*. 1985). It occupies high energy sandy beaches over the southern african

coastal range which extends from Namibia (west coast) to the Transkei (south coast) (Figs. 1.1 and 3.1., Table 3.1) (Kilburn and Rippey 1982). Within this range, the west coast is known for its climatic variability and erratic food supply (Brown 1982), factors which nonetheless have not inhibited the successful exploitation of this environment by the whelk.

Populations are restricted to relatively gently-sloping beaches with fine to medium sand, isolated from one another by intervening streches of rocky shores. The reproductive cycle is initiated during late spring or summer (southern hemisphere), the female possibly mating with several males. Gametogenesis occurs between March and May, vitellogenesis and egg storage taking place from June or July until December or January, when spawning begins (see Brown 1982). Clumps of eggs can be contained in a single large sheath and deposited in the sand, or each clump of 150 eggs or more may be contained in its own capsule and held on the ventral surface of the maternal foot (da Silva & Brown 1985). Embryonic development takes place over the next few weeks and there is no pelagic larval stage. Instead the juvenile whelks hatch from the egg capsules as miniature crawling adults (Brown 1982). Thus, the reproductive cycle in this species is possibly crucial in the determination of future population trends, suggesting that descrete and isolated whelk populations may occur.

In this study the genetic structure of populations of four species of *Bullia*, namely *Bullia digitalis*, *Bullia*

rhodostoma, Bullia pura and Bullia laevissima, located on the west and south coasts of South Africa, in the vicinity of the Cape Peninsula, was examined using inherited biochemical variants detected by eletrophoresis. Samples collected ranged from Lambert's Bay on the west coast to the Strand on the south coast, spanning 400 Km of shore. A significant change in the intertidal marine fauna occurs between these two coasts, that is mainly due to differences in sea water temperature (Stephenson 1948). Any differences due to geographical isolation may be enhanced by selection for alternate forms in the two faunal domains. The gene products of 33 loci were identified, 14 polymorphic, of which six were sufficiently polymorphic to test hypotheses of geographic structure. If gene flow between sites is restricted by intervening barriers of rocky shoreline, while larval stages are suppressed, then genetic differences among populations may be expected.

The correlation of enzyme function with environmental events is a most important yet often negleted aspect (Blackstock 1984); though to expect physiological adaptations to result directly from the effect of the environment on the genome is naive for gene activation can be a lengthy process and require more than one triggering event (Hunt 1980). Survival, during changing environmental conditions, could depend on a pre-existing adaptive mechanism reflected by certain enzyme properties, such as enzyme variants.

Biochemical differences amongst allozymes have often been reported (Place & Powers 1979, Koehn & Siebellaner 1981, McDonald et al. 1981, Hoffman 1981), though seldom have the physiological implications been demonstrated (Powers et al. 1979, Cavener & Clegg 1981, Koehn et al. 1980). Their role in adaptation to a varying environment has been the subject of much debate (Levins 1968, Willis 1973, Somero & Soule 1974, Somero 1975). The importance of natural selection in maintaining heterozygosity in natural populations has been stressed by Koehn (1983) as a pre-requisite for successful biochemical adaptation. This has been opposed by neutralists (Ayala 1975), who advocate that point mutations are random, yet may have adaptive value.

Examination of genetic profiles may reflect the latent ability of a cell to modify its physiological response to a changing environment. By relating the observed enzyme patterns amongst the tissues examined it may be possible to infer some physiological relevance, in addition to calculation of genetic distances amongst closely related species (Nei 1978, Sarich 1977).

MATERIALS AND METHODS

Collection of biological material

The whelks were collected at low tide at 6 locations as indicated in the map (Fig. 3.1.) and Table 3.1. Individuals were attracted with crushed mussels (a source of food) or were collected in shallow surf. Collections at any one site were

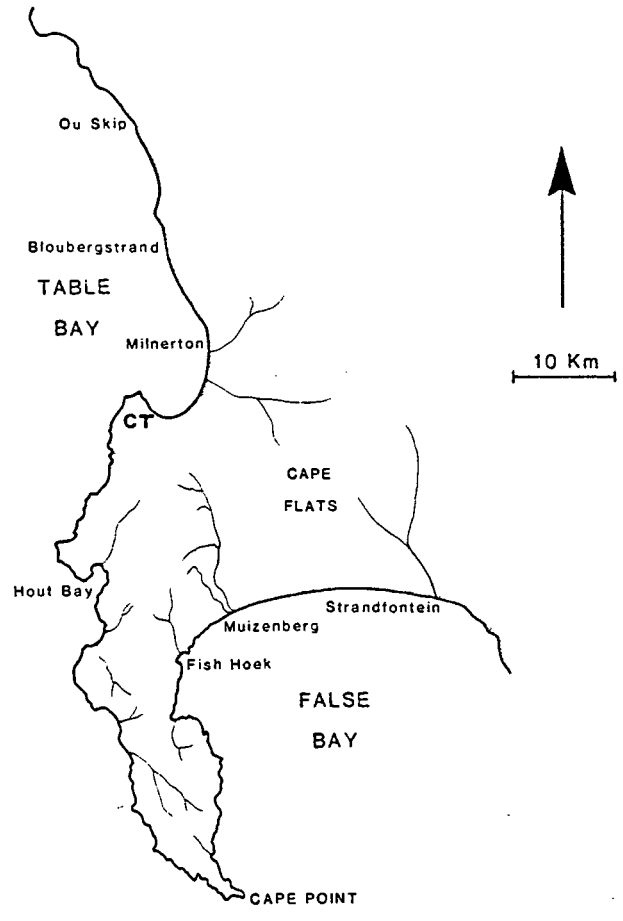


Figure 3.1. Map of the Cape Peninsula, South Africa, showing the main sites of collection of the four Bullia species (after Brown & da Silva 1984). Locations such as Yzerfontein and Lambert's Bay (not shown) are situated 80 and 250 Km north of Cape Town, respectively. Mnandi is situated between Strand and Muizenberg. (see also Fig. 1.1. and Table 3.1).

TABLE 3.1. Collection sites of *Bullia* samples used in study

LOCATION
False Bay
1. Strand
2. Mnandi
3. Muizenberg
West Coast
4. Ou Skip
5. Yzerfontein
6. Lambert's Bay

undertaken over a distance of 20 to 50 metres, depending on whelk availability and type of beach. Once in the laboratory, the animals were kept in an aerated sea water tank maintained at 15°C. They were sacrificed within a few days of collection, and the tissues immediatly isolated for electrophoretic analysis.

Determination of tissue specific gene expression

A pilot study was carried out in which tissues of 10 individuals were examined as an index of gene expression. Tissues included foot, mantle, digestive gland, brain and gonad (Table 3.2.). These were homogenised in aliquots of 0.1 M Tris-HCl buffer, pH 8.00, followed by centrifugation at 1000 x g, for 10 minutes. Supernatants were absorbed into thick filter-paper wicks and inserted into a starch block, approximately 1.5 cm from the cathodal terminal. Gels consisted of 12% hydrolysed potato starch (Sigma Chemical Co., St Louis, Missouri, U.S.A.). Electrophoresis (according to the method of May *et al*, 1979) of samples (supernatant in the wicks) was allowed for 5 minutes to ensure transfer of soluble proteins from the filter-paper to the stach medium. On completion of the pre-electrophoretic period, the wicks were removed and electrophoresis was allowed to proceed for 3 to 5 hours, depending upon the buffer system selected.

Three buffer systems were used for electrophoresis: (1) Electrode (pH 8.5), lithium hydroxide 0.06 M, boric acid 0.3 M; Gel (pH 8.5), Tris-HCl 0.03 M, citric acid 0.005 M, lithium hydroxide 0.0006 M, boric acid 0.03 M (after Rigway *et l*. 1970).

(2) Electrode (pH 6.9), Tris 0.15 M, citric acid 0.05 M; Gel, 1:14 dilution of electrode buffer (after Whitt 1970). (3) Electrode (pH 8.7), Tris 0.18 M, boric acid 0.1 M, NaEDTA 0.004 M; Gel, 1:4 diluton of electrodebuffer (Markert and Faulhaber 1965).

Gene products were identified using histochemical protocols of Shaw & Prasad (1970) and Harris & Hopkinson (1976).

Classification of identified loci, statistical treatment and analysis

Since the whelks do not breed in captivity, and the gravid female looses interest in its brood soon after settling into the laboratory aquarium, it was not possible to perform breeding experiments. Thus, in order to demonstrate Mendelian inheritance of the banding variation, the criteria of Allendorf & Utter (1979) were used to infer the genetic nature of the variants. In cases where several functionally similar loci appeared on a gel, loci were numbered beginning from the cathodic end of a gel. Alleles were designated by the electrophoretic mobility of the proteins they represented, relative to the most common allele, which was designated 100. Alleles encoding proteins that migrated cathodally from the origin, were prefixed with a minus sign.

Phenotypic frequences of polymorphic loci were tested for fitting to Hardy-Weinberg proportions with a chi-square test. This test, however, may be inflated in cases where there are low

expected phenotypic frequencies. Therefore, Yates correction for continuity was applied to the chi-square statistics and the G-test was used (Sokal & Rohlf 1969). Additionally, low frequency phenotypes were pooled according to Swofford & Selander (1981) and tests for deviation from proportions expected with random mating were recalculated. For each locus and each sample, departures of phenotypic proportions from Hardy-Weinberg expectations were expressed as

$$\underline{D} = (\underline{H}_O - \underline{H}_E)/\underline{H}_E$$

where $\underline{H}_O$ and $\underline{H}_E$ are observed and expected phenotypic proportions, respectivelly. A negative value denotes a deficit of heterozygotes and a positive value denotes their excess.

Significant differences in allelic frequencies between samples were tested using a nested contingency-table analysis. Samples were divided into groups, depending on the location of collection: (a) west coast and (b) south coast, so that a comparison could be made. The significance levels of the rejection criteria were modified to account for the increase in type I error when multiple tests are made on the same samples (Cooper 1968). Tests were considered significant if G exceded a value in a chi-square table associated with probability of 0.05/6=0.008, where 6 was the number of polymorphic loci tested. Thus the overall probability of rejecting $\underline{H}_O$ by chance was $1-(1-0.5/10)^{10}=0.05$.

Nei's unbiased estimator of genetic distance $\underline{D}$ for small sample sizes (Nei 1978) was calculated from allelic frequencies for 33 loci. The standard error of $\underline{D}$ was calculated

according to Nei & Roychoudhury (1974). Heterozygosity $\underline{h}$ per locus was calculated by

$$h = 1 - \Sigma\, p^2_i$$

where p_i is allelic frequency. Average sample heterozygosity H is defined as the mean of locus heterozygosities over all loci examined, including the monomorphic ones. Total heterozygosity or gene diversity (heterozygosity of alleles pooled over samples) was partioned into its geographic components (Nei 1973), using the algorithm of Chakraborty (1980) and the same experimental design of population subdivision used to test for allele-frequency differences. All calculations were carried out with FORTRAN programmes, written for a microcomputer by Dr WS Grant.

RESULTS

Determination of suitably polymorphic loci

Tissue specific gene expression was surveyed to identify suitably polymorphic loci, so that the genetic structure of the sample (99 whelks) collected at Ou Skip and that of other locations could be resolved. From the sample collected at Ou Skip 33 specific proteins, each apparently encoded by a single locus, were identified by electrophoresis (**Table 3.2**). Using a criterion of polymorphism, where the common allele has a frequency of 0.95 or less, the proportion of polymorphic loci found was 0.182, though when a 0.99 criterion was applied the proportion of polymorphic loci increased to 0.303. Three additional loci (GPD, LDH-1 and MDH-1) showed rare variants. Monomorphic loci were not

TABLE 3.2. Enzymes, locus abbreviations, and tissue type used in the study of Bullia genetic structure.
Tissues: foot = F; digestive gland = DG; mantle = M.

Enzyme (Enzyme Commission No.)	Locus Abbreviation	Tissue	Buffer†
Acid phosphatase (3.1.3.2)	ACP	DG	3
Adenosine deaminase (3.5.4.4)	ADA	DG	3
Adenylate kinase (2.7.1.20)	ADK	F	2
Aldolase (4.1.2.7)	ALD	F	3
Aldehyde oxidase (1.2.3.1)	AO	DG	1
Arginine kinase (2.5.3.3)	ARGK	F	3
Aspartate amina transferase (2.6.1.1)	AAT-2	F	1
Esterase (3.1.1.1)	EST-1	M	3
	EST-2	M	3
	EST-3	M	3
Fumerate hydratase (4.2.1.2)	FUMH	M	3
Glucose-phosphate isomerase (5.3.1.9)	GPI	F	1
Glucose-6-phosphate dehydrogenase (1.1.1.49)	G6PD	DG	1
Glyeraldehyde-phosphate dehydrogenase (1.2.1.12)	GAP	F	2
Glycerol-3-phosphate dehydrogenase (1.1.1.8)	GPD	F	2
Hexokinase (2.7.1.1)	HK	F	3
Isocitrate dehydrogenase (1.1.1.42)	IDH-1	DG	2
	IDH-2	F	2
Lactate dehydrogenase (1.1.1.27)	LDH-1	F	1
	LDH-2	DG	1
Leucine amino peptidase (3.4.11.-)	LAP	DG	2
Malate dehydrogenase (1.1.1.37)	MDH-1	F,DG	2
	MDH-2	F	2
Mannose-phosphate isomerase (5.3.1.8)	MPI	F,M	3
Peptidase (3.4.11.-)			
Substrate: Glycyl-Leucine	GL-1	M,DG	2
	GL-2	M,DG	2
Leucyl-glycyl-glycine	LGG	DG	2
Phenylalanyl-proline	PHP	M,DG	2
Octopine dehydrogenase (1.5.1.11)	ODH	M	1
Phosphogluco mutase (2.7.5.1)	PGM	M,DG	
Phosphogluconate dehydrogenase (1.1.1.44)	PGD	F	2

(continued)

TABLE 3.2. (continued)

Enzyme (Enzyme Commission No.)	Locus Abbreviation	Tissue	Buffer†
Sorbitol dehydrogenase (1.1.1.14)	SDH	DG	1
Superoxide dismutase (1.15.1.1)	SOD-1	DG	1
Xanthine oxidase (1.2.3.2)	XO	DG	3

† See Materials and Methods

examined further and were assumed monomorphic also in other samples, during the computation of genetic distance and average heterozygosity.

Of the loci resolved, 22 appeared to be monomorphic or showed only a few variant individuals. These included ACP, AAT-2, ALD-1, ADA, ADK, AO, EST-2, EST-3, FUM, GAP, G6PD, HK, LDH-1, LDH-2, MDH-1, MDH-2, GL-2,LGG-2, SOD-1, XO and PGD. The proteins encoded by ARGK, MPI and ODH, showed two-banded heterozygotes, expected of monomeric enzymes. The primary bands of ODH were accompanied by as many as 4 satellite bands, with decreasing intensity towards the anodic end of the gel. The proteins encoded by EST-1, GPI, IDH-1, IDH-2, MIP, GL-1 and PGD showed three banded heterozygous phenotypes typical of dimeric enzymes. The allozymes encoded by PHP showed broad-banded and three-banded heterozygous phenotypes where the broaded-banded phenotypes appeared for allozymes whose electrophoretic mobilities were close to the common allele.

Allelic frequencies and observed locus heterozygosities of 14 polymorphic loci in B.digitalis are presented in Table 3.3. In addition **Table 3.4** contains concise information on allelic frequencies of polymorphic proteins amongst the four species. No deviations from Hardy-Weinberg proportions were detected and there were no systematic large heterozygote deficits or excesses. A nested contingency-table analysis of absolute allelic frequencies using modified rejection criteria (see Materials and Methods) detected a single significant (G_4 = 19.17; p = 0.05)

Table 3.3. Allelic frequencies and proportions of heterozygotes of 14 loci in Bullia digitalis. h_o = observed locus heterozygosity; D = deficit (-) or excess (+) of heterozygotes; N = number of genomes sampled.

		Location					
Locus	Allele	1	2	3	4	5	6
Argk	100	0.907	0.939	0.929	0.910	0.929	0.943
	125	0.093	0.061	0.071	0.090	0.071	0.057
	h_o	0.160	0.160	0.143	0.138	0.121	0.115
	D	-0.045	-0.049	0.077	-0.159	-0.078	0.061
	N	162	98	196	188	198	174
Est-1	50	--	0.011	0.011	--	0.010	0.005
	65	0.006	0.011	--	--	--	--
	75	--	0.011	--	--	0.005	0.005
	80	0.045	0.053	0.033	0.010	0.051	--
	85	0.013	0.011	0.027	0.045	0.010	0.058
	90	0.051	0.021	0.005	0.010	0.015	0.012
	95	0.013	--	0.005	0.016	0.005	0.012
	100	0.654	0.628	0.603	0.631	0.621	0.628
	102	0.135	0.106	0.158	0.101	0.152	0.116
	104	--	--	--	--	0.005	--

	105	0.064	0.106	0.082	0.106	0.101	0.047
	107	--	0.010	0.054	0.005	--	0.064
	110	0.019	0.032	0.022	0.076	0.020	0.041
	125	--	--	--	--	0.005	0.012
	$\underline{h}_o$	0.564	0.595	0.598	0.586	0.545	0.558
	$\underline{D}$	0.035	0.030	-0.002	0.025	0.095	-0.038
	$\underline{N}$	156	94	184	198	198	172
Gpi	-120	0.006	--	--	--	--	--
	- 80	0.006	--	--	--	--	0.006
	10	--	--	--	0.010	--	--
	100	0.981	1.000	0.985	0.990	0.990	0.989
	180	0.006	--	0.005	--	--	--
	220	--	--	0.010	--	0.010	--
	$-_o$	0.004	--	0.030	0.020	0.020	0.023
	$\underline{D}$	0.013	--	0.012	0.010	0.010	0.012
	$\underline{N}$	162	98	200	198	200	174
Gpd-1	100	1.000	1.000	1.000	0.995	1.000	1.000
	115	--	--	--	0.005	--	--
	$\underline{h}_o$	--	--	--	0.010	--	--
	$\underline{D}$	--	--	--	0.005	--	--
	$\underline{N}$	162	98	200	198	200	174
Idh-1	25	--	--	--	--	--	0.006
	50	--	0.010	0.010	--	--	--
	60	0.038	0.031	0.045	0.076	0.055	0.040
	95	--	--	0.005	--	--	--

	100	0.925	0.888	0.935	0.924	0.920	0.954
	110	0.020	0.030	--	--	0.020	--
	125	0.019	0.041	0.005	--	0.005	--
	h_o	0.125	0.204	0.110	0.111	0.160	0.092
	D	-0.121	-0.020	-0.110	-0.207	0.066	0.0427
	N	160	98	200	198	200	174
Idh-2	100	0.975	0.980	0.995	1.000	0.985	1.000
	115	0.006	0.020	0.005	--	0.005	--
	125	0.012	--	--	--	0.010	--
	130	0.007	--	--	--	--	--
	h_o	0.049	0.041	0.019	--	0.030	--
	D	0.017	0.021	0.005	--	0.012	--
	N	162	98	200	198	200	174
Ldh-1	100	1.000	1.000	1.000	0.995	1.000	1.000
	105	--	--	--	0.005	--	--
	h_o	--	--	--	0.010	--	--
	D	--	--	--	0.005	--	--
	N	162	98	200	198	200	174
Mdh-1	100	0.994	1.000	0.990	1.000	1.000	1.000
	150	0.006	--	--	--	--	--
	200	--	--	0.010	--	--	--
	h	0.012	--	0.020	--	--	--
	D	0.006	--	0.010	--	--	--
	N	162	981	200	198	200	174
Mpi	80	--	--	0.005	--	0.005	--

	90	0.013	0.020	--	0.005	--	--
	95	--	0.011	0.005	0.010	0.015	0.011
	100	0.975	0.969	0.975	0.970	0.970	0.948
	105	0.012	--	0.015	0.015	0.010	0.029
	108	--	--	--	--	--	0.012
	$\underline{h}_o$	0.049	0.030	0.051	0.061	0.060	0.092
	$\underline{D}$	0.019	0.024	0.019	0.022	0.022	-0.078
	$\underline{N}$	162	98	198	198	200	174
Odh	100	1.000	0.990	0.995	0.985	1.000	0.994
	200	--	0.010	0.005	0.015	--	0.006
	$\underline{h}_o$	--	0.020	0.010	0.030	--	0.011
	$\underline{D}$	--	0.010	0.005	0.015	--	0.006
	$\underline{N}$	162	98	200	198	200	174
Gl-1	75	0.025	0.020	0.005	0.017	0.005	0.017
	80	--	--	--	--	0.010	--
	95	--	--	--	--	0.005	0.006
	100	0.648	0.633	0.576	0.522	0.565	0.569
	125	0.037	0.020	--	--	0.015	--
	145	0.265	0.276	0.338	0.372	0.375	0.391
	170	0.025	0.051	0.081	0.089	0.025	0.017
	$\underline{h}_o$	0.543	0.632	0.454	0.644	0.660	0.593
	$\underline{D}$	0.037	0.142	-0.096	0.110	0.095	0.121
	$\underline{N}$	162	98	198	180	200	174
Php	75	0.031	--	0.005	--	0.005	--
	90	0.006	--	0.010	--	0.025	0.017

	95	--	0.010	--	--	--	--
	100	0.864	0.908	0.899	0.843	0.890	0.920
	107	0.031	0.031	0.030	0.066	0.050	0.023
	115	0.068	0.051	0.056	0.086	0.030	0.040
	122	--	--	--	0.005	--	--
	$\underline{h}_o$	0.247	0.163	0.202	0.454	0.200	0.161
	$\underline{D}$	0.001	-0.049	0.076	0.099	-0.019	0.059
	$\underline{N}$	162	98	198	198	200	174
Pgd	- 20		--	--	--	--	0.006
	30	0.216	0.229	0.150	0.214	0.268	0.155
	60	--	--	0.05	--	--	--
	100	0.772	0.771	0.825	0.786	0.732	0.839
	120	--	--	0.005	--	--	--
	150	0.006	--	0.005	--	--	--
	$\underline{h}_o$	0.333	0.417	0.310	0.387	0.394	0.276
	$\underline{D}$	-0.067	0.179	0.045	0.091	0.005	0.015
	$\underline{N}$	162	96	200	196	198	174
Sdh-1	10	--	--	--	--	--	0.011
	70	--	--	--	--	--	0.006
	100	0.988	1.000	0.969	0.970	0.975	0.977
	180	0.012	--	0.021	0.025	0.025	0.006
	200	--	--	0.010	--	--	--
	300	--	--	--	0.005	--	--
	$\underline{h}_o$	0.025	--	0.062	0.061	0.050	0.046
	$\underline{D}$	0.013	--	0.025	0.027	0.026	0.016

N	162	98	194	198	200	174
Average Heterozygosity						
H	0.065	0.064	0.063	0.073	0.067	0.058
SE	0.022	0.023	0.023	0.025	0.024	0.021

Table 3.4. Allelic frequencies of polymorphic proteins among four species of *Bullia*. Sample sizes were 35 for *B. digitalis*, 35 for *B. rhodostoma*, 20 for *B. pura* and 4 for *B. laevissima*.

Locus	Allele	*Bullia digitalis*	*Bullia pura*	*Bullia laevissima*	*Bullia rhodostoma*
Acp	90	0.000	0.000	1.000	1.000
	100	0.591	0.000	0.000	0.000
	150	0.318	0.000	0.000	0.000
	160	0.061	0.075	0.000	0.000
	190	0.000	0.125	0.000	0.000
	200	0.000	0.800	0.000	0.000
	220	0.030	0.000	0.000	0.000
Adk	50	0.000	0.000	1.000	0.000
	100	1.000	1.000	0.000	1.000
Argk	75	0.000	1.000	0.000	0.000
	80	0.000	0.000	0.000	1.000
	85	0.000	0.000	1.000	0.000
	100	0.971	0.000	0.000	0.000
	125	0.029	0.000	0.000	0.000
Est-1	90	0.000	0.000	0.000	1.000
	94	0.000	0.025	0.000	0.000
	96	0.014	0.975	0.000	0.000

	100	0.657	0.000	0.000	0.000
	102	0.186	0.000	0.000	0.000
	105	0.014	0.000	0.000	0.000
	107	0.043	0.000	0.000	0.000
	110	0.057	0.000	0.000	0.000
	115	0.029	0.000	0.000	0.000
Est-2	95	0.000	0.000	1.000	0.000
	100	1.000	1.000	0.000	1.000
Est-3	85	0.000	0.000	0.000	1.000
	100	1.000	1.000	1.000	0.000
Fum	100	1.000	0.000	0.000	0.000
	120	0.000	1.000	1.000	0.000
	125	0.000	0.000	0.000	1.000
G6p	95	0.000	1.000	0.000	0.000
	100	1.000	0.000	1.000	1.000
Gl-1	100	0.729	0.000	0.000	0.000
	145	0.243	1.000	0.000	0.000
	150	0.014	0.000	0.000	0.000
	170	0.000	0.000	1.000	1.000
Gl-2	85	0.000	0.000	1.000	0.000
	100	1.000	0.000	0.000	1.000
	110	0.000	1.000	0.000	0.000
Gpd	80	0.000	1.000	0.000	0.000
	90	0.000	0.000	0.000	1.000
	100	1.000	0.000	0.000	0.000

	120	0.000	0.000	1.000	0.000
Gpi	100	1.000	0.000	0.000	0.000
	400	0.000	1.000	0.000	1.000
	1000	0.000	0.000	1.000	0.000
Idh-1	70	0.014	0.000	0.000	0.000
	100	0.986	0.000	0.000	0.000
	105	0.000	0.000	1.000	0.000
	110	0.000	1.000	0.000	0.000
	130	0.000	0.000	0.000	0.029
	145	0.000	0.000	0.000	0.045
Lap-1	100	1.000	0.000	0.000	0.000
	150	0.000	1.000	0.000	0.000
	250	0.000	0.000	1.000	0.000
	300	0.000	0.000	0.000	1.000
Lgg-1	60	0.000	0.000	0.500	0.000
	90	0.000	0.000	0.500	0.000
	100	1.000	0.000	0.000	1.000
	105	0.000	1.000	0.000	0.000
Mdh-1	100	1.000	0.425	0.000	1.000
	200	0.000	0.000	1.000	0.000
	250	0.000	0.525	0.000	0.000
	350	0.000	0.050	0.000	0.000
Mdh-2	0	0.000	1.000	0.000	0.000
	30	0.000	0.000	1.000	0.000
	60	0.000	0.000	0.000	1.000

	100	1.000	1.000	1.000	1.000
Mpi	70	0.000	0.000	0.000	0.943
	75	0.014	1.000	0.000	0.000
	77	0.000	0.000	1.000	0.057
	95	0.014	0.000	0.000	0.000
	100	0.971	0.000	0.000	0.000
Odh	30	0.000	1.000	0.000	0.000
	80	0.000	0.000	0.000	1.000
	100	1.000	0.000	1.000	0.000
Php	95	0.000	1.000	0.000	0.000
	100	0.986	0.000	0.000	0.000
	107	0.014	0.000	0.000	0.000
	115	0.000	0.000	1.000	1.000
Sdh-1	100	1.000	0.000	1.000	0.000
	160	0.000	0.000	0.000	1.000
	200	0.000	1.000	0.000	0.000
Aat-2	60	0.000	1.000	0.000	1.000
	85	0.000	0.000	1.000	0.000
	100	1.000	0.000	0.000	0.000
Pgd	20	0.000	0.000	1.000	0.000
	30	0.086	0.000	0.000	0.000
	60	0.000	1.000	0.000	0.000
	100	0.900	0.000	0.000	0.000
	110	0.000	0.000	0.000	1.000
	120	0.014	0.000	0.000	0.000

Pgm-1	90	0.286	0.000	0.000	0.000
	95	0.000	0.000	0.000	0.129
	97	0.000	0.000	1.000	0.000
	100	0.567	1.000	0.000	0.871
	107	0.057	0.000	0.000	0.000
Sod-1	-700	0.000	1.000	0.000	0.000
	-300	0.000	0.000	0.000	1.000
	100	1.000	0.000	1.000	0.000
Sod-2	70	0.000	0.000	0.125	0.000
	80	0.000	0.000	0.875	0.000
	85	0.000	0.000	0.000	1.000
	100	0.632	0.000	0.000	0.000
	110	0.000	1.000	0.000	0.000
	115	0.368	0.000	0.000	0.000
Xo	95	0.000	0.000	1.000	0.000
	100	0.914	0.000	0.000	1.000
	103	0.086	0.000	0.000	0.000
	105	0.000	1.000	0.000	0.000

frequency difference for GL-1 among the three samples from False Bay. There were no significant allele-frequency differences between the pooled samples on the south and west coasts for any of the loci.

Average population heterozygosity, H, ranged from 0.063 (S.E. 0.025) to 0.073 (S.E. 0.0246). The gene diversity analysis showed that this represented on average 98.7% of the total gene diversity in Bullia digitalis ; 1.0% of the total variation was due to allelic frequency differences between populations within each coastal region and 0.3% was due to differences between the west coast samples and those from False Bay on the south coast. Pair-wise estimates of Nei's genetic distance ranged from 0.0000 to 0.0007 and were not significantly different from zero. There was no geographic pattern in the genetic distances.

Tissue distribution of enzyme variants

Enzymes of the glycolytic, Krebs and fructose metabolic cycles were identified by the use of histological activity stains and are represented in Tables 3.5., 3.6. and 3.7. Since staining methodology is not available for all enzymes, the examination of components of each cycle is not complete. The intensity with which each enzyme is present is represented by (*), where (****) denotes maximal intensity. The loci were defined as monomorphic (M) or polymorphic (P) and differences recorded.

TABLE 3.5. Enzymes of fructose metabolism identified in Bullia digitalis. Identification key: (M) or (P):
M = Monomorphic; P = Polymorphic.
$(\ )_1$ or $(\ )_2$ refers to the number of different isozymes present, irrespective of polymor- or monomorphicity.

Enzymes	Type	Tissue specific enzyme expression				
		Foot	Mantle	Digestive Gland	Brain	Reproductive System
PGD	$(M)_1$	+	++	+++	±	+
PGM	$(M)_1$	++++	++++	+++	+++	+++
	$(P)_1$	+	++	++	+	+
PGI	$(M)_1$	++++	++++	++++	++++	++++
HK	$(M)_1$	++++	++	-	+	-
SDH	$(P)_3$	++	++	+++	+	+
		-	+	+	-	-
		-	-	++	-	-
MPI	$(P)_1$	++++	++++	+++	+++	+++

TABLE 3.6. Enzymes of glycolysis.
Identification key: (M) or (P):
M = Monomorphic; P = Polymorphic.
$(\ )_1$ or $(\ )_2$ refers to the number of different isozymes present, irrespective of polymor- or monomorphicity.

Enzymes	Type	Tissue specific enzyme expression				
		Foot	Mantle	Digestive Gland	Brain	Reproductive System
HK	$(M)_1$	++++	++	-	+	-
PGM	$(M)_1$	++++	++++	+++	+++	+++
	$(P)_1$	+	++	++	+	+
PGI	$(M)_1$	++++	++++	++++	++++	++++
PFK	$(M)_1$	+++	++	-	+	-
GPD	$(M)_1$	++	++	++	+	++±
	$(P)_1$	+	+	+	+	+
PK	$(M)_1$	++	+++	+	-	+±
LDH	$(M)_2$	++	++	+++	+	+
ODH	$(M)_1$	++++	+++	++	++	++

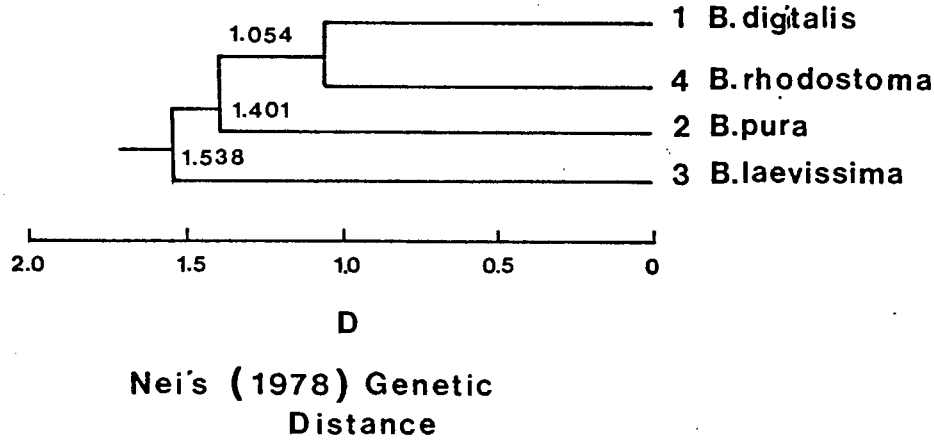
TABLE 3.7 Some enzymes of the KREBS cycle.
Identification key: (M) or (P):
M = Monomorphic; P = Polymorphic.
$(\ )_1$ or $(\ )_2$ refers to the number of different isozymes present, irrespective of polymor- or monomorphicity.

Enzymes	Type	Tissue specific enzyme expression				
		Foot	Mantle	Digestive Gland	Brain	Reproductive System
IDH						
(NADP depend.)	$(P)_1$	-	+	+++	+	+
(NAD depend.)	$(P)_1$	++	-	+	+	+
FUM	$(M)_1$	+	++++	+++	±	++
MDH	$(M)_2$					
(cytosol and mitochondrial fractions)		+++	+++	+++	+++	+++
		+++	++	+	++	++

Table 3.8. Nei's (1978) genetic distances (unbiased) and standard errors (Nei and Roychoudhury 1974) between four species of Bullia. 1 = B. digitalis, 2 = B. pura, 3 = B. laevissima, 4 = B. rhodostoma.

	2.	1.4455			
		0.3302			
Species	3.	1.3475	1.7239		
		0.3269	0.4114		
	4.	1.0539	1.3559	1.5423	
		0.2600	0.3161	0.3637	
		1	2	3	
			Species		

Figure 3.2. Cladogram of Nei's genetic distance of four species of Bullia using unpaired-group method of cluster analysis with arithmetic means (UPGMA) (Sneath and Sokal 1973).



The genetic distance of the four species examined

These results are summarised in Table 3.8. and Fig. 3.2. The genetic distance amongst species is large, where D=1 is equivalent to 20 million years. Of the four species studied, B.digitalis and B.rhodostoma are the more closely related, followed by B.pura and B.laevissima respectively. This is not surprising since B.digitalis, B.rhodostoma and B.pura are intertidal and surfing species, whereas B.laevissima is sub-tidal and non-surfing.

DISCUSSION

Little is known of the extent of migration between populations of Bullia. Unless otherwise disturbed, the whelks in many localities spend up to 90% of the tidal cycle buried (Brown 1981), the remaining period comprising their emergence during the incomming tide in order to feed. It is during this active period that animals may be washed below the intertidal zone, and it is possible that welks may be swept further from their habitat. It may be noted that B.digitalis has been found on sandy substrata to a depth of 20 metres and more (Brown 1961, 1982). These factors are responsible for the difficulties in estimating population parameters. Thus, the intent of this study was to use geographic distributions of electrophoretically-detectable protein variants to infer indirectly how populations of Bullia digitalis are genetically structured. Population structure is

principally influenced by gene flow between populations and random genetic drift. The possibility that natural selection may be acting upon the biochemical genetic population markers must also be considered. Strong selection may complicate geographic patterns in allelic frequencies, hindering the possibility of adequately determining population structure. The selection of samples across a strong environmental gradient around Cape Point would enhance the probability of detecting the effects of natural selection, if these were an important influence on allelic frequencies.

Sandy beaches that are suitable habitats for B.digitalis are often isolated from one another by intervening long stretches of rocky shores. Even along a single beach the distribution of this gastropod is patchy. Some patchiness may be attributed to recent feeding events, where large numbers of individuals congregate on food that has been washed up on the shore.

Brown (1971) found that Bullia cannot successfully burrow in gravelly beaches having particle sizes larger than 3.2 mm. Juveniles are even more affected by sand particle size. A whelk of 0.5 cm shell length, for instance, cannot burrow into the sand where the particle size exceeds 1.4mm. The amount of wave action that a beach receives also influences the distribution of Bullia. This whelk tends to be most abundant on beaches with moderate wave action and is absent from both very calm beaches and those with heavy surf.

The mode of reproduction may also retard movement between adjacent and distant locations. As indicated above the gravid female is highly protective of its brood. Furthermore, a great deal of care is provided by the female in ventilating the eggs, a function which is somewhat lost when the egg cases are buried; however, this may occur at an embryonic stage which is self-sufficient. Such care is in any case rare among gastropods. This behaviour may contribute to the maintenance of population homogeneity and low heterezygosity. This, together with the selectively different breeding times of Bullia species (especially those occupying closely situated habitats), may ensure species preservation. Gene flow would therefore be limited to non-pelagic migration, which could be aided by wave action and currents along a beach. Migration between sandy beaches in the intertidal zone seems unlikely, but may be accomplished by slower movement of deep water populations. However, because of the inaccessibility of outer coastal waters, subtidal distributions of Bullia along the west coast are highly speculative.

Estimates of genetic variation in natural populations have not been consistent (Redfield et al. 1980). A number of hypotheses have been proposed to explain the degree of genetic variation, namely: the "trophic-resource stability" of Ayala & Valentine (1979), the "niche-variation" hypothesis of Levins (1968) and Selander & Kaufman (1973) and the "time-divergence" model of Soulé (1976).

The first of these hypotheses was used to explain clines in heterozygosity in benthic marine invertebrates from the tropics (considered trophically stable) to the polar regions (trophically unstable). In an environment characterised by wide temporal variation in the abundance of food availability there is a tendency for animals to generalise their food consumption. This would imply the fixation of a small number of highly adaptive alleles and low heterozygosity (Redfield et al. 1980). However, estimations of trophic stabilities of most habitats are often difficult and complicated. In addition, the life cycles of most marine species include a larval stage (whose trophic demands are often widely different from those of adults (Gabbott 1983) and thus the typical conditions of an environment may in fact not be totally favourable throughout the animals life.

The niche-variation hypothesis has been used to correlate thermal environments with allelic variants, though with relatively little success. Somero & Soulé (1974) found no correlation between the annual thermal range experienced by a species and the respective variation in allelic polymorphisms. These authors suggested instead that high levels of polymorphism should be found in species whose environment changed little over a long period of time. Their findings had indicated that of all species of fish so far investigated, those inhabiting the tropics and the deep sea were the most polymorphic. A hypothesis was subsequently developed by Soulé (1976), the time-divergence model, which requires the following conditions in order for a population to exhibit high levels of heterozygosity: (1) the

population or lineage must be large; (2) it must be old (3) must be evolving quite slowly, since directional selection possibly erodes heterozygosity.

From the genetic distance data it is apparent that the genus Bullia is certainly old, in addition to having diverged into separated species a long time ago. In fact the genetic distances obtained between species are more typical of individual genera (see Nei 1978, Sarich 1977). These authors calculated genetic distance "D", taking into consideration the degree of protein polymorphism as well as its dominance, proposing that a genetic distance D= 1 is equivallent to 20 million years. As is evident from Fig. 3.2. the speciation distance of B.laevissima is 1.538, which is equivalent to just over 30 million years. Though the populations are large, the possibility of pockets of subpopulations has not been excluded. However, information on the evolutionary rate of Bullia is not available, and it is thus not possible to fit the necessary requirements of the time-divergence model. The populations of Bullia examined, exhibited a very low degree of heterozygosity, which could have been erased by fast development and specialisation. It is possible that in this genus the third requirement of Soulé' hypothesis (1976) plays a significant role in the determination of population genetic structure.

The overall genetic homogeneity observed in this study, suggests that populations of Bullia digitalis are not genetically subdivided to any large degree, even across the faunal boundary

around Cape Point. Hence, either the better equipped populations have survived and multiplied, proliferating in suitable environments, with the resultant extinction of less well adapted groups, or the whelk carries a number of silent genes. These are triggered by selected and specific environmental stimuli (Currie & White 1981), and are thus not detectable in the laboratory, due to the inability to simulate appropriate environmental conditions. Should environmental conditions be reproducible in the laboratory, the genetic profile of Bullia might have appeared different.

The disadvantage of such a mechanism is the requirement of a large genome. The latter are infrequent (Hochachka & Somero 1984), and may in fact be unnecessary. On the other hand, a too specific genome might be too limiting in the event of ecological catastrophy, and might not allow for specific environmental fluctuations. This may be of great importance on the west coast (where upwellig frequently results in sudden changes in water temperature, often as large as 10°C) (Brown 1982). A more generalised genetic profile is therefore advantageous and more flexible. Since the south coast is not characterized by the same degree of upwelling, it is not apparent why the animals belonging to the two coasts do not show any differences. Nonetheless there may be other factors that have been overlooked, both at an ecological and physiological level. The interaction of the environment with the organism's physiology might be crucial in determining certain physiological responses, such as post-translational modifications induced only in the presence of

relevant external stimuli.

Genetic heterogeneity was, however, detected for GL-1 among the three samples collected from False Bay. Similar small scale heterogeneity, as opposed to large scale heterogeneity, has also been described for Siphonaria sp., an intertidal pulmonate limpet with pelagic larvae (Johnson & Black 1982). In this instance, genetic mosaics along the shore were caused by temporal variation in the numbers and genotypes of recruits in to the adult population. A similar genetic mosaic may exist in Bullia on a finer scale than could be recognised in the sampling scheme used in this study. If such fine structure were a prominent feature of Bullia populations and if samples used in this study included more than one homogeneous patch, then consistent heterozygosite deficits, due to the Wahlund effect may be expected. None were however observed.

In spite of expecting a subdivided population structure, based on life-history considerations, the results of this study show that populations of Bullia are genetically homogeneous over a large geographic scale. It may be that migration of juveniles or adults between beaches, by water currents is substantial. Alternatively, there may be considerable isolation between populations over short periods of time, but in the long term populations on local beaches may be subjected to extinction and founder events in response to gradual changes in sea level and temperature. Populations of Bullia may be sufficiently large over these periods of time for changes via

genetic drift to be unimportant.

Results given in Tables 3.5., 3.6. and 3.7. summarise data in B.digitalis only. These indicate a low level of heterozygosity, though differential enzyme expression occurs in different tissue. Of the enzymes examined 13 were monomorphic and 5 polymorphic.

Enzymes of fructose metabolism (Table 3.5.), such as PGM and GPI are the most active, displaying no significant tissue expression differences. Since pathway flux is dependent on the summation effect of each enzyme reaction velocity, it is possible that PGD, SDH and MPI play a role not necessarily in control of flux, but contribute to regulation of carbon flow. This would be a consequence of reduced enzyme working capacity. Terminology such as rate limiting and flux generating steps of Newsholme & Crabtree (1979) have been avoided, since in recent years a new approach to metabolic control has been suggested by Kacser & Burns (1981). However, this approach has seldom been used in molluscan metabolic control. In the present analysis, if the approach of Kacser & Burns (1981) had been used, comparisons to metabolism of other groups would be somewhat invalid, as most studies still use the more traditional approach of Newsholme & Crabtree (1979).

Glycolytic enzymes (Table 3.6.) on the other hand are characterised by more varied expression over the five tissues examined. PGM and GPI are highly active irrespective of tissue. An alternative less active form of PGM is present, suggesting

differential expression of another locus. Enzymes such as PK and HK are not as active (**) and may also indicate points at which carbon flow is diminished. At the pyruvate branch point two enzymes are present, D(-)LDH and D(+)ODH. Both are monomorphic and the latter is more active than D(-)LDH. Competition for common substrates has been suggested between these 2 enzymes (Baldwyn et al. 1981), yet since in Bullia the acivity of one enzyme is in excess of that of the other, this situation may not occur. It is possible that selective expression of these loci is of some advantage to the whelk, especially since D(-)LDH produces D(-) lactate, which is cleared less efficiently from the tissues (Drury & Wick 1965), and thus its limited production could be favoured. Limited information is available on the Krebs cycle enzymes (**Table** 3.7.), except that in general the activities of the enzymes examined are higher in the digestive gland.

It should be noted that absence or low intensity of an enzyme form could be the result of: selective expression, susceptibility to substrate concentration which may enhance or diminished activity, thus producing different intensities in staining. This may not be a function of selective gene expression, however. In addition, temperature lability and overall stability could influence the observed genetic pattern. In consequence, inferences should only be used as an index of activity and conclusions should be drawn from more rigorous biochemical treatment.

Since metabolic fluxes depend on the overall rate of

the pathway and this is determined by the summation effect of each enzyme, where an enzyme is less active, limited flux could result. This may impose some regulatory constraint on pathways. From these results it is apparent that different isozymes are expressed differently in different tissues, representing possible selective forms of metabolic control designed especially for each tissue.

Multiple enzyme forms or variants were "discovered" independently by Meister (1950) and Neilands (1952), though their significance was not realised until later, when Vessel & Bearn (1958) and Hess (1958) became aware of their value in clinical medicine. Increases in enzyme variants can be produced via 2 mechanisms:

(1) Multiple allelic forms of an enzyme, designated allozymes are generally coded by a single locus.

(2) When several gene loci code for an enzyme, isozyme are formed.

The expression of multiple enzyme forms relates directly to control of dominant and recessive variants, as well as to the mutational rate with which a modified protein is produced, bearing in mind that the relationship between enzyme structure and function is vital in the maintenance of phsiological stability and often depends on the properties of enzymes to accomodate to changes in the surrounding matrix (resultant from external stimuli). These are controlled by strict regulation of enzyme binding affinities needed to achieve the

correct conformational changes so that reactants are properly oriented within the active site (Kacser & Burns, 1981).

The structural-functional diversity of enzymes implies their use in different reactions, depending on the properties of the tissues where these are expressed. Since enzyme-substrates affinities are often interdependent, the optimal substrate concentration for an enzyme and other kinetic parameters may differ with the tissue type. This may have important regulatory consequences resultant from the specific features of each pathway in the respective tissue, where variants of an enzyme (isozymes) may give rise to radically different products. The activity of these variants can also be influenced by cellular pH by modification of the properties of the active site (Somero 1981); in consequence conditions that may favour the activity of one enzyme form, may be disadvantageous to others.

Reliance on allozymes would only provide survival advantage if parental heterozygosity of every affected locus could be transmitted to the next generation, an unlikelly phenomenon in terms of genetic segregation mechanisms. In addition simplistic positive correlations between environmental and genetic variability do not take into consideration the mode of evolution of both the habitat and species (Somero & Hochachka 1976). However enzyme phenotypes are species specific and within the same species, variant phenotypes pertaining to different tissues may indicate differences in metabolic function.

By using electrophoretic techniques, functional differences in enzymes are seldom detected (due to saturating substrate conditions and the temperature at which the gels have to be run), yet these studies provide a qualitative index pointing towards possible directions for further kinetic analysis, which may disclose selective enzyme catalytic properties.

CHAPTER 4

MOLECULAR HETEROGENEITY OF D(-)LDH IN THE FOOT AND MANTLE OF THE WHELK BULLIA DIGITALIS

INTRODUCTION

The concept of multiple molecular forms of an enzyme was introduced by Markert and Møller in 1959, as a general enzymological phenomenon of great biological significance (Ogita 1968). The degree of heterogeneity of these multiple molecular enzyme forms is a consequence of differential tissue or cell expression, capacity to use analogues, sensitivity to inhibition, responses to physiological modulators and cellular compartmentation (Allen 1961). Thus the adequate regulation of these properties is of paramount relevance to maintenance of physiological homeostasis.

The discovery of multiple enzyme forms in molluscs has been associated with developmental, seasonal and temperature changes, especially in the case of LDH (Goldberg & Cather 1963, Coles 1969, Rodrick et al. 1971, Pesch 1972, Narang 1974, Storey 1977, Gill 1978, Long et al. 1979, Ottaviani & Ferrari 1982). Analysis of the physicochemical properties of this isozyme system throughout the animal kingdom, revealed the presence of several stereospecific and structurally descreet

enzyme complexes of LDH, depending on the tissue type or animal species (Long & Kaplan 1968, Michejda et al. 1969, Gleason et al. 1971, Ewing & Clegg 1972, Storey 1977, Eichner & Kaplan 1979, Gade 1979). Apart from variable quaternary structure (tetra or dimeric), differences in substrate specificity have resulted in distinct D(-) and L(+) lactate dehydrogenases (both of which are present in molluscs). Yet, no correlation appears to exist between substrate stereo-specificity and the degree of subunit organization, with the result that evidence points towards the independent emergence of these parameters in the course of the evolution of the lactate dehydrogenases (Ellington & Long 1978).

LDH catalyses the reoxidation of NADH to NAD^+, an important cellular redox function at the pyruvate branch point, where pyruvate is the terminal electron acceptor (Somero 1978) and lactate is the major anaerobic end product. However in some molluscs lactate is not found to accumulate and is reconverted to pyruvate (Storey 1977). In addition, LDH has often been reported to display temperature-adaptive changes of its properties (Somero 1978), as well as differential thermal responses of some of its isozymes, due to environmental temperature changes (Hochachka & Somero 1968). In invertebrates, particularly in the more active molluscs, this enzyme has either been replaced or coexists with other enzymes, namely ODH, ALADH or STDH (Fields et al 1980, Gäde 1980, Dando et al. 1981, Baldwyn et al. 1981), involved in the regeneration of phosphagen levels.

Bullia digitalis (Dillwyn) is a surf dwelling

prosobranch, which has sucessfully exploited high energy beaches around South Africa and particularly the west coast, characterized by frequent temperature fluctuations and erratic food supply (Brown 1982). The whelk is able to maintain a virtually flat rate-temperature curve at all levels of activity (Brown & da Silva 1983), a phenomenon considered to be an adaptation to its unpredictable environment.

Possible explanations for this phenomenon include the development of isozymes characterized by specific features, which allow for selectivity of thermal optima (Somero & Hochachka, 1976).

The aim of the current investigation was to examine some of the physicochemical properties of D(-)LDH of B.digitalis, in the foot and mantle. This knowledge may contribute to a better understanding of the relationship between phenotypic expression and physiological function, especially during adjustment to changing environmental conditions.

MATERIALS AND METHODS

Reagents

D(-)Lactate, NAD+, NADH, nitrobluetetrazolium (NBT), phenazinine methosulphate (PMS) and DL-Dithiothreitol were

purchased from Sigma, St. Louis, USA. Sephacryl-S 200, DEAE A-50, the low molecular weight calibration kit for SDS-gel electrophoresis and the chromatofocusing kit were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The molecular weight calibration kit for gel filtration was obtained from Boehringer Mainheim GmbH, Biochemica and all other reagents (analytical grade) were purchased from BDH Chemicals Poole, Great Britain.

Biological material and preparation of crude extract

Animals were collected from the west coast of South Africa and kept in an aerated sea water tank at 15°C. The whelks were not fed in the laboratory and were sacrificed by freezing in liquid nitrogen within 2 or 3 days of collection. The feet and mantles of 160 whelks (shell length 3.48±0.32 cm) were dissected immediatly after thawing at 4°C and kept on ice until homogenization. The combined feet (136.99 gm) and mantles (36.50 gm) mass were homogenized separately in 4 parts of 0.02 M Tris HCl buffer pH 7.40, containing 0.03 M β-mercaptoethanol, 0.15 M sucrose and 0.0001 M EDTA. The suspension was centrifuged at 25000 g for 30 minutes, in a Sorval RC2-B using an SS34 fixed angle rotor. The supernatant was either used for further purification or stored at -70°C in 30% glycerol. All preparative and analytical steps were carried out at 4°C.

Enzyme purification:

(a) **Ammonium sulphate precipitation.** Ammonium

sulphate was added to the crude extract, with agitation over a period of 30 minutes, until 40% saturation was attained. The suspension was centrifuged as before and enzyme activity was found in the precipitate which was resuspended in a minimum volume of 0.005 M Na_2HPO_4/NaH_2PO_4 buffer pH 6.90, containing 0.005 M β-mercaptoethanol and the same molarity of EDTA. The sample was dialysed against 4 litres of the same buffer for 5 hours (2 changes). On completion of dialysis the sample was centrifuged as indicated previously to remove precipitated protein and the supernatant saved for chromatographic analysis.

(b) **Sephacryl S-200.** A 20 ml volume of the previous sample was applied to a Sephacryl S-200 column (1.5 x 90 cm, 160 ml), which had been pre-equilibrated with 0.02 M Na_2HPO_4/NaH_2PO_4 pH 6.99, containing 0.1 M NaCl. Fractions were monitored for protein and enzyme activity. The active fractions were pooled (35 ml) and dialysed against 4 litres of 100% ammonium sulphate (including 0.005 β-mercaptoethanol), for 5 hours. The resultant precipitate was spun as before, dialysed against a 0.02 M phosphate buffer for 5 hours and centrifuged as indicated previously.

(c) **DE-52 ion exchange cellulose.** The above sample was applied to a DE-52 cellulose column (2.5 x 40 cm, 200 ml) pre-equilibrated with a 0.02 M sodium phosphate buffer, pH 6.99 and eluted with 75 ml of the same buffer. A linear KCl gradient (0.0 to 0.2 M) was started while 2 ml fractions of elutent were collected. The active peak was pooled and concentrated against a

solution of ammonium sulphate as before and the dialysed precipitate was resuspended in a minimum volume of 0.025 M ethanolamine HCl pH 7.5 and dialysed against 20 times its volume in the same buffer. Denatured protein was removed by centrifugation and the supernatant stored at $4^{o}C$.

(d) **PBE 94/Polybuffer 96 Chromatofocusing.** The proceedure followed was that given in the instructions included in the Pharmacia Fine Chemicals Chromatofocusing kit. An eluting pH gradient of 8.50 to 6.80 was chosen, where the start buffer comprised 0.025 M ethanolamine HCl, pH 8.50; gradient elution was initiated by 25 ml of Polybuffer 96-HCl pH 6.50, diluted 13 times in double distilled, degassed water. A flow rate of 40 ml h^{-1} was maintained throughout the column run and 2.5 ml fractions were collected.

Molecular weight determination

Molecular weight determinations were performed according to the procedure of Whitaker (1963) and Andrews (1964), using a Sephacryl S-200 column. Protein markers were applied in descending order of molecular weight to prevent elution overlapping and protein associations. Proteins were eluted in a sodium phosphate buffer, pH 6.99, while 0.80 ml fractions were collected. Absorption was followed at 280 nm. A standard plot of log molecular weight (Mr) vs elution volume was constructed using the following markers: catalase, from beef liver (MW= 240Kd); aldolase, from rabbit muscle (MW= 158Kd);

albumin, from bovine serum (MW= 68Kd); albumin, from hen's egg (MW= 45Kd); chymotrypsinogen A, from bovine pancreas (MW= 25Kd) and cytochrome c, from horse heart (MW= 12.5Kd).

Gel electrophoresis

(a) Non denaturing gel electrophoresis

A method modified after Ornstein (1964) and Davis (1964) was used, in which slab gels were used in place of tubes. A gel-forming chamber was constructed: 6 cm high, 12 cm wide and 0.15 cm thick. Wells were formed on the large pore gel with a gel comb, allowing equal spaces between the loaded samples. Samples were subjected to electrophoresis for 4 hours at 4°C (constant voltage of 90 volts per gel), followed by staining for activity at 40°C in the dark.

Staining for enzyme activity

The stain composition used was that of Fine & Costello (1963): 0.1 M Tris HCl pH 8.5, 2 M D(-) lactate, 30 mg ml^{-1} NAD^{+}, 5 mg ml^{-1} PMS and 10 mg ml^{-1} NBT (25 ml). This staining solution was incubated with the gel slab at 40°C in the dark and colour development of the zymogram occured within 30 to 45 minutes.

(b) SDS-gel electrophoresis (vertical slabs):

The procedure followed was that of Laemmli (1970), using a discontinuous acrylamide gradient (5 to 20%) as the

resolving gel. Molecular weight standards used consisted of: phosphorylase b (MW= 94 Kd); albumin (MW= 67 Kd); ovalbumin (MW= 43 Kd); carbonic anhydrase (MW= 30 Kd); trypsin inhibitor (MW= 20 Kd) and α-lactalbumin (MW= 14.4 Kd). Electrophoresis was allowed to proceed for 16 hours, at the end of which the gel slabs were stained with 0.5% Page Blue 83 (BDH dye) in methanol : acetic acid : water (25:10:65) and destained by diffusion in several changes of the same diluent.

Enzyme assay for D(-)LDH activity

Routine analysis of LDH activity was done at room temperature, using a Unicam SP 1800. The rate of change of NADH was measured at 340 nm. In the direction of lactate production, the assay contained in 3.00ml: 0.25 M triethanolamine HCl pH 7.5, 0.24 mM NADH and 1.00 mM pyruvate. In the direction of pyruvate production, the assayed mixture contained: 0.05 M Tris HCl pH 9.00, 0.40 mM NAD^+ and 0.04 M D(-) lactate. One unit of enzyme activity is defined as that amount of enzyme which elicts a change in absorbance at 340nm of 1.0 unit min^{-1} in the assay system given; this is equivalent to the oxidation of 0.40 umol of NADH per minute. The effect of temperature on enzyme activity was tested by incubating the enzyme and determining the % loss of activity with time at 10, 20 and 30°C.

Assessment of pH profile

Enzyme assays as described above for routine analysis were carried out at room temperature from pH 5.00 to 10.00, at

0.5 pH unit intervals.

Protein determination

The method of Lowry et al (1951). was used to determine the sample protein concentration. Known concentrations of bovine serum albumin were used as standards.

RESULTS

Enzyme Purification

A summary of the purification statistics is given in **Table 4.1.**(A & B). Both the foot and mantle enzymes are susceptible to degradation and are temperature labile. The degree of purity is shown in **Fig. 4.1** where the preparation is represented by one band after SDS PAGE.

The profile of the chromatofocusing column reveals 2 peaks corresponding to pI values of 7.20 and 6.75, for D(-)LDH in the foot (Fig. 4.2.) , whereas only one peak is present in the mantle at pI of 6.98, though this peak is not as sharply defined (Fig.4.3.). The final isolated enzyme is unstable even at -70°C, losing 40% activity after 3 weeks stored in 20% glycerol which contained 30 mM β-mercaptoethanol. The specific activity of the purified enzyme is higher in the foot (8.174 U mg^{-1}) than in the mantle (3.645 U mg^{-1}), though lower than that reported for other gastropods (Gäde 1980).

TABLE 4.1. A

Summary of Bullia digitalis foot muscle D(-)LDH purification procedure.
(AMS† = ammonium sulphate precipitation; E.U.†† = enzyme units)

	Vol. (ml)	Total E.U.††	Total Protein (gm)	Sp. Act. E.U. mg^{-1}	Purification Level	% Recovery
Cell free cytosol	632.10	5160	14.530	0.172	1.0	100.00
40% AMS† ppt. (after dialysis)	60.20	3311	2.750	0.516	3.0	64.17
Sephacryl-S200 column	116.10	1626	0,240	2.924	17.0	31.51
DE-52 ion exchange column (after concentration + dialysis)	50.74	1320	0,115	43.000	250.0	25.58
PBE94/Poly Buffer 96 chromatofocusing						
pI 6.75	12.00	73.96	0.258	89.210	519.0	1.43
pI 7.20	6.300	156.79	0,503	122.430	711.0	3.04

TABLE 4.1. B

Summary of Bullia digitalis mantle muscle D(-)LDH purification procedure.
(AMS† ppt. = ammonium sulphate precipitation; E.U.†† = enzyme units)

	Vol. (ml)	Total E.U.††	Total Protein (gm)	Sp. Act. E.U. mg^{-1}	Purification Level	% Recovery
Cell free cytosol	204.30	396.36	6.696	0.018	1.0	100.00
40% AMS† ppt. (after dialysis)	221.40	408.96	5.094	0.036	2.0	103.10
Sephacryl-S200 column	63.00	341.55	0.175	0.702	39.0	86.17
DE-52 ion exchange column (after concentration + dialysis)	6.66	256.95	0.022	4.176	233.0	64.83
PBE/Poly Buffer 96 chromatofocusing						
pI 6.98	11.50	85.14	0.003	65.484	3638.0	21.48

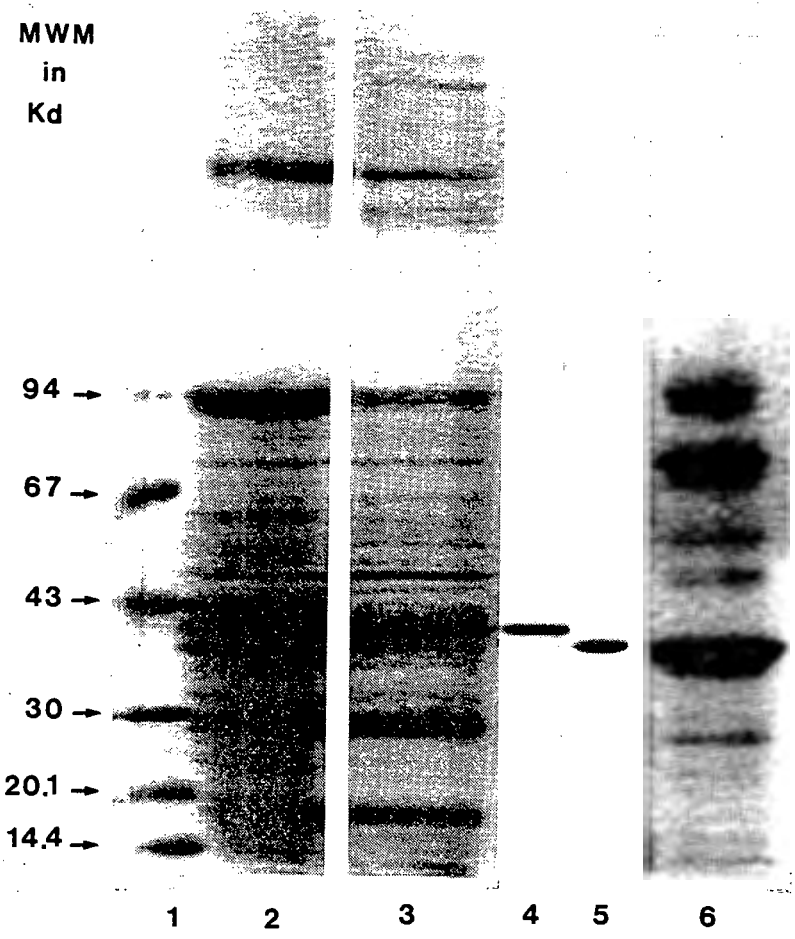


Figure 4.1. Gradient (5 to 20%) SDS-PAGE (sodium dodecyl sulphate -polyacrylamide gel electrophoresis) of LDH in Bullia digitalis foot and mantle muscle. **Lane 1:** Molecular weight (MW) markers (see Materials and Methods); **Lane 2:** Cytosol recovered from crude extract; **Lane 3:** D(-) LDH active cytosol fractions pooled from S-200 Sephacryl column run, prior to loading onto a DE-52 ion-exchange cellulose column; **Lane 4:** Foot D(-)LDH, subunit MW 38.5±2.9 Kd; **Lane 5:** Mantle D(-)LDH, subunit MW 36.2±3.7 Kd; **Lane 6:** D(-)LDH of Lactobacillus leichmannii, subunit MW 36 Kd (Boehringer Mainnheim West Germany), contaminants present at 90, 71, 52, 46 and 30 Kd). Note, owing to difficulties in photographic reproduction, blank areas occur on the gels, though none interfere with bands mentioned above.

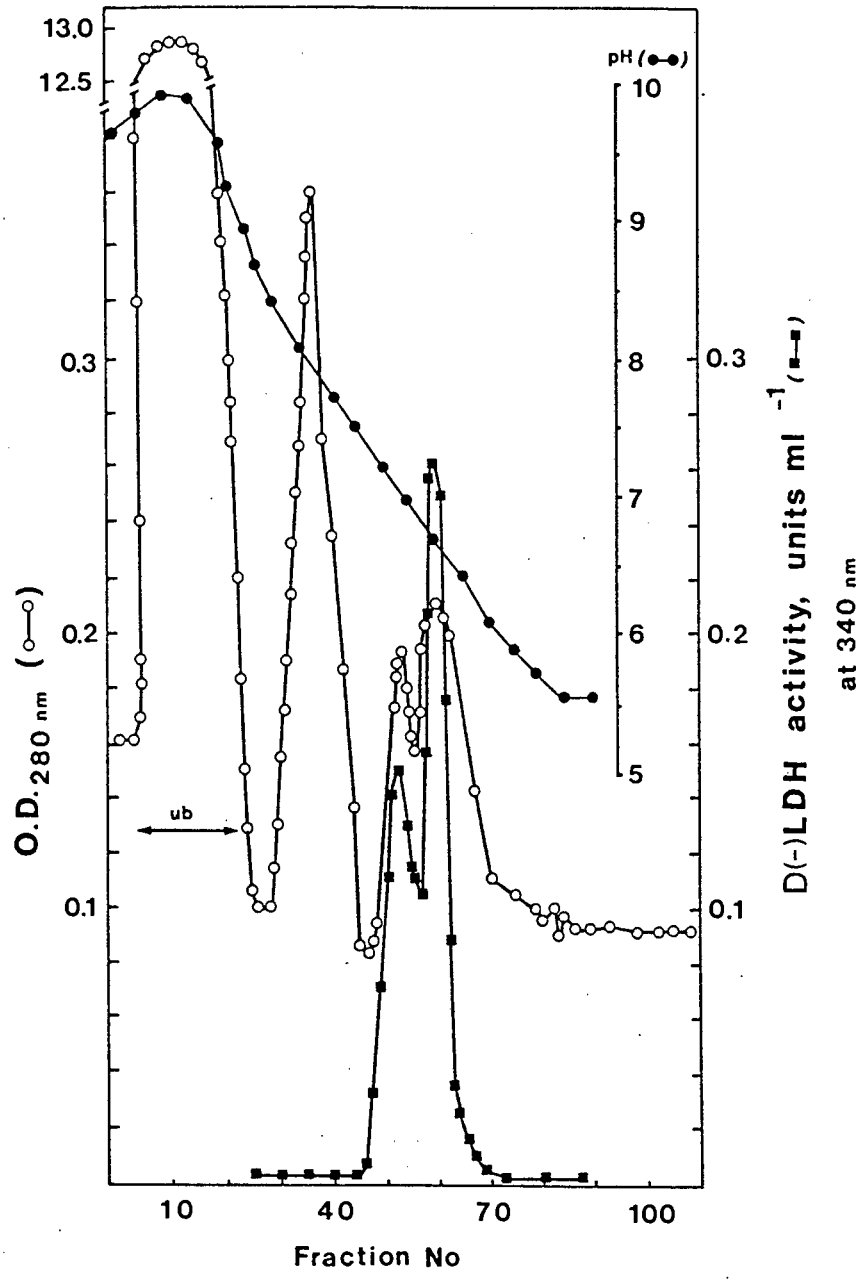


Figure 4.2. PBE 94/Polybuffer 96 Chromatofocusing column profile of foot D(-)LDH. Two D(-)LDH active peaks occur at pI 7.20 and 6.75. Protein content of column fractions as detected by u.v. light at 280 nm (O—O); D(-)LDH enzyme activity in column fractions (■—■); eluting pH gradient (●—●); ub: unbound protein fractions, whose pI fall outside of the pH range above (See Figure 4.1. for index of purity).

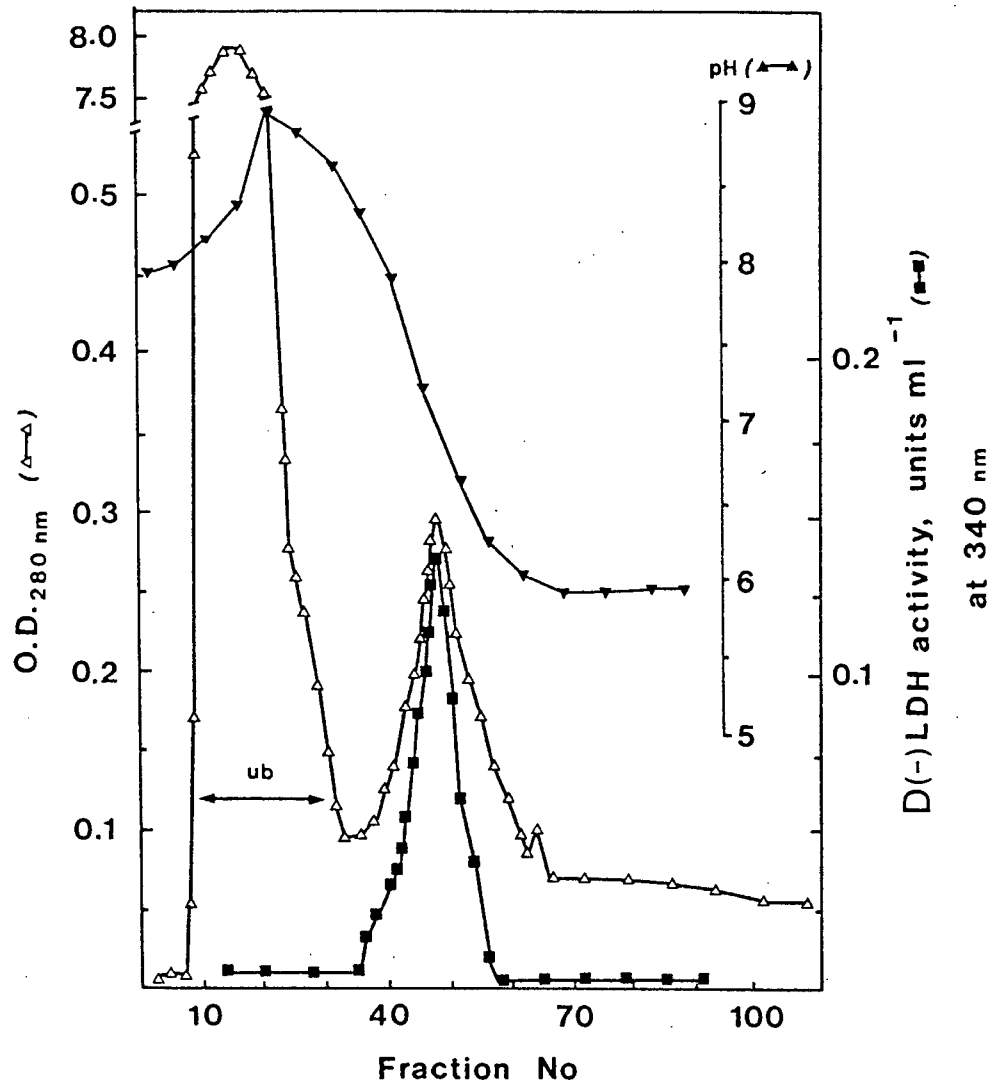


Figure 4.3. PBE 94/Polybuffer 96 chromatofocusing column profile of mantle D(-)LDH. This enzyme is represented by one active peak at pI 6.98. Protein content of column fractions, as detected by u.v. light at 280 nm (△—△); D(-)LDH enzyme activity of column fractions (■—■); eluting pH gradient (▼—▼); ub: as for Figure 4.2. (See Figure 4.1. for index of purity).

Physicochemical properties

(A) Molecular weight determination

The molecular weight of foot LDH (150±3.5 Kd) is slightly higher than that of mantle (140±2.8). Determination of subunit molecular weight gave a value of 38.5±2.9 Kd for the foot and 36.2±3.7 Kd in the case of mantle (Figs. 4.1. and 4.4.). This indicates that both enzymes are tetramers in their native conformation.

(B) Catalytic properties

Both foot and mantle LDH are D(-) specific and no reaction occurred in the presence of L(+) lactate. At the temperatures tested, foot LDH is more heat labile than the mantle equivalent. Mantle LDH displays substrate inhibiton at pyruvate concentrations above 5 mM, though this phenomenon is not observed in the foot enzyme.

Differential tissue expression of LDH

In the foot two isozyme forms predominate (Fig. 4.5.). These have been designated LDH-I and LDH-V, resembling H-type (LDH-1) and M-type (LDH-5) LDH of vertebrates, on the basis of their electrophoretic motility. Intermediate to these, are three other bands of lower intensity (LDH-II, LDH-III and LDH-IV), sensitive to denaturation at higher ambient temperatures. Of the major bands, LDH-V is the slowest and most active (as indicated by the intensity of the colour of the zymogram). This band is also the most stable regarding high or low ambient temperatures.

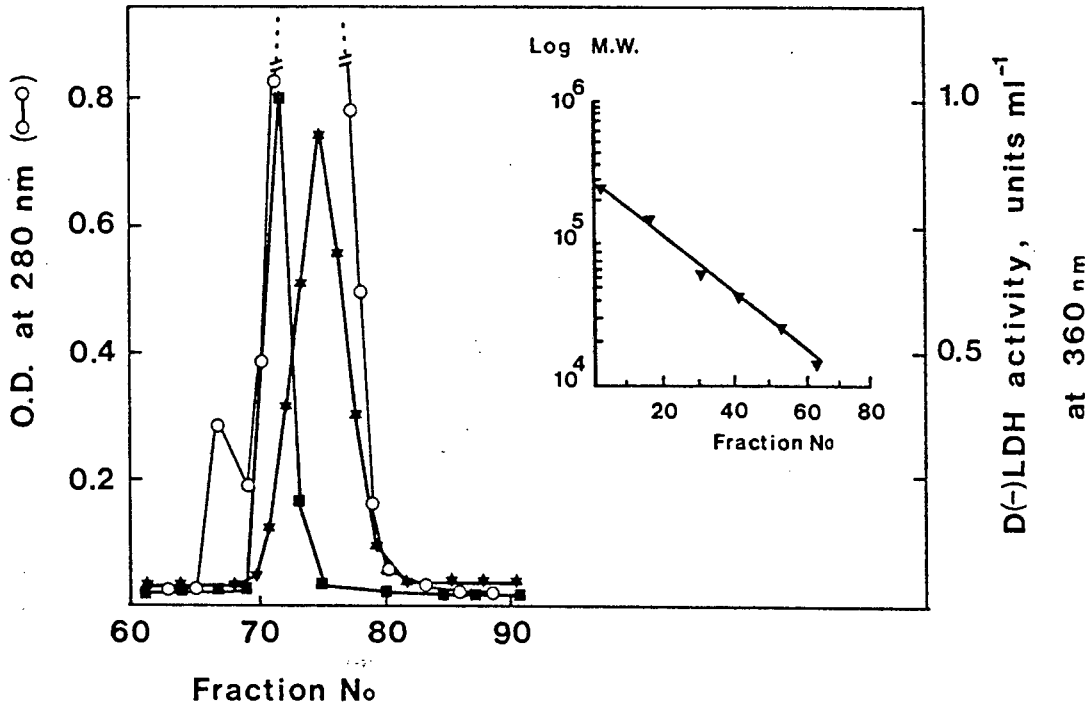


Figure 4.4. Determination of molecular weight of intact foot and mantle D(-)LDH on a Sephacryl S-200 column. Protein profile of foot enzyme (O—O); (that of mantle enzyme was omitted to simplify diagram). Foot D(-)LDH, active peak corresponding to a molecular weight of 150±3.5 Kd (■—■); mantle D(-)LDH, active peak corresponding to a molecular weight of 140±2.8 Kd (✱—✱). Inset: Molecular weight calibration curve (see Materials and Methods).

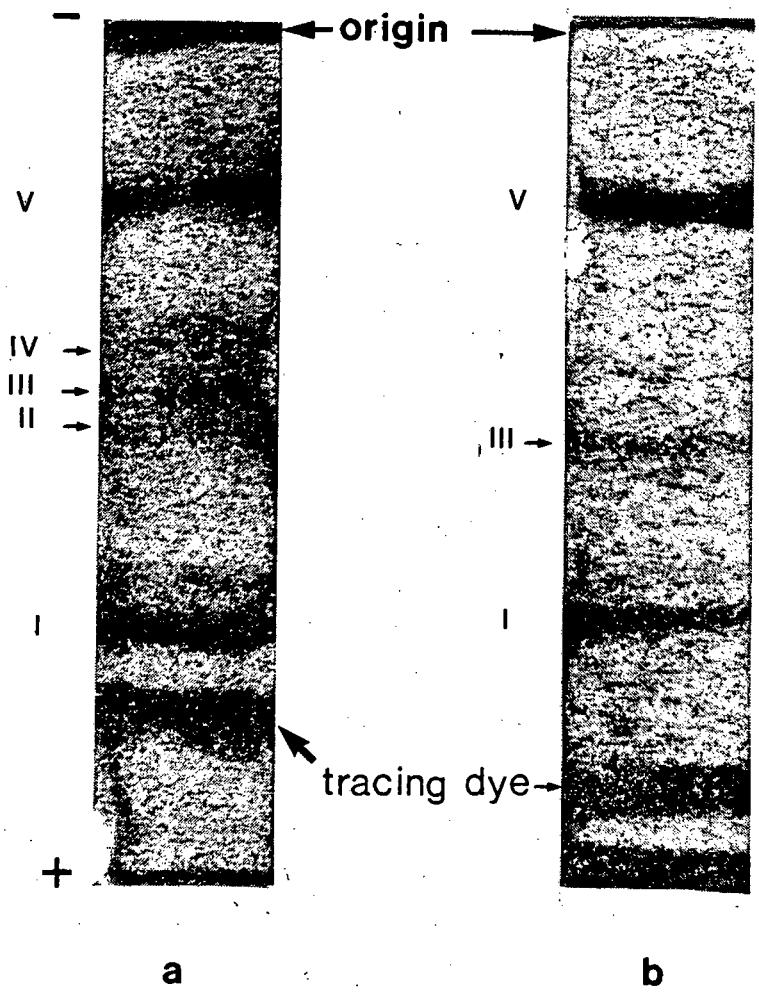


Figure 4.5. Non denaturing gels of foot and mantle muscle D(-) LDH. (a) D(-)LDH in the foot is represented by 2 major and 3 intermediate (hybrids, indicated by arrows) isozymes. (b) D(-)LDH in mantle is represented by 2 major and 1 intermediate isozymes.

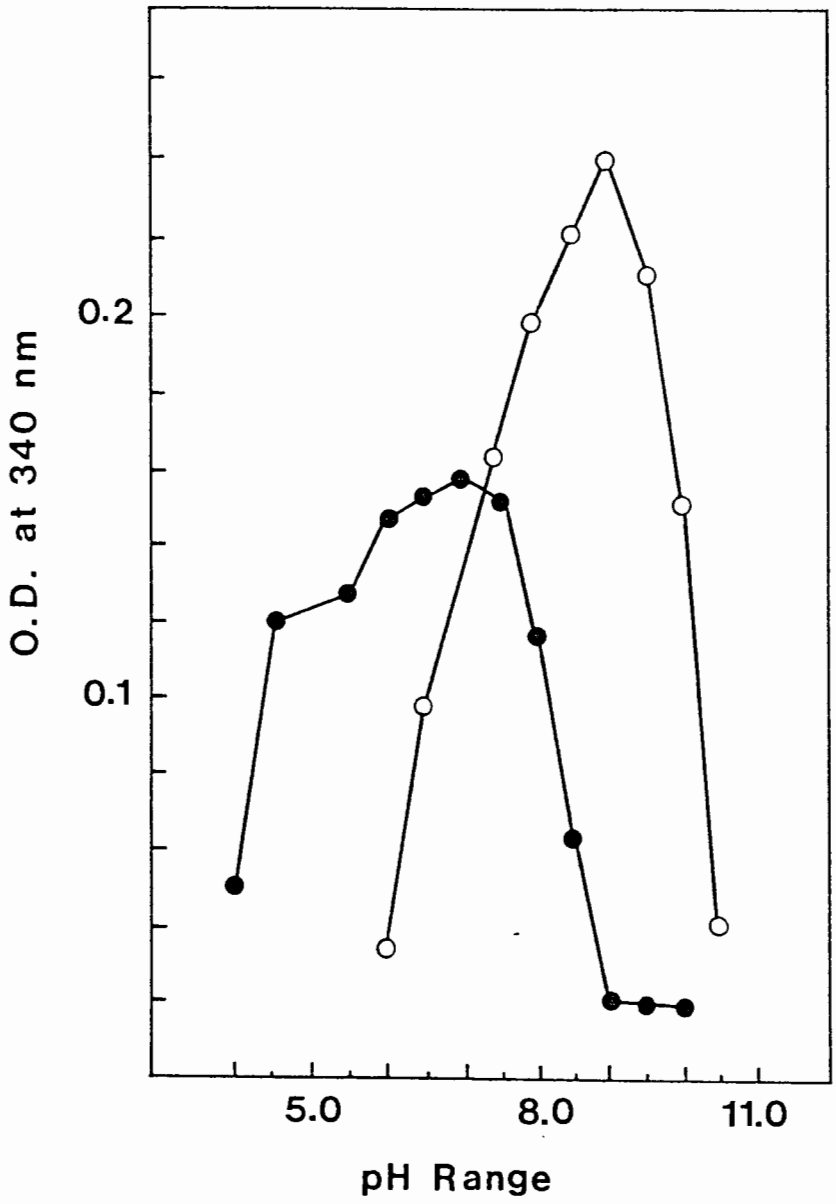


Figure 4.6. The pH profile of D(-)LDH (no significant difference between foot and mantle). The effect of pH on enzyme reaction velocity of D(-)LDH, in the pyruvate using direction (●—●). The effect of pH on enzyme reaction velocity of D(-)LDH in the lactate using direction (○—○). Reaction catalysed by D(-)LDH: PYRUVATE + NADH + $H^+ \rightleftharpoons$ D(-)LACTATE + NAD^+.

In the mantle, generally only two isozyme forms are expressed (LDH-I and LDH-V), yet an intermediate band is sometimes present. Unlike the phenotypic profile of foot LDH, in the mantle, LDH-I is the most active and the least temperature labile of the 2 (or 3) isozymes.

The pH profile

The pH profile of both reaction directions is given in Fig.4.6. There are no significant differences between the two enzymes and a pH optimum for both enzymes occurs at pH 9.00 in the pyruvate forming reaction. In the direction of lactate production the pH optimum occurs at pH 7.30.

DISCUSSION

Five electrophoretically distinct isozymes displaying different LDH activities have been shown to be present in the foot and two (possibly three) in the mantle of B.digitalis. This pattern is unlike that of most molluscs, in which LDH tends to be characterized by 2 isozyme bands (Coles 1969, Pesch 1972, Storey 1977, Ottaviani & Ferrari 1982). Nevertheless, 5 LDH bands have been shown to occur in Helisoma antrosum (Rodrick et al. 1971), in Biomphalaria glabrata (Narang 1974) and in Argobuccinum origonenses (Goldberg & Cather, 1963).

If homology between the isozymes of B.digitalis and those of vertebrates is assumed, then LDH-I and LDH-V (in the whelk) correspond to LDH-1 and LDH-5 of vertebrates, respectively. The phenotypic profile of LDH in the foot of B.digitalis indicates that of the 5 isozymes LDH-V is the most active. In contrast, LDH-I is predominant in the mantle. Besides these differences, the enzymes in the mantle are more temperature labile, especially LDH-I which becomes inactive as the temperature increases. This is contrary to the findings of Vesell & Yielding (1966) on rabbit skeletal muscle, who established that LDH-5 was the most heat labile isozyme. Vesell & Yielding (1966) attributed the differences in temperature response to the effects of pH and ionic strength on the isozymes, since these factors appeared to be instrumental in the determination of the conditions providing enzyme stability and permanency of specific gene expression. Since β-mercaptoethanol or PMSF were used in the preparative stages it is unlikly that enzyme instability is a consequence of proteolytic action. Instead it is more likely that this enzyme's instability is an intrinsic in vivo property, which may reflect fast turnover; fast turnover may contribute to regulation of appropriate enzyme numbers and might thus be a metabolic adaptation.

In mammals LDH is a tetramer which results from the association of two different types of subunits, designated M (muscle type) and H (heart muscle type). This association gives rise to 2 major homotetramers, namely LDH-1 (H_4) and LDH-5 (M_4) and 3 hybrids, LDH-2 (MH_3), LDH-3 (M_2H_2) and LDH-4 (M_3H), each

displaying different electrophoretic mobility and catalytic properties (Cahn et al. 1962, Markert 1963). Cahn et al. (1962) and Dawson et al. (1964) proposed the differences in substrate inhibition between LDH-1 and LDH-5 to be physiologically significant. LDH-1 is known for its sensitivity to substrate inhibition by pyruvate and favours glycolytic carbon flow into the Krebs cycle and lactate into the Krebs cycle or gluconeogenesis (Cahn et al. 1962, Kaplan et al. 1968, Everse & Kaplan 1973). LDH-5, on the other hand is most active in channeling glycolytic carbon into lactate during anaerobic stress. The regulation of selective enzyme expression, allowing for isoenzyme segregation is a crucial, yet poorly understood phenomenon. It has been suggested that part of the answer to this problem relies on post-translational mechanisms, such as rates of catabolism of each gene product (Vesell & Yielding 1966). Since in mammalian cells the rate of catabolism is vital in determining the steady state of enzyme levels, in addition to gene activity (Schrimke et al. 1965), it is possible that the same mechanism applies in molluscs.

In B.digitalis, LDH-I of mantle is not as susceptible to pyruvate inhibition as is the H-type of mammals (Vesell and Pool 1966).

By contrast LDH-V in the foot is able to handle high loads of pyruvate. This may be made possible by its conversion into lactate, which in turn can be reconverted to pyruvate (Storey 1977) channeling into other pathways, i.e. fatty acid

biosynthesis (Oudejans & van der Horst 1974); use by octopine dehydrogenase, which is 10 times as active as LDH in this species (da Silva et al. in prep.) and shares pyruvate as a common substrate, which interacts also with phosphoarginine kinase in the regulation of phosphagen levels.

It is difficult to establish whether the presence of 5 isozymes in the foot of B.digitalis is physiologically significant. These could have resulted from accidental dissociation and reassociation of the enzyme's subunits in vitro and may not be related to the random recombination of the two dissimilar LDH subunits in vivo, as suggested by Appella & Markert (1961). Freeze-thaw hibridization in high salt between individual forms of the slow and fast migrating proteins did not result in reassociation, unlike the results reported by Storey (1977) with LDH of Helix aspersa; however this could have been due to the unstable nature of the isozymes, whereby a modification on the surface of the protein (change in or loss of group polarity) could render it unable to recombine with other subunits.

The foot of B.digitalis is peculiar in that although it resembles white muscle, morphologically it also displays characteristics typical of red muscle. These include a high mitochondrial content, close association of these with the myofibrils and the degree of ramification of the sarcoplasmic reticulum (da Silva et al. 1985). These characteristics may have

arisen as a physiological adaptation, essential to the whelk's mode of life. Of even more relevance is the functional differentiation of the pedal musculature into 2 regions: the small propodium (anteriorly) and the much larger metapodium, each distinguished by a different muscle fiber types (da Silva et al. 1985), an unusual feature in gastropods (Brown et al. 1985a).

The inability to hybridize the subunits of the fast and slow LDH isozymes may be a consequence of enzyme compartmentization (possibly into different fiber types). Thus in vivo, the expressed subunits may be physically isolated from each other. Therefore, subunit association into heterodimers may not be a frequent occurance. This still leaves the problem of the intermediate bands especially in the case of foot LDH. However, if not resulting from sporadic subunit reassociation, they could have arisen by post-translational modifications of one gene product. To verify this hypothesis it would be necessary to dissect out the two distinct fibers characteristic of the foot and to isolate the cytosolic fraction of each. An electrophoretogram of both samples would reveal wether or not both fibers share common phenotypic expression.

In mantle, a large contribution to the phenotype of LDH may result from contamination of surrounding tissues, such as heart, kidney and osphradium, these being virtually impossible to remove. Hence, the pattern observed on the electrophoretogram may be that of the pallial complex rather than that of the mantle and it is possible that if complete removal of contaminating tissues

were possible the pattern for the mantle tissue alone would be different. However, in vivo the pallial complex works as a single unit and thus the current observations may be just as relevant.

Temperature fluctuations are known to have dramatic effects on intertidal species with respect to reproductive behaviour, feeding and locomotory activity (Thébault 1984). To date little understanding exists of the relationship between environmentally altered behaviour and the respective metabolic changes, despite a concerted effort from researchers throughout the world (Hochachka & Somero 1984). The effects of temperature on enzyme reaction rates are well documented. They are influenced by local pH, substrate concentration, enzyme sensitivity to denaturation and the cellular capacity to resynthesise damaged enzyme (Ferdinand 1976). B.digitalis is exposed to rapid and sudden temperature changes, often up to 10°C within only a few hours. Yet, irrespective of these temperature fluctuations, no impairment and little change is observed in any of the whelk's activities (Brown 1982). The likelihood of D(-)LDH isozymes in B.digitalis, at the pyruvate branch point, was considered primarily due to these enzymes' involvement in the aerobic-anaerobic transition, during muscle work.

The presence of a series of isozymes, characterized by a set of specific features, would enable them to function optimally, depending on the status of the surrounding cellular environment (Somero & Hochachka 1976). A wide range of temperature sensitivities of these isozymes may allow selective

use of structural elements with the desired biochemical features so that unpredictable cellular responses are rapidly compensated without disruption of the cellular homeostasis. The physiological selection of the correct isozyme may in addition gain from naturally high enzyme turnover, whereby an enzyme is immediately destroyed (and its amino-acids recovered in the synthesising pool) on completion of its task.

Although the physicochemical properties of the two tissue isozymes differ, this may not necessarily be due to differential gene expression. It could be the result of a post-translational modification, which may be needed for adequate cellular function. In vitro the situation is possibly quite remote from the physiological status quo; even though the reaction mixture simmulates in vivo conditions, there is no guarantee that these are of any relevance to the optimal conditions in vivo.

The effects of temperature on LDH may in fact be more pronounced in vitro, for in the cell, natural buffering conditions are provided as well as interactions with other components; these may protect the enzymes from extreme responses. Temperature effects may result in mere shifts of enzyme activity or metabolite concentration, which are restored as soon as possible. Thus cellular homeostasis is maintained.

CHAPTER 5

THE EFFECT OF TEMPERATURE ON THE ACTIVITY OF FOOT MUSCLE LDH IN THE WHELK *BULLIA DIGITALIS*

INTRODUCTION

Fluctuations in environmental temperature are amongst the most critical problems encountered by ectotherms. Since the latter have no control over this phenomenon and their body temperature equilibrates with that of the surrounding environment (Hazel & Prosser 1974), most such species have evolved specific physiological adaptations to tolerate and compensate for the chemical and metabolic changes which accompany these thermal inconsistencies (Somero & Low 1977). As a result most ectotherms are able to survive a wide range of temperatures (Hazel & Prosser 1974).

The nature of these physiological compensatory mechanisms differs, depending on whether or not they are instataneous, as is the case with intertidal species; this is especially true of those that do not acclimate. However, acclimation can also give rise to the development of metabolic compensations over a longer time scale and if environmental temperature alterations exhibit some permanency, evolutionary selectivity occurs towards the features that enable the organisms

to cope with new thermal regimes (Hochachka & Somero 1973; Somero 1969, Somero & Hochachka 1969).

Behavioural regulation of body temperature is often of relevance during short lived thermal fluctuations; on the other hand adaptation to seasonal or irregular diurnal changes requires more extensive biochemical and phenotypic modifications (Hazel & Prosser 1972).

The consequences of temperature fluctuations for intertidal organisms are reflected both physiologically and behaviourly (Hazel & Prosser 1970, 1972, Thébault 1984). Though one might predict that the metabolism of invertebrates varies significantly with temperature, data collected on the sea anenome Actinia equina, the winkle Littorina littorea, the cockle Cardium edule, the barnacle Balanus balanoides and the mussel Mytilus edulis have shown that a standard rate of activity, characterized by a Q_{10} which approximates unity, is maintained during quiescent periods over most of the environmental temperature range (Newell 1969, Newell & Northcroft 1967, Newell & Pye 1970). This temperature independence of whole body metabolism appears to be a consequence of some mitochondrial properties, as indicated by the low Q_{10} values obtained for pyruvate and succinate oxidation, at physiological concentrations (Newell 1967).

B.digitalis, which inhabits the high energy exposed sandy beaches of the west coast of South Africa, where the water temperature is subject to frequent and sudden changes due to

upwelling, is able to maintain an almost flat rate of oxygen consumption, over a temperature range of 12°C (between 10 and 22°C), at all levels of activity (Brown & da Silva 1979, 1983). This phenomenon is possibly the result of a physiological adaptation to a constantly changing thermal environment as the whelk does not acclimate (Brown et al. 1978, Brown & da Silva 1979).

Temperature adaptive changes have been known to occur at the pyruvate branch point, in LDH (Hochachka & Somero 1968), an enzyme generally associated with the regulation of the cellular redox function (Somero 1978). Though the relevance of LDH in molluscs has been questioned (Gäde 1980, Dando et al. 1981, Baldwyn et al. 1981), in B.digitalis it is represented by five isozyme forms, two of which exhibit different temperature sensitivities (da Silva et al. in prep.). Thus it was decided to investigate the kinetic properties of this multiple LDH isozyme system in B.digitalis over the temperature range encountered in the field.

MATERIALS AND METHODS

Biological material and preparation of foot muscle extracts of LDH

Animals were collected from the west coast of South Africa, just north of Cape Town and maintained in an aerated sea water tank at 15°C. The whelks were not fed in captivity.

Adult B.digitalis (shell length 3.48±0.32 cm) were sacrificed by freezing in liquid nitrogen and the foot excised immediatly after thawing. The feet of 30 individuals (28.00 gm) were homogenized in 4 parts of a 0.020 M Tris-HCl buffer pH 7.40, containing 0.030 M β-mercaptoethanol, 0.100 M sucrose and 0.001 M EDTA. The suspension was centrifuged at 25000 g for 30 minutes using a Sorval RC2-B centrifuge in an SS-34 fixed angle rotor. The supernatant was stored at 4°C, prior further analysis. A weekly LDH extract was prepared in view of loss of activity even at -70°C.

Enzyme assays for D(-) LDH activity

Routine assays were carried out at 20°C, though initial reaction rates were measured between 5 and 30°C, at 5 degree intervals, using a Varian 637 spectrophotometer linked to a temperature-regulated water bath. The rate of change of NADH was followed at 340 nm. Plots of initial velocity vs. substrate concentration (Michaelis plots) were constructed. Values of V_{max}, K_m and K_i were evaluated by non-linear fitting of data to the relevant steady state equation: either the Hill equation

$$V = \frac{V_{max} \cdot (S)^n}{K_m + (S)^n}$$

$$V = \frac{V_{max} \cdot (S)^n}{K_m + (S)^n + \frac{(S)^2}{K_i}}$$ ← or the Haldane equation

using a program written in BASIC for a Techtronix 4051 micro-computer. Data were treated by single factor analysis of variance (ANOVA) and a Neuman-Keuls multiple range test ($p<0.05$) was used

to differentiate significantly different kinetic parameters at the temperatures indicated.

The assay in the direction of lactate production contained in 3.00 ml: 0.25 M Triethanolamine HCl pH 7.40, 0.001 M pyruvate and 0.00024 M NADH. In the lactate using direction , the assay mixture contained 0.050 M Tris-HCl pH 9.00, 0.040 M D(-) lactate and 0.004 M NAD^+. Reaction was initiated by the addition of 50 ul of enzyme extract (2.5 mg ml^{-1}). One unit of enzyme activity is defined as that amount of enzyme which elicits a change in absorbance at 340 nm of 1 unit min^{-1} in the assay system given. This is equivalent to the oxidation of 0.40 umol of NADH per unit.

Non denaturing gel electrophoresis

A method modified after Ornstein (1964) and Davis (1964) was used, in which slab gels were used in place of tubes. A gel-forming chamber was constructed: 6 cm high, 12 cm wide and 0.15 cm thick. Wells were formed on the large pore gel with a gel comb, allowing equal spaces between the loaded samples. The sample was subjected to electrophoresis for 4 hours at 4°C, followed by activity staining of a 1 cm wide strip of the gel, to determine the relative location of the major isozyme bands.

Staining for enzyme activity

The stain composition used was that of Fine & Costello (1963): 0.1 M Tris HCl pH 8.5, 2 M D(-) lactate, 30 mg

ml^{-1} NAD^+, 5 mg ml^{-1} PMS and 10 mg ml^{-1} NBT (25 ml). This staining solution was incubated with the selected gel strip at 40°C in the dark. Colour development of the zymogram occured within 30 minutes.

Enzyme assays of single isozyme bands separated electrophoretically on acrylamide gels

Once the isozyme band was localized on the non-denaturing gel by activity staining of the gel strip, the area on the unstained gel corresponding to the desired band (including a 2 mm margin above and below the band) was cut and immersed in the minimal volume of assay buffer required to cover the slice. This was kept on ice and in the dark until the sample was used in the spectrophotometer, where the additional buffer and the remaining assay components were added and equilibrated to the desired temperature. The gel slice was positioned in the cuvette so that there was no interference with the light pathway.

Protein determination

The method of Lowry et al. (1951) was used to determine the sample protein concentration. Known concentration on bovine serum albumin were used as standards.

RESULTS

The response of LDH activity to temperature changes

The relationship between enzyme activity and temperature is shown in Fig. 5.1. In the pyruvate using direction, enzyme activity is lowest below 10°C, increasing three fold to 15°C and forming a platteau at this level up to 25°C; above this temperature the enzyme activity decreases. Enzyme activity is temperature independent below 10 and between 15 and 25°C, being highly temperature dependent between 10 and 15°C and inversely proportional to temperature above 25°C. In the reverse direction, LDH activity is low below 10°C (possibly temperature independent), becoming temperature dependent above this temperature and reaching a maximum between 25 and 30°C.

Measurement of maximal velocity with increasing pyruvate concentrations (Fig. 5.2.), shows that not only does the maximal activity rise with increasing substrate concentration, but also its thermal optimum shifts proportionally to temperature. At physiological concentrations, maximal activity occurs at 20°C, though as the substrate levels increase to 1.50 mM the thermal optimum shifts to 25°C. In the presence of 2.50 mM pyruvate, at 25°C, enzyme activity decreases rapidly. This could be due to thermal denaturation or accelerated denaturation by substrate binding (Robb et al. 1971) or possibly to substrate inhibition.

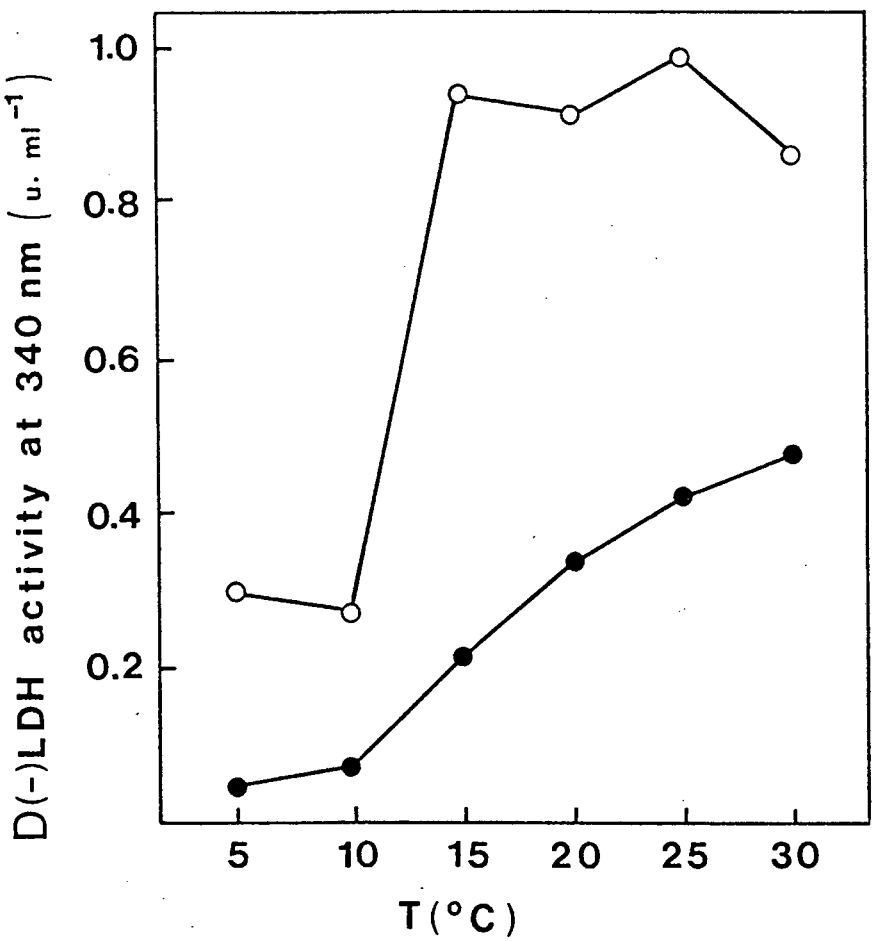


Figure 5.1. The effect of temperature on the pyruvate using (○—○) and forming (●—●) reaction of foot D(-)LDH. (See Materials and Methods for assay conditions).

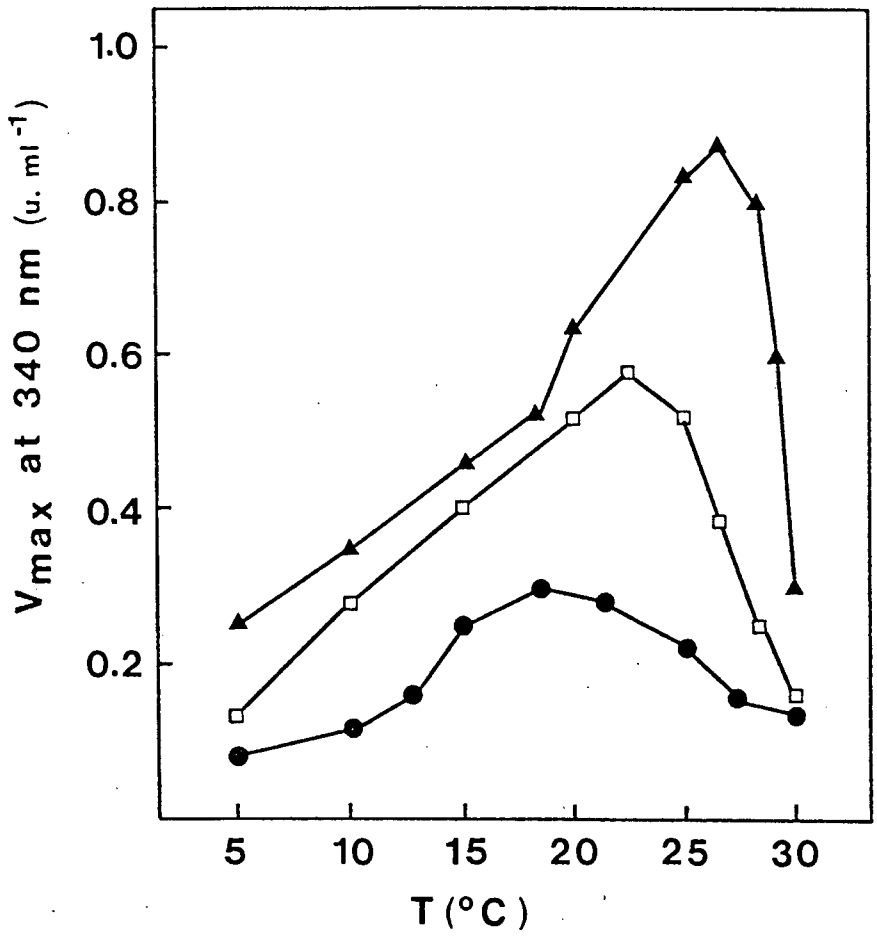


Figure 5.2. The effect of temperature on foot D(-)LDH reaction velocity, with increasing pyruvate concentrations. Reaction conditions as stated in Materials and Methods, except for pyruvate concentration, which varied from 0.50 mM (●—●), 1.50 mM (□—□) to 2.50 mM (▲—▲).

The K_m for pyruvate decreases from 5.10 mM (at 5°C) to 2.52 mM (at 20°C), which is its minimum value (Fig. 5.3). Above this temperature it rises steadily to 8.37 mM at 30°C. This implies that at 20°C the enzyme-substrate affinity is at least 2 fold higher than that at 5 or 30°C. In contrast the K_m for lactate is independent of temperature between 5 and 15°C (attaining its lowest values from 2.12 to 2.50 mM), though temperature dependent above 15°C. No significant temperature effect is observed on the K_m of either co-factor (Table 5.1.). Pyruvate inhibition (K_i) is present only below 15°C; above this temperature, the pyruvate concentrations necessary to cause inhibition are in excess of 40 mM, a level which was not considered physiologically significant and it was therefore assumed that above 15°C substrate inhibition is absent *in vivo* (Table 5.1.). Of interest is the fact that the K_i for pyruvate is several folds lower than the corresponding K_m. This implies firstly that the active site is not exposed to concentrations above K_i levels, with the consequence that V_{max} is never attained. Furthermore, since the active site is only exposed to concentrations below K_m, small fluctuations in substrate result in large changes in enzyme activity. No significant inhibitory effects occured with either excess NADH or NAD^+ (Table 5.1.).

The effect of temperature on isozyme bands I and V of LDH

Results are shown in Table 5.2. At 25°C, though the K_m of LDH-I for pyruvate (2.95 mM) is slightly higher than that of LDH-V (2.03 mM), this is not true for K_i. At this temperature,

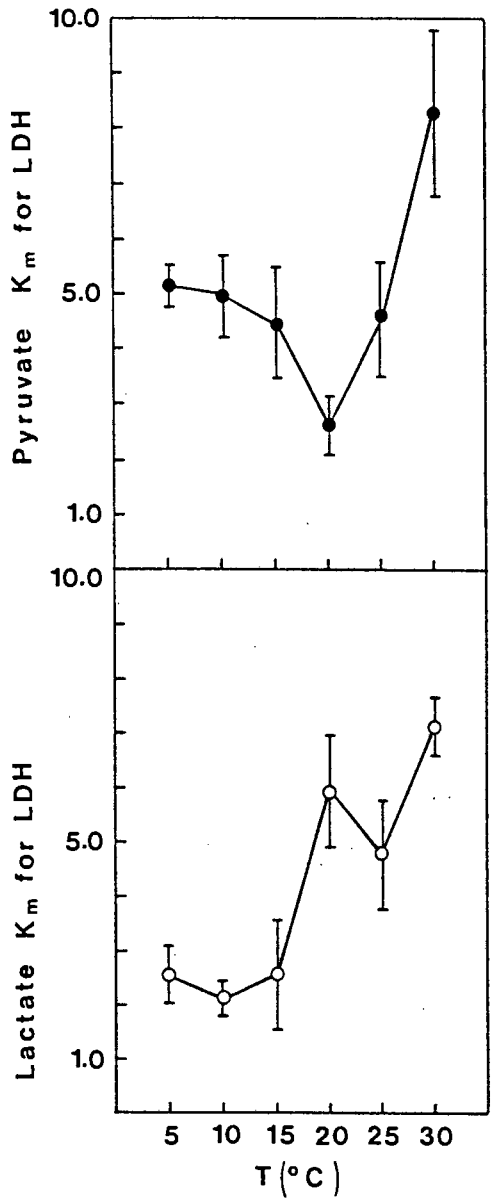


Figure 5.3. The effect of temperature on the K_m of pyruvate (●—●) and lactate (○—○), of D(-)LDH from Bullia digitalis foot.

TABLE 5.1. The effect of temperature on some kinetic parameters of the combined isozymes of foot D(-)LDH in Bullia digitalis. (All values within 10% error calculated from 6 kinetic analysis at each temperature.)

	Temperature (°C)					
	5.0	10.0	15.0	20.0	25.0	30.0
K_m						
NADH	0.120	0.220	0.195	0.180	0.099	0.203
NAD†	*	2.284	1.923	2.403	2.310	2.30
K_i						
PYRUVATE	2.70	3.46	4.00	52.00	**	**
D(-)LACTATE	10.80	12.52	**	**	**	**
NADH	0.245	0.289	0.312	0.282	0.305	0.261
NAD	**	**	**	**	**	**

* At this temperature it was not possible to establish a km value for NAD†.

** No inhibition observed with physiological substrate concentrations.

pyruvate inhibition of LDH-I (K_i= 1.95 mM) is present with relatively low concentrations of substrate; in order to elicit a similar response on LDH-V, much higher concentrations of the same substrate are necessary (K_i= 8.06 mM).

A decline in LDH-I activity is present at this temperature due to either substrate or cofactor inhibition, except that lactate does not give rise to this phenomenon. No inhibition was observed with LDH-V at this temperature (Table 5.2.).

Decreasing the temperature to 10°C results in a 10 fold decrease in the K_m value for pyruvate of LDH-I (from 2.95 mM at 25° to 0.27 mM at 10°). This is accompanied by a rise in K_i, thus releasing LDH-I from the constraints of pyruvate inhibition, present at 25°C. This has given rise to increased E-S affinity at 10°C. A slight decrease in the K_m and K_i of NADH has also occurred, though its significance is not clear. In contrast, the K_m and K_i for pyruvate of LDH-V increases at 10°C, representing at least a three fold loss in E-S affinity. Thus it is concluded that at the lower temperature LDH-I activity is favoured, in contrast to that of LDH-V. The reverse applies at the warmer temperature.

TABLE 5.2. The effect of temperature on some kinetic parameters of isolated foot D(-)LDH bands (I and V) of *Bullia digitalis*.

Temperature (°C)	Band I				Band V			
	PYR.	NADH	D(-)LAC	NAD+	PYR.	NADH	D(-)LAC	NAD+
10								
K_m (mM)	0.27	0.15	3.50	0.43	7.20	0.20	2.20	2.41
K_i (mM)	1.51	0.10	-	1.08	10.00	-	-	-
25								
K_m (mM)	2.95	0.20	5.00	0.45	2.03	0.20	11.32	2.59
K_i (mM)	1.95	0.28	-	0.75	8.06	-	-	-

DISCUSSION

Enzyme reaction rates in ectotherms may be highly temperature independent, at physiological substrate concentrations (Cowey 1967, Hochachka & Somero 1968, Somero & Hochachka 1968, Newell & Pye 1971). This appears to be the result of an inverse relationship between E-S affinity and temperature (within the thermal range characteristic of the species habitat). In addition, E-S affinity is often maximal at the environmental temperatures most frequently encountered by the species, even at the lower thermal range (Hochachka & Somero 1969, Somero 1968). Paradoxically, the increase in E-S affinity at these temperatures is sufficient to compensate for the accompanying decrease in catalytic rate, predicted on thermodynamic grounds (Atkinson 1966).

The temperature response of the K_m of pyruvate for LDH in B.digitalis, is analogous to that reported for some fishes by Hochachka & Somero (1968). The thermal optima, defined as the temperature range at which K_m values are at a minimum, occur at the temperature most commonly encountered by the whelk in the field. Despite the fact that whole LDH extract exibits a thermal optimum for the pyruvate K_m at 20°C, the situation is quite different for isolated LDH-I and LDH-V. These isozymes are distinguishable by their kinetic properties as well as by temperature sensitivities, indicating that LDH-I favours the lower thermal range in contrast to LDH-V. Since LDH-II, LDH-III and LDH-IV were not examined kinetically, due to their

instability and close proximity during electrophoretic separation, it is impossible to comment on the extent to which these other isozymes contribute to the overall properties displayed by whole LDH extract. However, one can suggest that at 20°C, a major contribution may result from LDH-V due to the resemblance of its properties at 25°C, to those of the whole extract at 20°C.

The temperature dependence of the pyruvate K_m above 20°C is represented by a 3 fold decrease in E-S affinity, resulting in deceleration of V_{max} as the temperature rises. This implies that under limiting substrate concentrations, the catalytic rates tend to be higher in the lower thermal range. By contrast, when substrate concentration is in excess of K_m values, the catalytic rates will increase proportionaly to temperature. The result is a family of temperature curves whose thermal optima shift upwards, towards the higher temperatures, as illustrated in **Fig. 5.2.** Denaturation may be present at the more elevated temperatures, since at 30°C the V_{max} decreases considerably. Similar thermal effects have been observed by Hochachka & Somero (1968) for the South American lung fish and by Thébault (1984) for the shrimp Palaemon serratus.

This mechanism, as suggested by Hochachka & Somero (1968), has a number of advantages. It means that the reaction velocity of LDH (and possibly that of other enzymes) is maintained independent of temperature, provided substrate concentrations remain below that of K_m levels. Thus, during

periods of routine activity, when energy demands are easily met, the whelk's metabolism involving LDH would be freed from temperature dependance, though when exposed to extreme thermal ranges, especially grossly elevated temperatures, the enzyme would respond to higher substrate concentrations and activity would increase as predicted thermodynamically. Even if substrate levels are below half saturation, a significant alteration in enzyme activity can still be obtained, since unsaturated systems are highly sensitive to substrate changes. A phenomenon similar to this has been observed by Newell (1966) on routine and active rates of metabolism in certain ectotherms.

It thus appears that the control of E-S affinity may be crucial to the sucessful adjustment of an enzyme to differing thermal regimes. Studies focusing on short term acclimation have generally revealed the presence of either of two strategies: (a) control of enzyme levels and type in a cell; (b) control of enzyme variants (isozymes), with suitable kinetic requirements, enhanced as a function of temperature (Hochachka 1967, Smith 1967). B.digitalis does not acclimate (Brown et al. 1978) and is characterized by 5 LDH isozymes, two of which are dominant. It is therefore assumed that there must be 2 subunits which can generate 5 different tetrameric LDH as is the case with most organisms (Markert 1963). However, the 2 major isozymes may not be expressed in the same cell (da Silva et al. in prep.), as examination of the fine structure of the pedal musculature has revealed two distinct types of fibers with differing morphological and possibly contractile properties (da Silva et

al. 1985) Although isozymes are frequently encountered in molluscs, generally only 2 or 3 are expressed (Ottaviani & Ferrari 1982). Expression of greater isozyme numbers have, however, been reported (Goldberg & Cather 1962, Rodrick et al. 1971, Narang 1974). Thus it is possible that this enzyme multiplicity in B.digitalis is a physiological adaptation to a constantly changing environment. However, isozyme multiplicity is not as common as expected (Somero 1975, Shaklee et al. 1977) and generally requires large genomes (Ohno 1970); the role of allozymes in environmental thermal adaptation has been reviewed by Hedrick et al. (1976), though few are those of proved significance. Two additional mechanisms employed during temperature adaptation include quantitative enzyme changes (Hazel & Prosser 1974, Shaklee et al. 1977, Sidell et al. 1973) and the enhancement of protein stability (Alexandrov 1977), though both are beyond of the scope of this discussion.

LDH in molluscs has been largely replaced by ODH (Baldwyn et al. 1981), which is closely associated with PAK (Vollmer et al. 1978) in the restoration of the phosphagen levels. In B.digitalis, LDH co-exits with ODH, thereby sharing common substrates (see Chapter 6). In vitro LDH is free of constraints from other enzymes, such as competition for common substrates; though the LDH extract is a milieu containing other enzymes, the system is controlled so that only the necessary substrates are present; hence other enzyme systems will be rendered inoperative or crippled. In consequence, the situation in vitro is far removed from complex interconnected pathways.

Therefore, the in vivo temperature responses of LDH may differ, because of the enzyme's interaction with other cellular components. Adaptation to a new situation is thus best described by the summation of all the altered effects, in response to an external factor such as temperature.

CHAPTER 6

UNUSUAL RESPONSES OF D(+)OCTOPINE DEHYDROGENASE TO TEMPERATURE IN THE WHELK *BULLIA DIGITALIS*

INTRODUCTION

Bullia digitalis is the only marine invertebrate so far known to maintain a virtually flat metabolic rate-temperature curve at all levels of activity (Brown & da Silva 1983). This ability may represent an adaptation to radically fluctuating temperatures on South Africa's west coast, coupled with erratic food availability; together these contribute to the unpredictability of the whelk's environment (Brown 1982).

Biochemical adaptation to the environment and its physiological implications have been reviewed by Hochachka & Somero (1984) and some of the related thermodynamic aspects have been considered by Brandts (1967), Lumry & Beltonen (1969) and Lowy *et al.* (1973). The direct effect of ambient temperature on the body temperature of ectotherms as well as its effects on physiological mechanisms are well described in the literature. Somero & Hochachka (1976) and Hochachka & Somero (1976, 1984) have focused attention on understanding the physico-chemical nature of the regulatory mechanisms which may have arisen in the course of evolution, to compensate and protect key enzymatic

reactions from the effects of temperature and other environmental parameters. No universal mechanism exists to counteract undesirable temperature effects but it appears that each affected species has developed a suitable solution. For instance a decrease in ambient temperature can give rise to increased E-S affinity (Hochachka & Somero 1971, Baldwyn & Hochachka 1970, Somero & Hochachka 1969, Hochachka & Somero 1968). In some situations more than one enzyme is present, with different temperature sensitivities (Somero 1969, Hoskin & Aleksiuk 1973), while in some cases enzymes are temperature independent (Somero & Hochachka 1968, Behrisch 1969, Suzuki & Imahory 1973, Luisi et al. 1975).

B.digitalis is remarkably active for a gastropod and amongst the Mollusca appears to be surpassed only by the Cephalopoda (Brown 1982). ATP required for the contractile cycle can be derived from substrate level phosphorylations coupled to the catabolism of carbohydrates through glycolysis (Gäde 1980, Baldwyn et al. 1981). The role of LDH in reoxidizing NADH during the final step of this pathway has been replaced by ODH in several molluscan groups (Gäde 1980, Grieshaber 1978, Koorman & Grieshaber 1980, Baldwyn et al. 1981, Grieshaber & Gäde 1976, Hochachka et al. 1977, Storey & Storey 1979). As a result, D(+)octopine (formed by the reductive condensation of pyruvate and L-arginine in the reaction PYRUVATE + L-ARGININE + NADH $\rightleftharpoons$ D(+)OCTOPINE + NAD^+), rather than D(-)lactate, accumulates as the end product of glycogen breakdown (Koorman & Grieshaber 1980).

The co-existance of D(+)ODH and D(-)LDH in B.digitalis could result in competition for common substrates. Although this phenomenon seldom occurs, as in general one substrate greatly exceeds the other (Regnouf & van Thoai 1970, Zammit & Newsholme 1976 a, Storey 1977b), at least one exception is known, in Nassarius coronatus (Baldwyn et al. 1981), where high activities of both enzymes occur simultaneously. High ratios of ODH to LDH are indicative of a high anaerobic capacity (Gäde 1980). Since ODH in B.digitalis is ten times as active as LDH, it is suggested that anaerobiosis must be a fairly common physiological state, even if both enzymes are characterized by low activities, when compared to related species (Gadë 1980).

Detailed kinetic studies and assessment of physical properties of muscle ODH have been undertaken by Thoai et al. (1969), Luisi et al. (1975), Doublet & Olomucki (1975), Doublet et al. (1975) and Monneuse-Doublet et al. (1978) in the genus Pecten (Bivalve), demonstrating that the enzyme obeys a bi-ter sequential mechanism, in which NAD^+ binds first, followed by D(+) octopine, while the products are released in the order of L-arginine, pyruvate and NADH. This was consistent with the mechanism of mneumonical transition suggested by Ricard et al. (1974), whereby cooperation between different free enzyme forms is assumed. In consequence ODH became known as a two substrate three product mneumonical enzyme (Monneuse-Doublet et al. 1975). Recently, the ordered product release of this reaction has been questioned by Schrimsher & Taylor (1984), who proposed that some degree of randomness exists in addition to a slight

modification of the established pathway.

Multiple forms of ODH, analogous to those of LDH in vertebrates, have been demonstrated by Storey (1977 b), Storey & Storey (1979) and Zettlmeissl et al. (1984). The possibility that ODH exists in more than one form was examined by investigating its genetic profile in several of the gastropod tissues.

The purpose of the present study on B.digitalis was firstly to elucidate the effect of temperature on ODH activity; secondly to verify the extent of genetic variation of this enzyme and thirdly to discuss possible physiological implications, with respect to the flat rate-temperature curve of intact whelks and their relationship to a constantly changing environment.

MATERIALS AND METHODS

Reagents

Octopine, NAD+, NADH, nitrobluetetrazolium (NBT), phenazinine methosulphate (PMS) and DL-Dithiothreitol were purchased from Sigma, St. Louis, USA. Sephadex G-100, DEAE A-50 and the low molecular weight calibration kit for SDS-gel electrophoresis were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). The calcium phosphate gel, hydroxylapatite (Bio gel HT-fully hydrated) was supplied by Bio Rad, Richmond California. The molecular weight calibration kit for gel filtration was obtained from Boehringer Mainheim GmbH, Biochemica

and all other reagents (analytical grade) were purchased from BDH Chemicals Poole, Great Britain.

Biological Material

Live material was collected off the west coast of South Africa, just north of Cape Town and maintained in aerated sea water at 15°C. Animals were not fed in the laboratory and were sacrificed within 2 or 3 days of collection.

Assay for ODH activity and enzyme kinetics

All enzyme assays were performed in a Varian Techtron 635 recording spectrophotometer, linked to a temperature-regulated water bath. Initial reaction rates were measured between 5°C and 30°C, at 5°C intervals, though routine assays were done always at 18.0±0.1°C. The rate of change of NADH concentration was followed at 340nm. Plots of initial velocity vs. substrate concentration (Michaelis plots) were constructed and the steady-state kinetics analysed by the Eadie and Lineweaver-Burk plots. Data were treated by single factor analysis of variance (ANOVA) and a multiple range test ($p>0.05$) was used to distinguish between significantly different kinetic parameters at the temperatures indicated.

Enzyme assays

Assay in the direction of octopine synthesis: 100 mM

imidazole buffer, pH7.00, containing 5 mM arginine (omitted for control), 5 mM pyruvate and 0.24 mM NADH ; Assay in the direction of octopine oxidation: 100 mM Tris-HCl, pH 9.00, containing 5 mM octopine (omitted for control) and 1 mM NAD+. Reactions were initiated by adding 50 ul of pure enzyme (1.20mg ml^{-1}) to a final assay volume of 2.40ml. The concentration range of substates used was as follows: 0.25 to 40.00 mM for pyruvate, L-arginine and D(+)octopine; 0.01 to 5.00 of mM NAD^+ and 0.01 to 0.75 mM of NADH.

Enzyme purification

Since the method of Thoai et al. (1969) was followed strictly, only a brief methodological description is provided. Purification steps were always carried out at 4°C.

(a) Crude extract:

Animals were sacrificed by immersion in liquid nitrogen, where they remained for 5 minutes. After thawing (at 4°C) the feet of 62 gastropods (shell length 3.20 ± 0.53 cm) were excised and kept on ice until homogenization. A total of 45.70 gm of foot muscle was obtained. To 10 gm of material, 40 ml of iced water were added; this was homogenized using a Janke & Keunkel ultraturrax homogenizer (Ika-Werk TR 50). The extract was spun at 27000 g for 30' (2x) and the supernatent stored for further purification.

(b) Ammonium sulphate precipitation, gel filtration and preparative electrophoresis

Enzyme purification was accomplished by a sequence of ammonium sulphate precipitations, resulting in the accumulation of a protein pellet, subsequently treated by Tiselius gel (Tiselius et al. 1956). The stabilized enzyme was further purified by Sephadex DEAE A-50 and by G-100. The final elutent underwent a preparative electrophoresis step and its purity was verified by SDS-gel electrophoresis.

Molecular weight determination

(a) Gel filtration:

Molecular weight determinations were performed according to the procedure of Whitaker (1963) and Andrews (1964), using a Sephadex G-100 column (60x2.5 cm). All steps were carried out at 4°C. Protein markers were applied in descending order of molecular weight to prevent elution overlaping and protein associations. Proteins were eluted in a sodium phosphate buffer, pH 7.00, while 0.80 ml fractions were collected. Absorbtion was followed at 280 nm. A standard plot of log molecular weight (Mr) vs elution volume was constructed using the following markers: catalase, from beef liver (MW= 240Kd); aldolase, from rabbit muscle (MW= 158Kd); albumin, from bovine serum (MW= 68Kd); albumin, from hen egg (MW= 45Kd); chymotrypsinogen A, from bovine pancreas (MW= 25Kd) and cytochrome c, from horse heart (MW= 12.5Kd).

(b) SDS-gel electrophoresis (vertical slabs):

The procedure followed was that of Laemmli (1970), using a discontinuous acrylamide gradient (5 to 20%) as the resolving gel. Molecular weight standards consisted of: phosphorylase b (MW= 94 Kd); albumin (MW= 67 Kd); ovalbumin (MW= 43 Kd); carbonic anhydrase (MW= 30 Kd); trypsin inhibitor (MW= 20 Kd) and α-lactalbumin (MW= 14.4 Kd). Electrophoresis was allowed to proceed for 16 hours, at the end of which the gel slabs were stained with 0.5% Page Blue 83 (BDH dye) in methanol:acetic acid:water (25:10:65) and destained by diffusion in several changes of the same diluent.

Assessment of polymorphic properties

(a) Non-denaturing electrophoresis (starch gels):

Horizontal starch gel electrophoresis was carried out according to the method of Rigway et al. (1970). Cytosol extracts of foot, mantle, digestive gland, brain and gonad, were electrophoresed for 4 hours, after which staining for enzyme activity using NBT (an electron transfer dye) followed. The standard staining solution contained: 1 M D(-) octopine, 10 mg ml^{-1} NAD+, 5 mg ml^{-1} PMS and 10 mg ml^{-1} NBT in 23 ml of 0.1 M Tris-HCl buffer, pH 8.90; to this solution a 2 ml volume of 3% agarose was added so that the staining solution was fixed on the surface of the starch gel. Colour development of the zymogram occured after exposure for 10 minutes in an incubator at 40°C.

RESULTS

Enzyme Purification

From the original 45.70 gm of material 1.03 mg of pure enzyme were obtained, corresponding to 13.25% recovery (Table 6.1.). Material required for kinetic analysis was accumulated from 9 purification sequences and stored at -20°C, in 60% ammonium sulphate. This enzyme is characterized by a high specific activity (84.62 umol min^{-1} mg^{-1}) in the foot, corresponding to a single band on SDS-PAGE, (Figs. 6.1. and 6.2.).

Physicochemical Properties

Molecular weight determination and assessment of purity

Both gel filtration and SDS-PAGE indicate ODH to be a monomer. As shown in Figs. 6.1. and 6.2., the molecular weight determined by gel filtration is slightly higher (MW= 40.50 + 2.80 Kd) than that given by gel electrophoresis (MW= 39.80 + 1.80 Kd), but this difference was not considered significant and possibly resulted from experimental variation due to proteolytic degradation and/or limitations of either technique. Furthermore, specific properties of the protein, namely shape, size and surface charges contribute to particular interactions of ODH with its surrounding medium and therefore one expects some variation with different analytical techniques. For a more comprehensive review see Rowe, Mantle & Thomas (1978).

TABLE 6.1. Summary of purification procedure of D(+)ODH of Bullia digitalis in the foot*.

	Vol. (ml)	Total E.U.††	Total Protein (gm)	Sp. Act. E.U. mg^{-1}	Purification Level	% Recovery
Cell free cytosol	622.6	2029.68	4.669	1.739	1.00	100.00
28.8% AMS† ppt (after dialysis)	166.0	1945.93	0.425	1.875	1.07	95.87
23.7% AMS† ppt	94.0	1073.48	0.212	2.030	2.92	52.89
Tiselius gel treatment (calcium phosphate column)	45.0	1061.2	0.179	27.200	39.10	52.28
DE-52 ion exchange column (after concentration + dialysis)	135.0	921.38	0.155	32.490	46.71	45.39
G-100 column	23.0	361.25	0.625	67.830	39.00	17.79
Preparative electrophoresis	6.0	268.87	0.003	163.55	163.55	13.25

* (AMS† ppt = ammonium sulphate precipitation; E.U.†† = enzyme units)

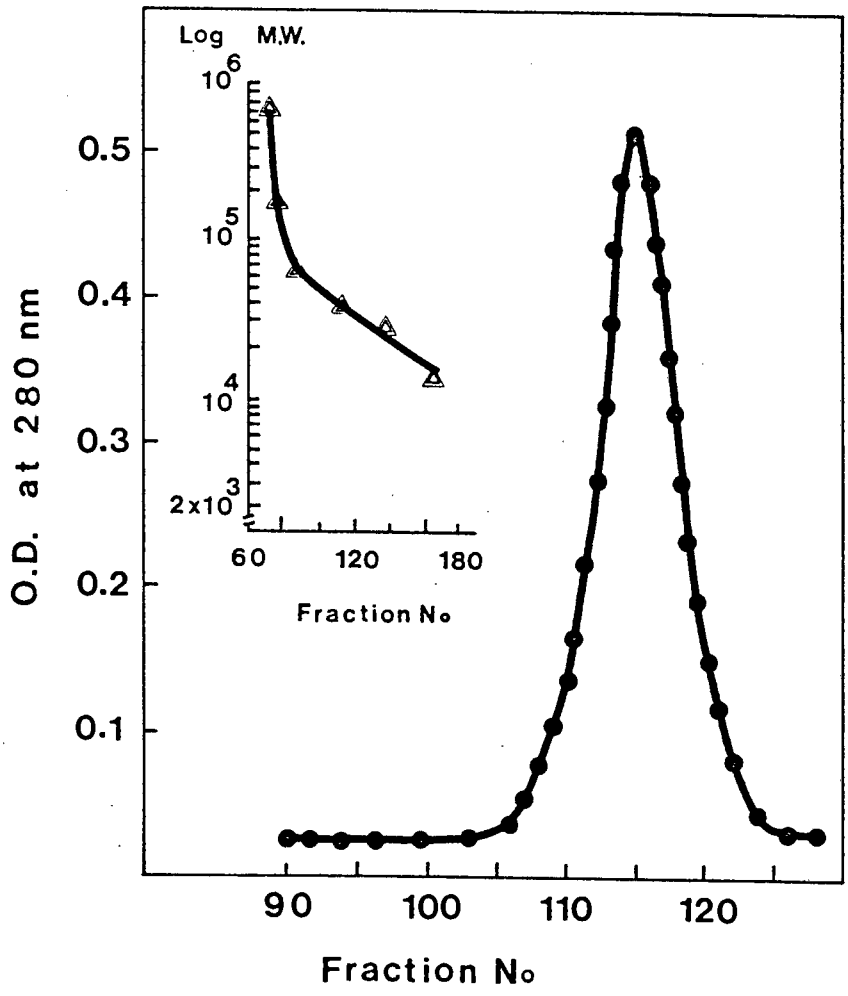


Figure 6.1. Determination of molecular weight of intact D(+)ODH of foot on a Sephadex G-100 column. Protein profile of purified D(+)ODH (●—●), as detected by u.v. light at 280 nm. Inset: Molecular weight calibration curve (see Materials and Methods).

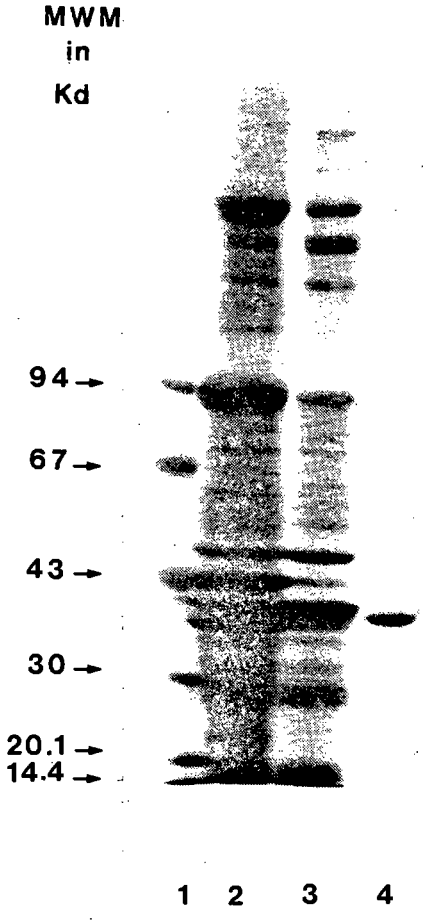


Figure 6.2. Gradient (5 to 20%) SDS-PAGE (sodium dodecyl sulphate - polyacrylamide gel electrophoresis) of foot D(+)ODH of Bullia digitalis. Lane 1: Molecular weight markers (see Materials and Methods); Lane 2: Cytosol extract following the first ammonium sulphate precipitation step; Lane 3: Cytosol extract recovered from the fourth ammonium precipitation, prior to loading into a DE-52 ion-exchange cellulose column; Lane 4: Purified enzyme after preparative electrophoresis (MW= 39.80±1.80 Kd) .

The effect of temperature

Catalytic properties

The enzyme is D(+) octopine specific and is inactive in the presence of L(+)-allo-octopine. Temperature has distinct effects on both the octopine forming and oxidation reactions (Table 6.2.). In the direction of octopine synthesis the enzyme is most active at 20°C, though the same reaction at 15°C is much slower than that of octopine oxidation at the same temperature. This may be an indication of different temperature optima for each reaction, at the pH used.

The pH profile in the direction of octopine synthesis

The pH profile illustrates the extent to which the forward and reverse reaction are affected differently by the same parameter, (Fig. 6.3.). With rising temperatures (from 10 through to 30°C) an activity shift occurs resulting in a 30% loss of enzyme activity in the vicinity of pH 6.00, despite a 25 to 30% activity gain above pH 7.00. Enzyme activity is almost absent at pH 8.00 (for 10 and 18°C); by raising the temperature to 30°C, at this same pH, the enzyme capacity to work increases to 54% that of maximal activity.

The pH profile in the direction of octopine oxidation

An upward shift in temperature induces some enzyme activity loss in the acidic pH range tested, in addition to which maximal activity for this reaction is not attained even at the basic pH, where activity is normally found to be optimal. Hence whereas at 10°C, at pH 9.00 enzyme activity is maximal, by

TABLE 6.2. Relative proportion of octopine forming and octopine using reactions of D(+)ODH in Bullia digitalis foot, with respect to temperature change.

Reaction V_{max} (μmol min^{-1} mg^{-1})	Temperature (°C)					
	5.0	10.0	15.0	20.0	25.0	30.0
octopine forming	1.073	1.217	0.909	4.353	0.359	0.479
octopine oxidation	0.368	0.404	3.961	0.841	0.196	0.396
% octopine forming/ % of octopine oxidation	291.57	301.23	22.95	534.77	212.46	120.96

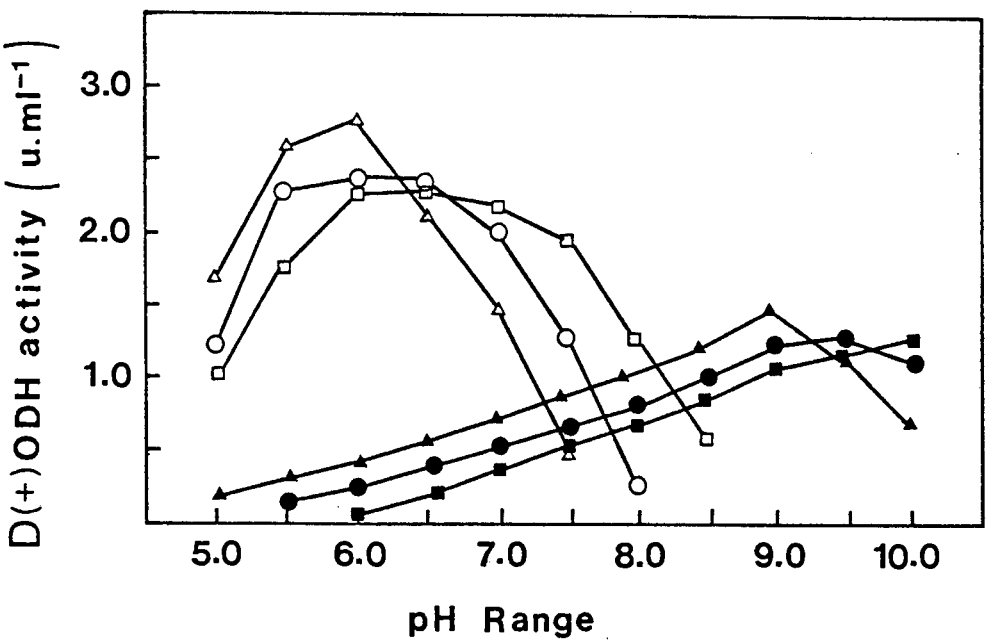


Figure 6.3. The effect of temperature on the reaction velocity of D(+)ODH, with respect to pH changes; in the octopine forming direction, reaction velocities at 10°C (△—△), 18°C (○—○) and 30°C (□—□); in the octopine using direction, reaction velocities at 10°C (▲—▲), 18°C (●—●) and 30°C (□—□).

increasing the temperature to 18°C activity decreases by 20%; a further temperature increase of 12°C (to 30°C) results in an additional 10% loss in activity (Fig. 6.3.).

Fig.6.3. illustrates further that although at acidic to neutral pH the octopine forming reaction is 4 to 5 times faster than its counter-part, it is inactive at basic pH. In contrast, the reaction in the direction of octopine oxidation is most active at these basic pH.

Enzyme kinetic properties of foot muscle ODH

(a) Substrate inhibition

Fig.6.4. shows the effect of increasing substrate concentration (pyruvate, L-arginine and D(+)octopine) on the activity of ODH. It represents also typical examples of substrate inhibition. Although it was not possible to determine true K_i, an index of substrate/product inhibition is given by I_{50} in Table 6.3. Both Fig. 6.4. and Table 6.3. indicate that enzyme inhibition can be triggered by relatively low concentrations of L-arginine, in analogy to the characteristic effect of pyruvate on H-LDH (see Long & Kapplan 1973, Storey 1976). The inhibitory effect of pyruvate on ODH is, however, milder than that of L-arginine, with the result that to reduce enzyme activity by 50%, higher pyruvate concentrations are required. Nonetheless, the enzyme is even less sensitive to octopine inhibition and especially high octopine concentrations were employed at 10 and 15°C to attain 50% inhibition (see Table 6.3.). This inhibitory phenomenon is present with most substrates, irrespective of assay

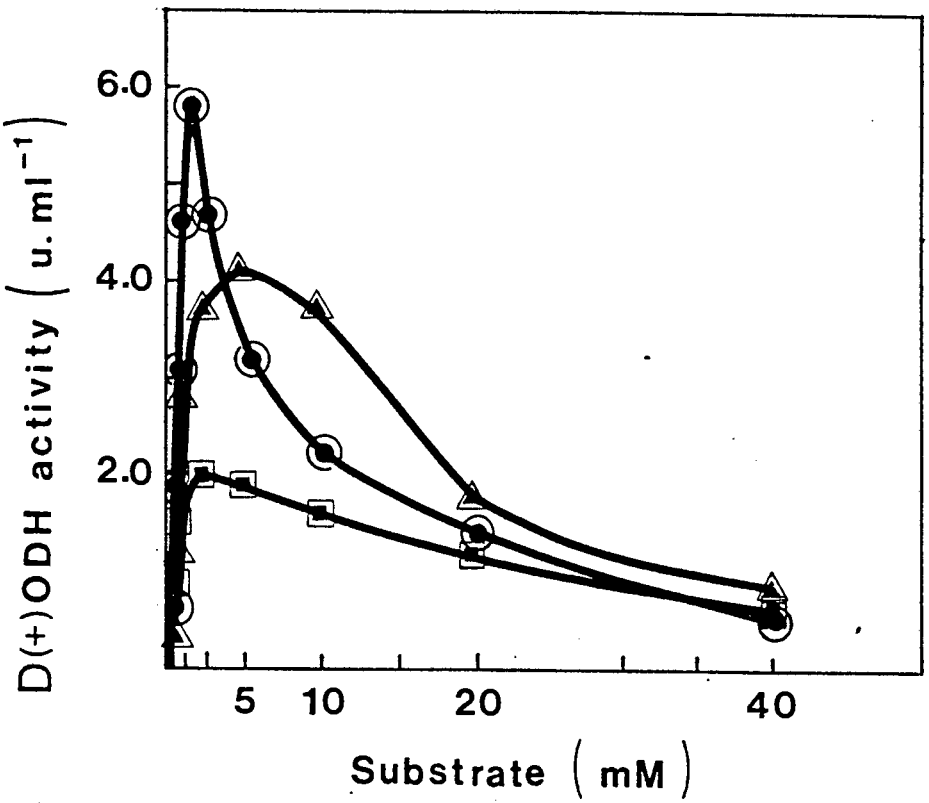


Figure 6.4. The effect of increasing substrate concentration on the activity of foot D(+)ODH at different temperatures. Substrate: Arginine at 20°C (⊙—⊙); Pyruvate at 25°C (△—△) and Octopine at 10°C (⊡—⊡). Substrate inhibition is more pronounced with excess arginine (above 2.99 mM), followed by that of pyruvate and octopine. (See Material and Methods for assay conditions).

TABLE 6.3. The effect of temperature on I_{50} of D(+)ODH of Bullia digitalis foot: Substrate concentration inducing 50% inhibition.

Temperature (°C)	Substrate (mm)				
	Pyruvate	Arginine	NADH	Octopine	NAD†
5.0	7.50	2.60	0.42	14.50	-
10.0	8.08	3.12	0.05	30.00	-
15.0	10.84	4.50	0.05	30.50	-
20.0	8.93	3.87	0.40	16.95	-
25.0	12.50	12.50	0.40	15.51	-
30.0	7.84	-	0.22	15.00	-

temperature (see **Table 6.3.**), though an exception occurs with L-arginine above 25°C, where twice as much substate (as that used at this temperature) was required in order to reach an I_{50} level. Nevertheless, even with concentrations as high as 40.00 mM enzyme activity decreased only by 30%.

NADH is also capable of inducing an inhibitory effect, yet high NAD+ concentrations (up to 5 mM) did not give rise to any decrease in enzyme activity.

These properties may be reflected in the features pertaining to the substrate inhibition curves in **Fig. 6.4.**, whereby excess of each substrate affects the enzyme in a unique manner. Apart from independent substrate effects on ODH, interactions between L-arginine, pyruvate and D(+)octopine may also occur on the enzyme, such that the behaviour observed at any one time results from the combined effects of all three substrates.

(b) The effect of temperature on apparent Michaelis constants.

Temperature affects the properties of each substrate in a distinctive way, as represented on **Fig. 6.5.** The determination of Michaelis constants for a two substrate enzyme requires special kinetic treatment (Ferdinand 1976); since the necessary computer programs (as used by Schrimsher & Taylor 1984) were not available, it was decided to calculate apparent constants, rather

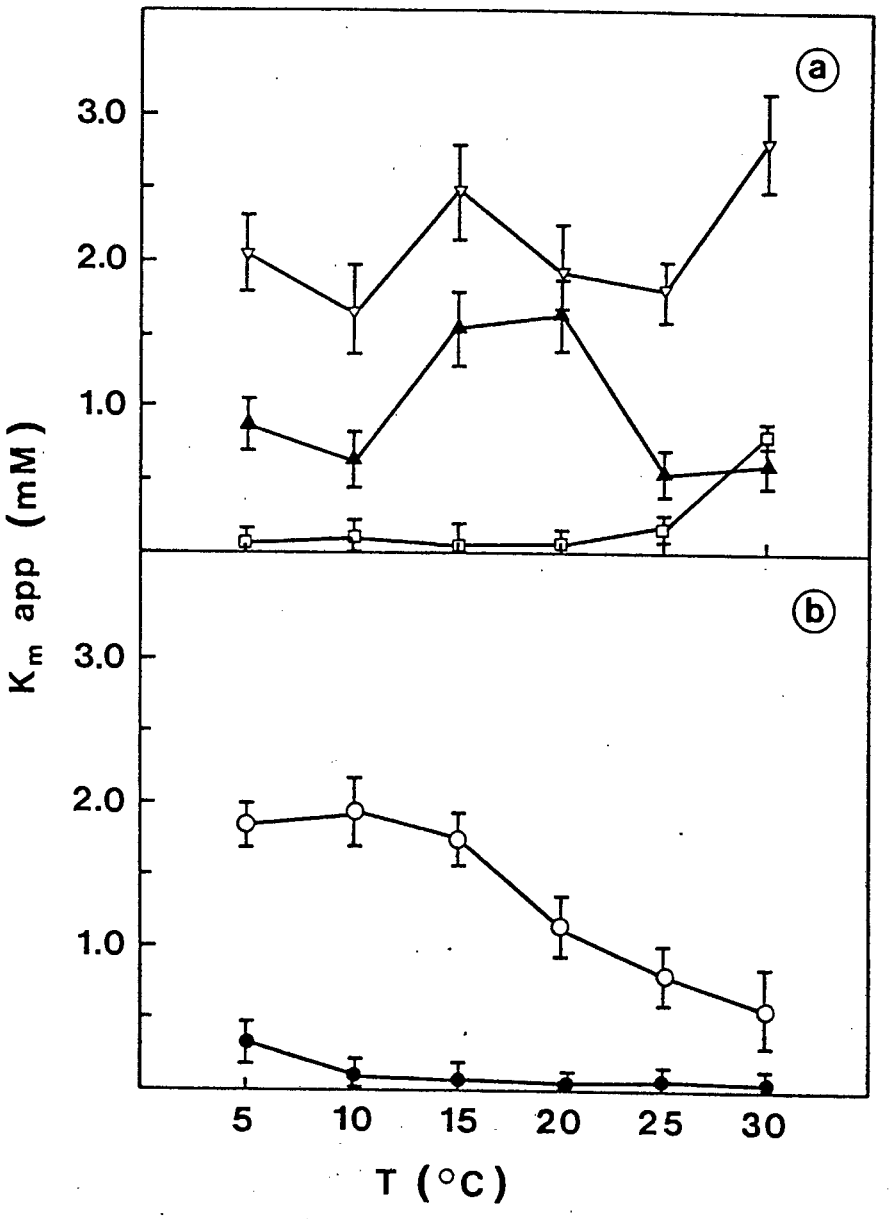


Figure 6.5. The effect of temperature on the K_m of pyruvate (▽—▽), arginine (▲—▲), NADH+H$^+$ (□—□), octopine (○—○) and NAD$^+$ (●—●) of foot D(+)ODH in Bullia digitalis: (a) Octopine forming reaction; (b) Octopine using reaction. Each point is a mean of 6 measurements.

than the true ones, as an index of the kinetic properties of the enzyme.

The $K_m$app profile of pyruvate is temperature independent. This was confirmed by statistical analysis. Concerning L-arginine the situation is more complex, since the highest $K_m$app values occur at 15°C (1.52mM) and at 20°C (1.62 mM), albeit above and below these temperatures this is 2 orders of magnitude lower. The $K_m$app profile for octopine resembles neither of the above, being inversely proportional to temperature.

The profiles relevant to NADH and NAD^+ disclosed some degree of complexity. The $K_m$app for NADH is temperature independent between 5 and 20°C, (approximatly 0.08 mM), though above this temperature it increases rapidly to 0.20 mM (at 25°C) and to 0.70 mM (at 30°C), representing a substantial decrease in E-S affinity at these temperatures. For NAD^+, like octopine, the $K_m$app decreases as the temperature rises, varying from 0.35 mM at 5°C to 0.05 mM at 30°C. Despite a significant decrease in $K_m$app between 5 and 10°C (from 0.35 to 0.14 mM), it is more gradual and less noticeable thereafter.

Genetic variation

In the foot, ODH is present in only one form ODH-1 (AA); this is generated by a single locus and despite its expression in other tissues (mantle, brain and gonad) it is absent in the digestive gland. Instead, a different locus is expressed in this tissue, namely ODH-2. Neither locus shows polymorphic

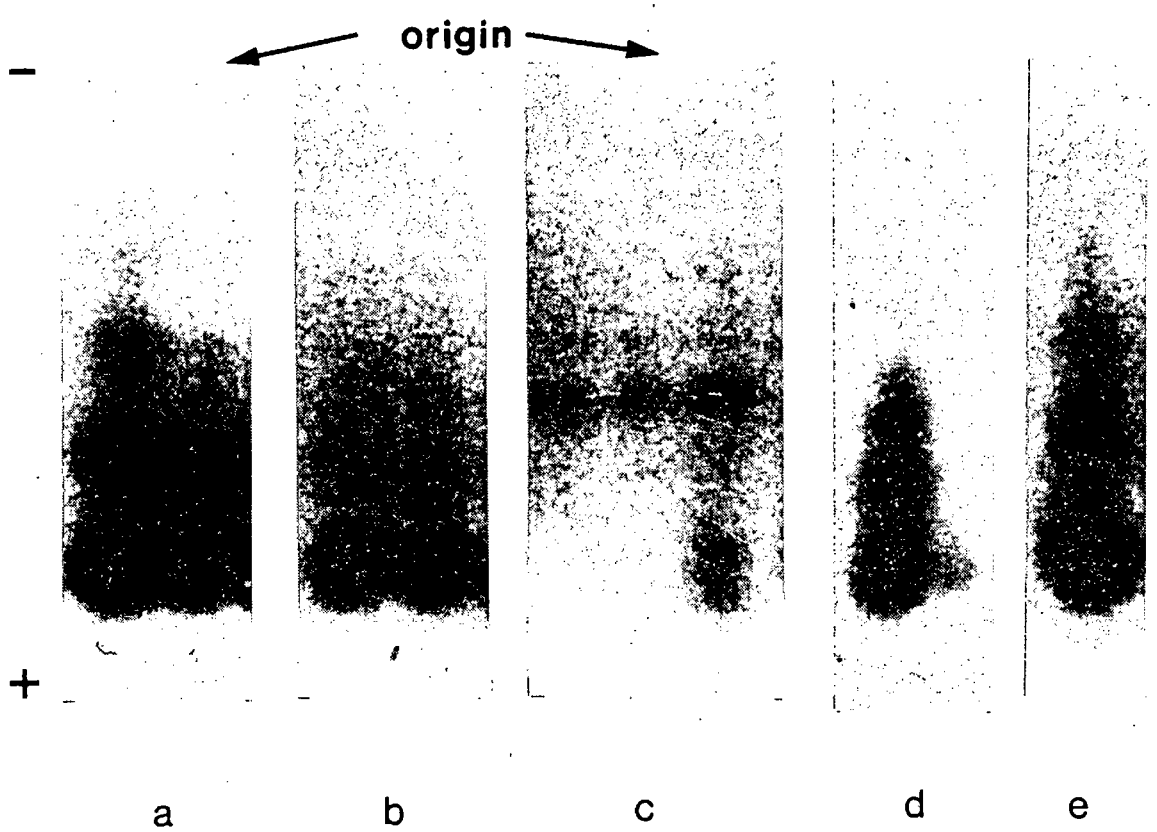


Figure 6.6. Non-denaturing starch gel of tissue specific D(+)ODH enzyme: (a) Foot; (b) Mantle; (c) Digestive gland; (d) Brain and (e) Gonad.

variation, though all appear to be extremely active, as indicated by the intensity of the zymogram colour (Fig. 6.6.).

Although ODH-2 is highly active in the digestive gland, it is present in relatively low concentrations. This, together with its rather unstable nature, impeded the successful purification of this enzyme.

DISCUSSION

The accumulation of D(+)octopine as the end product of anaerobic glycolysis, in most active molluscs (Grieshaber & Gäde 1976, Hochachka et al. 1977, Koorman & Grieshaber 1980), is analogous to the formation of L-lactate in working vertebrate muscle (Vollmer et al. 1981). Accumulation of D(+)octopine has also been associated with mechanisms of phosphoarginine kinase turnover, which is responsible for regeneration of phosphagen levels (de Zwaan 1983). In B.digitalis, where both D(-)lactate and D(+)octopine are produced concomitantly, the situation is far from simple. The co-existence of D(-)LDH and D(+)ODH may not necessarily lead to competition for common substrates, but instead may allow the selective use of each enzyme in the course of distinctive metabolic events. It was suggested by Baldwyn et al. (1981) that D(+) ODH functions primarily during strenuous exercise, whereas D(-) LDH is active in the course of metabolic recovery.

In squid mantle D(+)ODH is highly active in contrast to LDH, where the ratio of ODH/LDH is of the order of 100:1 (Storey & Storey 1983). The combined problem of octopine recycling and the interactions between octopine and arginine metabolism have only recently started to be unveiled. It appears that ODH is advantageous in tissue such as squid mantle, where in the event of bursts of activity, the hydrolysis of a large pool of arginine phosphate is allowed to proceed without the accompaning deluge of the cell by arginine, a highly basic and possibly damaging substance (Somero & Bowlus 1983).

Many molluscs that exhibit both ODH and LDH activity, exhibit also the activity of other imino-acid dehydrogenases such as alanopine dehydrogenase (Fields *et al.* 1980) and strombine dehydrogenase (de Zwaan & Zurburg 1981). These dehydrogenases catalyse the reductive condensation of pyruvate with alanine and glycine respectively, giving rise to the end products alanopine and strombine.

The reason for maintenance of multiple terminal glycolytic dehydrogenases in molluscs is somewhat obscure, but they may cooperate in sequence, so that different physiological states demand different dehydrogenases. This could give rise to selective fermenting pathways of glycogen, each resulting in a different end-product (Hochachka & Somero 1984). In poorly perfused muscle, the malleability of glycogen metabolism is a stylish *modus operandi* of availability of energy in the course of cell recovery. Since octopine oxidation is essential for the

regeneration of the arginine phosphate pool, during cell recovery, alternative terminal glycolytic enzymes have to be present. Thus simultaneous glycogen breakdown and cellular redox functions during recovery can occur. In B.digitalis, LDH may be involved in glycogen fermentation, while ODH may be vital in the regeneration of the cellular energy balance. Neither alanopine dehydrogenase, nor strombine dehydrogenase occur in this whelk.

ODH in B.digitalis is a monomer of approximately 40 Kd, and appears to be similar to that of other species studied (Thoai et al. 1969, Baldwyn et al. 1981, Storey & Storey 1981, Zettlmeissl et al. 1984). Irrespective of the temperature at which the pH profile was determined, it is clear that the enzyme is characterized by broad pH specificity. However, when the enzyme assays were carried out at the pH optima for each reaction direction, marked temperature selectivity occured. As indicated in Table 6.2., the octopine forming reaction is most active at 20°C, in contrast to the octopine using reaction which is favoured at 15°C; these differences are not only statistically significant, but in addition occur well within the temperature range encountered in the field.

The physiological significance of these findings has to be related to the effects of temperature on enzyme behaviour. These are directly related to the effect of temperature on reaction rates, to substrate concentration, to E-S affinity and to the properties of the enzyme's active site, as well as its

interaction with the surrounding micro-environment (Ferdinand 1976). The determination of temperature effects on initial reaction rates reflects the combined action of enzyme catalysis and denaturation, with the result that the optimum temperature (which dependends on assay methods in addition to pH and substrate concentration), is a reflection of the balance between efficient catalysis and the energy required to synthesise denatured enzyme. In the living cell, initial reactions are fleeting, while denaturation is a constant process; although *in vitro* attempts were made to minimize this phenomenon, its irradication is virtually impossible. Thus *in vitro* and *in vivo* temperature optima may bear little relationship to each other.

As shown in **Table 6.3.**, slight substrate concentration fluctuations have marked effects on the behaviour of ODH. Although it was not possible to determine K_i, measurements of I_{50} were taken as an index of substrate inhibition, bearing in mind that *in vivo* the concentrations of substate that are usually responsible for inhibition are much lower than indicated by I_{50}. A possible control mechanism of enzyme activity could arise if K_i were smaller than K_m, in which case the true Vmax would never be attained. From **Table 6.3.** this is evidenced in the concentrations of NADH at 10 and 15°C. However, in measuring I_{50} one is forced to take the observed Vmax as the highest activity level the enzyme will attain (which is not necessarily valid). In reality, because of the interaction of substrate/product with the enzyme, as well as the combined effect of substrate availability, E-S affinity and substrate/product inhibition, the value that

should be read as V_{max} is much larger than that observed. Since the error incurred in the determination of approximate inhibitory levels is constant, a relative cross examination of these levels is feasable.

Temperature independence of enzyme function is less common than expected, in ectotherms (Hochachka & Somero 1971, Somero & Hochachka 1968, Behrisch 1969, Behrisch & Hochachka 1969). In contrast to the findings of Luisi et al. (1975), in B.digitalis only the K_m for pyruvate appears to be temperature independent. The K_m for octopine on the other hand is directly proportional to temperature, resulting in an increase in E-S affinity with rising temperatures. This may seem paradoxical, as slight increases in temperature can give rise to a significant acceleration in the rate of a reaction (Hochachka & Somero 1984) and one would expect the enzyme to decrease its substrate affinity to compensate for the kinetic energy already gained and thus save its endogenous energy supplies. Our present lack of knowledge, however, may preclude a full understanding of this phenomenon. ODH does not work in isolation; its interaction with other cellular components is unquestionable and its behaviour cannot therefore be analysed without considering the possible effects that the kinetics and thermodynamics of other reactions may have on this enzyme. Enzymes such as LDH and PAK, which share common substrates with ODH, may have contributed towards the evolutionary selectivity of the properties the enzyme currently displays and therefore what one observes is the end result of a

physiological adaptation.

If, on the other hand, large I_{50} values occur, as is the case with octopine concentrations (Table 6.3.) and supposing that these are real, then this may allow the enzyme to handle greater loads of substrate which may have accumulated in the course of strenuous exercise. It may therefore be advantageous to be less sensitive to inhibition. In the case of octopine, the situation would suggest a delicate balance between E-S affinity and its catalytic efficiency, so that clearance of octopine via oxidation to pyruvate and L-arginine can occur and the phosphagen levels restored.

When the animal is faced with more unusual temperature regimes, such as the experimental extremes tested here, energy conservation may be a priority. Since pyruvate is unaffected by temperature changes, it is possible that arginine becomes the modulatory parameter, resulting in behaviour as is described in **Table** 6.3. and **Fig.** 6.5. The K_m for arginine is lowest above and below 15 and 20°C (the temperatures most frequently encountered in the field); the enzyme substrate affinity is under strict control, being two orders of magnitude lower at the above temperatures. In addition, whereas below 20°C the I_{50} is fairly small, it increases significantly above this temperature; this together with a fairly low K_m at these temperatures, may indicate that the octopine forming reaction is greatly favoured and that the phosphagen levels are being used.

All considered this could result in unequivocal behaviour of ODH, culminating in the sensitive and direct responses to temperature shifts. The Arrhenius plot (not shown) for either the forward or reverse reaction is not a straight line. Attempts at explaining such anomalies have been made by Talsky (1971), who suggested that the anomalies in the temperature dependence of reaction rates become more apparent with increasing substrate concentrations, as the equilibrium of formation of enzyme-substrate complex has less influence on the overall reaction, becoming more of a function of the rate constant of the last reaction forming the product (i.e. the decay of the final enzyme product complex).

It has been suggested that two classes of weak interactions are involved in the maintenance of a structurally viable protein (Somero & Hochachka 1976), namely electrostatic and hydrophobic interactions. The involvement of the first in substrate binding allows a positive correlation between K_m and temperature, whereas when hydrophobic interactions are present the tendency is towards an inverse relationship of these parameters. Where both interactions are implicated, and depending on the proportion of either, a complex situation may result and only a detailed analysis of the thermodynamic parameters would clarify the nature of the mechanisms involved. The present data illustrate the difficulties encountered in analysing an enzyme system which is both complex in itself and related to a myriad of other metabolic pathways.

Of some importance is the fact that ODH is represented by one form only in the foot, i.e. no isozymes, despite reports of multiple forms of ODH in Sepia officinalis (Storey & Storey, 1979). Hence, the unequivocal properties of this enzyme with changing temperature are entirely dependent on E-S interactions and interactions of the latter with the surrounding medium. Since it was not possible to isolate the alternative ODH form in the digestive gland, it is not possible to predict either its significance or differences of catalytic properties compared to the homologous foot enzyme.

It is suggested that if a situation arose whereby arginine, pyruvate or NADH were competed for, it is the overall kinetic determinant of an enzyme that regulates the direction of substrate flux. In other words, the presence of marked substrate inhibition and of differential substrate flux through the enzyme, depending on the temperature, may ensure that substrates do not accumulate, but are diverted to another enzyme system where these can be used. This raises the possibility that prior to full restoration of phosphagen levels, the cell's feed back mechanism could signal attenuation of enzyme activity, resulting in fuel production and or utilization only at the desired level. This signal could be provided by rising concentrations of octopine or by elevated levels of the pyruvate/arginine/NAD^+ complex (as an inhibitor effector), which could slow down glycolysis by preserving oxidation of generated NADH. Such a mechanism could contribute to the whelk's survival for long and strenuous

periods, with reduced energy expenditure.

CHAPTER 7

A BRIEF EXAMINATION OF SOME ASPECTS OF METABOLISM IN THE WHELK *BULLIA DIGITALIS*

NOTE: This chapter contains work of a preliminary nature which is not intended for publication.

INTRODUCTION

The physiological relevance of enzyme systems in metabolic control

Most of our current knowledge on enzymes stems from *in vitro* studies, though it is obvious that enzymes do not work in isolation and seldom under saturating conditions (as is necessary *in vitro*). Thus *in vitro* and *in vivo* responses may bear little relationship to each other (Ferdinand 1977). The diversity of enzyme function ranges from initiation and/ or suppression of metabolic processes, in which an "on-off" mechanism prevails to involvement in branch point systems, where the appropriate direction of substrate fluxes is crucial to avoid metabolic chaos; and to participation in catalytic steps devoid of regulatory properties. All of the above demand stringent *in vivo* control both at the cellular and whole body level, implying the presence of several levels of physiological control (Hochachka & Somero 1984).

Regulation of enzyme expression and function dawns at the DNA level, from which the quantitative and qualitative properties of a protein are determined. This process persists throughout translation, a mechanism which is only recently beginning to be fully appreciated (Hunt 1980, Clark 1980, Fersht 1980, Caskey 1980) and continues throughout the structural organisation of the forming peptide, exhibiting differential rates of control, up to the quaternary and quintinary structure, when proteins associate not only with each other, but also with other cellular components (without which they would become inactive) (Hochachka & Somero 1984). Thus, the properties reflected by any physiological system are determined both by the information contained within the genome and by the interactions which result from post translational-modifications.

The control of carbohydrate metabolism in molluscs

The regulation of glycolytic function is central to metabolism. In molluscs the most distinct feature of this system is the absence of lactate as the anaerobic end product (Gabbot 1976). Thorough metabolic investigations have been carried out on bivalves (de Zwaan 1977) and on cephalopods (Storey & Storey 1983), though much less is known about gastropods (Livingstone & de Zwaan 1983). In 1973 de Zwaan *et al.* proposed a scheme for anaerobic degradation of glucose (or glycogen) to alanine, succinate and glutamate. More recently, octopine has been found to be a major anaerobic end product (Grieshaber & Gäde 1976 a, b, 1977, Gäde *et al.* 1978, de Zwaan *et al* 1980, Gäde 1980 b), though other imino acids such as alanopine, strombine and lysopine

(Storey & Storey 1983) have also been isolated. A detailed glycolytic analysis on Mytilus edulis (Cameselle et al 1980), demonstrated that not only are PFK, FbP, PK and HK non-equilibrium enzymes, enzymes involved in flux regulation (Newsholme & Start 1973), but it also appears that the role of PK is crucial for the overall control of the pathway.

Atkinson & Walton (1967) developed the concept that ATP-generating processes and those involved in its regulation, are dependent not on nucleotide concentration, but rather on the energy balance of the cell. They proposed the concept of adenylate energy charge (A.E.C.), which can vary between 0 (only AMP present) and 1 (only ATP present). Interactions in effects of feed back modulators of enzyme activity and energy charge are highly important in the regulation of metabolism (Atkinson 1968). Atkinson (1968) also postulated that under adequate substrate availability, any trend towards a decrease in A.E.C. is counteracted by an increase in ATP production. However, with limited substrate availability, the rate of ATP production diminishes and a new steady-state is achieved. The importance of adenylates and feed-back regulation has been stressed by Newsholme & Start (1973), Atkinson (1977) and de Zwaan (1977), though more recently the use of A.E.C. in the assessment of cellular energy status has been questioned by Jacobus et al. (1982).

In the scheme proposed by de Zwaan etal. (1973) phosphoenol-pyruvate is converted to alanine (via pyruvate), with subsequent assimilation into the malate pool, which in turn enters the mitochondria and thus the Krebs cycle. Most workers have concentrated on the regulation of the phosphoenol-pyruvate branch point (Gabbot 1976), although extensive work has covered other branch points and pathways. A classical glycolytic pathway converting glucose-6-phosphate to pyruvate has been described in gastropods (Bennett & Nakada 1968, Goudsmit 1972, Marshall et al. 1974, Zammit & Newsholme 1976 a, Zammit et al. 1978, Avelar et al. 1978). The Krebs cycle has also been examined in this molluscan group (Goddard & Martin 1966, Coles 1969, Bacila 1970; Bolognari et al, 1979). However, no complete analysis of this cycle is available for any gastropod, except for the work of Zammit & Newsholme (1976 b) on the propertites of NAD^+-isocitrate dehydrogenase of the whelk Buccinum undatum. The pentose phosphate pathway has also been the subject of some analysis in this group (Horstmann 1960, Bennett & Nakada 1968, Hunger & Horstmann 1968, Hernandez et al. 1970, Fried & Levin 1973, Schilansky et al. 1979) but despite the demonstration of the pathway in vivo by Bennett & Nakada (1968), no information on its regulation is available (Livingstone & de Zwaan 1983).

Biochemical composition of molluscs and metabolic implications

The body tissue composition of any organism is a reflection of its diet and type of metabolism. Goddard & Martin

(1966) demonstrated that the percentages of constituents vary within the molluscan classes. However, since most of the determinations relate to seasonal changes and/or reproductive state (Meenaskshi & Scheer 1968, Blackmore 1969, Williams 1970, Holland et al. 1975), it is not always possible to compare or predict trends amongst the various groups studied. In addition, there are only weak links between the possible mechanisms of physiological regulation and body constituents (Livingstone & de Zwaan 1984).

A number of fresh water snails display carbohydrate dependent metabolism triggered by the transition from the aerobic to anaerobic state (von Brandt et al. 1950). Emmerson (1967) showed the metabolism of Planorbis corneus to be carbohydrate dependent but in addition a significant amount of protein was consumed simultaneously. The consumption of protein accompanying lipid oriented metabolism has been recognised in the fresh water pulmonate Australorbis glabrata (von Brandt et al. 1957). In contrast, the metabolism of the prosobranch Littorina was found to be lipid and carbohydrate oriented in the adult, though lipid oriented only, during larval development (Holland et al. 1975). The prosobranch Thais lamellosa was characterised by lipid oriented metabolism, irrespective of life cycle stage (Stickle & Duerr 1970).

Molluscs reveal some intriguing features with respect to glycogen content. The cephalopods, being the most active molluscan group, have the lowest glycogen levels (on average 24

umol gm^{-1} wet wt.) (Goddard & Martin 1966, Linvingstone & de Zwaan 1983). These determinations must be regarded with caution, due to the rapid catabolism of glycogen during capture (Storey & Storey 1979a). Glycogen concentration in gastropod tissues is generally high (from 38 to 320 mg gm^{-1} dry wt.) and similar to that of bivalves (de Zwann 1977, Livingstone 1982).

The tissue distribution of lipids has been extensively studied in gastropods and bivalves, and to a lesser extent in cephalopods (Voogt 1983), due to their economic value as a source of food. Interest in the determination of lipid source has also increased markedly, especially with regard to tracing food chains and as an index of marine polution. However, information on lipid metabolism is limited, apart from endocrinological determinations, sterol characterisation and the discovery that the majority of molluscan taxa are able to synthesise fatty acids from acetate (Voogt 1972).

These reports are but a small indication of the diversity of molluscan utilisation of energy resources. It appears that differences in the choice of energy source are related to the specific calorific value of each substance and the ease with which it can be metabolised. Despite a few cases reporting simultaneous consumption of protein and other energy source, in general the total percentage of nitrogen and phospholipid does not change during starvation. This is possibly a reflection of the tendency to maintain structural integrity of

body tissue (Holland et al. 1975).

Assessment of cellular dynamics by NMR spectroscopy

In recent years nuclear magnetic resonance (NMR) spectroscopy has been increasingly used to study cellular metabolite turnover, in whole body, isolated cells and organs, or in tissue homogenates (Norton 1978).

The non-invasive nature of intact or whole tissue measurements offers considerable advantages over conventional analytical determinations, though often limited by poor sensitivity, requiring fairly high concentrations of the molecules (0.5-1.0 mM) to be examined (Gaddian & Radda 1982).

^{1}H NMR (Daniels et al. 1976, Brown et al. 1977) and ^{31}P NMR (Houlet et al. 1977, Winkler et al. 1982) have been used due to the high natural abundance and good NMR sensitivity of these nuclei. Natural abundance of ^{13}C NMR has also been used (Robinson et al. 1972, Schaffer & Stejskal 1974, Kainosho 1976, Kainosho & Konish 1976, Sharp & Richards 1977) as well as the coupling of ^{13}C to ^{14}N (Norton 1978). Nonetheless the relatively low abundance of ^{13}C (1.1 %) and its poor sensitivity with respect to the other two nuclei are a great disadvantage, despite the good peak resolution, a function of its large chemical shift.

It is unlikely that currently accepted metabolic maps will undergo radical changes due to the use of this technique and so far it has confirmed already existing knowledge. However it

will no doubt bring more insight into the dynamics of cellular function, where energy-producing and -consuming reactions are controlled and the different forms of energy are tightly coupled (Radda 1979). In invertebrates NMR studies have been successful in illustrating metabolite changes during the course of fertilization (Winkler et al. 1982, Inoue & Yoshioka 1980).

Pilot investigations were carried out on B.digitalis, comprising the determination of the change in body constituents during a period of starvation. Glycolytic capacity was determined under resting and exercised conditions. The use of NMR was intended to provide some insight into the dynamics of cellular components of tissue homogenate, in response to addition of exogenous substrate.

MATERIALS AND METHODS

Biochemical analysis of body constituents

The method used was that of Holland & Gabbot (1971) and Holland & Hannant (1973). Mean values were calculated from triplicate determinations, extrapolated from standard curves of carbohydrate, lipid and protein levels. Dry body weight was determined by drying the body tissues at 60°C, for four days.

Collection of whelks and experimental design

Only adult females were captured. On arrival at the laboratory the 90 whelks were devided into 3 groups:

(1) 30 whelks were selected, their shell length recorded and marked. Following sacrifice by freezing in liquid nitrogen, each whelk was deshelled and the total tissue mass weighed and dried as above. No shell fragments were included. Once dried, the tissues were kept in glass vials in a desiccator awaiting further analysis.

(2) 30 animals were kept in a tank with sea water and 5cm sand. The water level was low (10 cm) and was not oxygenated. Conditons simulated those of stagnant water. Every day for 10 days 3 whelks were removed, measured, sacrificed and treated as in group (1).

(3) 30 animals were kept in a sea water tank as in (2), except that the water level was high (30 cm) and was strongly oxygenated, by maintaining constant, active water turbulance. Animals were treated as in (2).

Definition of terms

Protein nitrogen: nitrogen content of material insoluble in 5% TCA, and was determined by a micro-Kejeldahl method.

Total nitrogen: was determined in the same way, on a sample of the initial aqueous homogenate.

Total lipid: material extracted from a sample of aqueous homogenate into a mixture of chloroform:methanol (1:2) , and was determined spectrophotometrically after charring with H_2SO_4.

Neutral lipid: determined in the same way as total lipid after adsorption of phospholipid material from the total lipid extract onto silicic acid.

Phospholipid: this value was obtained by the difference of the above.

Total carbohydrate: all substances reducing alkaline ferricyanide after hydrolysis of the TCA-soluble extract with 1.0 N HCl at 95°C for 2 hours.

Free reducing sugars: was obtained from an unhydrolysed sample of the TCA extract and the value for polysaccharide obtained by difference. This polysaccharide is described as glycogen due to its high content in Bullia (da Silva et al. 1985), as well as innumerous other gastropods (Barry & Munday 1959, Emmerson 1965, Blackmore 1969, Holland et al. 1975).

Determination of glycolytic flux

A method modified from Rovetto et al. (1975) was used. The method is based on the assumption that products resulting from the breakdown of glucose accumulate as body H_2O and CO_2. In Bullia it was assumed further that these products equilibrate fast with the external pool, in the surrounding aqueous medium, thus reflecting the proportion of substrate incorporated into the anaerobic and aerobic pathway, respectively.

Two independent experiments were performed, one using

5-^{3}H-glucose and the other U-^{14}C-glucose. The whelks were injected with 250 ul of a 10.0 mM solution of glucose (250 uCi) of either isotope and immediatly introduced in a chamber with sea water (100 ml), containing also a magnetic stirrer bar. Continuous stirring creates water currents similar to those in Bullia's natural habitat. In response to these currents, the whelk extends its foot, maintaining the continual tension associated with surfing in the field. The chamber was sealed with parafilm once all air bubbles had been removed. The magnetic stirrer was switched on and water samples (0.1 ml) removed at the times indicated in Fig. 7.4., over a period of 4 hours. Utilisation of exogenous glucose was estimated by measuring the rate of breakdown of 5-^{3}H-glucose into $^{3}H_2O$ (anaerobic pathway) and that of U-^{14}C-glucose into $^{14}CO_2$ (aerobic pathway). The high level of radioactivity introduced into the animal was necessary due to the large dilution factor between the volume injected (250 ul) and the overall volume of the chamber used (100 ml), especially when small volumes were analysed (100 ul). Small volumes were used in order to reduce error during the experimental procedure, as reducing the chamber volume by 100 ul (x 44), did not justify the replacement of water, thus avoiding dilution errors as well as the disadvantage of introducing water with a different oxygen content, which may alter the animal's behaviour.

The measurement of oxygen consumption

Oxygen consumption was measured in a separate experiment (to avoid radioactive contamination of the oxygen

probe), though other conditions were identical to the previous experiments. The decrease in oxygen content of water was monitored by a Radiometer PHM 72 MK 2 Acid/Base analyser, using an oxygen probe.

Metabolite determinations

These were done according to the method of Bergmeyer et al. (1974), in a Unicam SP 1800, though only foot tissue was used. Estimations of the proportion of radioactive label accumulated in the tissues were carried out on 100 mg of each of the following organs: foot, pallial complex, digestive gland and gonad. These were homogenised and both cytosol and pellet counted.

Determination of Mass-Action ratios, $K_{eq.}$ and adenylate energy charge

Mass-Action ratios (MAR) and mass-action ratios/equilibrium ($MAR/K_{eq.}$) were determined according to the criteria of Rolleston (1972), whereby ($MAR/K_{eq.}$) values between 0.05 and 0.20 separate non-equilibrium and near equilibrium enzymes.

^{31}PNMR analysis of foot muscle homogenate

Foot muscle cytosol was prepared in a 0.1 M triethanolamine HCl buffer, pH 7.5, containing 0.001 mM EDTA and 0.1 M NaCl, in the ratio 1:1 parts of muscle and buffer. This was

homogenised with care to diminish protein denaturation, followed by centrifugation at 10 000 x g for 30 minutes, at 4°C using a Sorval RC2-B. The supernatant was stored at 4°C and used as soon as possible. The final protein concentration was in excess of 68 mg ml^{-1}, to compensate for the expected low level of phosphorous metabolites and due to the relatively low capacity of the instrument used. Two NMR runs were performed: one where no exogenous substrate was provided and the other in the presence of 0.02 M glucose. The ^{31}P-NMR experiments were performed at 36.44 MHz (maximum field capacity) with a Bruker WH 90 NMR System, using an iron magnet, at 28°C. Trimethylphosphate in a D_2O jacket was used as the external standard. No oxygenation or agitation was provided.

RESULTS

The biochemical composition of Bullia digitalis

Results are illustrated in Fig. 7.1., 7.2 and 7.3. The metabolism of this whelk is carbohydrate oriented (Fig. 7.1.) under conditions of limited oxygen availability (group 2), despite the simultaneous consumption of some protein (Fig 7.2). This strategy is far from simple as in the first 4 days of starvation protein levels rise 3 fold, with respect to levels of group (1) determined on arrival, but decrease subsequently to 20% of maximal value recorded. No significant changes were noticed in the lipid profile (Fig. 7.3.). Group (3) which was maintained in aerated conditions relies also on carbohydrate as an energy

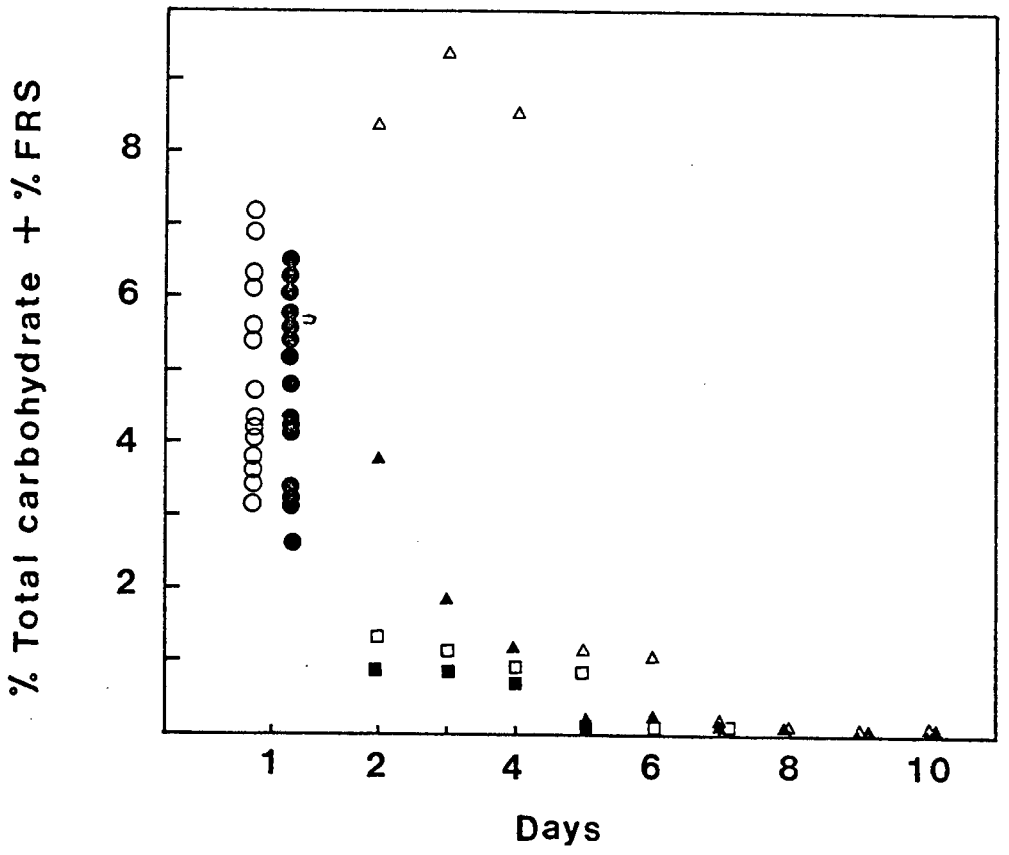


Figure 7.1. The carbohydrate consumption profile of Bullia digitalis during a period of starvation. Group 1 (○,●) was sacrificed on arrival at the laboratory (control). Group 2 (△,▲) was kept under conditions of limited oxygen availability. Group 3 (□,■) was maintained in fully aerated conditions. (○,△,□) Correspond to total percentange carbohydrate, whereas (●,▲,■) indicate the percentages of free reducing sugars, FRS).

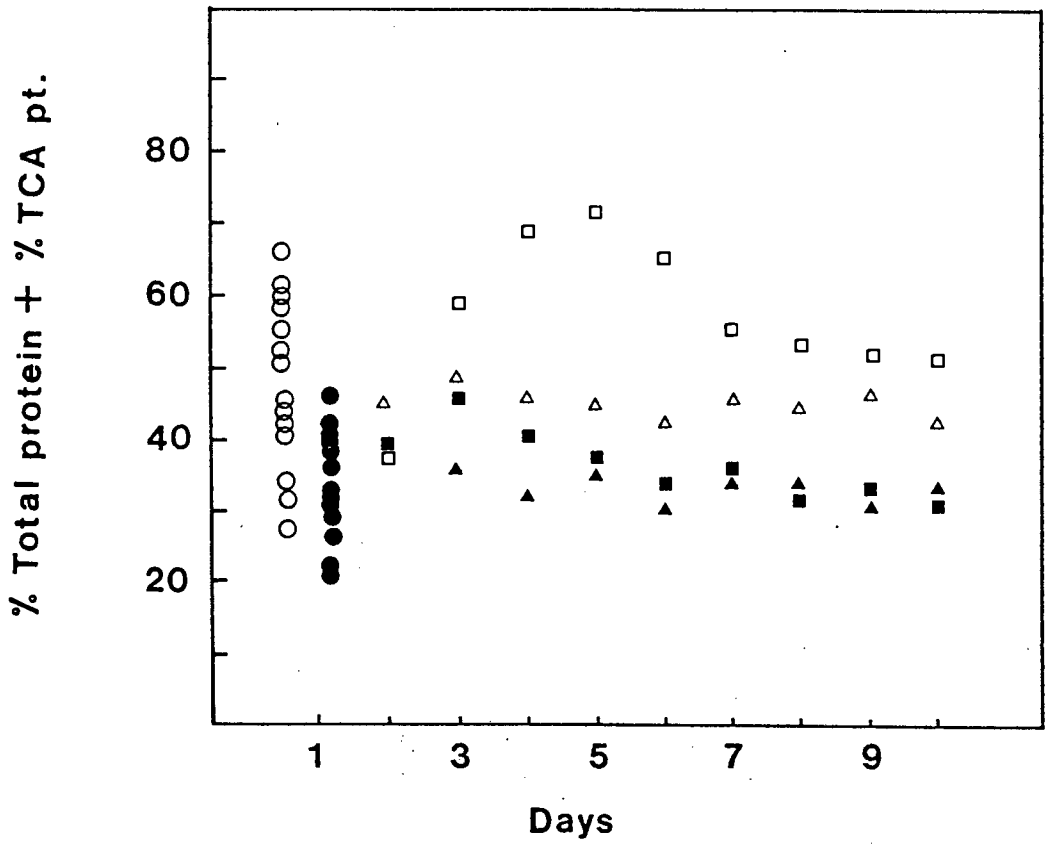


Figure 7.2. The protein consumption profile of Bullia digitalis during a period of starvation. Group 1 (○,●), Group 2 (□,■)and Group 3 (△,▲), as indicated in Figure 7.1.; (○,□,△) correspond to the percentage total protein, whereas (●,■,▲) indicate the percentage of TCA precipitated protein.

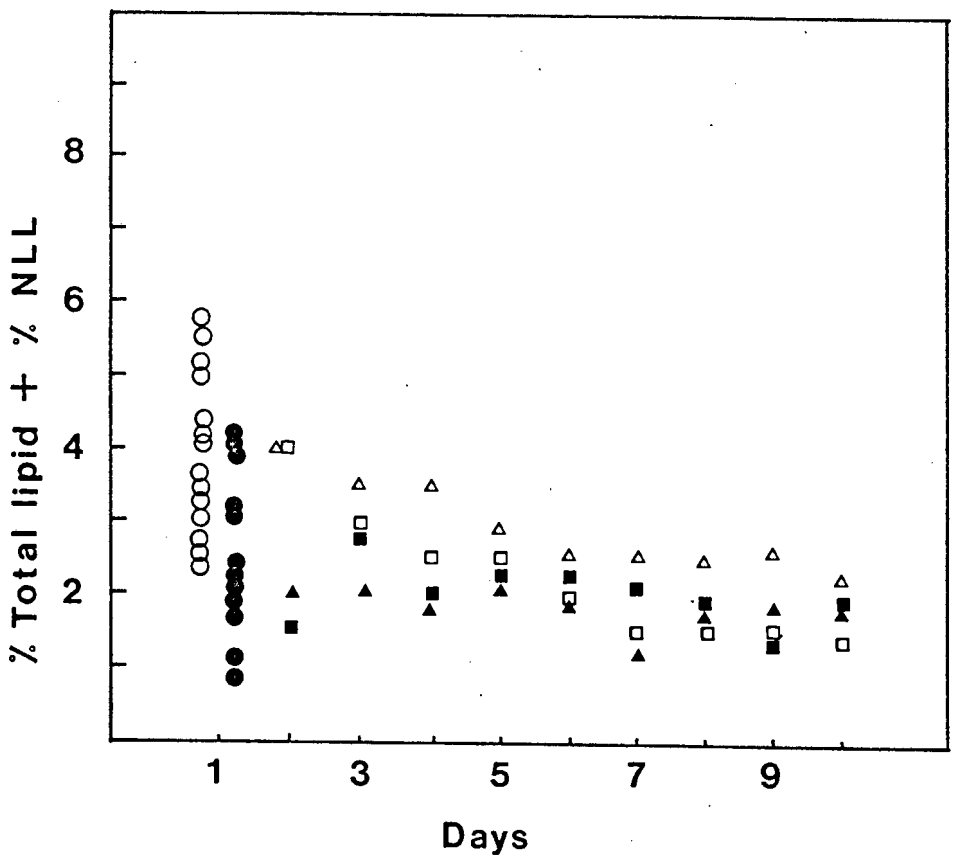


Figure 7.3. The lipid consumption profile of Bullia digitalis during a period of starvation. Group 1 (○,●), Group 2 (□,■) and Group 3 (△,▲), as in Figure 7.1. ; (○,□,△) correspond to the percentage total lipids, whereas (●,■,▲) indicate the percentage of neutral lipids, NLL).

source, albeit at a reduced rate (Fig. 7.1). There were no significant changes in protein or lipid profiles in this group (Figs. 7.2. and 7.3.).

Glycolytic flux, metabolite levels and adenylate energy charge

Results are summarised in Tables 7.1., 7.2. and 7.3. as well as on Fig. 7.4. The rate of glycolyis (consumptiom of exogenous 5-^{3}H-glucose) corresponds to 7.0 umol glucose min^{-1} sdt. dry $wt.^{-1}$, whereas the rate of U-^{14}C-glucose incorporation into the Krebs cycle is much slower, corresponding to 0.12 umol glucose min^{-1} sdt. dry wt. The rate of oxygen consumption during this period was calculated as 2.21 µgm O_2 min^{-1}. Table 7.1. represents the metabolite changes undergone during the exercise period in the experimental chamber. It shows a 5 fold increase in tissue octopine, though lactate does not accumulate as significantly. Metabolite concentration changes were also recorded for G6P, F6P, FDP, G3P, DHP and 3PG. The A.E.C. value decreases from 0.840 (unexercised state) to 0.667 (following exercise). Table 7.2. shows the calculation of mass action ratios (MAR) from which it appears that PFK and PK are not equilibrium enzymes. In Table 7.3. an indication of radioactivity accumulation is given in 4 tissues, from which relative percentage accumulation for each tissue with respect to total body mass was calculated.

TABLE 7.1. Determination of tissue levels of glycolytic intermediates in Bullia digitalis foot.

Glycolytic intermediate	Tissue levels (nmol/g fresh weight)	
	Unexercised Whelk	Exercised Whelk (surfing simulation for 3 hrs.)
G6P	38.0	108.3
F6P	8.2	20.4
FDP	15.0	45.0
G3P	7.0	16.3
DHP	5.9	48.4
3PG	16.2	18.5
2PG	-	3.3
PEP	4.9	5.1
Pyr.	16.6	10.8
Lact.	120.5	222.0
Oct.	1093.4	5145.0
ATP	874.7	703.4
ADP	233.8	392.3
AMP	124.3	251.7
A.E.C.*	0.840	0.667

G6P = Glucose-6-P
F6P = Fructose-6-P
FDP = Fructose-1,6-diphosphate
G3P = Glyceraldehyde-3-P
DHP = Dihydroxyacetone phosphate
3PG = 3-phosphoglycerate
2PG = 2-phosphoglycerate
PEP = Phosphoenolpyruvate
Pyr. = Pyruvate
Lact. = Lactate
Oct. = Octopine
ATP = Adenopine triphosphate
ADP = Adenosine diphosphate
AMP = Adenonine monophosphate
A.E.C. = Adenylate Energy Charge $= \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}$

TABLE 7.2. Calculated mass-action ratios (MAR)

Enzymes†	MAR Expressions	K_{eq}	MAR		MAR/K_{eq}	
			Unexercised	Exercised	Unexercised	Exercised
HK	$\frac{G6P \times ADP}{GLU \times ATP}$	320	$\frac{10.16}{GLU}$	$\frac{60.40}{GLU}$	-	-
PGI	$\frac{F6P}{G6P}$	0.49	0.21	0.91	0.43	0.39
PFK	$\frac{FDP \times ADP}{F6P \times ATP}$	331	0.49	1.22	0.0015	0.004
ALD	$\frac{DHP \times G3P}{FDP}$	48×10^{-5}	2.73	17.53	568.75	3652.0
TIM	$\frac{DHP}{G3P}$	32	0.84	2.62	0.026	0.070
PK	$\frac{PYR \times ATP}{PEP \times ADP}$	23×10^{4}	12.67	3.79	0.0006	0.0002

† Data available in Table 7.1 was used to calculate MAR.
HK = Hexokinase; PGI = Phosphoglucose isomerase; PFK = Phosphofructokinase;
ALD = Aldolase; TIM = Trioxphosphate isomerase; PK = Pyruvate kinase.

TABLE 7.3. Differential accumulation of radioactive label in body tissues. Values have been adjusted with reference to the ratio of organ to body wet weight.

Organ	% of label per organ		Equivalent % of body wet weight containing radioactive label
	^{3}H	^{14}C	
Foot	27.7	43.8	83
Mantle	26.8	22.9	4
Digestive gland	30.3	23.7	12
Gonad	15.2	9.6	1

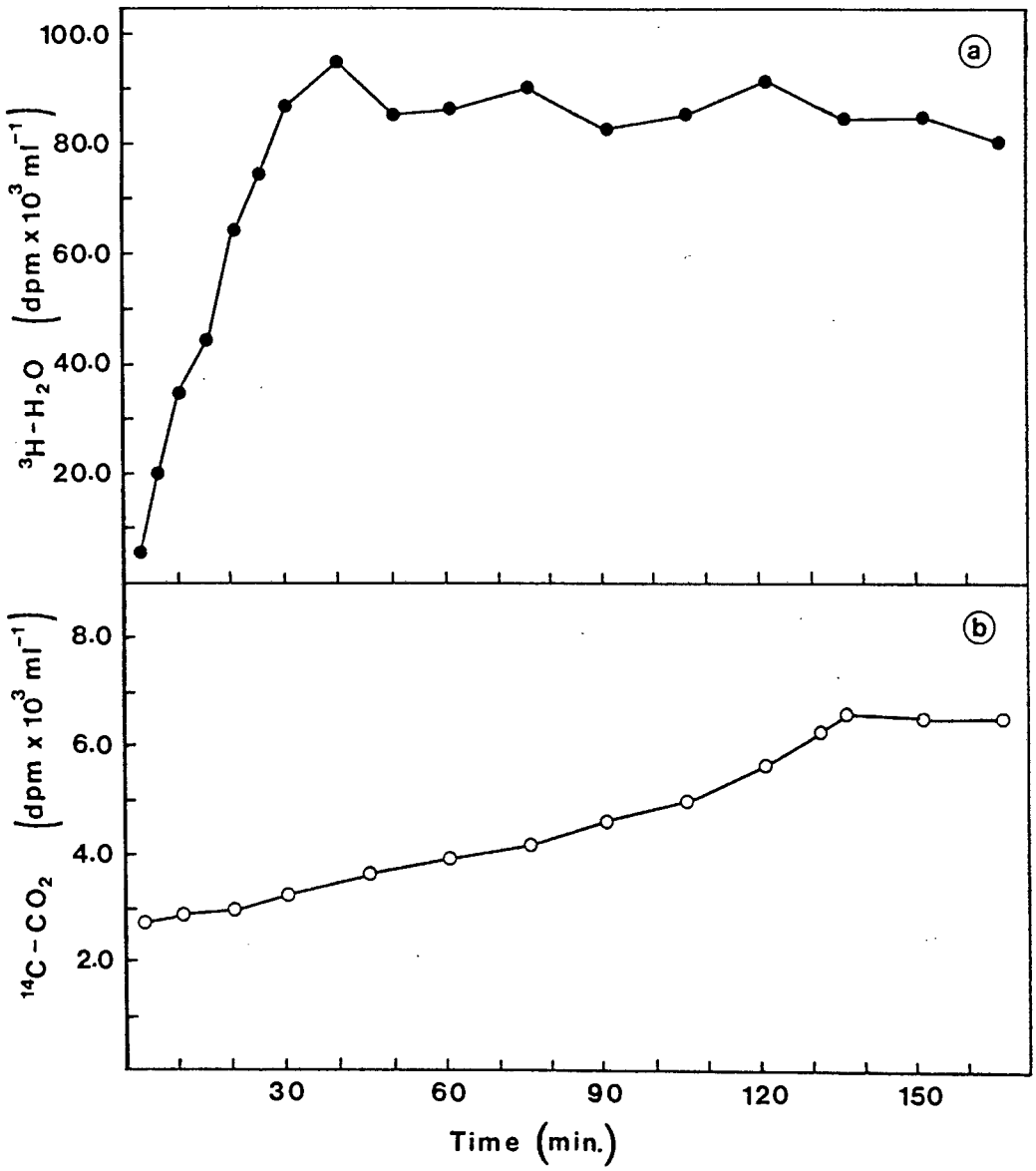


Figure 7.4. Anaerobic (a) versus aerobic (b) metabolism, in the course of a period of exercise (surfing). The glycolytic rate was determined from (a) as ^3H_2O, whereas (b) $^{14}CO_2$ release is indicative of carbon flow into the Krebs cycle. Though the experiments were carried out for a longer time period, no changes were observed after 150 minutes.

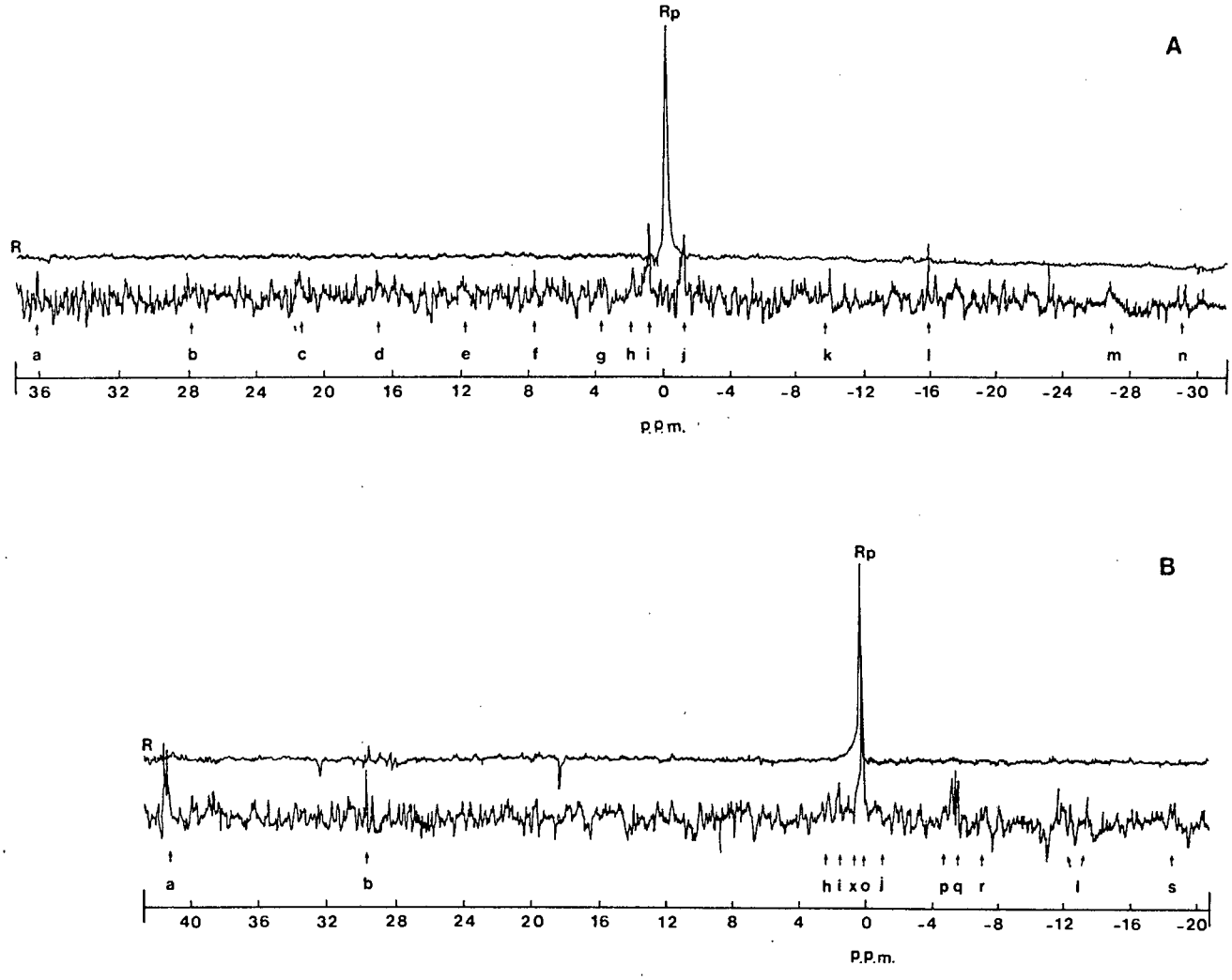


Figure 7.5. ^{31}P-NMR spectrum of Bullia digitalis foot cell free cytosol at ambient temperature, after 12706 scans: (A), in the absence of substrate; (B), in the presence of 20 mM glucose. Peak assignment: (a) imino-acid; (b) components of cell matrix and glutamate; (g) and (h) intermediates of glycolysis and Krebs cycle; (i), (o) and (x) phospholipid and phosphorous; (j) arginine phosphate; (k) β-ATP; (i) di- and trinucleotides; (p),(q) and (r) NAD^+, $NADP^+$; (l) and (s) ADP; (c), (d), (e) ,(f), (m) and (n) not identified; Rp, reference peak, which coincides with internal phosphorous peak.

^{31}Phosphorous NMR profile of foot cytosol

Results are given in Fig. 7.5. Differences between the two samples (presence and absence of exogenous substrate) are reflected in alterations of nucleotide concentrations: in the presence of 0.02 M glucose raised level of ATP and arginine phosphate; change in ADP was difficult to assess, yet slight decrease in the AMP and phosphorous peak also occur. Despite high background which impedes satisfactory identification of peaks, there are slight changes in glycolysis and Krebs cycle intermediates, as well as a significant change on a down field peak (40.0 ppm), corresponding to an imino acid, which in the present case is possibly octopine.

DISCUSSION

Since metabolic fluxes depend on the overall rate of the pathway and this is determined by the summation effect of each enzyme, where an enzyme is less active, limitation of flux could result. This may impose some regulatory constraint on pathways. From these results it is apparent that different isozymes are expressed differently in different tissues, representing possible selective forms of metabolic control designed especially for each tissue.

The effect of starvation on the biochemical composition of Bullia indicates that under stagnant water conditions, the whelk's metabolism is carbohydrate oriented and that despite an

initial increase of the proportion of protein, some protein consumption accompanies carbohydrate utilization. This initial rise in protein may reflect the conversion of other components into a nitrogenous product, though its significance is unknown. Protein and phospholipid levels have been observed to remain constant in the whelk Littorina, as an indication of maintenance of cellular integrity (Holland et al. 1975). Under oxygenated conditions, the changes observed are slight and may be an indication of the ability of the whelk to use its energy resources with great efficiency. Changes in other components are negligible. It is not surprising that a major component of B.digitalis is carbohydrate, since a commom source of food is stranded Cnidaria such as jelly-fish. These are composed of at least 80% carbohydrate on a dry weight basis, largely in the form of mucopolysaccharides (Barnes 1974).

The above results on metabolic carbohydrate dependence are confirmed by the glycolytic capacity of Bullia. The rate of anaerobic glucose utilisation is 7.0 μmol $min.^{-1}$ std. dry $wt.^{-1}$, far in excess of that of the rate of incorporation into the Krebs cycle (0.12 μmol $min.^{-1}$ std. dry $wt.^{-1}$). It is suggested that the difference between these values represents incorporation into other pathways, such as octopine and lactate pools, as well as into free fatty acids, as suggested by van der Horst (1974), de Zwaan (1977) and Hochachka & Somero (1984). Furthermore, since the activity of ODH exceeds that of LDH (see Chapters 5 and 6), it is possible that a much larger proportion of carbon flows into octopine than into lactate. This is further supported by

determination of metabolite levels, which indicate that following a period of exercise the percentage of octopine accumulated was 23 times higher than that of lactate. No information is available on the percentage synthesis of fatty acids in this whelk.

The major sources of energy for anaerobic metabolism are arginine phosphate and glycogen (Livingstone & de Zwaan 1983). Energy in the columellar muscle of Buccinum undatum, during escape reactions, is sustained by arginine phosphate (36%) and by the octopine pathway (64%) (Koorman & Grieshaber 1980), though under conditions of limiting levels of oxygen the ventricle of the whelk B.contrarium obtains its energy from arginine phosphate (37%), from the succinate pathway (50%) and by the octopine pathway (13%) (Ellington 1981).

The breakdown of glycogen during anaerobiosis in molluscs occurs via three mechanisms: (a) lactate pathway; (b) opine (octopine,alanopine and strombine) pathway and (c) succinate pathway. These differ in their energetic efficiencies and/or the rate at which the energy is produced (Livingstone 1982). The lactate and opine pathways are essentially linear and their efficiency is low (3 ATP per glucosyl unit); they tend to be employed when energy demands are high, such as during vigorous bursts of activity, recovery from aerial exposure (sessile bivalves), and swimming (free swimming bivalves). Unlike the above, the succinate pathway is a branched pathway of high energetic efficiency (6 to 7 ATP per glucosyl unit), which provides energy during anoxic survival (Livingstone & de Zwaan

1983). Rates of energy production of the above pathways (in μmol ATP equivalents gm^{-1} wet wt. min^{-1}) range from 0.13 in the succinate pathway, through to 0.46 in the lactate pathway, to 0.49 in the opine pathway (Livingstone 1982). Wieser and Right (1978) have proposed that the high rates of energy production result from the acceleration of glycolytic flux.

In the course of the period of exercise the levels of most metabolites were elevated with respect to quiescient animal. Since the level of DHP increased much more than that of G3P, it is suggested that this large increase in the phosphate associated with the reduction in glycolytic flux indicates that the rate of glyceraldehyde-3-P dehydrogenase may be limiting to glycolysis. The decrease in ATP level was less than expected; even the changes in ADP and AMP concentration were not very marked. This resulted in a slight decrease in AEC from 0.804 in control, to 0.667 in the exercised whelk. Brown (1982) reported that A.E.C. levels in natural populations varied between between 1.0 and 0.5, being particularly low if the whelks had been active in the surf. However, if the whelks were returned to the laboratory and allowed to remain buried in the sand without feeding, the A.E.C. levels rose, approaching 1, within 24 hours of capture. Brown (1982) suggested further that such natural A.E.C levels may represent a critical energetic situation for the whelk and that immediate energy availability from glycolysis may be insufficient, resulting in an ATP "debt". Surprisingly the cost to restore such a "debt" was found to be almost negligible, in

fact of the order of the expenditure involved in ATP turnover. Recently however, Jacobus et al. (1982) pointed out that cellular nucleotide changes are seldom of great magnitude and that the limiting factor is not the rate of ATP synthesis, but the availability of ADP to the mitochondria. In addition they demonstrated that the AEC often does not reflect real energetic changes and other mathematical treatment of energetic data should be used instead, in order to investigate cellular dynamics.

Despite the active nature of the motion in the experimental chamber, the whelk's oxygen comsumption (1.21 µl O_2 min^{-1} std. dry $wt.^{-1}$) is low when compared to that of other gastropods (Newell 1979). The initial percentage oxygen saturation of the water was high (close to 100%), but it decreased to circa 10% at the end of the four hour experimental run. Within 45 minutes of the start of the experiment, the whelk showed signs of stress: slowed movements, aquisition of a grey colouration on the surface of its body and limpness of the foot (nonetheless the animal was still alive and soon recovered when placed in fresh sea water). This was reflected in a shallower rate of oxygen uptake to 60% of the initial rate. This may be a function of the percentage oxygen remaining in solution, so that the whelk proportionally regulates its oxygen usage depending on the level of the surrounding medium. What is surprising is the fact that even at low oxygen concentrations (10%) B.digitalis can still use oxygen, considering that under normal circumstances no oxygen carrying or storage pigment can be demonstrated (Brown et al. 1985).

Of the enzymes examined, PFK and PK are clearly non-equilibrium enzymes, for their $MAR/K_{eq.}$ are well below 0.05 (criterion of Rolleston 1972), indicating their regulatory capacity of control points of glycolysis. This is in agreement with findings of Gabbot (1976) and Cameselle et al. (1980). $MAR/K_{eq.}$ for HK was not determined due to the lack of information on the concentration of glucose, as these were not consistent. Examination of other enzymes was also restricted due to the error encountered in determination of the respective K_{eq}.

Comparison of ^{31}PNMR metabolite profiles in the absence and presence of glucose indicates that the glycolytic pathway is being used to generate energy and is accompanied by the accumulation of an end product (an imino acid, probably octopine, though lactate accumulation was not observed). However, since the cellular system is disrupted, it is difficult to speculate on the potential use of the initial energy synthesised. For a number of technical reasons it was not possible to oxygenate the sample; thus the observations made were acquired shortly after the initiation of the experiment. Limiting oxygen conditions combined with high protein concentration, required to obtain qualitative changes, and the high ambient temperature in which the experiment took place (28°C) are known to be problematic. Iles et al. (1982) reported that the rise in temperature from 4°C to ambient (20°C) increases the kinetic energy of the cytosol molecules so markedly that the endogenous oxygen is used rapidly. In addition, the

high concentration of proteins gives rise to protein layers, whose colloidal properties induce settlement to the bottom of the NMR tube. In consequence only the surface protein layer is in contact with the gaseous environment above, resulting in sample death and distortion of the NMR signal. Winkler et al. (1982) have been able to overcome most of these problems by carefully bubbling oxygen into the NMR tube. Navon et al. (1977) used a PCA/TCA metabolite extraction procedure which obviates the necessity of keeping the sample alive. The current experiments were performed as pilot runs, with the full awareness of intrinsic experimental design problems, though it was felt that such a study was necessary to establish a working baseline, even if low capacity instrumentation had to be used.

In conclusion, Bullia appears to be a most efficient gastropod. Its metabolic organization is such that during routine environmental conditions it expends little of its energy stores; when stressed it is still able to survive, and remarkably shows little change in energy levels. Its metabolism is carbohydrate dependent, thus tending to maintain its cellular integrity. This may be one reason why such an animal is able cope with the harsh environment of the high energy sandy beaches.

CHAPTER 8

FINE STRUCTURE OF THE PEDAL MUSCLES OF THE WHELK *BULLIA RHODOSTOMA* REEVE: CORRELATION WITH FUNCTION

INTRODUCTION

Molluscan smooth muscle is extremely variable in its organisation and constituent fibre types (Chantler 1983, Nicaise & Amsellem 1983). Studies on the fine structure of the pedal musculature of gastropods are few and to date have been carried out either on terrestrial snails (Rogers 1969) or on snails employing ciliary locomotion (Plesch 1977). Intertidal members of the genus *Bullia* are exceptionally active, carnivorous whelks, adapted for inhabiting sandy beaches (Brown 1982). Unlike most gastropods, *Bullia* uses its foot for locomotion in a number of ways: for rapid crawling in search of food; for burrowing into sand (Trueman & Brown 1976); and for surfing in the waves (Brown 1982). Such activity is atypical of gastropods, is energetically expensive and implies a high degree of sophistication of the muscles.

Muscle arrangement within the foot of *Bullia* was described by Trueman & Brown (1976). There are two functionally distinct zones within this organ: (1) the small propodium (anterior end of the foot); (2) the very much larger metapodium. Preliminary investigations indicated that there are regional

differences in the fine structure of the pedal muscles of these zones (da Silva et al. 1985). These differences are further investigated in the present paper and attempts are made to relate fine structure to muscle function. Bullia rhodostoma Reeve was chosen as a representative member of the genus Bullia. It is a species which employs all three forms of locomotion (crawling, burrowing, surfing) and is common on many South African beaches (Brown 1982).

MATERIALS AND METHODS

Individuals of B.rhodostoma were collected from sandy beaches along the eastern Cape coast, South Africa, and transported to the laboratory where they were immediately prepared for transmission electron microscopy. Small portions of the foot were fixed overnight in 5% buffered glutaraldehyde (cacodylate buffer, pH 7.2) at 4°C, followed by postfixation in 1% osmium tetroxide for 90 minutes. The tissues were dehydrated in ethanol and embedded in TAAB 812 resin via propylene oxide. Thin sections (65 nm) were cut and stained in uranyl acetate (35 minutes) and lead citrate (3.5 minutes) and then examined using a JEOL 100 CXII electron microscope.

RESULTS

In a review of molluscan muscle Nicaise & Amsellem (1983) classified muscle according to six morphological criteria: the arrangement of dense bodies, dimension of thick filaments, thick to thin filament ratio, plasma membrane invaginations, sarcoplasmic reticulum organization and mitochondrial arrangement. In order to facilitate a comparison of Bullia pedal muscle with that of other molluscs we have followed this classification system.

Examination of muscle from several regions of the foot reveals that the muscle fibres are of two types. The first is almost exclusively restricted to the anterior of the foot, the propodium, and hereafter is referred to as Type A. The second type, Type B, is found predominantly in the metapodium, which comprises the largest area of the foot. A certain degree of overlap of the two types, just anterior to the head, has been observed.

Dense bodies

The dense bodies (homologous to the Z-lines of vertebrate muscle) of Type A are organized perpendicular to the long axis of the muscle fibre albeit with an occasional degree of obliqueness (**Figs. 8.1. and 8.2.**). This arrangement of the dense bodies has features of all of Z_2, Z_3 and Z_4, the muscle being a form of striated muscle where the typical organization of A and I

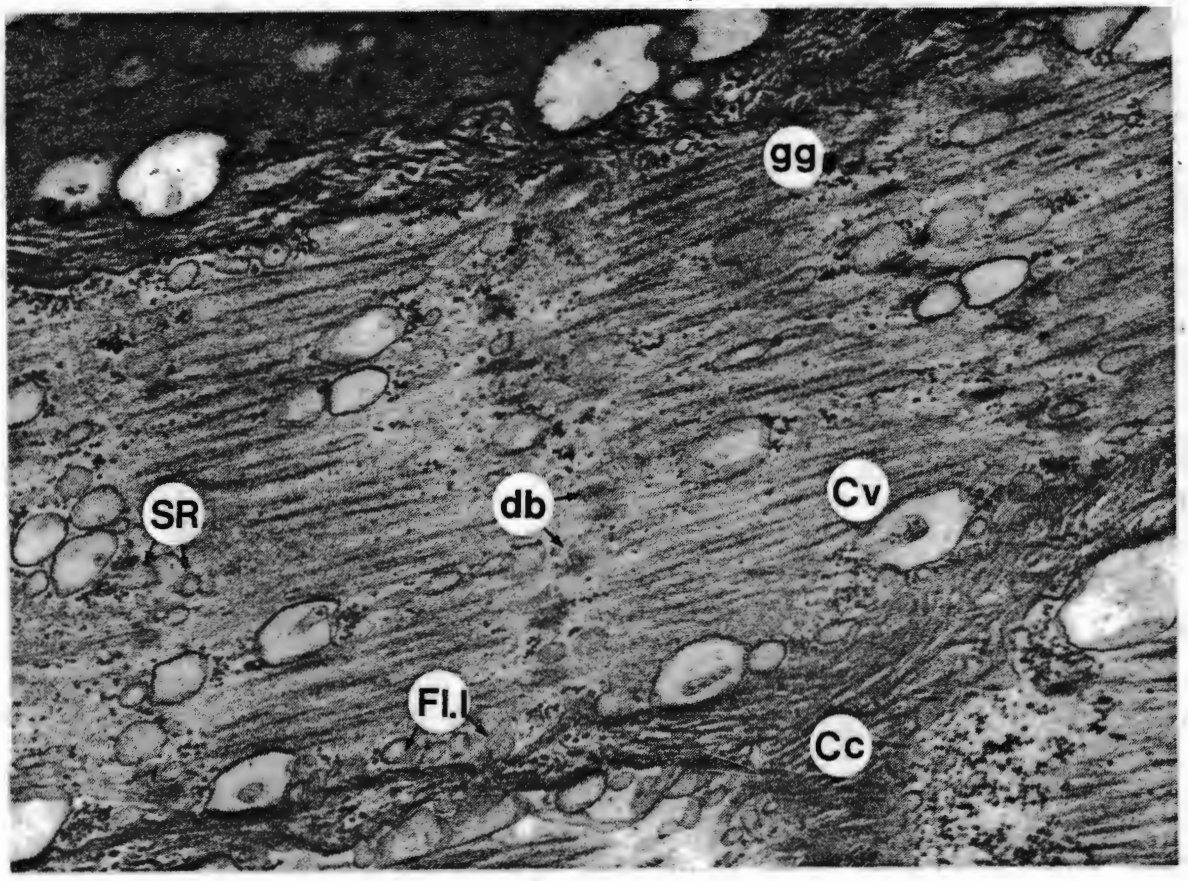


Figure 8.1. Longitudinal section through Type A muscle fibre. Dense bodies (db) are organised perpendicularly to the long axis of the muscle fibre. A layer of interstitial collagen (collagen capsule, Cc) surrounds these fibre types. Other features visible are: sarcoplasmic reticulum (SR); caveolae with everted membrane invaginations (Cv); finger-like plasmalemmal membrane invaginations (Fl.I) and glycogen granules of the β form (gg). Magnification: x 38000.

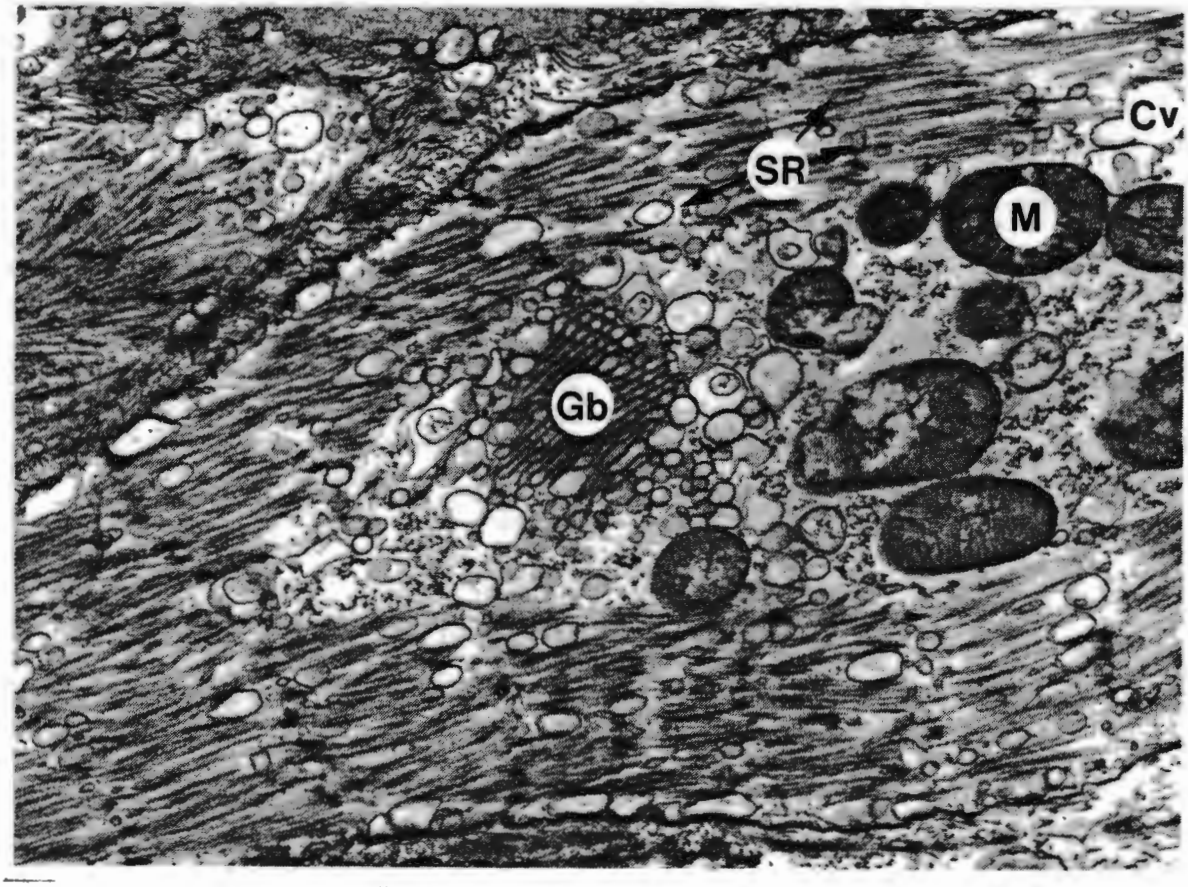


Figure 8.2. Longitudinal section through Type A muscle fibre, showing in addition to those features indicated in Fig.1, the mitochondria (M) confined to the inner core of the cell. Associated with these are organelles, namely Golgi body (Gb), sacoplasmic reticulum (SR) and caveolae (Cv). The pocketed nature of the localisation of these mitochondria is also evident. Magnification: x 20000.

bands is largely recognized. Sections of Type B fibres show them to have a poor organization of dense bodies (**Fig. 8.5.**). Striations are not apparent and the dense body distribution is more typical of smooth muscle (Z_5).

In both types attachment plates (specialised dense bodies) are present on the periphery of the plasmalemma, but are more pronounced in Type B than Type A (**Figs. 8.5.** and 8.6.).

Dimensions of filaments

Both types of muscle fibre have a thick filament length of less than 2 µm (Figs. 8.2. and 8.3.) (L_1 classification). In general, striated muscle is characterized by short sarcomeres (Nicaise & Amsellem 1983) and the pedal muscle of B.rhodostoma is no exception to this with a sarcomere length of 1.40 $\pm$ 0.16 µm (n= 17). A certain amount of error in estimating sarcomere length, caused by tissue contraction, cannot be excluded, this being inevitable in such preparations. The length of the filaments, however, should not be affected.

Although the filament length of Type A and B is similar, this is not true for the filament diameter. Fibre A has a filament diameter of 17.90 $\pm$ 6.40 nm (n= 85), categorizing it as F_1 (diameter of less or equal to 20 nm) (**Fig. 8.4.**); whereas in fibre B this same parameter is 26.80 $\pm$ 5.60 nm (n= 95) (**Fig. 8.6.**), characterizing it as F_2 (main diameter between 20 and 40 nm). The diameter of the dense bodies also differs between the

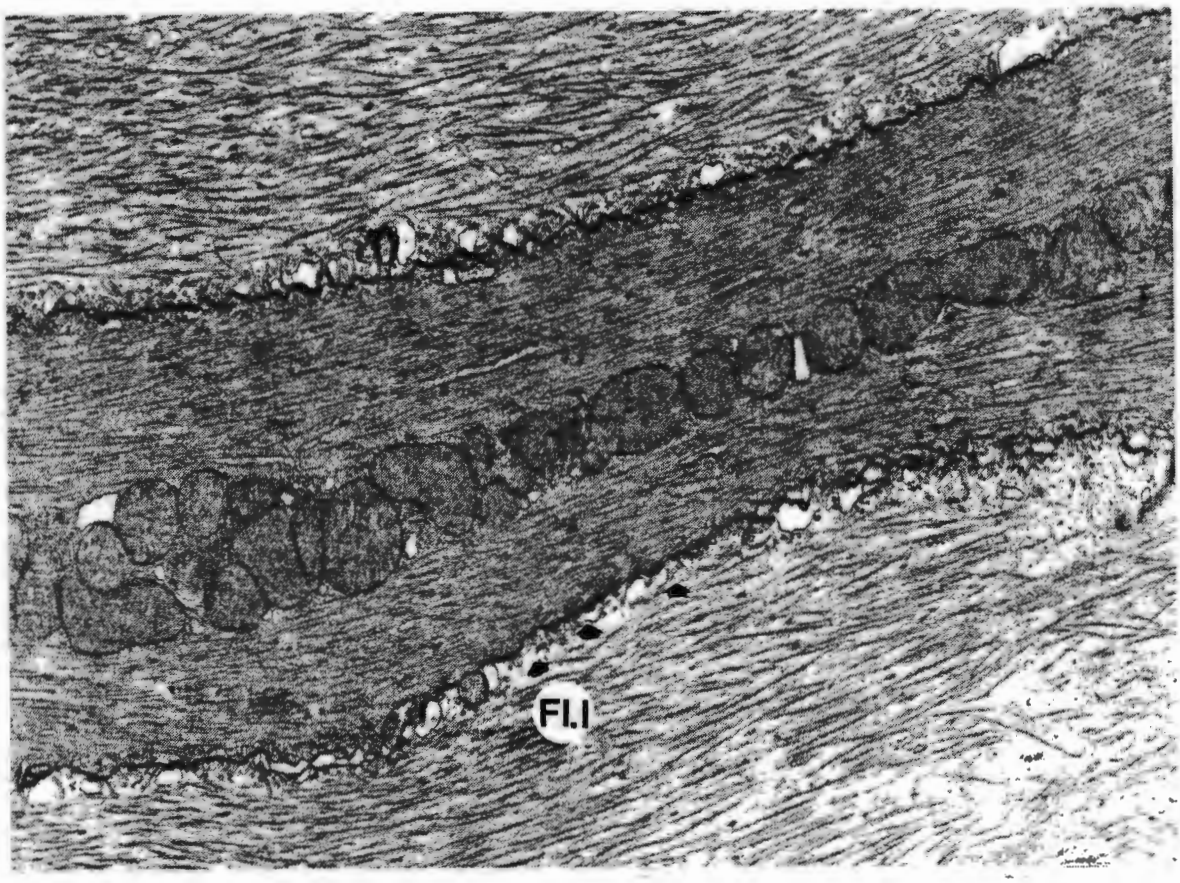


Figure 8.3. Longitudinal section through Type B muscle fibre. These fibres are closely packed with little or no collagen between them. Note the profuse array of finger-like invaginations (Fl.I) of the plasma membrane as well as the central core of densely packed mitochondria throughout the length of the cell, unlike the pocketed distribution of the organelles found in Type A. Magnification: x 14400.

two types, but the significance of this is unclear. In Type A, the diameter is 94.70 $\pm$ 7.50 nm (n=67) in contrast to B, where it is 84.40 $\pm$ 2.47 nm (n= 72) (**Figs. 8.4.** and **8.6.**).

The thick to thin filament ratio

In both types the ratio is greater than the usual 1:6 ratio characteristic of vertebrate muscle and of the muscles of higher molluscs (Chandler 1983). Each thick filament is generally surrounded by a double concentric array of thin filaments (**Figs. 8.4.** and **8.6**); in Type A the thin filaments are tightly packed, making it impossible to specify the thin to thick ratio exactly. It is, however, greater than 1:10. For Type B, the ratio ranges from 1:12 to 1:30.

Dimensions of the thin filaments are not included because of size irregularities and lack of confidence in the measurements.

Plasma membrane invaginations and sarcoplasmic reticulum

There are considerable differences between the two types of fibre in their membrane organization. The fibre types do not conveniently fit into any of the categories given by Nicaise & Amsellem (1983), with the result that neither cell can be graded as R_1 (presence of subsarcolemmal cisternae connected to a net of sarcoplasmic reticulum tubules which penetrate the

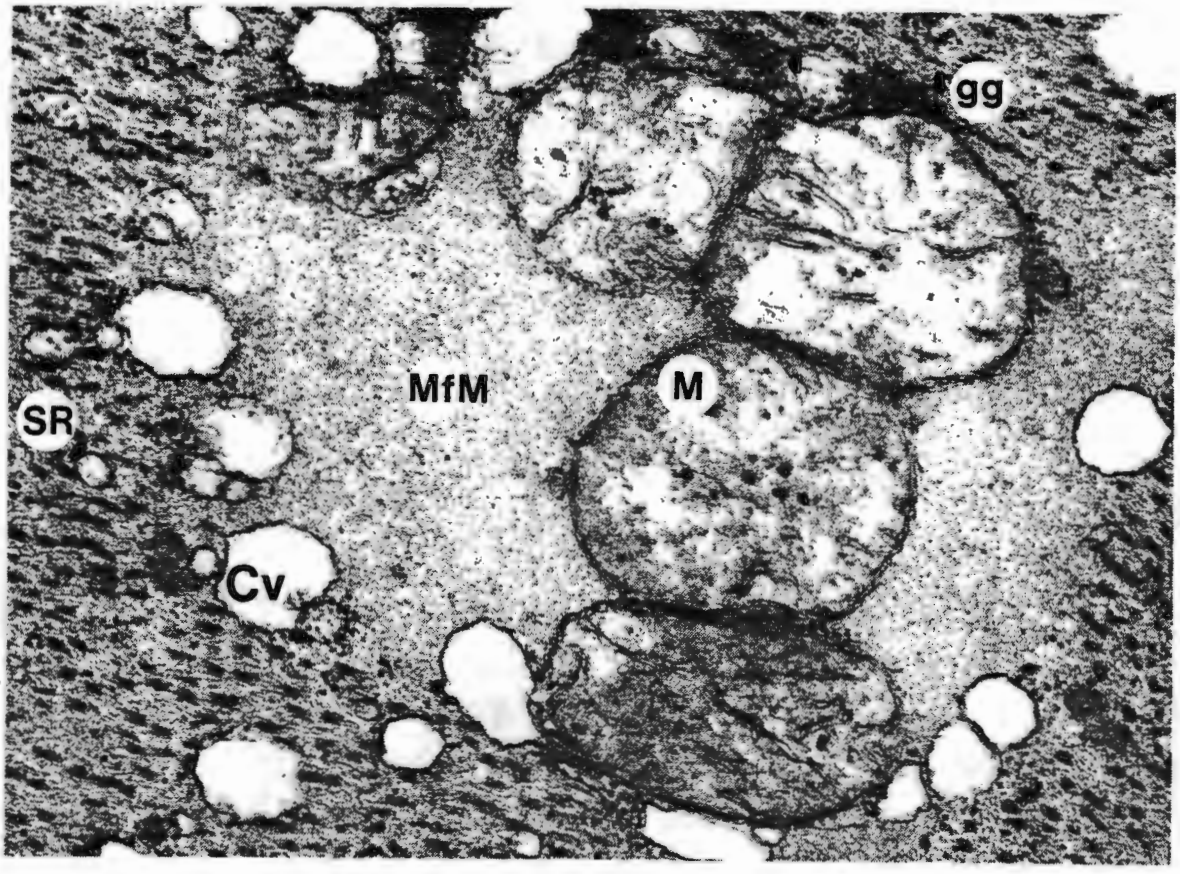


Figure 8.4 Cross section of Type A muscle fibre at a higher magnification illustrating the close association of the mitochondria (M) with caveolae (Cv) and sarcoplasmic reticulum (SR). These mitochondria lie in a myosin free myomatrix (MfM) and are often surrounded by glycogen granules (gg). Magnification: x 58000.

contractile myoplasma) nor R_2 (sarcoplasmic reticulum confined to the non-contractile periphery of the cell).

In Type A, the cell membrane exhibits inward projections forming caveolae, which extend further into the contractile myoplasma: these together with a profuse network of sarcoplasmic reticulum are closely associated with the inner mitochondria (Figs. 8.1., 8.2. and 8.4.). In addition to the more peripheral caveolae, there are finger-like invaginations, suggesting pinocytotic activity (Fig. 8.1.). The subsarcolemmal cisternae and thin reticular tubules in these fibres are extensive and proliferate throughout the myoplasma. Both tubules and caveolae often exhibit everted membrane invaginations , which are generally surrounded by glycogen of the β form (Figs. 8.1. and 8.4.).

In contrast, in Type B large caveolae are present almost exclusively on the periphery of the myoplasma, the latter being perforated by aligned rows of sarcoplasmic reticulum tubules and minor extensions of caveolae (Figs. 8.3. and 8.5.), which are less associated with the inner mitochondrial core. Finger-like invaginations of the plasma membrane are more profuse in Type B than in Type A, resulting in direct contact of pinocytotic vesicles/finger-like invaginations between juxtaposed cells (Figs. 8.3. and 8.5.). Of possible significance in the cell to cell contact system is the almost total absence of collagen surrounding these fibres. This is contrary to findings in Type A,

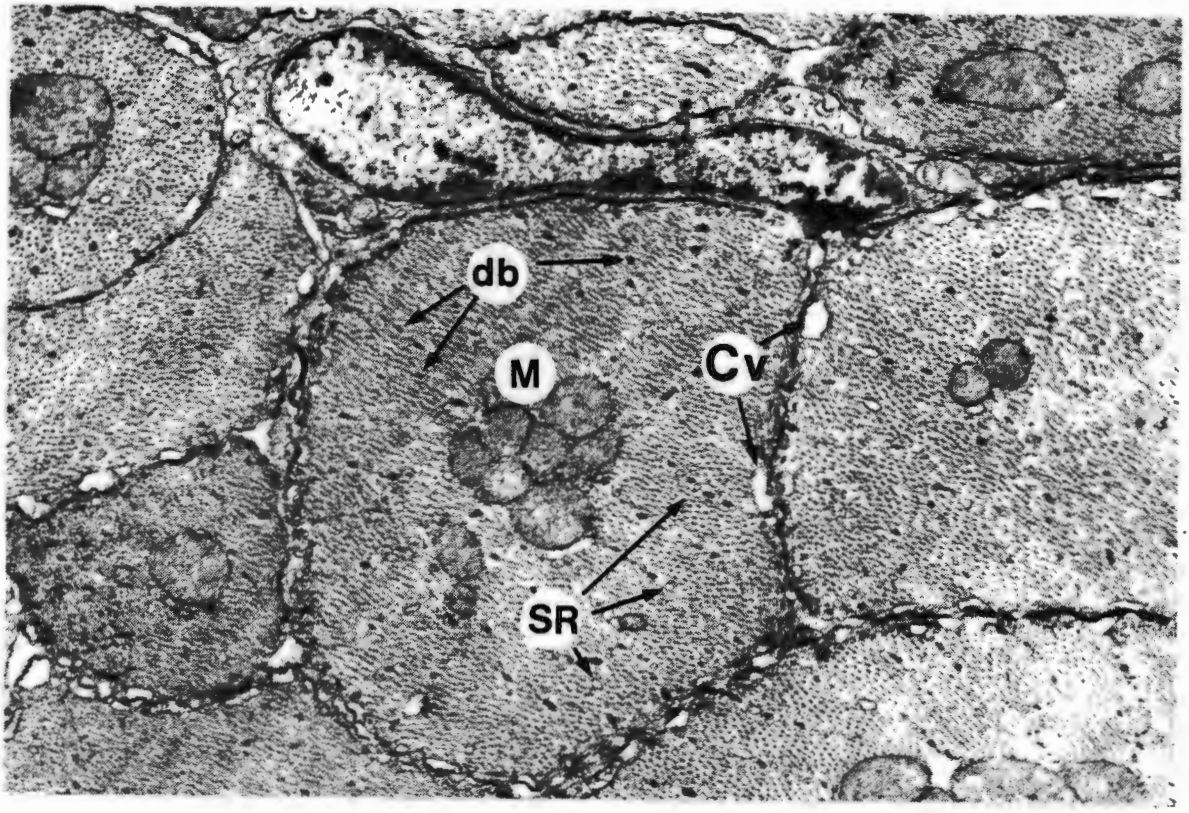


Figure 8.5. Cross section through Type B muscle fibre. Note the tightly packed mitochondrial core (M), the presence of caveolae (Cv) mainly on the periphery of the cell, the lessened SR ramification and the arrangement of the dense bodies (db). The cells are in close contact and lack a collagen boundary. Magnification: x 14400.

where collagen forms a capsule surrounding each cell (Figs. 8.1. and 8.2.).

Diameter of sarcoplasmic reticulum in Type A is larger than Type B (Figs. 8.4. and 8.5.); in both fibre types, connecting bridges from the plasma membrane to the subsarcolemmal system are represented by electron dense areas between these membranes (Figs. 8.1., 8.2. and 8.6.).

Mitochondria

An intermediate classification between M_2 and M_3 characterizes both Type A and B mitochondria. However, fundamental differences are exhibited between the two, despite the fact that in both types the organelle is confined to the inner core of the cell. In Type A the numerous, oval mitochondria (0.30 to 1.00 μm in diameter) reveal well developed cristae (perpendicular to the long axis of the cell and mitochondria) (Fig. 8.2.). These organelles are surrounded by a myomatrix of thin filaments composed of actin and α-actinin, there being a complete absence of the thick myofilaments (Fig. 8.4.), in addition to which their distribution is not uniform throughout the core of the cell, but rather confined to spaces within the myofibrils, together with other cellular components (Fig. 8.2.).

The total mitochondrial volume occupied within this fibre type amounts to at least 25% of the total cellular compartment. This differs from that of Type B, where between 30

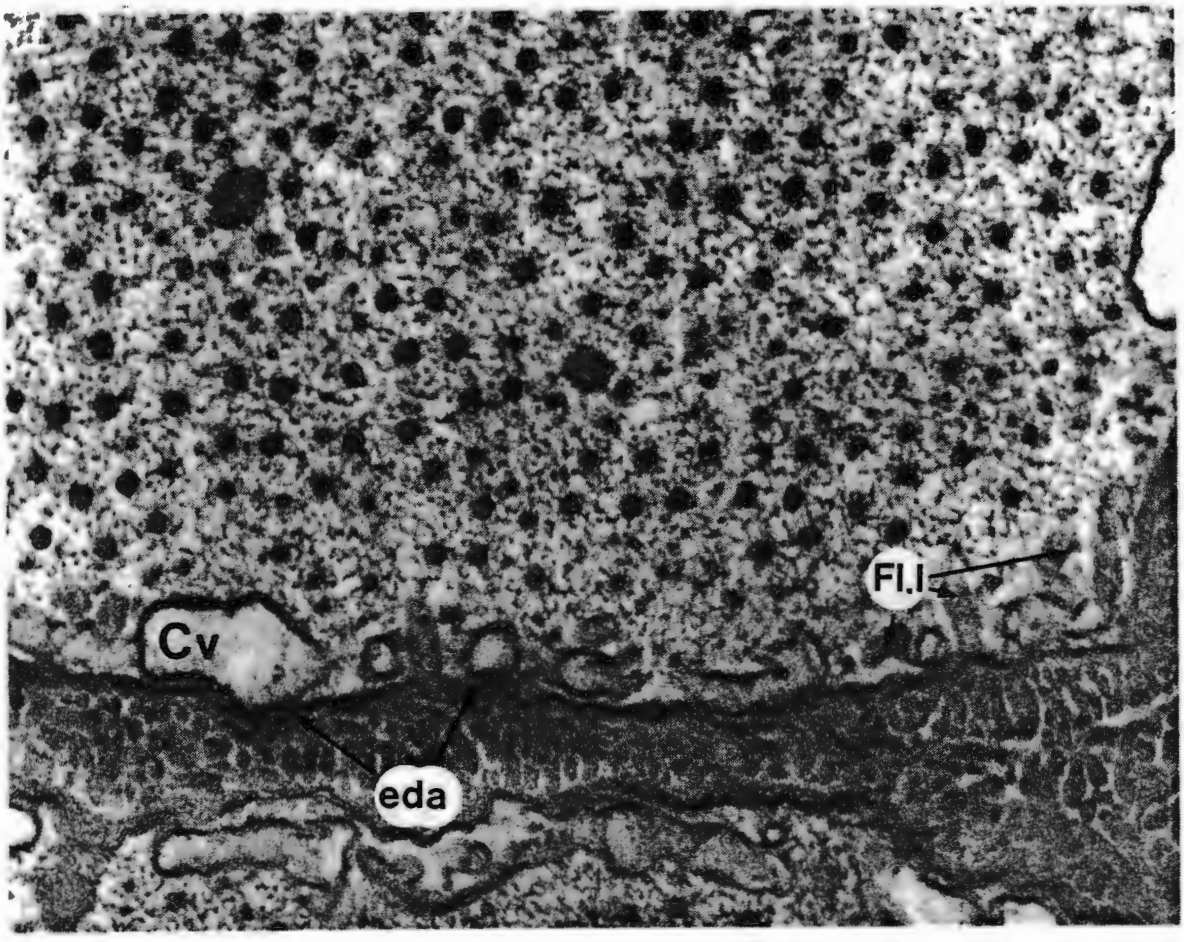


Figure 8.6. Detail of the cellular membrane of a Type B muscle fibre. This illustrates the myofibril arrangement, in addition to showing the presence of peripheral caveolae (Cv) and finger-like plasmalemmal invaginations (Fl.I). Electron dense areas (eda) are present indicating connecting bridges from the plasma membrane to the subsarcolemmal system. Magnification: x 116000.

and 35% of the cytoplasmic volume is occupied by the organelle.

In Type B, the mitochondria are closely packed and evenly distributed throughout the cellular core (Fig.8.3.). As in Type A, but to a lesser extent, a reticular system is associated with these organelles. The mitochondrial cristae are more numerous than those found in Type A, but show no fixed plan of orientation (Fig. 8.3.).

DISCUSSION

It is generally accepted that all molluscan muscles are mononucleate (Hoyle 1957). The fibres may be categorised as follows: cross-striated (large adductor muscle of scallops); oblique-striated (translucent adductor muscle of oyster); helical smooth (cephalopod mantle and siphon retractor); paramyosin smooth (lamellibranch catch muscle) and classic smooth (gastropod and pharynx retractor) (Chantler 1983).

Smooth muscle is characterized morphologically by the absence of a banding pattern, when viewed under the light or low power electron microscope (Chantler 1983). Banding on the other hand is the most distinct feature of striated muscle. Though the degree of order present in molluscan fibres is poor when compared to vertebrate skeletal muscle, a certain amount of organisation is seen in cross-striated fibres even in some smooth muscle cells (Lowy & Vibert 1967; Millman & Bennett 1967; Sobieszek 1973). The

characteristics of the thick filament are variable and unlike the equivalent in vertebrates (Hoyle 1957) and in addition to myosin, molluscan muscle (almost without exception) contains paramyosin (Chantler 1983).

The foot muscle of B.rhodostoma displays intermediate characteristics between a few established molluscan muscle types. Type A cannot however be placed in any of the categories already listed, yet it has features of several of these muscle types. Although no Z-lines are present, the organization of the dense bodies perpendicular to the long axis of the muscle fibre is typical neither of cross-striated nor of oblique-striated muscle fibres and one must refer to it as intermediate between the two.

By contrast, Type B shows characteristics of paramyosin smooth muscle, as described by Chantler (1983). Muscles that normally satisfy these requirements are known morphologically as "catch" muscles. In the typical paramyosin muscle, however, distribution of the dense bodies is random (Chantler 1983) whereas in the muscle of the metapodium of B.rhodostoma this is not so; a degree of organisation exists with resulting alignment of these structures from the centre of the cell, tending towards a radial or helical distribution (Fig. 8.5.).

Functional significance

It appears that the different localisations of these muscle fibres within the whelk's foot is not fortuitous. A

certain degree of striation (especially in Type A) is present; this is an indication of the speed of shortening (Nicaise & Amsellem 1983). Although striated muscle (Z_1) is typified by a greater ability to increase the rates of muscle shortening, smooth muscles are capable of generating greater tensions (Ruegg 1971). Type A possesses numerous contractile units in series, yet the length of the sarcomere is short.

Bullia is capable of selective use of the propodium and metapodium (Brown 1982). Despite the fact that both the propodium and metapodium are equally mobile, the former displays more subtle and agile movements e.g. initiation of burrowing and crawling (Trueman & Brown 1976), whereas the latter is used to its full power during surfing, where the foot is held rigid and muscle tension is maximal (Brown 1982).

The presence of extensive ramifications of the sarcolemmal membranes and tubules indicates the likelihood of the existence of a chemical mediator, i.e. calcium. As suggested by Hunt (1981) and by Presscott & Brightman (1976), the caveolae may have an analogous function to the subsarcolemmal system of vertebrate muscle. These cisternae may be associated with the source of calcium, while the sarcoplasmic reticulum ensures relaxation by calcium uptake (Nicaise & Amsellem 1983). The differential distribution of sarcoplasmic reticulum in both types of fibre indicates that Type A may relax faster, while Type B exerts greater tensions, providing the contractile apparatus uses the same principle.

Abundance of mitochondria is normally associated with endurance (Hoyle & McNeill 1968, Josephson 1975, Plesch 1977). In B.rhodostoma, where locomotory demands are energetically expensive, both types of muscle fibre (A and B) are typified by large numbers of mitochondria. This suggests that although metabolic anaerobiosis may play a significant role in the supply of energy, aerobic respiration is also of great importance; consequently, oxygen concentration cannot be a limiting factor. Supporting evidence has been gathered from determinations of oxygen diffusion rates through the pedal wall, these being more than adequate for the whelk's needs (Brown 1984). In general, large numbers of mitochondria are a characteristic of red muscle (Andrew & Hickman 1974), thus the muscle fibres of Bullia, although morphologically resembling smooth muscle, are atypical with respect to mitochondrial content; in essence the percentage of mitochondria in the whelk's muscles is more characteristic of red muscle. Information on ATPase activity, calcium accumulating properties, twitch speed and susceptibility to fatigue, of both Type A and B, is not available; it would contribute to a better understanding of the features pertaining to these muscle fibres.

Considering that ATP is essential for muscular relaxation (Katz 1977) and that the mitochondrion is the power house of the cell, as well being responsible for some source of calcium (Lehninger 1975), the abundance of the organelles in areas free of thick filaments may be of special significance. While the mitochondrion preferentially uses its endogenous ATP

(Jacobus & Lehninger 1973, Saks et al. 1980), the nucleotide is never limiting in the surrounding medium (Jacobus et al. 1982). It is the extramitochondrial concentration that bears directly on the control of the respiratory pathway and ATP synthesis (Jacobus et al. 1982). In consequence, myosin free areas within the myomatrix (surrounding the mitochondria) may enhance the access of ADP to the organelle. It follows that increasing return rate of ADP leads to more efficient rates of ATP synthesis; this results in higher turnover rates of the calcium induced contractile cycle.

The role of paramyosin in this system is unknown, as is its site within the muscle cell. In Limulus striated muscle, paramyosin has been associated with the A band of the sarcomere (Levine et al. 1972) of the catch muscle system (Bear & Selby 1956, Kominz et al. 1957, Elliott & Lowy 1961, Lowy & Millman 1963, Elliott 1964). Despite the fact that paramyosin has been found in virtually all molluscan muscle, not all show slow (tonic) relaxation or catch properties (Lowy et al. 1964). Likewise, these characteristics have not been observed in Bullia. This apparent paradox may be partly clarified by evidence suggesting that paramyosin preferentially aggregates within the core of the thick filament (Millman 1967). Thus, the larger the thick filament diameter, the greater its proportion of paramyosin. This may be of consequence to molecular packaging: paramyosin is a long, rod-like molecule (Lowey et al. 1963), which is likely to pack more easily into the inner zone of the thick filament, in contrast to myosin, which is not as uniformly

shaped at the quaternary structure level (Huxley 1963) and would be better suited on the outer surface of the thick filament.

In molluscs the thick to thin filament ratio varies from 1:6 in Crassostrea (Hanson & Lowy 1961) to 1:17 in the anterior byssus retractor muscle of Mytilus (Sobieszek 1973). This ratio is related to the ability to form cross-bridges and hence the maximal force a muscle is able to exert (Hoyle & McNeill 1968, Josephson 1975, Plesch 1977). It is possible that with increasing thick filament diameter, a greater number of cross-bridges are formed with the thin filaments; thus greater tension is attained. In addition, Lowy et al. (1964) have suggested that due to the length of the thick filament in molluscan muscle, the load carried at the centre is much greater than the equivalent in vertebrate cross-striated muscle.

Therefore the increased thickness of the thick filament, in some muscle fibres, may have evolved as a compensatory mechanism to support higher loads. B.rhodostoma has a thick to thin filament ratio between 1:12 to 1:30, which may be relevant when one considers that the diameter of the thick filament of Type A and B is not equal either. The difference in diameter is 10 nm (where Type A is smaller). This could result in greater tension in B, although A cannot be far behind with the added advantage of more effective relaxation, due to the myomatrix morphology. Where there are concentric layers of thin filaments, a larger number of cross-bridges can be formed, especially if staggering of the thick filament's heads occurs.

The difference in thick filament diameter may be significant at least on a functional basis. During burrowing, crawling and feeding, versatility of the propodium, whose major fibre type is A, is necessary in order to perform these activities satisfactorily. Conversely, the maintenance of turgidity of the metapodium (Type B fibre), is a prerequisite for the successful management of wave action during surfing. Therefore the properties of fibre Type A (thinner thick filament when compared to Type B) may have been selectively used as a functional and adaptational tool, providing a means to use the same organ for different activities. Consequently, the skillful manipulation of these anatomical features, combined with sophisticated behaviour, may play an integral part in the successful adaptation to the harsh sandy shore habitat.

CHAPTER 9

DISCUSSION AND CONCLUSIONS

Environmental adaptation may affect both homeostasis and enantiostasis. A classic example of such adaptation is temperature compensation in ectotherms, whereby metabolic rates are rendered relatively independent of temperature changes. Although a number of different mechanisms may be involved, the final result is maintenance of a steady state with regard to structure or function or, most commonly, both. Some adaptations provide organisms with marked and novel potential for change; these are the determinants of evolutionary development.

Both qualitative and quantitative enzyme changes are necessary to compensate for alterations in habitat conditions. Since the body temperature of ectotherms is usually in equilibrium with that of the surrounding environment, a decrease in environmental temperature may induce a reduction of enzyme activity, diminishing carbon flow through the metabolic pathways. This would result in alteration of the regulatory properties of enzymes. If environmental stresses interfere with enzyme function, then the cell has to devise alternative strategies to cope with the new situation. Changes occur in response to metabolic rate decreases both in flux and pathway choice. Changes in the peripheral environment such as temperature and pressure may interfere with enzyme function. Changes in the internal environment with reference to intracellular composition

and extracellular fluid may induce alterations in enzyme type and quantity (Hochachka & Somero 1984).

Metabolic thermal compensations fall into three categories, over different time scales, namely instantaneous compensation, acclimation/acclimatisation and changes over an evolutionary time span:

Instantaneous adaptations

Instantaneous adaptations are those which develop in response to sudden environmental changes. Metabolic compensation in these instances does not involve alterations in gene expression nor restructuring of the cellular make-up. These are the first defensive strategies of an organism (short of physical avoidance), though if these changes are frequent enough and show any sign of permanency, there may be a shift in the genetic structure of the affected population, resulting in more sophisticated responses.

Acclimation / Acclimatisation

This occurs in response to semi-long term or annual seasonal changes, which may be endured by the organism several times during its life span. Such metabolic compensations normally involve induction of new types of protein and restructuring of the immediate milieu of cellular components.

Genetic or evolutionary adaptations

Continual exposure over several generations to an environmental change may favour mutations in regulatory genes. These may lead to alterations in basal level concentrations of enzymes and associated molecules. In addition amino-acid substitutions in proteins are often reflected by the presence of new isozymes with different catalytic properties. The latter may contribute to an unique regulatory capacity permitting better responses to environmental stimuli and thus increasing survival rate.

The whelk Bullia digitalis provides an oustanding example of metabolic temperature-independence over the thermal range encountered by the organism in the field. It is unique among those ectotherms so far studied in displaying a virtually flat rate-temperature curve not only when inactive but at all levels of activity (Brown & da Silva 1979, 1983). The present work was motivated by a desire to unveil how the animal manages to assert this control at a biochemical level. That this question has not been fully answered is a direct reflection of the complexity of the biochemical systems and the diverse properties of enzymes responsible for catalysis. Indeed, although the details of temperature-independence have not been elucidated, the temperature relationships of those enzymes studied in the present work lead to the important conclusion that the metabolic temperature independence of the intact animal is by no means due to a single mechanism of control but is due to the summation of diverse properties of the enzyme systems concerned.

In the course of these investigations, relevant conclusions included the following:

1. The genetic population structure of the four Bullia species studied was shown to be conserved, characterised by low heterozygosity and absence of variations amongst spacially separated populations. In addition, electrophoretic data suggest that the proccess of speciation, from a commom ancestor, was initiated around 30 million years ago and that the genetic distance separating the species examined is more typical of genera than of species per se.

2. The molecular characteristics of foot and mantle D(-)LDH of Bullia digitalis are unique amonsgt the Mollusca so far studied. This enzyme is represented by five isozymes in foot muscle, in analogy to the five LDH isozymes of vertebrates. These show differential tissue expression and the two foot isozymes investigated exhibit distinct temperature sensitivities.

3. The kinetic properties of D(-)LDH are also unique. Of the five isozymes present in the foot, two exhibit distinct and selective thermal responses at 10 and 25°C. Though still operative throughout the thermal range tested, at these temperatures there is significant activity enhancement of one isozyme or the other. Nevertheless,

irrespective of temperature, "basal LDH activity" is present, so that this metabolic point is operative at all times.

4. Unlike LDH, D(+)ODH is expressed only in one form in the foot, mantle, brain and gonad, though an alternative form is present in digestive gland. Temperature optima of the forward and reverse reaction are distinct, ensuring that maximal activity of ODH and LDH are desynchronised. The effect of temperature on substrate inhibition in the octopine forming reaction is dependent on which substrate is available at higher concentrations.

5. The metabolism of Bullia digitalis is carbohydrate dependent, with RQ = 0.96, which also indicates preferential carbohydrate catabolism. This was further confirmed by the determination of glycolytic flux (anaerobic metabolism), whose carbon flow greatly exceeded that of aerobic metabolism. Information is however not available on the remaining pathways at the end of glycolysis, except that NMR studies of foot cytosol metabolism, in the presence of glucose, revealed changes in adenylate nucleotides as well as the accumulation of an imino-acid (presumably octopine). This is of special relevance, as it indicates that restoration of phosphagen levels may occur via the turnover of octopine, which contributes to the regeneration of arginine phosphate, the usual phosphagen store of

molluscs, as demonstrated by de Zwaan (1983) and Hochachka & Somero (1984). This is supporting evidence in favour of selective use of LDH and ODH.

6. Finally it appears that in the course of functional evolution, Bullia has evolved a specific type of musculature which ensures dexterity, maintenance of turgor for extensive periods of time and reduction of muscular fatigue. This is reflected by morphological differentiation of the foot, together with different spacial location of specific types of muscle fibres.

It is clear that the present work represents only a beginning to the investigation of the complex metabolic biochemistry of Bullia digitalis and that the next step should be a complete study of glycolytic and Krebs cycle enzymes. NMR studies of intact metabolism should also be considered as a valuable tool in comparing in vivo and in vitro results. Without such studies a number of assumptions have to be made, assumptions rendered still more precarious by lack of similar work on any other prosobranch gastropods. Nevertheless, it is apparent from the work so far undertaken that Bullia digitalis has achieved an intricate balance between maintenance of homeostasis and enantiostasis. It follows that the whelk is graced with the ability to deal with particularly unpredictable environments, especially with regard to temperature.

The most closely-related work is that on bivalve and cephalopd molluscs; the former with special relevance to the significance of anaerobiosis, during exposure to air at low tide; the latter due to the physiological consequences of bursts of activity. Many studies have been reported involving the above groups, as well as other marine ectotherms, with respect to temperature adaptations, though these tend to be adaptations to warm or cold environments. Bullia digitalis is different in that its over-riding temperature adaptation relates to a constantly-changing thermal regime, to unpredictable and extreme temperature fluctuations which the animals cannot escape but must endure. The adaptation thus represents mastery, over the course of evolution, of a highly unstable, erratic and stressful habitat, a domain over which the animals have no control but have, nevertheless, conquered in a most elegant fashion.

CLOSING NOTE:

This poem was written in 1956 by DR Rómulo de Carvalho, physicist, philosopher and poet, resident in Lisbon, Portugal. The poem was translated by the candidate.

THE PHILOSOPHISING STONE

They don't know that the dream
is a constant of life
so concrete and defined
like anything else,
like this grey stone
on which I sit and rest,
like this serene river
in serene turbulance,
like this tall pine forest
which shimmer green and gold,
like these crying birds
in a drunkenness of blue.

They don't know that the dream
is wine, is foam, is yeast,
vivacious and sedentary creature
of pointed face,
that searches through everything
in perpetual movement.

They don't know that the dream
is canvas, is colour, is a brush,
base, pillar, archway,
flying butress, stained glass window,
pinacle of a cathedral,

treble clef, symphony,
Greek mask, magic,
that is an alchemist's reflux vessel,
map of a distant world
Compass, Prince,
Renaiscance caravell,
that is of the Cape of Good Hope,
gold, cinnamon, ivory,
shining armour,
soldier, dancing step,
Colombine and Harlequin,
flying machine,
lightning conductor, locomotive,
decorated boat,
high furnace, generator,
cision of atom, radar,
ultrasound, television,
launching of a spaceship
on the surface of the moon.

They don't know that the dream
the dream commands life,
and when a man dreams
the world spins faster
as does a colourful ball
between children hands.

António Gedeão (pseudonim) 1956.

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THE EFFECTS OF TEMPERATURE ON OXYGEN CONSUMPTION IN *BULLIA DIGITALIS* MEUSCHEN (GASTROPODA, NASSARIDAE)

A. C. BROWN and F. M. DA SILVA
Department of Zoology, University of Cape Town, South Africa

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Abstract—1. The oxygen consumption of *Bullia digitalis* from South Africa's west coast, measured at a fixed activity level at 15°C, does not differ significantly between winter and summer.

2. The adult acute rate–temperature curve is flattened over the temperature range likely to be encountered in the field, there being no significant difference in oxygen consumption between 15 and 22.5°C.

3. Below this plateau the Q_{10} is normal, giving a value of 2.67 between 5 and 10°C, but at temperatures above 22.5°C the Q_{10} is less than 2 and oxygen consumption at 30°C does not approach that of the tropical *Bullia melanoides* at the same temperature.

4. Both field and laboratory acclimated animals provide evidence that the rate–temperature curve is unaffected by such acclimation, either to high or low temperatures.

INTRODUCTION

Brown *et al.* (1978) have recently reported the rate of oxygen consumption in the sandy-beach whelks *Bullia digitalis* from South Africa and *Bullia* (*Dorsanum*) *melanoides* from India, measured at temperatures which were consistent with those experienced by the animals in the field: 30°C in the case of the tropical Indian species and 15°C for the temperate *Bullia digitalis*. While the regression coefficients defining the relationships between size and oxygen uptake did not differ significantly between the two species, rate of uptake in *B. melanoides* was about an order of magnitude higher than that of *B. digitalis* of the same tissue weight. The authors considered this phenomenon to be an example of non-acclimation and attempted to explain the difference in metabolic rate in terms of differences in activity, life-span and reproductive frequency.

It is clearly necessary to test such hypotheses and to acquire additional data which may throw further light on the topic. To this end we have measured oxygen consumption in *B. digitalis* at a range of temperatures and have attempted to acclimate them to temperatures both higher and lower than those they normally experience on the west coast. This paper reports the results of the investigation.

MATERIALS AND METHODS

Individuals of *B. digitalis* were collected during November and December 1977, from the beach at Ou Skip, just north of Table Bay. The animals thus came from the same site as those used to gain the data presented by Brown *et al.* (1978). Only medium-sized to large adult females which had not recently taken a large meal were retained. They were returned to the laboratory and held at 15°C, the experiments being performed as soon as possible after collection. Oxygen uptake in the first batch collected was measured at 15 and 22.5°C. The lower temperature was employed because Brown *et al.* (1978) measured consumption at this temperature in mid-winter, whereas the material described here was collected in summer. The oxygen uptake of each animal was measured several times at both temperatures, the sequence being varied so that sometimes it was measured first at the lower temperature, sometimes at the higher. The data was thus ideal for the employment of statistical analysis based on the *t*-test for matched pairs.

Following the results of these tests, reported below, fresh animals were obtained from the same site and their oxygen uptake measured at 5 and 10°C, while for a later batch the temperatures were 25 and 30°C. The method used was identical to that employed for the first batch of animals, except that each individual was placed at the experimental temperature for half an hour before measurements were begun. This modification arose from the finding that animals placed at 30°C showed an immediate oxygen consumption higher than that measured subsequently, presumably due to overshoot, whereas repeated measurements made after about 20 min were consistent.

Oxygen consumption was measured using a YSI polarographic oxygen electrode and analyser, feeding into a pen recorder. Each animal was suspended in oxygen-saturated sea water by means of a thread attached to the apex of its shell with dental model cement ("sticky wax"). A magnetic stirrer set at 550 rev/min ensured a constant flow of water passed the electrode and also caused the animal to expand its foot and wave it from side to side in response to the current (Brown, 1961). The measurements thus took place at an activity level which was easily and consistently reproduced, leading to a routine rate of oxygen consumption (Brown, 1979). The apparatus was physically sealed, all air being excluded from the chamber. Rate of oxygen uptake was calculated from the rate of depletion of oxygen in the water, allowance being made for the different saturation concentrations at different temperatures. In no case was this depletion allowed to exceed 25%. Blanks were run at all temperatures to determine the rate of depletion of oxygen due to the electrode and other possible factors, in the absence of an experimental animal. After completion of the measurements, each animal was removed from its shell and the dry tissue weight (including the radula and the tiny operculum) determined after drying to constant weight at 65°C.

Two attempts were made to acclimate animals to 22.5°C by keeping them for 2 weeks in the laboratory at that temperature. Hochachka & Somero (1973) suggest that the induction of isozymes during thermal acclimation may

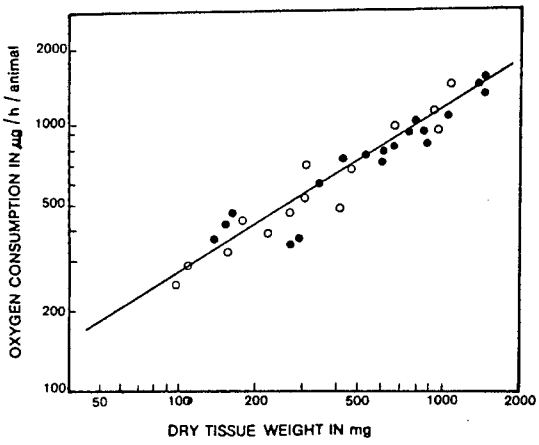

Fig. 1. Oxygen consumption of *Bullia digitalis* from Ou Skip at 15°C. ●, measurements made in mid-winter; ○, mid-summer.

take at least 1–2 weeks. At the first attempt, none of the animals survived, despite the fact that they can be kept for months at 15°C or lower (Brown, 1961). In view of the finding that nutritional state is important to metabolic rate and to successful acclimation (Newell & Bayne, 1973), another batch of animals was fed on the tunicate *Pyura stolonifera* immediately after collection and again on the seventh day of the attempted acclimation. Most of these animals survived for 2 weeks but were in poor condition and gave inconsistent results, which are thus not reported here. Instead we resorted to the possibility of natural acclimatization in the field, provided by the occurrence of *B. digitalis* in False Bay (Brown, 1971). While the waters of the west coast (including Ou Skip) vary from 8 to 14°C in summer, increasing to 16 or 17°C in winter (Andrews, 1974), False Bay ranges from 15°C or less in winter to 22 or 23°C in summer (Atkins, 1970; Brown & Jarman, 1978). The system thus provides a natural acclimation experiment. We collected animals from Muizenberg Beach, False Bay, in February 1978, when field temperatures had reached their peak, and measured their oxygen consumption at 5 and 30°C.

Meanwhile, in the laboratory, we attempted to acclimate *B. digitalis* from Ou Skip to a temperature of 7.5°C after feeding them. These individuals remained in good condition and after 15 days their oxygen uptake was measured first at 5°C and then at 30°C, after allowing the sea water to warm up to that temperature over a period of about 1 hr. In some cases measurements were also made at 15°C when the water reached this temperature. The animals were fed and returned to 7.5°C for a further seven days, after which the measurements were repeated.

RESULTS AND DISCUSSION

Analysis of covariance applied to the results gained from fresh, non-acclimated animals from Ou Skip at 15°C showed no significant difference from those reported by Brown *et al.* (1978) (see Fig. 1). The regression fitted to the combined data gives the following relationship:

$$\log R = 0.598 \log W + 1.3380,$$

where R is the oxygen consumption in µg/hr per animal and W is the dry tissue weight of the animal in mg.

Figure 2 shows the acute rate–temperature curve for unfed, adult females from Ou Skip. As the mean dry tissue weight of the animals was in the region of 750 mg, all values for oxygen consumption were transformed to that of a standard sized animal of 750 mg, following the formula of Newell *et al.* (1977):

$$V_{O_2(750\,mg)} = \left(\frac{750}{W}\right)^{0.598} \cdot V_{O_2(exp)},$$

where $V_{O_2(exp)}$ is the oxygen consumption of the individual per hour, W its dry tissue weight in mg and 0.598 is the regression coefficient of oxygen consumption against weight (see above). In Fig. 2 the means of these transformed values are shown against temperature, together with their standard deviations.

The powerful t-test for matched pairs, applied to the transformed results gained at 15 and 22.5°C, showed no significant difference in oxygen uptake at these two temperatures ($P > 20\%$). However, the same test applied to measurements at 5 and 15°C

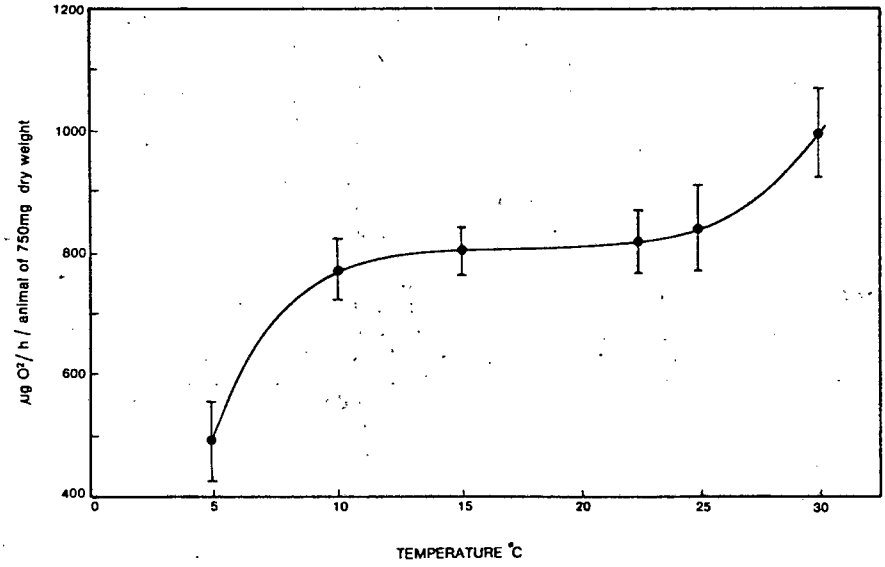

Fig. 2. Acute rate–temperature curve for *Bullia digitalis* from Ou Skip, all values being transformed to a standard-sized animal of 750 mg dry tissue weight. The vertical bars represent 1 S.D. on either side of the mean values.

showed a highly significant difference ($P < 1\%$) and a significant difference was also shown for the tests at 25 and 30°C ($P < 5\%$). The weaker *t*-test for unmatched pairs (again applied to "standard animals") failed to demonstrate significant differences between 10 and 15°C, or between 22.5 and 25°C, this being possibly due to the small number of animals used.

The standard deviations obtained at extreme temperatures are higher than those at more moderate temperatures. Nevertheless, the range of values obtained at the various individual temperatures is less than is commonly encountered in marine invertebrate respirometry (Newell & Northcroft, 1967; Newell & Pye, 1970; Griffiths, 1977) and this may be attributed largely to the successful maintenance of a fixed activity level during the measurements, so that the values all refer to a well-defined and reproducible routine rate. This rate does not approach the maximum of which the animal is capable (active rate) but is much higher than any assessment of standard rate (Brown, 1979).

The most striking feature of the acute rate–temperature curve shown in Fig. 2 is its departure from the logarithmic relationship often associated with ectothermic animals. Instead there is a flattening of the curve over the entire range of temperatures likely to be encountered by the animals in the field, not only at the site of collection but throughout the known distributional range of the species (see Brown, 1971). Similar acute metabolic adaptation has been demonstrated in certain other marine invertebrates, though it is often most marked in inactive animals (Newell, 1966, 1970; Newell & Northcroft, 1967; Newell & Pye, 1971; Griffiths, 1977). In the present example it is displayed by a marine whelk at a controlled level of activity well above the quiescent state, as has also been found in some other molluscs, though not to such a marked degree (Newell & Bayne, 1973).

The advantage of this rate–temperature relationship to an animal such as *Bullia* may be considerable. While it is not subjected to the temperature fluctuations which some rocky-shore intertidal gastropods must endure, it does come up on to the foreshore of the beach in search of food, exposing much of its body to the air. Perhaps more important is the fact that it must face rapid and considerable changes in water temperature (Brown & Jarman, 1978). As the animal must be capable of surviving long periods without food (Brown, 1964), conservation of energy is of the utmost importance, an aspect of this conservation demonstrated here being the ability to undergo an acute rise in temperature without a significant rise in metabolic rate.

Even outside this range the Q_{10} as measured between 5 and 10°C, and between 25 and 30°C is low. Between the former temperatures it is 2.67 but between the latter it is only 1.39. Thus oxygen consumption at 30°C in *Bullia digitalis* does not approach the values measured for the tropical *B. melanoides* at the same temperature (Brown *et al.*, 1978).

Measurements of oxygen uptake of eight animals kept for 15 and 22 days at 7.5°C did not differ significantly from the acute values shown in Fig. 2 ($P > 30\%$ for the 5°C group and $P > 30\%$ for the 30°C group). The few readings taken at 15°C all lay within two standard deviations of the mean of the acute group and were scattered on both sides of that mean. The values gained from animals collected in False Bay in mid-summer were somewhat ambiguous in that, while at 30°C there was no significant difference in oxygen uptake between these animals and those collected from Ou Skip ($P > 20\%$), at 5°C values for 10 False Bay animals were significantly lower than those for Ou Skip ($P < 5\%$), the mean being 372 μg O_2/hr per animal of 750 mg (S.D. = 60). However, we are dealing here with two populations which may well be genetically distinct. The False Bay animals probably never have to contend with the lowest temperatures experienced by the Ou Skip population and it might not be surprising if they no longer showed metabolic adaptation to such temperatures, quite apart from any ability to acclimate.

The weight of evidence thus indicates that, while *Bullia* shows marked metabolic adaptation to acute changes in temperature, it does not show acclimatory adaptation. Indeed, for an animal which can maintain its metabolic rate virtually constant over the full range of temperatures it is ever likely to encounter, the ability to acclimate would hardly be an advantage.

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ACUTE METABOLIC RATE: TEMPERATURE RELATIONSHIPS OF INTACT AND HOMOGENIZED *BULLIA DIGITALIS* (GASTROPODA, NASSARIIDAE)

By

A. C. Brown and F. M. da Silva

Department of Zoology, University of Cape Town

[*Read 13 April 1983*]

SUMMARY

The results of experiments to determine the temperature relationships of the active rate of oxygen uptake in the whelk *Bullia digitalis* are reported. These are compared with previously published data on the temperature relationships of 'inactive' and controlled activity routine rates. At all levels of activity the rate of oxygen consumption of intact animals is markedly temperature independent over the range 10 to 20 °C. This contrasts with results gained on the oxygen uptake of whole body tissue homogenates, the latter being temperature dependent. Possible explanations for this difference are discussed and proposals made for further research on the control of metabolism in this species.

INTRODUCTION

The rate of oxygen consumption of the sandy-beach whelk *Bullia digitalis* (Dillwyn) from the west and south coasts of South Africa has been the subject of several investigations in recent years and has been measured under a variety of controlled conditions (Brown 1979a, b, c, Brown & da Silva 1979, Dye & McGwynne 1980, Brown & Meredith 1981). The resulting data have been used to construct a time–energy budget for adult animals (Brown 1982a) and to calculate the cost of free existence (Brown 1981).

One of the striking features of this work has been the demonstration of a virtually flat rate:temperature curve within the range of temperatures normally encountered by animals in the field. This temperature independence has been shown not only in the case of relatively immobile or 'inactive' individuals (Dye & McGwynne 1980) but also for animals maintained at a constant, higher, routine level of activity (Brown & da Silva 1979). The marked depression of metabolic responses to rising temperature may clearly be advantageous to an animal which must conserve energy in an environment imposing rapid and considerable fluctuations in temperature while offering a highly erratic food supply (Brown 1982b). It may be noted, however, that the extremely flat rate:temperature curve is not a feature of all species of *Bullia* and that large *B. digitalis* exhibit greater control than small individuals (Dye & McGwynne 1980).

In the work presently reported, it was attempted to answer two questions. First, is the metabolic temperature independence shown by inactive and routinely active adult *B. digitalis* maintained at levels which approach the active rate, or is some loss of control apparent? Secondly, what are the acute rate:temperature characteristics of

cell-free tissue homogenates? Data on prosobranch homogenates exist only for *Littorina* (Newell & Pye 1971a), and these show similar temperature independence to intact, inactive animals, in contrast to active individuals.

MATERIALS AND METHODS

Medium to large females of *B. digitalis* were collected from the beach at Ou Skip, on South Africa's west coast, and returned to the laboratory, where they were maintained at a temperature of 13 °C in sea water and sand from their natural habitat.

In experiments to determine the active rate:temperature curve of intact animals, individuals with a shell length of about 50 mm were suspended individually from the apex of the shell in a sealed container of oxygen-saturated sea water over a magnetic stirrer, and oxygen depletion measured by means of an oxygen probe, as described by Brown *et al.* (1978) and Brown & da Silva (1979). However, whereas these authors deliberately employed a constant rate of stirring (550 r.p.m. setting), giving a controlled and constant level of activity, in the presently reported series of tests the rate of stirring was continuously changed by rotating the knob on the stirrer from the off position to maximum revolutions (800 r.p.m.) and back again. This was known to result in a rate of oxygen consumption much higher than if the current was constant, a rate comparable to that measured during burrowing and thought to be similar to the rate occurring in the field during surfing (Brown 1979a, b, 1981). This probably approaches the active rate for the animal. The cycle of stirring was adjusted so as to reach a peak approximately every seven seconds, this being the average wave period on the shore. The experiments were conducted at 5 °C intervals from 5 to 35 °C. At the lower temperatures some animals were used more than once, after being allowed a period of recovery, but above 20 °C each individual was used once only. Depletion of oxygen was not allowed to exceed 25 % of the saturation value and no animal was subjected to the experiment for longer than 30 min.

Following the tests, each animal was extracted from its shell and dried to constant mass at 70 °C. The results for oxygen uptake were then transformed to those of a standard animal of 750 mg dry tissue mass, using the formula developed by Brown & da Silva (1979) for the same population of the species.

For the experiments on the oxygen uptake of tissue homogenates, animals with a shell length of between 35 and 40 mm were chosen; shell length was measured accurately so that dry tissue mass could be calculated from figures available for the population (Brown, unpubl.). The whelks were held for between 12 and 18 hrs before being removed from their shells and the tissues weighed wet. To 20 gm of tissue, 100 ml of a 20-mM imidazole buffer of pH 7,40 containing 0,25 M sucrose, 5 mM $MgCl_2$, 2 mM EDTA and 0,1 mM DTT, was added. This was kept on ice. The tissue was minced and homogenized in the buffer and the resulting suspension spun at 1 000 × g so as to sediment nuclei and debris. The supernatent was stored on ice for use in a Gilson Respirometer.

Oxygen consumption of the cell-free homogenate was measured at 5 °C intervals from 5 to 30 °C, over a period of 30 min at each temperature. Each vial contained

0,5 ml 15 % KOH in the side arm and 2,0 ml of buffered homogenate in the main compartment. Equilibration was allowed for 10 min at the test temperature, after which readings of oxygen uptake were made at 5-min intervals. The protein concentration in each sample was subsequently determined by the method of Lowry *et al.* (1951).

RESULTS AND DISCUSSION

Intact animals tested under the conditions described above proved to have a rate of oxygen uptake which was markedly temperature independent between 10 and 20 °C (Fig. 1C). The degree of control was, however, not quite as great as was found for less active animals by Brown & da Silva (1979). Below 10 °C the Q_{10} was 2,3, which is similar to that of the routine rate. Between 20 °C and 30 °C some degree of control appeared to be lost, the Q_{10} being 0,26 as compared with 0,12 for the routine rate (Brown & da Silva 1979). The rate continued to rise between 25 and 30 °C, with a Q_{10} of 0,49. Between 30 and 35 °C, however, there was a drop in the rate of oxygen uptake. It is unlikely that maximum consumption actually occurs at exactly 30 °C, the real peak probably occurring on one side or the other of this temperature; this part of the curve must thus be treated with some caution. It is also unfortunate that neither Brown & da Silva (1979) nor Dye & McGwynne (1980) ran tests at these higher temperatures on less active animals, so that comparisons are impossible. It should also be noted that the inactive animals studied by the latter authors were smaller than those used in the present study and that the curve reproduced in Fig. 1A is taken from the largest size-class studied (about 200 mg dry tissue mass).

Many marine invertebrates are now known to have very flat rate:temperature curves while they are inactive, at least over part of the appropriate temperature range (Newell & Branch 1981). However, temperature independence at routine rates appears to be far less common and *B. digitalis* would seem to be the only marine ectotherm so far studied to possess a high degree of temperature independence at all levels of activity.

The results gained with cell-free homogenates contrast with those obtained from intact animals (Fig. 1), the rate of oxygen consumption being temperature dependent throughout the range 5 to 25 °C, with an overall Q_{10} of 2,51. A marked drop in the rate of consumption occurs between 25 and 30 °C. At first sight this drop does not accord well with the curve gained for intact, active animals (Fig. 1C), which rises between 25 and 30 °C, only then falling. This might, of course, be explained on the grounds that the homogenate represents an unnatural system with different characteristics from those of the intact animal. An alternative explanation presents itself, however; it may be that the actual peak rate occurs at a temperature somewhere between 25 and 30 °C both in homogenates and in intact animals. This suggestion could only be tested by repeating the experiments at smaller temperature intervals within this range.

However this may be, it is clear that intact animals enjoy a remarkable control over their metabolic rate with rising temperature, a control not exhibited by cell-free homogenates. The energy saved by the animal may be considerable. Brown (1981) has shown that a 'standard' animal (750 mg dry tissue mass) expends on average some 52

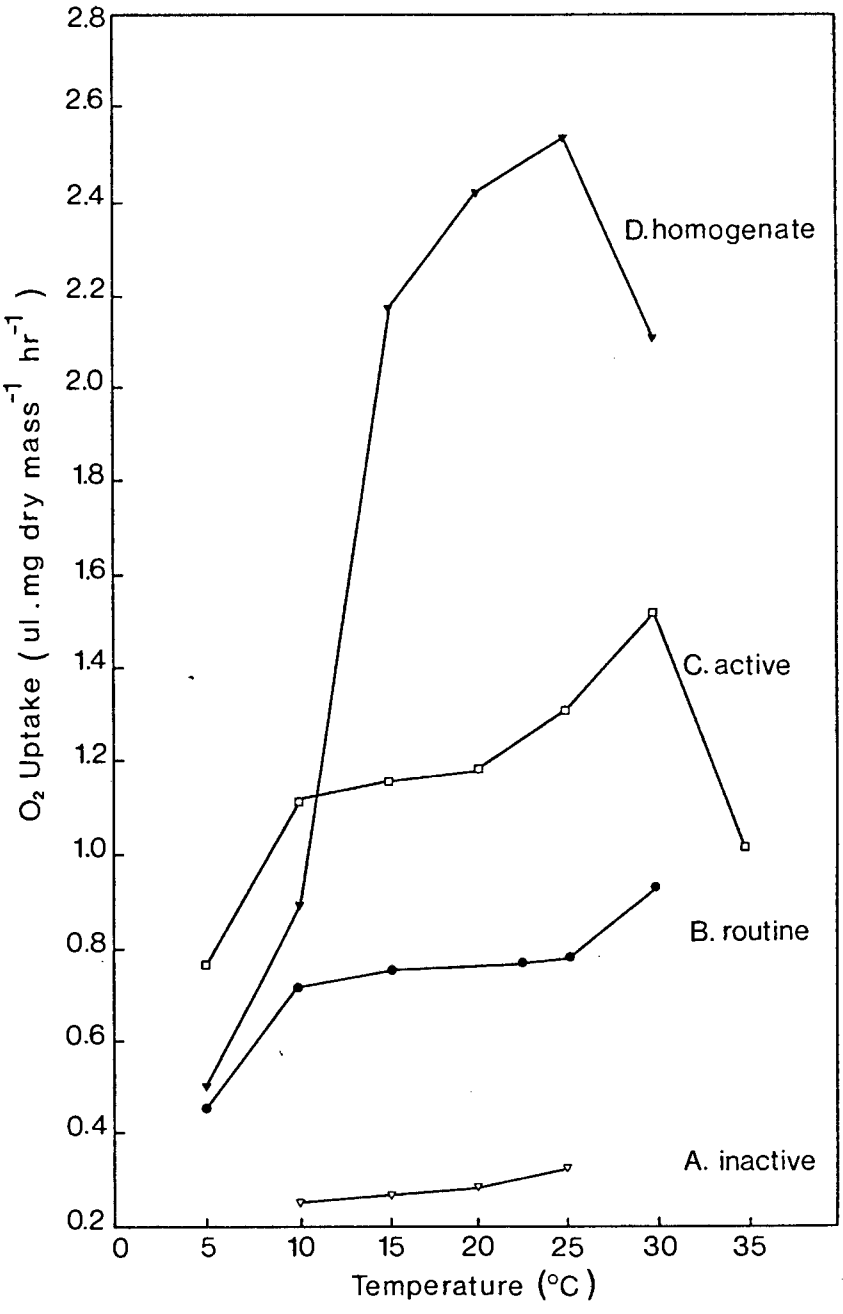

Fig. 1. Acute rate:temperature curves for the whelk *Bullia digitalis*: A: Inactive animals (after Dye & McGwynne 1980); B: Routine rate for animals in a constant current (after Brown & da Silva 1979); C: Active animals in a constantly changing current (this paper); D: Whole tissue homogenates (this paper).

cals in 24 hrs of free existence at 15 °C. This may rise to 53 or 54 cals at 20 °C; but the same mass of homogenate at 20 °C consumes just over 43,5 μl of oxygen in 24 hours, this being equal to 212 cals, or approximately four times as much. This estimate must, in fact, be considered conservative as in the preparation of the homogenate some enzyme denaturation must have occurred even although EDTA and DTT were added, so that the results obtained for oxygen uptake are likely to be too low.

The results gained with *Bullia digitalis* homogenates contrast not only with those obtained from intact animals but also with the data of Newell & Pye (1971a) on homogenates of *Littorina littorea*. The latter workers deliberately simplified the medium in which their homogenates were prepared and suspended, using sea water instead of the more sophisticated medium employed in the present work, on the grounds that it presented the cell-free system with more natural conditions (Newell, pers. comm.), a view to which the present authors do not subscribe. Whether this accounts in any substantial way for the differences in results is not clear at this stage. It certainly seems unlikely that the addition of sucrose as a stabilizer in the present work could have presented the homogenate with a substrate (see Lehninger 1975) and so the data gained here as well as those of Newell & Pye (1971a) on *Littorina* must be regarded as being for homogenates in the absence of additional substrate. It should, however, be mentioned that, prior to our experiments, three students working under the direction of the first author obtained very similar results for *B. digitalis* homogenates in the presence of excess substrate to those reported here; they suspended the homogenates in a simple gastropod ringer and added glucose as a substrate. The degree of temperature dependence they obtained was virtually identical to that shown in Fig. 1D.

A number of mechanisms have been proposed to account for the relative temperature independence of some poikilothermic animals. Atkinson (1966) envisaged competition for common substrates between the citrate synthetase responsible for the catalysis of acetyl CoA into the Krebs cycle, on one hand, and acetyl CoA carboxylase, concerned with fatty acid synthesis, on the other. Another possibility is an increase in the rate of glycogen synthesis with increasing temperature (Atkinson 1966, Hochachka 1968), which would also have the effect of limiting the supply of substrate to the Krebs cycle. In either of these possible systems, the marked temperature dependence of *B. digitalis* homogenates could have arisen by making available to the mitochondria an excess of substrate, not necessarily by its direct addition to the system but by freeing it during the process of homogenization. There is, however, also evidence from certain fish that enzyme–substrate affinities may decrease with increasing temperature (Hochachka & Somero 1968, Somero 1969). In fact Somero (1969) suggests that saturating levels of substrate have little or no effect on reaction rate V_{max} and are therefore of little biological significance.

In order to investigate more fully the nature of temperature effects on oxygen consumption, we are at present considering the respiration of mitochondrial isolates. Some work has already been performed on crude mitochondrial suspensions in *Littorina* (Newell & Pye 1971b) but in view of the very different temperature responses of homogenates of *Bullia* and *Littorina* we suspect that the mitochondria may also behave differently. We also propose to study the system in more detail, investigating not

only oxidative phosphorylation (states I, II, III and IV of respiration), but also ADP/ATP ratios together with P/O ratios in the presence of a variety of substrates. Results from these studies may elucidate possible relationships between Krebs cycle intermediates and other pathways, such as gluconeogenesis, glycogenolysis, protein synthesis and lipid metabolism. In consequence it is hoped to construct a metabolic map for *Bullia digitalis* and related species, in much the same manner as has been presented by de Zwaan (1977) for sessile Bivalvia.

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EFFECTS OF TEMPERATURE ON OXYGEN CONSUMPTION IN TWO CLOSELY-RELATED WHELKS FROM DIFFERENT TEMPERATURE REGIMES

A. C. BROWN and F. M. DA SILVA
Department of Zoology, University of Cape Town, Rondebosch, 7700 South Africa

Abstract: The rate of oxygen consumption of the sandy-beach whelk *Bullia rhodostoma* Reeve from Muizenberg, on the east coast of the Cape Peninsula, South Africa, has been measured at two levels of activity at temperatures between 5 and 40 °C. This rate is temperature-dependent throughout the range, unlike that of *B. digitalis* (Dillwyn) from the west coast, although the relationship tends to be linear rather than logarithmic. The rate–temperature curves at all levels of activity are lower in summer than in winter, indicating the presence of temperature acclimation, again unlike *B. digitalis*. The rate of consumption also increases up to a higher temperature in *B. rhodostoma* than in *B. digitalis*. These marked differences between the two species may be correlated with the different temperature regimes of which the animals are characteristic and, in particular, with the extreme and rapid fluctuations in temperature to which the west coast is subject, as compared with the Peninsula's east coast. Differences in food availability and in the behaviour of the animals may, however, be significant additional factors in determining their distribution.

Key words: gastropods; respiration; thermobiology

INTRODUCTION

Temperatures in the sea around South Africa, and particularly around the coast of the Cape Peninsula, have received much attention and have long been considered to play a major part in determining the distribution of the marine fauna and flora (Gilchrist, 1905; Stephenson, 1944; Ekman, 1953; Day, 1964; Briggs, 1974; Brown & Jarman, 1978). In general, the Peninsula's west coast is influenced by the relatively cold waters of the Benguela Current, while the water on its east (False Bay) coast is derived partly from the Benguela Current but particularly from the warm Agulhas Current and, being shallow and having a long residence time, is heated to a considerable extent by the sun (Day, 1970). Stephenson *et al.* (1937) wrote that "the meeting places of warm and cold water masses... vary in position with season and weather. It is, nevertheless, correct to say that during summer the northern part of the False Bay coast of the Peninsula is chiefly affected by the warmer water and the northern part of its Atlantic (west) coast by the colder; and the difference between the temperature of the sea at specific localities on opposite sides, on the same day, may exceed 8 °C". In the opinion of these pioneer writers, "possibly there is no small area of land in the world where water of such different temperature is separated by so little land".

In fact, the differences in temperature regimes between the west and east coasts of

the Peninsula are more complex than these observations might lead one to believe. This arises largely from the fact that the west coast is subject to considerable upwelling (Andrews, 1974), relatively warm coastal water being blown away from the shore by the southeasterly wind of summer, to be replaced by cold, upwelling water. Thus, west coast temperatures tend to be lower in summer than in winter. As the wind, however, is erratic, so is the upwelling it generates, with the result that the surface temperature varies greatly from day to day and even from hour to hour, in contrast to the relatively gentle rise and fall in temperature experienced along the northern shores of False Bay. Indeed on one occasion a change from 17 to 8 °C was recorded on the west coast over a 7-h period (Branch & Branch, 1981). There can be little doubt that these extreme and rapid fluctuations in temperature have a far greater significance for the flora and fauna of the west coast than do the average temperatures of winter or summer.

Ever since it was recognized that the west and east coasts of the Peninsula display

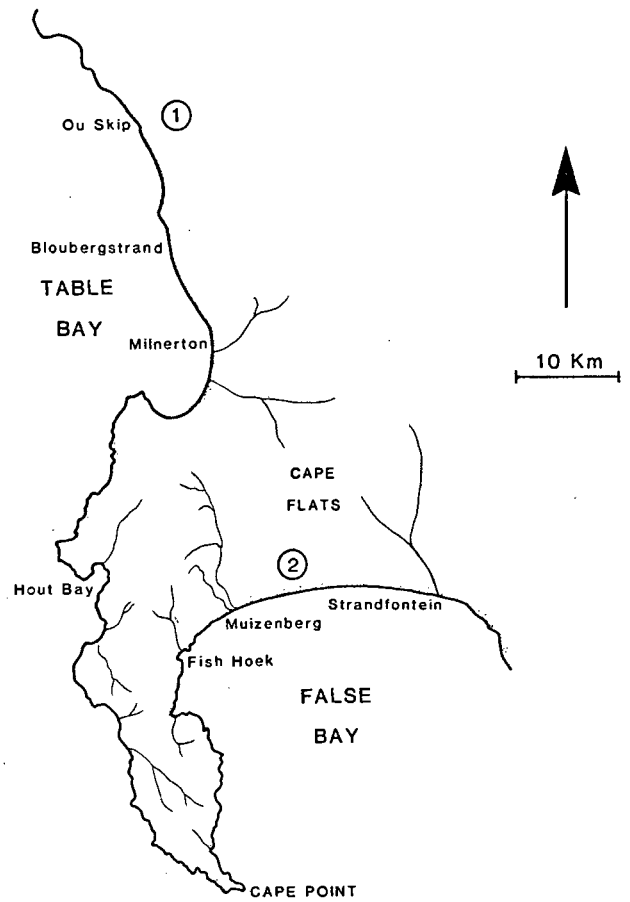

Fig. 1. Map of the Cape Peninsula, South Africa, showing the sites of collection of (1) *Bullia digitalis* and (2) *B. rhodostoma*.

different temperature regimes, it has repeatedly been suggested that the area is ideal, a natural laboratory, for the study of the effects of temperature on littoral organisms. In fact, however, although differences in the distribution of organisms between the two coasts are well known (Stephenson & Stephenson, 1972; Brown & Jarman, 1978), the opportunity to study temperature-dependent physiological differences has been largely ignored.

The whelk *Bullia* is a common and characteristic animal on intertidal sandy beaches around the Cape Peninsula. On the west coast the only intertidal species of consequence on high-energy beaches is *B. digitalis*. It extends round Cape Point into False Bay, where it is joined by two other common species, *B. rhodostoma* and *B. pura* (Brown, 1982a), all three being common along the whole length of the south coast. Thus it appears that, while conditions east of Cape Point are perfectly acceptable to the west coast *B. digitalis*, conditions on the west coast are suboptimal for the essentially south coast *B. rhodostoma* and *B. pura*. The west coast's temperature regime would seem to present the most likely factor limiting distribution and it therefore appeared appropriate to attempt a comparison between *B. digitalis* (Dillwyn) and *B. rhodostoma* Reeve with regard to the effects of temperature on their respiratory rates.

Much attention has, in fact, already been paid to rates of oxygen uptake in *B. digitalis* from the west coast, including the effects of temperature (Brown *et al.*, 1978; Brown, 1979a,b,c, 1981, 1982b; Brown & Da Silva, 1979, 1983; Brown & Meredith, 1981). There are also some observations in this regard on *B. digitalis* from False Bay (Brown & Da Silva, 1979) and from Algoa Bay, on South Africa's south coast (Dye & McGwynne, 1980). Rates of oxygen uptake in *B. rhodostoma*, however, have been studied previously only in relatively inactive animals from Algoa Bay (Dye & McGwynne, 1980).

The present paper reports the effects of temperature variation on rates of oxygen uptake at two levels of activity in *B. rhodostoma* from False Bay, using methods identical to those employed previously for *B. digitalis* by Brown & Da Silva (1979, 1983) and compares rate–temperature relationships in the two species.

Material and Methods

Individuals of *B. rhodostoma* were collected from Muizenberg Beach, on the Peninsula's False Bay coast (see Fig. 1), in July 1980 and 1982, and in January 1982 and 1983. Large, adult females were chosen for study, having a dry tissue weight estimated from shell length > 500 mg and as close as possible to 750 mg, the standard value chosen for the experiments on *B. digitalis* by Brown & Da Silva (1979, 1983). These animals were held unfed at 15 °C in sea water over sand from their natural habitat and were used as soon as possible after collection.

Each animal was suspended individually in a sealed chamber by a cotton thread attached to the apex of its shell by means of dental "sticky wax". The oxygen-saturated,

bubble-free sea water in the container was stirred by a magnetic stirrer whose rate of stirring could be adjusted between 0 and 1000 r.p.m. The water was maintained at a fixed temperature, either by working in a temperature-controlled room or by means of a thermostatically regulated water jacket. Intervals of 5 °C were chosen, ranging from 5 to 40 °C. The animal was kept for between 15 and 30 min at the test temperature before being introduced into the chamber.

Oxygen depletion was measured by means of a YSI oxygen probe wired to an oxygen metre and a pen recorder, so that a continuous and permanent record was obtained from each test run. Oxygen depletion was not allowed to exceed 25% of the saturation value at each temperature, the test being continued for between 30 and 60 min.

In some cases, individual animals were used more than once, after a period of recovery, either at the same temperature or at temperatures 5 °C apart. At temperatures of 30 °C and above, however, most individuals were used only once. Two types of test were conducted. In one series, the magnetic stirrer was set to a constant rate of 550 r.p.m. In *B. digitalis* this procedure was known to result in a constant level of activity, the animal extending its foot fully and waving it gently from side to side (Brown & Da Silva, 1979). This also proved to be true of *B. rhodostoma*. In the second series of experiments, the rate of stirring was continually changed, the dial on the stirrer being rotated from 0 to 1000 r.p.m and back again, with a frequency of ≈ 7 s, the approximate frequency of waves on the shore. In *B. digitalis* this results in a considerably higher rate of oxygen consumption, thought to be similar to that involved in surfing and to approach the active rate for the animal (Brown, 1979a, Brown & Da Silva, 1983).

The whelks were preserved in 5% neutral formalin and were later removed from their shells and the tissues dried to constant weight at 75 °C. Preservation in formalin does not significantly change the dry tissue weight of marine invertebrate animals (J. G. Field, pers. comm.). The results gained from each animal were then transformed to those of a standard whelk of 750 mg dry tissue weight, using the formula developed by Brown & Da Silva (1979) but substituting the value for the regression of oxygen consumption against weight calculated by Dye & McGwynne (1980). This regression may, of course, differ at different levels of activity, at different temperatures or for different populations of *B. rhodostoma*. These and other possible errors in the calculations were minimized, however, by having chosen test animals as close as possible to the standard weight.

Results

The results of the experiments are shown graphically in Fig. 2, together with the curves gained from relatively inactive animals from Algoa Bay by Dye & McGwynne (1980). It should be noted that the latter came from a different population, that their level of activity was not controlled and that they averaged only 500 mg dry wt, rather than the 750 mg employed as a standard in the present work.

Neither the results gained by Dye & McGwynne (1980) nor those reported here

appear to be in any way exceptional when compared with data from other marine invertebrates (see Newell, 1979; Newell & Branch, 1980). Increasing activity leads to increasing oxygen consumption and the rate is also temperature dependent at all levels of activity. Some acclimation occurs between winter (July) and summer (January) and this, too, is apparent at all activity levels.

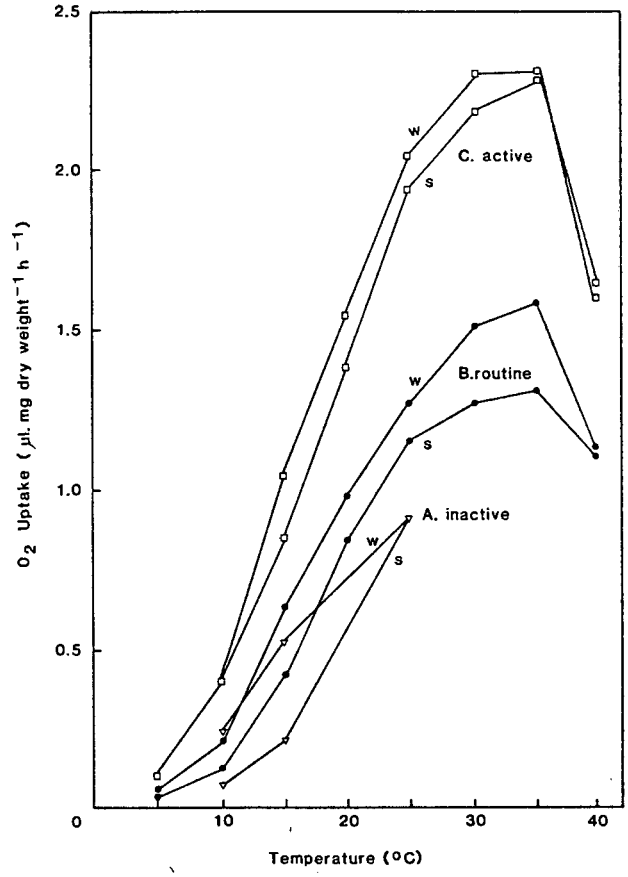

Fig. 2. Acute rate–temperature curves for *Bullia rhodostoma*: A, inactive (after Dye & McGwynne, 1980); B, routine rate in a constant water current (present paper); C, active rate (present paper), s, summer; w, winter.

Some of the points shown for routine and active rates in Fig. 2 are the averages of relatively few readings, due to the necessity of separating winter and summer readings. At 15 °C more than sufficient measurements were, however, made (80 readings on 48 animals) to allow simple *t*-tests to be undertaken; these show that winter and summer values for rates of oxygen uptake are significantly different at this temperature both for the routine and active rates ($P < 0.01$).

The Q_{10} for both rates reported is >2 at temperatures <20 °C but between 20 and 30 °C it is <2, the overall relationship being linear rather than logarithmic. Between 30

and 35 °C, increasing temperature leads to little increase in consumption and above 35 °C the rate of uptake falls rapidly. The seasonal rotation of the rate–temperature curves shown by Dye & McGwynne (1980) for smaller individuals from Algoa Bay was not apparent in the larger animals investigated here.

The results previously obtained for *B. digitalis* from the west coast are shown in Fig. 3, so that the curves for the two species may easily be compared. Unlike *B. rhodostoma*,

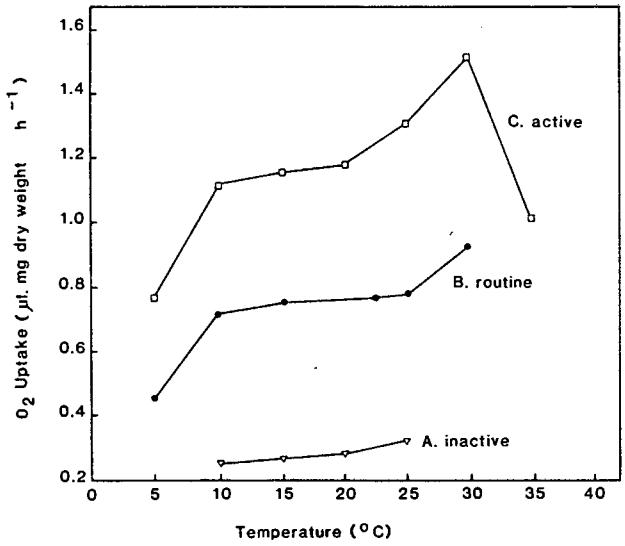

Fig. 3. Acute rate–temperature curves for *Bullia digitalis*: A, inactive (after Dye & McGwynne, 1980); B, routine rate in a constant water current (after Brown & Da Silva, 1979); C, active rate (after Brown & Da Silva, 1983).

this species does not appear to show temperature acclimation, at least on the west coast (Brown *et al.*, 1978; Brown & Da Silva, 1979). The most striking contrast, however, lies in the relative temperature independence of the respiratory rate, the Q_{10} for the routine rate between 10 and 25 °C being only 1.1 (Brown & Da Silva, 1979). Some loss of control is exhibited at the active rate but even here the rate is markedly independent of temperature when compared with the rate–temperature relationships of either *B. rhodostoma* (Fig. 2) or of whole-tissue homogenates of *B. digitalis* (Brown & Da Silva, 1983).

It should be noted that, although the graphic representation of the active rate–temperature curve for *B. digitalis* published by Brown & Da Silva (1983) and reproduced here in Fig. 3 was correct, their reporting of the Q_{10} values was erroneous. The correct values are as follows. Below 10 °C the Q_{10} of the active rate is 2.3, between 10 and 20 °C it is 1.2 and between 20 and 30 °C it is 1.7. Thereafter the rate drops rapidly with increasing temperature, in contrast to *B. rhodostoma*, whose rate drops only after a temperature of 35 °C is reached.

Discussion

The first person to consider seriously the rates of oxygen uptake in closely related marine animals from different temperature regimes appears to have been Fox (1936), who measured the oxygen consumption of pairs of species, representing a number of groups, from two latitudes in the northern hemisphere. He concluded that in general the rate of metabolism is somewhat higher in relatively warm waters than in colder waters. This has, in general, been supported by subsequent work, although inevitably exceptions have been found, and in some cases the respiratory rate in tropical species is very markedly higher than in their temperate counterparts. The case relevant to the present study is that of *B. melanoides* from tropical India which, at its ambient temperature of 30 °C, displays a rate of oxygen uptake a full order of magnitude higher than that of *B. digitalis* from South Africa's west coast at 15 °C (Brown *et al.*, 1978).

In the present work, the comparison is between two species from the same latitude, the populations being separated by only a few kilometres, but subjected to different temperature regimes. As already pointed out, these differences in regime are more complex than would be expected from a simple consideration of relatively warm and relatively cold water masses. The west coast *B. digitalis* displays a temperature independence in oxygen uptake which is clearly advantageous in a habitat where temperature fluctuations are rapid, extreme, and unpredictable. That this temperature independence is not typical of all species of the genus is demonstrated by the results gained from *B. rhodostoma* from False Bay, oxygen consumption being temperature-dependent throughout the temperature range.

The absence of temperature acclimation in *B. digitalis* is also consistent with life in an area where not only are mean summer temperatures frequently lower than the winter means, but both maximum and minimum temperatures for the year are likely to occur in summer. On the other hand, *B. rhodostoma*, facing more gradual changes in temperature and consistently higher summer than winter values, shows clear, although not particularly marked, acclimation between summer and winter. It should also be noted that Dye & McGwynne (1980), working on relatively inactive *B. digitalis* from Algoa Bay, much further east, found significant differences in the rate–temperature curves between summer and winter, so that the lack of acclimation displayed by this species on the west coast is not necessarily a feature of all populations.

The fact that the rate of oxygen uptake declines only above 35 °C in *B. rhodostoma* as compared with 30 °C in *B. digitalis* is also consistent with the different regimes to which the animals are exposed, higher temperatures being experienced in False Bay than on the west coast. The slightly different behaviour of the two species may, however, also be a factor here, for *B. rhodostoma* tends to come higher up the shore than does *B. digitalis* (McLachlan, 1980; Brown, 1982a) and on some beaches is often left behind by the retreating tide, buried in the sand (Brown & Da Silva, unpubl.). It may thus be exposed to higher temperatures than *B. digitalis*, even on the same beach, and particularly at the height of summer.

While it is apparent that the different rate–temperature curves obtained for the two species reflect, in every respect, adaptations to the different temperature regimes to which they are subject, it should be noted that for both species the individuals chosen for study were large adults. As far as distribution is concerned, temperature is more likely to be limiting to the eggs, embryos and newly-hatched young than to the adults, and of this we have as yet no knowledge. It must also be stated that other factors may well be relevant with regard to differences in the rate–temperature curves. Chief among these are differences in food availability between the two coasts. *Bullia* is primarily a scavenger of carrion (Brown, 1982a), although algal material supplements this diet (Da Silva & Brown, in press). The supply of carrion to False Bay beaches, and along the whole length of South Africa's south coast, is considerable during summer, due to the prevailing southeasterly wind, but poor during winter. The supply of carrion to Algoa Bay beaches has been quantified by McGwynne (1980). Observations of west coast beaches indicate, however, that the supply of carrion is poor at all seasons of the year. This is to some extent confirmed by the observations of Shannon & Chapman (1983) on the siphonophore *Physalia*, which is a staple food for *Bullia* east of Cape Point but is relatively rare on the west coast. It would, therefore, seem that energy conservation associated with a very limited food supply may be more critical for a *Bullia* population on the west coast than in False Bay.

The extremely flat rate–temperature curve of *B. digitalis* at all levels of activity may be seen as an important factor in such energy conservation. Brown & Da Silva (1983) comparing intact animals with homogenates, have calculated that this metabolic control represents a saving of energy of no less than 75% at 20 °C, although the energy saved would be less at lower temperatures. Comparing intact *B. digitalis* with *B. rhodostoma* it is apparent that below 15 °C *B. digitalis* actually uses more energy than *B. rhodostoma* for the same amount of activity. The rates are comparable at 15 °C and above that temperature *B. digitalis* will use less energy than *B. rhodostoma*. Such direct comparison is valid for False Bay, where both species occur on the same beaches. Comparison between species on different coasts is obviously more complex because of the generally lower and more erratic temperatures of the west coast. In principle, however, it may be stated that *B. digitalis* on the west coast enjoys a real saving in energy expenditure over *B. rhodostoma* in False Bay, when the activity patterns are the same.

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CHROMOSOME NUMBERS IN THE GENUS *BULLIA* (NASSARIIDAE)

F.M. da SILVA and A.C. BROWN

Department of Zoology, University of Cape Town, South Africa

The literature on molluscan cytogenetics has been reviewed by Patterson[1] and by Murray[2]. The phylum displays a wide range of chromosome numbers: n = 6 for some Amphineura, n = between 10 and 23 in Bivalvia, n = 28 in those Cephalopoda which have been examined and n = 5 to 44 in Gastropoda. Dolph & Humphrey[3] and Ahmed & Sparks[4] have presented tables of haploid chromosome numbers in the Mollusca. It is clear that the Gastropoda show by far the greatest range of haploid numbers. The actual number of chromosomes per cell is rendered even more variable by the occurrence of polyploidy in some forms[2].

Individuals of *Bullia digitalis* (Dillwyn) were collected from Ou Skip, on the west coast of South Africa, while *B. digitalis, B. rhodostoma* (Gray) and *B. pura* Melville were collected at Muizenberg, on the east coast of the Cape Peninsula. The animals were injected with 0.5% colchicine (10 μl. gm^{-1} wet tissue weight) and their tissues prepared for examination after the method of Stern[5]. They were placed in 0.56% KCl for up to 3 h in order to determine the optimum length of time and controls were placed in distilled water. Prior to fixation the tissues were washed gently in 3:1 ethanol:acetic acid; the cells were then centrifuged, decanted and resuspended in a small volume of fixative. They were stained in Giemsa stain. The tissues examined from each species were gonad, hepatopancreas and gill.

In all three species examined, the diploid number of chromosomes was found to be 64 (n = 32). Female *B. digitalis* from Muizenberg showed a tendency to polyploidy in both gill and hepatopancreas, a feature not observed in other individuals. The chromosomes of *B. rhodostoma* appeared to be more compact than those of *B. digitalis* and stained more deeply, as did those of *B. pura*. In all cases the chromosomes were small, with poorly developed arms.

It is not unusual for several, or all, members of a genus to display the same chromosome number; this is true not only of the Mollusca[2] but of other phyla as well[6]. Buch[7] was of the opinion that chromosome numbers do not necessarily reflect primitive or advanced condition among Mollusca, although they may have a tendency to increase with increasing specialization. A different view was expressed by Butot & Kiauta[8], who contended that primitive members of molluscan groups have large numbers of chromosomes, the number being reduced in more advanced forms. The results obtained in the present work suggest otherwise, or at least that exceptions occur, as *Bullia* is a specialized and advanced genus of the most advanced superfamily of the Neogastropoda[8], yet has a relatively high number of chromosomes.

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VERTEBRATE MUSCLE CHARACTERISTICS IN A MARINE INVERTEBRATE: SIGNIFICANCE FOR MODE OF LIFE

F.M. da Silva[1], A.N. Hodgson[2] and A.C. Brown[1]

Departments of Zoology, [1]University of Cape Town and [2]Rhodes University, Grahamstown, South Africa

Marine snails of the genus Bullia are adapted to the unpredictable and ever-changing environment of exposed sandy shores in a variety of ways which present a marked contrast to typical gastropods. Obvious adaptations include rapid crawling, burrowing and surfing with every tidal cycle, activities having high energy demands (1). Yet the supply of carrion on which Bullia feeds is highly erratic, so that energy conservation is a top priority in the lives of the animals. A high level of physiological maleability is thus implied, in addition to sophisticated morphological design of the foot, for this organ is responsible for all forms of locomotion. The pedal musculature has been described elsewhere (2); the present paper is concerned with its fine structure and attempts to relate this to the animal's life-style.

(I) MUSCLE ORGANISATION AND FIBRE TYPES. Two types of muscle fibre dominate the pedal musculature. At the anterior end of the foot (propodium) a fibre designated TYPE A prevails, while in the more extensive metapodium TYPE B dominates. Although both types present superficially a smooth muscle appearance, electron microscopy of their fine structure reveals, firstly, a dense body organisation (equivalent to Z-lines) which is more profound in Type A, where striation is present although somewhat oblique. Type B has a more disorganised framework, resembling a transitional stage between classical smooth muscle and obliquely striated muscle. Secondly, although the thick filament length is the same in both (somewhat less than 2um), the diameter in Type A (18nm) is less than in Type B (27nm). Thirdly, the thick-to-thin filament ratio is greater than 1:10 in A and always between 1:12 and 1:30 in B. Fourthly, while both types display subsarcolemmal and SR systems, these are more developed in A than in B. The SR penetrates the contractile myoplasma but, whereas in Type A the tubules are profuse and extend inwards (caveolae), in Type B finger-like plasmalemma invaginations are marked. Lastly, the numerous well-developed mitochondria (0.29 to 1um) are differently located: Type A has a central core of mitochondria in localised pockets where thick filaments are absent, so that the organelles are surrounded by myosin/paramyosin free myomatrix, in addition to the extensive tubular SR net. In Type B the central mitochondrial core is continuous along the axis of the fibre; both thick and thin filaments are present and there is less association of SR.

(II) FUNCTIONAL SIGNIFICANCE. The fibre types do not correspond to any of the categories of Chantler (3) or Nicaise and Amsellem (4). Type A is a form of striated muscle, although resembling smooth muscle while Type B has the characteristics of paramyosin smooth. Catch properties have not, however, been found. Also the degree of helical

organisation of the dense bodies in B has seldom been reported in the Mollusca. The different locations of these fibre types within the foot must be expected to have a functional basis. While the propodium is responsible for agile, complex movements during crawling, burrowing and feeding, the turgidity of the metapodium is the essential factor in surfing (1). Striated muscle usually displays a fast rate of shortening, whereas smooth muscle can generate much more tension (5), especially in bivalves, where the catch mechanism of the adductor muscle can operate continuously (3). Extensive SR and subsarcolemmal tubules suggest the presence of a chemical mediator ($Ca2^{+}$) and a cell communicating system. The caveolae of molluscs may be analogous to the subsarcolemmal system of vertebrates, associated with $Ca2^{+}$ stores (6), while SR is responsible for $Ca2^{+}$ uptake and hence relaxation. The The differences between the fibre types may thus result in faster relaxation in A (propodium) but greater tensions in B (metapodium), providing the contractile apparatus is the same in principle. Large numbers of mitochondria are unusual in white muscle and provide an index of endurance (7). This denotes the inability of metabolic anaerobiosis to provide energy and suggests that O_2 supply is not a limiting factor, a suggestion supported by O_2 diffusion measurements across the pedal wall (8). A contribution to mechanical efficiency may reside in the myosin/paramyosin free areas surrounding the mitochondria in Type A, where $Ca2^{+}$ and ATP may reach the contractile apparatus more easily. This may result in improved turnover rates of the contractile cycle. Thick:thin filament ratios are an indication of ability to form cross-bridges (4) and are related to the maximum force a muscle can exert (7). A ratio greater than 1:6 suggests enhancement of this ability, particularly if staggering of the thick filament heads occurs. In addition, concentric layers of thin filaments may result in multiple actin attachment and hence higher tension.

Thus differential dominance of these two fibre types promotes regional specialisation of the superficially uniform foot, while presenting features which approach those of vertebrate muscle.

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Proof

Regional differentiation of the foot in a sandy-beach whelk

A.C. Brown[1], F.M. da Silva[1] and A.N. Hodgson[2]
Departments of Zoology, [1]University of Cape Town and
[2]Rhodes University, Grahamstown

In the older morphological literature, much is made of the division of the gastropod foot into regions (propodium and metapodium, with possibly a mesopodium between), these regions being often clearly demarcated externally[1,2]. In recent decades, however, this differentiation has not been stressed, largely, no doubt, because internally there is little to indicate such divisions[2] and the pedal sinus is continuous from anterior to posterior[3]. Gross examination of the pedal musculature also indicates little differentiation into regions[1,4].

Recent observations on the foot of the sandy-beach whelk *Bullia*, however, strongly support a theoretical division of the foot into two regions, both on histological and on functional grounds. For example, Hodgson & Brown[5], working on contact chemoreception in connection with feeding, have shown that the propodium is sensitive to amino acids, while the rest of the foot is insensitive to all the chemicals tested. The distribution of chemoreceptors supports this conclusion[6].

A finding of greater significance is that two types of muscle fibre dominate the pedal musculature of *Bullia*, one being prevalent in the propodium, the other in the metapodium[7]. Their fine structure is described elsewhere[8]; suffice it to say here that the typical propodial fibre is striated and has many of the characteristics of vertebrate skeletal muscle, while the typical metapodial fibre is less organized and more closely resembles smooth muscle. These differences are reflected in different functions of the two regions during locomotion and other activities. Surfing, for example, is made possible by continuous turgor of the metapodium, maintained by tension of the metapodial muscles — a function more suited to smooth than to striated fibres. This is true also of copulation, during which the male grips the shell of the female for long periods with his metapodium[9]. In contrast, the propodium is the most active region of the foot during crawling and burrowing[4] and during feeding[5], rapid and complex movements being performed during these activities. Skeletal-type striated muscle fibres are implicated.

Bullia is extremely active for a gastropod mollusc and its movements are surprisingly rapid; moreover its versatility in crawling, burrowing and surfing is by no means typical of the class. Thus it may be that muscular and other differences between pro- and metapodium are exaggerated in this genus. Nevertheless, a reconsideration of morphological and physiological differences between the regions of the foot in gastropods is clearly called for.

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HAEMOCYANIN AND PROTEIN CONCENTRATIONS IN THE BLOOD OF THE SANDY-BEACH WHELK BULLIA DIGITALIS (DILLWYN)

A.C. Brown[1], F.M. da Silva[1] and M.J. Orren[2]

Departments of [1]Zoology and [2]Analytical Science,
University of Cape Town, South Africa, 7700

Sandy-beach nassariid whelks of the genus Bullia have been the subject of numerous experiments involving rates of oxygen uptake under various conditions. Results have been summarized by Brown[1] and subsequently extended[2,3]. Partial pressures of oxygen in the pedal sinus have also been assessed[4] and it has been shown that sufficient oxygen diffuses through the pedal wall to supply all the animal's needs if the surrounding sea water is approximately saturated[5]. The possible significance of the copper-containing pigment haemocyanin as an oxygen carrier is, however, an issue which has not been addressed, although it has been stated that the haemocyanin content is low[1,5].

About 1 ml of haemolymph was withdrawn from the pedal sinuses of each of eight large individuals of B. digitalis, through a hypodermic syringe introduced into the neck region. The animals came from the beach at Ou Skip, just north of Table Bay; half the extractions were performed in the field, the rest in the laboratory. Samples were stored for a few days at -20°C, thawed and analysed for copper on a Varian model AA-6 atomic absorption spectrophotometer, using standard air-acetylene flame conditions for copper. A pulse method[6] was used to minimize sample intake. Results were confirmed by injecting 20 µl aliquots of the haemolymph into a Perkin-Elmer model HGA 500 graphite furnace atomizer and determining copper with a Perkin-Elmer model 5000 atomic absorption spectrophotometer. All samples gave values well below

0.1 ppm copper, concentrations thus being of the same order as those usually found in sea water. We conclude that in normal circumstances the haemocyanin content of the blood is also very low and that it can play virtually no rôle in oxygen transport. This is confirmed by the colourless nature of Bullia haemolymph allowed to stand in contact with air[5].

This does not mean, however, that the animal is unable to produce substantial amounts of the pigment should they be required. Brown[7] conducted experiments using haemolymph extracted from B. digitalis collected in Hout Bay, when that area had for some time been heavily polluted with organic material from a fish-meal factory, and noted that the initially colourless haemolymph turned blue on exposure to air. At one time it was believed that haemocyanin concentrations did not alter in response to environmental changes; however, since the work of Jones[8], it has been recognised that such changes do occur in some invertebrates, including gastropods.

The protein concentrations of five of the samples described above were determined, using the method of Bradford[9]. Values ranged from 1.04 to 2.30 $\mu g.ml^{-1}$, with a mean of 1.48 $\mu g.ml^{-1}$, these being very low compared with the blood of other marine invertebrates investigated[10]. The low haemocyanin concentration is thus reflected in a low overall protein concentration, the latter not being boosted by proteins of other types.

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The gardens of the sandy-beach whelk *Bullia digitalis* (Dillwyn)

F. M. da Silva and A. C. Brown
Department of Zoology, University of Cape Town, South Africa

The nassariid whelk Bullia digitalis is an abundant and characteristic animal on the sandy beaches of South Africa's west and south coasts. Our knowledge of it has been summarized by Brown[1]. The whelk is an opportunistic scavenger, feeding mainly on dead or dying animal matter in the wash zone or, if wave action permits, in the surf[2-5]. It will also on occasion turn predator, capturing small animals by folding its foot over them[3,6], although such behaviour is rare and normally follows a long period of starvation.

There can be little doubt that the supply of carrion to the beach is in general inadequate to meet the nutritional needs of the vast populations of *Bullia* which occur. Most of this carrion consists of Scyphozoa and siphonophores such as *Physalia*, which may wash up in large quantities, but erratically and unpredictably, limited to a restricted season. McGwynne[7], working on beaches around Algoa Bay, on the south coast, found no carrion at all during winter and Brown (unpubl.) during some hundred visits to Ou Skip, on the west coast, has only twice encountered carrion on the beach or in the surf.

Bullia can consume food equal to a third of its own tissue weight in a single meal[2], yet it appears that such a meal of cnidarian tissue could supply the needs of the whelk for little more than two days.[1]. A meal of bivalve molluscs such as *Aulacomya* or *Choromytilus*, because of its far higher calorific value, may supply the whelk's energetic needs for a fortnight[8] but such food is rarely available except in very localized areas[1]. *Bullia* has not been seen to eat stranded plant material, although some members of the Nassariidae are known to graze plants[9,10] and some are obligate omnivores[11]. The possibility that newly-hatched *Bullia* may eat plant detritus has been suggested but not witnessed[1].

Recently, Colclough & Brown[12] have demonstrated the ability of *Bullia digitalis* to take up dissolved organic matter directly from sea water. This absorption exceeds losses to a highly significant degree and the authors conclude that such uptake may account for more than 15% of the energetic needs of the animal, although they did not take into account the possible cost of absorption. The question remains as to whether these various sources of nutrition — scavenging animal material, occasional predation and the absorption of organic matter — are adequate. Also to be explained is the fact that the whelks will live for months in the laboratory without feeding[2].

A further possible source of food has now come to our attention. It has several times been mentioned that *Bullia* frequently has an algal growth on the upper surface of its shell and especially on the last whorl[1,4]. This is the part of the shell receiving the most light, for it is closest to the surface of the sand when the animal is buried and is, in fact, frequently exposed. It had been thought that this occurrence of epiphytic algae was fortuitous, indeed inevitable in the circumstances. However, microscopic examination of the shells of individuals from Ou Skip shows that only a single species of alga is present and that in all cases it appears to have been cropped almost down to the shell. No small animals could be discovered which might be responsible for such cropping. Virtually all adult *B. digitalis* have been found to possess such an algal "garden", although to the naked eye the alga is often only apparent on light-coloured shells. Observations both in the field and in the laboratory show that from time to time the whelk's long, mobile proboscis is extended over the head to this algal garden, the tip moving over it in a systematic manner. Prof. G. M. Branch (pers. comm.) has also witnessed this behaviour. It appears to us that it is this activity which keeps the alga cropped and that the garden may be a food source for the animals. Additional evidence is that the usually sparse gut contents are often green in colour, a fact for which we have previously been unable to account.

Gardens have been studied in several other marine animals, the classic example being that of some species of *Patella*[13]. Here a stable rock surface is available for cultivation but clearly for a sandy-beach whelk living in a highly unstable substratum, the only possible site for such a garden is its own shell. The statement of Brown[14] that animals on high-energy sandy beaches can do nothing to modify their environment and "cannot find peace by cultivating their gardens" thus appears to be not entirely true, although our discovery lends weight to the same author's claim that it is a characteristic of sandy-beach animals that they have several methods of obtaining food.

What now has to be done is to determine if, how and to what extent *Bullia* cultivates its garden and the nutritional value such a garden may afford the gardner.

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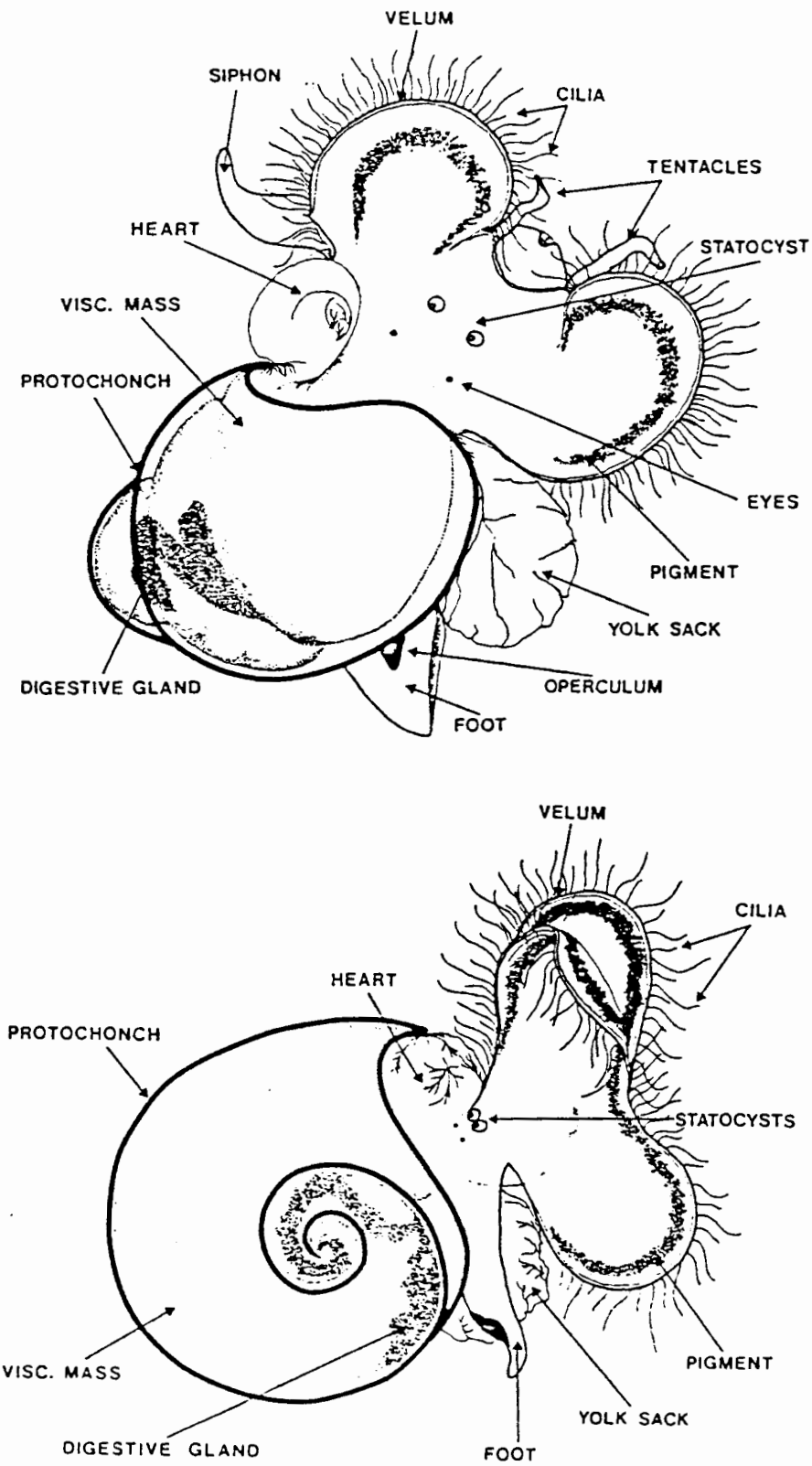

Figure 2

Veliger larva of *Bullia digitalis*. Above, dorsal view. Below, lateral view.

and a siphon were present, as was a ridge of dark orange pigment on the velum that probably represents the respiratory complex of the adult. A pair of eyes was apparent, despite the fact that the adults lack eyes (BROWN, 1982). Laterally and slightly ~~posterior~~ to the eyes, a pair of statocysts lay close to the actively pumping heart. Torsion had already occurred but it could not be determined whether torsion was complete. A small foot and operculum were present. A sac lying on the outside of the body and attached near the base of the visceral mass was tentatively identified as a yolk sac, as its contents dissolved rapidly on contact with acetone, indicating the presence of lipids. A veliger of *Bullia digitalis* is shown in Figure 2.

Attempts to rear the eggs failed, the veligers becoming lethargic and darkly pigmented; within five days they had become infested by larvae of a digenic trematode and died soon thereafter. Eggs in capsules that remained attached to the feet of other individuals also failed to develop.

DISCUSSION

Although the literature on planktonic prosobranch larvae is voluminous, descriptions of veliger stages passed within the egg are rare and no *Bullia* veliger has previously been described. THIRIOT-QUIÉVREUX (1980) described the planktonic veligers of *Nassarius*, a genus closely related to *Bullia*, but these differ considerably from the veligers described here. On the other hand, veligers of *Littorina littorea* are quite similar to those of *Bullia digitalis*, both in size and at least superficially in morphology (FISH & FISH, 1977), with the exceptions that the cilia on the velum of *Littorina* are 3 to 5 times longer and no tentacles are visible.

The eggs of all species of *Bullia* so far investigated produce crawling young, the larval stages being passed within the egg (BROWN, 1982). *Bullia digitalis* is no exception and the small size of the eggs of this species may thus be remarked upon, as one might have expected eggs of little more than 0.2 mm in diameter to hatch at a much earlier stage. It is also of interest that every egg we examined had within it a living veliger and that every egg in the capsule collected by Mrs. Connolly contained a miniature adult; there are thus no nurse eggs in this species, despite tentative previous suggestions (BROWN, 1971, 1982).

The numbers and size of young *Bullia digitalis* contrast with those of *B. tenuis*, a subtidal species whose egg cases and young have recently been described (BROWN, 1985). The adults of these two species are of similar size and appearance, but *B. tenuis* produces only about 60 egg capsules at a time and each capsule contains only one developing egg, although nurse eggs are also apparently present. By contrast, *B. digitalis* appears capable of producing up to 40,000 young at one time, but these are minute compared with the young of *B. tenuis*, which may attain a shell length of 5.3 mm before emerging from their capsules (BARNARD, 1959; BROWN, 1985). It is clear that these extremes represent very different strategies and it must be supposed that juvenile mortality is high in *B. digitalis* as compared with *B. tenuis*.

Finally, *Bullia digitalis* can package its eggs in two different ways—either with each clump of eggs contained in its own capsule, as reported here, or with all the clumps in a single all-embracing case or sheath, as described by Professor Omer-Cooper and subsequently by BROWN (1971). In the former circumstance, the tiny capsules are loosely attached to the undersurface of the foot, while if contained in a single large case they are deposited in the sand. It is clear that such a large case must be formed outside the body of the parent and it is logical to suppose that it is molded by the foot after the eggs have been extruded; its size and shape certainly support this explanation. Differences in egg packaging according to circumstances of food availability are not unknown among the Nassariidae (MCKILLUP & BUTLER, 1979) but the present example would appear to be the most extreme so far reported for any of the Prosobranchiata.

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ALGAL GARDENS AND HERBIVORY IN A SCAVENGING SANDY-BEACH WHELK

S.A. Harris[1], F.M. da Silva[1], J.J. Bolton[2] and A.C. Brown[1†]

Departments of [1]Zoology and [2]Botany, University of Cape Town, South Africa 7700

†from whom reprints may be obtained

Running head: Herbivory in a sandy-beach whelk

ABSTRACT: The shells of living Bullia digitalis, a nassarid whelk common on the west and south coasts of South Africa, are consistently invaded by a green, filamentous, boring alga (Chlorophyta), the morphology of which corresponds to Eugomontia sacculata Kornm. Behavioural observations suggest that this and/or other algae growing on the shell may supplement the predominantly carnivorous diet of the whelk. The gut contents and digestive glands of the animals are frequently green in colour, particularly during winter, and it is demonstrated that this is due to the presence of chlorophyll a. Cellulolytic symbiotic bacteria, as well as α-amylase, cellulase and laminarinase activity, are shown to be present in the gut. It is concluded that B. digitalis ingests and utilises green algal material and thus plays a more complex role than previously thought in the sandy-beach food web.

INTRODUCTION

Bullia digitalis (Dillwyn) is a nassarid whelk which is characteristic of high-energy sandy beaches along the west and south coasts of southern Africa. Our knowledge of this and other species of the genus has been reviewed by Brown (1982). The whelk is essentially a scavenger of washed-up animal matter, although it will turn predator on occasion. The supply of carrion to the beaches in question is highly erratic, however, and tends to be seasonal, while predation appears to be relatively uncommon. Thus, although this whelk can consume food up to one third of its own tissue weight on a single meal (Brown, 1961), it seemed unlikely that carrion and prey could supply all its requirements. Colclough & Brown (1984) therefore investigated the possibility of the animal making use of dissolved organic matter in the surrounding sea water to supplement its diet. The results proved positive but it is clear that this source of nutrition could not by itself supply the needs of the whelk for an extended period. The possibility that the animals may eat stranded plant material has never been completely rejected, as some members of the Nassariidae are known to graze plants (Kilburn & Rippey, 1982) and Nassarius obsoletus has been shown to be an obligate omnivore (Curtis & Hurd, 1979). Nevertheless, this possibility has not been confirmed in the field, nor has algal material offered to captive whelks in the laboratory ever been eaten.

Recently, da Silva & Brown (1984) have reported the consistent presence of an alga associated with the shells of living B. digitalis and have described behaviour suggesting that

the animal periodically crops this "garden" with its long, mobile proboscis and ingests the algal material. Contributory circumstantial evidence is the fact that the gut contents and digestive glands are frequently green in colour.

The aims of the present work were to establish the nature of the alga or algae associated with the shell, to discover whether the green colour of the digestive system is due to ingested chlorophyll and to assess the digestive capabilities of the animal with regard to utilising algal material.

THE ALGAL GARDEN

Examination of intact shells and shell fragments of Bullia digitalis by light microscopy revealed the consistent presence of an extensive growth of a green filamentous, boring alga embedded within the outer layer of the shell (Fig. 1). Contact between the alga and the exterior was maintained through numerous small holes, some 7 µm in diameter (Fig. 2). It was found possible to isolate the alga from the shell by treatment with dilute HCl. It consists of uniseriate filaments displaying an irregular but predominantly opposite pattern of branching. The diameter of the algal filaments is 5 to 7 µm. The vegetative features thus correspond to those of Eugomontia sacculata Kornmann, a boring species found in both living and dead shells in temperate regions in both hemispheres (Kornmann, 1960, Wilkinson & Burrows, 1972a, b, South & Adams, 1976).

Scanning electron microscopy of shell fragments fixed in 2% gluteraldehyde, critical point dried after dehydration and coated

with gold-palladium, showed that the algal filaments bore only into the outer prismatic layer and ramify throughout the crystalline matrix of this layer (Fig. 3).

It was attempted to culture the alga by keeping shell fragments in an enriched seawater medium (ES of Provasoli, 1968) at 15°C and a photoperiod of 16 hours at a light intensity of 50 to 60 $\mu Em^{-2}.s^{-1}$. This proved successful to the extent that after some weeks isolated patches of algae were observed growing on the bottom of the Petri dish, presumably resulting from released spores. Sporangia were not observed on the shell surface due to dense growth of other algae (Ectocarpus sp., Enteromorpha sp. and Ulva sp.) which, although present in low numbers initially, flourished in the culture medium.

DIGESTIVE GLAND CHLOROPHYLL ANALYSIS

The gut contents and digestive glands of B. digitalis vary considerably in colour. They are frequently brown or grey and are bright blue after the animals have been feeding on the siphonophore Physalia. The green colour already referred to is also common and appears to be most in evidence in individuals from the west coast during winter.

Green digestive glands from four whelks from the west coast were each homogenised in 12 ml 90% acetone and the extracts ultrasonified for 30 mins in the dark on ice. The samples were centrifuged at 9 000 rpm for 15 mins at 15°C and the supernatant analysed on a spectrophotometer linked to a Spectroprinter. The four samples gave very similar results, showing absorbance peaks in the 660-663 nm and 400-410 nm regions. These maxima correspond to

those of chlorophyll a (Bogorad, 1962, Round, 1973) and we conclude that the green colour observed is indeed due to chlorophyll resulting from the ingestion of GREEN algal material.

CARBOHYDRASES

Material and methods: To test for cellulolytic bacteria in the gut of B. digitalis, a modification of the method of Teather & Wood (1982) was employed. The whole gut was homogenised in 600 µl sea water and the crude supernatant subjected to a dilution series. 10 to 20 µl of the diluted bacterial solution were then plated onto a growing agar medium (GAM) (2% agar, 0.5% peptone, 0.1% yeast extract). Colonies of pure bacterial isolates were stabbed onto a final plate medium consisting of 2.5% w/v agar containing 0.1% w/v peptone and 0.01% carboxymethyl cellulose. The plates were incubated at 23°C for four days and then flooded with an aqueous solution of Congo Red for 15 mins. This stain reacts with β-D-glucans in the CMC substrate, providing a rapid and sensitive assay for bacterial strains possessing β-D-glucanohydrolases (Teather & Wood, 1982). The Congo Red was then poured off and the visualised zones of β-D-glucans hydrolysis stabilised by flooding with 1N HCl for 15 mins; this changes the dye colour to blue and inhibits further enzyme activity. Results were recorded photographically. Scanning electron micrographs were prepared both of bacteria from the colonies growing in culture and of the bacteria in situ in the animal's gut.

In a further series of experiments, homogenised whole guts were centrifuged in phosphate buffer (pH 7) and the supernatant subjected to cellulase, α-amylase and laminarinase determinations, using the Nelson-Somogyi colorimetric method (Nelson, 1944,

Somogyi, 1952). Attempts to first eliminate enzyme-producing bacteria with β-mercaptoethanol or Ampicillin had to be abandoned, as both interfered with the assay. In order to obtain comparative results, each sample was analysed for total protein content, using the Folin Ciocalteus (2N) reagent and following the method of Lowry et al. (1951). Crystalline bovine albumin was used as a standard. Results of the enzyme analysis were expressed in mg glucose evolved. mg protein^{-1}. hour^{-1}.

Results: Isolated bacterial colonies appeared on the GAM plates within one to three days. About 10% of the bacteria were cellulolytic, as was clear from the visualised zones of β—D-glucans. The bacteria were of the agarose-eating type, identifiable by depressions in the agar. SEM examination showed the pure bacterial isolates to be rod-like, while those photographed in the gut were predominantly coccoid. These could, however, be the same bacteria under different conditions.

Results of the carbohydrase assays are given in Table 1. All three activities tested were positive, at levels indicated by the amount of reducing sugar evolved from each substrate. Thus α-amylase activity (0.837 mg glucose.mg protein^{-1}.hr^{-1}) accounted for some 77% of the total activity measured, while cellulase and laminarinase accounted for only 14% and 9% respectively.

DISCUSSION

Among marine invertebrates, the hydrolytic capacity of the carbohydrases is generally greater with respect to reserve carbohydrates (starch, glycogen, laminarin) than to structural carbohydrates (cellulose and chitin) (Elyakova et al., 1981). The present

study supports this with regard to α-amylase and cellulase but not with regard to laminarinase. It is difficult to compare our work quantitatively with other published data, because of the variety of techniques employed, but qualitative comparisons can be made.

Stone & Morton (1958) studied the distribution of carbohydrases in molluscs having a wide range of feeding habits. They found α-amylase activity to be present among both herbivores and carnivores, in accordance with the ability to digest starch and glycogen respectively. There was minimal cellulase activity but high levels of laminarinase in the carnivorous whelks, a finding confirmed for Nassarius reticulatus by Kristensen (1972). Slight hydrolysis of alginic acid and alginate was also detected in this species, although the animal apparently never feeds on brown algae.

It was suggested by Stone & Morton (1958) that, far from being strictly functional, cellulases may form part of the basic digestive enzyme system in the Mollusca, and this concept has been supported by subsequent work. Yokoe & Yasumasu (1964) proposed that the distribution of cellulases in invertebrates generally is more closely correlated with phylogeny than with feeding habits, while Gianfreda et al. (1979) were unable to explain the significance of the β-1,4-glucanase (cellulase), found in most carnivorous molluscs examined, on functional grounds. They inclined to the view that it simply represents an evolutionary remnant from an originally herbivorous stock. This view is supported by Agnisola et al. (1981). The presence of these carbohydrases in Bullia digitalis is thus no proof, by itself, that the animal ingests plant material; on the other hand, it is a good indication that the whelk can utilise such material if it is ingested.

Production of cellulases by symbiotic bacteria in the gut is well known among the Mollusca. In most cases it is not certain, however, whether the host animal also produces such enzymes, although this is clearly the case in marine borers. Morton (1978) has suggested that in these animals the bacteria provide the enzymes for the initial breakdown and that this is followed by digestion within the cells of the animal's digestive diverticuli, using enzymes produced by the animal. In the present work, the presence of cellulase-digesting bacteria in Bullia digitalis has been demonstrated but whether the animal itself also produces such enzymes is not clear.

However that may be, the presence of chlorophyll in the digestive system, together with enzyme activity appropriate to the digestion of algal material, leads to the firm conclusion that the whelk can and does supplement its predominantly carnivorous diet with algae. It thus plays a more complex rôle than previously thought in the sandy-beach food web.

Less certain is the source of this algal material. Macrophytic algae do not occur on high-energy sandy beaches (Brown, 1964) and there is no hint, after 25 years of observation, that Bullia eats stranded plant material. The only alternative would appear to be an algal "garden" growing on the shell itself. Apparent cropping of such a garden has been witnessed (da Silva & Brown, 1984) but whether it is the embedded Eugomontia or other algae growing on the shell which are being cropped is not at all clear. The boring alga grew only very slowly in culture and according to the detailed description of the life history of E. sacculata by Kornmann (1960), as well as our own observations, only the reproductive structures of the alga protrude from the shell. It is thus questionable

whether the small amount of material made available could be of any importance in the diet of the whelk. The same applies to the non-boring algae discovered on the shell, for these were very sparse until they were cultured.

However, a culture medium provides very different conditions from those in the field and it may be that the buffetting of the shell by waves and sand on the beach provides a stimulus for growth or even that the whelk encourages the growth of the algae in some way. Only further experiments will solve the mystery.

ACKNOWLEDGEMENTS

Mr Klaus Schultes and Miss Jean Harris assisted with the preparation of scanning electron micrographs, while Miss Cathy Roberts gave advice on the bacterial section of the work. The project was supported by a postgraduate CSIR grant made available to the first author.

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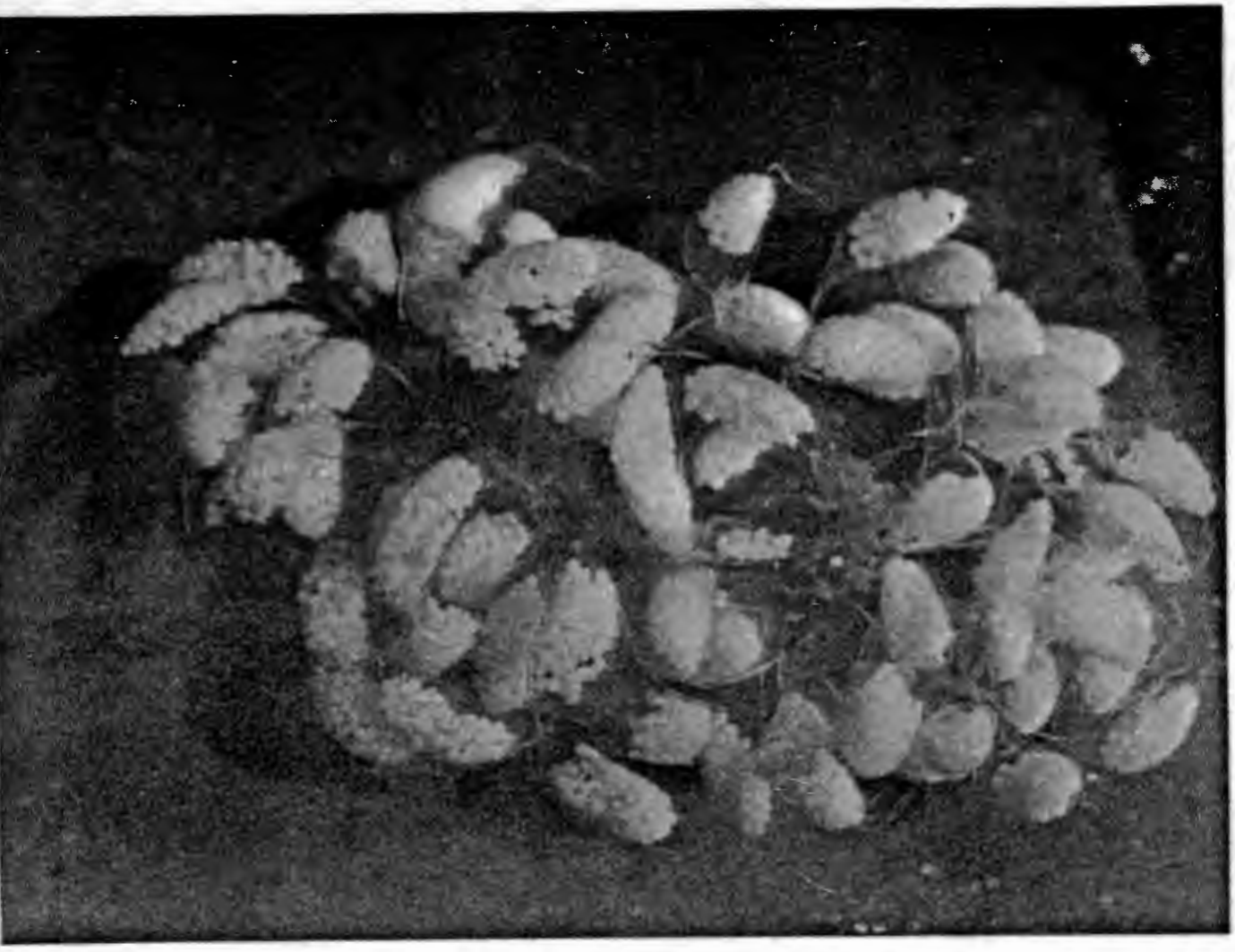

Figure 1

Egg capsules of *Bullia digitalis* removed from the foot of a gravid female (×12). The cases are typical except for one near the center of the picture, which contains few eggs.

RESULTS

Eggs and Egg Capsules

The number of capsules per female varied from 150 to 203, each capsule typically containing 150 to 200 eggs, although an occasional capsule had only 30 or 40. Each capsule measured 3.00 ± 0.05 × 1.5 ± 0.15 mm, had an extremely thin, transparent, membranous wall, and possessed an attachment thread at either end, one thread being more coiled than the other. The capsules were attached loosely to the undersurface of the maternal foot and to one another by a sparse but viscous mucous secretion and were further anchored to one another by their coiled attachment threads. A group of such egg capsules, removed from the foot and comprising about a quarter of those present, is shown in Figure 1. Each egg was about 220 μm in diameter, as were the eggs and newly hatched young collected by Mrs. Donnolly on Fish Hoek beach.

Gravid whelks in the laboratory protected their capsules by curling the foot over them to form a tubular brood pouch, in the manner described from *Bullia melanoides* by ANSELL & TREVALLION (1970), while during crawling only the margins of the foot were used. These protective behavior patterns did not appear to be entirely adequate, however, as the whelks tended to shed capsules.

The Veligers

Each veliger carried a very thin, transparent protoconch consisting of 1½ whorls. The veligers measured 205 ± 25 μm between the apex of the protoconch and the leading edge of the head. At its widest the shell diameter was 98 ± 7 μm. The head was bordered by a bilobed velum, which was heavily ciliated with cilia of two types; the longer (10 μm in length) exhibited metachronal rhythm, while the shorter cilia, only about half that length, showed a more random pattern of movement. Two well-defined tentacles

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TABLE I

The carbonydrase activities obtained from the Nelson-Somogyi enzyme assays (n=10).

		Enzyme activity ($mg\ glucose.mg\ protein^{-1}.hour^{-1}$)	
Substrate	Enzyme	$\bar{X}$ ± S.D.	%Total activity
Glycogen	α-amylase	0.4256 ± 0.0922	39.31
Starch	α-amylase	0.4113 ± 0.1016	37.99
CMC	cellulase	0.1511 ± 0.0634	13.96
Laminarin	laminarinase	0.0946 ± 0.0822	8.74

LEGENDS TO FIGURES

Figure 1: Light micrograph of a shell fragment of Bullia digitalis (X 200), showing branching filaments of the green alga Eugomontia embedded within it.

Figure 2: Scanning electron micrograph of the shell surface of B. digitalis, with holes caused by the boring alga (X 1670). The tips of the filaments are situated in the holes, just below the surface.

Figure 3: Algal filaments ramifying through the crystalline matrix of the outer prismatic layer of the shell of B. digitalis (SEM X 1670).

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Egg Capsules and Veligers of the Whelk *Bullia digitalis* (Gastropoda: Nassariidae)

by

F. M. DA SILVA AND A. C. BROWN

Department of Zoology, University of Cape Town, South Africa

Abstract. The sandy beach whelk *Bullia digitalis* can package its eggs in two different ways. Clumps of eggs may be contained in a single large sheath and deposited in the sand, or each clump of 150 eggs or more may be contained in its own capsule and held on the ventral surface of the maternal foot. In the latter case up to 40,000 eggs may be produced at one time. The eggs and capsules are described for the first time, as is the veliger stage, which is passed within the egg. The reproductive strategy of *B. digitalis* is contrasted with that of *B. tenuis*.

INTRODUCTION

Bullia digitalis (Dillwyn) is a nassarid whelk which is abundant on medium to high energy sandy beaches along the west and south coasts of southern Africa. Its biology, together with that of other species of the genus, has been reviewed by BROWN (1982). On beaches in the Eastern Cape Province of South Africa, gametogenesis occurs between March and May, vitellogenesis and egg storage taking place from June to December or January, after which the females spawn (MCGWYNNE, 1980). We believe that the timing of events on the west coast may be both different and more variable than in the Eastern Cape (BROWN, 1971) and in recent years we have discovered females with eggs from early July to late January at Van Riebeeck Strand (Ou Skip), just north of Table Bay.

Females of several intertidal species of *Bullia* tend to migrate offshore before producing their egg capsules (BROWN, 1982). *Bullia digitalis* appears to be no exception (MCGWYNNE, 1980), although this migration of females is more marked in some areas than in others. The gravid females found at Van Riebeeck Strand were all buried just below low water mark, the migratory tendency thus being poorly developed.

Egg cases of *Bullia digitalis* were first described by Professor J. Omer-Cooper in a letter to one of us (A.C.B.), this description being subsequently confirmed by BROWN (1971). A case measured about 2 cm in length and 1.2 cm in width and contained more than 1500 eggs arranged in clumps of 50 to 100 or more. Such egg cases were found 4 to 12 cm below the surface of the sand, usually in the presence of an adult female.

The present work was undertaken due to the discovery of *Bullia digitalis* eggs, from Van Riebeeck Strand, that were packaged differently, being held in numerous small capsules under the maternal foot, and also to the acquisition for the first time of eggs containing veligers.

MATERIALS AND METHODS

Of the several females of *Bullia digitalis* discovered carrying egg capsules beneath their feet, four were returned to the laboratory from Van Riebeeck Strand. The capsules and the eggs within them were counted and measurements made using a graduated eyepiece in a binocular microscope. In addition we had on loan from the South African Museum a female with capsules collected on Fish Hoek beach, False Bay, by Mrs. C. M. Connolly on 5 January 1961; in these capsules all the eggs had hatched or were on the point of hatching, as miniature adults.

More recently, a whelk collected at Van Riebeeck Strand produced a full batch of egg capsules in the laboratory. These were discovered on 19 January 1984, well over a month after the animal had been captured. It is almost certain that this whelk had copulated in the field, the sperm being stored in the spermatheca. A number of capsules shed from the parental foot were held over sand in flowing seawater at 14°C. In each egg a veliger larva could be observed, which swam actively in the water if mechanically released from the egg. Several such veligers were examined and photographed under light microscopy, using various types of illumination, first while they were swimming freely and later while held immobile under a coverslip.

This is a reduced version of a poster presented at "The Muscle and Membrane International Symposium", Cape Town, South Africa. March 1985.

Questions

How is the marine snail **Bullia** successfully adapted to the harsh unpredictable sandy beach environment?
(The animal crawls, burrows, surfs, etc)
What is the morphological disposition of the two white muscle fibres, with vertebrate red muscle characteristics, within the foot?
What are the selective advantages of a different muscular organization?

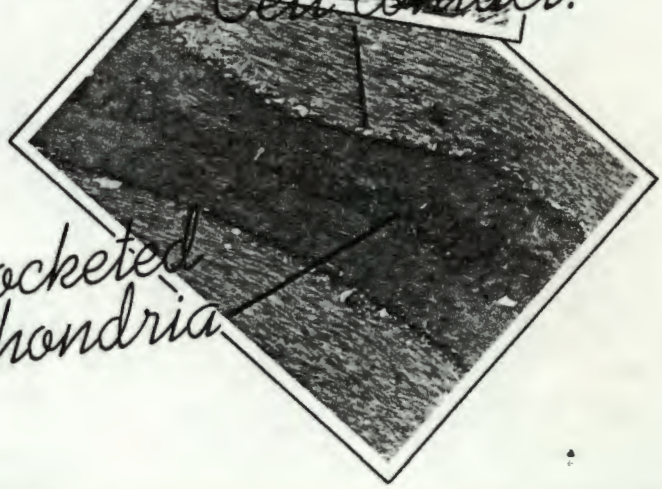
On Analysis...

Transverse

Longitudinal

Propodium

Metapodium



Some properties of white, intermediate & red muscle fibre WRT features of type A & B fibres.

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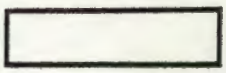
[Glycogen]			
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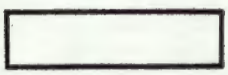
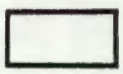
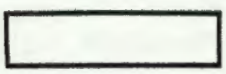
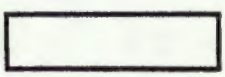
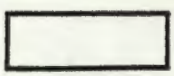
Neuromuscular junction			
% SR			
Index of fatigue			

(Size of boxes = proportion w.r.t. A or B)

Conclusions

Although ***Bullia*** pedal muscle superficially resembles smooth muscle, it in fact combines properties of rich red muscle and anaerobic-tolerant white skeletal muscle. The myosin/paramyosin free myomatrix may allow more efficient movement of Ca^{2+} and ATP to and from the SR and mitochondria. The extensive SR mesh may increase relaxation capacity in type A over type B, without affecting endurance aptitude — an important consideration in an ever changing environment imposing extraordinary physiological and behavioural demands.