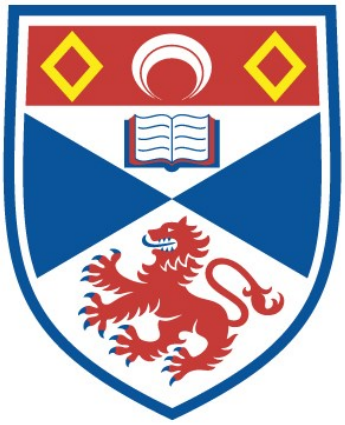
COMPARATIVE STUDIES ON THE SARCOPLASMIC RETICULUM OF FISH MUSCLE

Harry J. McArdle

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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A Thesis submitted to the
University of St. Andrews
for the degree of Doctor of Philosophy

by

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"Auf Grund unserer Forschungen ..... eine neue Zeit ist angebrochen, ein grosses Zeitalter, in dem zu leben eine Lust ist."

Bertolt Brecht.

ACKNOWLEDGEMENTS

There have been a great many people who have helped me, in one way or another, during the period of this research. I hope that I can remember them all, but apologise to anyone who I may have excluded. The omissions, I assure them, are accidental.

First I must acknowledge the enormous debt I owe to my supervisor Dr. I.A. Johnston for the support, encouragement and guidance that he has unfailingly given me.

I would like to thank Dr. P.S. Agutter and Dr. F.W. Flitney, who first interested me in research. I hope that they are not dissatisfied with what they started.

The support and assistance of my wife, Karen, has been of great value to me. She has given unselfishly of her time and interest, despite the imminence of her own final exams. I hope that this public acknowledgement will give her some idea of the gratitude I feel.

My thanks, too, to my parents, who first persuaded me to try a university career. I have not regretted the choice; I hope that neither have they.

Finally, my thanks to Mrs. M. Wilson for typing this manuscript and to Messrs. Bob Adam and Murray Coutts for their photographic assistance.

ACADEMIC RECORD

I first matriculated at the University of St. Andrews in October, 1970, and graduated with the degree of B.Sc. (Hons.) (1st Class) in Physiology in June, 1976.

I matriculated as a postgraduate student of the Department of Physiology, University of St. Andrews, in October, 1976.

CERTIFICATE

I hereby certify that Harry J. M'Ardle has spent twelve terms engaged in research work under my direction, and that he has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Court No. 1, 1967), and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

Dr. Ian A. Johnston.

DECLARATION

This is to certify that the thesis I have submitted in fulfilment of the requirements governing candidates for the degree of Doctor of Philosophy in the University of St. Andrews entitled "Comparative studies on the sarcoplasmic reticulum of fish muscle", is my own composition and is the result of work done mainly by me during the period of matriculation for the above degree. No part of this work has been previously submitted for a higher degree.

The research was conducted in the Department of Physiology and Pharmacology, United College of St. Salvator and St. Leonard, University of St. Andrews, under the supervision of Dr. I.A. Johnston.

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SUMMARY

1. The sarcoplasmic reticulum from fish red and white muscle has been isolated and compared with that of mammalian sarcoplasmic reticulum (SR). The white muscle SR is shown to be similar to mammalian fast muscle with the majority of the protein being accounted for by a band with molecular weights of approximately 100,000. This has been demonstrated in several species to correspond to the calcium pump protein. The red muscle SR pumps Ca^{2+} ions and hydrolyses ATP at approximately half the rate of the white SR. Gel electrophoresis of the SR vesicles shows that there is not sufficient 100,000 dalton protein in the red muscle SR to account for the relative rates of pumping. It is suggested that fish red muscle SR resembles mammalian slow fibres in that the pump protein's molecular weight is not the same as the white. Unlike mammalian SR, vesicles isolated from trout red muscle or not stimulated by commercially available cAMP dependent protein kinases.

2. The microsomal pellet of muscle is generally contaminated with mitochondrial fragments and sarcolemmal vesicles. Pure sarcoplasmic reticulum fractions have been obtained from plaice muscle and the characteristics of the Ca^{2+} ATPase and Ca^{2+} uptake studied. The protein composition of the vesicles, the dependence on Mg^{2+}, Ca^{2+}, K^{+} and ADP are demonstrated to be similar to that of rabbit muscle. A comparison of the levels of the phosphorylate enzyme intermediate with those obtained by other workers shows that the pump protein concentration is similar to rabbit fast skeletal muscle.

3. The effect of evolutionary temperature adaptation on the sarcoplasmic reticulum of different species of fish has been examined. The Ca^{2+}-ATPase activity shows an inverse correlation with the cell temperature of the species at $0^{o}C$, and this difference is reflected in the relative rates of Ca^{2+} uptake. Activation enthalpy increases with increasing environmental temperature, while activation entropy goes from negative values in the cold adapted to positive values in the hot adapted species. Unlike other enzyme systems, the activation energy shows no correlation with temperature. Acclimation to seasonal temperature changes has also been studied. Unlike adaptation on evolutionary time scales, acclimation does not affect the activation entropy of the ATPase, and there is no significant difference in the rates of ATP hydrolysis. It is concluded that the mechanisms of compensation are different in adaption to evolutionary and seasonal temperature changes. Evidence for acclimation to low temperatures involving an increase in the amount of sarcoplasmic reticulum is reviewed.

4. The enzyme-substrate affinity has been monitored by measuring the K_{Ca} of the Ca^{2+} ATPase at different temperatures for four different species of teleost. As with the Vmax estimations, compensatory changes are shown in the ATPase. K_{Ca} values, estimated at $0^{o}C$ are found to be inversely related to the animal's cell temperature. When estimated at that temperature, the values are similar. These results are discussed in relation to the observed prepondence of parvalbumins in fish muscle, and a possible role of the parvalbumins is considered.

5. In even the purest of SR preparations, i.e. those with no contaminating marker enzymes, a Ca^{2+} independent ATPase activity can be demonstrated. Unlike the Ca^{2+}-ATPase the Ca^{2+}-independent ATPase activity is not related to habitat temperature, and the activation entropy is not proportional to cell temperature. As shown in the rabbit, incubation of the SR in the presence of 0.1% Triton X-100 converts the basal ATPase activity in all except the lighest fractions to Ca^{2+} dependence. It is suggested that the Ca^{2+} independent ATPase represents a form of the pump protein uncoupled from Ca^{2+} transport, with the two forms of the ATPase in thermal equilibrium. Supportive evidence is provided for this theory. The ratio of Ca^{2+} dependent to total ATPase is temperature dependent, increasing to maximal value at and above the animal's cell temperature. For example, the ratio of approximately 0.25 at $0^{o}C$ in Tilapia mariae (a tropical species), increasing to 0.85 at $25^{o}C$. In contrast, the ratio for Myoxocephalus scorpius (North Sea) is only slightly affected by assay temperature and remains at about 0.95 throughout the range $0 - 33^{o}C$. The activation energy for the conversion has been estimated for two species and compared with that of rabbit. It is possible that the contribution of the activation energy for the conversion may account for the poor correlation of the $\Delta G^{\ddagger}$ of the Ca^{2+}-dependent ATPase with environment temperature.

CHAPTER I

THE SARCOPLASMIC RETICULUM

THE SARCOPLASMIC RETICULUM

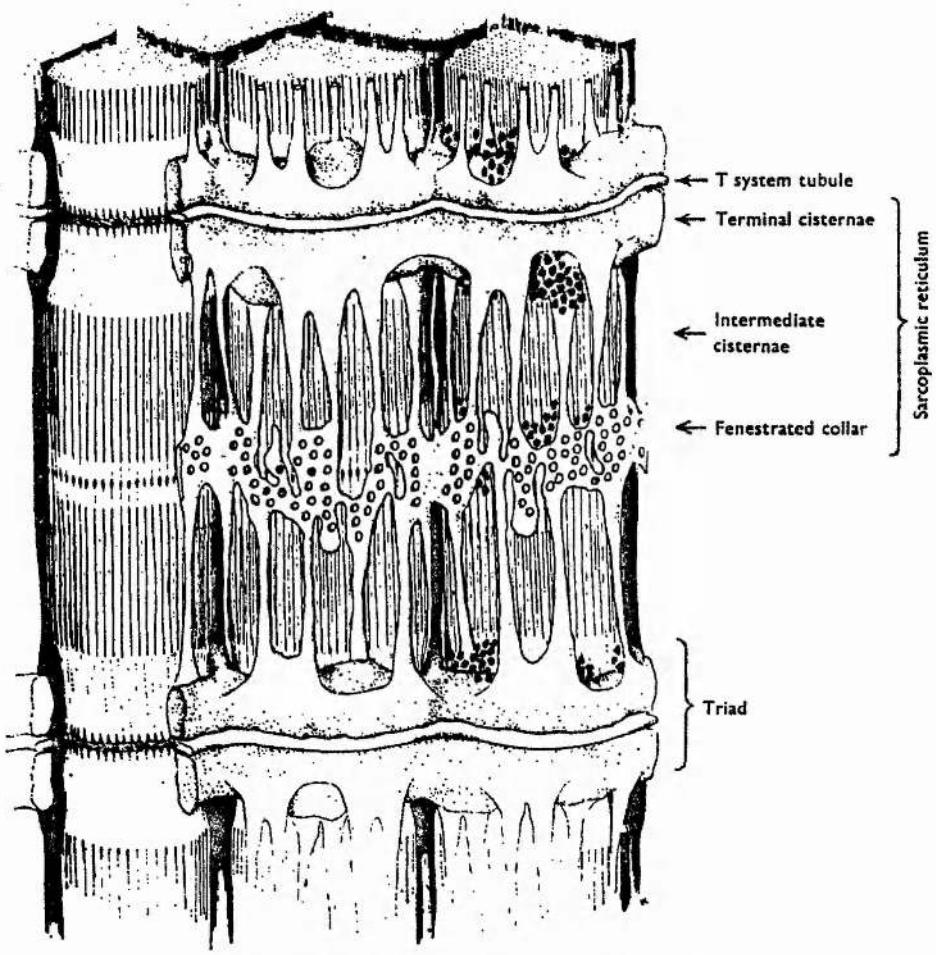
The sarcoplasmic reticulum (SR) is a membrane network found in the interior of skeletal and cardiac muscle cells. It was first seen under the light microscope by Dobie (1849) and then by Thin (1874). In 1902, Veratti performed a very careful study, in which he described the SR, but it was not clearly visualised until the advent of the electron microscope. (e.g. Bennett and Porter, 1953; Porter and Palade, 1957; Huxley, 1957, 1964; Andersen-Cedergren, 1958; Peachy, 1965). Essentially, the SR is a fine reticular structure of membrane limited elements (Franzini-Armstrong, 1973). There are many similarities between the sarcoplasmic and endoplasmic reticulum in other cell types (Franzini-Armstrong, 1973). For example, the interior of both membrane systems is separate from the cytoplasm, the lumen is continuous over large areas of the cell, the components of the system are not separated from each other, and the contents are probably in equilibrium. Both SR and ER have large surface area to volume ratios and both the SR and ER are continuous with and/or homologous to the nuclear envelope (Porter and Palade, 1957; Ezerman and Ishikawa, 1967; Franzini-Armstrong, 1973).

Under the electron microscope (EM), the SR can be seen to be divided into repeating segments, which recur apparently in register with the striations of the muscle. At regular intervals, the SR forms dilated sac like processes known as the terminal cisternae or terminal sacs. Associated with these, are the units of the T-system, or transverse tubular

system, which form "triads" or "diads", depending on whether there are two or one SR process per T-tubule. Where these triads or diads occur in relation to the striations of the muscle varies, depending on the species and muscle type concerned. For example, they occur at the level of the Z-line in (i) frog sartorius (Peachey, 1965), (ii) tail muscle of some amphibian larvae (Porter and Palade, 1957), and the white muscle of certain fish species (Franzini-Armstrong and Porter, 1964), and at the level of the A-I band junction in (i) lizard (Robertson, 1956), (ii) rat (Porter and Palade, 1957), (iii) toadfish swim bladder (Fawcett and Revel, 1961). (For further examples, see Martonosi (1971).) In frog, there also appears to be an intermediate flattened cisternal system between the triads and the longitudinal tubules of the SR (Peachey, 1965).

Opposite the H-zone, the SR elements fuse to form a flattened cisternae, which is oriented crosswise similarly to the lateral sacs of the triads. These are called the central cisternae. Characteristically, they have small pores, or fenestrae of diameter 150 - 200Å. Their function is unknown. Peachey (1965) coined the term "fenestrated collar" to describe this aspect of the system. For a three dimensional reconstruction of a muscle fibre showing the SR, see Fig. I.1.

Although the function of the cisternae is uncertain, the triads have been subjected to much speculation as to function. The local activation experiments of Huxley and Taylor, in the middle 1950's (Huxley and Taylor, 1955, 1958) established the T-system as the inward conducting mechanism



The internal membrane systems of a frog sartorius muscle fibre. (From Peachey, 1965.)

Fig. 1:1 A three dimensional reconstruction of a frog sartorius muscle fibre, showing the internal membrane system

of the muscle membrane, so presumably, the SR terminal cisternae are involved in excitation contraction coupling. The actual method of involvement is still uncertain, but recent work by Adrian, Chandler, Chalmers and others suggests that T-system membrane bound charge groups move during the action potential, initiating a movement of groups in the SR, allowing Ca^{2+} to be released from the SR.

Marsh (1951, 1952) suggested that there was a factor in skeletal muscle which was capable of reversing the super-precipitation of actomyosin induced by the addition of ATP or Ca^{2+} ions to the suspension. Portzehl (1957) found that if a relaxing factor extract is centrifuged at high speed for some time, the resulting activity is confined to the precipitate, which is particulate in nature, and the super-natant is without relaxing activity. Electron microscopy of the precipitate showed that it consisted of small membrane bound vesicles, which presumably derived from the sarco-plasmic reticulum (Nagai, Makinose and Hasselbach, 1960). It was later shown that the relaxing factor was capable of accumulating Ca^{2+} ions (Ebashi and Lipmann, 1962; Ohnishi and Ebashi, 1963, 1964; Hasselbach and Makinose, 1961, 1963) and had a Ca^{2+}-dependent ATPase which seemed to be the same as that described by Kielley and Meyerhof in 1948.

Ca^{2+} seemed to be the most likely ion to mediate the excitation-contraction process, with Jöbsis and O'Connor (1966) and Ashley and Ridgeway (1970) providing the first direct evidence for the involvement of the ion. However, Sandow (1965) calculated that, if all the Ca^{2+} for the E-C* coupling came from the external medium, then the influx

* excitation - contraction

demonstrated in toad sartorius by Bianchi and Shanes (1959) was much too small to cause activation. Further, Hill (1948, 1949) showed that the diffusion time from the exterior of the fibre was too great to account for the observed time to peak twitch tension. All these problems can be overcome if the sarcoplasmic reticulum acts as an intracellular Ca^{2+} store, and as a source of calcium ions during the excitation process. Constantin et al (1965) and Winegrad (1968, 1970) demonstrated directly that Ca^{2+} was located in the sarcoplasmic reticulum, and it is now accepted that the SR does mediate contraction and relaxation in vertebrate skeletal muscle.

Molecular Structure of the Sarcoplasmic Reticulum

The SR is a comparatively simple membrane. It consists of approximately seven proteins in a lipid matrix. Attached to the outside of isolated vesicles are particles of approximately 35Å in diameter, connected to the membrane surface by somewhat thinner stalks (Ikemoto et al, 1968, Inesi and Asai, 1968). Several lines of evidence indicate that these projections, and the inner membrane particles visualised by freeze etching (Scales and Inesi, 1976) correspond to the ATPase protein. Firstly, both these features appear in parallel with ATPase activity in developing muscle (Baskin, 1974; Tillack et al, 1974; Sarzala et al, 1975, 1979; Holland and MacLennan, 1976). Secondly, both ATPase activity and the projections are affected adversely by trypsin digestion (Inesi and Scales, 1974; Stewart and MacLennan, 1974), and thirdly, both the projections and the inner

particles appear in purified phospholipid vesicles following incorporation of pure ATPase protein. The number of the inner particles is only a third to a quarter of the outer projections. It has been suggested that the projections on the outer surface of the membrane represents individual ATPase molecules, which form oligomers in the hydrophobic core of the membrane (Scales and Inesi, 1976; Inesi, 1979).

The Proteins of the Sarcoplasmic Reticulum

Using immunofluorescent techniques, Jorgensen et al (1979) have established the in vivo position of the different proteins in rat sarcoplasmic reticulum. High concentrations of both the ATPase and calsequestrin were found in fast twitch fibres, and lower concentrations of both in slow twitch. The ATPase appeared to be uniformly distributed throughout the SR, while calsequestrin was mainly found in the terminal cisternae. This correlates well with the observations of Winegrad (1968, 1970) mentioned above.

Membrane proteins may be divided into two classes, the intrinsic and the extrinsic proteins. Those proteins which remain attached to the membrane after disruption are termed the intrinsic proteins, and those which are loosely associated, and are removed by the washing treatment, are called the extrinsic proteins. Margreth et al (1974) using ultrasonication and osmotic shock found that, in rabbit white muscle, only the ATPase protein could truly be called an intrinsic protein. There is some evidence that the extrinsic proteins, calsequestrin, the high affinity protein and the acidic proteins are bound to the membrane by divalent salt bridges (MacLennan, 1975).

The ATPase Protein

This is the largest of the SR proteins, with a molecular weight of approximately 100,000 (MacLennan, 1970; Ikemoto et al, 1971; Meissner et al, 1973).

MacLennan et al (1971) have determined the amino acid composition of the protein. They report that there are about 900 residues per mole, approximately 20% of which are acidic - either glutamic acid or aspartate. These hydrophilic amino acids may, they suggest, be involved in binding the Ca^{2+} ions to the membrane. Trypsin digestion of the SR for a short period ($\sim$ 10 min. at 25°C) causes an inhibition of Ca^{2+} uptake and an activation of the ATPase. Incubation with the proteolytic enzyme for longer periods results in a loss of ATPase activity. The loss of ATPase activity is accompanied by a loss of the granular appearance of the vesicles (Inesi and Asai, 1968; Ikemoto et al, 1968).

Several groups have attempted to characterise the protein through trypsin fractionation. Brief exposure of the SR to trypsin in the presence of 1M sucrose causes the ATPase to be degraded into two subfractions with molecular weights of 52,000 - 60,000 (fragment I) and 45,000 to 50,000 (fragment II) (Migala et al, 1973; Thorley-Lawson and Green, 1973, 1975; Inesi and Scales, 1974; Louis et al, 1974; Stewart and MacLennan, 1975; Stewart et al, 1976). The larger fragment contains the phosphorylation site, and probably the calcium binding sites. Both fractions can be nearly completely purified, using a variety of methods. It is found that the smaller fraction is somewhat more hydrophobic than the larger, and possibly corresponds to that part of the enzyme

that is buried in the membrane leaflet (Thorley-Lawson and Green, 1973; Rizzolo et al, 1976; Stewart et al, 1976).

Further proteolysis of fragment I led to the formation of two sub-fragments Ia and Ib. Fraction Ia was demonstrated to contain the ATPase active site, while fragment Ib seemed to have a Ca^{2+} ionophore activity (Stewart et al, 1976). Experiments with anibodies led MacLennan (1975) and Stewart et al (1976) to suggest that the active site of the ATPase is located on the membrane surface, while fragments Ib and II are buried in the membrane, and only poorly exposed at the membrane surface. Abramson and Shamoo (1978) have inserted the 45,000 subfragment into a black lipid membrane, and have clearly established that it can function as a non-selective divalent cation ionophore. They demonstrated that there are either one or two relatively inaccessible disulphide bonds which are essential for ionophoric activity. From their results, they suggest that the 45,000 and 55,000 fragments lie in series in the membrane.

Associated with the protein is a high lipid content (450-650 ug/mg protein) (MacLennan et al, 1975; Bertrand, Yu and Masoro, 1975). MacLennan and Holland (1975) suggest that the lipid composition associated with the ATPase is the same as that of the bulk phase. It is thought that the protein has about 30 phospholipid molecules per molecule associated with it as an immobilised annulus (Hesketh et al, 1976; Warren et al, 1975). Recent evidence suggests, however, that this may not be the case. Madden et al (1979) were able to introduce cholesterol into SR vesicles, and they noted that the ATPase activity decreased in inverse proportion to the amount of cholesterol incorporated. This inhibition

was completely reversible, indicating that the cholesterol was not affecting the ATPase protein directly. Cholesterol is known to restrict the movement of disordered fatty acyl chains of pure phospholipid dispersions or phospholipids in biological membranes at physiological temperatures (Ladbrooke et al, 1968; de Kruyff et al, 1971) and so, from these results, Madden et al suggest that either the lipids do not form an annulus around the ATPase, or if they do, then the lipids are freely permeable to cholesterol. Warren and co-workers (1975) had achieved contrary results, and as yet, no satisfactory explanation has been found to explain the difference between the two groups.

Other studies have also produced results that are inconsistent with the concept of an annulus (Martonosi et al, 1968; Dean and Tanford, 1977; Chapman et al, 1979). For example, Dean and Tanford (1977) have demonstrated that virtually full enzymatic recovery can be obtained after replacing almost all of the membrane phospholipids with suitably high concentrations of non-ionic detergents. In addition, Martonosi et al (1968) were able to reverse the inhibitory action of phospholipase on the SR ATPase and Ca^{2+} uptake by incubating with either lecithin, lysolecithin or phosphatidic acid (see also Hidalgo et al, 1976).

Moore et al (1978) have measured the ATPase activity and the "microviscosity" of partially delipidated ATPase preparations. The ATPase activity when, plotted against temperature as an Arrhenius plot, ahows a transition temperature. Plotting the activity between 20 and 40°C gives a straight line, parallel to that describing the change in

microviscosity. They suggest that this is supportive evidence for membrane fluidity being the rate determining factor in Ca^{2+} ATPase activity in the physiological temperature range. However, decreasing the lipid:protein ratio results in a decrease in specific activity, even before the critical value of 30:1 is reached. This suggests that perturbations in the bulk lipid can affect the protein, and mitigates against the idea of a lipid annulus. In addition, Moore et al (1978) preformed a mathematical analysis of the consequences of the lipid annulus concept, and concluded that "while this model may offer a description of Ca^{2+}-ATPase-lipid interactions at low temperatures, it is probably an oversimplification at physiological temperatures."

Calsequestrin

In 1971, MacLennan and Wong isolated a protein with very high Ca^{2+} binding capacity, which they have named calsequestrin.

On SDS gels, it has molecular weight of 44,000 daltons. It is strongly anionic; 37% of the residues are glutamate and aspartate, and only 9% are basic amino acids. None of it is soluble in organic solvents, suggesting that, unlike the ATPase, no fatty acids or proteolipid moieties are associated with it.

The protein remains membrane bound throughout the preparation of the SR, and treatment with deoxycholate in the presence of KCl will quantitively release it. Sonication and chaotropic salt treatment does not extract it, so that it is probably fairly firmly bound to the membrane (MacLennan and Wong, 1971).

Ikemoto et al (1971, 1972, 1973, 1974) were also able to isolate and identify this protein, which they called the Ca^{2+} precipitating protein (CPP). However, they found that the molecular weight was not 44,000, as reported by MacLennan's group, but was 55,000. Meissner, Connor and Fleischer (1973) showed that with minor changes in the electrophoretic procedure, they could change the apparent molecular weight of their CPP from, in their case, 50,000 to 65,000 daltons. In 1973, harmony was restored at a congress in Warsaw (Drabikowski et al, 1974). Ikemoto and his group had been given a sample of calsequestrin by MacLennan, and compared it's properties with their CPP (Ikemoto et al, 1973). They found that, using their electrophoretic procedure, MacLennan's protein had a molecular weight of 55,000 daltons, and its behaviour was in all respects, virtually identical to the CPP. MacLennan et al (1972) had followed Ikemoto's preparative procedure, because Ikemoto had reported different protein bands in the gel electrophoresis characterisation that he had performed, and said that, if they used their own electrophoretic method, they could see no difference between the two protein profiles.

Calsequestrin was calculated to be theoretically capable of binding up to 1270 nMol Ca^{2+}/mg pure protein, and an experimental value of 970 nMol/mg has been achieved (MacLennan et al, 1972). To determine whether the protein is capable of sequestering the Ca^{2+} which is taken up during the relaxation process, the following calculation may be made. In intact SR, calsequestrin comprises 7% of the total protein. 0.07 mg of calsequestrin can bind 89 nMol of Ca^{2+}. Thus, 1 mg total SR protein can bind 89 nMol of Ca^{2+}, if calsequestrin

is assumed to be the only binding protein. Since observed binding is never in excess of 80 nMol Ca^{2+}/mg protein, (Ebashi 1976; Sandow 1970) it may be seen that calsequestrin may account for all the *in vivo* anionic binding (MacLennan and Wong 1971; MacLennan and Holland 1975).

In the presence of physiological salt concentrations, the dissociation constant is about 800μM (Ostwald and MacLennan 1974). If the SR Ca^{2+} concentration is less than 1μM, then the protein would not bind significant amounts of Ca^{2+}, However, if the protein were located on the interior of the membrane, then the interior Ca^{2+} concentration (postulated to be 10-20μM MacLennan and Wong, 1971; MacLennan and Holland, 1975; Sandow, 1970) would be high enough to saturate the binding sites.

Thus, it is necessary to determine whether the protein is, in fact located on the interior. Evidence concerning this is equivocal.

It is not readily susceptible to trypsin digestion, and extraction procedures are such as to involve disruption of the membrane. MacLennan *et al* (1972) attempted to locate the protein using lactoperoxidase, with little success. King and Louis (1974) also used this procedure, and concluded that the protein is located on the inside of the membrane. Katsumata *et al* (1976) found that the CPP was unaffected by fluorescent probes attached to the membrane, and concluded that the protein was probably located on the inner surface.

In contrast, Hasselbach and his group (Hasselbach and Migala, 1975; Hasselbach, Migala and Agostini, 1975) considered that the protein was located on the exterior of the membrane. Using the fluorescent compound "Fluram", they

showed that the specific fluorescence of the calsequestrin did not change with increasing membrane disruption, indicating that the protein must be located on the outside of the vesicles. In order to explain the trypsin indigestibility, they suggest that the calsequestrin is located in crypts in the membrane surface, which have a diameter of 30A. Since the protein would be only 30% saturated at a Ca^{2+} concentration of 1μM, they consider it unlikely that it plays a significant role in Ca^{2+} storage.

In order to elucidate what happened when the Ca bound to the protein, Idemoto has used a technique called circular dichroism. This gives a measurement of the α-helical content of the protein. The result of such experiments is to show that the α-helical content increases dramatically, upon interaction with divalent cations.

Since the addition of Ca^{2+} and other cations will also cause quantitative changes in the spectral absorbance and fluorescence, the amount of change can also be estimated. This gives an order of affinities of the various ions, which was found to be as follows:-
$La^{3+} > Zn^{2+} > Cd^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+} > Sr^{2+} > K^{+}$. These results can be seen to agree with those of Carvalho and Leo (1967) who had performed binding studies on complete SR.

Since the affinities of the CPP and the intact SR are so similar, it would tend to indicate that the protein is involved in the <u>in vivo</u> storage of Ca^{2+} ions.

In addition, Meissner and co-workers found that in reconstituted functional vesicles, in the absence of calsequestrin and/or a precipitating anion, such as oxalate, Ca^{2+}

binding is only 25% of normal, suggesting that the calcium precipitating protein is important in Ca^{2+} binding.

The High affinity Ca^{2+} binding Protein

Preliminary descriptions of this protein were first given by MacLennan's group in 1972 (MacLennan et al, 1972). They noted that treatment of SR with DOC (deoxycholate) resulted in the isolation of five acidic proteins (MacLennan and Wong, 1971; MacLennan et al, 1972).

The five proteins were the three acidic proteins, calsequestrin, and a high affinity Ca^{2+} binding protein. Ostwald and MacLennan (1974) estimate the molecular weight of this protein to be 55,000 ± 1,000.

The isolation technique involves initial solubilization with DOC, and dialysis against Tris and EGTA for 22 hours. Centrifugation removes insoluble protein, and elution through DEAE cellulose columns results in the initial purification of the protein. Further purification was achieved by ultrafiltration and after further dialysis, the final yield is found to be about 1 mg from 800 g of wet muscle. Maximum binding amounted to 450 nmol/mg protein, equivalent to about 25 Mole Ca^{2+}/mole protein, and the dissociation constant is about 120μM. In the presence of 0.1 M KCl, the high capacity binding constant shifts to 5,000μM, but significant binding could be observed in the range 1-10μM Ca^{2+}. At this concentration range, the protein bound 16-22 nmol/mg protein, 1 mol/mol with a dissociation constant of between 2 and 4μM.

This protein differs from calsequestrin in the following manner. Calsequestrin's binding capacity does not change with the introduction of 0.1 M KCl, but the dissociation

constant shifts from 60μM to about 800μM suggesting that there is no high affinity site on calsequestrin.

The calsequestrin has 32% acidic amino acids, and 13% basic amino acids. This may explain why the high affinity protein has less capacity for total binding than calsequestrin, with 37% acidic residues, and only 8% basic amino acids.

The function of the protein is again uncertain, but it is possible that it is involved in *in vivo* Ca binding and storage.

The Low Molecular Weight Acidic Proteins

These also appear to bind Ca^{2+} ions (Ostwald and MacLennan, 1974).

The 20,000 MWt protein, which may be equivalent to the protein described by Kirchberger *et al*, binds a maximum of 11,000 nMol/mg in the absence of KCl, with a K_d (dissociation constant) of 150μM, and in its presence, binds 580 n mol/mg, with a K_d of 780μM. This protein also appears to have a high affinity site, with K_d's of 12 and 26μM and capacities of 430 and 23 nMol/mg in the presence and absence of KCl respectively.

Proteins of 33-3800 MWt also have two sites, which are KCl dependent. The low affinity site changes its K_d from 115 to 1400μM, and its capacity from 920 nMol/mg, in the absence of 0.1 MKCl to 1,000 in its presence - a somewhat anomalous shift. The high affinity site behaves in a more conventional manner, with the K_d going from 7-55μM, and the capacity from 880 to 45 nMol/mg protein.

The Proteolipid

In 1972, MacLennan *et al* reported that a sugar and

phospholipid free protein, containing 150-200 nmol fatty acid/mg protein could be isolated from SDS prepared purified ATPase. The fatty acids are apparently covalently bonded. The SDS estimated molecular weight is about 6,000 daltons. This may not be its real molecular weight, because amino acid analysis reveals anomalous results. If the molecular weight is 6,000, then there must be fractional residues of both lysine and histidine. For there to be intact residues of these amino acids, a minimum molecular weight of 12,000 must be assumed.

The role of the proteolipid is uncertain. It does not act as an innophore (Shamoo and MacLennan, 1974). It was thought that the proteolipid may be involved in the interaction between the ATPase and the membrane.
However, after removal of the proteolipid, the ATPase still exists as a 102,000 dalton subunit and is still globular in appearance (MacLennan et al, 1972). It is possible that the subunit is required in order for the ATPase protein to insert into the membrane properly (MacLennan et al, 1972).

Lipid composition of the SR Membrane

The lipid composition of the SR membrane has been characterised in several species ranging from lobster to man (Madeira and Antunes-Madeira, 1976; Marai and Kuksis, 1973b; Takagi, 1971).

Phospholipids

Phospholipids constitute the majority of the lipid membrane, with estimates varying from 90% to 98% (Meissner and Fleischer, 1971, 1972; Bertrand et al, 1975). Of the relative amounts of the different species of phospholipids,

estimates vary considerably in absolute amounts, but bear a close qualitative resemblance. For example, phosphatidylcholine is generally recognised as being the most abundant phospholipid, but absolute amounts vary from 50% in embryonic chicken (Sarzala et al, 1979) to 65-75% in adult rabbit (Martonosi et al, 1968; Meissner and Fleischer, 1971, 1972; Marai and Kuksis, 1973a,b; Sarzala et al, 1979), 53-65% in man (Marai and Kuksis, 1973b; Takagi, 1971), 62% in rats (Marai and Kuksis, 1973; Bertrand et al, 1975), and between 30% and 58% in chicken (Martonosi et al, 1973; Marai and Kuksis, 1973b). Species and experimental differences are found in the estimates given for the other phospholipid classes, although qualitative agreement is generally very good (see Table 1:1).

The fatty acid contents of the different lipids also vary according to species and worker. The qualitative values are, however, in good agreement. In rabbit, 16:0 seems to be the most abundant fatty acid, while in humans it is present in somewhat lower concentrations than is 18:2 (Fiehn and Hasselbach, 1970; Takagi, 1971; Marai and Kuksis, 1973a; Madeira and Antunes-Madeira, 1976). 18:2 is the next most frequent fatty acid in all homeotherms studied, except in the report of Cossins et al (1978), who found that in rat, 18:0 was more frequent than any other species.

In poikilotherms the situation is somewhat less clear. It is generally accepted that, in order to adapt to lower cell temperatures, poikilotherms increase the unsaturation of their fatty acids. Madeira and Antunes Madeira (1976) show that, in lobster, the relative unsaturation increases as predicted, as it does in the Arctic Sculpin (Cossins et al,

1978). However, in frogs, the change in the distribution is not as clear (Fiehn and Hasselbach, 1970). Further studies must be performed before a clear statement can be made.

Originally, it was thought that phospholipid were not necessary for formation of the phosphoenzyme intermediate (Meissner and Fleischer, 1972; Martonosi et al, 1968). However, Knowles et al (1976) demonstrated that this was not the case. They showed that phosphatidylcholine was more effective in reactivating PE formation than was PE, or monogalactosyldiglyceride. They point out that Meissner and Fleischer (1971) and Martonosi et al (1968) showed their results by progressive treatment with detergents or bile salts or phospholipases. This protocol degrades only 60-90% of the phospholipids, and the amount of phospholipid necessary to reactivate formation of the intermediate is very small (Knowles et al, 1976).

Using lipid substitution techniques, Knowles et al (1976) demonstrated that phospholipids were essential for all the processes of Ca^{2+} transport. Three levels of specificity could be differentiated:-

(a) Phosphoenzyme formation, which is the least specific.

(b) ATPase activity, which cannot be supported by acetyl dilauryl phosphatidylethanolamine (ADPE), and shows greatest stimulation with PC alone.

(c) Calcium translocation, which cannot be supported by PC alone, and needs both PC and PE for maximal activity.

Neutral lipids

Estimates of the relative amounts of the different

lipids vary. According to Meissner and Fleischer (1972), cholesterol comprises only 25% of the neutral lipids. Martonosi (1971), on the other hand, considers that cholesterol comprises 5-8% of the total lipid content, making it the most concentrated neutral lipid present. Meissner et al (1972) state that triglycerides make up 60% of the neutral lipid. During development in rabbit, the cholesterol levels fall from 0.25 mg/mg protein to an adult level of 0.05 mg/mg (Sarzala et al, 1975). Cholesterol has a complicated effect on lipid membranes. At low temperatures, below the transition temperature, it acts to decrease the viscosity, while at higher temperatures, it decreases the fluidity. Increasing the relative amount of cholesterol in a membrane eliminates the phase transition as visualised in an Arrhenius plot (Ladbrooke et al, 1968; Moore et al, 1978; Chapman et al, 1979). Thus, depending on the amount of cholesterol in the different preparations, the different groups of workers could achieve very different values for ATPase and Ca^{2+} accumulating activity. For example, Headon et al (1977) have shown that the Ca^{2+}-independent ATPase found in SR preparations is almost entirely associated with the cholesterol containing membranes, and suggests that pure SR membranes are actually free from any cholesterol.

The Structures of the SR Lipids

Information on the physical state of the membrane lipids is most clearly obtained by direct methods of structural analysis, such as nuclear manetic resonance spectroscopy (NMR) and X-ray diffraction. Davis et al (1976) have shown using X-rays that even at temperatures as low as 1°C, only

3% of the lipids are in a crystalline array, and this value falls to less than 1% at 9°C.

More detailed information can be gained using proton NMR (Davis and Inesi, 1971; Davis et al, 1976). At low temperatures, data suggests that most of the molecules are in a state of restricted anisotropic motion which, by 35°C, has changed such that nearly all the phosphatidylcholine N-methyl groups and approximately one quarter of the hydrocarbon chain protons have acquired isotropic motion (McConnell et al, 1972; Davis et al, 1976).

Inesi (1979) has calculated the total membrane area occupied by phospholipids, assuming an area of one phospholipid molecule of 50Å, and considering the number of lipid molecules per gram protein. The value that he achieves is $1.7 \times 10^{14} \mu m^2$, which is less than sufficient to cover a monolayer of the corresponding membrane area (2.8 to $3 \times 10^{14} \mu m^2$). This result suggests one membrane layer (the outer) consists mainly of lipoprotein complex, while the other (inner) layer consists predominantly of a monolayer of phospholipid.

Table 1:1

Lipid	Species	% total lipid	Reference
Phosphatidyl choline	Rabbit	73	Meissner and Fleischer, 1972
		70	Madeira and Antunes-Madeira, 1976
		75	Sarzala et al, 1979
		72	Meissner and Fleischer, 1971
		65	Martonosi et al, 1968
		66	Marai and Kuksis, 1973a,b
		65	MacLennan et al, 1971
		67	Owens et al, 1972
		70	Sanslone et al, 1971
		71	Waku et al, 1971
	Rat	69	Martonosi et al, 1968
		62	Marai and Kuksis, 1973b
	Man	53	Marai and Kuksis, 1973
		65	Takagi, 1971
	Chicken	58	Marai and Kuksis, 1973b
		30	Martonosi et al, 1973

Lipid	Species	% total lipid	Reference
Phosphatidyl ethanolamine	Rabbit	13	Meissner and Fleischer, 1972
		12	Martonosi et al, 1968
		17	Madeira and Antunes-Madeira, 1976
		15	Sanzala et al, 1979
		13	Meissner and Fleischer, 1971
		19	Marai and Kuksis, 1973a
		17	Marai and Kuksis, 1973b
		19	MacLennan et al, 1971
		18	Owens et al, 1972
		18	Sanslone et al, 1971
		17	Waku et al, 1971
	Rat	15	Martonosi et al, 1968
		18	Marai and Kuksis, 1973b
	Chicken	25	Martonosi et al, 1973
		20	Marai and Kuksis, 1973b
	Man	17	Takagi, 1971
		24	Marai and Kuksis, 1973b

Lipid	Species	% total lipid	Reference
Phosphatidyl inositol	Rabbit	0	Martonosi et al, 1968
		9	Meissner and Fleischer, 1972
		0	MacLennan et al, 1971
		(9	MacLennan and Holland, 1975)
		tr	Madeira and Antunes-Madeira, 1976
		7	Sarzala et al, 1979
		9	Meissner and Fleischer, 1971
		11	Marai and Kuksis, 1973b
		9	Owens et al, 1972
		9	Sanslone et al, 1971
		2	Waku et al, 1971
	Rat	8.8	Marai and Kuksis, 1973b
	Chicken	0	Martonosi et al, 1973
		10	Marai and Kuksis, 1973b
	Man	9	Marai and Kuksis, 1973b
		9	Takagi, 1971

Lipid	Species	% total lipid	Reference
Phosphatidyl serine	Rabbit	2	Meissner and Fleischer, 1972
		7	Madeira and Antunes-Madeira, 1976
		3	Sarzala et al, 1979
		2	Meissner and Fleischer, 1971
		6	Martonosi et al, 1968
		11	MacLennan et al, 1971
		2	Owens et al, 1972
		2	Waku et al, 1971
		11% (Phosphatidyl serine and inositol measured together)	Marai and Kuksis, 1973a
		0-8	Marai and Kuksis, 1973b
	Rat	6	Martonosi et al, 1968
		3	Marai and Kuksis, 1973b
	Chicken	10	Martonosi et al, 1973
		1	Marai and Kuksis, 1973b
	Man	2	Marai and Kuksis, 1973b
		3	Takagi, 1971

Table 1:1. The relative percent of total lipids of the major phospholipid classes, for different species estimated by different groups.

The Mechanism of Ca^{2+} transport

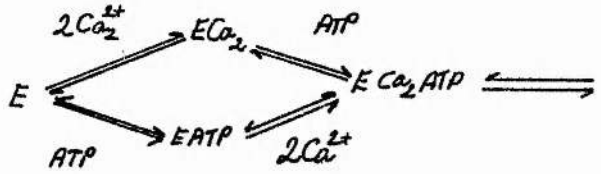
Pardee (1968a,b) has suggested that during ion transport, an enzyme must play at least three roles in a successive manner. First, it has to recognise, then translocate and then release the specific ion. In initiating transport, the carrier has to recognise and bind its particular substrate before all the other species present in the solvent system. It then has to transport the substrate across the membrane - generally thought to be the energy requiring step - before releasing it and returning to its original conformation.

Ca^{2+} ions will bind to the sarcoplasmic reticulum, and to the purified ATPase, replacing bound Mg^{2+} ions (Carvalho, 1974), even in the absence of ATP. Ikemoto (1974, 1975) showed that, at $0^{o}C$, there were three classes of binding site on the isolated ATPase, which he designated α, β, and γ. The β site, on increasing the temperature, converted to an α type of site, while the γ site appeared to be inhibitory. Ikemoto's observations supported Sumida and Tonomura (1974), who found that the molar ratio of Ca^{2+} transported : ATP hydrolysed decreased at low temperatures, becoming one at $0^{o}C$. The capacity of the SR for Ca^{2+} seems to be between 10 and 20µmol Ca^{2+}/gm protein, with a Kd of 0.5 to 2.5µM in the absence of ATP (Meissner, 1973; Meissner et al, 1973; Chevallier and Butow, 1971; Fiehn and Migala, 1971; Miyamoto and Kasai, 1976).

Assuming that the ATPase has a molecular weight of 100,000, and constitutes 60-80% of the total protein, then the protein binds approximately 2mol/mol ATPase (Meissner et al, 1973). This bears a striking relationship to the

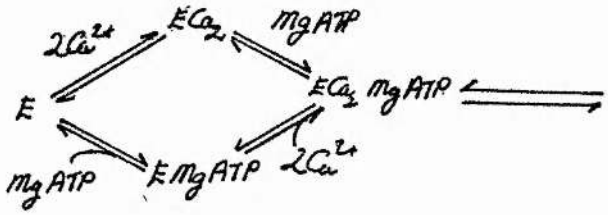
observed coupling ratio. Under a wide variety of conditions, and irrespective of the energy providing substrate, two ions of Ca^{2+} are transported per ATP hydrolysed (Weber et al, 1966; Hasselbach et al, 1973; Kurzmack and Inesi, 1977; Hasselbach, 1978).

Following, or preceding the binding of Ca^{2+} ions, ATP binds so that the first Michaelis-Menten complex is formed (Tada et al, 1978).



Scheme 1

This model is similar to that described by Inesi and Almendares (1968) and Kanazawa et al (1971). However, there is good evidence (Vianna, 1975) that the true substrate of the ATPase is MgATP, and not ATP, so that the scheme should be as follows:



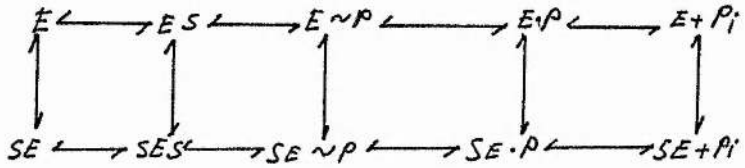
Scheme 2

Immediately following the formation of this complex, a phosphorylated enzyme intermediate (PEI) is formed. The absence of a lag phase, even at very low ATP concentrations, suggests that formation of the ECa_2ATP complex takes place on the outside of the membrane (Tada et al, 1978). Formation of the PEI complex can be prevented by the addition of large amounts of EGTA or ADP, adding further support to the idea that all these events take place on the exterior surface of the

leaflet (Tada et al, 1978).

The acid-stable phosphorylated enzyme intermediate was first described by Yamomoto and Tonomura (1967, 1968). It appears to be an acyl phosphate (Kanazawa, 1975), and one phosphorylation site is found per ATPase molecule (Meissner and Fleischer, 1971, 1973; MacLennan, 1970; Ikemoto et al, 1971; MacLennan and Holland, 1975). It has been shown that the phosphate is bound to an aspartyl residue (Degani and Boyer, 1973), which can be isolated in a seryl (or tyreonyl) phospho-aspartyl lysine tripeptide (Bastide et al, 1973). Several groups have shown that formation of the PEI complex is absolutely dependent on the presence of Ca^{2+} ions (Panet et al, 1970; Kurzmack and Inesi, 1977; Kurzmack et al, 1977).

An acid labile form of the intermediate has been postulated by Froehlich and Taylor (1975, 1976). Using rapid quench techniques, they found a transient decay followed by a secondary rise in labelled protein following the initial burst. After a lag, inorganic phosphate (Pi) production contained a burst of activity which coincided with the transient decay of the ^{32}P-protein. Froehlich and Taylor (1975) suggest that this burst was due to the formation of an acid labile form of the intermediate, E.P. Further, they produced evidence to show that the substrate must participate in the transport process other than simply as an energy donor. Therefore, they suggest that the intermediate reaction steps should be displayed as follows:



Scheme 3

Steps 1 and 2 have the same rate constants as 1' and 2'. $k_3:k_{-3} = 10s^{-1}:5s^{-1}$ $k'_3:k'_{-3} = 50s^{-1}:45s^{-1}$, $k_4 = 5s^{-1}$, $k'_4 = 10s^{-1}$. The binding constant for SE is assumed to be smaller than the constant for ES, so that, at high concentrations of S, the second pathway becomes more important, and the rate of EP decomposition and the steady state rate at each site increases. Further investigation required that a negative feedback loop be inserted, since the late rise in EP seen under some ATP and Ca^{2+} concentrations has the appearance of a damped oscillation:-

$$E + 2Ca^{2+} + S \underset{1}{\rightleftharpoons} ESCa_2 \underset{2}{\rightleftharpoons} E\sim PCa_2 \underset{3}{\overset{5}{\rightleftharpoons}} E.PCa_2 \underset{4}{\longrightarrow} E+Pi:2Ca^{2+}$$

Scheme 4

(see also Weber, 1971a,b).

In a series of papers analysing the effect of alkali metal salts on the ATPase, Shigekawa and co-workers (Shigekawa and Dougherty, 1978a,b; Shigekawa et al, 1978; Shigekawa and Akowitz, 1979) also suggest that there is more than one PEI species. The first of these, which they designate E_1P, is capable of reversal, and can transfer its phosphate group to ADP, generating ATP. The second species, called E_2P cannot do this. These observations may correspond to Froehlich and Taylor's (1975, 1976) observations on the movement of E P to E•P. However, there is no agreement on the rate constants proposed. For example, for step 3 in scheme 4, Froehlich and Taylor derive a rate constant for the forward reaction of $20s^{-1}$ ($k_{-2} = 10s^{-1}$). In contrast, Shigekawa and Akowitz give a rate constant for the step E_1P to E_2P of 5.24 min^{-1}. Several differences of technique may account

for these discrepancies. For example, Shigekawa et al (1978) admit that their vesicles could not transport Ca^{2+}, presumably because the isolation procedure had rendered them leaky. In addition, while Froehlich and Taylor were using rapid quench techniques capable of resolving millisecond responses, Shigekawa's group were using conventional pipetting methods, and extrapolating back to obtain their initial observations.

An alternative hypothesis has been mooted by Kanazawa et al (1971) and Sumida et al (1976). They suggest that the burst could be accounted for by assuming a transition of the dissociation constant (k_d). Sumida et al (1976) have fitted their results to the following equation:-

$$k_d = (k_{d,initial} - k_{d,steady}) \times \exp(-k_{tr} \times t) + k_{d,steady}$$

(see also the work of Shigekawa and colleagues), where $k_{d,initial}$ and $k_{d,steady}$ are the values of k_d at the initial and steady states of the reaction respectively, k_{tr} is the rate constant for transition in the k_d value during the initial phase, and t is time in seconds. The ratio of $k_{d,initial}$ to $k_{d,steady}$ was 7.3, and the value of k_{tr} was reported to be $0.5s^{-1}$ (Sumida et al, 1976). In addition, the time course of Pi liberation observed experimentally agreed well with the theoretically predicted results.

Tada et al (1978) consider this interpretation to be better than that of Froehlich and Taylor (1975, 1976) for three reasons viz. (a) the sum of the amounts of PEI formed and Pi liberated is equal to the amount of the decrease in ATP; (b) during the reverse reaction, the amount of ATP formed from PEI and ADP is equal to the amount of decrease in PEI, and (c) although the amount of the initial burst of Pi production

reaches 5 times that of PEI, the maximal molar concentration does not exceed the molar concentration of the ATPase protein.

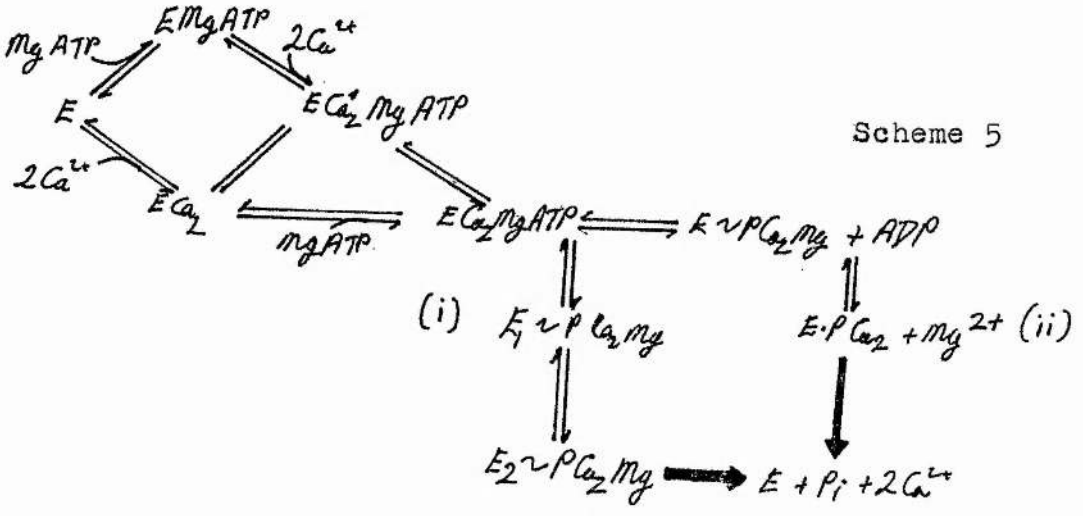
When the Ca^{2+} has been translocated into the interior of the vesicle, it must be released before the enzyme can return to repeat the cycle. This process is probably related to the decomposition of the PEI. Several groups have shown that Mg^{2+} ions are necessary for the decomposition of the intermediate (Martonosi, 1969a; Kanazawa et al, 1971; Panet et al, 1971; Inesi et al, 1974). When Mg^{2+} was removed after formation of the PEI, by adding EDTA, PEI decomposition did not stop immediately, suggesting decomposition was occurring in the interior (Kanazawa et al, 1971). Yamada and Tonomura (1972) have shown that Ca^{2+} competes with Mg^{2+} at the dephosphorylation site, and has the reverse effect. Under these circumstances, the relative affinities of Ca^{2+} and Mg^{2+} are 2.5:1, in contrast to the values they obtained for unsonicated vesicles, where the affinity for Ca^{2+} is 3,000 times that for Mg^{2+}. These results led Yamada and Tonomura to suggest that Mg^{2+} replaced Ca^{2+} when the site was translocated into the interior, with a concomitant breakdown of the PEI. Froehlich and Taylor (1975) argued that it was not necessary to postulate a conformational change to account for the affinity change. Instead they suggested that it was the change in phosphorylation state that caused the decrease in affinity. They do not, however, explain how the translocation process takes place in their model.

Yamomoto and Tonomura (1977) have investigated the incorporation of trinitrophenyl complexes into the ATPase when held in different conformational states. 2,4,6-trinitrobenzenesulphonate reacts with lysine residues to give

fluorescent trinitrophenyl complexes. These can be measured, and the number of lysine molecules exposed to the surface of the membrane may be estimated. Thus, 2,3,1, and 3 moles of lysine were exposed when the enzyme was in the states E, ECa, EATP and EP respectively. They suggested that the protein was rotating in the membrane, and that this conformational change was responsible for the transport of Ca^{2+}.

Considerable evidence exists to show that the final step in the translocation process, EP → E + Pi, is the rate limiting step. Early evidence was provided by Panet et al (1971), who found that the ATP-ATPase dissociation constant was equivalent to the Michaelis constant for ATP. Many other workers have analysed the rates of formation of the PE complex, and found that it greatly exceeds the rate of Pi production (Martonosi et al, 1974; Hasselbach and Suko, 1975; Froehlich and Taylor, 1975; Kurzmack et al, 1977; Inesi et al, 1978; Inesi, 1979).

In summary, therefore, the Ca^{2+} transport mechanism may be represented as follows with pathways (i) and (ii) representing alternative hypotheses.



Scheme 5

Recently, Inesi and co-workers have performed a thorough analysis of the partial reactions of the Ca^{2+} transport (Inesi et al, 1978; Verjovski-Almeida et al, 1978; Chaloub et al, 1979; Chiesi and Inesi, 1979; Coan et al, 1979; Verjovski-Almeida and Inesi, 1979). Using stopped flow and rapid quench techniques, this group have followed enzyme phosphorylation, inorganic phosphate production, H^+ liberation and Ca^{2+} transport on a millisecond timescale. On the basis of their observations, they suggest that the following scheme described the sequence of events that occur during Ca^{2+} transport:-

$$E + 2Ca_{out} \longleftarrow E.2Ca_{out} \quad (1)$$

$$E.2Ca_{out} + ATP \xleftarrow{(Mg^{2+})} ATP.E.2Ca \quad (2)$$

$$ATP.E.2Ca_{out} \xleftarrow{(Mg^{2+})} (ADP).E\sim P.2Ca_{out} \quad (3)$$

$$(ADP).E\sim P.2Ca_{out} \longleftarrow (ADP)*E\text{-}P.2Ca_{in} \quad (4)$$

$$*E\text{-}P.Ca_{in} \longleftarrow *E\text{-}P + 2Ca_{in} \quad (5)$$

$$*E\text{-}P \xleftarrow{(Mg^{2+})} *E.P_i \quad (6)$$

$$*E\text{-}P_i \longleftarrow *E + P_i \quad (7)$$

$$*E \longleftarrow E \quad (8)$$

$K_1 = 10^6 M^{-1}$, k_2 and k_{-2} are assumed to be 2 to $3.10^7 M^{-1}s^{-1}$ and $200s^{-1}$, k_{-3} k_3 = 85 to $150s^{-1}$, k_4 k_6 = 6 to $12s^{-1}$, $K_5 = 10^3 M^{-1}$ and $K_7 = 10^3 M^{-1}$ (Chiesi and Inesi, 1979). Other steps that may be included are the release of a proton when the phosphate is liberated, and the possible feedback inhibition of the transport system by accumulated Ca^{2+} ions (Verjovski-Almeida et al, 1978).

In essence, the scheme is similar to that proposed by other groups, and may be considered as an amalgam of the best aspects of these models.

CHAPTER 2

A COMPARISON OF THE PROPERTIES OF THE SARCOPLASMIC RETICULUM OF RED AND WHITE MUSCLE FROM THE TROUT, *S.gairdneri*

A COMPARISON OF THE PROPERTIES OF SARCOPLASMIC RETICULUM FROM RED AND WHITE MUSCLE OF THE TROUT, S. GAIRDNERI

Introduction

In the trunk muscles of fish, the different fibre types are arranged in anatomically discrete regions. This arrangement lead to the first description of different muscle fibre types in vertebrates by Lorenzini, in 1678. There are several early studies on the anatomy, histology and chemical composition of fish red and white muscle (Stirling, 1885; Arloing and Lavocat, 1875; Patton, 1898; Green and Green, 1913). In the majority of fish, the red muscle is found in a triangle running under the lateral line canal.

Greer-Walker and Pull (1975) examined 84 species of fish, and found that the red fibres occupied between 0 and 29% of the total musculature. Boddeke et al (1959) found that the amount of red muscle reflects the life style of the animal. Thus, the best swimmers, the "stayers", had the most red muscle, and the "crawlers" the least.

Red muscles are multiply innervated, do not conduct action potentials, and hence are comparable to the tonic muscle of amphibia. They differ, however, in having very high mitochondrial content, nearly approaching that of the mammalian ventricle (Bossen et al, 1978; Bone, 1978; Johnston, 1980). The remaining bulk of the musculature consists of white fast twitch muscles which rely mainly on anearobic glycolysis for their energy requirements. In

some species, the carp for example, fibre types other than the red and white have been described (Johnston et al, 1977).

The separation of the fibre types allows electrodes to be inserted selectively into either the red or white muscle, and the muscle function to be studied electromyographically (Rayner and Keenan, 1967; Grillner, 1974; Hudson, 1973; Johnston et al, 1977; Johnston and Moon, 1980). There appears to be an orderly recruitment of muscle types. At slow swimming speeds, only the red muscle is active. The threshold speed for recruitment of the white fibres varies from species to species, and with body size. Estimates vary from 0.5 body lengths/sec (Bone et al, 1978) for carp, 1.5 lengths/sec for trout (Johnston and Moon, 1980) and 4.5 lengths/sec for the bluefish Pomatomus saltatnix (Freadman, 1979). Table 2:1 summarises the main differences between red and white muscle in fish.

Teleost muscle fibres are not arranged in an orderly array as they are in mammals, but rather in a series of complex helices, which allow constant shortening at different body flexions (Bone, 1966; Alexander, 1969). They also differ from mammalian muscle in that instead of joining onto rigid bone through tendons, fish fibres insert into myosepta. Thus, it is very difficult to isolate either whole muscles or single muscle fibres, and experiments using strips of muscle are very difficult to interpret in terms of the twitch characteristics.

One exception to the above is the adductor operculi muscle. It is a mixed muscle, and it is possible to isolate fibre bundles which consist of either red or white

muscle (Flitney and Johnston, 1979). The white muscle has several unusual characteristics; firstly, it is capable of generating graded tetani over a considerable range of stimulation frequencies, and secondly, external Ca^{2+} ions are required for the generation of tension. In the slow muscle, single stimuli elecit no response, suggesting that the red muscle fibres are incapable of conducting action potentials, but as in the white, multiple stimulation result in graded fused tetani. In summary, the properties of the red muscle are similar amphibian tonic muscles, but the white muscle differed in its requirement for external Ca^{2+} and in its ability to produce graded tetani.

Morphological studies have shown that, unlike mammalian muscles, the relative volumes of SR are not dissimilar in fast and slow muscles. For example, SR forms 4.9% of the slow fibre volume in the elasmobranch Etmopterus spinax, and only 6% of the fibre volume in the fast muscle (Kryvi, 1977). Nag showed that the relative volume of SR in the red muscles of rainbow trout (Salmo gairdneri) was nearly half that of the fast muscles; 5.1% as opposed to 13.7% (Nag, 1972). Similar results were found for the marine teleost Pollachius virens (Patterson and Goldspink, 1972), the tench eye muscle (Kilarski, 1973), and the hagfish (Korneliussen and Nicolaysen, 1973).

For the reasons given above, no studies have been performed on the relaxation rate of red and white fish muscle. Thus, it is not possible to say whether the similarity in SR fractional volume is reflected in a similar rate of relaxation. However, assuming that the relative

concentration of pump sites is the same, and that the SR surface area to volume ratio is the same in both fibre types, then the rates of relaxation may be similar. There is some evidence to support these assumptions; for example, the SR occupies nearly 80% of the fibre volume in the bat cricotyroid (Revel, 1962) and the lobster antenna remoter muscle (Rosenbluth, 1969), which are two of the fastest muscles studied, and in the frog sartorius, the SR volume is only about 12% (Peachey, 1965). Josephson (1976) has gathered all the data available, and plotted relative volume of the SR against the $t_{\frac{1}{2}}$ for relaxation. Although there is considerable scatter in the points, there is a clear correlation between SR relative volume and the speed of the muscle.

This chapter examines the characteristics of SR isolated from red and white muscle of Salmo gairdneri, and compares them with each other and with data on other species obtained from the literature.

Materials and Methods

Trout (Salmo gairdneri) (weight ~600g) were obtained from the North East Fife Fish Farm, and kept in tanks of recirculating aerated fresh water at 10°C until use.

The fish were killed by stunning and decapitation. The muscles were rapidly excised, with care being taken to avoid contamination with other muscle types. The muscle was chopped with scissors, then homogenised in 4 vols. (w/v) of 0.3M sucrose. 10mM imidazole, pH 7.3, using an Ultra-Turrax blade homogeniser, at three quarters full speed, for 3 x 30s. Myofibrils and cellular debris were pre-

cipitated by centrifuging at 2,500g for 30 min. Centrifuging at 15,000g removed mitochondria from the supernatant, which was then spun at 95,000g for 1.5h, to precipitate the microsomal fractions. The microsomes were resuspended at a protein concentration of 5mg/ml in isolation buffer, and were used within three hours of isolation.

For those results obtained for the 30/35% sucrose fraction, the pellet was layered onto a discontinuous sucrose gradient as described ~~in Chapter 4~~ on page 74.

ATPase activity was measured in a medium containing 60mM KCl, 5mM $MgCl_2$, 5mM Na azide, 40mM imidaxole, pH 7.2, and either 0.3mM EGTA and 0.3mM $CaCl_2$ (free Ca^{2+} concentration = 8.78μM) or 1mM EGTA. For experiments determining the K_{Ca} for Ca^{2+}, the free Ca^{2+} concentration was derived using an iterative computer programme. Reactions were started by adding ATP (final concentration 2mM) and terminated with the addition of an equal volume of 10% TCA.

Ca^{2+} uptake was measured in the same medium (containing 0.02μCi/ml $^{45}CaCl_2$), except that 5mM K oxalate was included, using a Millipore filtration technique.

Gel electrophoresis was carried out using the method of Weber and Osborne (1969) on 7.5% gels.

For further details, see the Materials and Methods section of Chapter 4.

Ca^{2+} uptake into cryostat sections was measured by the method of Mabuchi and Sreter (1978). Blocks of muscle (approx. $1mm^3$) were mounted on cryostat chucks and deep frozen rapidly in iso-pentane cooled in liquid nitrogen. They were allowed to warm up to the same temperature as the

cryostat (approx -18°C) before being cut. About 10 10μ sections were mounted on each cover slip and allowed to dry in air at 4°C for about three hours. Failure to allow adequate time for drying resulted in sections floating off the slides. Sections were incubated in incubation medium described above containing 0.02μCi/ml $^{45}Ca^{2+}$. The reaction was stopped by adding 5% acetic acid saturated with Ca oxalate.

The sections were washed in acetic acid - oxalate, air dried, and counted in a scintillation counter. Blanks were obtained both by incubating sections without ATP and by incubating cover slips without any sections. No difference was found between these two types of blank.

Results

Since the amount of red muscle that could be dissected from any one fish was small (approx 4 - 6gm), most of the experiments reported in this chapter were performed on the microsomal fraction of the muscle. 5mM Na azide was included in the incubation media to inhibit any ATPase activity by contaminating mitochondria or mitochondrial fragments.

Ca^{2+} uptake in both red and white muscle can be recorded in the homogenate. Capacities of the different muscles appears to be similar (Fig. 2:1), but initial uptake rates are higher in the white (Table 2:2).

Mabuchi and Sreter (1978) have described a method for measuring Ca^{2+} uptake in cryostat sections. Results obtained from this method are shown in Fig. 2:2 and Table 2:2.

Again, the capacities of the two muscles are similar, but the white muscle displays a significantly higher rate of Ca^{2+} accumulation (Table 2:2). In the membrane fraction, similar results are obtained, with white muscle accumulating Ca^{2+} at a rate of 32 ± 4nmol Ca^{2+} $mg^{-1}min^{-1}$, as opposed to the red muscle, which shows an uptake rate of 8 ± 1nmol $mg^{-1}min^{-1}$.

ATPase activity in the white muscle is also higher than the red. Sucrose gradient fractionation increases the Ca^{2+}-dependent ATPase activity of both red and white muscle, but does not change the relative activities (Table 2:3) with fast muscle ATPase activity still being higher than the slow. Figs 2:3 and 2:4 show the effect of Ca^{2+} on the ATPase activity of red and white muscle microsomes as a Hill plot. From this graph may be derived the K_{Ca}, that Ca^{2+} concentration which gives half maximal activation of the ATPase. Values for the K_{Ca} (0.21 - 0.29 μM) for both red and white muscle are similar, and resemble those found for the 30 - 35% fraction of other species when measured at the appropriate environmental temperature (see Chapter 6) (Table 2:3).

Although no conclusions can be drawn regarding the relative volumes <u>in vivo</u> of the sarcoplasmic reticulum of the different types of muscle, it is interesting to note that significantly more of the white muscle microsomal pellet than of the red is found in the 30 - 35% fraction (Table 2:3).

Polyacrilamide gels of the 30 - 35 fraction show considerable differences. The protein composition of this fraction was analysed rather than that of the microsomal pellet because it was felt that the higher levels of mito-

chondria in the red muscle would lead to greater contamination of the pellet by mitochondria and mitochondrial fragments, resulting in an artefactual distortion of the protein composition. There is very little, if any Ca^{2+} pump protein in the red muscle (Fig. 2:5), but more protein of molecular weight 48,000 daltons. Table 2:4 gives the molecular weights of the major protein bands in the red and white muscle, those marked with an arrow in Fig. 2:5. It may be seen that there are considerable numbers of lighter bands in the red muscle. Attempts to phosphorylate the membrane proteins have not yet been attempted, so that it is not possible to say which of these proteins, if any, are involved in the Ca^{2+} transport cycle.

FSR was incubated in the presence of 20µg/ml Peak I, Peak II or beef heart protein kinase, 20µM ATP and 40µM cAMP (Pette and Heilmann, 1979) to see if the fish SR could be stimulated in the same manner as is rabbit slow muscle SR. The results are shown in Table 2:5.

Discussion

The differences between fast and slow muscle sarcoplasmic reticulum have been investigated extensively in mammals (Sreter, 1969; Fiehn and Peter, 1971; Harigaya and Schwartz, 1969, 1973; Margreth et al, 1972, 1973, 1974, Heilmann et al, 1977; Van Winkle et al, 1978; Van Winkle and Schwartz, 1978; Pette and Heilmann, 1977, Pette and Heilmann, 1979), using both native slow muscles and muscles artificially made slow by electrostimulation.

Comparing their data with that described in this

chapter shows very clearly that, while there are qualitative similarities between the slow muscles of fish and mammals, the fish muscle appears to be about twice as active as the mammalian. For example many workers on mammalian muscle have been unable to detect any Ca^{2+}-dependent ATPase activity in the slow muscle of rabbit and rat (Sreter, 1969; Margreth et al, 1972; Heilmann et al, 1977). In contrast, ATPase levels are nearly half of the white values (Table 2:3).

Mabuchi and Sreter (1978) have described a method for estimating Ca^{2+} uptake which involves a minimum of disruption to the muscle. Under these conditions, the fish red muscle is about half as slow as the white, while in the mammals, the slow muscle is only one fifth as fast as the fast muscle.

The difference in Ca^{2+} uptake rates is less in the fish than in mammals (Sreter, 1969; Harigaya and Schwartz, 1973; Margreth et al, 1973) (Table 2:2).

The K_{Ca} for the red and white muscles were similar, as has been found by workers on mammalian muscle (Fiehn and Peter, 1971; Heilmann and Pette, 1979).

Van Winkle and Schwartz (1978) suggest, on the basis of the Ca^{2+} binding data, that uptake proceeds in qualitatively the same manner in fast and slow muscle. The final amounts of Ca^{2+} bound were similar in all three muscles studied (70 - 100nmol Ca^{2+}/mg protein in the anterior tibialis, soleus and caudofemoralis of the cat), but the rates of binding varied from fast to slow muscle. In the caudofemoralis, binding was complete in 20 - 25 sec, while in the soleus, the binding required 60 - 75 sec. Rates of

uptake were also correlated with muscle speed. Caudofemoralis SR had the fastest uptake rate (1.8μmol $mg^{-1}min^{-1}$) and soleus the slowest (580nmol $mg^{-1}min^{-1}$). Similar results were obtained in the cat and rat soleus and extensor digitorum longus (Briggs et al, 1977).

These results are usually taken to mean that the SR from both red and white muscle transport Ca^{2+} ions in essentially the same manner. However, there are certain difficulties that must be resolved before this can be definitely stated.

Heilmann et al (1977) have performed a comprehensive study of the fast and slow muscles of the rabbit. They found that there was no band on their polyacrilamide gels which could be denoted the Ca^{2+} pump protein. Further, under the electron microscope, they could not find any of the 4nm projections which are thought to be the ATPase protein. In the soleus, two proteins, of molecular weights 47 and 57,000 daltons, phosphorylated in the presence of ^{32}P. Heilmann et al (1977) suggested that these proteins are involved in the transport of Ca^{2+} in the slow muscle. They were also able to subdivide the SR from the fast muscle into two fractions, the light one of which displayed similar characteristics to the slow SR, and they suggested that the fast muscle light vesicles accumulated Ca^{2+} in the same way as the slow, but the fast muscle had developed an additional mechanism which allowed it to be more efficient than the slow muscle.

Other groups have found that the E-P levels are much higher in the fast muscle, indicating that the concentration

of pump protein is much greater in the white than in the red (Sreter, 1969; Fiehn and Peter, 1971). However, these workers did not investigate whether it was the 100,000 dalton protein or some other component of the slow SR which was phosphorylated.

In summary, it is unclear whether or not the SR from red muscle transports Ca^{2+} in the same way as white. In the carp, the absence of a 100,000 dalton component seems to support Heilmann's contention that uptake in the slow muscle proceeds in a different manner from in the fast. Since uptake rates are generally $\frac{1}{2}$ to $\frac{1}{4}$ of the white muscle, it might be expected that the 100,000 component would constitute $\frac{1}{2}$ to $\frac{1}{4}$ of the density of that of the white. As may be seen in figure 2:5 and table 2:4, there is very little of the 100,000 dalton protein in the red muscle gels, certainly not enough to account for the observed uptake rates.

Recently, several workers have noted that slow muscle Ca^{2+} uptake can be stimulated by incubating in the presence of peak 1 protein kinase (Pk1) and cAMP (Heilmann and Pette, 1979; Pette and Heilmann, 1979).

However, Ca^{2+} uptake in fish SR is not stimulated by commercially available peak I, peak II or beef heart protein kinases (Table 2:5). The inhibition induced by the beef heart PK may be a function of the ammonium sulphate in which it was lyophilised.

There is some evidence that cAMP dependent protein kinases are not found in fish muscle. For example, while rabbit muscle phosphorylase kinase is activated or inhibited

by cAMP dependent protein kinases or specific phosphatases, the dogfish enzyme is dependent only on Ca^{2+} for its activity (Cohen et al, 1971; Fischer et al, 1975, 1978). The activation of lipolysis is also reported to be independent of cAMP dependent protein kinases (Farkas, 1969). It is possible, therefore, that the SR in fish can be modulated, but that the conditions thus far used have not been those required for stimulation of uptake.

Table 2:1 Differences between Fast and Slow Muscle Fibres in Fish.

Characteristic	Slow	Fast
Morphology	Stored lipid and glycogen	Glycogen stored, usually little lipid
	Myosatellite cells abundant	Fewer myosatellite cells
	Sarcotubular system usually less in volume than in fast fibres	Relatively larger sarcotubular system
	Z-lines broader than fast fibres in some cases	Z-lines usually thinner than in slow fibres
	Distributed cholinergic innervation	Focal or distributed cholinergic innervation
	Subjunctional folds usually absent	Subjunctional folds usually present
	Smaller diameter (20-25% of fast)	Larger diameter (may be more than 300μm)
	Well vascularised	Poorly vascularised
	Usually abundant myoglobin, red colour	No myoglobin, usually white
Electrical properties	Lower resting potentials than fast fibres	Higher resting potentials
	No propagated muscle action potentials, except under experimental conditions	Propagated muscle action potentials usual; may not always occur during activity of multiply innervated fibres
	Long-lasting contractions evoked by depolarising agents	Brief contractions evoked by depolarising agents
Contractile properties	Low activity Ca^{2+}-activated myofibrillar ATPase	High activity of enzyme
Energy supply	Abundant large mitochondria	Few smaller mitochondria with fewer cristae
	Oxidative enzyme systems	Enzymes of anaerobic glycolysis

Table 2:2 Ca^{2+} uptake by red and white muscle SR under different conditions. FSR = fragmented sarcoplasmic reticulum. 600g SN = 600 g supernatant. Values are the mean ± SEM of (n) observations.

	Ca^{2+} uptake (nmol Ca^{2+} $mg^{-1}min^{-1}$)		
Muscle	FSR	600g SN	Cryostat sections
Red	8 ± 1 (5)	13 ± 3 (4)	2.75 ± 1.1 (3)
White	32 ± 4 (8)	10 ± 4 (4)	5. 4 ± 1.0 (3)

Table 2:3 ATPase activities of FSR and purified sarcoplasmic reticulum (30 - 35% fraction - see chapter 4) from red and white trout muscle. The yield was calculated by dividing the total protein present in the 30 - 35% fraction by the total protein in the FSR. Results are the mean ± SEM of at least 3 observations.

Muscle	Fraction	ATPase activity (nmol $mg^{-1}min^{-1}$)			KCa(μM)	Yield
		Total	Basal	Ca^{2+} dependent		
Red	FSR	125 ± 5	80 ± 1	46 ± 8	0.24 ± 0.02	0.40 ± 0.07
	30 - 35%	147 ± 8	40 ± 3	107 ± 6		
White	FSR	187 ± 40	20 ± 2	169 ± 41	0.29 ± 0.02	0.60 ± 0.03
	30 - 35%	228 ± 44	0	228 ± 44		

Table 2:4 The Molecular weights of the major protein components of red and white muscle 30-35% sucrose fraction. The proteins referred to are marked by arrows in Fig. 2:5. Results are the mean ± SEM of 6 preparations. Molecular weight is in daltons ($x10^{-3}$)

Red FSR	White FSR
116.8 ± 0.2	146.5 ± 0.7
97.5 ± 0.8	94.0 ± 0.4
85.3 ± 1.0	84 ± 0.7
60.2 ± 1.5	58.2 ± 1.0
54.8 ± 1.2	44.6 ± 0.6
48.2 ± 0.6	38.0 ± 0.6
31.2 ± 0.7	30.8 ± 0.4 ($n=4$)
	19.8 ± 1.2

Table 2:5 Effect of protein kinase (20µg/ml), cAMP (40µm) and ATP(20µM) on Ca^{2+} uptake by red and white FSR of S. gairdneri. Results are expressed as a percentage of the control values. Values are mean ± SEM of (n) observations. p values * = n.s.d. ** =<0.01 *** =<0.001.

Condition	Red muscle	White muscle
Control	100	100
+ Peak I	93 ± 8 (5) *	81 ± 16 * (7)
+ Peak II	67 ± 9 (5) **	74 ± 23 * (5)
+ Beef heart	58 ± 3 (6) ***	30 ± 8 ** (7)

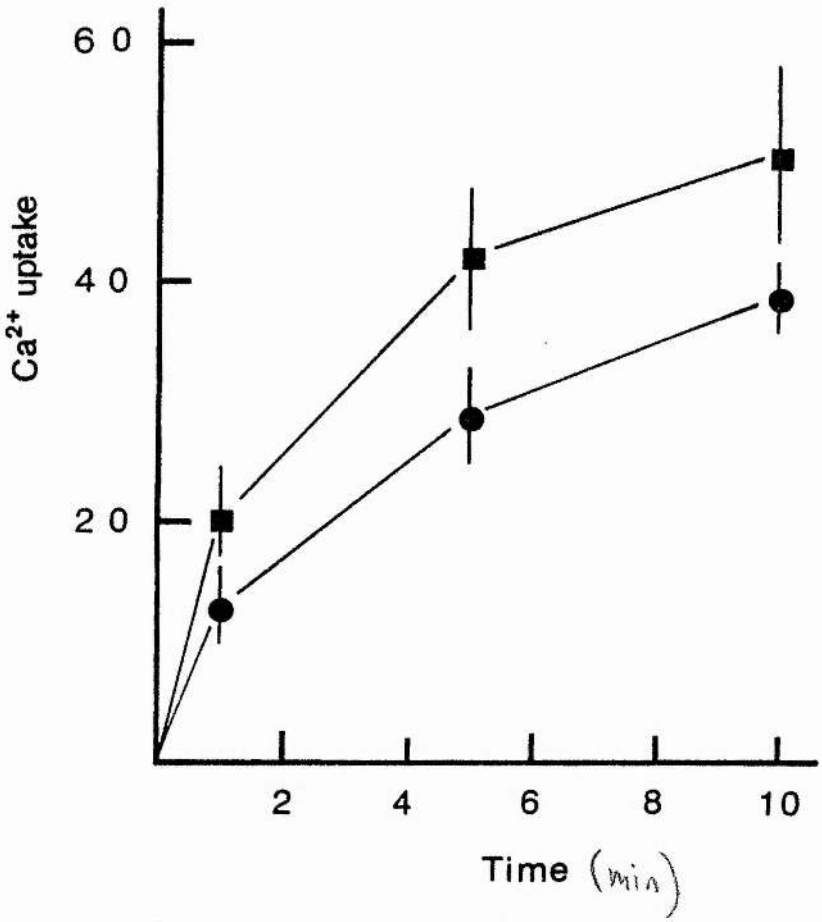


Fig. 2:1 Ca^{2+} uptake (nmol Ca^{2+}/mg protein) by red (●) and white (■) 600g supernatants. Values represent mean ± S.E.M. of 4 observations.

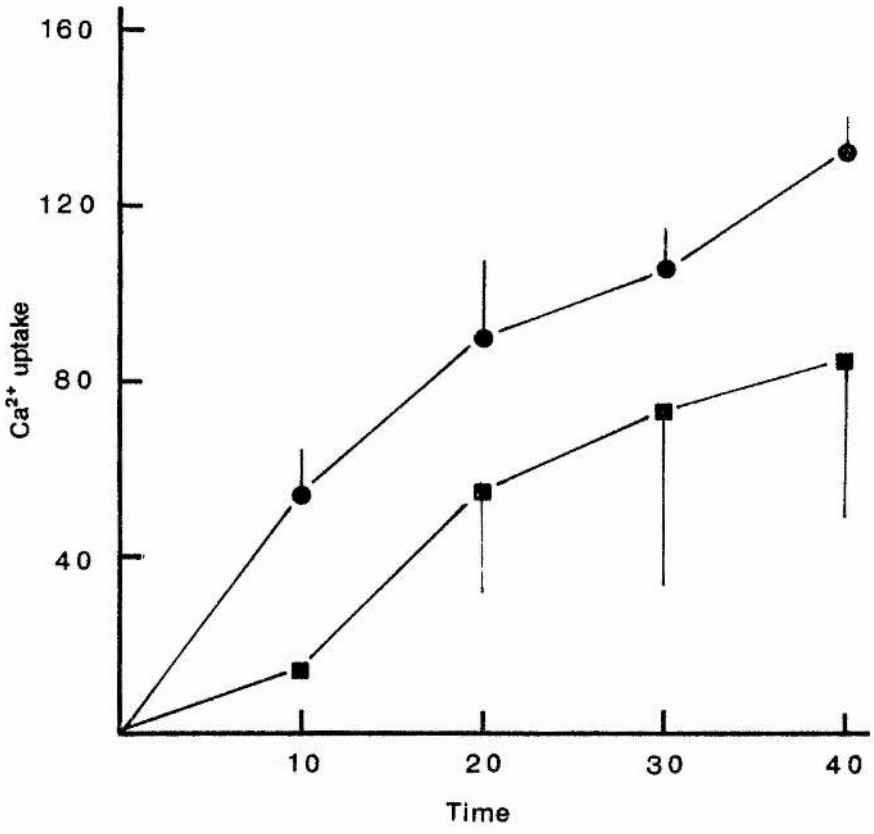


Fig. 2:2 Ca^{2+} uptake (nmol Ca^{2+}/mg protein) by red (■) and white (●) cryostat cut 10μ sections. Values represent the mean ± S.E.M. of at least 5 observations.

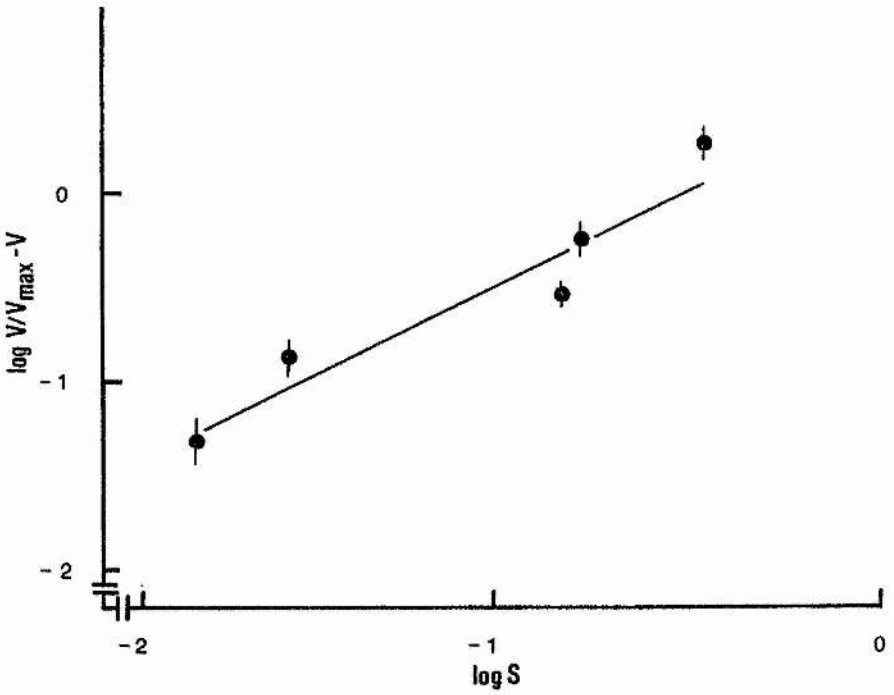


Fig. 2:3 A Hill plot of the Ca^{2+} dependent ATPase of red muscle microsomes. S, the free Ca^{2+} concentration, is in μM. Values represent the mean ± S.E.M. of three observations.

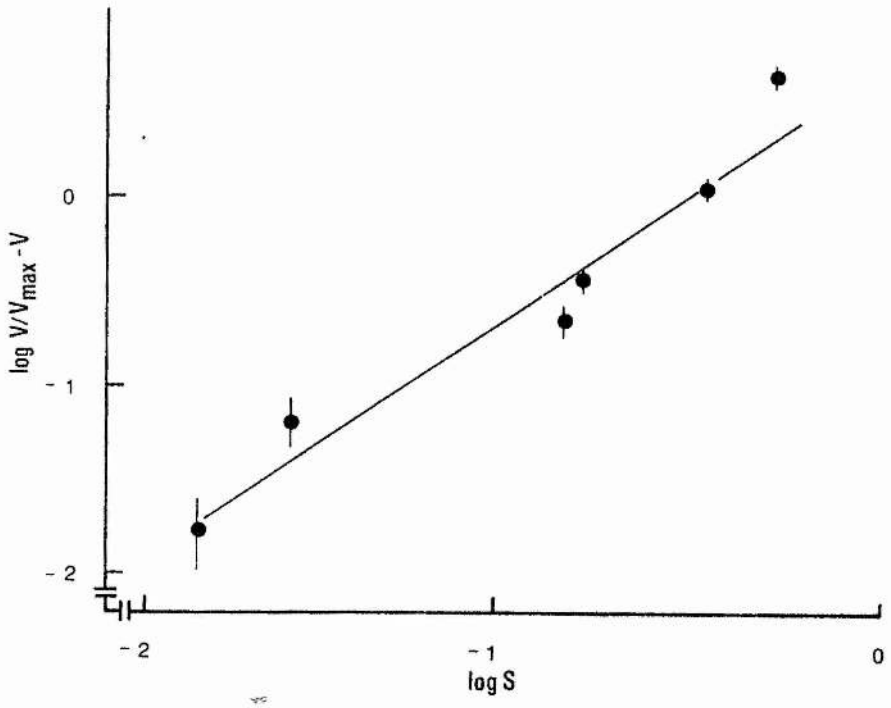


Fig. 2:4 The effect of Ca^{2+} on the Ca^{2+}-ATPase activity of white muscle FSR plotted according to the Hill equation. Values represent the mean ± S.E.M. of 6 observations.

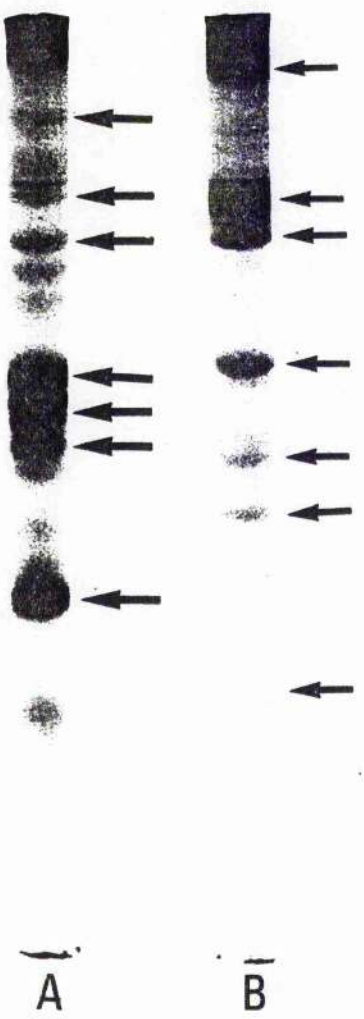


Fig. 2:5(a). 7.5% acrylamide gels of (A) red muscle and (B) 30/35% white vesicles. The arrows indicate the bands whose molecular weights are given in table 2:4. Only these bands which were found at significant levels in at least 75% of the gels were estimated. Thus, the low molecular weight band found in the red muscle, being present in only 2 out of 6 preparations, was not estimated.

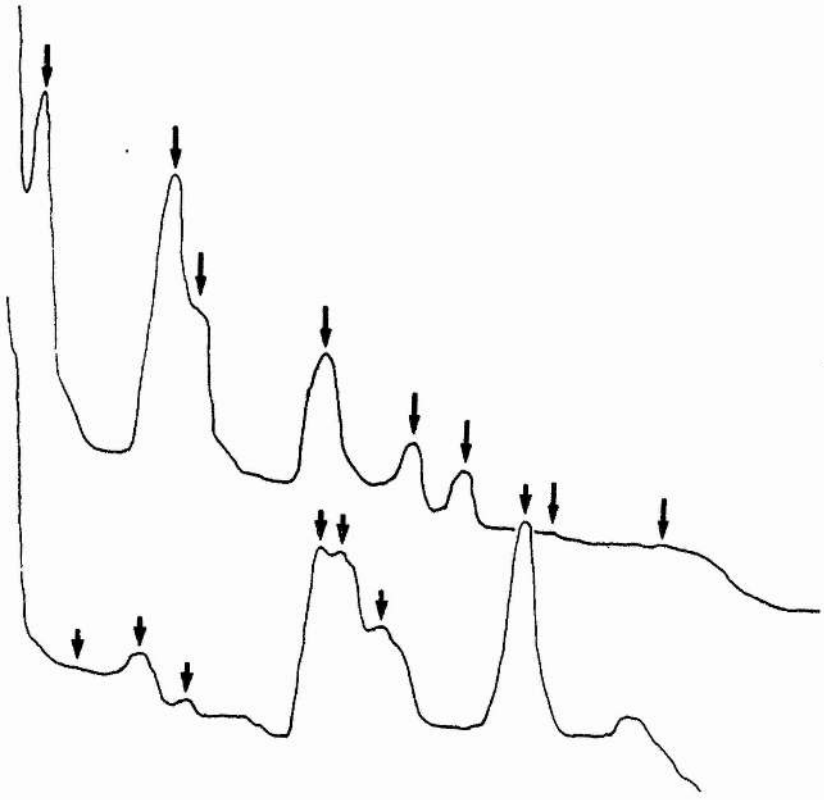


Fig. 2:5(b). A densitometric scan of the gels shown in fig. 2:5(a). The upper trace shows white muscle vesicles and the lower the protein composition of red muscle vesicles.

CHAPTER 3

TEMPERATURE ADAPTATION IN MEMBRANES

TEMPERATURE ADAPTATION IN MEMBRANES

Temperature affects the rate of all biological processes. Indeed, it has been estimated that if the average daily temperature prevailing at the surface of the earth were increased or decreased by only 20°C, all life would disappear (Florkin and Schoffeniels, 1969). Therefore, it is important for animals to become as temperature independent as possible. In homeotherms, temperature independence is achieved by maintaining the body temperature constant, while in ectotherms, the cellular and molecular processes have adapted to confer as great a degree of temperature independence on the animal as is feasible. Thus, despite displaying very different body temperatures, ectotherms from different thermal environments may display similar levels of physiological activity.

Thermal stress can be imposed over a variety of time periods. For example, a fish living in the Antarctic may have a body temperature of as little as -2°C, but this will change very little throughout the year. An animal living in African hot springs, at a temperature of about 40°C, will also experience little variation. On the other hand, some freshwater species experience annual temperature changes of as much as 30°C. Inter-tidal animals experience this kind of temperature change over a diurnal time period. These three types of stress often result in different responses. For example, in adaptation over the first of the three periods, evolutionary adaptation, there is evidence that adaptation in fish myosin ATPase occurs by selected modifications of the myosin light chains, while during seasonal adaptation, the

changes occur in the troponin complex (Johnston et al, 1977; Johnston, 1979).

In membranes, however, the response seems to be similar, irrespective of the nature of the time course of the stimulus. It seems that cold adaptation, whether occurring over an evolutionary time period or an acclimatory period, results in an increase in the unsaturation of the membrane fatty acids. This increase in the unsaturation of the membrane fatty acids results in a decrease in the membrane viscosity. In Escherichia coli grown at different temperatures, the fatty acid content of the membrane is manipulated in such a fashion as to maintain the membrane viscosity constant (Sinensky, 1974). Sinensky has termed this response the "homeoviscous response". Although, in eukaryotic cells, the primary goal of adaptation may not be homeoviscosity, there is little doubt that it is a significant factor in determining acclimatory responses.

Adaptation in Protista

In crustacean plankton, the iodine number varied inversely with water temperature, with colder temperature resulting in increased proportions of tetra-, penta and hexaenoic acids of the C_{20} and C_{22} fatty acids (Farkas and Herodek, 1964). This observation has been corroborated in many micro-organisms, for example, Chlorella sorokiniana, (Patterson, 1970), Pseudomonas fluorescens (Cullen et al, 1971), many Escherischia coli strains (see Cronan and Gelmann, 1975, for a review), Acholeplasma laidlawii (Steim et al, 1969; Engelman, 1970), and Tetrahymena pyriformis (Fukushima et al, 1976; Martin

et al, 1976).

A thermotolerant strain of Tetrahymena pyriformis, designated NT-1 by Fukushima et al (1976) was described by Phelps (1981). A comprehensive study of the mechanisms of adaptation of this species is of interest, since it is probably one of the simplest eukaryotic systems available. Varying the growth temperature altered the fatty acid saturation of the membrane. On cooling the cells, there was a lag in the growth. During this period, phase separation of the membranes occurred, which was visible under the electron microscope (Martin et al, 1976) as discrete aggregations of membrane particles. This phenomenon has also been reported by other groups working on other strains of Tetrahymena (Erwin and Bloch, 1963; Wunderlich et al, 1973; Nagel and Wunderlich, 1977). After the lag phase the cells started to grow again. Analysis of the membranes showed an increase in the unsaturation of the fatty acids and a change in the different amounts of phospholipids. For example, increasing the growth temperature increased the ethanolamine phosphoglyceride concentration, while the phosphatidylcholine concentration was unaltered (Fukushima et al, 1976). If the cilia were isolated and analysed, a different pattern was obtained, with the phosphatidylcholine levels increasing and the phosphatidylethanolamine levels remaining more or less constant (Fukushima et al, 1976). As stated above, chilling the cells resulted in an increase in the unsaturation of the fatty acids, with the relative percentage of γ-linolenic acid increasing most.

The increase in unsaturation of the membranes did not seem to be controlled by any alteration in the O_2 content of

the water (Skriver and Thompson, 1976), nor by the temperature as such (Martin et al, 1976), but by the membrane fluidity itself (Martin et al, 1976). Further support for this hypothesis was given by Nandini-Kishore et al (1977), who noted that the anaesthetic methoxyflurane, which has a reversible fluidising effect on the membrane, had a temporary inhibitory effect on the fatty acid desaturase system. In addition, cells grown isothermally with supplemented diets tend to resist the increased fluidity caused by the exogenously applied fatty acids by increasing de novo synthesis of saturated fatty acids and incorporating them into the membrane (Kasai et al, 1976).

Fukushima et al (1977) have produced evidence to suggest that the key enzyme in this acclimatory process may be palmitoyl coenzyme A desaturase, which is located on the endoplasmic reticulum of the cell. They suggested that the order of recovery of the different membrane fractions concurred with the idea of the new membrane being manufactured in the endoplasmic reticulum and then flowing out, with the oldest membrane being located in the cilia. Further support came from Kitajima and Thompson (1977), who noted that the endoplasmic reticulum was the first membrane system to recover from cooling, the recovery then spreading out, with the cilia the last to adapt.

Direct evidence that the acclimatory response was an increase in the membrane fluidity was produced by Martin and Thompson (1978), using the fluorescent probe DPH (1,6 - diphenyl hexatriene). However, Martin and Foyt (1978), in a companion paper, point out that the Tetrahymena response

does not support the "homeoviscous response" theory suggested by Sinensky (1974). Although the qualitative evidence of Kitajima and Thompson (1977) does support the idea, direct measurement of the "viscosity" of the membranes at their respective temperatures shows that the viscosity of the 39.5°C grown membranes at 39.5°C is far greater than their 15°C counterparts at 15°C. Thus, it seems likely that there are other factors than the viscosity which are more directly related to the observed in vivo separation temperatures and are under regulatory control.

Adaptation in Multicellular Animals

Adaptation in Organs

In multicellular animals, adaptation in membranes has been studied both in the organs and on the subcellular organelles of the species.

Liver fatty acids of cold acclimated frogs showed a greater degree of unsaturation than their warm adapted counterparts. Most of the increase was due to a rise in the relative percentage of polyunsaturated rather than monoenoic fatty acids (Baranska and Wlodawer, 1970). Hazel (1979) found the same trend in the liver of rainbow trout acclimated to 5°C and 20°C. In addition, Hazel (1979) found a significant decrease in sphingomyelin, phosphatidylserine, cardiolipin and unidentified phosphatides, no change in phosphatidylcholine (PC) or lysoPC, and a rise in phosphatidylethanolamine (PE) when the fish were cold acclimated. All the phospholipids with the exception of lysoPC experienced an increase in the relative proportion of their unsaturated fatty acids.

The increase in PE levels with cold acclimation has been observed in other systems (Roots, 1968; Miller *et al*, 1976), but is contrary to the decrease seen in *Tetrahymena* by Fukushima *et al* (1976).

Cold adaptation in the goldfish intestine results in an increase in the percentages 20:1, 20:4 and 22:6 fatty acids, while decreasing the amount of 18:0 and 22:3 recovered (Kemp and Smith, 1971). The relative amounts of PE and PC did not change on acclimation, although the change in 18:0 fatty acids was greatest for PE, as has also been noted in goldfish synaptosomes by Cossins (1977).

Friedlander *et al*, (1976) correlated physiological with behavioural acclimation in the goldfish. They found that the electrical activity of the Purkinje neurons changed in the same thermal range as the behaviour. Increasing the acclimation temperature conferred an increased resistance to thermal stress on the animal. Fish adapted to 5°C showed irritability and hyperreflexia after about 5 min exposure to water at 25°C, while fish adapted to 25°C had to be exposed to water at 32°C to elicit the same response in the same time. Local heating of the cerebellum produced the same effect as immersion of the goldfish in water of the same temperature. Using this data, and information on the neutral pathways presented by Kotchabhakdi (1976a,b), Friedlander *et al* (1976) constructed a circuitry model to account for the effect of temperature on the cerebellum of the goldfish.

Later work with Cossins (Cossins, 1977; Cossins *et al*, 1977) showed that there was a biochemical correlate of the observed changes. Cossins (1977) demonstrated that the

viscosity of synaptosomal preparations was less at all temperatures studied in the cold adapted fish than it was in the warm adapted animals. This change in viscosity was reflected in an increased unsaturation of the fatty acids of the phospholipids of the cold adapted fish synaptosomes. The changes in the fatty acid distribution was different for different phospholipids. For example, on transfer of the fish from 25 to 5°C, there was a fall in 18:0 in phosphatidyl ethanolamine, with a corresponding increase in the amount of 18:1 present, but no changes were observed in the fatty acid profile of phosphatidyl choline apart from a small but consistent increase in the proportion of 16:1.

Adaptation in sub-cellular organelles

Adaptation in Mitochondria

"Adaptations clearly defined at the organismic level should be analysed at the molecular level" (Schoffe-Neils, 1971). The rate of oxygen consumption shows thermal compensation in many species of fish, e.g. Carassius auratus (Smit et al, 1974), Salmo gairdneri (Evans et al, 1962), Xiphophorus helleri (Precht, 1962), Anguilla vulgaris (Jankowsky, 1966), and Rhodeus amarus (Kruger, 1962). This compensation has also been observed in the oxygen consumption of tissue homogenates in many species and organs e.g. in brain tissue of goldfish (Carassius auratus) (Freeman, 1950) and rainbow trout (Salmo gairdneri) (Evans et al, 1962), in muscle tissue of goldfish (Smit et al, 1974), swordtail (Xiphophorus helleri) (Precht, 1962) and bitterling (Rhodeus amarus) (Kruger, 1962).

Cold acclimation leads to an increase in the fractional

volume of the muscle occupied by the mitochondria in carp (Johnston and Maitland, 1980), and in two species of insect, Periplenata americana and Musca domestica (Thiessen and Mutchmor, 1967). Sidell (1977) noted that cold acclimation results in a decrease in the levels of synthesis of cytochrome c, but an even greater decrease in the levels of degradation of the enzyme. It is unlikely that there is any alteration in the relative concentration of cytochrome c, since Hazel, (1972a), Caldwell and Vernberg (1970) and Wodtke (1978) all agree that the protein:phospholipid ratio does not alter, irrespective of adaptation temperature. One of the major arguments against a strategy of quantitative change in response to environmental temperature stress has been that synthesis of increased amounts of enzyme at low temperatures would be "maladaptive", since such a response would place a large demand on the energy resources of the organism at a time when it could least afford it. However, Sidell's results indicate that the fish is able to increase the amount of enzyme while actually decreasing the rate of synthesis (Sidell, 1977). There is, however, a considerable body of evidence to indicate that the adaptation does have molecular as well as morphological correlates.

Pye et al (1976) have investigated mitochondrial respiration in the tench, Tinca tinca. They found that the rate temperature curves showed characteristic changes in slope which depended on the tissue, the substrate and the season. For example, in the winter, respiration was driven more efficiently at low temperatures by succinate in the liver, and by α-keto glutarate in the muscle. The reverse was true

at high temperatures in the summer in muscle, while in the liver, there was little difference in the efficiency of the substrates. There was no difference in the ADP:O ratios in either summer or winter muscle or liver mitochondria. An unusual adaptation was found in the tench muscle mitochondria in winter. Measuring the Km for succinate resulted in a sharp increase in the Km between 5 and 6°C. They suggested that the tench may have developed anaerobic mechanisms for winter respiration. This result is somewhat surprising in view of the increased oxygen solubility in cold water.

Adaptation to seasonal temperature changes does not appear to have any effect on the activation energy (Ea) of succinate oxidation in carp (Cyprinus carpio) (Wodtke, 1976, 1978), rainbow trout (Salmo gairdneri), (Smith, 1973a, 1973b, 1976, 1977) and goldfish (Carassius auratus) (Hazel, 1972a, 1972b; Van den Thillart and Modderkolk, 1978). There is, in addition, some doubt as to whether there is or is not a transition temperature in the SDH Arrhenius plot. Trout (Smith, 1977) and the channel catfish, Ictalurus punctatus (Lyon and Raison, 1970) have linear plots, while rat has a break in the line (Raison et al, 1971; Smith, 1977). According to Hazel (1972a), the goldfish plot is linear, but Van den Thillart and Modderkolk (1978) consider that there is a break in the line. Wodtke has recorded a transition temperature in mitochondria isolated from carp liver which alters depending on the animal's cell temperature (Wodtke, 1976, 1978), and Irving and Watson (1976) investigating adaptation in fish from different thermal environments also found transitions in the slope of the Arrhenius plot.

Although there does not appear to be any adaptive change in the Ea during acclimation, a comparison of Ea values for succinate oxidation from different species indicates that adaptation may have occurred over an evolutionary time course. For example, mitochondria from rat liver shows a break in the Arrhenius plot between 18.5 and 24°C, with an Ea of 19.5kcal/mol below the transition temperature, and values between 2 and 12kcal/mol above it (Raison et al, 1971; Smith, 1977). In contrast to rat, Arrhenius plots of the enzyme from liver are linear, and have Ea values between 8 and 12kcal/mol for trout (Lyon and Raison, 1970; Smith, 1977), goldfish (Hazel, 1972a), channel catfish, Ictalurus punctatus (Lyon and Raison, 1970), all of which experience approximately the same temperature regime. These values are considerably greater than the values of 4.4kcal/mol reported for the frog muscle enzyme (Vroman and Brown, 1963) and 3.7kcal/mol for Trematomus, an Antarctic fish (Somero et al, 1968). Irving and Watson (1976) investigated succinic dehydrogenase activity in several different fish species. In these fish, which experienced only a small annual temperature fluctuation, they found a transition temperature in the Arrhenius plot, and that there was no significant difference in the energy of activation of either SDH or MDH isolated from hot or cold adapted animals. However, rate compensation did seem to have occurred; with the hot adapted fish having higher specific activities than their cold adapted counterparts.

Smith (1977) has suggested that in acclimation, at least, changes in the activation entropy may have a greater adaptive significance than alterations in the activation

enthalpy.

Reactivation of the soluble enzyme (lipid-free) by mitochondrial lipid extracts showed that the cold adapted lipids were more effective, irrespective of the origin of the enzyme protein (Hazel, 1972b). Studies with purified phosphatides indicated that while the affinity of the enzyme for the lipid was largely dependent on the phosphatide species, the magnitude of the reactivation was dependent primarily on the degree of unsaturation of the fatty acid residues. This result has also been obtained in beef heart (Cerletti et al, 1967, 1969). The most effective reactivator (apart from the 5°C lipids) was found to be bovine brain phosphatidylserine, which contains highly unsaturated fatty acids in the No 2 position; 60% 22:6 and 8% 22:4 (Hill and Lands, 1970). This observation corroborates the observation of Caldwell and Vernberg (1970), who showed that the lipids from 5°C acclimated fish were more unsaturated than those from fish acclimated to 30°C.

Cold adaptation in the mitochondria follows approximately the same pattern as described for Tetrahymena. Irving and Watson (1976) found that the mullet, a cold adapted fish, had higher unsaturated fatty acid levels than a hot adapted fish, the coral trout. In acclimation, too, the same pattern has been observed. Cold acclimated carp have lower cholesterol: phospholipid ratios than warm acclimated animals (Caldwell and Vernberg, 1970; Wodtke, 1976, 1978). Phosphatidylcholine (PC) decreased on cold acclimation, with a concomitant increase in the phosphatidyl ethanolamine (PE) concentration. Cold adaptation in the carp led to an

increase in the unsaturated fatty acid concentration of both PC and PE, and a fall in the saturated fatty acid levels (Wodtke, 1976). The resulting increase in membrane fluidity may account for the decrease in transition temperature of the Arrhenius plot for succinate oxidation found when carp are cold acclimated (Wodtke, 1978).

Van den Thillart and Modderkolk (1978) found that the evidence for a straightforward increase in unsaturation of the fatty acids with decreasing acclimation temperature was somewhat equivocal. Although the fatty acids of the 5°C acclimated goldfish were more polyunsaturated than their 20°C counterparts, so too were the fatty acids of the 30°C acclimated animals, and the liver mitochondrial preparations of the 30°C fish were more unsaturated than even the 5°C group (Van den Thillart and Modderkolk, 1978).

An interesting phenomenon has been observed in the adult tuna (Thunnus thynnus). The tuna is a fish which, because of its extensive rete mirabile is able to maintain its muscle temperature at a higher level than the ambient water temperature (Carey and Teal, 1969; Carey et al, 1971). However, blood coming from the gills is virtually at ambient temperature. Thus, the tuna heart is in the unique position of receiving blood of two different temperatures; cold coronary blood into the outer compact layer, and warm venous blood perfusing the inner spongy layer. Maresca et al, (1976) have exploited this property to examine the thermal characteristics of mitochondria from the different layers of the heart. They found that the mitochondria from the inner, warm layer exhibit higher succinoxidase activity, higher

temperature dependence and higher NADH-oxidase activities than the mitochondria from the outer, cold adapted layer. Disrupting the mitochondrial membrane reversed this result, indicating that integrity of the membrane was essential for the functioning of the adaptive processes.

Apart from an increase in the amount of the enzyme, or a conformational change resulting in an alteration in the Km and Vmax values, adaptation can occur by altering the isozyme pattern of the enzyme. This has, in the past, been considered a fairly frequent method of adaptation, but recent evidence suggests that this may not be the case (Shaklee et al, 1977). In the green sunfish Lepomis cyanellus, thermal acclimation has been shown to occur. Out of the 15 enzymes studied, only the non specific esterases exhibited isozymic changes. Further, initial examination of phosphoglucomutase indicated that there were isozyme changes, but on increasing the number of observations, it was found that the difference arose from a genetic polymorphism of two alleles at the single PGM locus in these fish (Shaklee et al, 1977). Shaklee et al (1977) suggest that many of the previous observations that had been thought to be due to isozyme induction were, in fact, due to too small a sample being taken, and that the experimenters were merely seeing polymorphism of the enzyme.

In those fish with a polyploid ancestry, however, isozymes may play a more significant role (Shaklee et al, 1977). Isocitrate dehydrogenase (IDH) is a rate limiting enzyme in Kreb's cycle (Lehninger, 1970). Moon and Hochachka (1971a,b, 1972) have investigated the properties of this enzyme in rainbow trout. In this species, there appears to be three isoenzymes. In the cold adapted fish,

most of the enzyme is present as the slow moving isomer, while in the warm adapted animal, 95% of the enzyme is found in the fast moving band. Activation energies are similar for both warm and cold acclimated fish. In the warm adapted trout, the minimum Km occurs at 15°C, while in the cold adapted animals, the minimum occurs at about 5°C.

The enzyme has an absolute requirement for either Mg^{2+} or Mn^{2+} ions, with a Ka for Mg^{2+} between 3.3 and 3.8×10^{-5} for the cold enzyme, and between 2.3 and 2.6×10^{-5} for the warm enzyme. Moon and Hochachka suggest that alteration of the Mg^{2+} or Mn^{2+} concentration may act as the compensatory mechanism in temperature acclimation.

In 1972, Moon and Hochachka extended their investigations to a group of fish which appeared to have a considerable heterogeneity in their IDH phenotypes. There were 6 types, generated by 4 subunits, designated A,A',B and C. The A_2 type seemed to be temperature independent, but the other types acted conventionally, i.e. as the temperature increased, so too did the Km.

The substrate dependence of the Q_{10} value meant that compensation occurred normally in all the phenotypes except the A_2 one. The fact that the Q_{10} remained the same would seem to mitigate against survival in low temperatures for these fish. However, this does not appear to be the case, for they survive as well as the other types.

A possible explanation for this was indicated when it was observed that if the NADP or isocitrate concentrations were altered, independently, then the A_2 Km became temperature dependent. Thus, perhaps the changes in metabolite levels

may be more important to these individuals than to the others, who will rely more on the relative amounts of isozyme present. If this is so, then it may be that the A_2 individuals are better fitted for survival, because they will be able to cope with sudden changes in the environment, irrespective of the amount of enzyme present. Individuals with the multi-enzyme system will be relying on the amount of each type of enzyme present for adaptation to occur. Richmond and Zimmerman (1978) found that the supernatant MDH in Notropis lutrensis, the red shiner, also had different allozymic forms exhibiting similar temperature preferendia. McCorkle et al, (1979) found that winter acclimation in the channel catfish, Ictalurus punctatus, resulted in an increase in the levels of MDH in brain, gill, liver and muscle.

Hochachka and Lewis (1970) have investigated thermal acclimation of citrate synthase in trout liver. They used a purified preparation of the enzyme, and showed that the eurythermic enzyme was unusual, in that two kinetically distinguishable forms existed, one of which they designated the C (cold) type, and the other the W (warm) type. The Km - temperature profiles for both substrates, oxaloacetate and acetyl-CoA are similar for both enzyme types, except that at 10°C, the cold enzyme had a significantly lower Km for oxaloacetate than the warm type. However, the cold enzyme is more sensitive to an increase in temperature than is the warm. A consequence of the temperature dependence of the Km for the cold enzyme is that, at sub-saturating substrate concentrations, the enzyme becomes almost entirely temperature independent, with a Q_{10} of 1 at 0.025mM acetyl-CoA, and less than one at a concentration of 0.01mM. Unfortunately, since

the preparation is purified, it is not possible to say what modulating effects, if any, the mitochondrial lipids may have had. ATP inhibits both forms, and the effect that temperature has on the inhibition is similar in both enzymes. Several other enzymes of the citric acid cycle have been studied. Cytochrome oxidase levels increase on cold acclimation in brain, gill and muscle homogenates of goldfish (Caldwell, 1969) and in muscle and the suboesophageal ganglion of three species of Uca crabs from different thermal environments (Vernberg and Vernberg, 1968). Succinate- cytochrome-c reductase and NADH-cytochrome-c reductase activities also increase in the cold, and to a similar degree as cytochrome oxidase, suggesting that acclimation has occurred in the whole electron transport chain (Caldwell, 1969).

During hibernation similar changes are observed in the mitochondria. In both the ground squirrel (Platner et al, 1976) and the European hamster (Cremel et al, 1979) the amount of polyunsaturated fatty acids increases either just prior to or during hibernation, and then falls again in the spring, with the break temperature of the Arrhenius plot of protein release from the mitochondria falling from 29°C at the beginning of spring, to a minimum of 14°C at the end of summer (Cremel et al, 1979).

Adaptation of the Na^+-K^+-ATPase (NaK ATPase)

Many experiments have been performed on the Na K ATPase, isolated from different tissues. Charnock and Simonson (1978a,b) investigated seasonal changes in the Na K ATPase activity and the Mg ATPase activity of the renal cortex and brain from the ground squirrel, which is a hibernating animal.

They found that the properties of the Na K ATPase did not change, but that there was a decrease in the absolute amount during hibernation. They also found that there was an increase in the amount of the Mg ATPase, but that this could only be seen after the animal had been hibernating for about 75 days.

Lagerspetz et al (1973) found that the E_a for the Na K ATPase in the ventral nerve cord of Lumbricus terrestris decreased when the worm was acclimated to 14°C. For animals acclimated to 25°C, the E_a was 26kcal/mol, while in the 14°C acclimated animals, it was only 12kcal/mol. They suggested that enzyme induction might have occurred. In the frog (Rana temporaria) brain, there was no difference in either rate or temperature dependence of the microsomal NaK-ATPase, nor was there any correlation between the transition temperatures of the Arrhenius plots and acclimation temperature (Lagerspetz, 1977).

Kohonen et al (1973) have investigated temperature adaptation in two fish species. In a cold adapted fish, the roach, the NaK-ATPase activities from brain homogenates displays two temperature maxima, at 20° and 41°C. The gourami, however, shows one activity maximum, and is insensitive to temperature between 20 and 35°C. The Mg^{2+}-dependent ATPase displays similar properties, but the roach enzyme is less sensitive to temperature.

Smith and co-workers have studied acclimation in the intestinal NaK-ATPase of goldfish. Depending on the acclimation temperature, the relative amounts of ouabain-sensitive and ouabain-insensitive enzyme differs (Smith, 1967). The total

ATPase activity did not change with acclimation temperature, but the ouabain sensitive fraction of the enzyme was higher in the cold acclimated animals. The Arrhenius plots of the enzymes showed discontinuities that varied with the acclimation temperature of the fish. The activity of the NaK-ATPase was lower at all temperatures in the warm adapted fish, and the activation enthalpy appeared to be higher at low temperatures in the hot adapted animal. It is suggested that there are different forms of the enzyme in the fish adapted to different temperatures (Smith, 1967; Smith et al, 1968; Smith and Ellory, 1971). Double reciprocal plots of the effect of Na^+ ions on the activity of the enzyme give Km values that are very similar in both the hot and the cold acclimated fish. However, the Vmax for the cold adapted enzyme was considerably higher than the warm, suggesting that there was an increase in the amount of enzyme present in the cold acclimated fish (Smith et al, 1968). Similar results have been recorded in the rainbow trout gill and kidney (McCarty and Houston, 1977), steelhead (Salmo gairdneri) trout gills (Adams et al, 1973) and juvenile coho salmon (Zaugg and McLain, 1976).

The lipid changes which occur in the intestinal membrane during adaptation have been monitored by Miller et al,(1976). The proportion of PE increased on cold adaptation, with a concomitant increase in the levels of 22:6 and 20:4 fatty acids. There was no change in the relative amounts of the different lipid classes, with phospholipids accounting for 58%, neutral lipids for 26% and cholesterol for 17% of the total lipid. PE levels increased in the cold adapted fish,

but there is no apparent change in the levels of PC. Cold acclimation leads to an increase in the levels of unsaturated fatty acyl chains in both PE and PC, although the position of the changes depends on the phospholipid. For example, increases in the relative amounts of 22:6 in the cold adapted fish were found in both chains of PE, but only in chain 1 of the phosphatidyl choline.

A rather different situation exists in the eel. Acclimation to warm water results in an increase in the transition temperature of the gill NaK-ATPase Arrhenius plot (Thomson et al, 1975; Thomson et al, 1977, Sargent et al, 1975). In this case, the relative amounts of saturated mono- and polyunsaturated fatty acids are similar in cold, warm, sea and fresh water animals. Acclimating naturally caught sea and fresh water fish gives an interesting result; only those fish whose lipids are rich in (n-3) unsaturated fatty acids are capable of altering the transition temperature of the Arrhenius plots. Freshwater eels, with membranes rich in (n-6) unsaturates can only decrease the transition temperature by incorporating essential fatty acids (n-3) into the membrane. From their data, Thomson et al (1977) suggest that, since the membrane lipids are in a gel state at the fish's normal cell temperature, then the ions cannot be transported by rotation of the protein carrier in the membrane.

Adaptation in Amino acid Transport Processes

Together with his studies on the NaK-ATPase and adaptation, Smith has investigated the effect of temperature

on transport of amino acids across the goldfish intestine (Smith and Kemp, 1970).

After 20 days acclimation at 30°C, the rate of transfer of three amino acids, valine, methionine and phenylalinine had fallen from the 16°C value. The time course of the adaptation process closely paralleled the change in unsaturation, when measured by the docosahexaenoic/stearic acid ratio. Smith and Kemp suggest that, since it is only the amino acid transport which seems to follow the time course of acclimation demonstrated by the fatty acids, these results indicate an interdependence of amino acid transport and fatty acid composition of the intestinal membrane.

Adaptation in Cholinesterases

Acetyl cholinesterase (AChE) is an enzyme located on sub-synaptic membranes, involved in the degradation of acetyl choline, a neurotransmitter in many central nervous systems. It is unusual, in goldfish brain, at least, in that it displays inverse acclimation to temperature (Hazel, 1969; Hazel and Prosser, 1973). In the killifish, Fundulus heteroclitus, there is no adaptation to temperature, with the enzyme activity remaining independent of acclimation temperature (Hazel, 1969). Conversely, in rainbow trout, there appears to be different isozymes, which have minimal Km's at the animal's acclimation temperature. Thus, AChE isolated from the brain of a 17°C acclimated trout displays a minimal Km at 17°C, while that from the 2°C acclimated fish displays its minimum Km at 2°C (Baldwin and Hochachka, 1970). Although the slopes of the Arrhenius plots are not significantly different, there is a considerable degree

of rate compensation.

It seems, therefore, that there is some degree of uncertainty as to the extent of acclimation in AChE, and further studies are needed to clarify the problem.

Conclusions

In prokaryotic cells, the mechanism of adaptation to temperature is to alter the fatty acid unsaturation mechanism. Sinensky (1974) has produced evidence to suggest that the rational behind the mechanism is to maintain a constant membrane viscosity.

In the eukaryotes, the response to decreased cell temperature also involves increasing the relative amount of unsaturated fatty acids in the membrane. However, it does not seem to be "homeoviscosity" that is the primary goal of these adaptive changes. The evidence of Fukushima and coworkers suggested that the increases in unsaturation did not result in homeoviscosity in the tetrahymena, nor does it appear to do so in the SR or in the NaK-ATPase. In the SR, evidence has been produced to suggest that the bulk lipid phase may not be important in determining ATPase activity (Davis and Inesi, 1971; Davis et al, 1976), but other workers have found that increasing the unsaturation results in an increase in ATPase activity at any temperature (Hidalgo et al, 1976, 1978).

This may also be the case in mitochondria (Wodtke 1978) and the NaK-ATPase (Thomson et al, 1975, 1977), since from the Arrhenius plots the lipids may be in a gel state at the animal's normal cell temperature, suggesting that their physical state is not important in regulating activity.

Further work in this area would be of value not only in elucidating the mechanisms of adaptation, but also in providing information about the fundamental processes involved in the catalytic cycle.

CHAPTER 4

ISOLATION AND CHARACTERISATION OF SARCOPLASMIC RETICULUM FROM THE FAST MUSCLE OF TELEOST FISH

ISOLATION AND CHARACTERISATION OF SARCOPLASMIC RETICULUM FROM THE FAST MUSCLE OF TELEOST FISH

Introduction

A number of methods have been described for the isolation of sarcoplasmic reticulum (SR). Most of these methods involve homogenisation of the muscle to disrupt membrane components, which subsequently reseal to form microsomal vesicles. Larger cellular components (e.g. mitochondria, myofibrils, nuclei) are removed by low speed centrifugation. The supernatant contains microsomes which consist, in fast muscle at least, primarily of sarcoplasmic reticulum vesicles.

Early workers found that their SR preparations contained a high proportion of a myosin-like protein which was thought to be an integral part of the membrane. Subsequently, Uchida et al (1965) demonstrated that the presence of the protein was a function of the preparation method. Myosin free SR can be obtained by isolating the membrane in sucrose (Uchida et al, 1965) or by washing the SR in 0.6M KCl (MacLennan, 1970).

The microsomal vesicles can be collected by high speed centrifugation. The resulting pellet is generally referred to as fragmented sarcoplasmic reticulum, and has been used by numerous workers for their studies (Weber et al, 1966; MacLennan, 1970; Huddart et al, 1974; Price, 1976). Further purification of the crude microsomes involves centrifugation on sucrose gradients of varying complexities and densities (Frank and Sleator, 1975; Inesi et al, 1970; Meissner, 1974).

Heterogeneity in the microsomal pellet has been noted by several different groups of workers (Inesi et al, 1976; Meissner, 1975; Meissner and Fleischer, 1973; Seraydarian and Mommaerts, 1965). Seraydarian and Mommaerts were able to obtain microsomal fractions sedimenting between 15-41,000g and 41-150,000g which could be further subdivided by sucrose density centrifugation. The 15-41,000g pellet separated into two fractions, the lighter of which demonstrated high "sarcotubular activity", and kept its activity for several weeks in the cold, while the heavier seemed to consist of the same material in a less well preserved state. The 15,000g pellet could be resolved into three subfractions. The lighter ones displayed "relaxing activity"in terms of inhibition of the myofibrillar ATPase, while the heaviest fraction activated the enzyme. All fractions showed Ca^{2+}-ATPase activity, but the heavier fractions accumulated Ca^{2+} ions less efficiently, suggesting loss of stored Ca^{2+} by leakage.

Meissner (1975) isolated two populations of vesicles from a purified preparation of rabbit SR. The "light" vesicles were obtained from the top and the "heavy" vesicles from the bottom of a linear sucrose gradient (25-45% w/w). Each fraction accounted for about 15% of the total protein. The remainder of the vesicles were located between these two fractions, and were termed the "intermediate" fraction. In addition to the Ca^{2+} pump protein, the heavy vesicles contained two other major protein components; the Ca^{2+} binding protein, and the M_{55} protein, which accounted for 20-25% and 5-7% of the protein of these vesicles respectively. The vesicles formed phosphoenzyme levels proportional to their Ca^{2+} pump

levels, but displayed similar Ca^{2+} ATPase activities (.97 and 1.28 umol/mg/min respectively). The intermediate vesicles displayed intermediate properties. Using electron microscopy, Meissner (1975) located electron dense material in both the heavy vesicular fraction and in the terminal cisternae of the intact muscle fibres. He suggests that the heavy vesicles may be derived from the terminal cisternae, and the light fractions from the longitudinal tubules. The distribution of the Ca^{2+} binding protein appears to support this theory, and also to substantiate the hypothesis of Winegrad (1968, 1970), in that the greatest amount of the protein is found in the vesicles which appear to derive from the terminal cisternae. Thus, the Ca^{2+} is taken up from the myoplasm into the longitudinal SR, diffuses to the terminal cisternae, and is stored there, ready for release.

Lau and co-workers (Caswell et al, 1976; Lau et al, 1977; Lau et al, 1979) have isolated T system membrane from rabbit muscle, using the microsomal pellet as a starting material.

Headon's group (Headon et al, 1977) have shown that it is possible to separate the Basal from the Ca^{2+}-dependent ATPase by isopyonic centrifugation.

It seems evident, therefore, that some further purification of the microsomal pellet is necessary, both to avoid the presence of contaminating subcellular organelles, and to standardise the fraction which is subjected to the experimental procedures. In this chapter, I consider the ATPase activities of various fractions isolated by sucrose gradient centrifugation, and partially characterise those fractions

which appear most suitable for further investigation.

Materials and Methods

Fish

Plaice (*Pleuronectes platessa*; length~35cm) and cod (*Gadus morhua*; length~50cm) were caught by local fishermen from the Firth of Forth. Prior to use, they were maintained for up to 1 week in tanks of filtered recirculated sea water at 10°C. *Notothenia rossii* (length~25cm) were caught by light trap at South Georgia (British Antarctica) and transported to the U.K. in tanks of recirculating sea water at 2°C. The *Notothenia* were kept for up to a year in tanks regulated to 2°C. Unless otherwise stated, all experiments were performed on plaice (*P. platessa*).

Preparation of Sarcoplasmic Reticulum

All operations were performed between 0°C and 4°C. Fish were killed by stunning and decapitation, and the white epaxial muscle was rapidly excised, taking care to remove all traces of red and intermediate muscle. The chopped muscle was homogenised in 3 vols. (w/v) of 0.3M sucrose, 10mM imidazole, pH 7.3, using an Ultra-Turrax blade homogeniser, at 3 full speed for 3x20s. Myofibrils and cellular debris were pelleted by 30 minutes centrifugation at 2,500g. The supernatant was centrifuged at 15,000g for 30 minutes to precipitate the mitochondria. The microsomal pellet was obtained from the supernatant by centrifuging at 95,000g for 1.5h. The microsomal pellet was resuspended in the homogenising medium (10mls) and layered on to a sucrose

gradient consisting of 5mls 40% sucrose, 10mls 35% sucrose and 10mls 30% sucrose, in 10mM imidazole, pH 7.3 (Carsten, 1969). The gradient was centrifuged at 95,000g for 2h. The final microsomal distribution is shown in Fig. 4:1.

Measurement of ATPase Activities

"Total" ATPase activity was measured in a medium of 40mM Tris-HCL, 25mM KCl, 5mM $MgCl_2$, 500μM $CaCl_2$, 300μM EGTA (Free Ca^{2+} concn ≈ 50μM), 0.2-0.4mg ml^{-1} SR protein, pH 7.2 at 10°C. Ca^{2+}-independent ("Basal") ATPase activity was measured in the same medium, except that 1mM EGTA replaced the Ca^{2+}-EGTA buffer. Ca^{2+}-dependent ATPase activity was obtained by subtracting the Basal from the Total ATPase activities. Incubation temperatures were controlled to within ± 0.1°C. in a thermostatted water bath.

The reaction was initiated by the addition of ATP (final concentration 2mM) to the pre-incubated incubation medium, and terminated by adding an equal volume of 10% Trichloroacetic acid (TCA). Denatured protein was precipitated by centrifugation. Phosphate released was assayed by the method of Rockstein and Herron (1951).

Measurement of the Phosphorylated Enzyme Intermediate

Steady state levels of the acid-stable phosphorylated enzyme intermediate were measured in a medium containing 25mM KCL, 5mM $MgCl_2$, 500μM $CaCl_2$, 300μM EGTA, 40mM Tris-HCl, 0.2-0.4 mg ml^{-1} SR protein, pH 7.2 (10°C). The reaction was started by the addition of (γ ^{32}P)-ATP (final concentration 2mM, 0.4μCi ml^{-1}), incubated for five minutes, and quenched by the addition of an equal volume of 10% TCA. The

precipitated material was washed twice in 50 vols. of acidified incubation medium by centrifugation and resuspension. The final sediment was dissolved in 10mls.of scintillant (100:100:5 toluene; Triton X-100: Scintol 2 (Koch-Lite Labs)), and counted in a Packard Tri-Carb scintillation counter.

Aliquots of blank [illegible] incubated at 0°C [illegible]

Calcium Uptake

Calcium uptake into isolated vesicles was measured using a Millipore filtration technique (Tume and Hunington, 1974). Vesicles were pre-incubated in standard Total ATPase medium containing 10mM oxalate and 0.02μCi/ml $^{45}Ca^{2+}$. The reaction was started by the addition of ATP to a final concentration of 2mM. Aliquots were taken at various times (0.5-10 minutes) and filtered through 0.45μm Millipore filters. The filters were washed with 2x2mls of cold incubation medium, dissolved in 10ml of scintillant (see above) and counted. Appropriate controls were included in all experiments.

Cytochrome Oxidase Assay

Cytochrome oxidase (EC 1.9.3.1.) was used to indicate the extent of contamination of microsomal fractions by inner mitochondrial fragments. Activities were measured by following the oxidation of reduced cytochrome c in 50mM phosphate buffer, pH 7.6. Enzyme activity was calculated using E_{mM} redox = 19.1.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was carried out in

7% gels in the presence of sodium dodecyl sulphate as described by Weber and Osborn (1969). Microsomal samples were solubilised by addition of a 5-fold excess of SDS by weight, 1% 2-mercaptoethanol, 25% glycerol, and boiled for 3 minutes.

Aliquots of 50-100µg of protein were applied to gels, and run at a constant current (1mA/cm/tube 6mm in diameter) for about 4 hours. Gels were stained in 1.2% Coomassie Brilliant Blue in 50% methanol, 10% acetic acid for 2hrs. Destaining was initially in the same solvent and then in 5% methanol, 10% acetic acid. Molecular weights were calculated by running proteins of known molecular mass under identical conditions (BDH Chemicals, Poole).

Electron Microscopy

Microsomes were fixed with 1% osmium tetroxide (OsO_4) in 120mM NaCl, 2.4mM $CaCl_2$, 28mM veronal acetate buffer, pH 7.2 for 24h at 4°C. Following washing in the above medium, omitting the OsO_4, samples were stained for 2h in 0.5% uranyl acetate, 120mM NaCl, 2.4mM $CaCl_2$, 28mM veronal acetate buffer pH 6.0.(Meissner and Fleischer, 1972; Fleischer, Fleischer and Stoeckenius, 1967). The samples were dehydrated in a graded ethanol series. Subsequently, samples were treated as follows.

They were immersed in a 1:1 mixture of 100% ethanol: epoxypropane for 40 min. Following this, they were immersed in epoxypropane for 3 changes of 40 minutes each, and then in epoxypropane and araldite (see below) overnight. The following morning, the samples were put into the Araldite ABCD mix (see below) then embedded by putting into a 60°C

oven for at least 36h.

Following embedding, ultra thin sections were cut, double stained with uranyl magnesium acetate and lead citrate (Frasca and Parks, 1965) and viewed in a transmission electron microscope.

Araldite Preparation

Solutions : Araldite AB mixture: mix 10g CY212 Resin and 10g DSSA Hardener by rotation for at least 1h.

Araldite CD mixture: 3g of Accelerator DMP30 and 3 drops of dibutylphthalate are mixed for at least 1h.

Araldite/Epoxy propane mixture: Add 5g Araldite AB mix, 0.125g CD mix and 5 mls epoxypropane and mix for 1h.

Araldite mix: 15g of Araldite AB and 0.375g CD mix are mixed by rotation for 1h.

Protein Estimation

Protein concentrations were estimated using the Maddy and Spooner (1970) modification of the Lowry method (Lowry et al, 1951) as follows:

Solutions: (1) 4% Na_2CO_3

(2) 0.1N NaOH

(3) 2% NaK tartrate

(4) 1% $CuSO_4$

(5) 2% Na deoxycholate in 0.25N NaOH

Mixture A: 100mls soln 1 plus 100mls soln 2 plus 2mls soln 3 and 2mls soln 4 are added in that order.

Procedure: To 0.45mls soln 5 are added 0.05ml of sample and mixed. After leaving to solubilize, 2.5ml mixture A is added. The solution is left for 5 min. then 0.25ml diluted Folin Ciocalteau reagent (1:2 Folin's:water) is added and mixed immediately. Tubes are incubated at room temperature for 60 min. and the optical density read at 695nm.

Statistical Analyses

Statistical analyses were carried out using the Student's t-test.

Results

Sucrose density fractionation of microsomes

Table 4:1 shows the distribution of ATPase activity across a sucrose gradient after centrifuging for 2h at 95,000g (see also Fig. 4:1). Fractions II and III showed the lowest Ca^{2+}-independent ATPase activities, and the highest Ca^{2+}-dependent ATPase levels. Although fractions IV and V show high Ca^{2+}-ATPase activity, they were generally particulate in nature, presumably consisting of aggregated membrane particles. For this reason, they were considered unsuitable for further investigation. A similar aggregation of membrane particles was observed by Seraydarian and Mommaerts (1965).

In the presence of oxalate, vesicles from fractions II and III show a high ATP dependent Ca^{2+} uptake ability (Fig. 4:2). The ratio of calcium transported to ATP split could

not be determined accurately, since the method used for monitoring Ca^{2+} uptake precludes the possibility of taking samples sooner than 30s following initiation of the reaction.

Characterisation of fractions II and III

Both the mitochondrial ATPase and cytochrome oxidase are located on the inner mitochondrial membrane (White et al, 1973). Consequently, cytochrome oxidase was used as a marker for the presence of inner mitochondrial membranes. By this criterion fractions II and III showed negligible contamination.

Table 4:2 shows the effect of 0.5mM ouabain on the ATPase activities of these fractions. No statistically significant inhibition was found, indicating a lack of contamination with Na^{+}-K^{+} ATPase, a sarcolemmal membrane indicator.

Under the electron microscope, the vesicles appear as flattened lipid bilayers (Fig. 4:3). No contamination with mitochondria or myofibrils is apparent (see Pretorius et al, 1969, for comparison).

Gel electrophoresis of fractions II and III yielded the patterns shown in Fig. 4:4A. Following hypo-osmotic washing, only the integral membrane proteins (the "intrinsic" proteins) remain (Fig. 4:4B). The molecular weights were estimated by running proteins of known molecular weight concurrently.

Properties of the Ca^{2+}-dependent ATPase

The effect of altering the Ca^{2+} concentration on the ATPase activity is shown in Fig. 4:5. The curve is sigmoid in shape, concurring with the observations of other workers

(Weber et al, 1966; Panet et al, 1971; Shigekawa et al, 1976). The curve can be linearised using the Hill plot transform (Segel, 1975):-

$$\log\left(\left(v/(Vmax) - v\right)\right) = h \log S - \log K \qquad (1)$$

where v = specific activity

Vmax = maximum specific activity, which was usually determined graphically

h = the Hill number, which is approximately equivalent to the number of binding sites of the enzyme.

K = an apparent dissociation constant.

The Ca^{2+} concentration which gives half maximal velocity of the Ca^{2+}-ATPase (K_{Ca}) can be determined as follows:-

when v = Vmax

$$\text{Log}\left(v/(Vmax) - v\right) = \log 1 = 0 \qquad (2)$$

$$h \log K_{Ca} = \log K \qquad (3)$$

$$\log K_{Ca} = (\log K)/h \qquad (4)$$

From the Hill plot transform, shown in Fig. 4:6, $K_{Ca} = 1.02 \pm 0.07\mu M$ at $10^{o}C$.

Increasing the K^{+} concentration increased the specific activity of the Ca^{2+}-ATPase at all temperatures studied (Fig. 4:7).

The effect of increasing Mg^{2+} concentration is shown in Fig. 3:8. The Ca^{2+}-dependent ATPase activity increases markedly between concentrations of 0 and 5mM, when the increase slows, reaching a maximal value at about 10mM Mg^{2+}. Above 1mM Mg^{2+}, the basal ATPase activity is independent of the magnesium concentration (Fig. 4:8).

Increasing the ADP concentration inhibits the Ca^{2+}-

dependent ATPase slightly (Fig. 4:9), but has no effect on the basal ATPase activity. At an ADP concentration of 2mM, activity of the Ca^{2+}-dependent ATPase has dropped by about a third of its initial value.

The effect of pH on the Ca^{2+}-ATPase activity is shown in Fig. 4:10. The Ca^{2+}-independent ATPase is essentially pH insensitive, a reduction in activity only occurring at pH 8.5 (Fig. 4:11).

Levels of the phosphorylated intermediate were measured at three temperatures (Table 4:3). There was no significant difference between the P-E levels measured at different temperatures. In the presence of 1mM EGTA, no E-P was detectable.

The effect of temperature on the Ca^{2+}-ATPase activity is shown in Fig. 4:12, as a semi-logarithmic Arrhenius plot. The activation enthalpy of 59.45 $\pm$ 1.47 kJ mol^{-1} is derived from the slope of the line ($-E_a/2.303R$). The value used for the Gas constant, R, was 8.3143 joules mol^{-1}.

The effect of altering the K^+ concentration on the activation enthalpy of the ATPase is shown in Fig. 4:13. There is no significant difference between the values at different K^+ concentrations.

As the assay temperature is lowered, the pH optimum of the enzyme increases (Fig. 4:14). Between 10 and 30°C, for cod, Gadus morhua, the $\Delta pH/\Delta T$ is -0.04, while for Notothenia rossii, $\Delta pH/\Delta T$ is -0.027.

Discussion

Highly purified sarcoplasmic reticulum would be expected

to have the following characteristics: (a) a high Ca^{2+}-dependent ATPase activity; (b) the ability to accumulate calcium ions; (c) an affinity for calcium similar to that reported by other workers for other vertebrates; (d) form an acid stable phosphorylated intermediate; (e) should be ultrastructurally homogenous; (f) show negligible contamination with mitochondrial and sarcolemmal ATPases, and (g) a protein composition comparable to that of other SR preparations.

Sucrose Density Fractionation of Microsomes

Myofibrils, cellular debris, mitochondria and nuclei were sedimented by centrifugation. The distribution of the microsomes after separation on a discontinuous sucrose gradient (2h:95,000g) is shown in Fig. 4:1. ATPase activities of the different fractions, collected by aspiration with a Pasteur pipette, is shown in Table 4:1. Fraction I demonstrated no Ca^{2+}-dependent ATPase activity, and was therefore discarded. Fractions II to V all show some Ca^{2+}-ATPase activity, with the highest levels being found in fractions II and III. The heaviest fractions, however, consisted largely of particulate material, and hence were considered unsuitable for further analysis. Consequently, all experiments reported in this and later chapters will refer, unless stated otherwise, to vesicles combined from fractions II and III.

This observation, that most Ca^{2+}-ATPase activity is concentrated in the 30-35% fraction, agrees with those of many other workers on many different muscle types. For example, the highest levels of Ca^{2+}-dependent ATPase activity are

found in those microsomes which collect above the 35% fraction from muscles as diverse as rabbit skeletal (Seraydarian and Mommaerts, 1965; Meissner et al, 1973; Meissner, 1974; Davis et al, 1976; Kim et al, 1976), locust flight (Volmer, 1978), pig atrial (Frank and Sleator, 1975) and coronary muscle (Wuytack et al, 1978), rabbit colon muscle (Nilsson et al, 1978) and uterine muscle (Carsten, 1969).

Vesicles from fractions II and III accumulate Ca^{2+} ions in the presence of oxalate (Fig. 4:2). The ratio of Ca^{2+} pumped to ATP hydrolysed is nearer 1:1 than the most commonly accepted value of 2:1 (Weber et al, 1966; Hasselbach and Makinose, 1966). This is probably because the method used was not capable of monitoring uptake over the first 30s of the reaction, so that the initial burst was not recorded, and only the steady state rate of filling was observed (see also Makinose, 1975).

Characterisation of microsomal Fractions II and III

Most workers report the presence of mitochondrial enzyme markers in the high speed pellet (Meissner, 1975; Heilmann et al, 1977; Wuytack et al, 1978; Volmer, 1978). Methods of monitoring impurity levels in the final "pure" SR fraction vary. Huddart (1978) working on crab and cockroach muscle, used electron microscopy to determine whether mitochondria or mitochondrial fragments were present. Volmer (1978), investigating cockroach muscle, utilised cytochrome oxidase as an inner mitochondrial membrane marker. Cytochrome oxidase activity in the microsomal pellet amounted to 15% of the activity in the mitochondrial pellet, indicating the

presence of considerable amounts of mitochondria or mitochondrial fragments. Further fractionation on a sucrose density gradient improved the separation. Although there was still cytochrome oxidase activity in all the fractions, the greatest amount was located in the 37.2% sucrose band, while the greatest Ca^{2+}-ATPase activity and the greatest Ca^{2+} uptake ability were located in the middle layers (31.6% and 29.6%). Using succinate cytochrome c reductase, Meissner and Fleischer (1972) were able to detect only 1-2% contamination with mitochondria in a fraction sedimenting at the same sucrose concentration. In the present study no cytochrome oxidase activity in fractions II and III was detected.

Examination of the vesicles under the electron microscope also failed to reveal mitochondrial or myofibrillar contamination (see Pretorius et al, 1969 for comparison) (Fig.4:3).

Incubation of the vesicles in the presence of 0.5mM ouabain did not significantly affect the ATPase activities (Table 4:2), indicating that it is unlikely that these fractions contain sarcolemmal membrane vesicles.

Some workers suggest using Na azide and ouabain in the SR incubation medium routinely, in order to inhibit the plasmalemmal and mitochondrial ATPases (Heilmann et al, 1978; Briggs et al, 1977). Although this procedure would permit measurement of the SR ATPases only, it is possible that the presence of contaminating protein would lead to an artefactual reduction in the ATPase specific activities.

Gel electrophoresis of fractions II and III yielded the patterns shown in Fig. 4:4A. The protein composition is

comparable to that described for rabbit sarcoplasmic reticulum (Meissner and Fleischer, 1971; MacLennan et al, 1972; Margreth et al, 1974; Madeira, 1977). There are major bands corresponding to molecular weights of 195,000, 100,000 65,000, 63,000, 55,000, 38,000 and 34,000 daltons (Fig. 4:4a). Washing in hypo-osmotic medium removes extrinsic protein from the SR membrane (Margreth et al, 1974). The effect of osmotic shock on the protein composition of plaice SR is shown in Fig. 4:4b. The residual protein is accounted for mainly by components of molecular weight 195,000,100,000 and 55,000 daltons. It has been established that the 100,000 daltons component corresponds to the Ca^{2+}-ATPase in SR from such diverse animals as rabbit (MacLennan, 1970), chicken (Martonosi et al, 1973) and lobster (Madeira et al, 1974). Although no attempt was made to identify the 55,000 component, it is possible that it corresponds to calsequestrin, or to the 55,000 dalton protein described by MacLennan et al (MacLennan, 1970; MacLennan and Wong, 1971; MacLennan et al, 1972). There is evidence that the loosely associated proteins correspond in part to various glycolytic enzymes (Margreth et al, 1974).

Properties of the Ca^{2+}-ATPase

The effect of altering Ca^{2+} concentration on the ATPase activity is shown in Fig. 4:5. The curve is sigmoidal, and may be linearised using the Hill plot transformation (Segel, 1975) (Fig. 4:6). From the computed regression the Ca^{2+} concentration that gives half maximal activation of the ATPase (K_{Ca}) may be derived. The K_{Ca} values obtained for plaice SR are similar to those values found by other workers

(Table 4:4). The slope of the Hill Plot is 1.39±0.09, which indicates that more than one Ca^{2+} ion is bound per ATP split (Hasselbach, 1978).

Increasing the K^+ concentration increased the ATPase activity (Fig. 4:7). Whether this was an effect of increasing K^+ alone, or an increase of the ionic strength, was not investigated. However, Duggan (1977) has shown that the Ca^{2+}-ATPase is stimulated by K^+ ions. This effect was seen at all temperatures studied.

Mg^{2+} ions have at least two important roles in Ca^{2+} transport. First, the true substrate for the translocation process is Mg-ATP; Vianna (1975) noted that the Lineweaver-Burke plot of the Ca^{2+}-dependent ATPase activity, in the presence of equimolar concentrations of Mg^{2+} ions and ATP was linear only when plotted against the reciprocal of the Mg-ATP concentration. Further, more indirect evidence is given in that the ATPase activity is optimal when the Mg^{2+} concentration is in excess over that of the ATP, and the Ca^{2+} dependency of the enzyme is not altered over a wide range of Mg^{2+} concentrations (Fig. 4:9; Weber *et al*, 1966; Yamomoto and Tonomura, 1967). Secondly, the decomposition of the phosphorylated enzyme intermediate is Mg^{2+}-dependent (Inesi *et al*, 1974; Martonosi, 1969; Kanazawa *et al*, 1971; Panet and Selinger, 1970). The implications of this finding is discussed in greater detail in Chapter 1.

Increasing the ADP concentration decreases the Ca^{2+}-ATPase activity (Fig. 4:9). In contrast the basal ATPase activity is unaffected.

It is possible to reverse the Ca^{2+} pump, generating ATP

from ADP and P_i, by incubating Ca^{2+} loaded vesicles in the presence of ADP, and inorganic phosphate, and it seems likely that increasing the ADP concentration shifts the equilibrium towards this reverse reaction. In all experiments, therefore, care was taken to ensure that the ADP concentration did not rise above 0.5mM, which, from Fig. 4:9 gives only approximately a 5% decrease in activity.

The effect of pH on the Ca^{2+}-dependent and Ca^{2+}-independent ATPases are shown in Figs. 4:10 and 4:11 respectively. The Ca^{2+}-independent ATPase activity is virtually pH independent, decreasing only at a pH of 8.5.

Phosphorylated enzyme intermediate levels are also comparable to those achieved by other workers (Table 4:5). In recent years, it has been realised that there may be several phosphorylated intermediates. There is good evidence for the formation of more than one acid stable intermediate (Ikemoto, 1976; Hasselbach, 1978), and an acid-labile intermediate (Froelich and Taylor, 1975; Kurzmack and Inesi, 1977). Recently, Shigekawa and co-workers have suggested that there is also an ADP independent phosphorylated complex (Shigekawa and Dougherty, 1978; Shigekawa and Akowitz, 1979). It is likely that it is this last complex that is measured under the experimental conditions used. For a more detailed discussion of the intermediate, see the introductory chapter of this thesis. The present finding, that the levels of the phosphorylated intermediate do not alter with temperature is consistent with the observations of others (Hidalgo et al, 1976; Inesi et al, 1976).

The effects of temperature on the activity of the

Ca^{2+}-ATPase is shown in Fig. 4:12. In contrast to the mammalian SR, there is no apparent transition in the slope of the Arrhenius plot. The activation enthalpy value ($\Delta H^{\ddagger}$), of 60kJ mol^{-1} (14.4 kcal mol^{-1}) is lower than that reported for rabbit (19.5 kcal mol^{-1}) below the transition temperature, and somewhat higher than rabbit above the transition temperature (10 kcal mol^{-1}) (Madeira et al, 1974). Hidalgo et al (1976) report higher values both above and below the transition temperature, of 21.9 and 27.4 kcal mol^{-1} respectively. This result is not surprising, since the plaice has a cell temperature significantly lower than that of rabbit. Adaptation to temperature will be discussed in the following chapter.

Altering the ionic strength does not alter the $\Delta H^{\ddagger}$ value for the Ca^{2+}-ATPase (Fig. 13). In the myofibrillar ATPase, $\Delta H^{\ddagger}$ increases with ionic strength in tropical fish, while in Antarctic fish, $\Delta H^{\ddagger}$ increases to a maximum at about 0.15u, and then levels off (Johnston and Walesby, 1977).

The ATPase has temperature pH interdependence characteristics similar to those of other poikilothermic enzymes (Fig. 4:14). Two species of fish were investigated in these experiments, Notothenia rossii (British Antarctica, Environmental temperature -2° - $4^{\circ}C$) and Gadus morhua (North Sea, Environmental temperature 2 - $15^{\circ}C$). As the assay temperature is decreased, the pH optimum increases. The $\Delta pH/\Delta T$ is -0.04 pH units per $^{\circ}C$ for Gadus morhua, and -0.027 for N. rossii. Hazel et al (1978) have investigated five enzymes from rainbow trout muscle and liver, and have found similar results. Yancey and Somero (1978a) also report that

the temperature dependence of the K_m of LDH for pyruvate is dependent on pH (see also Wilson, 1977). Poikilotherms actively regulate their blood, and, in many cases, their intracellular pH such that the OH^-/H^+ ratio remains constant, irrespective of temperature (Howell et al, 1970; Randall and Cameron, 1973; Heisler et al, 1976; Malan et al, 1976). Thus the intracellular pH follows the change in the pH maxima of the enzymes of the cell, ensuring that the catalytic rate is optimal at all temperatures. In all subsequent experiments, in order to approximate the physiological conditions, the buffer solutions were adjusted to pH 7.2 at 10^oC, and allowed to vary with temperature.

Table 4:1. The distribution of the ATPase activities (nmol P_i mg^{-1} min^{-1}) of microsomal fractions on a discontinuous sucrose gradient. Densities of the different fractions are illustrated in Fig. 4:1. Values represent mean ± S.E.M. of determinations.

Fraction No.	% sucrose		Total ATPase	Basal ATPase	Ca^{2+} dependent ATPase
I	11 - 30	6	706 ± 90	701 ± 164	0
II	30	6	709 ± 103	218 ± 66	499 ± 61
III	30 - 35	6	648 ± 81	198 ± 57	466 ± 13
IV	35	6	425 ± 64	77 ± 22	348 ± 73
V	35 - 40	6	420 ± 118	82 ± 38	337 ± 94

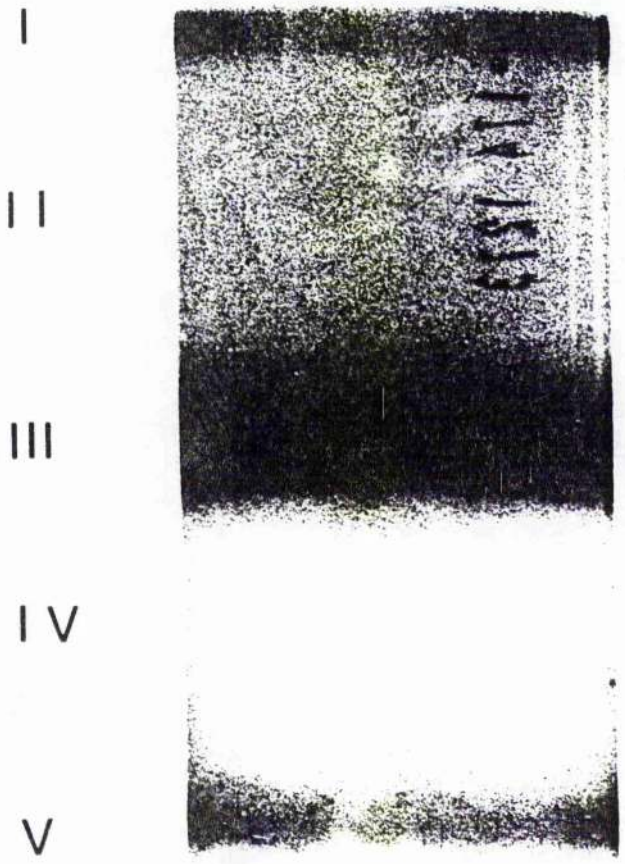


Fig. 4:1 The distribution of microsomes from plaice white muscle across a discontinuous sucrose gradient. Densities of the different fractions are given in Table 4:1.

Table 4:2. The effect of ouabain (0.5mM) on ATPase activities (nmol P_i mg^{-1} min^{-1}) of microsomal fractions on a discontinuous sucrose gradient. Ouabain has no statistically significant effect on any fraction.

Fraction No.	n	Control			+ 0.5mM Ouabain		
		Total ATPase	Basal ATPase	Ca^{2+} dependent ATPase	Total ATPase	Basal ATPase	Ca^{2+} dependent ATPase
I	5	283 ± 42	97 ± 13	186 ± 30	297 ± 46	103 ± 11	176 ± 40
II	5	681 ± 47	135 ± 17	546 ± 32	694 ± 44	130 ± 15	564 ± 30
III	5	807 ± 20	93 ± 14	714 ± 27	857 ± 26	110 ± 13	747 ± 26
IV	5	892 ± 93	59 ± 20	769 ± 108	873 ± 70	20 ± 10	768 ± 79
V	5	737 ± 65	32 ± 13	705 ± 72	584 ± 122	45 ± 24	539 ± 127

Table 4:3. Steady state levels of the acid stable phosphorylated enzyme intermediate (PEI) of plaice fast muscle sarcoplasmic reticulum.

Temperature °C	No. of fish	Phosphoenzyme Intermediate Levels (mean ± SEM) (nmol P_i mg^{-1})
0	6	3.75 ± 0.8
14	6	4.30 ± 1.2
29	6	3.25 ± 1.4

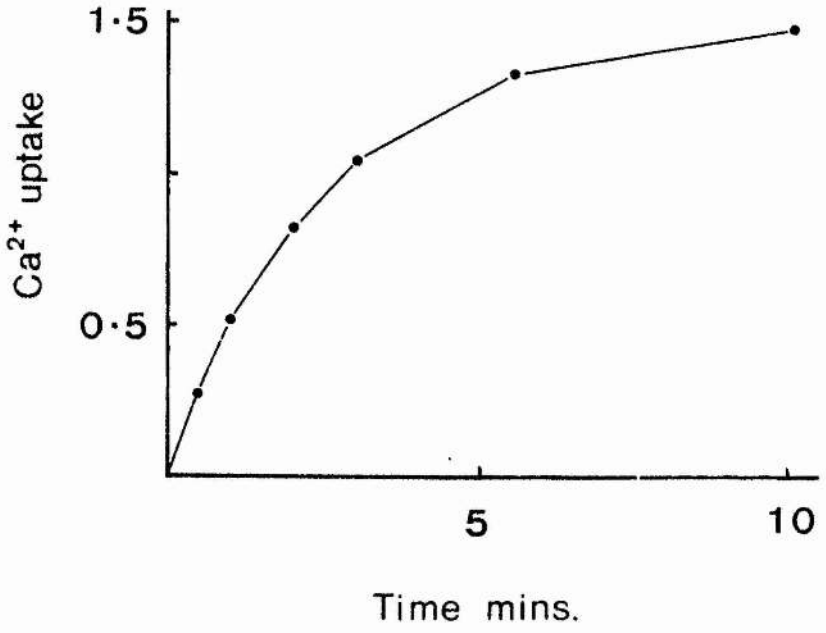


Fig. 4:2 Ca^{2+} uptake (μmol/mg/min) by plaice SR, in the presence of oxalate, at 20°C.

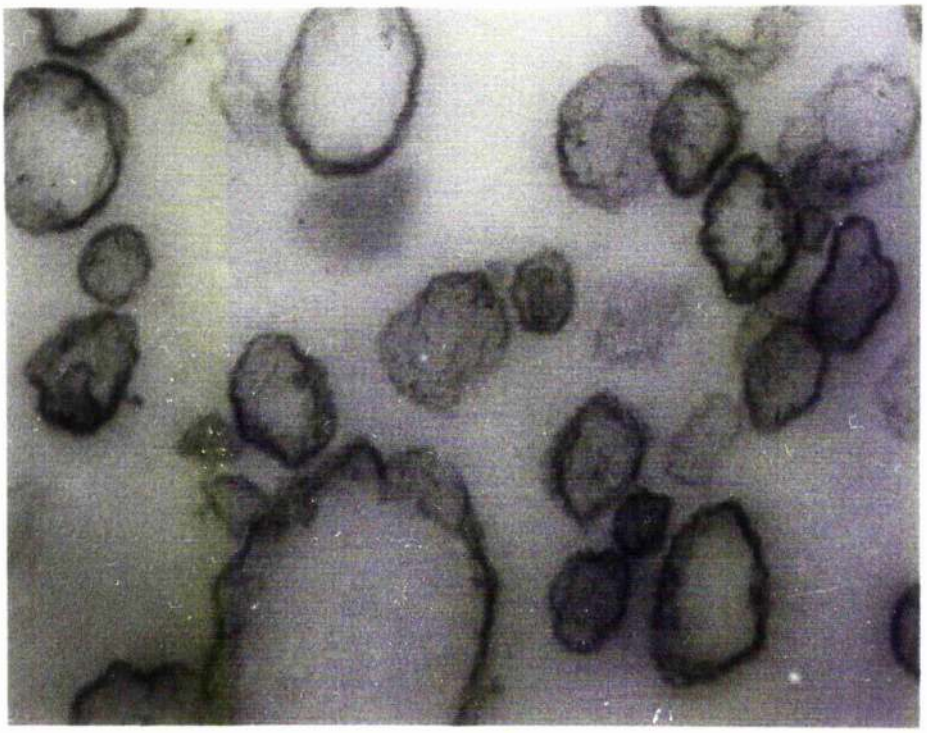


Fig. 4:3 Electron micrographs of vesicles from fractions II and III. Vesicles were prepared as described in Materials and Methods. Magnification x150,000.

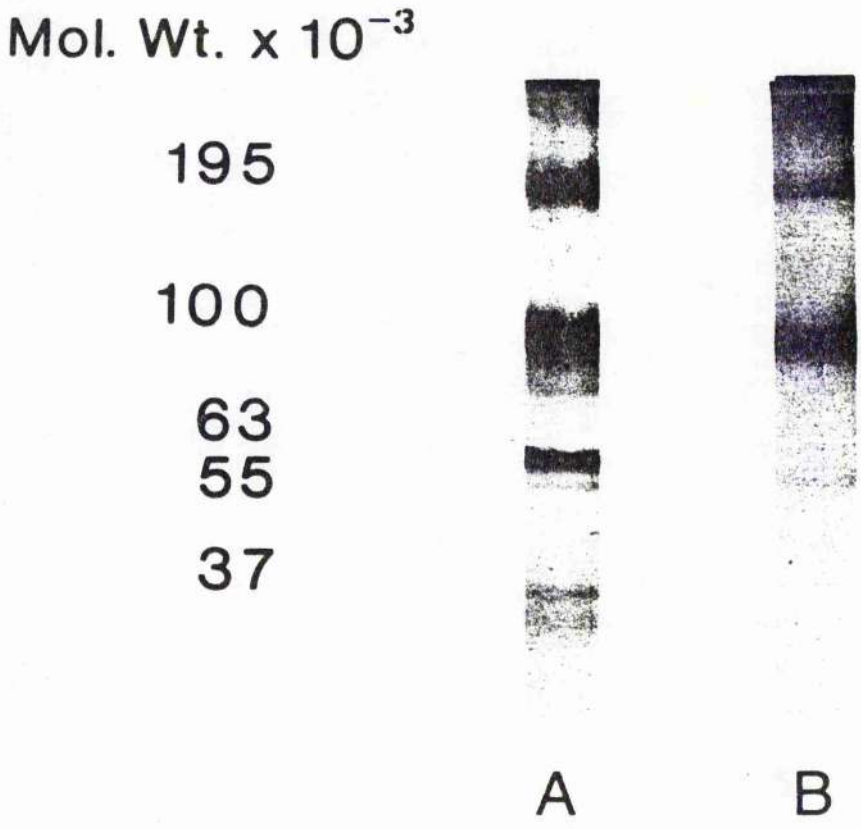


Fig. 4:4 Gel electrophoresis of fraction II and II (A) before and (B) after hyper-osmotic washing. Molecular weights were estimated by running known standards concurrently.

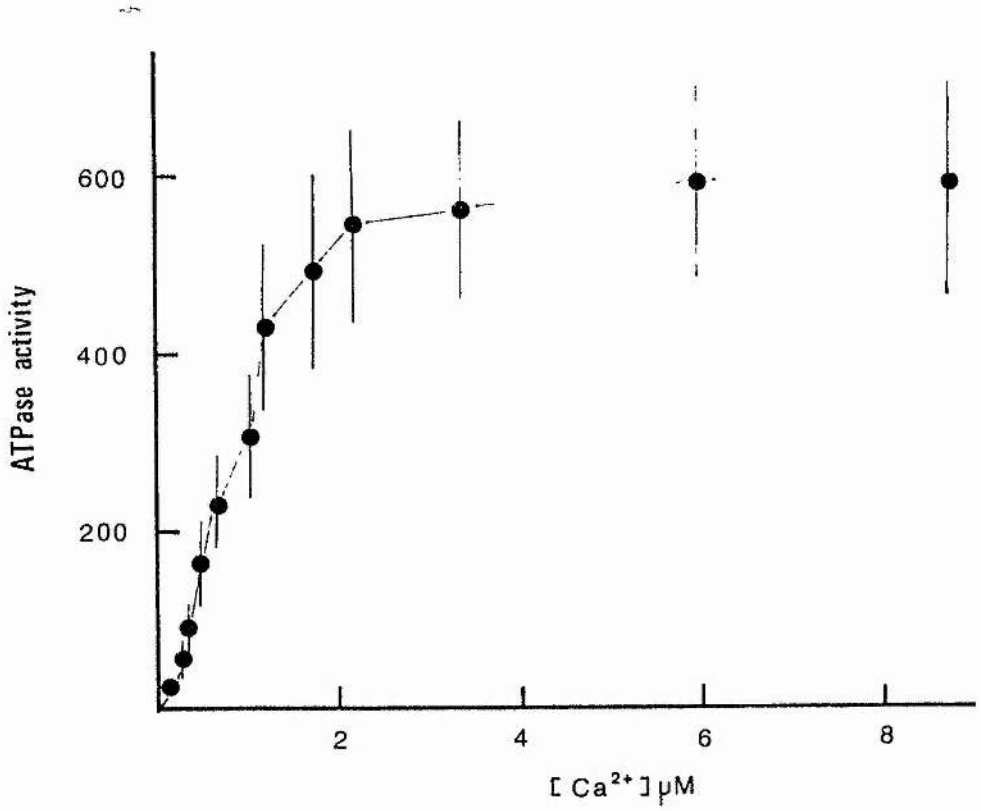


Fig. 4:5 The effect of altering the free Ca^{2+} concentration on the Ca^{2+} ATPase activity (nmol Pi/mg/min) of vesicles isolated from fractions II and III. Values represent mean $\pm$ S.E.M. of 6 observations.

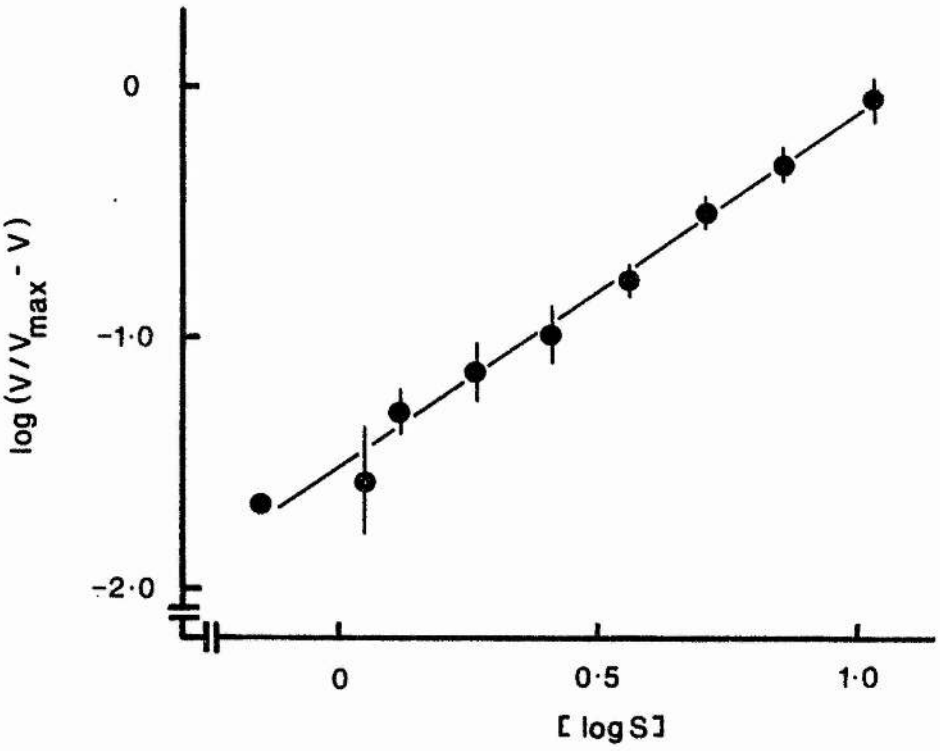


Fig. 4:6 A Hill plot transform of Fig. 4:5.

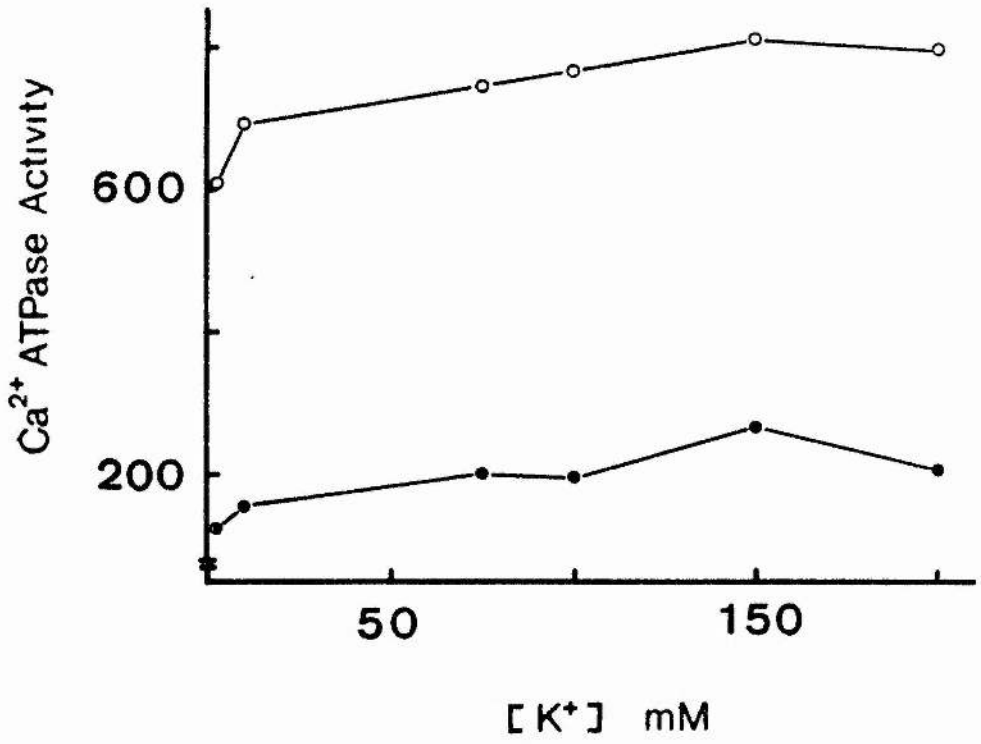


Fig. 4:7 The effect of increasing K^+ on Ca^{2+} ATPase activity (nmol Pi/mg/min) of plaice white muscle SR at 0°C (●) and 25°C (○). Values represent the mean of three observations.

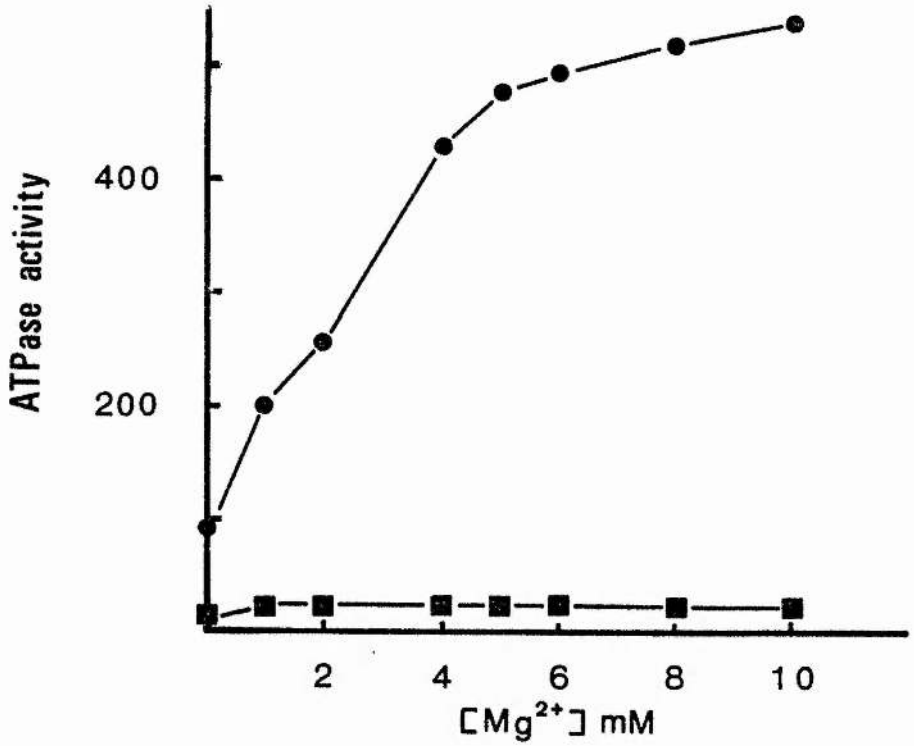


Fig. 4:8 The effect of Mg^{2+} on basal (■) and Ca^{2+} dependent (●) ATPase activity (nmol Pi/mg/min) Values are the mean of duplicate observations of two preparations.

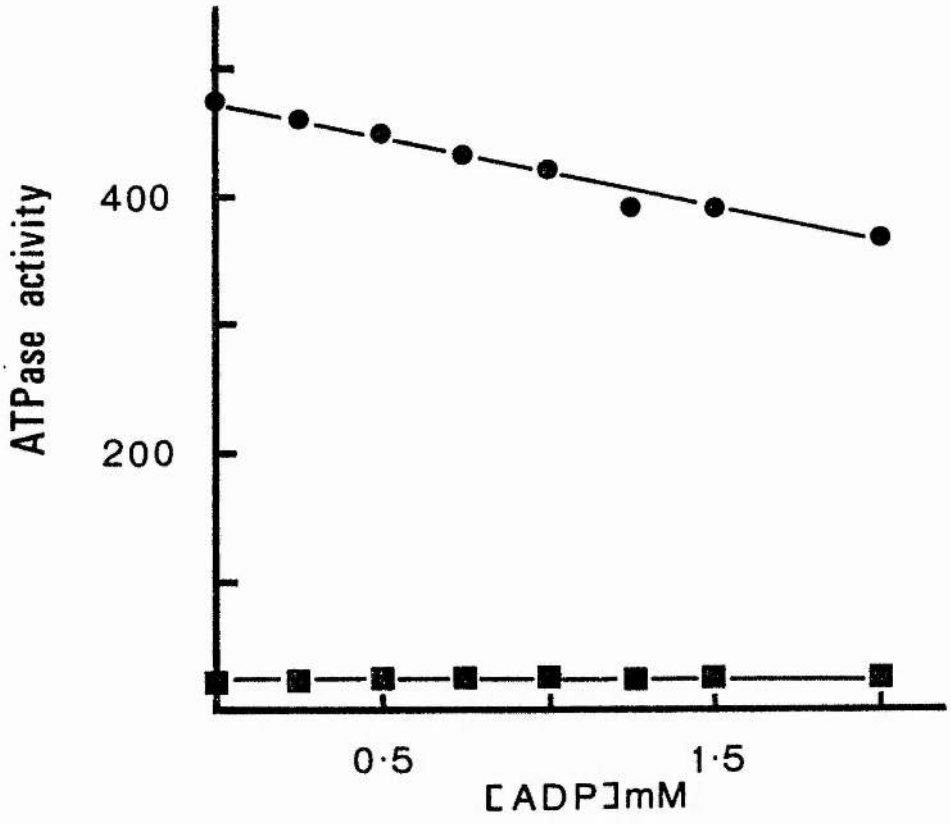


Fig. 4:9 The effect of increasing ADP on basal (■) and Ca^{2+}dependent (●) ATPase activity (nmol Pi/mg/min). Values are the mean of duplicate observations of two preparations.

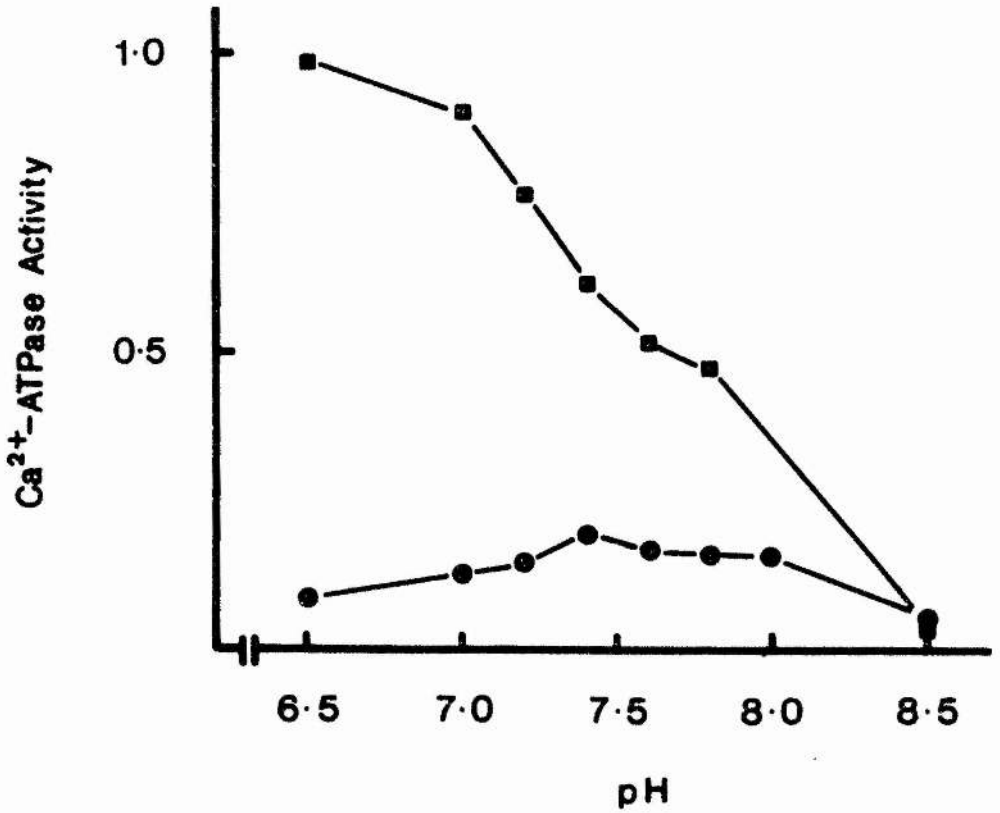


Fig. 4:10 The effect of pH on the Ca^{2+}-dependent ATPase activity (nmol Pi/mg/min) at 0°C (●) and 25°C (■) of plaice white muscle FSR.

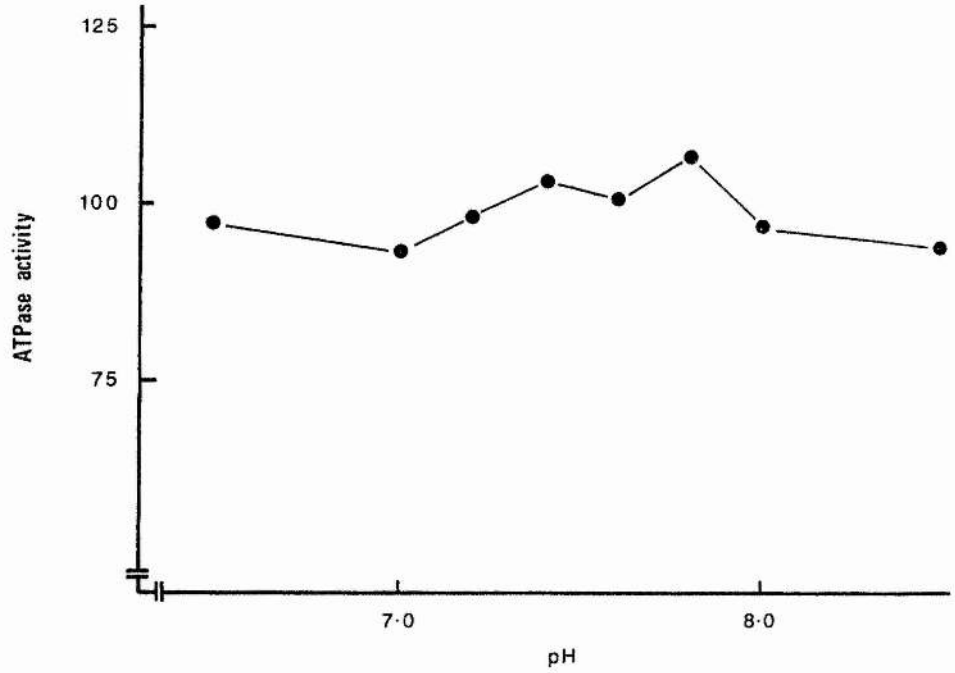


Fig. 4:11 The effect of pH on the basal ATPase activity (μmol Pi/mg/min) of vesicles from fractions II and III from cod white muscle estimated at 10°C.

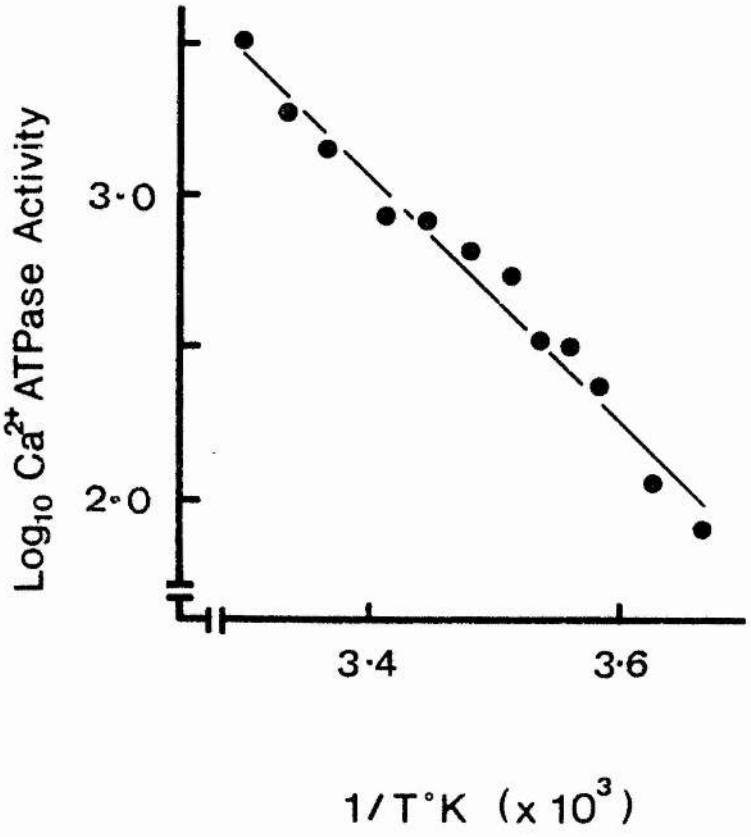


Fig. 4:12 The effect of temperature on the Ca^{2+}-dependent ATPase activity of plaice SR, shown as an Arrhenius plot. Values represent the mean of four observations.

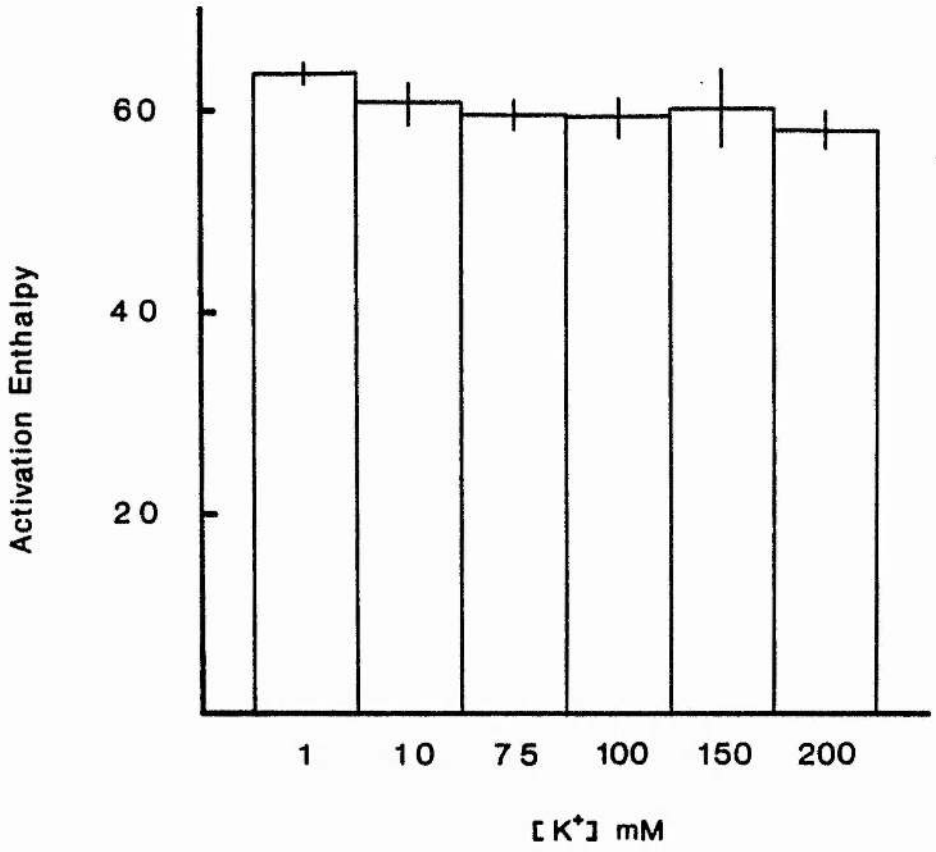


Fig. 4:13 The effect of altering $[K^+]$ on the $\Delta H^{\ddagger}$ values of the Ca^{2+} dependent ATPase of plaice SR. Values represent the mean $\pm$ S.E.M. of 6 observations.

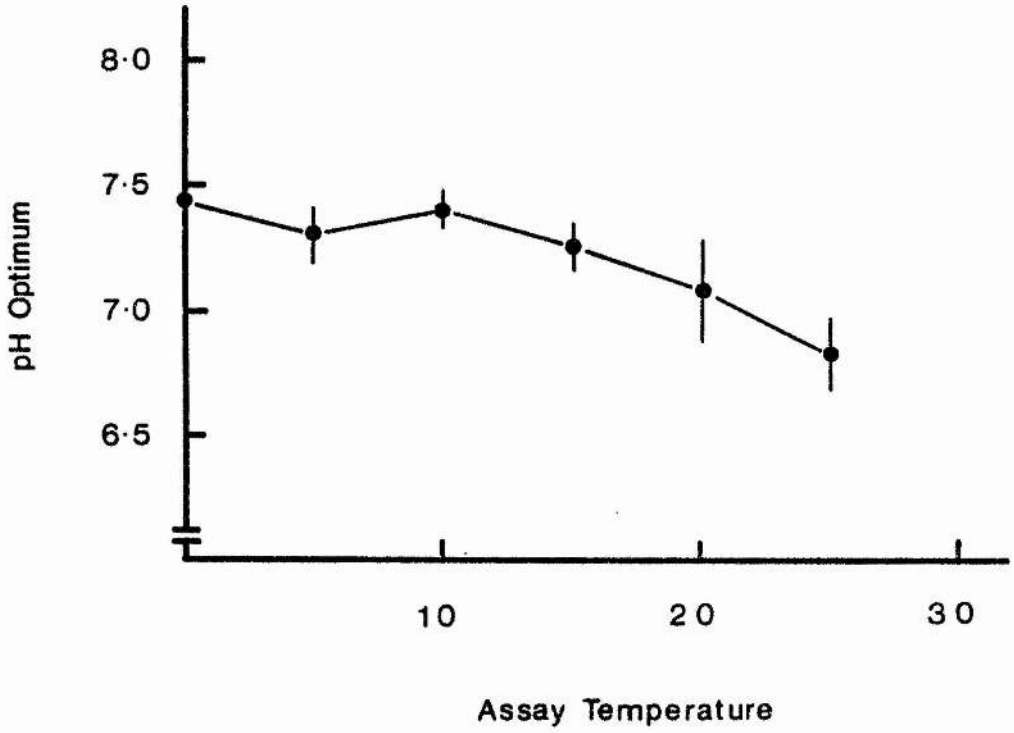


Fig. 4:14 The effect of temperature on the pH optimum of the Ca^{2+} ATPase of SR isolated from the white muscle of the cod. Values are the mean ± S.E.M. of 5 observations.

Table 4:4 K_{Ca} values for SR from skeletal muscle of rabbit and dog. Values from Weber et al (1966) and Hangaya and Schwartz (1969) are calculated from binding data.

Species	K_{Ca} (μM)	Reference
rabbit	1.3 ± 0.1	Shigekawa et al, 1976
	0.1	Martonosi, 1971
	~0.3	Weber et al, 1966
	0.8	Panet et al, 1971
	~0.2	Hangaya and Schwartz, 1969
	0.1	Chevallier and Butow, 1971
dog	0.1	Pretorius et al, 1969

Table 4:5 Levels of the acid-stable phosphorylated intermediate achieved by different workers on rabbit SR. Values from Panet et al (1971) show the range of PE levels found at a Ca^{2+} concentration of (x) and differing Mg^{2+} and ATP concentrations. The PE concentration was found to be temperature independent by Inesi et al (1976) and Hidalgo et al (1976).

P-E Level	Reference
3.1 ± 0.8 nmol/mg	Inesi et al, 1976
2.7 - 5.4 (10μMCa^{2+})	Panet et al, 1971
5.3 - 6.5 (10mMCa^{2+})	
4.0	Makinose, 1969
3	Hidalgo et al, 1976
3-5	Martonosi, 1972

CHAPTER 5

THE EFFECT OF TEMPERATURE ON THE SARCOPLASMIC RETICULUM

THE EFFECT OF TEMPERATURE ON THE SARCOPLASMIC RETICULUM

INTRODUCTION

As outlined in chapter 3, a number of studies have shown that cold adaptation in fish is associated with an increase in incorporation of unsaturated fatty acids into membrane phospholipids (Johnson and Roots, 1964; Knipprath and Mead, 1968; Hazel, 1973; Cossins, 1977; Cossins et al, 1977). This is thought to provide a mechanism for controlling membrane fluidity and hence conserving membrane function at different temperatures (Hockachka and Somero, 1973; Sinesky, 1974; Hazel and Prosser, 1974; Chapman, 1975). For example, Cossins and Prosser (1978) have shown a correlation between phospholipid unsaturation, membrane viscosity, and adaptation temperature for brain synaptosome preparations from a variety of species of fish and small mammals. However, there was only a poor correlation between membrane viscosity and cell temperature for SR from rat, desert pupfish and Arctic sculpin (Cossins, 1977; Cossins et al, 1978). Using the polarization of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH) as an indication of membrane fluidity shows that there is very little difference between the three species at 25°C. The polarization/temperature graphs are very similar for the three different species.

However, Madeira et al (1974) noted physiological differences between an ectothermic and a homeothermic vertebrate. Measuring the Ca^{2+}-ATPase activity, they found

that, although the activation energies were similar, there was a significant difference in the transition temperature of the Arrhenius plot; 11.5°C for the lobster, and 16.7°C for the rabbit.

An analysis of the fatty acid constituents of the membranes showed that the Arctic Sculpin had the highest levels of unsaturated fatty acids, the Desert Pupfish (34°C) second and the rat (37°C) third. Similarly, rabbit SR fatty acids were approximately 50% unsaturated, and lobster fatty acids were about 65% unsaturated (Madeira and Antunes-Madeira, 1976).

The extent to which Ca^{2+} transport by SR has undergone evolutionary modification for function at different temperatures is unknown. This chapter investigates the effects of temperature on calcium transport and the Ca^{2+}-dependent ATPase activity of native SR vesicles isolated from fish adapted to a wide range of thermal environments.

In the second part of this chapter, the effect of acclimation, as opposed to adaptation, on the Ca^{2+}-dependent ATPase is investigated. Cossins et al(1977) have suggested that the adaptation does not occur on a functional level but did not investigate ATPase or Ca^{2+} uptake activity. In a recent study, Penney and Goldspink (1980) have suggested that the acclimatory mechanism involves an increase in the amount of SR in the muscle on cold acclimation. Johnston and Maitland (1980) have demonstrated that such acclimatory responses are found in the mitochondria of goldfish muscle, but did not measure the amount of sarcoplasmic reticulum. Despite the increase there is still functional acclimation

in mitochondria (Wodtke, 1976, 1978) so that it would seem that a compromise between two strategies of acclimation has been reached in mitochondria.

Penney and Goldspink agreed with Cossins *et al* that there was no functional acclimation, but did not measure the temperature dependence of the ATPase. These experiments extend those of Penney and Goldspink (1980) and Cossins *et al*, (1977) by investigating the effect of acclimation on the ATPase activity of carp white muscle sarcoplasmic reticulum.

MATERIALS AND METHODS

Fish

The species used in these experiments, their geographical locations, habitat and environmental temperature range are listed in Table 5:1. Where necessary, fish were maintained in filtered, recirculated water at their habitat temperature.

For the acclimation studies, carp (*Carassius carassius*, average length 25cm, were kept at their acclimation temperature (2° or $30^{\circ}C$) for five weeks prior to sacrifice, with a 12-12 photoperiod, in tanks of recirculating fresh water.

Table 5:1

SPECIES	NO. OF FISH USED	CODE NO.	HABITAT	GEOGRAPHICAL LOCATION	ENVIRONMENTAL TEMPERATURE (°C)
Notothenia rossii	2	1	Marine	British Antarctica	0 - 4
Gadus morhua	6	2	Marine	Balsfjørd, Norway	2 - 10
Hippoglossoides platessoides	2	3	Marine	Balsfjørd, Norway	2 - 10
Gadus aeglifinus	2	4	Marine	Balsfjørd, Norway	2 - 10
Pleuronectes platessa	6	5	Marine	Firth of Forth, Scotland	2 - 18
Myoxocephalus scorpius	6	6	Marine	Firth of Forth, Scotland	2 - 18
Dascyllus melanarus	2	7	Marine	Indian Ocean	22 - 28
Echidna nebulosa	2	8	Marine	Pacific Ocean	22 - 28
Salvelinus alpinus	6	9	Fresh-water	Ringvassøy, Norway	2 - 8
Salmo salar	4	10	Fresh-water	Almondbank, Perthshire	2 - 12
Ostronotus ocellatus	6	11	Fresh-water	South America	15 - 28
Osphronemus guramy	2	12	Fresh-water	South America	15 - 28
Colossoma spp.	2	13	Fresh-water	South America	15 - 28
Tilapia mariae	2	14	Fresh-water	African lakes	24 - 30
Tilapia mossambicca	2	15	Fresh-water	African lakes	24 - 30

Isolation and preparation of SR, and measurement of physiological parameters

Isolation and preparation of SR, the measurement of ATPase activities, Ca^{2+} uptake and the levels of the phosphorylated enzyme intermediate were all carried out as described in the previous chapter.

Determination of rate constants and thermodynamic activation parameters

An apparent rate constant k (s^{-1}) was obtained by dividing the Ca^{2+}-dependent ATPase activity by the corresponding steady state concentrations of the phosphoenzyme intermediate.

Activation energies (E_a) of the Ca^{2+}-dependent ATPase were calculated from Arrhenius plots of log k against 1/T (^{o}K). Thermodynamic activation parameters were obtained according to transition state theory from the following (Hidalgo et al, 1976).

$$k = (\bar{k}.T/h)e^{-\Delta G^{\ddagger}/RT} \qquad (1)$$

$$\Delta H^{\ddagger} = E_a - RT \qquad (2)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \qquad (3)$$

where $\bar{k}$ and h are Boltzmann's and Planck's constants respectively. Values were computed at $2^{o}C$ (275 K). Best-fit lines were computed using linear regression analysis.

Statistical Analyses

Statistical analyses were carried out using the Student's t-test.

RESULTS

The effects of temperature on calcium uptake and Ca^{2+}-dependent ATPase activities

The Ca^{2+}-dependent ATPase activity of SR from 15 species of fish has been determined at a series of temperatures between 0 and 30°C. Fig. 5:1 shows the Ca^{2+}-ATPase activity at 0°C plotted against the environmental temperature range experienced by each species. In general, cold adapted fish have higher activities than warm-adapted species. Similarly at 0°C, the SR from two cold-adapted species Notothenia rossii (ET* 0-4°C) and Pleuronectes platessa (ET 2-18°C) accumulate calcium at six times the rate achieved by Tilapia mossambicca (ET 24-30°C) (Fig. 5:2). At 25°C similar rates of Ca^{2+}-uptake are obtained for all three species (Fig. 5:3).

Thermodynamic activation parameters

With the exception of Tilapia mariae Arrhenius plots of the SR Ca^{2+}-ATPase were linear over the temperature range at 0-30°C (Fig. 5:4). Presence or absence of a discontinuity in the Arrhenius plot was not, therefore, correlated with any particular environmental temperature. Steady state levels of the phosphoenzyme intermediate are independent of assay temperature, in the range 0.8 - 3.6 nmole Pi/mg protein, and show no significant correlation with adaptation temperature. Activation enthalpies ($\Delta H^{\ddagger}$) of the ATPase ranged from 53-190 kJ/mole and were positively correlated with environment temperature (Fig. 5:5). Activation entropy ($\Delta S^{\ddagger}$) varied from negative values in cold adapted species to positive values in the tropical fish (Fig. 5:6). Values for

activation free energy ($\Delta G^{\ddagger}$) were not strongly correlated with environmental temperature and were in the range 64-69 kJ/mole (Fig. 5:7).

The effects of acclimation or ATPase activities of the SR of carp

Arrhenius plots of the Ca^{2+}-dependent ATPase activities of hot and cold acclimated carp are shown in Figs 5:8 and 5:9 respectively. It may be seen that there is no significant difference in the slopes of the graphs, indicating that there is little difference in the activation enthalpies (Table 5:2). Since the phosphoenzyme levels have not been determined, it is not possible to say whether there are significant differences in the relative amounts of the Ca^{2+} pump protein.

ATPase activities measured at $0^{\circ}C$ show no significant differences (Table 5:2), which is markedly different from the adaptation results (Fig. 5:1).

DISCUSSION

It appears that fish sarcoplasmic reticulum has undergone evolutionary modification for function at different temperatures. The higher rates of calcium transport and ATP hydrolysis at low temperatures in cold adapted species parallels functional adaptations in catalytic efficiences observed for other enzymes of energy metabolism in fish muscle (Johnston et al, 1973; Low et al, 1973; Somero and Low, 1976; Johnston and Walesby, 1977, 1979). Activation enthalpy ($\Delta H^{\ddagger}$) of the Ca^{2+}-ATPase is positively correlated

with environmental temperature for the 15 species of teleost fish investigated. Similar correlations between $\Delta H^{\ddagger}$ and cell adaptation temperature have been demonstrated for fish muscle pyruvate kinases (Low and Somero, 1976) and Mg^{2+}-Ca^{2+}-myofibrillar ATPases (Johnston et al, 1977; Johnston and Walesby, 1977, 1979).

Adaptations in $\Delta H^{\ddagger}$ are associated with energetically unfavourable but biologically advantageous adjustments in activation entropy (Low and Somero, 1976; Johnston et al, 1977). Activation entropy ($\Delta S^{\ddagger}$) varies from negative values in cold adapted fish to positive values in more warm adapted species (Fig. 5:6). Mechanistic interpretations of these results is made difficult by a lack of information concerning the detailed kinetics of the reaction. It has been suggested that these adjustments may result from differences in weak bond formation during the activation process (Somero and Low, 1976). In the case of the Ca^{2+}-ATPase of SR, weak bond formation might include protein-protein, protein-phospholipid and membrane-solute interactions. In membrane-bound enzymes, the physical state of associated lipids may play an important role in stabilising protein structure. It might be expected, therefore, that adaptations in catalytic efficiencies of cold adapted SR ATPases would result from modifications both in protein structures and of the lipid microenvironment of the enzyme. In the SR, however, the evidence for this is somewhat contradictory. For example, Cossins et al (1977) have shown that the fatty acid composition of SR phospholipids becomes unsaturated in the order rat, desert pupfish (ET 28-34°C), and artic sculpin (ET 0.5-2°C), but that there was no corresponding changes in membrane viscosity. In contrast,

other studies have shown lobster SR to have a higher ATPase activity at low temperature, and be more fluid than SR from rabbit muscle (Morse et al, 1975; Madeira and Antunes-Madeira, 1976).

Recent evidence, however, tends to indicate that the phospholipids may not be as important as once thought. For example, Anzai et al (1978) have found that the transition in the Arrhenius plot of rabbit at 20°C may be associated with conformational changes in the protein rather than the lipid. In support, Davis et al (1976) have reported that, at 1°C, only 9% of the phospholipid chains are in a rigid formation, and that by 5°C, the percentage has dropped to just over 1% (Kirino et al, 1977).

In summary, the role of the lipids in the Ca^{2+} transport process does seem to be somewhat equivocal, and the possibility that it is not crucial in determining the rate of the Ca^{2+} pumping cannot be excluded. These observations may explain the lack of acclimatory changes in the SR. If the physical state of the lipids is not crucial to the SR function (Cullis and de Kruijff, 1979), then it may be more economical simply to manufacture more or less SR depending on the temperature.

It is known that enzyme induction is an important acclimatory response in prokaryotic cells, and may be invoked under many conditions; e.g. in the type of nutrients present, or the introduction of an antibiotic (Hochachka and Somero, 1973). There are two ways that the cell can increase the amount of enzyme. Either it can increase the rate of synthesis, or it can decrease the rate of degradation. Sidell (1977) measured the rates of synthesis and degradation of

the cytochrome oxidase system, and found that, in spite of decreased levels of synthesis, the absolute amounts of enzyme increased. The explanation was that, although synthesis declined, so too did the rates of degradation, and to a greater extent. Thus, the animal was able to compensate by increasing the cytochrome oxidase levels, at the same time as conserving energy by decreasing the rate of synthesis.

In support, Johnston and Maitland (1980) studying ultrastructural changes in the muscle of the carp during cold acclimation, found that the relative amount of the fibre occupied by mitochondria increases with cold acclimation. Cold acclimation resulted in an increase in the depth of the subsarcolemmal mitochondrial zone in all fibre types. The subsarcolemmal mitochondria are thought to provide energy for the active transport of amino acids, salts and metabolites across the cell membrane, while the intrafibrillar mitochondria provide the ATP for the contractile process (Pette, 1966; Kubista et al, 1971; Muller, 1977).

Recently, Penney and Goldspink (1980) have suggested that acclimation occurs by increasing the relative volume of SR in the muscle fibre. In cold acclimated animals, the SR forms 14% of the fibre volume, decreasing to 9% in the warm acclimated animals (Penney and Goldspink, 1980). As stated above, this mechanism may not be adaptively disadvantageous if the increase occurs through a decrease in the breakdown of the membrane, rather than an increase in the rate of synthesis (Sidell, 1977). Interestingly in the SR, the ATPase has the characteristics of the colder adapted animals, with an activation enthalpy of 62.76 $\pm$ 0.08kJ/mol, and a

Ca^{2+}-ATPase activity at 0°C of 80 ± 10nmol/mg/min (Fig. 5:9). From the $\Delta H^{\ddagger}$ value, it would be estimated that the enzyme was adapted to a habitat temperature of approximately 10°C, as would also be estimated from the specific activity at 0°C (see Figs. 5:5 and 5:1 respectively. It is possible, therefore, that the carp adapts to seasonal temperature changes by adopting a strategy of compromise, i.e. it adapts to an intermediate temperature, and compensates for changes from that mode by increasing or decreasing the amount of sarcomplasmic reticulum.

Thus, it would seem that the sarcoplasmic reticulum employs different mechanisms of adaptation depending on the time scale of the thermal stress. This has also been demonstrated in the Mg^{2+}-Ca^{2+}-myofibrillar ATPase, where adaptation involves changes in the myosin light chains, and acclimation proceeds through alterations in the troponin fraction of the thin filament (Johnston, 1979).

Table 5:1 ATPase activities at 0°C and activation enthalpy of the Ca^{2+}-ATPase of cold and hot acclimated carp.

	ATPase activities(nmol $mg^{-1}min^{-1}$)			H of Ca^{2+}
	Total	Basal	Ca^{2+}dependent	dependent ATPase
Cold acclimated	114±11	34±7	80±10	62.76±0.08
Hot acclimated	207	71	136	57.46

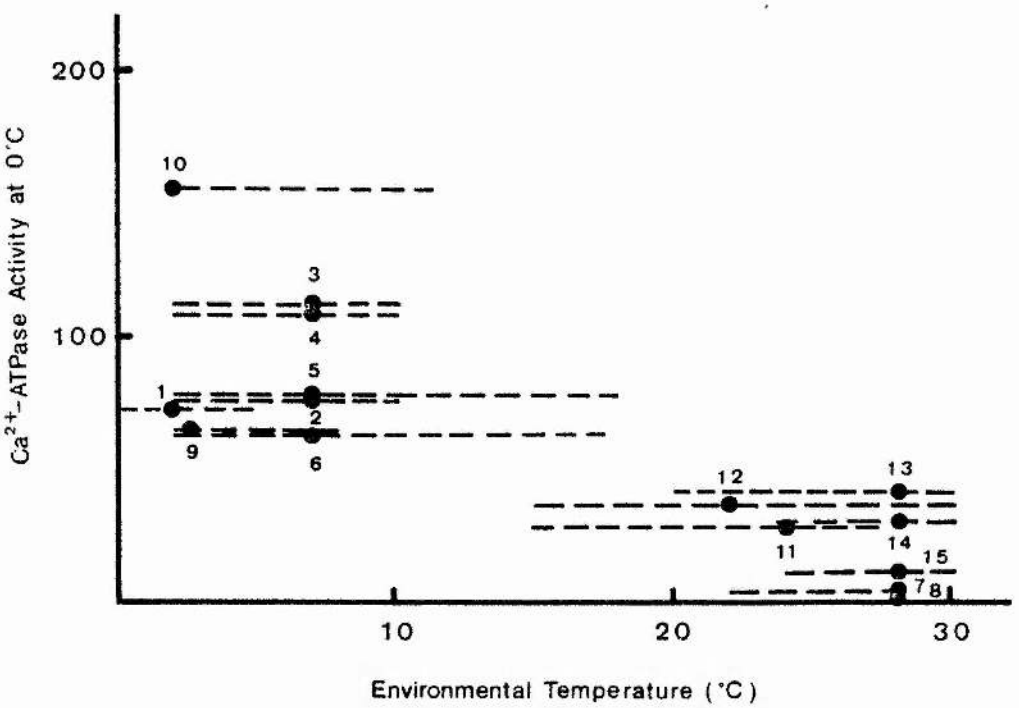


Fig. 5:1 Ca^{2+} ATPase activity (nmol Pi/mg/min) at $0^{\circ}C$ of SR isolated from different species of fish, (See table 5:1 for key) plotted against environment temperature. The dotted lines represent the approximate annual temperature range experienced by each species.

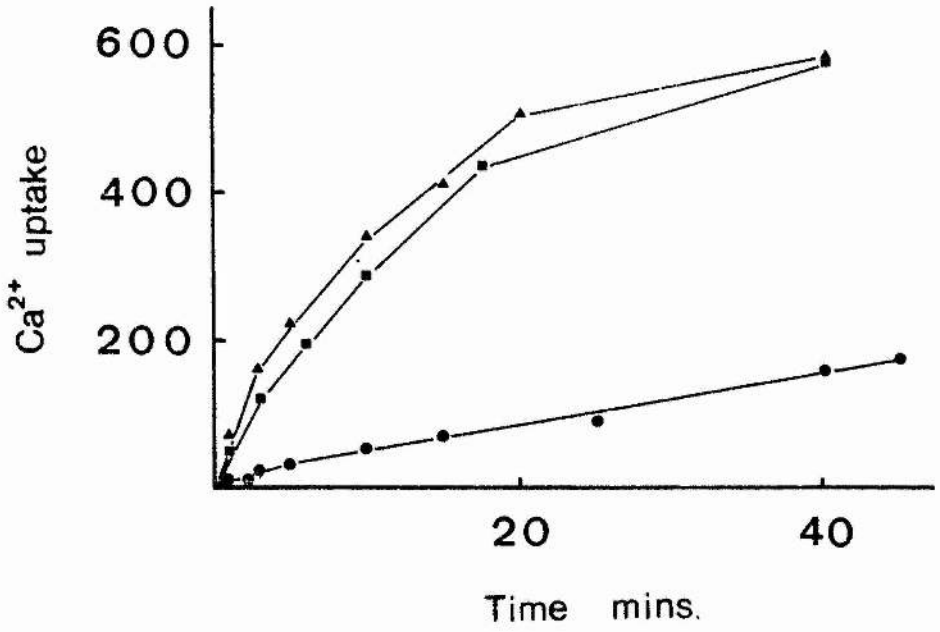


Fig. 5:2 Ca^{2+} uptake (nmol Ca^{2+}/mg/min) by SR from three different fish species, (●) Tilapia mossambicca, (▲) Pleuronectes platessa, (■) Notothenia rossii, at $0^{o}C$. Values represent the mean of three observations.

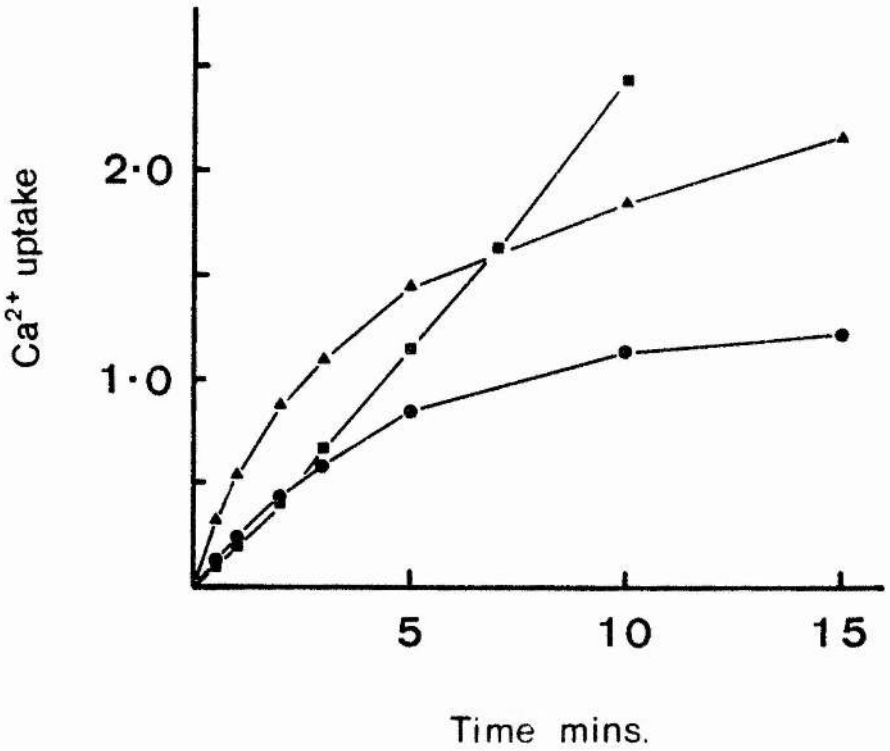


Fig. 5:3 Ca^{2+} uptake (μmol/mg/min) by SR from three different fish species, (●) Tilapia mossambicca, (▲) Pleuronectes platessa, and (■) Notothenia rossii, at 25°C. Values represent the mean of three observations.

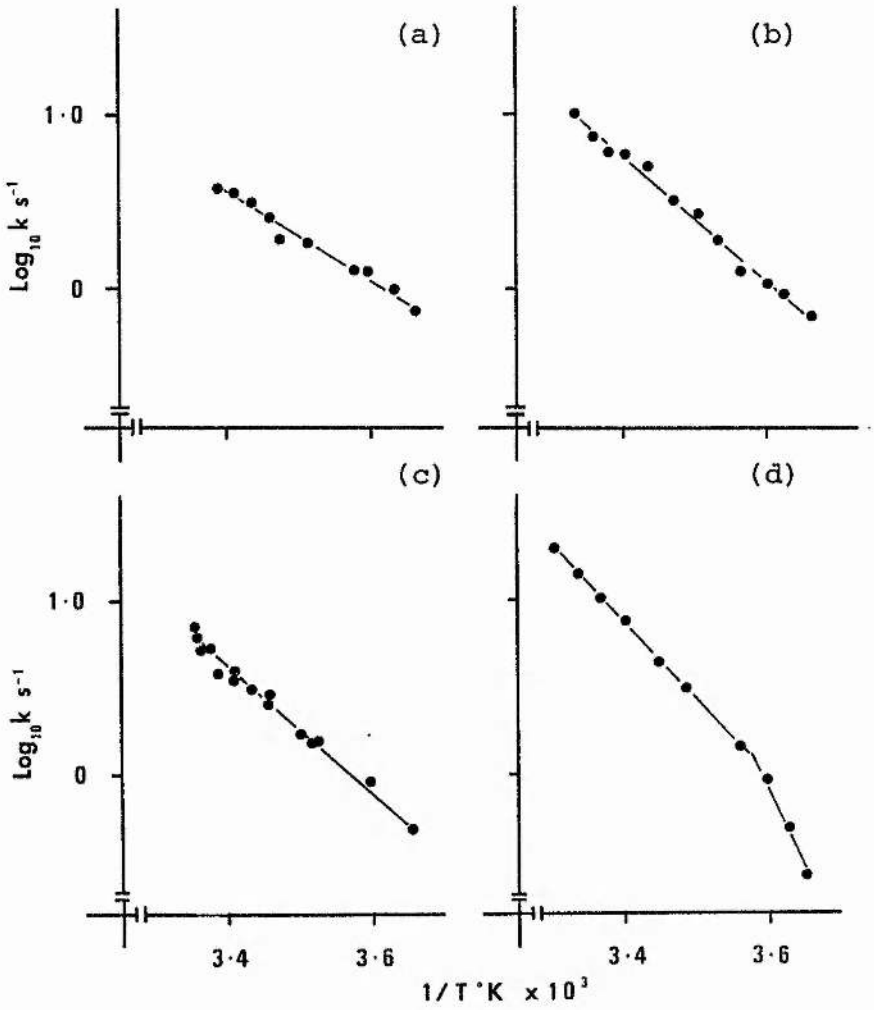


Fig. 5:4 Arrhenius plots of four different species.
(a) N. rossii; (b) Salvelinus alpinus;
(c) Astronotus ocellatus; (d) Tilapia mariae.
K values were derived as described in the text.

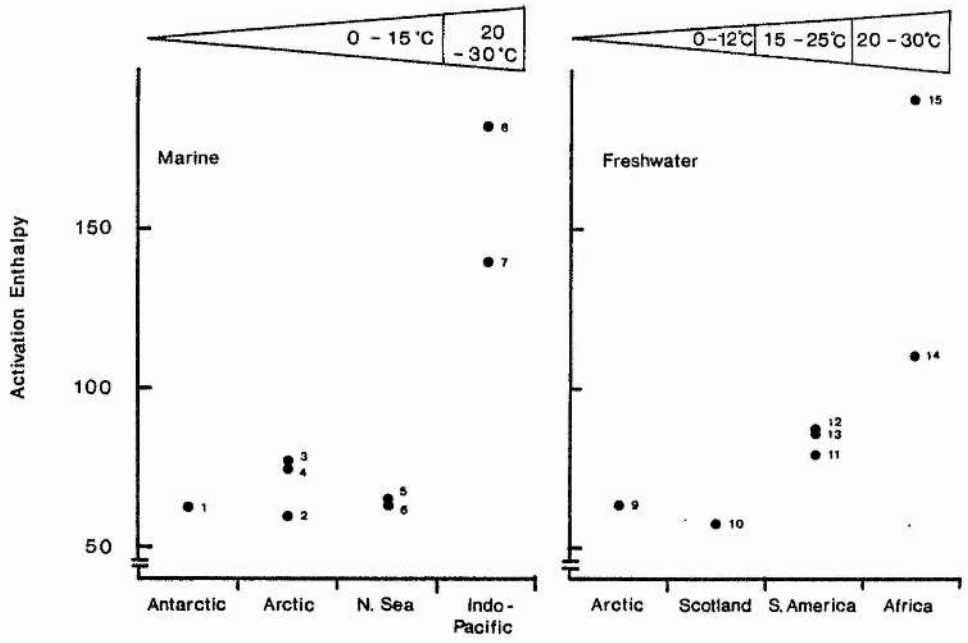


Fig. 5:5 The effect of adaptation temperature on the activation enthalpy (KJ/mol) of the Ca^{2+} ATPase. Activation enthalpy is plotted against geographical location of the species. The triangles at the top represent the approximate temperatures experienced. The numbers 1 - 15 refer to the numbers allocated in table 5:1.

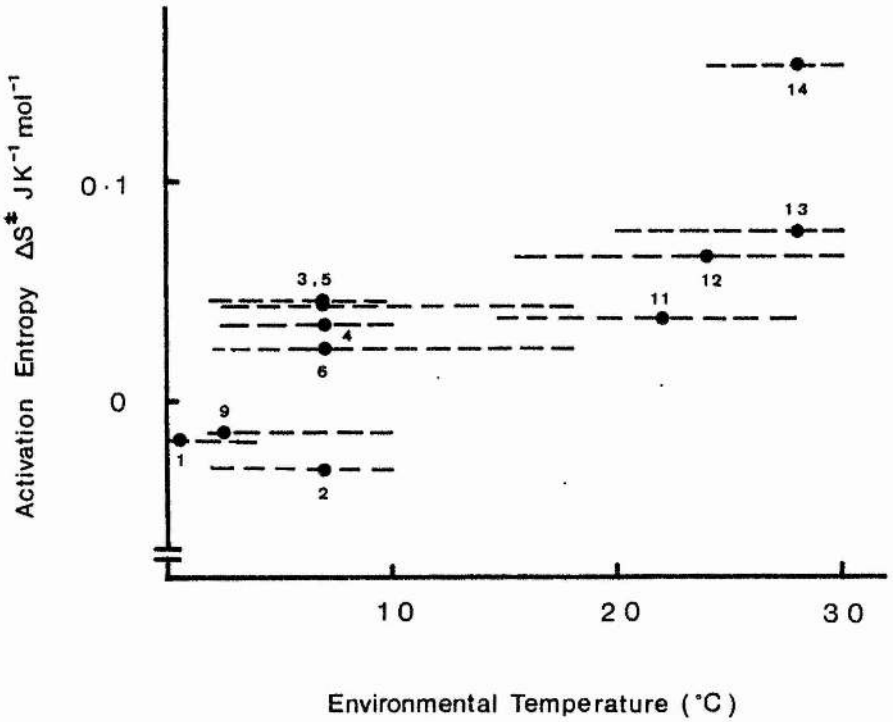


Fig. 5:6 Activation entropy ($\Delta S^{\ddagger}$) in KJ/deg/mol is shown plotted against environment temperature. For details see Fig. 5:1 and Table 5:1.

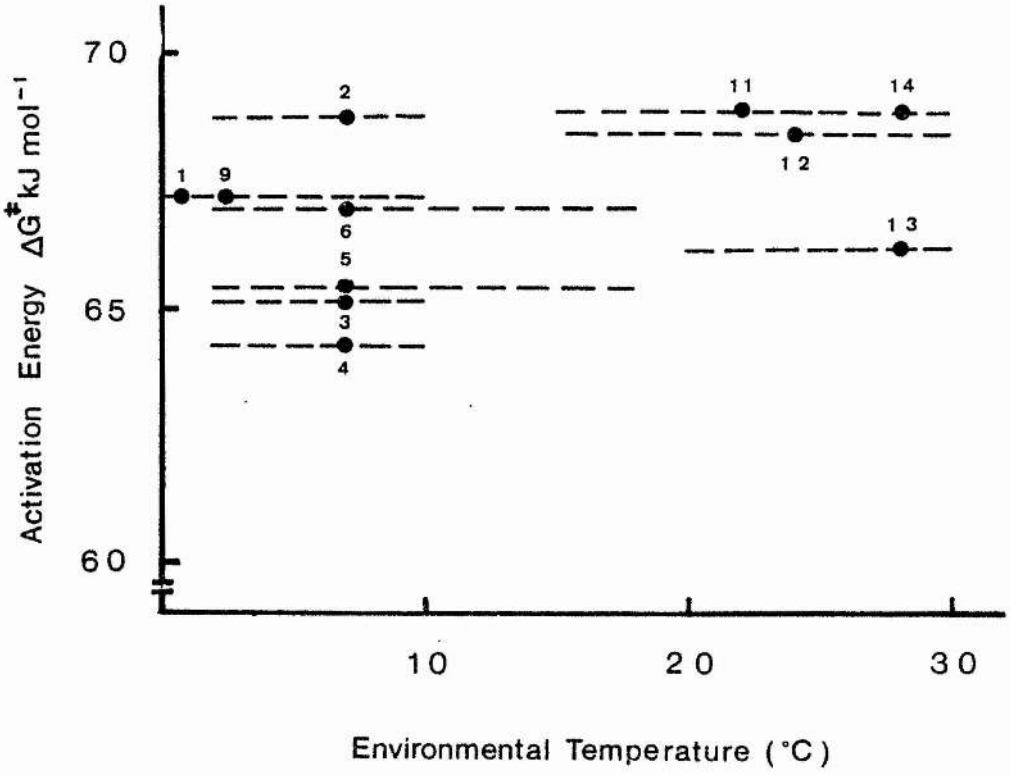


Fig. 5:7 Activation energy ($\Delta G^{\ddagger}$) in KJ/mol is shown plotted against cell temperature. For full details see Fig. 5:1 and Table 5:1.

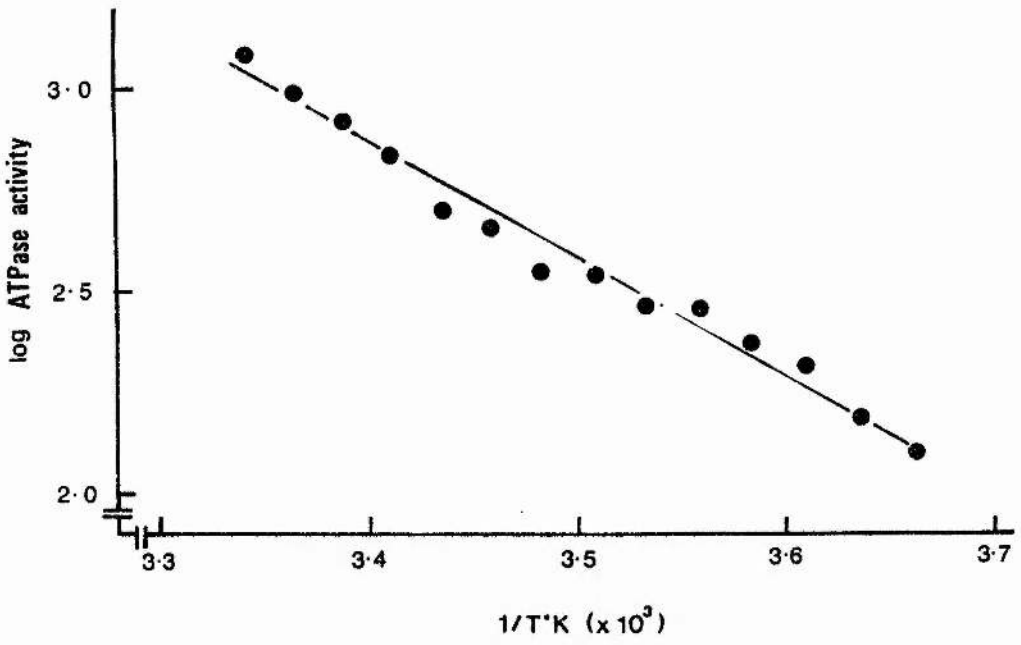


Fig. 5:8 An Arrhenius plot of the Ca^{2+} dependent ATPase activity of hot acclimated carp (Carassius carassius). Values represent the mean of three observations.

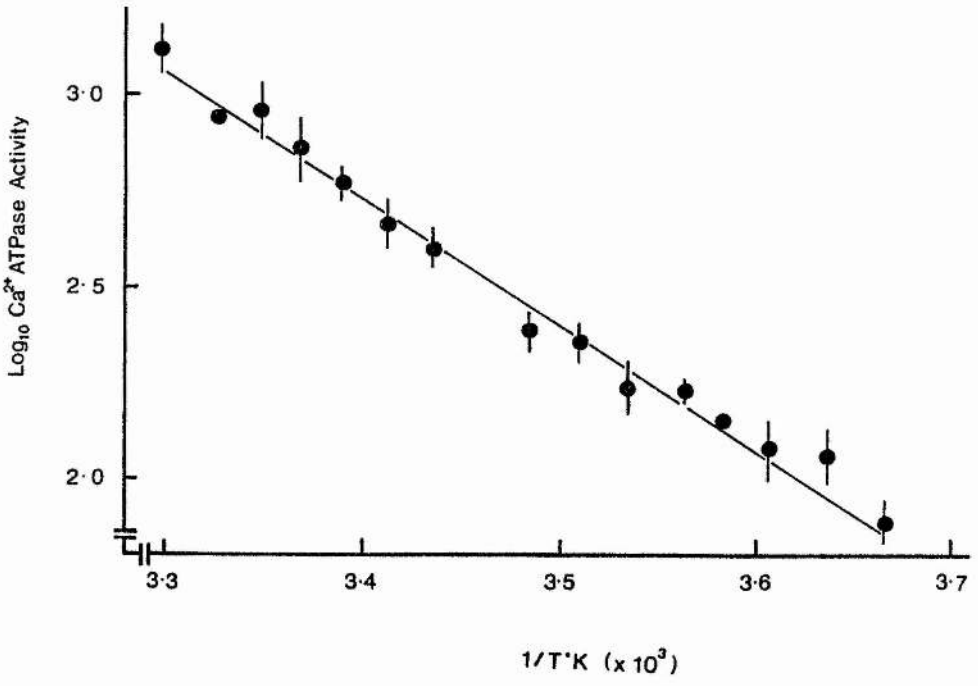


Fig. 5:9 An Arrhenius plot of the Ca^{2+} ATPase activity of SR isolated from cold acclimated carp. Values represent the mean $\pm$ S.E.M. of four observations.

CHAPTER 6

EVOLUTIONARY TEMPERATURE ADAPTATION OF THE K_{Ca} OF THE Ca^{2+} DEPENDENT ATPASE OF SARCOPLASMIC RETICULUM

EVOLUTIONARY TEMPERATURE ADAPTATION OF THE K_{Ca} OF THE Ca^{2+}-DEPENDENT ATPASE OF SARCOPLASMIC RETICULUM

Introduction

The previous chapter discussed the effect of temperature on the Vmax, and hence the K_{cat} of the Ca^{2+}-dependent ATPase. However, in terms of enzyme functional properties, two traits appear to be exceptionally important in evolutionary adaptation to different environmental conditions. These are the K_{cat}, as discussed previously, and the apparent Michaelis constant, the Km of the enzyme. High degrees of conservation of Km values have been observed in enzymes of intermediary metabolism such as M_4-LDH's (Yancey and Somero, 1978a) and pyruvate kinase (Low and Somero, 1976) and in a membrane bound enzyme, acetyl cholinesterase (Baldwin, 1971; Baldwin and Hochachka, 1970).

Enzymes are not only important in catalysing reactions, but are also very important physiological regulators of metabolism. The regulatory functions are diverse, and may involve complex allosteric effects as well as more simple changes in concentration of substrates or modulators. Somero and Yancey (1978a) suggest that the simplest type of response which can be realistically termed a regulatory response is the ability of an enzyme to increase its metabolic rate in response to an increase in the substrate concentration. In order to do so, the enzyme must possess a reserve capacity which allows it to alter its rate (Atkinson, 1976). The

reserve capacity is achieved by either maintaining the substrate concentration in the same range as the Km, or maintaining the Km in the same range as the substrate concentration. This conservation is very clear when Km's are measured under physiological conditions. For example, different species from thermal environments as diverse as -2°C and 37°C have pyruvate Km values for LDH in a very narrow range, approximately 0.15 to 0.35mM (Yancey and Somero, 1978a).

Further circumstantial evidence for the importance of the conservation of Km lies in the fact that observed metabolite concentrations from different species are often very similar (Yancey and Somero, 1978a,b). For example, pyruvate concentrations in resting white muscles of vertebrates are very similar, varying from 0.11mM in trout (Black et al, 1962) and frogs (Sacks et al, 1954) to 0.37mM in starving eels (Mayerle and Butler, 1971) and goldfish (Freed, 1971).

The sarcoplasmic reticulum Ca^{2+} pump is a regulatory enzyme in the sense that it maintains a low concentration of Ca^{2+} in the myoplasm. During contraction, however, there is a very rapid increase in the internal Ca^{2+} concentration (10^{-7} to approximately 10^{-5} (Sandow, 1970; Ashley, 1970)) in the myoplasm. Thus, since it appears that for a large part of the excitation-contraction cycle the Ca^{2+} pump would be working at or close to Vmax, it was thought that adaptations in Km would not be so important in the SR.

Rather than measuring the Km for Ca^{2+}, by measuring Ca^{2+}-uptake, at different Ca^{2+} concentrations, it was

decided to measure the K_{Ca}, the Ca^{2+} concentration which gives half maximal activation to the Ca^{2+}-dependent ATPase.

There were several reasons for the choice. Firstly, using the Millipore filtration method involves the use of Ca^{2+}-precipitating agent such as oxalate or phosphate. This introduces several possibilities for artefactual results (Tume and Hunnington, 1974). Several authors have shown that Ca^{2+}-ATPase activity is closely related to Ca^{2+} transport, with two Ca^{2+} ions being pumped for each ATP molecule hydrolysed (Hasselbach, 1964; Weber, 1966; Hasselbach, 1978). In addition, it has been demonstrated that the Km for Ca^{2+} is identical numerically to the K_{Ca} of the ATPase (Inesi, 1979). Consequently, it is felt that, although the experiments described in this chapter describe the effects of temperature on the ATPase, they also reflect the effect on the Ca^{2+} transport process.

Materials and Methods

Four species of fish were investigated in these experiments; Notothenia rossii (Environment temperature, ET, -2 - +4°C), rainbow trout, Salmo gairdneri (ET 2 - 16°C), plaice, Tilapia mossambicca (ET 24 - 30°C) and Sarocherodon niloticus (ET 24 - 30°C).

Fish were killed by stunning and decapitation, and the white muscle was rapidly excised, ensuring that there was no contamination with other fibre types. Highly purified sarcoplasmic reticulum (SR) was prepared as previously described (Chapter 4). Briefly, a partially purified microsomal pellet was isolated from the homogenised muscle by

differential centrifugation, resuspended in isolation buffer (0.3M sucrose, 10mM imidazole, pH 7.2), and layered onto a sucrose density gradient. The fraction sedimenting between 30 and 35% sucrose has been shown to be free of contamination with other membrane components (McArdle and Johnston, 1979, 1980), and was used in all experiments described.

ATPase activity was assayed in a medium containing 60mM KCl, 5mM $MgCl_2$, 40mM imidazole, pH 7.2 (10°C), and a Ca^{2+}-EGTA buffer which was varied to give different free Ca^{2+} concentrations. Free Ca^{2+} concentrations were estimated using an iterative computer programme.

Thermodynamic activation parameters were estimated from transition state theory according to the following equations:

$$k = (KT/h) \exp - \Delta G^{\ddagger}/RT \quad (1)$$

$$\Delta S^{\ddagger} = (\Delta H^{\ddagger} - \Delta G^{\ddagger})/T \quad (2)$$

$$\Delta H^{\ddagger} = Ea - RT \quad (3)$$

where K = Boltzmann's constant, h = Planck's constant and Ea = the energy of activation derived from the slope of the Arrhenius plot according to the equation

$$m = Ea/RT \quad (4).$$

k, the substrate turnover number, was derived by dividing the specific activity of the enzyme by the molecular weight of the ATPase (100,000 daltons) and the relative proportion of the total protein that the ATPase represents (70%) (Inesi et al, 1976).

K_{Ca}, the Ca^{2+} concentration which gives half maximal activation of the ATPase, was determined from the Hill plot of

$$\log v/(Vmax - v) = n \log S - \log K \quad (5).$$

Prior to initiating the reaction, SR (0.2 - 0.5 mg/ml)

was preincubated in the incubation medium for three minutes at the required temperature (10°C). The reaction was started by adding ATP (final concentration 2mM), and terminated with the addition of an equal volume of 10% trichloro acetic acid (TCA). The denatured protein was precipitated by centrifugation, and phosphate released was measured using the method of Rockstein and Herron (1951).

Protein concentration was estimated by the Maddy and Spooner (1970) modification of the Lowry method (Lowry et al, 1951).

Statistical analyses were performed using the Student's t-test.

Results

Table 6:1 shows the ATPase activities of fast muscle sarcoplasmic reticulum isolated from four species of fish, estimated at a free Ca^{2+} concentration of 1.07μM and at two temperatures. At 0°C, the Ca^{2+}-dependent ATPase activity increases with decreasing adaptation temperature, varying from 2± 2 nmol/mg/min in Sarocherodon niloticus to 128 ± 11 nmol/mg/min in Notothenia rossii. In contrast, the Basal ATPase does not display a clear correlation with temperature. At 30°C, the differences in ATPase activity have largely disappeared (Table 6:1).

The effect of Ca^{2+} concentration on the Ca^{2+}-dependent ATPase can be expressed as a Hill plot (Figs. 6:1 and 6:2). The Ca^{2+} concentration which gives 50% of Vmax (K_{Ca}) can be determined from these graphs, according to the equation given in the Materials and Methods. K_{Ca} has been estimated

for four fish species from different thermal environments at seven temperatures. The results are plotted in Fig. 6:3. It may be seen that, at 0°C, there is a positive correlation between habitat temperature and K_{Ca}. When measured at the animal's cell temperature, however, there is a considerable degree of conservation between K_{Ca}s, with values falling in the range 0.4 to 0.7μM (see inset to Fig. 6:3).

Assuming that the Ca^{2+}-ATPase has a molecular weight of 100,000, and constitutes 70% of SR protein, an apparent substrate turnover for the enzyme may be derived at different Ca^{2+} concentrations (Inesi et al, 1976). Measuring the slope of the Arrhenius plot at different Ca^{2+} concentrations gives an apparent $\Delta H^{\ddagger}$ for the Ca^{2+}-dependent ATPase. Fig. 6:4 shows the $\Delta H^{\ddagger}$ for the Ca^{2+}-ATPase at different Ca^{2+} concentrations for N. rossii, and Fig. 6:5 shows the same parameter for a tropical species, S. niloticus. The reason for the shape of the curve is not known, but it is possible that it represents the sum of two curves, one of which is due to the Ca^{2+}-dependent ATPase, and one arising from the postulated interconversion of the basal to the Ca^{2+}-dependent form of the enzyme. This possibility will be discussed in the following chapter.

Measurement of the activation enthalpy ($\Delta H^{\ddagger}$) at 1μM Ca^{2+} gives the result shown in Table 6:2. As has been previously demonstrated, there is a good correlation between environment temperature and $\Delta H^{\ddagger}$. Activation entropy ($\Delta S^{\ddagger}$) measured at the K_{Ca} increases in tropical species, varying from about -28 ± 9 $JK^{-1}mol^{-1}$ in N. rossii, to $+23 \pm 11$ $JK^{-1}mol^{-1}$ in S. niloticus (Fig. 6:6).

Again, as was observed at Vmax, there is no correlation between ΔG^{*} and adaptation temperature (Fig. 6:7).

Discussion

Studies of enzymes of intermediary metabolism have shown that the Km or $S_{0.5}$ are temperature sensitive, but over the normal range of temperature experienced, Km's are similar and highly conserved between species. For example, Yancey and Somero (1978a) demonstrated that the M_4-LDH's from a variety of ectotherms and homeotherms had minimum Km's at their adaptation temperatures and, further, that a considerable degree of conservation of absolute values of the Km is exhibited (a range of 0.15 to 0.35mM for pyruvate). This result is not unexpected, since the substrate concentrations for many enzymes are often similar between different species, and are generally about half that required to saturate the enzyme (Atkinson, 1976).

Plots of SR ATPase versus Ca^{2+} concentration are sigmoidal, and transform according to the Hill equation (Figs. 6:1 and 6:2). The K_{Ca}, which is defined at the Ca^{2+} concentration required to give half maximal activation of the Ca^{2+}-dependent ATPase, may be derived from these graphs. When assayed at different temperatures, there is found to be a close correlation between cell temperature and the minimum K_{Ca} level (Fig. 6:3). In Notothenia rossii, an Antarctic species (ET -2 to +2°C) the K_{Ca} is 0.67 ± 0.07μM at 0°C, decreasing to 0.34 ± 0.02μM at 25°C, while in a hot adapted species, Tilapia mossambicca, K_{Ca} has a value of 1.94 ± 0.15μM at 0°C, decreasing to 0.48 ± 0.03μM at 25°C. The trout, Salmo gairdneri, a species from water of intermediate

temperature, displays a minimum K_{Ca} at 15°C (Fig. 6:3).

Thus it is clear that modifications in substrate binding are as important in temperature adaptation of SR as they are in non-membrane bound enzymes. At first sight, this seems somewhat surprising, since, in skeletal muscle, the free Ca^{2+} concentration during the contraction relaxation cycle increases from a resting value of 10^{-7}M to a maximum of 10^{-5}M (Ashley, 1970). Therefore, for a considerable part of the cycle, the SR ATPase might be expected to be operating at Vmax and under these circumstances, adaptations in the Km would not be so important.

Using Ca^{2+} sensitive dyes and luminescent proteins, several groups have studied Ca^{2+} transients during twitches in isolated frog (Blinks, 1973; Miledi et al, 1977), and barnacle (Ashley and Ridgeway, 1970) muscle fibres. Contraction is preceded by a rapid rise in Ca^{2+} concentration, which decays almost to the resting level prior to the development of tension. Ca^{2+} released from the SR binds very rapidly to Troponin-C (TnC) which has a very high affinity for Ca^{2+} ions ($K_d = 10^{-7}$M). Ashley and Moisescu (1977) have suggested that the release from TnC is very slow compared to uptake of Ca^{2+} by the SR, so that the free Ca^{2+} concentration is actually maintained at a comparatively low level (approximately 0.5μM). In fish muscle, the situation is further complicated by a class of Ca^{2+} binding proteins known as the parvalbumins (Gillis and Gerday, 1977). These are low molecular weight acidic proteins (11,000 to 12,000 daltons) distributed throughout the muscle myoplasm which bind Ca^{2+} with a K_d of 10^{-6}M (Gillis and Gerday, 1977).

They are an unusual group of proteins, in that they share common properties, but seem to be derived from very different antecedents (Gosselin-Rey and Gerday, 1977). For example, Gosselin-Rey and Gerday (1977) found that parvalbumins IVA and IVB from frog muscle did not interreact antigenically. The same conclusions were reached by Pironf and Gosselin-Rey (1975) working on Gadidae, and Gerday (1976) has analysed the amino acid sequences of two pike parvalbumins, providing very good evidence of early gene differentiation.

The function of the parvalbumins is uncertain (Gerday and Gillis, 1976), but since they occur in such high concentration, and bind Ca^{2+} with such high affinity, it seems likely that they could act as Ca^{2+} buffers in the myoplasm (Gerday and Gillis, 1976; Gillis and Gerday, 1977; Gillis et al, 1979).

Ca^{2+} released from the sarcoplasmic reticulum, therefore, causes only a transient activation of cross bridges, since large proportions of the ion released become bound to the PA's, and are resequestered in the SR before being bound to the TnC and causing activation. The result is that the free Ca^{2+} concentration is not as high as was previously thought, and the SR Ca^{2+}-dependent ATPase is therefore likely to be working at sub-saturating concentrations in vivo, which explains the necessity for temperature induced modifications in K_{Ca}'s.

As noted in the preceding chapter, there is a close correlation between activation enthalpy and environment temperature. Interestingly, $\Delta H^{\ddagger}$ values are also dependent on the free Ca^{2+} concentration (Figs. 6:4 and 6:5). Activation enthalpy values calculated for the Ca^{2+} concentration

which gives half maximal activation of the enzyme are shown in Table 6:2.

Earlier reports suggest that the lowered $\Delta H^{\ddagger}$ was biologically advantageous in reducing the temperature sensitivity of the activation process (Hochachka and Somero, 1973). However, $\Delta H^{\ddagger}$ seems to be correlated with the absolute temperature of the animal, and not with the range experienced. For example, $\Delta H^{\ddagger}$ for N. rossii is significantly lower than that of the trout, although the Antarctic fish will experience less annual temperature variation than the temperate S. gairdneri. Until more information is known concerning the details of the reaction mechanism, little can be said concerning the different contributions of the activation enthalpy and entropy to the activation process.

Table 6:1 Ca^{2+}-ATPase activities of SR from fish white muscle at 107μM free Ca^{2+}.

Species	Environmental temperature	Assay temperature	ATPase activity (nmol Ca^{2+} mg protein^{-1}) Basal	Ca^{2+} dependent	Total
Notothenia rossii	Antarctica (-2 - +2°C)	0	9 ± 2	128 ± 11	137 ± 12
		30	179 ± 13	1495 ± 165	1674 ± 16
Salmo gairdneri	Temperate river (2 - 8)	0	39 ± 5	71 ± 14	110 ± 16
		30	219 ± 60	3370 ± 324	3590 ± 332
Tilapia mossambicca	African lake (20 - 30)	0	20 + 4	16 ± 2	35 ± 3
		30	404 ± 27	2676 ± 444	2878 ± 67
Sarocherodon niloticus	African lake (20 - 30)	0	38 ± 5	2 ± 2	40 ± 5
		30	43 ± 5	1219 ± 90	1237 ± 168

Table 6:2 $\Delta H^{\ddagger}$ values of the Total and Ca^{2+}ATPases of SR from fish fast muscle measured at a free Ca^{2+} of 1.06μM.

Species	ET	n	$\Delta H^{\ddagger}$ Total ATPase	$\Delta H^{\ddagger}$ Ca^{2+}ATPase
N. rossii	-2 - +2°C	4	51 ± 5	59 ± 4
S. gairdneri	2 - +8°C	6	65 ± 2	93 ± 4
T. mossambicca	20 - 30°C	3	81 ± 4	100 ± 7
S. piloticus	20 - 30°C	3	78 ± 3	162 ± 20

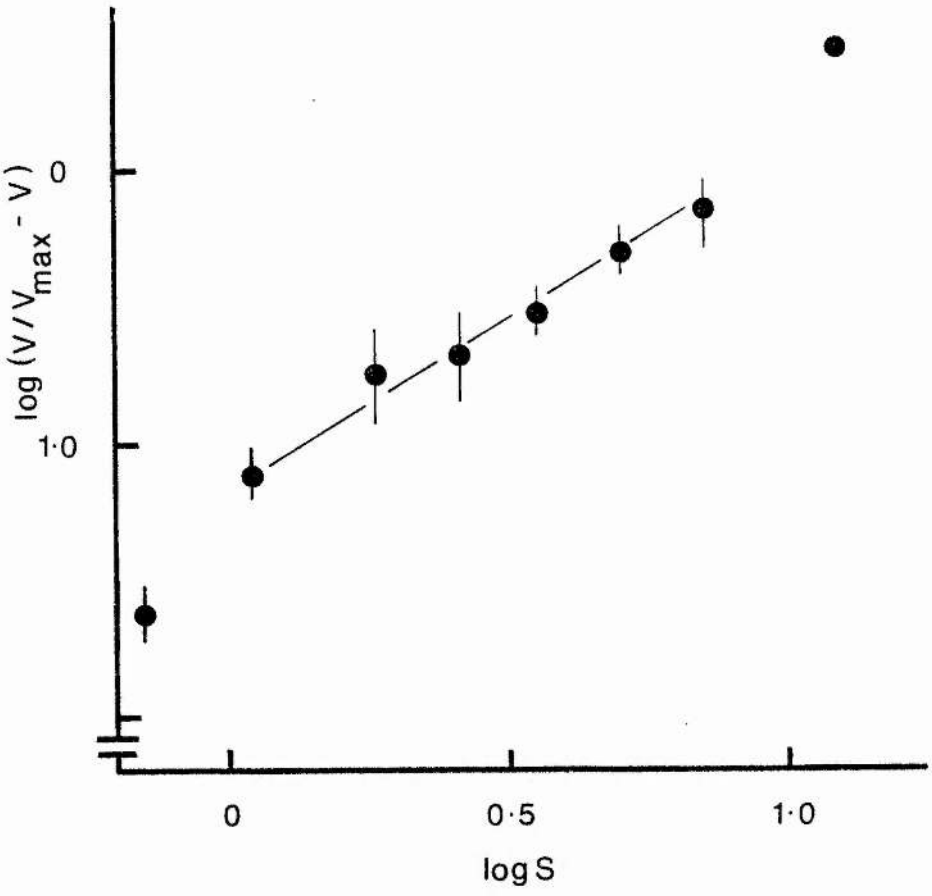


Fig. 6:1 A Hill plot of the Ca^{2+} dependent ATPase activity of SR isolated from Notothenia rossii at 5°C. Values represent the mean ± S.E.M. of four preparations.

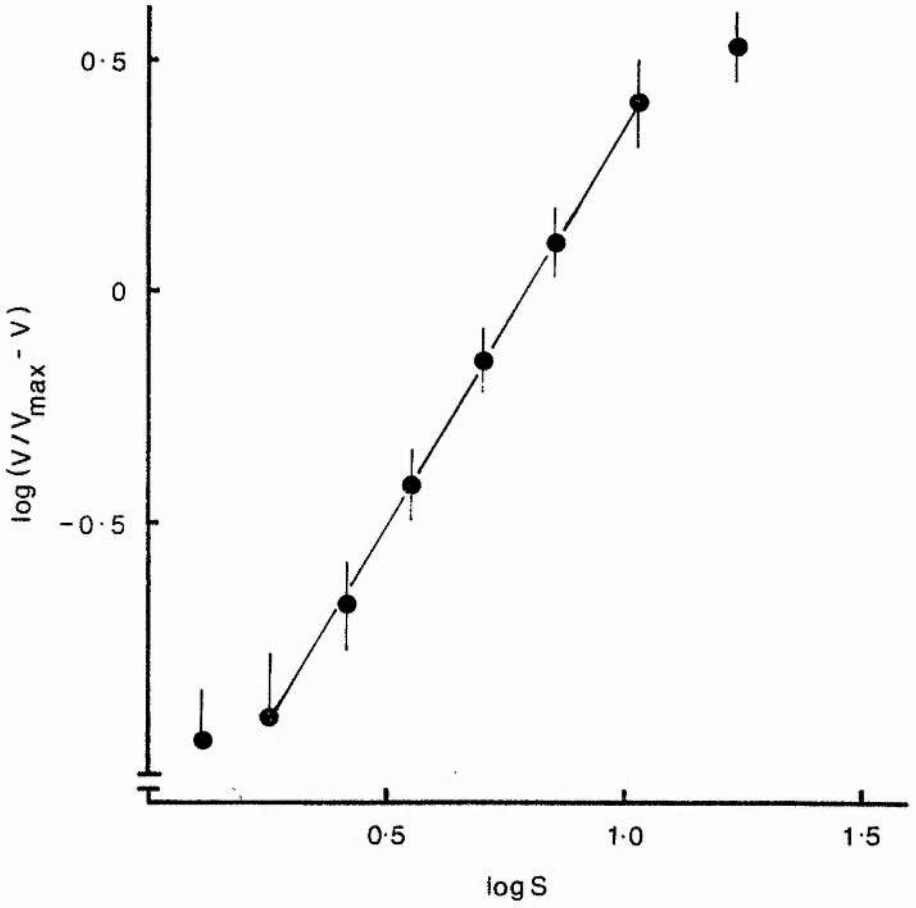


Fig. 6:2 A Hill plot of the Ca^{2+} dependent ATPase activity of SR isolated from trout, S. gairdneri, measured at 25°C. Values represent the mean ± S.E.M. of 6 observations.

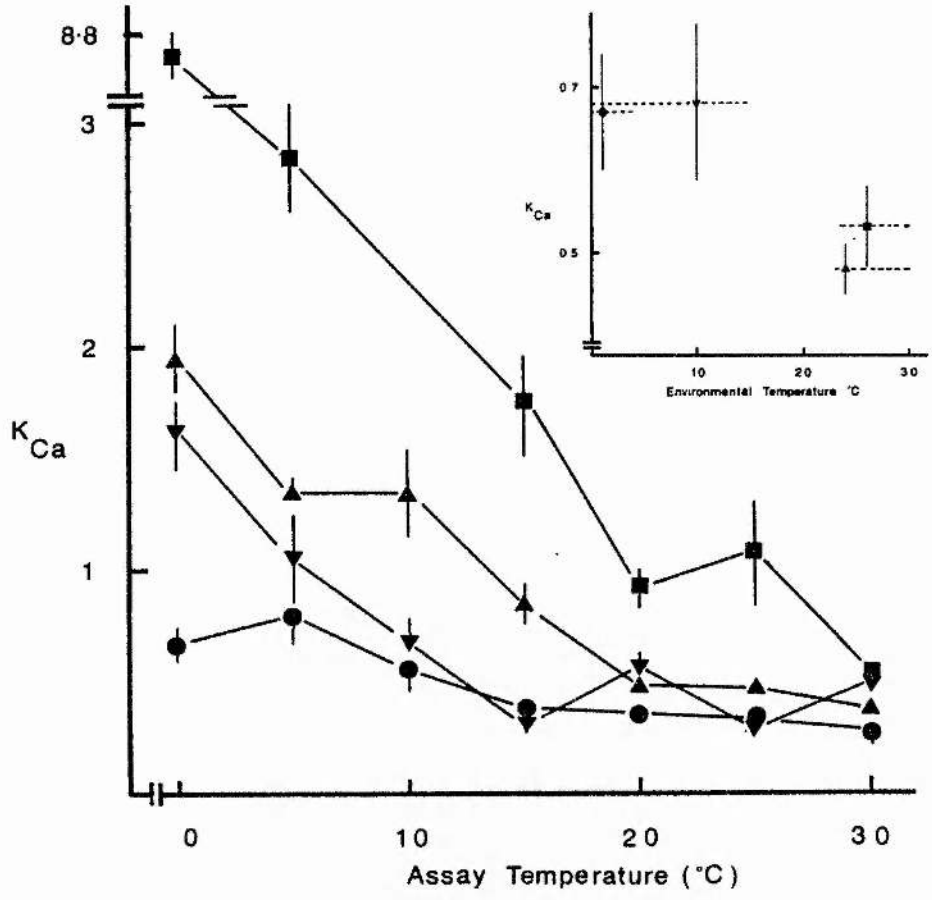


Fig. 6:3 The K_{Ca} (μM) of N. rossii (●), S. gairneri (▼), Tilapia mossambicca (▲) and Sarocherodon niloticus (■) has been measured at different temperatures. The inset shows the K_{Ca} at the animals normal habitat temperature. Values are the mean ± S.E.M. of at least 3 duplicate observations.

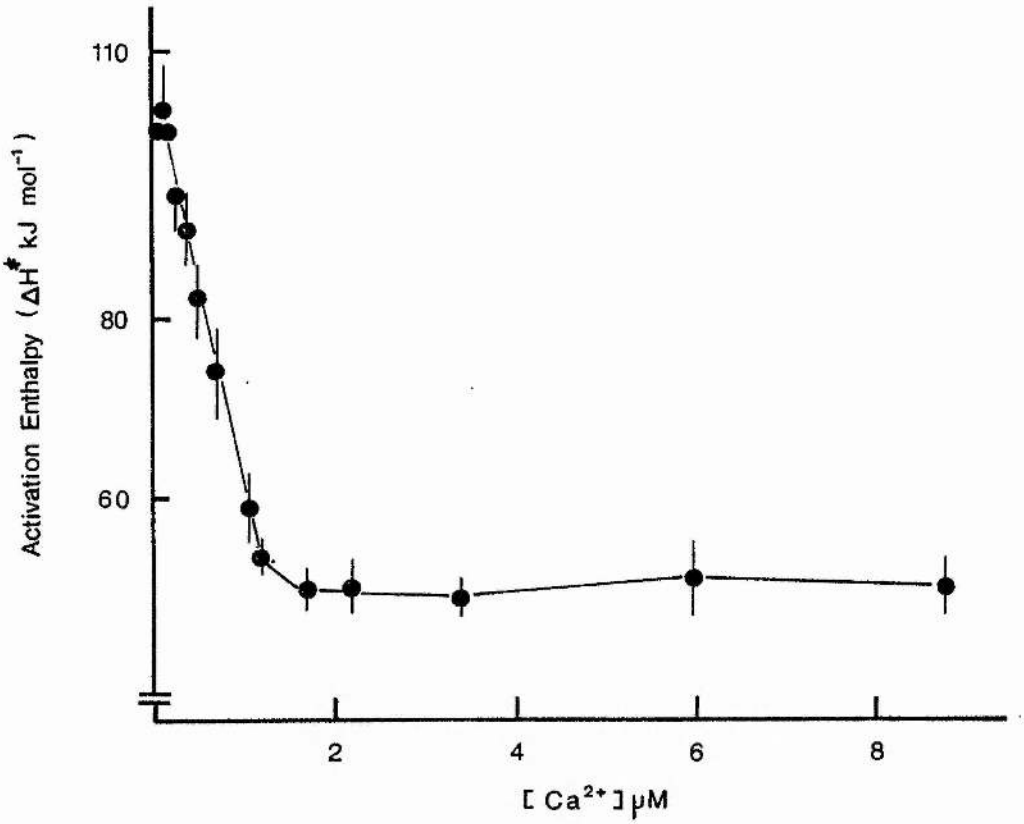


Fig. 6:4 The effect of Ca^{2+} on apparent ΔH^* (KJ/mol) for the Ca^{2+} dependent ATPase of Notothenia rossii. Values represent the mean ± S.E.M. of four preparations.

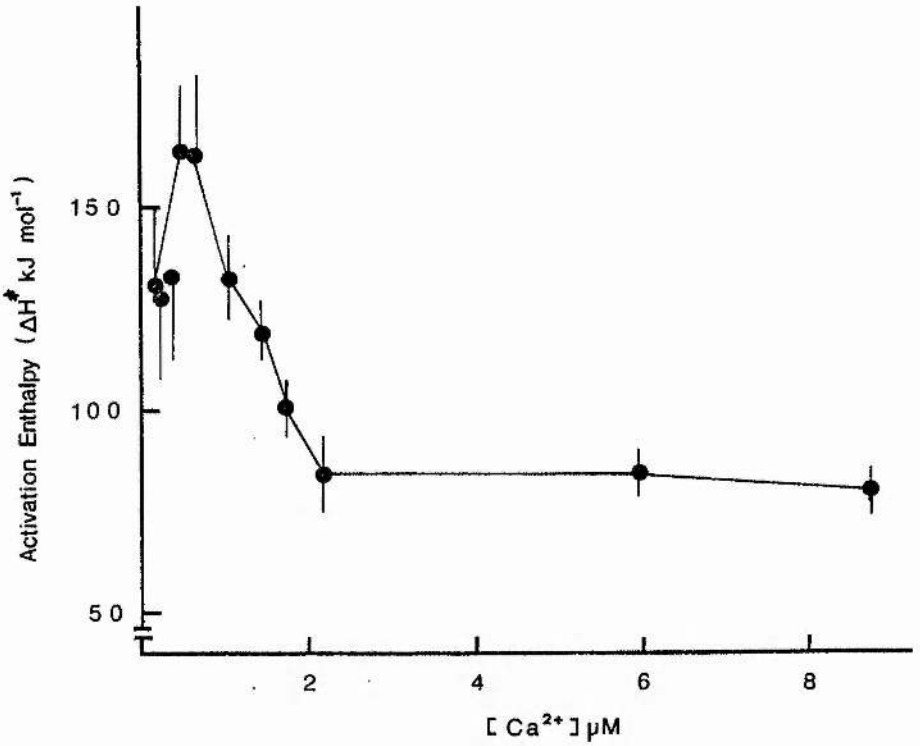


Fig. 6:5 The effect of Ca^{2+} on the apparent $\Delta H^{\ddagger}$ (KJ/mol) of the Ca^{2+} dependent ATPase of Sarocherodon niloticus. Values represent the mean ± S.E.M. of duplicate observations of 3 preparations.

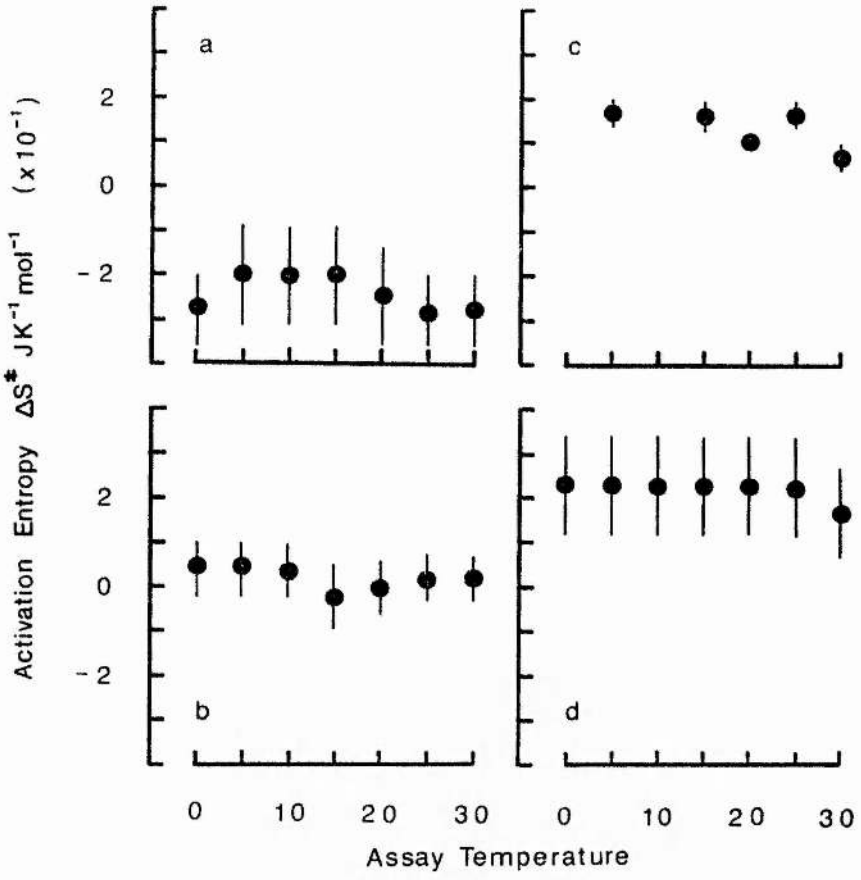


Fig. 6:6 The effect of assay temperature on the apparent ΔS^* (J/K/mol) measured at the K_{Ca} of the Ca^{2+} dependent ATPase of (a) N. rossii, (b) S. gairdneri, (c) T. mossambicca, (d) S. niloticus.

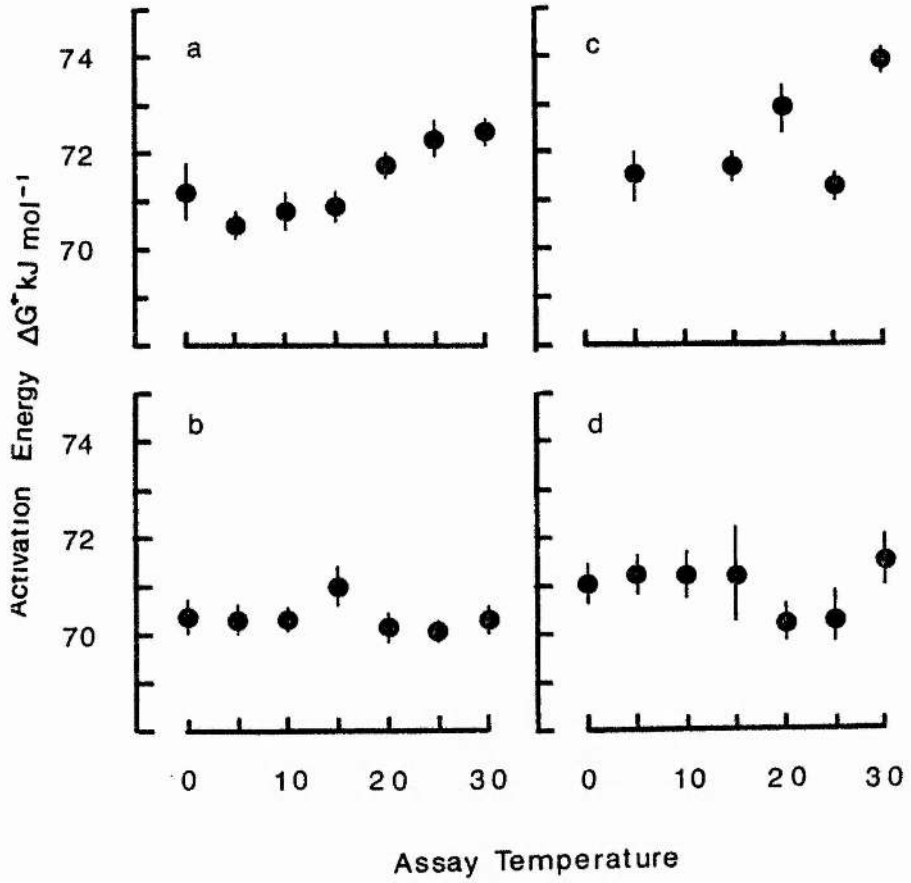


Fig. 6:7 The effect of assay temperature on the apparent $\Delta G^{\ddagger}$ (KJ/mol) measured at K_{Ca} of the Ca^{2+} dependent ATPase of (a) N. rossii, (b) S. gairdneri, (c) T. mossambicca, (d) S. niloticus.

CHAPTER 7

ON THE NATURE OF THE BASAL ATPASE

ON THE NATURE OF THE BASAL ATPASE

Introduction

In the absence of Ca^{2+} ions, membrane vesicles isolated from muscle hydrolyse ATP at low levels. The enzymic activity associated with this hydrolysis is known as the "basic" activity, and the enzyme (or enzymes) responsible for the activity is referred to as the basal ATPase (Hasselbach, 1964). Despite its prevalence, few attempts have been made to analyse its nature.

Headon and his group (Barrett and Headon, 1975; O'Flaherty et al, 1975; Headon et al, 1977) have investigated the distribution of cholesterol in muscle membranes and, from their results, suggested that the basal ATPase could be an enzyme located in contaminating vesicles of sarcolemma or T-tubules. The role of cholesterol in Ca^{2+} transport is somewhat equivocal. For example, digitonin, which binds specifically to 3-β-hydroxy steroids such as cholesterol, abolished Ca^{2+} accumulation (Martonosi, 1968). Further, extracting vesicles with diethyl ether results in a loss of cholesterol from the SR, and a decrease in Ca^{2+} uptake ability and ATPase activity (Hasselbach and Makinose, 1963; Fiehn and Hasselbach, 1969). However, later experiments demonstrated that cholesterol could be removed without prejudicial effect from lyophilised SR using ether, or from normal SR using heptane (Drabikowski et al, 1972). In addition data from Fiehn et al, (1971)

and Kidwai et al (1973) suggested that cholesterol was associated with the sarcolemma rather than any other membrane systems.

Barrett and Headon (1974) used density perturbation techniques to demonstrate that cholesterol and the Ca^{2+}-ATPase were not coincident in a sucrose gradient. Using digitonin (see above) as a perturbing agent, resulted in a density shift of 0.028 for both the cholesterol and the basal ATPase, while the distribution of the Ca^{2+} dependent ATPase was unaffected.

When oxalate was the perturbing agent, thus increasing the density of Ca^{2+} accumulating vesicles, and separating them from other factions, it was found that there was still a significant, but lower, amount of cholesterol in the precipitated vesicles. Headon et al (1977) suggested that the Ca^{2+} and cholesterol containing membranes derived from sealed vesicles of sarcolemma with a Ca^{2+} accumulating capability. Thus, since the cholesterol is thought to derive from the sarcolemma or T-system and not the SR, and since the basal ATPase is associated with the high cholesterol containing membranes, then the basal ATPase activity must be a result of contamination with either sarcolemma or T-system membranes.

In contrast, Sarzala and Michalak (1978) noted that the "heavy" SR fractions, which were capable of accumulating Ca^{2+} ions, and which displayed no contamination with other membranes, had levels of cholesterol about 150% that of the "light" microsomes, which did not accumulate Ca^{2+}, and which displayed low and variable levels of Ca^{2+}-ATPase activity.

In addition the presence of contaminating membranes can usually be demonstrated using marker enzymes; Na-K-ATPase or 5'-nucleotidase in sarcolemma, for example, and azide sensitive ATPase or TCA enzymes in mitochondria, and fractions which have no marker enzyme activity can still be demonstrated to possess a basal ATPase activity (Meissner, 1973; Inesi, 1976).

An alternative hypothesis has been proposed by Inesi et al (1976), who isolated fractions of SR from rabbit muscle, using sucrose density centrifugation, separating the microsomal pellet into four fractions. The protein composition of the four fractions was very similar (see also Sarzala and Michalak, 1978), except that the highest density fraction contained somewhat higher amounts of calsequestrin. No contamination was evident in any of these fractions. Treating the S_1 fraction, which had the highest basal ATPase activities, and the highest cholesterol levels, with Triton X-100 results in the basal ATPase becoming Ca^{2+}-dependent.

Using a fraction which was further purified by another step gradient centrifugation, Inesi and colleagues demonstrated that it was possible to alter the ratio of Ca^{2+} to basal ATPase simply by changing the assay temperature. Thus, at 37°C, the Ca^{2+}-ATPase accounts for 89% of the total ATPase activity, but at 0°C, it accounts for only 38%.

In addition, during development of the SR in rabbit, the total ATPase levels do not alter very much (1.35μmol/mg/min at day 1 to 1.17μmol/mg/min in the adult), but the basal ATPase activity decreases by more than five times, and the Ca^{2+}-ATPase increases by more than twice (Sarzala et al,

1975). This suggests that the SR can interconvert basal and the Ca^{2+}-dependent ATPase such that the ratio changes while the total ATPase activity remains roughly constant.

From this data, Inesi et al (1976) have constructed a model whereby the ATPase exists in two functional states, which are in a thermal equilibrium, and have derived thermodynamic values for the conversion of one state to the other.

If their theory is correct, it might be expected that cold adapted fish would display maximal ratios of Ca^{2+}-ATPase to total ATPase at low temperatures, and that hot adapted animals would behave more like the rabbit, and display low levels of coupling at low temperatures, increasing the relative amounts with increasing temperature.

This chapter describes the effect of temperature and Ca^{2+} concentration on the basal ATPase activity and the total ATPase activity of several different species of teleost fish, and discusses how the results obtained relate to the theory proposed by Inesi et al (1976).

The data used in this chapter is derived from that given in chapters 5 and 6, and the species of fish, the materials and the methods used are described in the relevant sections in those chapters.

Results

Fig. 7:1 shows the activation enthalpy values for the basal ATPase for animals from different thermal environments plotted against their cell temperature. Unlike the Ca^{2+} ATPase, there is no correlation between habitat temperature and $\Delta H^{\ddagger}$.

In Fig. 7:2, the basal ATPase activity at 0°C for the different species of fish is shown plotted against habitat temperature. Again, unlike the Ca^{2+}-dependent ATPase, there is no clear correlation between ATPase activity and thermal environment.

The effect of temperature on the ratio of Ca^{2+} to total ATPase activity for four different species of fish is shown in Figs 7:3 and 7:4. In the cold adapted species, there is little difference in the ratio over the temperature range 0 to 30°C. In the tropical species, on the other hand, the ratio is much lower at 0°C than it is at 30°C, providing supportive evidence for Inesi's theory. The relationship between adaptation temperature and ratio at 0°C for fifteen species of teleost fish is shown in Fig. 7:5. It is clear that there is an inverse correlation between cell temperature and the ratio of Ca^{2+} to total ATPase activity, with cold adapted species having ratios in the range 75-98% at this temperature compared with only 2-45% for the tropical species. Above 20°C, all species had similar ratios in the range 80-98%.

Inesi et al (1976) had demonstrated that perturbation of the SR vesicles with mild detergent treatment (0.1% Triton X-100) resulted in a conversion of basal to Ca^{2+}-dependent ATPase. Table 7:1 shows the effect of 0.1% Triton on the ratio of Ca^{2+} to total ATPase at 20°C. The vesicle fraction sedimenting between 11 and 30% sucrose is unaffected by the treatment, but the basal ATPase in all other fractions is nearly completely converted into a Ca^{2+} dependent form. The detergent has no effect on the total ATPase of the 30-35% or the 35-40% fractions, though it

does inhibit the total ATPase of the 11-30% fraction (363 ± 75 without Triton and 187 ± 43 nmol/mg/min in the presence of 0.1% Triton X-100; $p < 0.01$).

Fig. 7:6 demonstrates the effect of different Ca^{2+} concentration on the ratio of Ca^{2+} to total ATPase activity, for four species of fish. Again, it may be seen that the hot adapted species have lower ratios at low temperatures than their cold adapted counterparts, but that the difference disappears at higher temperatures. It is of interest to note that decreasing the free Ca^{2+} concentration results in a change in the ratio at different temperatures. For example, in Sarocherodon niloticus the ratio at 0°C is 60 ± 12% at a Ca^{2+} concentration of 8.78μM, and at a Ca^{2+} concentration of 0.7μM, it is 0% at 0°C (Fig. 7:6).

Inesi and co-workers had treated the conversion of basal to Ca^{2+}-ATPase analogously to the process of protein denaturation, and used the equations given by Joly (1965) to derive thermodynamic values for the conversion $E_1 \rightleftharpoons E_2$ (Ca^{2+}-independent to Ca^{2+}-dependent ATPase), viz:-

$$K_E = \exp\left(-\Delta G^{\circ}_{1\rightarrow 2} / RT\right) \qquad (1)$$

the enthalpy and entropy of conversion are defined by

$$\Delta G^{\circ}_{1\rightarrow 2} = \Delta H^{\circ}_{1\rightarrow 2} - T\Delta S^{\circ}_{1\rightarrow 2} \qquad (2)$$

and their values obtained by plotting $\ln (V_{Ca}/V_{basal})$ against $1/T^{\circ}K$, since

$$\ln(V_{Ca}/V_{basal}) \backsimeq \ln K_E = \Delta S^{\circ}_{1\rightarrow 2}/R - (\Delta H^{\circ}_{1\rightarrow 2}/RT) \qquad (3)$$

(Inesi et al. 1976).

Values for $\Delta H^{\circ}_{1\rightarrow 2}$, $\Delta S^{\circ}_{1\rightarrow 2}$ and $\Delta G^{\circ}_{1\rightarrow 2}$ are given in Table 7:2. Because there was little change in the ratio in cold adapted animals, it was not always possible to derive the

conversion parameters, so that only data from trout and S. niloticus are presented. These values compare favourably with those of Inesi given for rabbit sarcoplasmic reticulum. Inesi and co-workers suggest that the high positive entropy of conversion indicated that the conversion is accompanied by conformational changes in the protein and/or its environment.

$\Delta G^{\ddagger}$, measured at K_{Ca}, shows a similar lack of correlation with habitat temperature to the results shown in chapter 5 for the V_{max} estimations (Fig. 7:7).

Discussion

Unlike the Ca^{2+}-dependent ATPase, the activation enthalpy of the basal ATPase does not increase with increasing adaptation temperature (Fig. 7:1). This observation tends to mitigate against the enzyme being of physiological significance, since virtually all enzyme systems thus far studied show adaptive changes of one form or another to thermal stress, especially over evolutionary time periods (see Hazel and Prosser, 1974; Alexandrov, 1977 for reviews).

In addition, and again unlike the Ca^{2+}-dependent ATPase, the basal enzyme does not show rate compensation. Fig. 7:2 shows that there is no correlation between the enzyme's activity and cell temperature at $0^{o}C$. Hazel and Prosser (1970) have suggested that only enzymes of degradation, e.g. peroxidase and catalases, show no or inverse compensation. Adaptations in the Na-K-ATPase of fish have been demonstrated (Thomson et al, 1977) as have adaptive changes in the TCA enzymes of mitochondria (see e.g. Wodtke, 1976, 1978; Smith, 1977). Thus, if the basal ATPase were due

to contamination by these vesicle fractions, one would expect to see some form or degree of compensation between the different species. The fact that no such changes are evident is, to some extent, supportive evidence to the contention that contamination is not responsible for the ATPase activity.

From Inesi's theory, it would be predicted that SR adapted to cold temperatures, having an optimal configuration at low temperatures, would show high Ca^{2+}/total ratios at low temperatures, while their warm adapted counterparts would more closely resemble the rabbit, and would show lower ratios at $0^{o}C$. Figs. 7:3, 7:4 and 7:5 show this to be the case. Warm adapted species have a lower ratio at $0^{o}C$ than cold adapted species, presumably because the latter's SR is already in an optimal configuration at the low temperatures. At higher assay temperatures, the enzymes of all the species are maximally coupled.

As recorded in the rabbit (Inesi et al, 1976), perturbation of the membrane with a mild detergent can reverse the temperature induced interconversion (Table 7:1). It is interesting that the 11 - 30% fraction was unaffected by the Triton treatment. We suspect that this fraction represents contamination by, for example, sarcolemmal fragments, but that the Ca^{2+}-independent ATPase activity present on the other fractions is most likely to be an uncoupled form of the Ca^{2+} pump.

Table 7:2 gives $H^{o}_{1\rightarrow 2}$, $S^{o}_{1\rightarrow 2}$, and $G^{o}_{1\rightarrow 2}$ values for two species of fish and the rabbit results obtained by Inesi and co-workers. The high positive entropy suggests that

the conversion is accompanied by conformational changes in the protein and/or its environment (Inesi et al, 1976). The conversion may also be dependent on the free Ca^{2+} concentration, since changing the Ca^{2+} concentration alters the ratio of Ca^{2+} to total ATPase at any one temperature (Fig. 7:6).

Studies with pyruvate kinases, glyceraldehyde-3-phosphate dehydrogenases and Mg^{2+}-Ca^{2+}-myofibrillar ATPases from skeletal muscle have shown that, in animals from cold environments, natural selection has favoured the evolution of enzymes with lower activation free energies ($\Delta G^{\ddagger}$) than temperate or tropical species (Johnston et al, 1973; Johnston and Walesby, 1977, 1979; Hazel and Prosser, 1974). For example, there is an almost linear correlation between $\Delta G^{\ddagger}$ for fast muscle Mg^{2+}-Ca^{2+}-myofibrillar ATPases and the habitat temperature of the fish species from which they were isolated (Johnston et al, 1977; Johnston, 1980). However, as seen in Fig. 7:7, there is no such relationship evident for the Ca^{2+}-ATPase of fish sarcoplasmic reticulum.

Examination of the $\Delta G^{\circ}_{1\rightarrow 2}$ values for the interconversion of the ATPases indicates that there is an inverse correlation with environment temperature. The rabbit, with the highest body temperature, having the lowest $\Delta G^{\circ}_{1\rightarrow 2}$. Assuming that the $\Delta G^{\circ}_{1\rightarrow 2}$ contributes towards the total observed $\Delta G^{\ddagger}$ for catalysis, then the lack of correlation between $\Delta G^{\ddagger}$ and environment temperature may be explained. Subtracting $\Delta G^{\circ}_{1\rightarrow 2}$ from the $\Delta G^{\ddagger}$ would markedly improve the correlation.

In summary, it would seem that both theories are, to some extent, correct. It is clear that the fraction which

precipitated in the 11-30% interface is not an interconvertible form of the Ca^{2+}-dependent ATPase (Table 7:1), and it is possible that the enzyme activity is derived from contaminating vesicles, possible sarcolemmal in origin.

There is a large body of evidence to show that the microsomal pellet of muscle does have other fractions than SR present. For example, Volmer (1978) has shown that in a muscle with many mitochondria, 15% of the mitochondrial pellet's activity may be found in the microsomal pellet. In contrast, fractions which are further purified by sucrose density centrifugation can be shown to be free of vesicles deriving from either the sarcolemma or the mitochondria of the muscle (Meissner, 1973; Inesi, 1976; Hidalgo *et al*, 1976; this thesis, chapter 4).

In these fractions, there is still found a Ca^{2+} independent ATPase activity, and it is this enzyme which we suggest is a form of the Ca^{2+} pump protein uncoupled from Ca^{2+} transport.

Table 7:1 The effect of Triton X-100 (0.1%) on the ratio of Ca^{2+}-dependent ATPase/total ATPase activity of microsomal fractions isolated from Tilapia mossambica on a discontinuous sucrose gradient. The assay was performed at 20°C under the conditions described in chapter 4, except that HEPES buffer replaced Tris buffer. n.s. = not significant at P=0.05 level.

Fraction % sucrose	Triton X-100	n	Ca^{2+} dependent ATPase / total ATPase	P
11 - 30	-	5	0.16 ± 0.05	
11 - 30	+	5	0.19 ± 0.13	n.s.
30 - 35	-	5	0.63 ± 0.06	
30 - 35	+	5	0.97 ± 0.02	< 0.001
35 - 40	-	5	0.80 ± 0.03	
35 - 40	+	5	0.94 ± 0.03	< 0.05

Table 7:2 Thermodynamic conversion parameters for the change $E_1 \rightleftharpoons E_2$ for two species of fish. The data for the rabbit has been calculated from Inesi et al (1976).

Species	$[Ca^{2+}]$	$\Delta H^{\circ}_{1\rightarrow 2}$	$\Delta S^{\circ}_{1\rightarrow 2}$	$\Delta G^{\circ}_{1\rightarrow 2}$
	(μM)	kJ/mol	J/deg/mol	kJ/mol
S. gairdneri	0.11	95.4±15	143.5±21	56±9
S. niloticus	8.78	71	114.4	39.7
rabbit		52	184	1.4

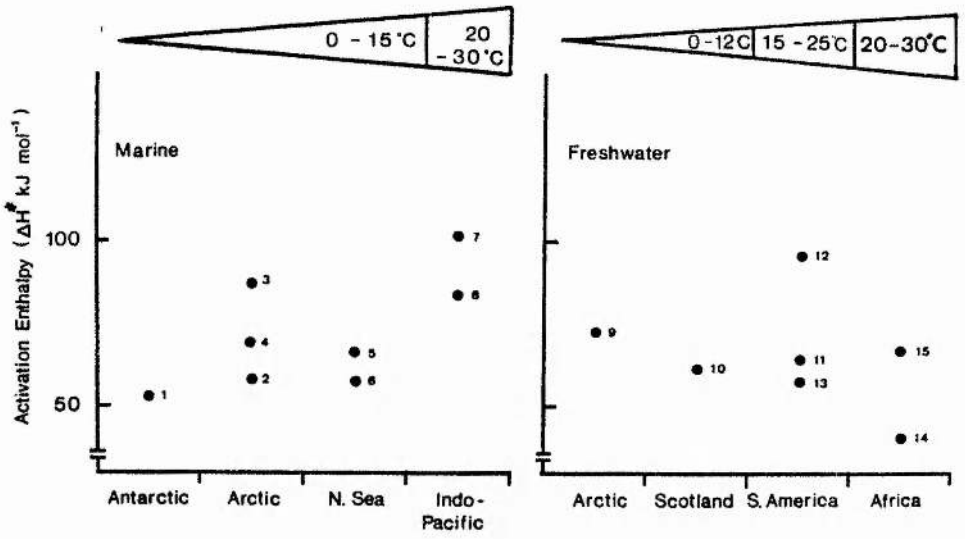


Fig. 7:1 Apparent $\Delta H^{\ddagger}$ values for the basal ATPase activities for different species of fish. See table 5:1 for identification. The species have been divided into marine and freshwater, and the triangles above the graph represent the approximate temperature at which each species is found.

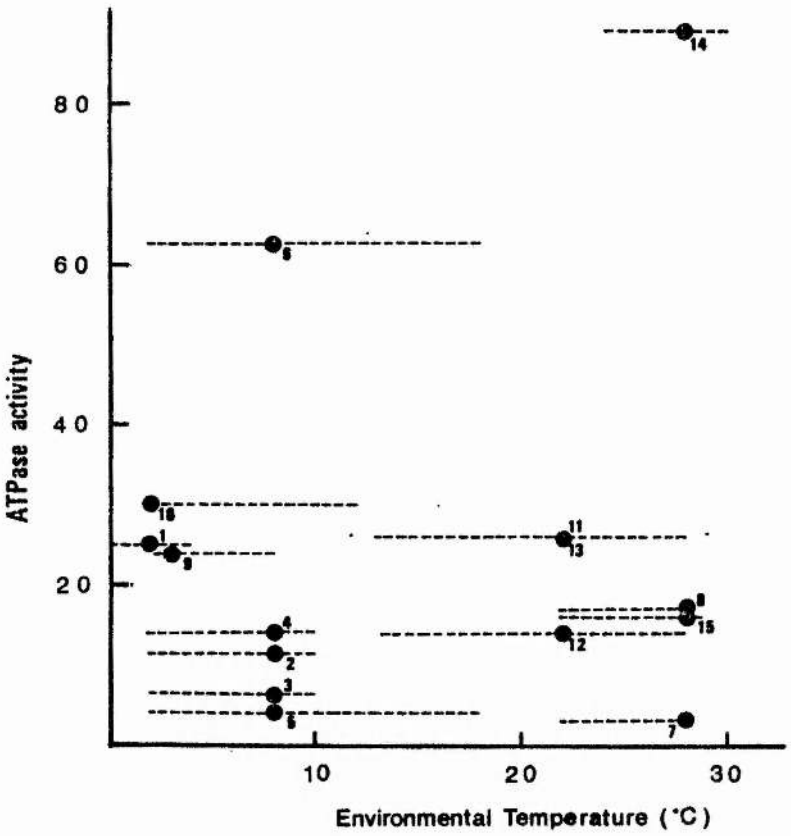


Fig. 7:2 The effect of adaptation on basal ATPase activity (nmol/mg/min) measured at 0°C. The numbers 1 - 15 refer to the animal species (see Table 5:1).

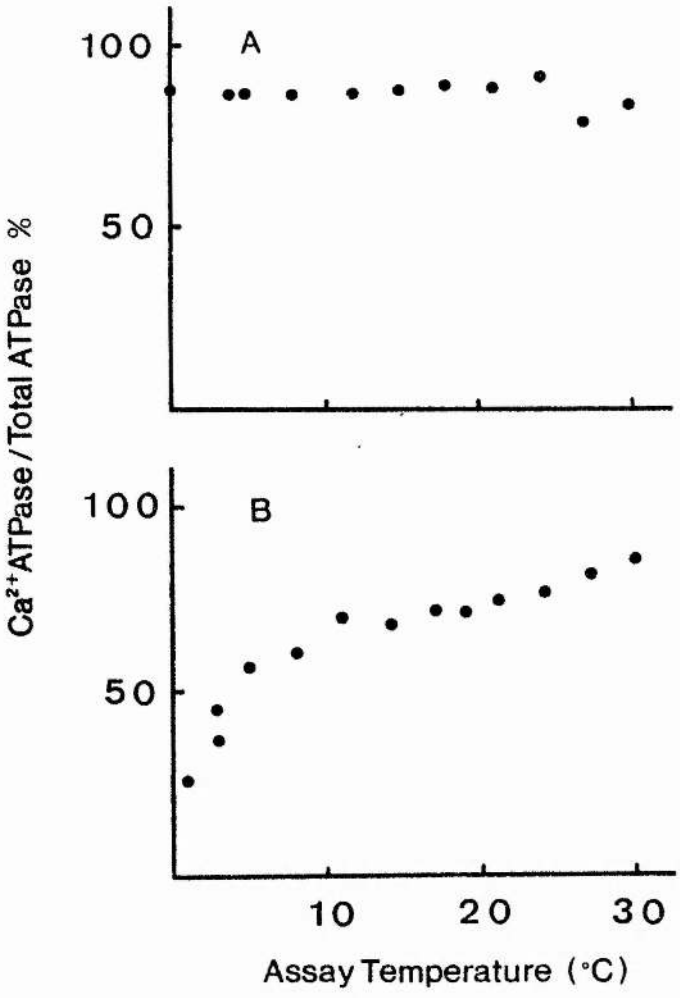


Fig. 7:3 The ratio of Ca^{2+} ATPase/Total ATPase activity is measured against assay temperature for A. Myoxocephalus scorpius and B. Tilapia mariae.

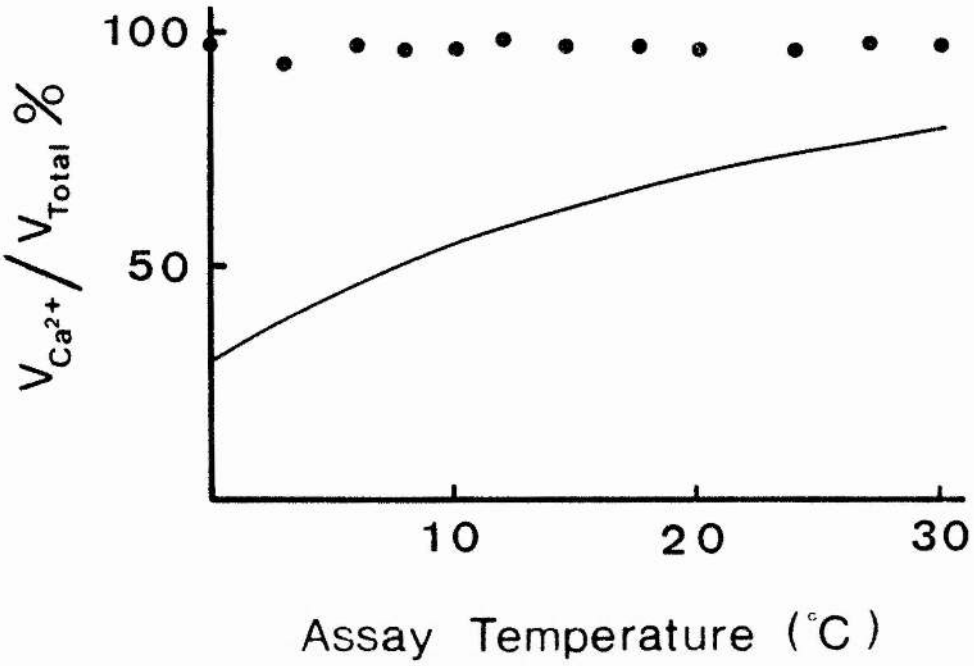


Fig. 7:4 The effect of assay temperature on the ratio of Ca^{2+} dependent/Total ATPase activity is shown for plaice (dotted line) and for rabbit (continuous line : data from Inesi et al (1976)).

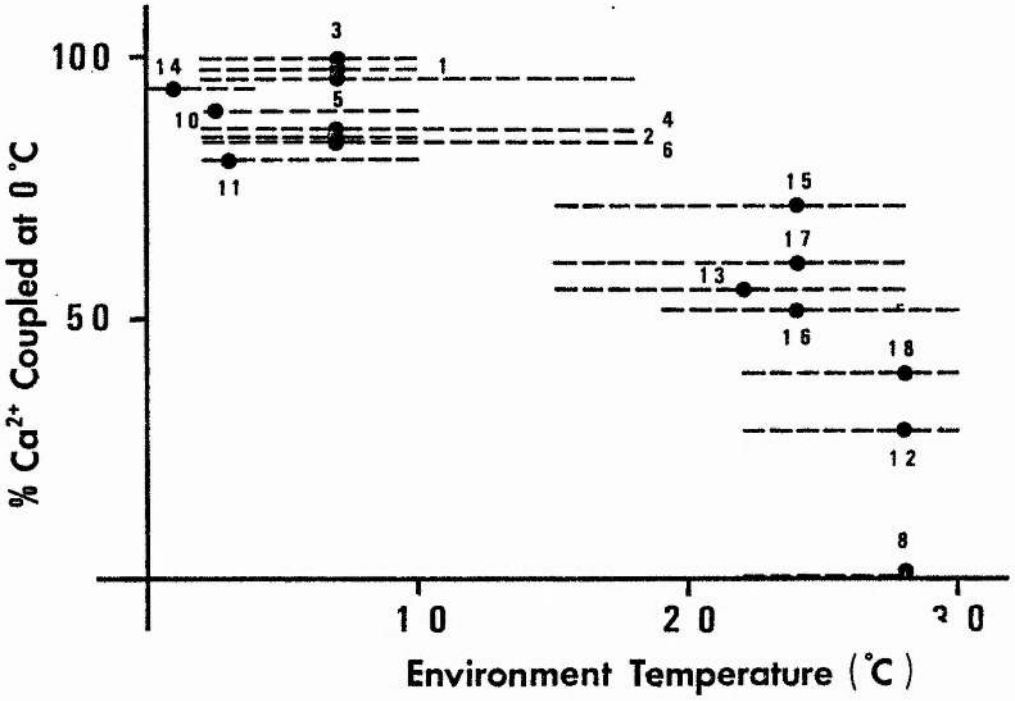


Fig. 7:5 The ratio of Ca^{2+}/Total ATPase activity measured at $0^{o}C$ is shown plotted against cell temperature. For an explanation of the different numbers, see Table 5:1.

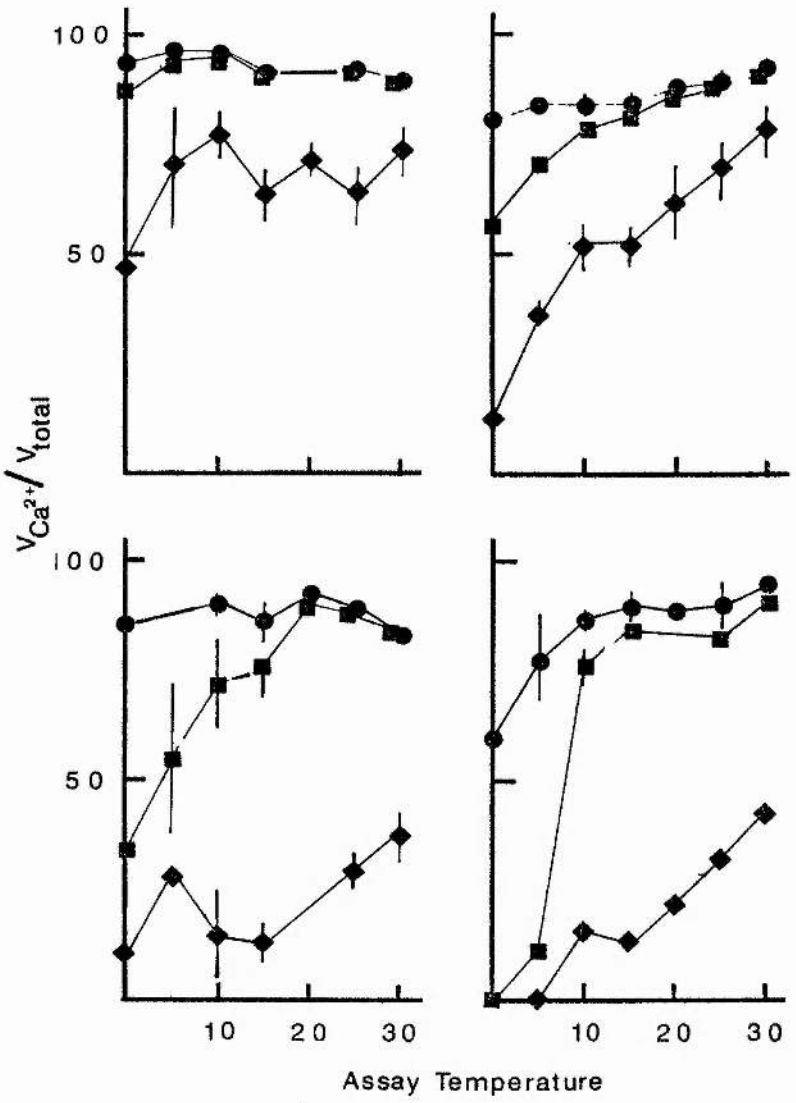


Fig. 7:6 The effect of temperature on the ratio of Ca^{2+}/Total ATPase activity at (◆) 0.11μM, (■) 1.06μM and (●) 8.78μM free Ca^{2+} for four species of fish: (a) N. rossii, (b) S. gairdneri, (c) T. mossambicca and (d) S. niloticus. Values represent the mean ± S.E.M. of at least 3 observations in duplicate.

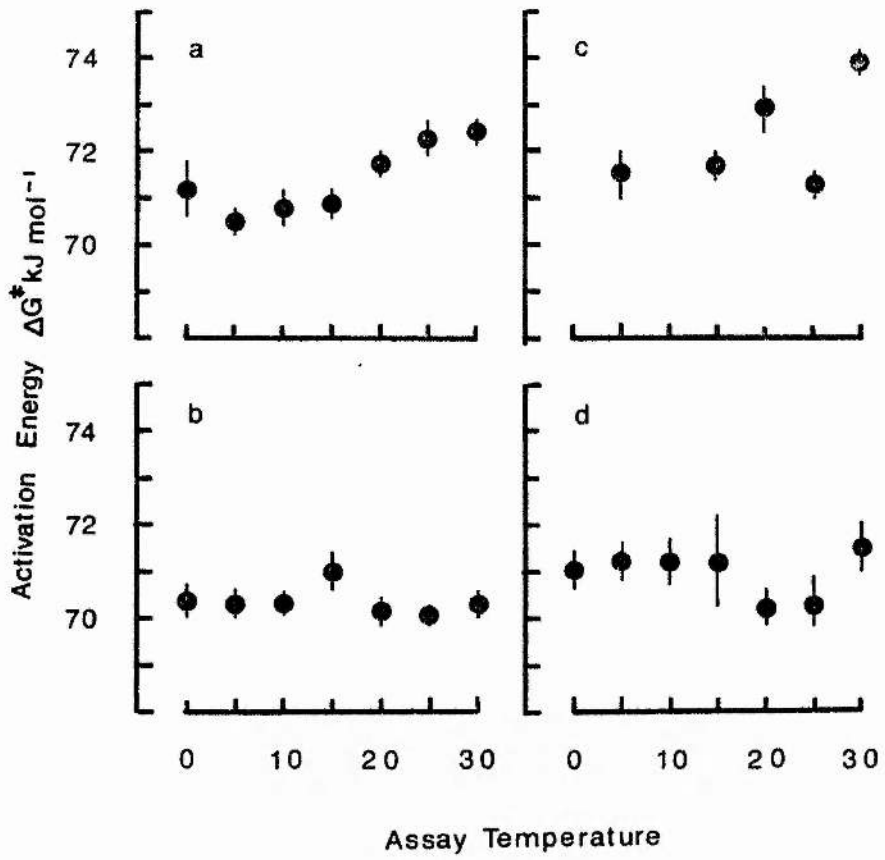


Fig. 7:7 The effect of assay temperature on the apparent activation energy (kJ/mol) of the Ca^{2+}-ATPase of SR from (a) N. rossii, (b) S. gairdneri, (c) T. Mossambicca, and (d) S. niloticus measured at K_{Ca}. Values represent the mean ± S.E.M. of at least three duplicate observations.

GENERAL DISCUSSION

GENERAL DISCUSSION

As was discussed in the introductory chapter on temperature and membranes, ectothermic animals usually adapt to temperature stress by altering the relative amount of unsaturated fatty acids in the membrane, maintaining, to varying extents, the "homeoviscosity" of the membrane (Sinensky, 1974).

The sarcoplasmic reticulum is an ideal system in which to study adaptation since it serves only one major function, that of releasing and storing Ca^{2+} ions during the excitation-contraction cycle. Furthermore, there have been extensive studies on the chemical composition of the membrane and on the kinetics of the reaction system (see Chapter 1). It is a fairly simple structure, consisting of about seven proteins, with only one directly involved in the Ca^{2+} translocation process (Hasselbach, 1978). The studies reported in this thesis provide clear evidence that the sarcoplasmic reticulum has undergone adaptation to temperature, at least over an evolutionary time period. The mechanism of adaptation is, however, far from clear. As with other membranes, the relative amounts of unsaturated fatty acids increases in the order rat, desert pupfish and Arctic sculpin, but there are no compensating changes in the viscosity (Cossins et al, 1977).

In goldfish acclimated to 25°C, the relative amounts of mono-unsaturated fatty acids is much higher than in fish acclimated to 5°C. These increases are offset mainly by a decrease in the relative amounts of polyunsaturates. In this study, no significant differences in the proportions of saturated fatty acids were found.

The mechanisms involved in altering the lipid composition of a membrane are complex and energetically expensive, so

that it would seem more economical for the cell to alter the properties of the transport protein rather than the lipids. A similar mechanism has been demonstrated by Sidell (1977) in cytochrome oxidase, when cold acclimation leads to an increase in the amount of the enzyme. Energy expenditure is further decreased by decreasing the rate of catabolism of cytochrome oxidase rather than increasing the rate of synthesis. Thus, acclimation leads to the cell producing more enzyme, which is less energetically expensive than producing an entirely new population of efficient enzyme. In evolutionary adaptation the system has had time to allow lipid changes and to produce an enzyme which is optimally efficient at the cell temperature.

Following the muscle membrane depolarisation, Ca^{2+} is released from the SR into the myoplasm. The actual mechanism of release is unknown, but it is likely that the final event is a marked increase in the permeability of the membrane to Ca^{2+}, and some sort of "facilitated diffusion" into the myoplasm. Altering the lipid composition of a membrane results in dramatic changes in it's permeability properties. For example, liposomes from *E. coli* grown at 20°C are more fragile and susceptible to osmotic swelling than from bacteria grown at higher temperatures (Haest *et al*, 1969). Smith and Kemp (1970) found that the active uptake of alanine into the intestinal mucosal cells does not depend on the acclimation temperature, while the net transport of alanine across the cell does. They suggest that the passive efflux of amino acids may be regulated by temperature induced changes in the membrane

permeability. The situation in the SR is somewhat more complicated since adaptation and acclimation appear to proceed via different mechanisms. The cold adapted membranes are more viscous at their cell temperature (Cossins et al, 1977) and there appears to be no acclimatory functional changes (Cossins et al, 1977; Penney and Goldspink, 1980; Chapter 5). Penney and Goldspink (1980) determined that acclimation results in the synthesis of more membrane, albeit less efficient. The results recorded in Chapter 5 suggest that the carp SR is adapted to an intermediate temperature, and compensates by altering the relative amount of membrane. Since an increase results in an increased relative volume, the problems introduced by temperature dependent changes in Ca^{2+} diffusion rates are avoided. In adaptation, some other mechanism has been adopted.

Cossins et al have suggested that any alterations which occur in the lipid moeity do not affect the physical properties of the membrane, even in adaptation, but other workers have found that this is not the case (Madeira and Antunes-Madeira, 1976). In this thesis, functional adaptation has been shown in both the Vmax (K^{cat}) and the K_{Ca} of the Ca^{2+}-dependent ATPase of the sarcoplasmic reticulum of fast muscle in different species of fish. The V_{max} of the enzyme is inversely related to the habitat temperature. Activation enthalpy is positively correlated with cell temperature. As has been demonstrated with several enzymes of intermediate metabolism, the changes in $\Delta H^{\ddagger}$ are offset by energetically disadvantageous but biologically valuable changes in the activation entropy $\Delta S^{\ddagger}$ (Low and Somero, 1976; Somero and Low,

1976). Measurements of Ca^{2+} uptake rate of SR vesicles shows that cold adapted SR operates more efficiently at low temperatures than the hot. Finally, estimates of the substrate binding affinity (K_{Ca}) also show adaptive changes. For example, K_{Ca} values at $0^{o}C$ range from 8μM to 0.6μM, but when measured at the environment temperature, the range is much less, between 0.3μM and 0.6μM (Chapter 6).

Having demonstrated that adaptation has occurred, it remains to elucidate the mechanisms involved. Again the SR proves to be an ideal system for such experiments, for the following reasons.

The Ca^{2+} ATPase was first successfully isolated in 1970, by Maclennan, and more recent work has managed to produce an enzyme which retains a very high activity (see Maclennan and Holland, 1975). The vesicles are solubilized in deoxycholate after first washing in low osmotic strength medium to remove the other proteins. Removal of the detergent results in spontaneous re-aggregation of functional vesicles. The purified ATPase can then be suspended in controlled dispersions of phospholipids, and incorporated into vesicles which will pump and store Ca^{2+} ions.

Using this technique many workers have been able to demonstrate the importance of the phospholipids in the Ca^{2+} transport process (see Chapter 1). Knowles *et al* (1976) have shown the importance of the different lipids in the various levels of the transport process, and Hidalgo and her group (Hidalgo *et al*, 1976; 1978) have shown the relevance of the lipids to the thermodynamics of the transport process.

These techniques could well be applied to the mechanisms

of adaptation. For example, the lipids from cold adapted SR ("cold" lipids) could be incubated with the transport protein of a hot adapted animal, and the functional vesicles examined for their temperature characteristics. Such experiments would determine the extent to which the lipids can modify the protein's properties. Examination of the isolated protein would also yield information on the adaptive mechanisms employed.

Various workers (e.g. Warren et al, 1975) have suggested that, associated with the ATPase, there is a rigid annulus of about 30 phospholipids/ATPase molecule. Others have suggested that if such an annulus exists, it must be in a dynamic equilibrium with the bulk lipids (Madden et al, 1975). Progressive substitution of hot adapted lipids with cold could provide information relevant to this controversy. If the lipids were capable of affecting the protein, and it had previously been demonstrated that the protein itself was not the temperature sensitive moiety, then the lipids must be interacting with the protein in such a manner as to obviate the possibility of an immobile annulus.

Inesi and co-workers have developed fast reaction techniques which allow the determination of individual steps in the transport process. Examination of the thermodynamics of these different steps in cold and hot adapted animals would provide information as to how the physical membrane changes affect the actual process of transport. Substituting the hot and cold adapted lipids and monitoring the changes on the partial reactions would also provide an insight into the relative importance of adaptation in the protein and lipid components.

The large body of information concerning the SR makes this a promising model system to investigate membrane adaptation in ectotherms. Moreover, such studies provide the investigator with a powerful experimental tool to employ in the elucidation of ion transport mechanisms.

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