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Resistance Adaptation of the Freshwater Crayfish and Thermal Inactivation of Membrane-Bound Enzymes

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Summary. Heat death and resistance adaptation of freshwater crayfish are thought to be properties of its muscle membranes. The inactivation at high temperatures of a membrane-bound enzyme, the Ca^{++}-stimulated ATPase of crayfish abdominal muscle sarcoplasmic reticulum, and the effect of thermal acclimation of crayfish upon the inactivation kinetics have been investigated. In the absence of KCl, the Ca^{++}-stimulated ATPase is irreversibly inactivated with pseudo-first order kinetics at temperatures that cause heat death in the whole animal. 0.1–10.0 mM KCl resulted in slower inactivation, while 100 mM KCl activated the enzyme to 120–180% of its original activity. Enzyme activation by KCl and heat involved a shift in the enzyme concentration/activity curve. Thermal acclimation of crayfish had no significant effect upon the kinetics or Arrhenius activation energy for enzyme inactivation (100.6 ± 10.5 and 92.3 ± 14.6 kcal/mole for preparations from 4 °C and 25 °C acclimated crayfish).

Ca^{++}-stimulated ATPase isolated from heat dead crayfish exhibited normal in vitro activity due presumably to the high intracellular K^+ concentration. Nevertheless, the close correspondence between heat death temperatures and inactivation temperatures for several membrane-bound enzymes of muscle is thought to reflect some perturbation of muscle structure that occurs during heat death.

Introduction

Living organisms show cellular heat injury and heat death at temperatures only slightly above their normal range. The precise cause(s) of cellular heat

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Abbreviations: ATP = Adenosine 5′-Triphosphate; EGTA = Ethyleneglycol-bis [β-amino-ethyl ether] N, N′-tetraacetic acid; Hepes = N-2-Hydroxyethylpiperazine-N′-2-ethanesulphonic acid; FSR = Fragmented sarcoplasmic reticulum; Tris = Tris (hydroxymethyl)aminomethane

injury and heat death, as well as those factors involved in resistance adaptation, remain unclear, although recent work has indicated that cellular membranes of excitable tissues play an important role (Bowler et al., 1973; Grainger, 1973; Friedlander et al., in preparation; Cossins, 1976).

Current concepts of the structure and function of biological membranes emphasize an important role for the physical state of the hydrophobic interior of the membrane. It has been suggested (Cossins, 1976) that at high stressful temperatures the molecular motion of the alkyl chains of membrane phospholipids becomes so great that lipid-lipid and lipid-protein interactions are disrupted through reduction of London-Van der Waals forces between the hydrophobic moieties. This may result not only in an increased leakiness of the membrane but also exposure of intrinsic membrane-bound enzymes to various denaturing stresses, both of which may contribute to the observed membrane perturbation during heat stress. In addition, modification of the lipid composition of the membrane during thermal acclimation provides an attractive mechanism to account for resistance adaptation.

In this communication we examine the effect of exposure to temperatures that cause heat death in crayfish upon the activity of a membrane-bound enzyme from crayfish muscle.

Materials and Methods

Materials

All salts were "AnalaR" grade and were obtained from British Drug Houses Ltd. (Poole, Dorset, U.K.). Bovine serum albumin (fraction V), disodium adenosine triphosphate (Sigma grade), EGTA and Hepes were obtained from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). Tris ATP was prepared from disodium ATP by treatment with acid ion exchange resin (Dowex 1-X8, 20–50 U.S. mesh) and stored at −20 °C at pH 7.0. Lubrol W and Cirrasol ALN-F were a gift from I.C.I. Ltd., Dyestuffs Division.

Treatment of Crayfish

Crayfish (*Austropotamobius pallipes*, Lereboullet) were collected locally in streams and reservoirs of the Newcastle and Gateshead Water Company. They were maintained for several weeks at 15 °C as described previously (Cossins, 1976), and were subsequently acclimated to 4±0.5 °C or 25±0.1 °C with an 18L:6D photoperiod for at least 21 days prior to sacrifice. Moulting animals were excluded from the study.

Preparation of Sarcoplasmic Reticulum Vesicles

The abdominal flexor and extensor muscles were rapidly excised from the animal and were finely minced in 10 volumes ice-cold extraction medium (100 mM KCl, 10 mM imidazole-HCl, pH 7.1) using a top-drive macerator (M.S.E. Ltd., London). The suspension was then homogenized with 10 passes of a Potter-Elvehjem homogenizer and the crude homogenate was centrifuged at 1,000 g for 30 min. The pellet was resuspended in 5 volumes of extraction medium and recentrifuged at 1,000 g for 30 min. The combined supernatants were spun at 13,000 g for 30 min and the supernatant was centrifuged at 35,000 g for 60 min. The pellet was resuspended by homogenization in 0.6 M

KCl, 10 mM imidazole-HCl, pH 7.1 (Martonosi and Feretos, 1964) and then centrifuged at 35,000 g for a further 60 min. The pellet was resuspended by gentle homogenization in 0.3 M sucrose, 10 mM imidazole-HCl, pH 7.1 at a protein concentration of 40–80 μg microsomal protein/ml. The preparation was used immediately.

Pyruvate kinase was extracted from crayfish abdominal muscle by homogenization in 0.25 M sucrose, 2.5 mM EDTA, 25 mM Tris-HCl (pH 7.5) and centrifuged at 30,000 g for 20 min. The protein from the supernatant was precipitated between 25 and 45% ammonium sulphate, resuspended in the homogenization medium and used immediately.

Thermal Inactivation and Assay of Ca^{++}-Stimulated ATPase

The inactivation process was followed by assaying residual enzyme activity at 25 °C after different periods of exposure ("preincubation") to an inactivation temperature. Aliquots (0.5 ml) of the FSR preparations were incubated in pre-equilibrated borosilicate glass test-tubes at appropriate temperatures for known times, and then rapidly transferred to a 25 °C water bath. After exactly 5 min equilibration, the residual total (Ca^{++}, Mg^{++}) ATPase activity was initiated by addition of 1.5 ml assay medium to give final concentrations of 100 mM KCl, 0.5 mM EGTA, 0.5 mM $CaCl_2$, 3 mM $MgCl_2$, 25 mM imidazole-HCl, pH 7.1 and 3 mM ATP. After 5–10 min, depending upon the activity of the preparation the enzyme reaction was stopped and the inorganic phosphate liberated was measured, both using the method of Atkinson et al. (1973). Separate experiments demonstrated that the reaction was linear with time. Protein was determined using the method of Lowry et al. (1951), modified to permit accurate determination of less than 100 μg microsomal protein/ml. "Basal" Mg^{++}-dependent ATPase was assayed simultaneously in the same reaction medium but omitting the $CaCl_2$. Ca^{++}-stimulated ATPase activity was calculated as the difference between ATPase activity in the presence and absence of $CaCl_2$. All preincubation and assay temperatures were controlled at the required temperature ± 0.1 °C.

Pyruvate kinase was exposed to 50, 55 and 65 °C for varying periods of time and residual activity was assayed at 25 °C using the method of Susor and Rutter (1968).

The rate of enzyme inactivation was conveniently described by the half-time, which was defined as the time for enzyme activity to decay to half its original activity. It was estimated graphically from a plot of log % original activity against preincubation time. The half-time (LT_{50}) is simply related to the rate constant for inactivation k, by

$$\text{Half-time} = \frac{0.693}{k}$$

The Arrhenius activation energy for the inactivation process was calculated by regression analysis of log k against the reciprocal of the preincubation temperature (°A).

Results

The Effects of Ionic Conditions upon Thermal Inactivation

Figure 1A illustrates the effects of exposure to 32 °C for varying periods of time upon the Ca^{++}-stimulated ATPase activity of an FSR preparation which had been resuspended in different ionic media. When incubated in 10 mM imidazole-HCl, pH 7.1 or 10 mM Hepes-KOH, pH 7.1, the enzyme was rapidly and irreversibly inactivated with pseudo-first order kinetics, since a semilog plot of % Ca^{++}-stimulated ATPase activity remaining was linear with preincubation time (not shown). Preincubation in the presence of 100 mM KCl, 10 mM imidazole-HCl, pH 7.1, however, led to increased enzyme activity. Replicate experiments indicated that there was considerable variability in the maximal

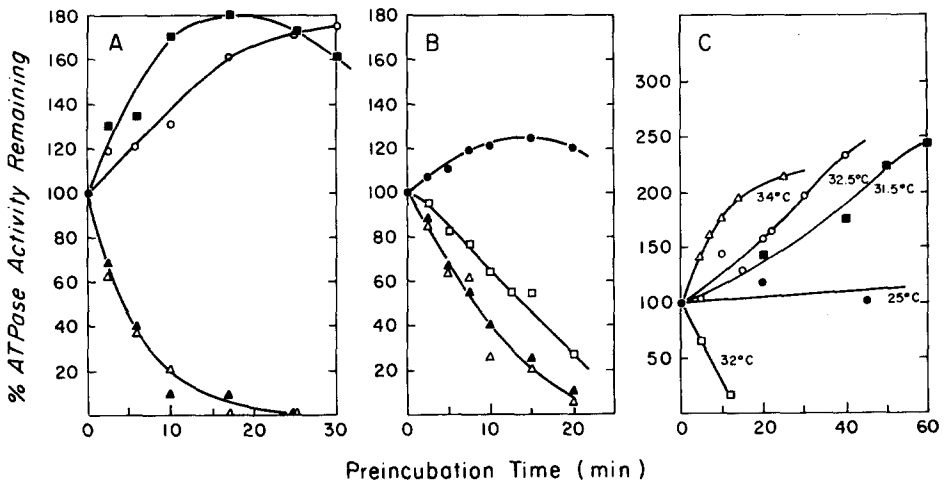

Fig. 1A. Effect of different ionic conditions upon the thermal stability of the Ca^{++}-stimulated ATPase of crayfish abdominal muscle FSR. A FSR preparation was preincubated at 32 °C for varying times and the % ATPase activity remaining was assayed at 25 °C. Media: (△) 10 mM Hepes-KOH, pH 7.1; (▲) 10 mM imidazole-HCl, pH 7.1; (■) 100 mM KCl; 10 mM imidazole-HCl, pH 7.1; (○) 133 mM KCl, 33 mM imidazole-HCl, pH 7.1, 0.66 mM $CaCl_2$, 0.66 mM EGTA, 2 mM $MgCl_2$; **B** Effect of increasing the KCl concentrations upon the thermal stability of the Ca^{++}-stimulated ATPase of crayfish abdominal muscle FSR. Preincubation media contained 10 mM imidazole-HCl, pH 7.1 with the following KCl concentrations: (●) 100 mM; (□) 10 mM; (▲) 1 mM; (△) 0.1 mM; **C** Effect of preincubation at 25, 31.5, 32.5 and 34 °C in the presence of 100 mM KCl, upon the thermal stability of the Ca^{++}-stimulated ATPase of crayfish abdominal muscle FSR. (□) A portion of the same untreated microsomal preparation initially suspended in 100 mM KCl, 10 mM imidazole-HCl, pH 7.1, was washed with 10 mM imidazole-HCl, pH 7.1 and exposed to 32 °C

activation, usually varying between 120–180%, compared to an untreated preparation. The Ca^{++}-stimulated ATPase showed similar behaviour when suspended in the ionic media used for routine enzyme assay, but omitting the substrate ATP, although the rate of enzyme activation was slower (Fig. 1A). A similar experiment in the presence of 0.5 mM $CaCl_2$ and 0.5 mM EGTA (8.1 μM free calcium, Cossins and Bowler, in preparation), 10 mM imidazole-HCl, pH 7.1 indicated that the rate of inactivation was not significantly different from a control preparation also exposed to 32 °C but in the presence of 10 mM imidazole-HCl, pH 7.1 (not shown).

The effects of exposure of the Ca^{++}-stimulated ATPase to 32 °C in media with increasing concentrations of KCl are shown in Figure 1B. Whilst 100 mM KCl caused an increase in enzyme activity with preincubation, 10 mM KCl or less resulted in pseudo-first order inactivation; the lower the KCl concentration the more rapid the inactivation process. It was not possible to reduce KCl concentration of the enzyme suspension much below 0.1 mM by repeated washing in KCl-free media, since a sizeable contamination was introduced with the microsomal preparation, presumably in a tightly-bound form. The rate of enzyme activation increased with higher preincubation temperatures

(Fig. 1C). Suspension in 100 mM KCl by itself was not sufficient to protect or activate the enzyme, since the Ca^{++}-stimulated ATPase of a microsomal preparation that was suspended in 100 mM KCl and subsequently washed with 10 mM imidazole-HCl, pH 7.1, was rapidly inactivated by exposure to 32 °C (Fig. 1C).

The "basal" Mg^{++}-dependent ATPase activity was negligible (Cossins and Bowler, in preparation) and was not affected by prior exposure to 31–35 °C.

Dependence of Enzyme Activity upon the Concentration of Microsomal Protein

During the course of these experiments it became clear that the Ca^{++}-stimulated ATPase activity of any one FSR preparation was not a linear function of the concentration of microsomal protein (Fig. 2). However, preincubation at 32 °C for 10 min in the presence of 100 mM KCl caused not only the described increase in enzyme activity (usually 120–180%) but also a shift of the enzyme activity/concentration curve from a curvilinear form to a straight line which passed through the origin (Fig. 2).

Separate experiments indicated that the concentration of microsomal protein or presence of BSA had no effect upon the rate of inactivation at 32 °C in the presence of 5 mM KCl.

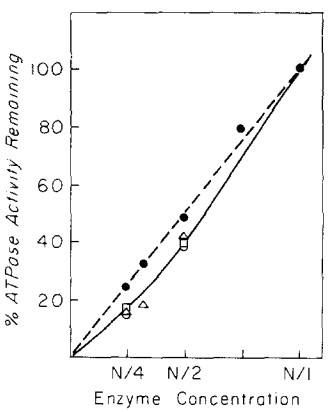

Fig. 2. Effect of enzyme concentration upon the specific activity of Ca^{++}-stimulated ATPase of crayfish abdominal muscle FSR. The microsomal preparation (approximately 40 µg protein/ml) was suspended in 100 mM KCl (○) or 10 mM KCl (△□) and was diluted 2, 3 and 4-fold with the respective media and the relative activity of each was determined at 25 °C. One preparation (●) suspended in 100 mM KCl, 10 mM imidazole-HCl, pH 7.1 was exposed to 32 °C for 20 min before dilution and assay. The protein concentration is expressed as a fraction of the original preparation to facilitate comparison between preparations with different protein contents and different specific enzyme activities

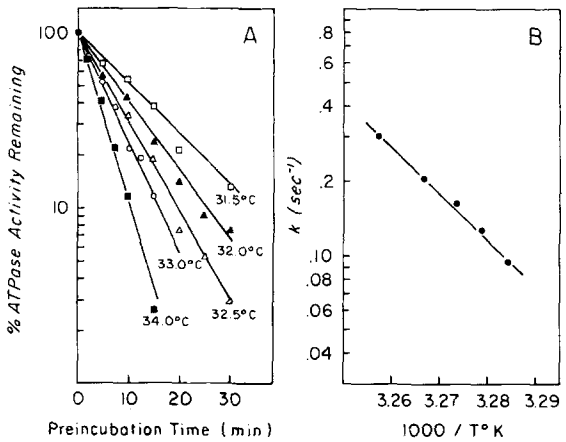

Fig. 3. A Typical inactivation curves for the Ca^{++}-stimulated ATPase of crayfish muscle FSR. A microsomal preparation was resuspended in 5 mM KCl, 10 mM imidazole-HCl, pH 7.1 and preincubated for varying periods at temperatures between 31.5 °C and 35 °C. Residual activity was assayed at 25 °C. **B** Arrhenius plot of rate constant for the inactivation process, k

Effect of Thermal Acclimation upon Thermal Inactivation Kinetics

For the comparison of the thermal sensitivity of FSR preparations from differently acclimated crayfish the microsomes were preincubated at various temperatures between 31 and 35 °C in the presence of 5 mM KCl, 10 mM imidazole-HCl, pH 7.1 since this KCl concentration was small enough to allow rapid inactivation to occur without inducing an activation phenomenon, and large enough to make variations in the quantity of bound K^+ insignificant to the inactivation process. The results of a typical experiment are illustrated in Figure 3A where it is apparent that the Ca^{++}-stimulated ATPase is sensitive to exposure at 31–34 °C; the higher the preincubation temperature the more rapid is the inactivation process. All preparations exhibited linear plots of log % Ca^{++}-stimulated ATPase activity remaining versus preincubation time. The rate constant was determined for each preincubation temperature and the Arrhenius plot for the inactivation process is shown in Figure 3B.

The results of similar experiments on several FSR preparations from each acclimation group were averaged and the results are presented in Table 1 and Figure 4. Although FSR preparations from 25 °C acclimated crayfish exhibited a greater degree of thermal resistance at all preincubation temperatures than equivalent preparations from 4 °C acclimated crayfish, the difference was statistically significant only at 34 °C ($P=0.02$–0.05). The relatively large standard error of the 25 °C acclimation group was attributable to a particularly heat resistant preparation. The remaining preparations showed similar heat sensitivities as the 4 °C acclimation group. It was concluded that there was no demonstrable difference between the two classes of preparations. For comparison, pyruvate kinase was inactivated by exposure to 50–65 °C (Fig. 4).

Table 1. The average half-times for inactivation at 31–34 °C and the Arrhenius activation energies (E_a) for the thermal inactivation of the Ca^{++} stimulated ATPase of crayfish acclimated to 4 °C and 25 °C. n = number of preparations. Mean values ± SEM

Preincubation temperature (°C)	Half time (minutes)	
	4 °C acclimated crayfish (n=4)	25 °C acclimated crayfish (n=5)
31	13.70 ± 1.36	27.94 ± 10.24
32	8.03 ± 0.42	14.50 ± 4.04
33	4.73 ± 0.13	7.70 ± 1.54
34	2.82 ± 0.18	4.16 ± 0.52
Average E_a (kcal. mole^{-1})	100.58 ± 10.47 (69–133)[a]	92.29 ± 14.63 (63–112)[a]

[a] Range of values

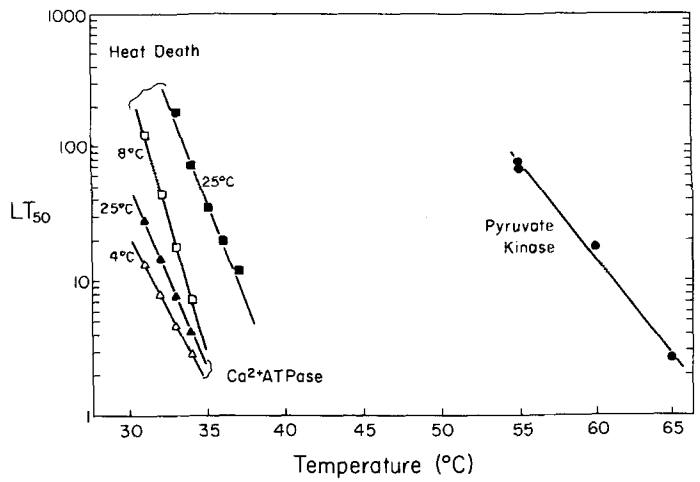

Fig. 4. Comparison of the temperatures of inactivation of the Ca^{++}-stimulated ATPase of the FSR and muscle pyruvate kinase with the process of heat death in the freshwater crayfish. Data for inactivation of Ca^{++}-stimulated ATPase was obtained from Table 1; and for heat death, from Bowler (1963)

The Arrhenius activation energies for the inactivation process in FSR preparations from both acclimation groups are compared in Table 1. The difference in mean activation energy was not statistically significant (P=0.6–0.7).

Effect of Heat Death of Crayfish upon in vitro Ca^{++}-stimulated ATPase Activity

The protective effects of high KCl media upon the in vitro inactivation of the microsomal Ca^{++}-stimulated ATPase suggested that this enzyme would

not be inactivated in vivo during heat death, since the bulk muscle K^+ concentration is approximately 120 mM (Gladwell, 1976).

Three crayfish of approximately equal size were exposed to 15 °C (control), 35 °C and 55 °C for 10 min, respectively. The latter two animals were heat dead by the criteria of Bowler (1963) and Gladwell (1976). However, the FSR preparations from animals exposed to 15 °C and 35 °C showed vigorous Ca^{++}-stimulated ATPase activity when assayed at 25 °C, while the preparation from the 55 °C treated crayfish was totally inactive. It was concluded that heat death of the whole animal is not accompanied by an in vivo irreversible inactivation of the Ca^{++}-stimulated ATPase.

Discussion

The fact that the Ca^{++}-stimulated ATPase of crayfish FSR and the Mg^{++}-dependent ATPase and (Na^+ and K^+) stimulated ATPase of muscle microsomes (Bowler and Duncan, 1967; Gladwell et al., 1976) were inactivated in vitro over the same temperature range (31–35 °C) and with high activation energies suggests that there may be some common factor in the inactivation process. This correspondence is made more dramatic by consideration of the half-time/temperature curve for the thermal inactivation of pyruvate kinase from crayfish muscle (Fig. 4) which occurs between 55 °C and 65 °C. In general, soluble enzymes are thermally inactivated at temperatures well above those characteristic of heat death (Read, 1967; Ushakov, 1967). Brandt (1967) has emphasized that the configurational state of a protein molecule is thermodynamically controlled and that the solvent environment has important effects on intramolecular forces of proteins and hence enzyme structure. In this case, the solvent environment of these membrane-bound ATPases is thought to be the hydrophobic core of the membrane and this may be the common factor that limits the thermal stability of intrinsic membrane-bound enzymes. In this context it may be significant that Laggner and Barratt (1975) have recently shown that the temperature gradients of the mobility of spin-labelled fatty acids incorporated into intact sarcoplasmic reticulum of rabbit skeletal muscle and into liposomes composed of their membrane phospholipids, converge with increasing temperature. They suggest that the immobilization of membrane lipids due to the membrane-bound protein (principally the Ca^{++}-stimulated ATPase) decreases with increasing temperature leading presumably to the disruption of at least some lipo-protein interactions.

If enzyme inactivation is a consequence of a breakdown in lipid bilayer integrity then membranes from warm-acclimated animals (which presumably contain a reduced proportion of unsaturated fatty acids [Cossins, 1976; Hazel and Prosser, 1974]) should be more resistant to the deleterious effects of high temperatures than the equivalent membranes of cold-acclimated animals. The absence of any demonstrable effect of thermal acclimation of crayfish upon the kinetics of in vitro thermal inactivation of the Ca^{++}-stimulated ATPase indicates that this hypothesis does not apply in this case. Either the inactivation kinetics for this enzyme is not influenced by the composition of its lipid

environment, or the composition of FSR phospholipids is not modified during thermal acclimation.

The necessity of a hydrophobic environment for vigorous ATPase and Ca^{++} uptake functions has been well documented (Meissner and Fleischer, 1972), and other investigators have shown that the Ca^{++}-stimulated ATPase of rabbit skeletal muscle (Madeira and Antunes-Madeira, 1975; Inesi et al., 1973) and lobster abdominal muscle (Madeira and Antunes-Madeira, 1975) exhibits an inflexion in its Arrhenius plots at temperatures that correspond to a change in slope of the Arrhenius plot of the motion of a hydrophobic probe, although these observations have been disputed by other workers (Laggner and Barratt, 1975; Becker and Willis, 1975). On the other hand, several lines of evidence indicate that the sarcoplasmic reticulum does not show significant adaptation of functional properties to changed environmental temperature due presumably to a lack of changes in FSR lipid composition during temperature acclimation. Becker and Willis (1975) failed to discover differences in the Arrhenius plots of the Ca^{++}-stimulated ATPase isolated from hibernating and non-hibernating mammalian species. Cossins and Bowler (in preparation) similarly failed to demonstrate any significant differences in the activity, Ca^{++}-stimulation kinetics, apparent K_m (ATP) over a range of assay temperatures and Arrhenius activation energy of the Ca^{++}-stimulated ATPase isolated from the muscle of differently acclimated crayfish muscle. Finally, Tume et al. (1973) failed to observe changes in various ATPase and Ca^{++}-sequestration kinetics in FSR preparations isolated from rats fed lipid-free and lipid-supplemented diets, despite changes in the fatty acid composition of the FSR membranes.

The presence of Ca^{++}-stimulated ATPase activity in the FSR isolated from heat-dead crayfish indicates that thermal inactivation of this enzyme does not occur during heat death, in contrast to the situation with the Mg^{++}-dependent ATPase and (Na^+, K^+)-stimulated ATPase of crayfish muscle microsomes (Gladwell et al., 1976). The protective effect of high KCl concentrations during in vitro exposure to high temperature provides an explanation for the lack of in vivo inactivation, since crayfish muscle is known to contain K^+ concentrations of approximately 120 mM (Gladwell, 1976). According to Dixon and Webb (1964) the curvilinear form of the enzyme activity/concentration curve observed for unheated preparations (Fig. 2) is characteristic of progressive enzyme activation by some factor with increasing enzyme concentration. The mechanism of this phenomenon is unknown and may represent a direct effect on the membrane phospholipids, membrane-bound proteins or on the interactions between the two. However, it is tempting to speculate that protection and activation of the Ca^{++}-stimulated ATPase in the presence of high KCl concentrations can only occur when membrane structure is sufficiently disrupted to permit access of KCl to the protein molecule.

Together with the neurophysiological observations of Gladwell et al. (1976) these results are taken to reflect some perturbation of muscle membrane function at temperatures which also cause heat death. In the case of the sarcoplasmic reticulum this phenomenon is not modifiable by thermal acclimation, whilst these observed properties of the sarcolemma (Gladwell et al., 1976; Gladwell, 1976) show some degree of resistance adaptation.

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