The effect of high lethal temperatures on the muscle of the crayfish, astacus pallipes lereboullet; a biochemical and electrophysiological

Gladwell, R. T.

THE EFFECT OF HIGH LETHAL TEMPERATURES ON THE MUSCLE OF THE CRAYFISH, ASTACUS PALLIPES LEREBOULLET; A BIOCHEMICAL AND ELECTROPHYSIOLOGICAL STUDY.

by R. T. GLADWELL.

A thesis submitted to the University of Durham for the degree of Doctor of Philosophy, March 1973.

Abstract.

The phenomenon of temperature adaptation, as measured by the mobility of the heat death point, was demonstrated in Astacus pallipes Lereboullet. Analysis of muscle, haemolymph and environment during lethal high temperature exposure showed dramatic Na^+ and K^+ concentration changes in all three compartments. In all cases the monovalent cations showed a net movement down their respective electro-chemical gradients, although this was sometimes only observed after a brief initial period during which the ions tended to exhibit a net movement up their electro-chemical gradients. The results indicated that heat death occurred when the haemolymph K^+ concentration rose to about 9 mM. No relationship between the haemolymph Na^+ concentration and the heat death point was observed.

Electrophysiological experiments indicated that lethal high temperatures caused an increase in the conductivity (= permeability) of the sarcolemma following comparable exposure times to those causing heat death in the whole animal. The Mg^{++}activated ATPase (EC 3.6.1.3.) which is involved in the control of sarcolemmal permeability was shown to be inactivated by lethal high temperatures. It was therefore concluded that the increased sarcolemmal permeability was caused by the thermal inactivation of this enzyme.

The spontaneous activity of the CNS was only slightly affected by exposure to lethal high temperatures. Raising the extracellular K^+ concentration to the levels observed in animals at the time of heat death caused a massive increase in nervous activity.

The sarcolemmal Mg^{++}activated ATPase from warm adapted

crayfish was shown to be more resistant to high temperatures than that from cold adapted crayfish. This could be correlated with the greater heat resistance of the sarcolemmal Mg^{++}activated ATPase and conductivity seen in the warm adapted animals.

It is proposed that the primary lesion of heat death in Astacus pallipes Lereboullet is the thermal inactivation of the sarcolemmal Mg^{++}activated ATPase which allows muscle K^{+} to move into the haemolymph. The activity of the CNS is increased markedly by the high haemolymph K^{+} resulting in loss of co-ordination and death. The thermal sensitivity of the Mg^{++}activated ATPase is modified by the previous thermal history of the crayfish and is involved in the mechanism of temperature adaptation.

ACKNOWLEDGMENTS.

I wish to express my gratitude to Professor C. J. Duncan and Doctor K. Bowler for their advice and encouragement in the preparation of this thesis.

I should also like to thank the staff of the Northumberland and Gateshead Water Company for their kind permission to collect crayfish from their streams and reservoirs. In particular I wish to express my thanks to Mr Tulip of Hallington Reservoir and Mr Palmer of Whittledene Reservoir for their help and advice concerning these collections.

I also wish to express my thanks to Professor D. Barker and the staff of the Department of Zoology for their help and advice during my time in their Department.

This study was supported by a grant from the Science Research Council for which I am most grateful.

CONTENTS.

GENERAL INTRODUCTION.

It is a matter of common observation that the activity of cold blooded animals is influenced by the environmental temperature. Increased body temperature leads to increased activity up to a temperature limit at which the viability of the organism is adversely affected by further temperature increases. The earliest studies of the effects of temperature on organisms were hampered by two factors. Firstly the thermodynamic principles were not coherently formulated until 1848 (Lord Kelvin) and secondly, in order to understand these temperature effects it was necessary to have some concept of the relationship between an organism and its environment. This concept was developed and refined in the 19th century. Claude Bernard made an important contribution by explicitly stating that preservation of the internal stability and constancy of an organism in the face of changes in the external environment was an essential characteristic of life.

This emphasis on the ability of organisms to maintain a constant internal environment stimulated considerable enquiry, including investigations into the effect of changes in the environmental temperature on poikilotherms. The rate of reactions in organisms increase with temperature up to a maximum, a further temperature increase bringing about a decline in the rate. It is therefore necessary to compare reaction rates over similar temperature intervals if meaningful comparisons are to be made, this interval is usually $10^{o}C$. The ratio of the rate of activity of a reaction at two temperatures $10^{o}C$ apart, called the temperature coefficient, is designated the Q_{10}. The Q_{10} may be derived from the van't Hoff equation (Belehrádek, 1935):-

$$Q_{10} = \left(\frac{K_1}{K_2}\right)^{10/(t_1 - t_2)}$$

where K_1 and K_2 = velocity constants which correspond to the temperatures

t_1 and t_2. The Q_{10} value of thermochemical and comparable thermo-biological reactions is usually about 2, indicating that the rate of reaction doubles or more for a $10^{o}C$ rise in temperature. The reason for this dramatic temperature effect appears to be an increase in the kinetic energy of the molecules involved in the multiple reactions which constitute metabolism. Arrhenius (1889) quantified the effect of temperature on chemical reactions. He pointed out that not all the molecules of a given population will possess the same kinetic energy, some being relatively 'energy-poor' and others correspondingly 'energy-rich' in comparison with the mean energy level. Arrhenius suggested that it is the energy-rich molecules which are most likely to react on collision, and that the rate of reaction might depend upon the concentration of such molecules. In order to react a molecule must possess a certain amount of kinetic energy in order to undergo a thermochemical reaction. This critical amount of energy is known as the 'energy of activation'. Arrhenius derived an equation for experimental determination of the temperature characteristic, $\bar{\mu}$, which is related to the energy of activation:-

$$\frac{d}{dT} \ln k = \frac{\bar{\mu}}{RT^2}$$

Where T = absolute temperature,

k = reaction velocity constant,

R = the gas constant,

and $\bar{\mu}$ = the temperature characteristic.

The Arrhenius equation has often been used by biologists instead of the van't Hoff equation, the $\bar{\mu}$ being considered to be the energy of activation of the rate limiting reaction, and hence a valuable index of the order of magnitude of the energy of activation required for a particular reaction. Subsequent thermodynamic considerations indicated that $\bar{\mu}$ is an unprecise value which is not a simple activation energy, but incorporates several other terms (see for example Johnson,

Eyring and Polissar, 1954). In most reactions the Arrhenius equation successfully describes the relative rates at different temperatures. Deviations are observed when the temperature range is extended, but generally experimental data conforms to the Arrhenius equation within the temperature range permitting convenient rate measurements. Homeotherms are able to regulate their body temperature within close limits over a wide range of environmental temperatures, terrestrial poikilotherms are often able to regulate their body temperature by selecting particular microclimates and by evaporative cooling, aquatic poikilotherms in general, however, are unable to use evaporative cooling to regulate body temperature. On the other hand the necessity of gills for respiration, which also act as efficient heat exchangers means that their body temperature follows that of the environment extremely closely (see Gunn, 1942). This is not too important for animals living in large bodies of water in which the temperature fluctuation is small, but in smaller water bodies such as most fresh water environments are, poikilothermic organisms may be exposed to wide variations in body temperature throughout the year. Thus the relationships between the rate of metabolism and temperature are extremely important for fresh water poikilotherms.

The effects of temperature on metabolic rates described above were found to only hold if no time was allowed for the organism to adapt to the new temperature regime. When a poikilotherm is moved from a temperature to which it has become acclimatised to another temperature an immediate change in its metabolic rate occurs (Grainger, 1958). Acclimatisation to the new temperature regime, however, brings about a compensatory adjustment in the metabolic rate. Precht (1957) has classified a number of variations in the way in which acclimatisation can alter the metabolic rate, he related these patterns to various physiological parameters, namely changes in enzymic concentrations and activity and alterations in substrate and cofactor concentrations. Precht called

called this form of adaptation "Capacity adaptation", thus distinguishing it from the other form, "Resistance adaptation". In this type of acclimatisation the resistance of the organism to exposure at extremes of its temperature range is altered. Thus acclimatising an animal to a higher temperature was found to increase its heat tolerance and correspondingly reduce its cold tolerance, adaptation to a lower temperature having the reverse effect.

The compensatory changes in reaction rates and thermal tolerance described above are evident at all levels of organisation from the molecular to the whole animal levels. Much of the earlier work on temperature acclimatisation was performed on intact animals, measuring their respiratory rates (see Precht, 1957) or thermal tolerance (see Belehrádek, 1935). Temperature acclimatisation has also been shown to bring about compensatory changes in the physiology of several poikilotherm tissues, e.g. nerve (see, for example, Roots and Prosser, 1962) and Anderson and Mutchmor, 1967, muscle (see Roberts, 1957 and Bowler 1963 b for example) and osmoregulatory systems (see for example Woodhead and Woodhead, 1959 and Lockwood, 1960). It now seems likely that these compensatory changes at the whole animal, and tissue, levels of organisation are reflections of changes in cellular processes. Many adaptive changes have been explained in terms of enzyme changes. The kinetic properties of an enzyme may alter during thermal acclimatisation. Somero (1969) has suggested that this may be brought about by both qualitative and quantitative changes of the enzyme. Quantitative changes in the amount of enzyme, substrate or enzyme cofactor will all effect the rate of an enzymically catalysed reaction. There is some evidence in poikilotherms that there is an increase in both substrate (see Hazel and Prosser, 1970) and enzyme concentrations (Eckberg, 1962; Baslow and Nigrelli, 1964; Freed, 1965) during cold adaptation. Qualitative changes in the enzyme during thermal acclimatisation have been reported. Hochachka and Somero

(1968) have shown that changes in the isoenzyme patterns can have major adaptive importance. Due to the central role which enzymes play in the functioning of every cell, changes in the enzymic activity are of basic importance to the whole organism. The changes which have been observed during thermal acclimatisation were of a compensatory nature, thus acclimatisation of poikilotherms to low environmental temperatures has been shown to result in an increased enzyme/substrate affinity (Hochachka, 1967) which increases the reaction velocity. Changes in enzyme kinetics of this nature, in combination with increased substrate concentrations at low environmental temperatures (Hazel and Prosser, 1970) will tend to compensate for the reduction in reaction velocity brought about by a fall in body temperature.

The mechanisms of heat injury are not understood. In the older literature death by exposure to heat has been attributed to protein coagulation, enzyme destruction and asphyxiation whilst Heilbrunn (1924) suggested that lipid liberation is the cause of heat death in poikilotherms. Later, Belehrádek (1931) suggested that the melting of lipids at higher temperatures destroyed essential cellular structures. There is considerable evidence of changes in the degree of cellular lipid saturation in poikilotherms, e.g. blowfly larvae (Fraenkel and Hopf, 1940); micro-organisms (Gaughran, 1947); goldfish brain (Johnson and Roots, 1964), muscle (Knippath and Mead, (1968) and intestine (Kemp and Smith, 1969); and in frog nerves (Elkes and Finean, 1953; Chapman, 1965). The experimental evidence, however, fails to explain the differing heat resistance of related species possessing the same lipid constituents (Fraenkel and Hopf, 1940).

More recently Ushakov (1964) proposed that denaturation of cellular enzymes was of greater significance during heat injury than lipid changes. He noted that a relationship existed between the thermostability of cell proteins and protein complexes and the cells

from which they were obtained. This has been shown in bacteria, (Langridge, 1963), for collagenous proteins (Gustavson, 1956, Rigby 1968), myosins (Cornell, 1961) and several enzymes (Kusakina, 1963). Ushakov (1964) went on to point out that most protein preparations tested had a greater heat resistance than did the cells from which they were obtained. He suggested that the heat resistance of a cell was limited by its least resistant protein system, though these have yet to be identified. Rosenberg, Kemeney, Switzer and Hamilton (1971) have produced evidence on thermodynamic grounds that heat death in yeasts, bacteria and viruses is due to denaturation of proteins. They found that the "compensation" law constants a and b in the equation below for these two processes were the same:-

$$\Delta S^{\ddagger} = a\,\Delta H^{\ddagger} + b$$

where $\Delta S^{\ddagger}$ = the entropy of activation

$\Delta H^{\ddagger}$ = the heat of activation

and a and b = constants.

Banks, Damajanovic and Vernon (1972) and Evans and Bowler (1973) have separately questioned whether the "compensation" law can be applied to such a complex phenomenon as heat death, and have consequently questioned the interpretation of the data of Rosenberg, Kemeney, Switzer and Hamilton (1971) that thermal denaturation of proteins is the cause of heat death.

The growing realisation in recent years of the frequent close association between cellular enzymes and membranes has resulted in the idea that heat death may result from a combination of lipid and protein damage. Cellular membranes consist of proteins and lipids in a particular orientation which is important to the physiology of the cell, e.g. in the mitochondrion (Lehninger, 1964); or in some cases enzymes probably exist as an integral part of the membrane (see Weiss, 1970 a, and 1970 b. There is some evidence that heat injury may be a co-

operative effect of both protein and lipid changes. Ling (1967) has reported a dramatic change in the permeability of frog striated muscle plasma membrane to sucrose which occurs over a relatively narrow thermal range. This range, 35 - 45°C, corresponded to the temperature at which the muscles were inactivated by heat rigor. Such a change in membrane permeability might well be ascribed to a co-operative breakdown of the membrane phospholipid/protein structure. Recently Davison (1971) has described ultrastructural changes in the mitochondrial membranes of blowfly flight muscles following exposure to lethal heat. This was correlated with a reduction in the efficiency of oxidative phosphorylation which occurred under similar heat treatment (Davison and Bowler, 1971). Davison and Bowler (1971) have suggested that the impairment of membrane/enzyme complexes is a major lesion during heat death in the adult blowfly, Calliphora erythrocephala.

The preceding synopsis of the present situation concerning the effects of temperature on poikilotherms reveals that there is much to be learnt. The present study was initiated with the intention of examining the effects of high lethal temperature on a poikilotherm. The animal selected to work on was the crayfish, Astacus pallipes, which appeared to be well suited to such studies for several reasons. It is an aquatic animal which is normally found in small bodies of fresh water, it is therefore exposed to wide seasonal variations in environmental, and hence body, temperature. In order to withstand such fluctuations the crayfish has a wide physiological temperature range which is coupled to a notable ability to adapt to temperature (see Bowler, 1963 a). The study had two main objectives, firstly to determine the primary lesion of heat death in this animal, and secondly to investigate the physiological basis of resistance adaptation.

CHAPTER 1.

THE EFFECT OF HIGH LETHAL TEMPERATURES ON THE INTACT ORGANISM.

INTRODUCTION.

Resistance adaptation is a common phenomenon in decapod crustaceans. It has been reported in crabs, lobsters and crayfish by several authors (e.g. Emerita talpoida Say., Edwards and Irving, 1943; Pachygrapsus crassipes, Roberts, 1957; Uca spp,, Vernberg, 1959; Hemigrapsus spp., Dehnel, 1960 and Todd and Dehnel, 1960; Homarus app., Huntsman, 1924 and McLeese, 1956; Orconectes propinquus and Cambarus fodiens, Bovjberg, 1952; Orconectes rusticus, Spoor, 1955; and Astacus pallipes, Bowler 1963 a). The results of these studies indicate that resistance adaptation in these organisms develops over a period of 7 to 21 days, thus indicating that resistance adaptation is a physiological event rather than an immediate negative thermal modulation effect or a long term genetic modification. The findings of Spoor (1955) and Bowler (1963 a) are of particular relevance here since these authors both worked with fresh water crayfish. Spoor (1955) reported that Orconectes rusticus adapted to 22 - 26^{o}C lost their adaptation gradually over a period of 16 days when maintained at 4^{o}C. He also found that the gain of heat tolerance was more rapid than loss in that the heat tolerance lost by transfer to 4^{o}C for 13 days was regained within 1 day of returning the animals to 22 - 26^{o}C. Bowler (1963 a) found the gain of heat tolerance in Astacus pallipes to be quite rapid. Crayfish adapted to 8^{o}C were virtually completely adapted to 25^{o}C within 3 days. It is therefore apparent that the physiological events which lead to the increased heat tolerance seen on warm adaptation of fresh water crayfish are quite rapid.

The measurement of heat tolerance/resistance raises some problems. Should the body temperature be raised gradually in order to avoid heat trauma, or would this allow a significant gain in heat tolerance? There is no clear answer to this question and both methods of

heat application have been used and justified (e.g. Spoor, 1955, Bowler, 1963 a). Both methods give consistent results, however, though their absolute relevance might be open to question. A more important consideration is the criterion of heat death. Bowler (1963 b) has shown that the tissues of crayfish exposed to lethal temperatures for periods far beyond any possibility of recovery still respired at rates which were comparable to those of tissues taken from unheated crayfish, thus indicating that physiological activity proceeds after the animal has irreversibly ceased to function. Because of this ambiguity it is necessary to use an index of heat death, the important point being to select an index which indicates an irreversible failure of the organism as a functioning unit. The index which appeared best suited to fit these criteria in the crayfish was the cessation of scathognathite beat. This was suitable since the scathognathite could be relatively easily observed during heating, and scathognathite beat cessation indicates that active circulation of water over the respiratory surfaces has ceased.

The problem of the actual moment of heat death can be avoided by measuring the percentage survival of animals exposed to a high temperature for a known period, Davison (1970). This method requires relatively large numbers of animals, however, which are not always available. Alternatively the highest temperature at which 50% of the animals can survive for a predetermined period may be measured, e.g. Spoor (1955). Probably the simplest method is to measure the survival time of an animal at a lethal temperature, e.g. McLeese, (1956) and Bowler (1963 a). All of these methods give consistent results, and the method selected must depend to a large extent upon the experimental animal. The latter method was selected in the following work because it is the most suitable for relatively low numbers of experimental animals and a good index of heat death was available.

The experiments described in this Chapter were designed to determine the heat tolerance of Astacus pallipes adapted to either 10 or 25°C at temperatures above their viable range. Detailed observations of the animals' behaviour during heat death and an analysis of the survival times at various lethal temperatures were made in order to correlate heat death in the intact organism with the results obtained at tissue and cellular levels in later chapters.

MATERIALS AND METHODS.

The experimental animals used throughout the following work were obtained either from a commercial source (L. Haig & Co. Ltd., Newdigate, Surrey) or from feeder streams of the Hallington and Whittledene reservoirs in Northumberland. They were identified using Freshwater Biological Association, Publication Number 19. Since the availability of crayfish from these sources was seasonal it was necessary to maintain stocks in the laboratory from late September to early May. They were kept in flowing, aerated tap water in shallow, stainless steel or polypropylene tanks. Animals which were to be adapted to 10°C were kept in tanks in a 5°C constant temperature room and animals which were to be adapted to 25°C were kept in a 15°C constant temperature room. No attempt was made to regulate the temperature of the storage tanks accurately, but over a twelve month period water temperature was found to vary in these tanks between 7 and 11°C in the 5°C room and between 14 and 17°C in the 15°C room. The animals were fed weekly on small pieces of liver, meat or bread, uneaten food was removed about 15 hours later. The animals were also noted to forage in the vegetation (Elodea spp.) which was present in the storage tanks.

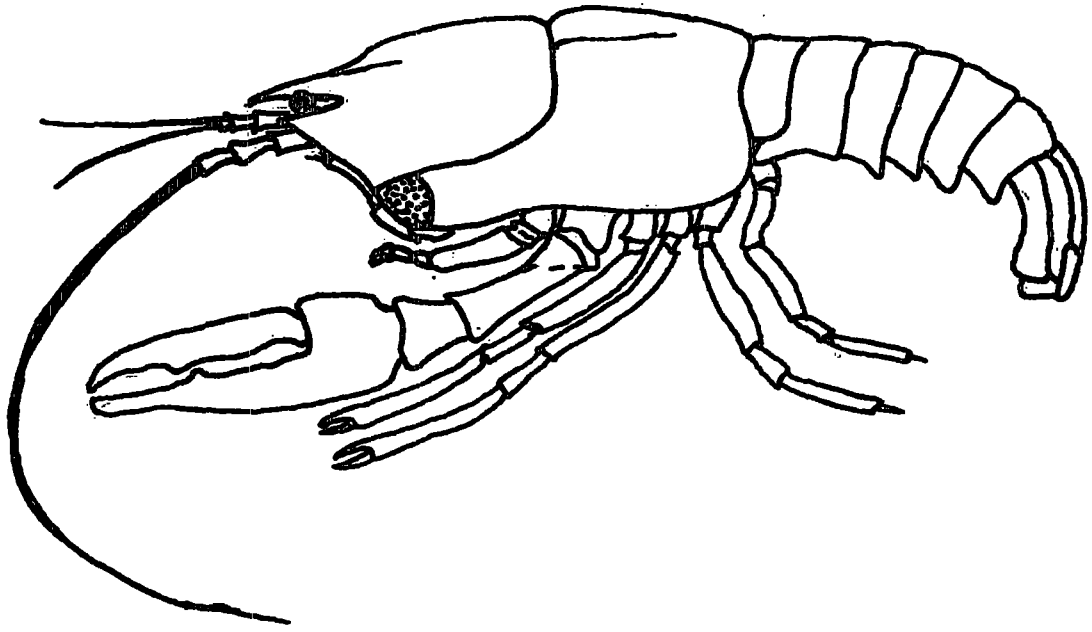
Experimental animals were adapted to 10 or 25°C in glass or polypropylene aquaria containing aerated tap water. In order to avoid fouling of the water the aquaria were cleaned and refilled with fresh water every two days. The temperature in these aquaria was maintained at $\pm$ 0.1°C using an immersion heater controlled by a Jumo thermoregulator and a hotwire vacuum relay switch (A.E.I. Ltd.). The water in the aquaria was stirred by continual aeration. The animals to be adapted were taken from the storage tanks and maintained for a minimum of 7 days at the adaptation temperature before each experiment. This was considered sufficient time for adaptation to occur since Bowler (1963 a)

Figure 1.

Diagram of *Astacus pallipes*.

The stippled region of the cephalothorax indicates the cuticle covering the scathognathite. This part of the cuticle was removed prior to heat treatment so that the scathognathite could be observed.

Figure 1.



and Spoor (1956) found that crayfish gained heat tolerance quite rapidly. In the majority of cases, however, experimental animals were maintained at their adaptation temperature for periods of 10 - 14 days. Experimental animals were not fed during the adaptation period.

Measurements of heat tolerance were performed in glass aquaria (dimensions 0.457 m long x 0.305 m wide x 0.152 m deep) containing fresh tap water. The water temperature was maintained at $\pm$ 0.1°C using the same apparatus as described earlier. The water was stirred with a mechanical stirrer and aerated. Experimental animals were dried by careful blotting with absorbent paper tissues, weighed, sexed and a small piece of the thoracic carapace was removed on one side to reveal the scathognathite (see figure 1) at least 60 minutes before the animal was exposed to the lethal temperature. The experimental animals were placed into the heated aquarium two or three at a time. They were not restricted in any way, though care was taken that they should not come into contact with the mechanical stirrer. Observations were made through the glass sides of the aquarium.

Water temperatures were measured with either a mercury thermometer or a copper-constantin thermocouple. A thermometer calibrated by the National Physics Laboratory was used to calibrate both the thermocouple and the mercury thermometer used. The potential generated by the thermocouple was measured with a 'Scalamp' galvonometer.

In the following experiments 10 animals were used to determine the heat tolerance of 10 or 25°C adapted animals at each lethal temperature investigated. Only mature animals weighing more than 7 g were used. The time taken for 50% of the animals to die was estimated by plotting the time from immersion to scathognathite beat cessation against the percentage survival. In this way the 50% survival time (LD_{50}) was obtained. Observations were made of the behaviour of crayfish during heat treatment.

TABLE 1.

The Effect of Temperature Adaptation on the Survival Times of *Astacus pallipes* at High Temperatures.

The LD_{50} times of groups of 10 adult crayfish exposed to lethal temperatures between 32 and 35°C were determined from figures 2 and 3 for 10° and 25°C adapted crayfish. Arrhenius μ values for the thermal inactivation of the whole animals were calculated from an Arrhenius plot of these LD_{50} times (see figure 4).

TABLE 1.

Adaptation Temperature (°C).	Exposure Temperature (°C).	LD_{50} (minutes).	Arrhenius μ (K cals $mole^{-1}$)
10	32.0	16.0	70
	32.5	13.3	
	33.0	11.3	
	34.0	7.2	
25	32.0	205	143
	33.0	113	
	34.0	43.3	
	35.0	19.3	

RESULTS.

A preliminary experiment with ten 25°C adapted crayfish was performed to investigate whether the cessation of scathognathite beat was a suitable index of heat death. In normal animals the scathognathite was observed to beat irregularly, but quite frequently, at their adaptation temperature. In experimental animals, exposure to high lethal temperature (32°C) resulted in an almost immediate increase in the rate of scathognathite beat such that the beat was continuous rather than intermittent. After a period the scathognathite beat became more erratic, with some strokes being incomplete, until it finally ceased. This phase of irregular beating occurred quite suddenly, and was followed within 1 to 2 minutes by the cessation of scathognathite beat. Five of the animals were left in the aquarium at 32°C for at least 10 minutes following this phase and in no case was any further scathognathite beat observed. Five other animals were removed from the high temperature aquarium immediately scathognathite beat ceased and returned to their adaptation temperature (25°C). None of these animals recovered. Scathognathite beat cessation was therefore taken as an acceptable index of heat death signifying that some irreversible process had occurred. In agreement with the results obtained by Spoor (1955) and Bowler (1963 a) no correlation was found between size or sex and survival time of crayfish at high lethal temperatures.

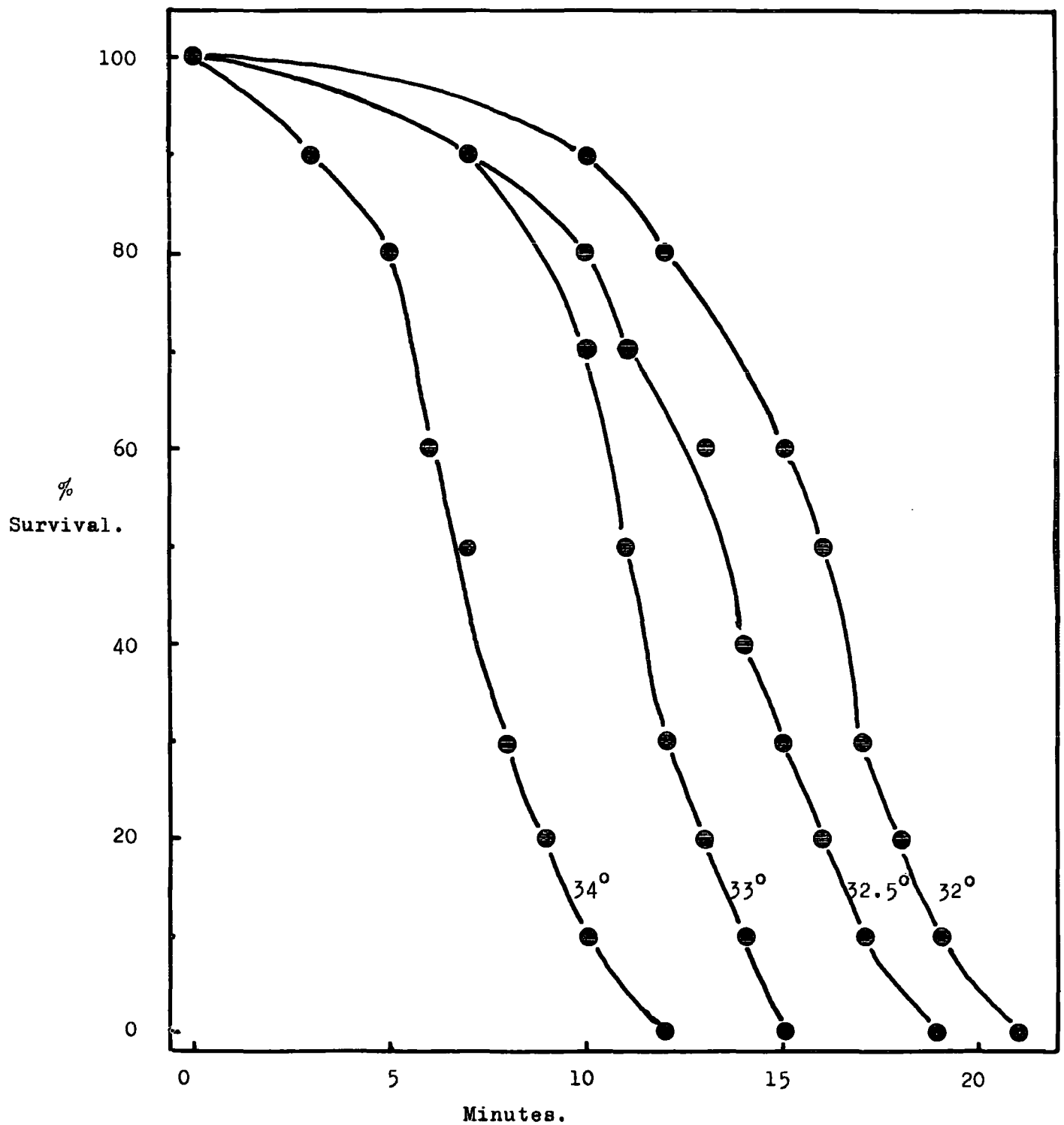
The behaviour of these and subsequent experimental animals was observed during heat exposure. It was found to follow a set pattern which varied only in its time course with animals of different heat tolerance at different temperatures. On being introduced to the warm water the crayfish typically displayed high motor activity with frequent, forceful abdominal flexures. This phase was quite brief and was followed by a period during which the crayfish moved around the aquarium in a co-ordinated, exploratory fashion. Abdominal flexure was rare during this phase. This controlled 'walking' behaviour was

Figure 2.

Determination of LD_{50} Times of $10^{o}C$ Adapted Crayfish.

Four groups of ten $10^{o}C$ adapted Astacus pallipes were exposed to lethal temperatures of 32.0, 32.5, 33.0 and $34.0^{o}C$ respectively and the time of scathognathite beat cessation recorded as described in the Methods. The percentage survival of each group was plotted against the time of exposure to lethal temperature, and the LD_{50} determined from the 50% survival time (see table 1).

Figure 2.



followed by increasingly long periods of locomotory quiescence during which the crayfish showed no desire to move. The rate of scathognathite beat during the latter phases was higher than that observed in the animals at their adaptation temperature.

The next phase was an increasing loss of co-ordination as evinced by the frequent inability of the animals to remain upright. This phase was accompanied or succeeded rapidly by a marked increase in the rate of scathognathite beat, though the amplitude of beat appeared to be somewhat shortened. The cessation of scathognathite beat followed soon after. It was accompanied,or closely succeeded,by twitching of the distal segments of the limbs,depending upon the actual lethal temperature used. Rapid twitching occurred before scathognathite beat cessation in most animals treated at relatively high lethal temperatures (34^{o} and $35^{o}C$) and followed scathognathite beat cessation at the lower lethal temperatures (32^{o}, 32.5^{o} and $33^{o}C$). Fibrillation of the scathognathite itself was also observed at or about the time of scathonathite beat cessation.

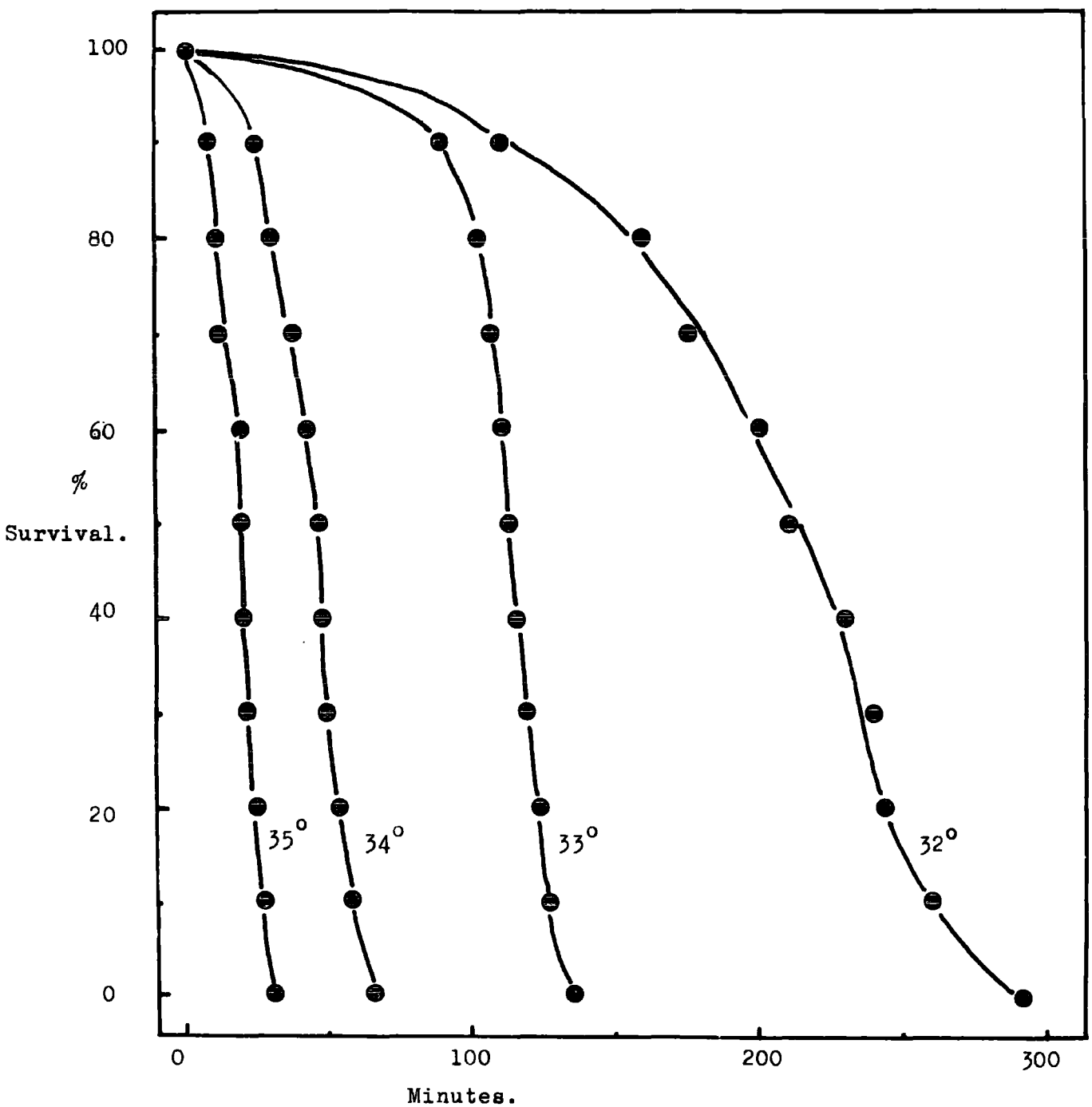
Figures 2 and 3 show the time of cessation of scathognathite beat plotted against the percentage survival for 10^{o} and $25^{o}C$ adapted crayfish respectively. The LD_{50} times obtained from figures 2 and 3 are presented in table 1. These results clearly indicate that the resistance of $25^{o}C$ adapted crayfish to high lethal temperatures was greater than that of the $10^{o}C$ adapted crayfish. For example, at $32^{o}C$ the LD_{50} of $10^{o}C$ adapted crayfish was 16 minutes but for $25^{o}C$ adapted animals was 205 minutes. The exponential relationship between survival times and increasing lethal temperatures is shown in figure 4 where the $logarithm_{10}$ of the LD_{50} times from table 1 have been plotted against the reciprocal of the absolute temperature. The slope of the two curves gives the Arrhenius $\bar{\mu}$ value for the heat death of 10^{o} and $25^{o}C$ adapted crayfish. Arrhenius $\bar{\mu}$ was 70 and 143 Kilocaries $mole^{-1}$ for 10^{o} and $25^{o}C$ adapted crayfish respectively.

Figure 3.

Determination of LD_{50} Times of $25^{o}C$ Adapted Crayfish.

Four groups of $25^{o}C$ adapted Astacus pallipes were exposed to lethal temperatures of 32.0, 33.0, 34.0, and $35.0^{o}C$ respectively and the time of scathognathite beat cessation recorded as described in the Methods. The percentage survival of each group was plotted against the time of exposure to lethal temperature, and the LD_{50} determined from the 50% survival time (see table 1).

Figure 3.



DISCUSSION.

The increased heat tolerance of warm adapted crayfish with respect to cold adapted animals has been known for some time. Bovjberg (1952) observed that two species of crayfish, Orconectes propinquus, became increasingly tolerant of temperatures between 34° and 35°C as the advancing season warmed their environment, or after they had been maintained in warm water (18° - 28°C) in the laboratory for five to six weeks. Spoor (1955) also observed that Orconectes rustious had a higher 24 hour median heat tolerance following 7 days' acclimatisation at 30°C than 22 - 26°C adapted animals; and further that transferring crayfish from 22 - 26°C to 12° or 4°C resulted in a loss of heat tolerance. Bowler (1963 a) found that Astacus pallipes acclimatised to 25°C had a higher heat tolerance than animals adapted to 8°C. The experimental results presented above therefore agree with the findings of previous workers in that crayfish heat tolerance varies with the previous thermal history of the individual in a predictable fashion, warm adapted animals having the greater heat tolerance.

Direct comparison of the experimental results with those of Bovjberg (1952) and Spoor (1955) is not possible. Neither of these authors accurately controlled the acclimatisation temperature of their experimental animals and they brought the animals to the lethal temperature over an extended period. Bowler (1963 a) used similar methods to those described in the Methods, adapting his crayfish to 8° or 25°C ($\pm$ 0.1°C) and transferring the animals directly from their adaptation temperature to the lethal temperature. Bowler's index of heat death differed from that used in this work in that he considered the animal to be dead when all visible movements had ceased. His mean survival times are therefore somewhat longer than those given in table 1. For example, he found that 25°C adapted crayfish exposed to 34°C survived for about 80 minutes compared to an LD_{50} of 43.3 minutes (see table 1) at this

Figure 4.

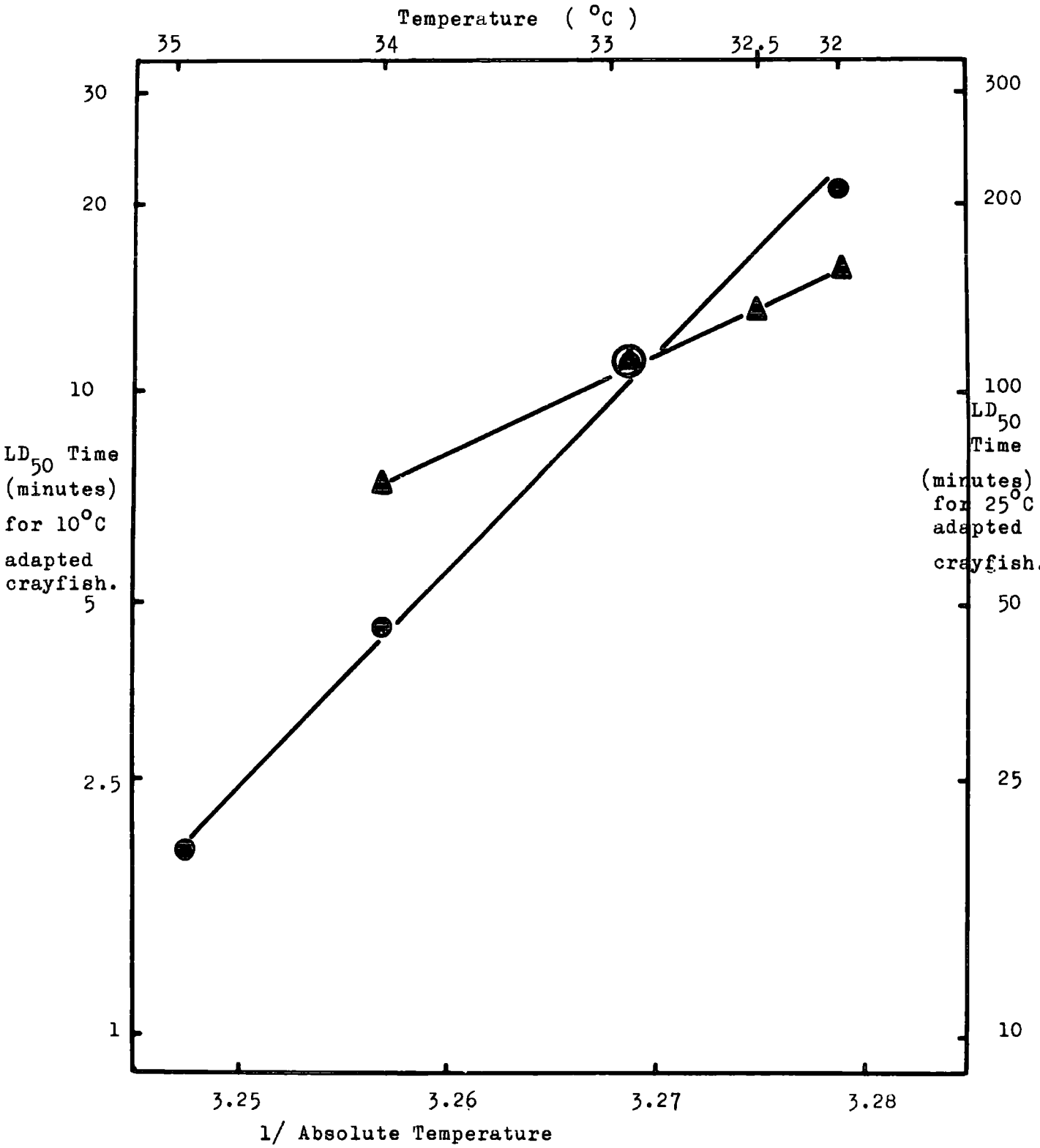
Arrhenius Plot of the LD_{50} Times of 10^{o} and $25^{o}C$ Adapted Crayfish.

The logarithms of the LD_{50} times for Astacus pallipes adapted either to 10^{o} or $25^{o}C$ was plotted against the reciprocal of the absolute temperature to which the crayfish were exposed. The ordinate on the left refers to the 10^{o} adapted crayfish and the ordinate on the right to the $25^{o}C$ adapted crayfish.

$10^{o}C$ adapted crayfish..... ▲

$25^{o}C$ adapted crayfish..... ⊜

Figure 4.


Temperature (°C)
35
34
33
32.5
32
30
20
10
5
2.5
1
300
200
100
50
25
10
LD50 Time (minutes) for 10°C adapted crayfish.
LD50 Time (minutes) for 25°C adapted crayfish.
3.25
3.26
3.27
3.28
1/ Absolute Temperature

temperature for similarly adapted crayfish using cessation of scathognathite beat as the index of heat death. The differences appear to be quantitative rather than qualitative, however, and the results are similar in nature to those reported above. One direct comparison between Bowler's work and this is possible, the Arrhenius $\bar{\mu}$ value obtained from Bowler's results for 25°C adapted crayfish was found to be 140 kilocalories $mole^{-1}$ (Bowler and Duncan, 1968). The Arrhenius $\bar{\mu}$ value for 25°C adapted crayfish calculated from figure 4 is 143 kilocalories $mole^{-1}$. In view of this very close agreement and the qualitative similarity of the effects of cold and warm adaptation it is concluded that these results agree with those of Bowler (1963 a).

CHAPTER 2.

THE EFFECTS OF HIGH TEMPERATURE ON ION DISTRIBUTION.

INTRODUCTION.

Crayfish experience the same osmotic problems as do other fresh water organisms, that of dilution of internal fluids by the inflow of osmotic water. They overcome this by excreting a copious hypotonic urine via the green gland and by reducing the permeable body surfaces. The urine has been shown to contain salts (Riegel, 1968; Sharma, 1968) and this continual loss of ions is made good by the active uptake of cations, particularly sodium from the environment against a large concentration gradient (Shaw 1959 and 1960 a, b, and c). This is the basis of the physiological regulation of the ion content of crayfish haemolymph which has been demonstrated by Bryan (1960 a, b, and c) and Shaw (1960 a, b, and c).

The permeability of crustaceans to water and salt may be affected by hormones. Neuroendocrine regulations of water permeability has been demonstrated in *Carcinus maenas* (Carlisle, 1955), *Hemigrapsus nudus* (Lehman and Scheer, 1956) and *Geocarcinus lateralis* (Bliss, Wang and Martinez, 1966). Sodium outflow from the body of the shore crab *Hemigrapsus nudus* is reduced by an extract of prawn cephalothorax (Ramaurthi and Scheer, 1967), and crayfish eyestalk extracts have been shown to affect the water balance of frogs (Heller and Smith, 1948), although this may be a pharmacological rather than a physiological effect. Recently Kamemoto, Kato and Tucker (1966) and Kamemoto and Ono (1969) have produced evidence of neuroendocrine regulation of salt and water balance in the fresh water crayfish, *Procambarus clarkii*. They suggested that eyestalk removal, and thus removal of the sinus gland and 'X' organ, affected the ability of crayfish to control water influx. They further reported that the injection of eyestalk extracts relieved the symptoms brought about by eyestalk removal or ligation.

In general, multicellular organisms also maintain ionic gradients between extra and intracellular compartments, cells having high concentrations of potassium, magnesium and calcium ions and low concentrations of sodium ions with respect to the extracellular fluid. The maintenance of the correct ionic composition of the extracellular fluid is of particular importance to excitable cells, for the generation of bioelectric potentials requires the maintenance of specific ionic distributions across their plasma membranes.

The ionic requirements for the maintenance of the resting potential and the production of the action potential in crustacean muscles have been widely investigated (e.g. Atwood, 1967; Fatt and Katz, 1953; Fatt and Ginsborg, 1958; DeMello and Hutter, 1965; Gainer, 1968; Gainer and Grundfest, 1968; Girardier, Reuben, Brandt and Grundfest, 1963; Hays, Lang and Gainer, 1968; Obaru, 1968; Reuben, Brandt, Garcia and Grundfest, 1967). The muscle fibre resting potential appears to depend upon the permeability of potassium, sodium and chloride ions in accordance with the Goldman Constant Field Equation (Hinkle, Heller and Van der Kloot, 1971), permeability being highest for potassium and lowest for sodium. The muscle fibre action potential has been shown to depend upon the presence of high potassium concentrations inside the cells to carry the outward current, with sodium normally carrying the inward current (Lockwood, 1968) though divalent ions such as calcium, strontium and barium can replace sodium (Fatt and Ginsborg, 1958).

The crayfish axon has been demonstrated to have high potassium and chloride permeabilities, potassium being the more permeable ion (Strickholm and Wallin, 1967). The nerve axons are depolarised by high external potassium ion concentrations (Strickholm, Wallin and Shrager, 1969) as are crayfish muscle fibres (Fatt and Katz, 1953). Alterations in extracellular sodium concentrations do not appreciably alter the resting potential of nerve axons (Strickholm and Wallin, 1967) or muscle fibres

(Fatt and Katz, 1953). The concentration of calcium ions in the extracellular fluid has been shown to affect the membrane resistance of muscle fibres (Fatt and Ginsborg, 1958) and the form of the action potential in crayfish giant axons (Machne and Orozco, 1967; Takenaka and Yumoto, 1966). The extracellular ionic concentrations therefore exert considerable influence on the functioning of the excitable tissues.

The unequal distribution of sodium and potassium ions about the plasma membrane of excitable cells is thought to be maintained by the cation pump. This is a $Na^+ + K^+$ activated adenosine triphosphatase (EC 3.6.1.3) which exchanges intracellular sodium with extracellular potassium in many cells. Since these movements are against the concentration gradients of the respective ions, this is an energy requiring system which is powered by the metabolic energy of the cell. Bowler and Duncan (1968) have demonstrated the presence of a $Na^+ + K^+$ activated adenosine triphosphatase in the abdominal muscle of Astacus pallipes.

The concept of Na^+ being actively removed from muscle cells was first introduced by Dean (1940). Later, Conway and Hingerty (1948) produced evidence for active Na^+ extrusion in mammalian muscle cells in vivo. Direct linkage of Na^+ and K^+ movements was proposed by Steinback (1940) from his studies on frog muscle and was demonstrated by Glynn (1959) in nerve, muscle and red cells. The current view is that the plasma membranes of excitable cells are leaky and thus allow permeable ions to move slowly down their concentration gradients, this leakage being corrected by the sodium pump. A more detailed account of the mechanism and functions of the cation pump will be given in later sections. It is relevant to note at this point that the cation pump which Bowler and Duncan (1968) observed in crayfish abdominal muscle was markedly inactivated by incubating the enzyme preparation at 33^o to 36^oC.

The effect of high lethal temperatures on crayfish haemolymph sodium and potassium ion concentrations was investigated by Bowler (1963 b). Bowler found that there was a dramatic increase in haemolymph K^+ and decrease in haemolymph Na^+ concentrations during heat death. The fact that these changes were initiated long before heat death led to the suggestion that these ion movements were in some way involved in the process of heat death. Bowler and Duncan (1968) in a later study suggested that the observed ion movements were a result of a breakdown in the passive rather than the active component of membrane permeability. Bowler (1963 b) also found that the changes in haemolymph cations, which he observed during the latter stages of heat death in the crayfish, were sufficient to affect the functioning of isolated, perfused crayfish claw preparations. He found, for example, that the preparation ceased to respond to stimulation earlier when perfused with a saline adjusted to have the same concentrations of Na^+ and K^+ as the haemolymph of heat dead crayfish, than with saline containing normal levels of Na^+ and K^+.

Finally, Grainger (1968) has described an exchange of Na^+ and K^+ in the body fluids of the snail, Arianata arbustorium during heat death similar to that described by Bowler (1963 b) in the crayfish. His findings led him to suggest that this effect might be widespread in poikilotherms undergoing heat stress. Davison and Bowler (1971) reported that in the adult blowfly, Calliphora erythrocephala ion movements did not occur at temperatures which just killed the animal, but only at much higher temperatures.

The previous work indicates that increased cellular permeability may be a primary lesion in heat death. The earlier work by Bowler (1963 a and b), Bowler and Duncan (1968) and Grainger (1968) was concerned with changes in the Na^+ and K^+ of extracellular fluids. It is considered that a more detailed examination which investigated Ca^{++}

and Mg^{++} as well as Na^{+} and K^{+} in the tissues and haemolymph of heat stressed animals is particularly important. A comparative study of cation movements in animals of differing adaptation temperatures would also provide further information upon the mechanisms of thermal adaptation. The following experiments are designed to confirm and extend the work reported by Bowler (1963 a and b).

MATERIALS AND METHODS.

Crayfish used in these studies were adapted to either 10 or 25°C as described in Chapter 1.

Concentrations of cations in tap water, haemolymph and muscle were determined using a Pye-Unicam SP.90 Atomic Absorption Spectrophotometer. Na^+ and K^+ concentrations were measured by emission, typical calibration curves for standard solutions are shown in figure 5. Ca^{++} and Mg^{++} concentrations were measured by absorption and typical curves for standard solutions are shown in figure 6. Standard solutions of Na^+, K^+ and Ca^{++} were made by diluting BDH Concentrated Volumetric Solutions of sodium and potassium hydroxide and calcium chloride appropriately. Standard solutions of Mg^{++} were made by dilution of a stock solution of magnesium chloride, the BDH 'Analar' magnesium chloride being dried to constant weight to make up the stock solution. All standards and samples for calcium determinations contained 5,000 ppm lanthanum chloride (Hopkin and Williams special low calcium atomic absorption grade) to eliminate phosphate interference. Control studies indicated that when the standard solutions were stored at 5°C in clean 'Pyrex' glassware there was no change in the calibration curves for at least three weeks. Fresh standard solutions were made up every three weeks. The methods of obtaining samples for assay are described below:-

1. Tap water.

This experiment required the assay of sequential samples of water in which the animal was held during exposure to high lethal temperatures. Each animal was carefully blotted dry and weighed and placed into a known volume (200 ml) of tap water in a crystallising dish. The dish stood in a water bath the temperature of which was controlled to within $\pm$ 0.1°C. The temperature in the crystallising dish was constantly monitored by a thermocouple.

One ml samples of the water were taken at known intervals and

Figure 5.

Typical Calibration Curves for the Determination of Sodium and Potassium Concentrations.

Standard solutions of sodium and potassium were made up and used to calibrate the Pye-Unicam SP90 Spectrophotometer as described in the Methods. Figure 5 (a) illustrates a sodium calibration and figure 5 (b) illustrates a potassium calibration.

Figure 5(a)

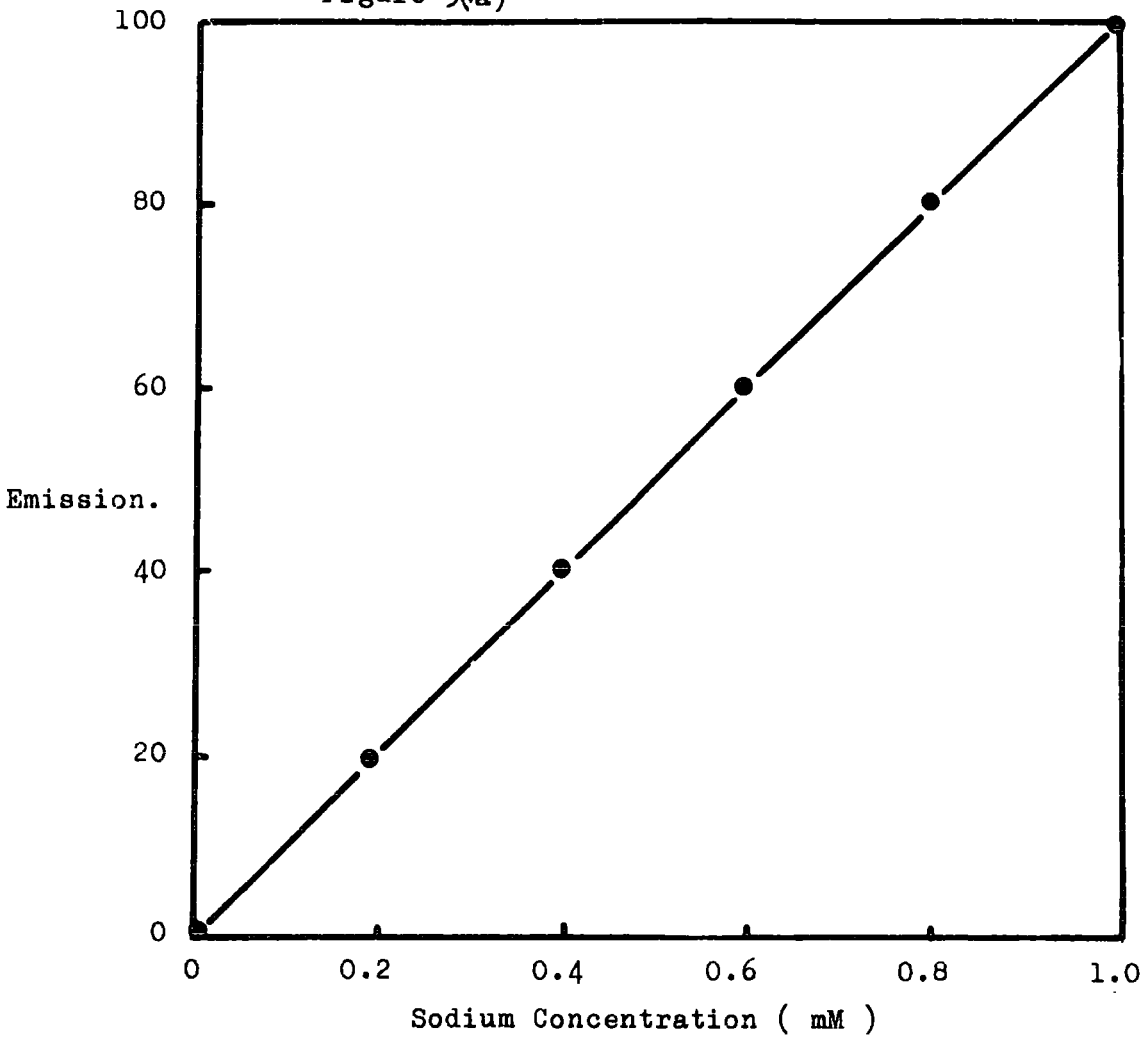


Figure 5(b)

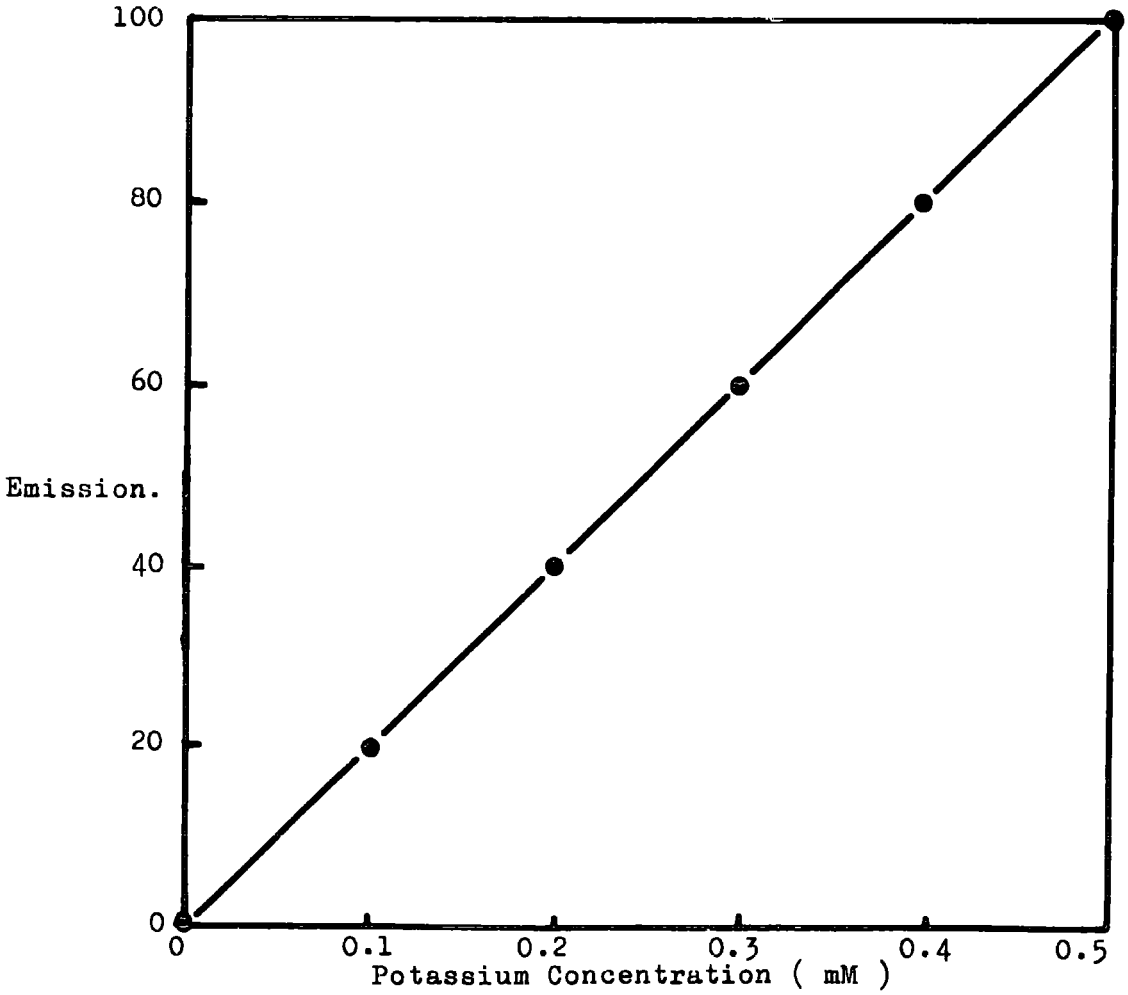


Figure 6.

Typical Calibration Curves for the Determination of Calcium and Magnesium Concentrations.

Standard solutions of calcium and magnesium were made up and used to calibrate the Pye-Unicam SP90 Spectrophotometer as described in the Methods. Figure 6 (a) illustrates a calcium calibration and figure 6 (b) illustrates a magnesium calibration.

Figure 6(a)

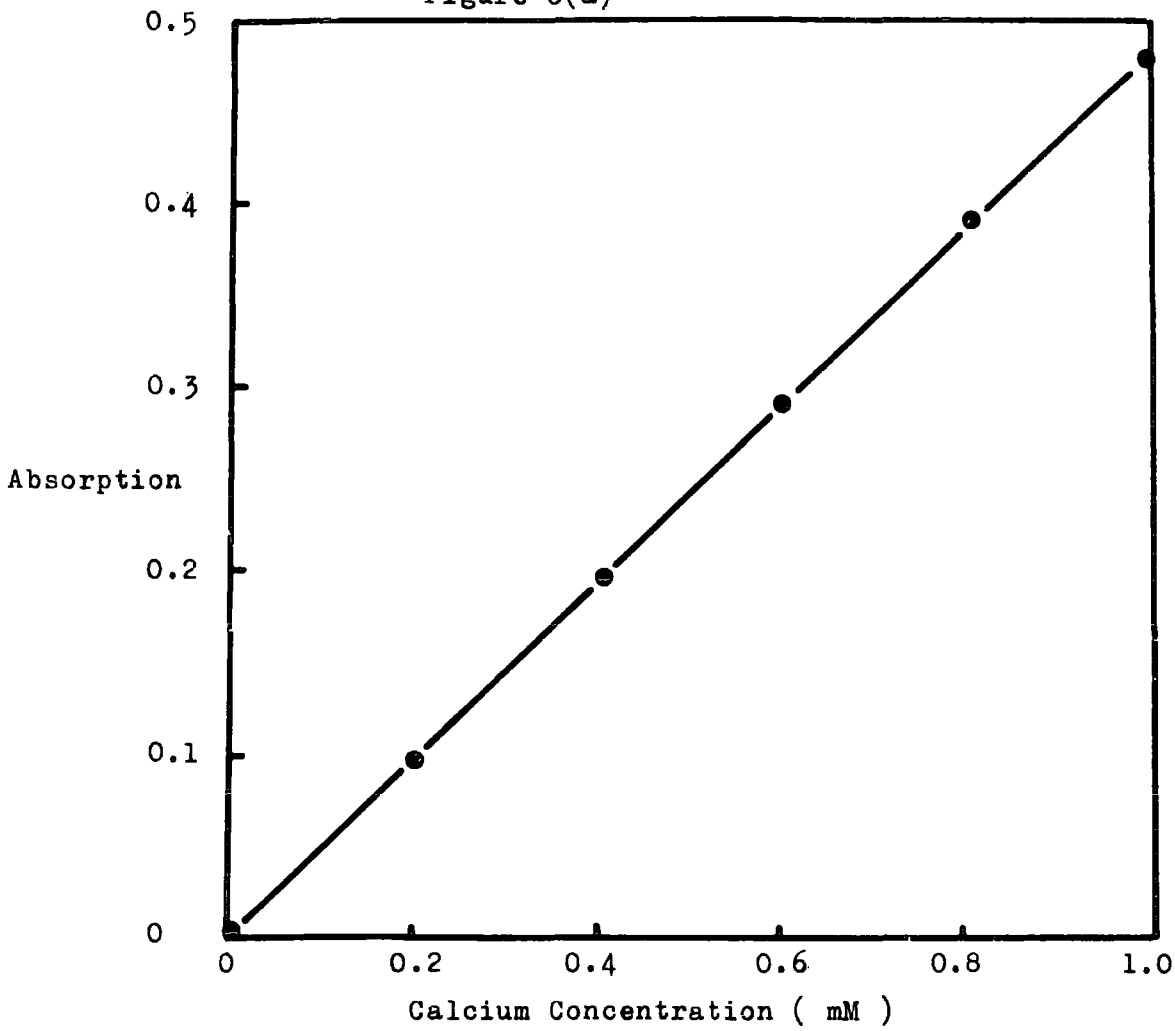
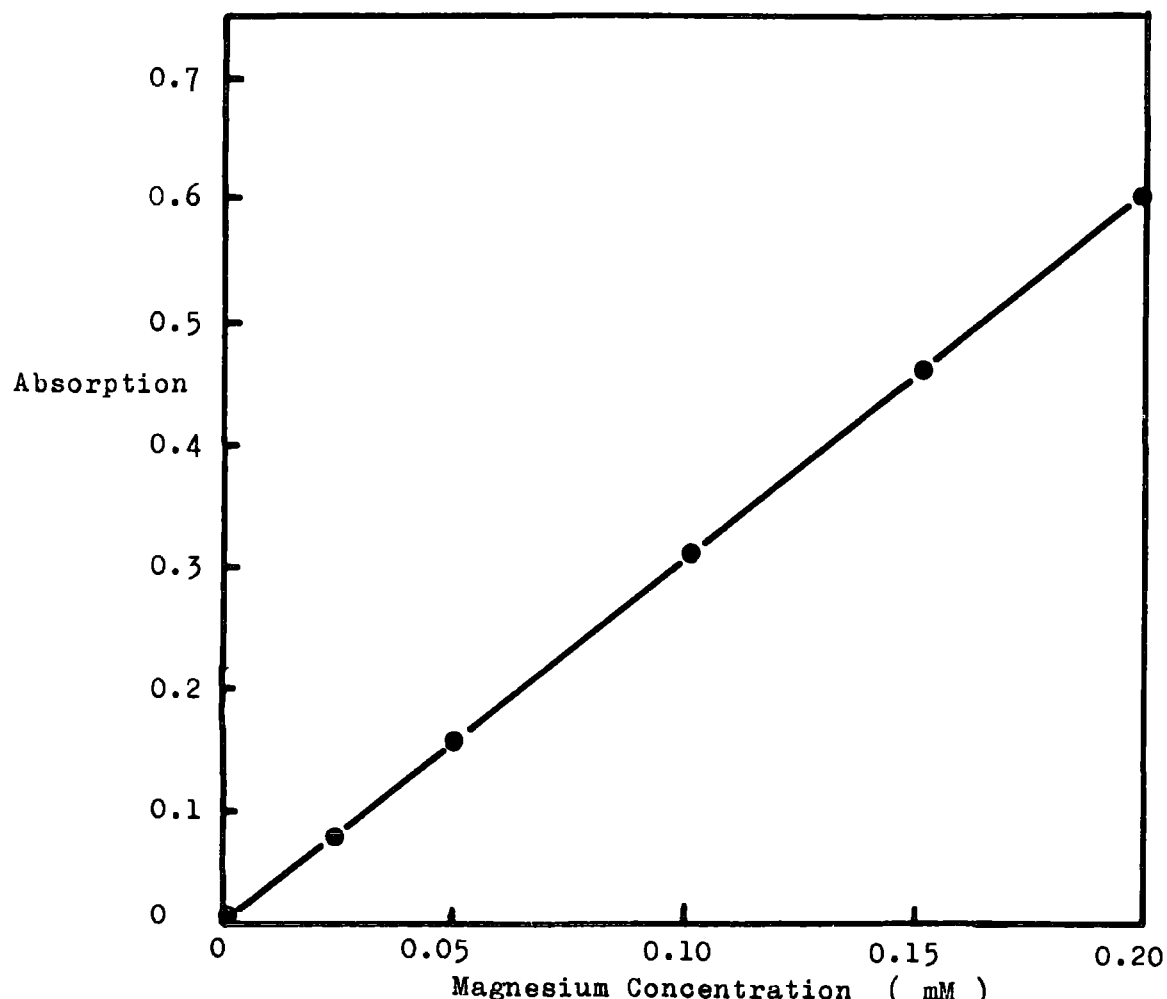


Figure 6(b)



stored in clean 'Pyrex' centrifuge tubes for analysis. Control experiments were performed to show that no net exchange of cations occurred between the 'Pyrex' crystallising dish and the water at any of the experimental temperatures for the duration of the experiments.

2. Haemolymph Cations.

Either 100 µl (for mono and divalent cations) or 50 µl (for monovalent cations) samples of haemolymph were obtained by dorsal penetration of the articular membrane between the cephalothorax and the abdomen with the tip of a Shandon-Terumo 100 µl Micro-syringe. To avoid clotting, the samples were drawn into a 5% formalin ('Analar') solution. They were then diluted with deionised water for analysis. Diluted samples were kept in clean stoppered 'Pyrex' centrifuge tubes and were analysed immediately after the experiment. Control experiments were performed to ensure that there was no net exchange of cations between the glassware and the sample during storage.

3. Muscle Cations.

Portions of the abdominal flexor muscle were rapidly dissected taking care to damage the fibres as little as possible. They were rinsed quickly in deionised water, blotted and weighed on clean aluminium foil. A minimum of four samples were taken from each experimental animal. These samples were treated independently to give four separate results for each animal. Muscle samples were dried in a vacuum oven at 60°C for 24 hours, reweighed and ashed in a muscle furnace for 3 hours at 500°C. The ash was dissolved in 0.2 ml dilute nitric acid ('Analar') and then diluted appropriately with deionised water for analysis.

4. Estimation of Extracellular Space.

In order to calculate the ion content of muscle cells it was necessary to estimate the extracellular space of the tissue. This was determined by the method of Van der Kloot (1966). Muscle samples were obtained as described and equilibrated for 2 hours at 5°C in Van

Harreveld's saline containing ^{3}H-Inulin (Radio-Chemicals, Amersham). The tissues were then blotted, weighed and dissolved in 0.2 ml 5 M KOH. The muscle solution was then added to 18 ml scintillation fluid (30% methanol in toluene containing 6.0 g/l 2,5-diphenyl oxazole (PPO) and 0.1 g/l 2-p-phenylenebis (5-phenyloxazole) (POPOP)) and counted on a Nuclear Chicago liquid scintillation counter. The PPO and POPOP were obtained from Sigma Ltd., Missouri, U.S.A.

5. Haemolymph Volume.

The method used to determine the haemolymph volume was that of Prosser and Weinstein (1950), 0.1 ml saline containing 0.2 mg of Evans Blue (T-1824) (T.G.Gurr Ltd.) was injected slowly into the pericardium. Sixty minutes later 0.3 ml haemolymph was withdrawn from the base of the rear walking leg, 0.25 ml of this was diluted in 3.0 ml saline and well mixed. A further 0.3 ml sample was withdrawn 180 minutes after the initial injection and treated similarly. The animal was then exposed to a high lethal temperature, the time of death recorded, and a final 0.3 ml sample taken and treated as described above. The optical density of the samples were then measured at a wavelength of 620 nm on a Hilger-Watt 'Uvispek' spectrophotometer. The amount of Evans Blue present was then determined by comparison with the optical densities of calibration dilutions of the dye. Experimental animals were weighed prior to the initial injection.

Method of calculating data for table 15.

The calculations for the data presented in table 15 were performed as follows:-

(a). Haemolymph.

The initial haemolymph volume = $\frac{\text{mean weight of animal} \times 25}{100}$ ml.

If the initial concentration of monovalent ion C^+ in the haemolymph

$= [C^+]_1$ mM

and the final concentration of monovalent ion C^+ in the haemolymph

$= [C^+]_2$ mM

then the initial haemolymph content of the monovalent ion, C^+

= initial haemolymph volume in ml $\times [C^+]_1 \times \frac{\text{atomic weight of C}}{1{,}000}$ mg

and the final haemolymph content, allowing for a 10% reduction of haemolymph volume (see table 11)

= initial haemolymph volume in ml $\times \frac{90}{100} \times [C^+]_2 \times \frac{\text{atomic weight of C}}{1{,}000}$ mg

The change in net haemolymph content in mg was then obtained by subtraction.

(b). Muscle.

The weight of muscle = 50% weight of animal = $\frac{W}{2}$ g (see table 10).

The volume of muscle = $\frac{W}{2 \times \text{density}} = \frac{W}{2 \times 1.26}$ ml. (see table 13).

The wet muscle is 84.97% water (see table 12) of which 18.74% is extracellular water (see table 7), therefore the cell water is 66.23% of the muscle volume. The net change of muscle cell water monovalent ion C^+ content is thus

$$\frac{W}{2 \times 1.26} \times \frac{66.23}{100.0} \times ([C^+]_2 - [C^+]_1) \times \frac{\text{atomic weight of C}}{1{,}000} \text{ mg}$$

where $[C^+]$, and $[C^+]_2$ are the initial and final muscle fibre concentrations of cation C^+.

(c) Water.

The initial volume of water was 200 ml. Assuming an increase in monovalent ion C^+ concentration of $[C^+]_{inc}$ mM, then the increase

$$= 200 \times [C^+]_{inc} \frac{\text{atomic weight of C}}{1,000} \text{ mg}$$

RESULTS

1. Net Exchange of Sodium and Potassium between Animal and Water.

In control experiments in which crayfish were kept at their adaptation temperature, no net exchange of Na^+ or K^+ was observed. Net exchanges were observed when the animals were exposed to lethal temperatures (see table 2a and b). Ten 10^oC adapted crayfish were exposed to 32^oC and six 25^oC adapted crayfish were exposed to 34^oC. These temperatures were chosen as a compromise, since they bring about heat death fairly rapidly, but allow reasonable time for sampling.

Table 2a shows the changes in the amounts of Na^+ and K^+ in the water which occurred during heat death of 10^oC adapted crayfish at 32^oC. The expected heat death time is 16 minutes (see table 1). In general it can be seen that about 75% of the total increase in sodium in the water occurred during the first four minutes of exposure, i.e. 0.0294 ± 0.0017 mg sodium per g wet weight of animal. The total sodium lost after 12 minutes rose to 0.0388 ± 0.0075 mg and rose only to 0.0394 ± 0.0013 mg sodium per g wet weight of animal in a further 3 minutes, a time which approximates the LD_{50}. The gain of Na^+ by the water which occurred during heat death of 25^oC adapted crayfish at 34^oC is shown on table 2b, the expected heat death time of this group of animals is 43 minutes (see table 1). The gain of Na^+ by the environment during the first 10 minutes of exposure was about 50% of the total increase, 0.034 ± 0.014 mg sodium per g wet weight of animal. The total sodium lost in 25 minutes was 0.046 ± 0.006 mg and rose to 0.064 ± 0.009 mg sodium per g wet weight of animal. The sodium loss to the water is thus of a similar pattern in both adaptation groups, the initial loss was rapid slowing as the animal approached death.

The gain of K^+ by the water showed a similar pattern in both adaptation groups. There was an initial, slower, rate of loss from the

TABLE 2.

The Effect of Temperature Adaptation on the Net Exchange of Na^+ and K^+ between Crayfish and their Environment at Lethal Temperatures.

The net gain or loss of Na^+ or K^+ from the beginning of the experiment by a known volume of water in which crayfish of known weight were immersed was measured. Values given represent the gain or loss by the water of an ion expressed as mg ion per g wet weight of animal. Values without a bar represent a net gain of the ion, and values with a bar represent a net loss of the ion by the water.

The initial Na^+ and K^+ concentrations of the water in the experimental chambers were;-

Na^+ ... 1.284 mM (n = 18, S.E. of Mean 0.036 mM)

K^+ ... 0.053 mM (n = 18, S.E. of Mean 0.0048mM).

TABLE 2.

(a):- 10°C Adapted Crayfish at 32°C.

Animal	mg Na^+			mg K^+		
	Time of Sampling (minutes).					
	4	12	15	4	12	15
1	0.037	0.050	0.047	0.0010	0.0039	0.0020
2	0.013	0.013	0.045	0.0020	0.0005	0.0045
3	0.062	-	0.075	0.0008	-	0.0035
4	0.016	-	0.029	0.0007	0.0055	0.0067
5	0.022	0.041	0.016	0.0011	0.0011	0.0012
6	0.045	0.043	0.061	0.0010	0.0030	0.0078
7	0.030	-	0.032	0.0038	0.0018	0.0048
8	0.045	0.064	0.039	0.0010	0.0010	0.0093
9	0.022	0.022	0.023	0.0004	0.0004	0.0021
10	0.002	-	0.057	0.0000	0.0000	0.0032
$\bar{x}$	0.0294	0.0388	0.0394	0.0008	0.0018	0.0045
S.E.	0.0017	0.0075	0.0013	0.0009	0.0009	0.0006

(b):- 25°C Adapted Crayfish at 34°C.

Animal	mg Na^+			mg K^+		
	Time of Sampling (minutes).					
	10	25	45	10	25	45
X 1	0.035	0.066	0.093	0.0011	0.0022	0.0064
2	0.028	0.032	0.040	0.0004	0.0022	0.0046
3	0.035	0.035	0.040	0.0013	0.0031	0.0090
4	0.028	0.032	0.067	0.0013	0.0001	0.0079
5	0.031	0.060	0.058	0.0049	0.0077	0.0085
6	0.046	0.052	0.087	0.0030	0.0102	0.0195
$\bar{x}$	0.034	0.046	0.064	0.0012	0.00425	0.0093
S.E.	0.014	0.006	0.009	0.0017	0.0016	0.0021

Figure 7.

Net Sodium and Potassium Loss from Crayfish during Heat Death.

The net Na^+ and K^+ loss from crayfish during heat death was determined from the net gain of these ions by the surrounding water. The mean loss of each ions was expressed as mg loss per g wet weight of animal was plotted against the time of high temperature exposure. Ten 10^o and six 25^oC adapted crayfish were used, the lethal temperatures were 32^o and 34^oC respectively. The data was obtained from table 2.

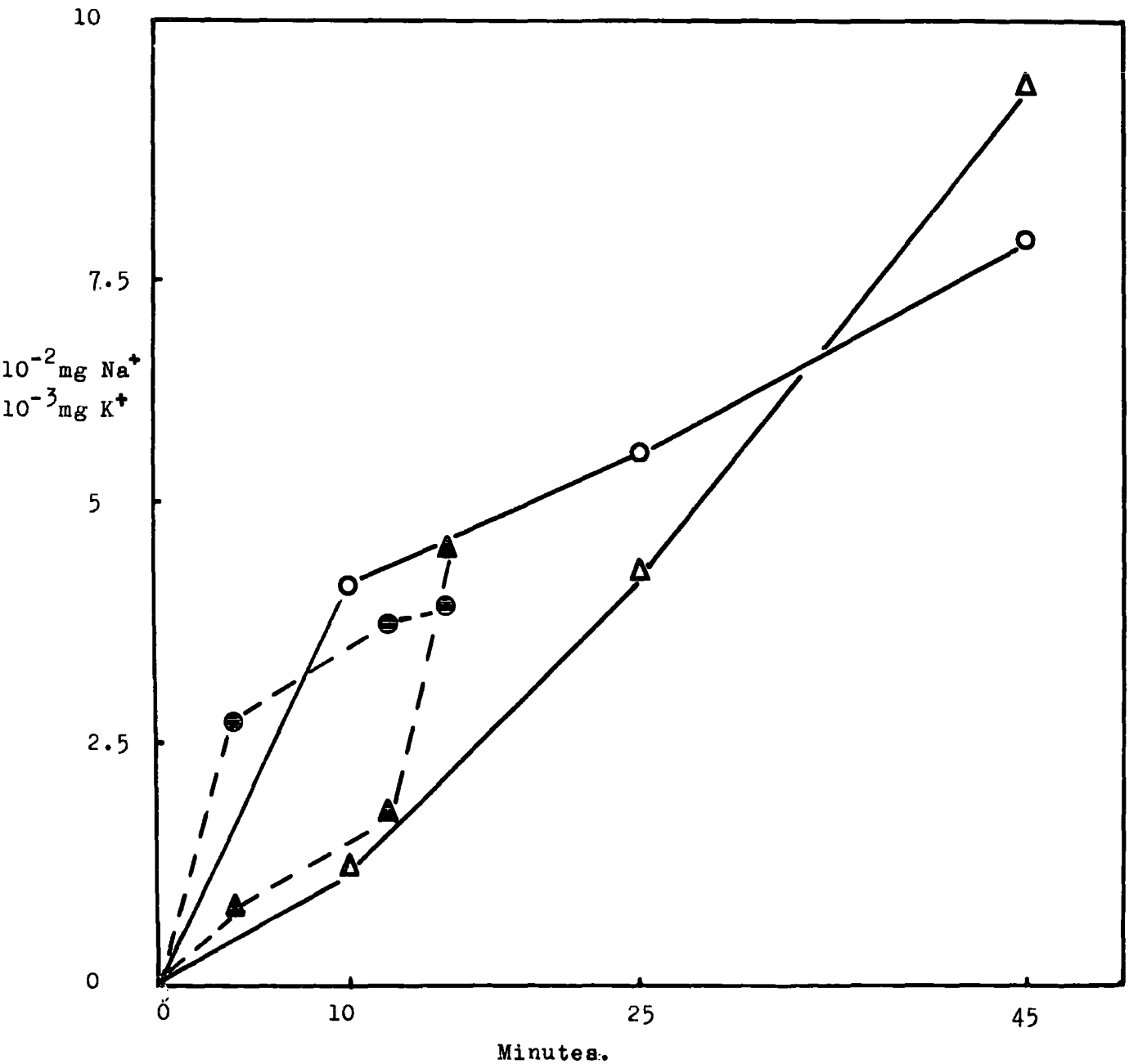
Mean net Na^+ loss from 10^oC adapted crayfish .....●——●

Mean net K^+ loss from 10^oC adapted crayfish .....▲——▲

Mean net Na^+ loss from 25^oC adapted crayfish .....○——○

Mean net K^+ loss from 25^oC adapted crayfish .....Δ——Δ

Figure 7.



animals during the first 12 minutes at 32°C and the first 25 minutes at 34°C amounting to about 40-45% of the total loss; 0.0018 ± 0.0009 and 0.00425 ± 0.0016 mg potassium lost per g wet weight for 10 and 25°C adapted animals respectively. A much more rapid loss of potassium occurred during the last 3 minutes at 32°C and 20 minutes at 34°C as can be seen from table 2. In both adaptation groups the sodium movement was more rapid initially; the potassium movement more rapid terminally (see figure 7), though the absolute amounts of potassium lost were much less than that of sodium.

2. Haemolymph Cation Concentrations during Heat Stress

As has been shown above, animals undergoing heat death lost monovalent cations to the environment. The following experiments were designed to assess the effect of heat on haemolymph cation levels. An initial control experiment was performed to establish whether the sequential haemolymph sampling technique used affected haemolymph cation concentrations. The results of this experiment are shown in table 3, they show that there was no significant change in haemolymph cation content when 100 µl samples of haemolymph were withdrawn at 5 minute intervals. The normal concentrations of haemolymph cations were similar to those reported by previous authors for Astacus, as may be seen from the table below:

Author	Concentration of haemolymph cations (mM)				Species
	Na^+	K^+	Ca^{++}	Mg^{++}	
Berger (1931)	-	4.1	10.7	-	A. fluviatilis
Scholles (1933)	-	5.2	10.4	2.6	"
Bogucki (1934)	152	3.1	12.0	2.5	"
Huf (1934)	185	7.8	7.7	1.5	"
Shaw (1959)	186	-	-	-	A. pallipes
Bryan (1960 a)	203	4.6	-	-	A. fluviatilis
Bowler (1963 b)	211	5.1	-	-	A. pallipes

TABLE 3.

The Effect of Sequential Haemolymph Sampling on Haemolymph Cation Concentrations.

Sequential haemolymph samples were taken and analysed as described in the text. Concentrations of Na^{+}, K^{+}, Ca^{++}, and Mg^{++} were expressed in mM; the temperature was maintained at the adaptation temperature of the crayfish.

TABLE 3 [a]. 10°C Adapted Crayfish.

Animal.		Time of sampling (minutes). 0	5	10	15
1		173	166	171	165
2	mM	169	165	181	177
3	Na^+	157	165	161	159
4		193	190	177	197
5		201	193	210	205
$\bar{x}$		179	176	180	183
S.E.		7.0	5.4	6.1	5.1
1		5.4	5.0	5.5	5.3
2	mM	5.1	5.1	5.3	5.0
3	K^+	5.8	5.8	5.6	5.6
4		4.9	5.0	4.8	4.8
5		5.0	5.3	5.1	5.1
$\bar{x}$		5.2	5.2	5.3	5.2
S.E.		0.36	0.36	0.53	0.29
1		9.7	9.5	9.5	9.5
2	mM	10.9	11.4	11.1	11.1
3	Ca^{++}	8.6	8.6	8.6	8.6
4		12.0	12.3	12.2	12.1
5		13.1	13.0	12.9	13.2
$\bar{x}$		10.9	11.0	10.9	10.9
S.E.		0.65	0.57	0.59	0.78
1		3.7	3.8	3.8	3.8
2	mM	3.2	2.9	3.1	3.3
3	Mg^{++}	3.5	3.5	3.5	3.4
4		4.0	3.8	4.0	4.1
5		3.1	3.2	3.2	3.1
$\bar{x}$		3.5	3.4	3.5	3.5
S.E.		0.17	0.33	0.26	0.32

TABLE 3 [b]. 25°C Adapted Crayfish.

Animal		Time of sampling (minutes).				
		0	5	10	30	45
X 1		181	179	172	172	174
2	mM	211	214	214	220	215
3	Na^{++}	185	179	175	176	178
4		190	185	187	185	194
5		195	199	191	194	187
$\bar{x}$		192.4	191.4	187.8	189.1	189.6
S.E.		4.5	6.8	7.5	8.5	7.2
1		5.9	5.4	5.5	5.8	5.6
2		5.5	5.1	5.1	5.3	5.5
3	mM K^{+}	5.5	5.6	5.4	5.5	5.3
4.		5.9	6.0	5.9	6.0	5.7
5		5.7	5.8	5.7	5.9	5.8
$\bar{x}$		5.7	5.6	5.5	5.7	5.6
S.E.		0.09	0.16	0.14	0.13	0.09
1		12.9	12.9	12.9	12.9	12.9
2	mM	8.8	8.8	8.8	8.8	8.8
3	Ca^{++}	11.0	11.0	11.0	10.9	10.8
4		11.0	11.0	11.0	11.0	11.0
5		12.2	12.2	12.2	12.3	12.2
$\bar{x}$		11.18	11.18	11.18	11.18	11.10
S.E.		0.70	0.70	0.70	0.71	0.70
1		3.2	3.3	3.1	3.2	2.9
2	mM	3.2	3.4	3.4	3.4	3.2
3	Mg^{++}	4.8	4.5	4.5	4.4	4.5
4		4.6	4.5	4.5	4.4	4.6
5		3.9	4.0	4.0	3.8	3.9
$\bar{x}$		3.94	3.94	3.90	3.84	3.82
S.E.		0.34	0.26	0.28	0.25	0.34

My results for normal animals may be grouped as follows:-

Cation	$25^{o}C$ adapted animals	$10^{o}C$ adapted animals
Na^{+}	196.8 ± 1.9 (n = 35)	181.6 ± 3.2 (n = 15)
K^{+}	5.15 ± 0 48 (n = 35)	5.20 ± 0.19 (n = 15)
Ca^{++}	9.80 ± 0.23 (n = 30)	10.89 ± 0.71 (n = 5)
Mg^{++}	2.35 ± 0.19 (n = 30)	3.50 ± 0.16 (n = 5)

where n = number of crayfish sampled

It can be seen from the data of table 4 that exposing $25^{o}C$ adapted crayfish to temperatures of 30, 31 and $32^{o}C$ resulted in very small changes in the haemolymph concentrations of Ca^{++} and Mg^{++}. These small fluctuations were considered to be the result of sample variations. At 34 and $35^{o}C$, however, small but consistent increases of these ions were observed (see table 4). The increases were small, in no case exceeding 0.3 mM magnesium or 0.7 mM calcium which represented a 12.7 and 7.1% increase respectively over the normal levels. Comparison of these changes with the control values, and the consistency of the direction of change indicate that they are probably significant.

The changes in haemolymph Na^{+} and K^{+} concentrations were more clear cut, the results are shown in table 4 and figure 8. These results indicated that exposing $25^{o}C$ adapted crayfish to water at $31^{o}C$ and above resulted in increasingly drastic alterations of haemolymph Na^{+} and K^{+}. The percentage changes in haemolymph Na^{+} concentrations are shown in figure 8. It is clear that the extent of the mean percentage change from zero time depends not only on exposure time but also on the temperature. At $30^{o}C$ there was an 8% increase in haemolymph Na^{+} after 30 minutes, rising to about 17% after 60 minutes (these animals all survived this exposure). An initial movement of Na^{+} into the haemolymph also occurred at 31 and $32^{o}C$, although subsequently a net loss was observed (table 4).

TABLE 4.

The Effect of High Temperatures on the Haemolymph Cation Concentrations of 25°C Adapted Crayfish.

Sequential haemolymph samples were taken from crayfish exposed to temperatures between 30° and 35°C. Samples were analysed as described in the text (Methods section, Chapter 2) and cation concentrations expressed in mM. The temperature at which the crayfish were exposed is denoted at the top of each section.

TABLE 4 [a]. T = 30°C.

Animal		Time of sampling (minutes).		
		0	30	60
1		214	242	278
2		190	215	230
3	mM Na^+	190	200	204
4		185	190	200
5		199	210	234
$\bar{x}$		196	211	229
S.E.		5.1	8.8	13.9
% change		0%	+7.7%	+16.8%
1		4.5	5.1	6.4
2		5.2	5.2	4.6
3	mM K^+	6.0	5.5	5.0
4		5.1	5.1	4.9
5		4.7	5.1	5.0
$\bar{x}$		5.1	5.2	5.2
S.E.		0.26	0.08	0.31
% change		0%	+2%	+2%
1		9.5	9.5	9.6
2		10.6	10.9	10.9
3	mM Ca^{++}	9.6	9.8	9.8
4		8.6	8.1	8.0
5		9.2	9.2	9.2
$\bar{x}$		9.5	9.5	9.5
S.E.		0.33	0.45	0.47
% change		0%	0%	0%
1		1.7	2.0	2.2
2		1.8	2.9	2.9
3		2.1	2.1	2.2
4	mM Mg^{++}	2.0	2.0	1.8
5		1.9	1.9	2.0
$\bar{x}$		1.9	2.2	2.2
S.E.		0.07	0.18	0.19
% change		0%	+1.6%	+1.6%

TABLE 4 [b]. T = 31°C.

Animal		Time of sampling (minutes).				
		0	5	10	15	30
1		181	188	196	212	205
2	mM	224	225	223	212	208
3	Na^+	203	199	207	200	194
4		199	195	212	199	194
5		186	192	205	182	173
$\bar{x}$		199	200	209	201	195
S.E.		7.5	6.6	4.4	5.5	6.1
% change		0%	+0.5%	+4.8%	+1%	-2%
1		4.4	5.0	5.1	5.5	6.0
2	mM	5.2	5.25	5.5	5.0	5.5
3	K^+	5.0	5.4	6.0	6.0	5.9
4		5.6	5.8	6.0	5.9	5.6
5		5.0	5.1	5.3	5.5	5.8
$\bar{x}$		5.04	5.31	5.60	5.60	5.76
S.E.		0.19	0.14	0.18	0.18	0.09
% change		0%	+4%	+12%	+12%	+14%
1		7.8	8.4	8.4	8.4	8.4
2	mM	9.9	10.0	9.4	9.9	9.6
3	Ca^{++}	8.7	8.9	8.6	8.7	8.7
4		10.2	10.2	10.2	10.0	10.0
5		11.3	11.4	11.4	11.4	11.3
$\bar{x}$		9.6	9.8	9.6	9.7	9.6
S.E.		0.61	0.53	0.55	0.53	0.51
% change		0%	+2.1%	0%	+1%	0%
1.		1.5	1.5	1.7	1.6	1.5
2	mM	1.3	1.2	1.2	1.2	1.2
3	Mg^{++}	2.1	2.0	2.2	2.2	2.2
4		1.9	1.8	1.8	1.8	1.8
5		1.6	1.5	1.7	1.7	1.7
$\bar{x}$		1.7	1.6	1.7	1.7	1.7
S.E.		0.15	0.14	0.17	0.16	0.17
% change		0%	-5.9%	0%	0%	0%

TABLE 4 [c]. T = 32°C.

Animal		Time of sampling (minutes).				
		0	5	15	30	45
1		184	194	196	168	128
2		190	204	208	184	181
3		220	218	225	179	182
4		186	190	197	168	174
5	mM	186	199	199	154	146
6	Na^+	201	214	221	206	167
7		198	216	214	180	175
8		194	213	216	195	182
9		196	213	213	174	167
10		212	212	226	201	190
$\bar{x}$		196.7	207.3	211.5	181.1	169.2
S.E.		3.7	3.1	3.6	5.2	6.0
% change		0%	+5.4%	+7.5%	-7.9%	-14.0%
1		5.1	5.2	6.2	8.6	7.8
2		5.2	5.6	6.9	6.8	7.0
3		4.6	4.6	5.3	8.7	14.2
4		4.2	4.2	5.4	7.9	9.9
5	mM	5.3	5.2	6.5	8.8	10.4
6	K^+	5.6	6.1	7.0	8.9	13.2
7		4.9	5.2	6.0	7.2	9.1
8		5.3	5.4	7.5	9.4	12.3
9		5.9	6.3	7.8	10.3	11.3
10		5.6	5.9	6.1	6.0	6.4
$\bar{x}$		5.17	5.37	6.47	8.26	10.16
S.E.		0.16	0.20	0.26	0.41	0.83
% change		0%	+3.9%	+25.1%	+59.8%	+96.5%
1		8.7	8.6	8.6	8.8	8.8
2		9.4	9.4	9.4	9.4	9.4
3	mM	11.1	10.8	10.8	10.8	11.1
4	Ca^{++}	9.2	9.4	9.4	9.4	9.4
5		9.0	9.2	9.4	9.2	9.2
$\bar{x}$		9.48	9.48	9.52	9.52	9.58
S.E.		0.65	0.34	0.27	0.34	0.14
% change		0%	0%	+0.4%	+0.4%	+1.1%
1		3.5	3.7	3.7	3.7	3.7
2		1.7	1.8	1.7	1.7	1.7
3	mM	1.5	1.8	1.7	1.7	1.8
4	Mg^{++}	2.1	2.1	2.1	2.1	2.1
5		3.3	3.6	3.5	3.6	3.4
$\bar{x}$		2.42	2.60	2.54	2.56	2.54
S.E.		0.41	0.43	0.44	0.54	0.42
% change		0%	+7.4%	+5.0%	+5.8%	+5.0%

TABLE 4 [d]. T = 34°C

Animals		Time of sampling (minutes).				
		0	5	10	15	20
1		209	207	201	221	159
2	mM	203	207	207	194	190
3	Na^+	196	201	194	194	167
4		187	190	181	170	172
5		204	201	196	175	161
$\bar{x}$		200	201	196	191	170
S.E.		3.8	3.1	4.3	9.0	5.5
% change		0%	+0.5%	-2%	-4.5%	-15%
1		5.4	-	5.5	6.8	8.9
2	mM	4.7	5.4	6.1	7.1	9.2
3	K^+	5.0	5.3	5.7	7.4	8.9
4		4.6	5.0	5.5	7.0	8.7
5		5.3	5.5	6.0	7.2	8.7
$\bar{x}$		5.0	5.3	5.8	7.1	8.8
S.E.		0.16	0.13	0.12	0.10	0.15
% change		0%	+6%	+16%	+42%	+76%
1		9.8	9.8	9.8	10.7	10.7
2	mM	8.0	8.0	8.6	8.6	9.3
3	Ca^{++}	10.3	10.3	10.7	10.7	10.7
4		10.6	10.4	10.6	10.6	11.0
5		9.4	9.6	9.6	9.8	9.6
$\bar{x}$		9.6	9.6	9.9	10.1	10.3
S.E.		0.45	0.43	0.38	0.41	0.34
% change		½%	0%	+3%	+5.2%	+7.3%
1		1.8	-	1.8	1.8	1.8
2	mM	1.7	1.6	1.6	1.7	1.8
3	Mg^{++}	2.1	2.1	2.2	2.2	2.2
4		1.5	1.6	1.6	1.6	1.6
5		1.0	1.1	1.1	1.1	1.1
$\bar{x}$		1.62	1.60	1.67	1.68	1.70
S.E.		0.18	0.18	0.19	0.18	0.18
% change		0%	-6.9%	+2.2%	+3.2%	+5.5%

TABLE 4 [e] T = 35°C

Animal		Time of Sampling (minutes)				
		0	5	10	15	20
1		199	184	177	170	156
2		212	185	189	175	168
3	mM Na^+	189	173	171	165	154
4		186	161	165	148	134
5		203	182	179	171	174
$\bar{x}$		198	177	176	166	157
S.E.		4.7	4.5	4.0	4.7	6.9
% change		0%	-10.6%	-11.1%	-16.2%	-22.0%
1		4.6	5.8	7.3	8.3	9.1
2		4.4	4.8	7.2	8.4	9.6
3	mM K^+	5.4	5.5	5.9	7.8	8.9
4		5.3	5.6	8.0	9.7	10.4
5		4.7	5.0	6.9	8.5	9.2
$\bar{x}$		4.9	5.3	7.1	8.5	9.4
S.E.		0.20	0.19	0.34	0.31	0.27
% change		0%	+8%	+45%	+73.5%	+92%
1		8.5	8.8	8.0	7.9	8.6
2		8.0	7.8	8.6	8.6	8.7
3	mM Ca^{++}	10.3	10.4	10.4	10.7	10.7
4		9.1	9.0	9.5	9.5	9.7
5		11.2	11.2	11.7	11.3	11.6
$\bar{x}$		9.4	9.4	9.6	9.6	9.9
S.E.		0.59	0.58	0.66	0.63	0.58
% change		0%	0%	+2%	+2%	+5%
1		3.5	3.5	3.5	3.5	3.5
2		3.8	3.8	3.8	3.2	3.6
3	mM Mg^{++}	2.1	2.4	2.4	2.2	2.3
4		1.4	1.2	1.2	1.4	1.4
5		1.9	2.0	2.0	1.9	2.0
$\bar{x}$		2.5	2.6	2.6	2.4	2.6
S.E.		0.47	0.48	0.48	0.40	0.43
% change		0%	+4%	+4%	-4%	+4%

Figure 8.

Haemolymph Sodium and Potassium Concentrations during Exposure to High Temperature.

The mean change in haemolymph Na^+ and K^+ concentrations of 25^0C adapted crayfish during exposure to a temperature between 30 and 35^oC was expressed as the percentage difference from the control level and plotted against the time of exposure. Five crayfish were used to obtain the data at each experimental temperature except 32^oC when 10 crayfish were used. The results of table 4 were used to obtain the mean Na^+ and K^+ concentrations.
Figure 8 (a) illustrates the percentage change of haemolymph Na^+, and figure 8 (b) illustrates the percentage change of haemolymph K^+.

Figure 8(a)

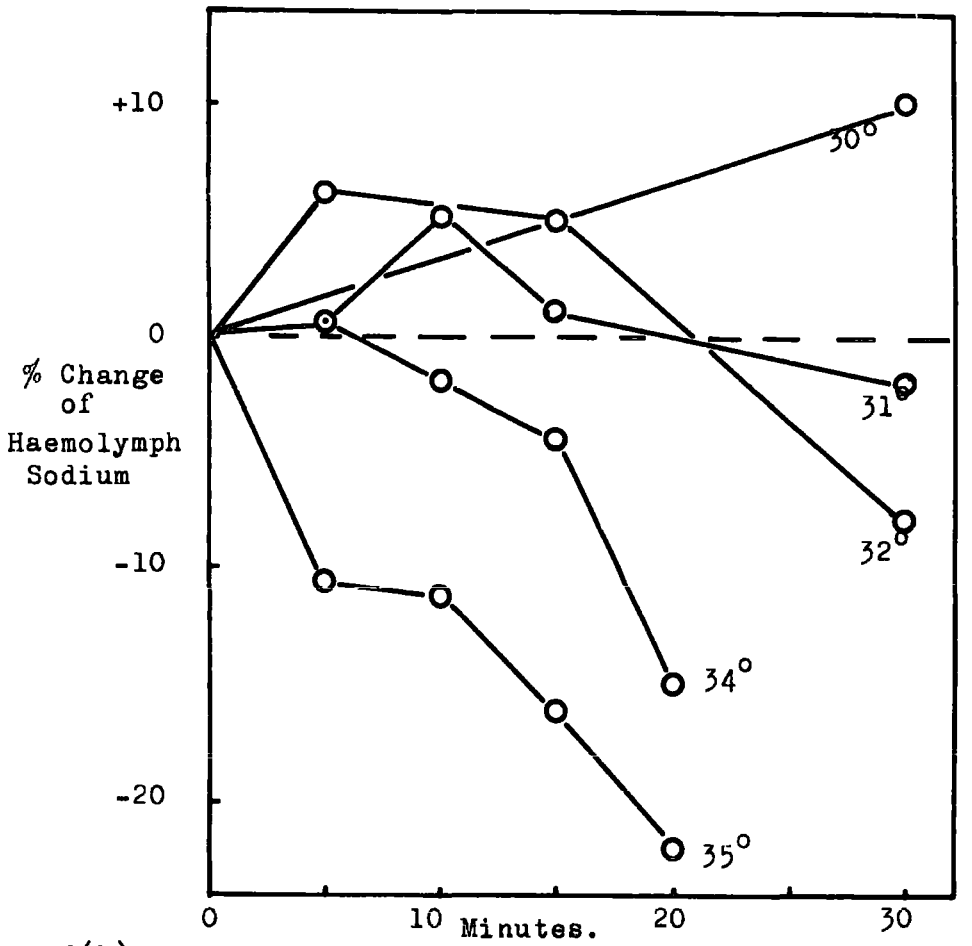
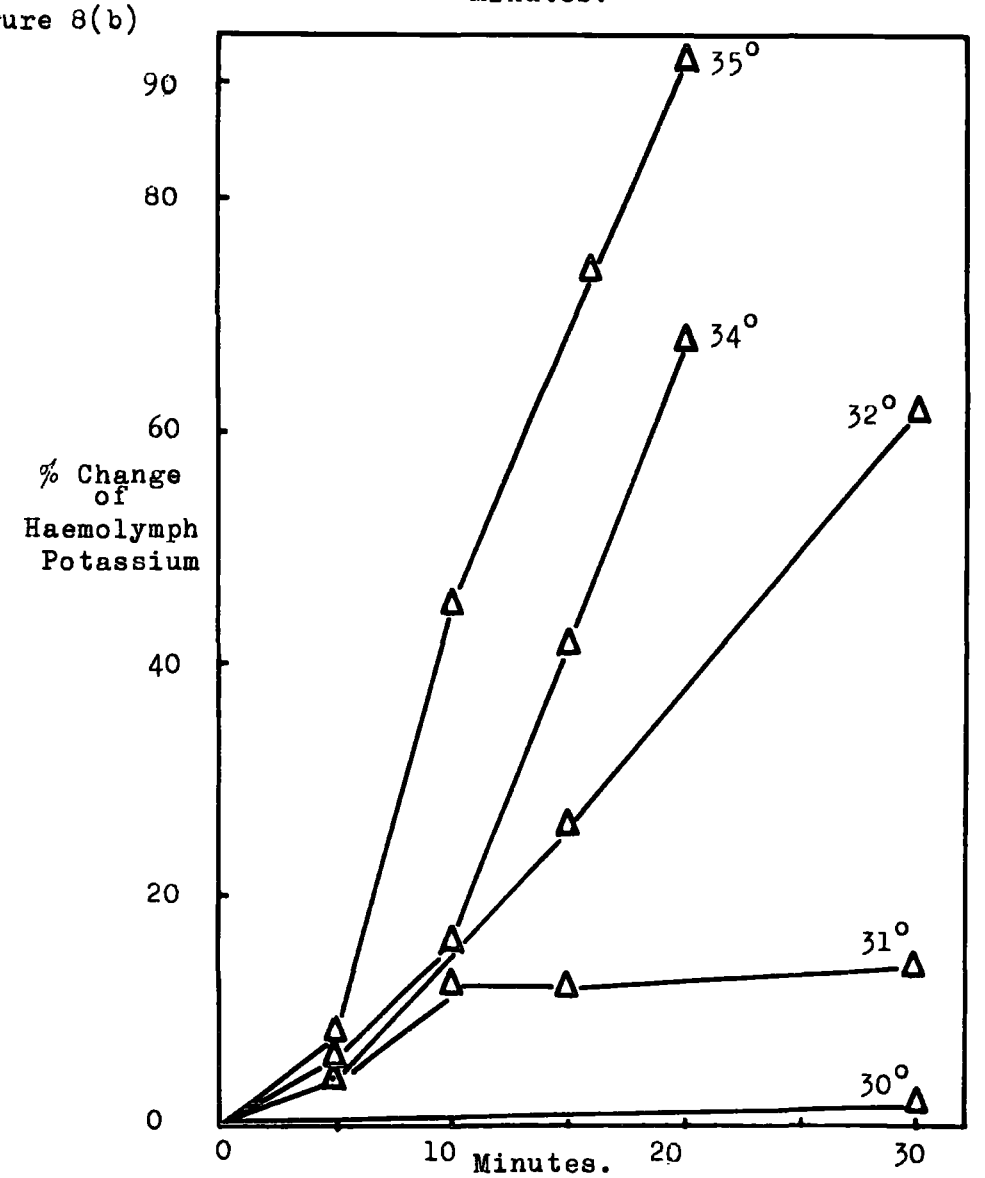


Figure 8(b)



At 34 and 35°C, however, there was no significant initial increase in haemolymph Na^+, the concentration decreased more rapidly in the 35°C exposed animals. (No animals exposed to 34 or 35°C survived the experiment.)

The changes in haemolymph Na^+ therefore suggested that haemolymph sodium was increased by short exposure to sublethal and the lower of the lethal temperatures, but that this situation was reversed by longer exposure times or higher temperatures (figure 8). Thus a decrease was noted only after 30 minutes exposure to 31°C, but had already occurred after 5 minutes exposure to 35°C (see table 4).

The changes in haemolymph K^+ are shown on table 4. These results show that the extent of haemolymph K^+ concentration changes also depended upon temperature and time of exposure. At 30°C there was a small increase of 2% after 30 minutes which did not change after a further 30 minutes exposure. There was an increase in haemolymph K^+ after exposure to 31°C for 30 minutes, the change being more rapid during the initial stages of exposure so that a 12% increase occurred after 10 minutes (table 4). All the experimental animals survived 60 minutes exposure to 30°C and 30 minutes exposure to 31°C. When the animals were exposed to 32°C a considerably greater change occurred, haemolymph K^+ increased by about 25% after 15 minutes, 60% after 30 minutes and 100% after 45 minutes (table 4). Of the 10 experimental animals used, only two survived this experiment (animals number 2 and 10, table 4). Haemolymph K^+ rose to about 70 and 90% above the normal level following 20 minutes exposure to 34 and 35°C respectively (see table 4), no animals survived these experiments.

In order to investigate the effects of temperature adaptation a group of ten 10°C adapted crayfish were exposed to 32°C and haemolymph

TABLE 5.

The Effect of $32^{o}C$ on the Haemolymph Na^{+} and K^{+} concentrations of $10^{o}C$ adapted crayfish.

Sequential haemolymph samples were obtained from $10^{o}C$ adapted crayfish exposed to $32^{o}C$ and analysed as described in the methods (Chapter 2), Concentrations were expressed in mM.

TABLE 5.

Animal		Time of Sampling (minutes).			
		0	5	10	15
1		177	193	186	184
2		171	180	176	165
3		189	200	189	195
4		203	190	199	187
5	mM	183	196	200	182
6	Na^+	182	187	185	167
7		185	186	221	182
8		181	184	190	186
9		188	193	201	195
10		172	178	179	178
$\bar{x}$		183.1	188.7	192.6	182.1
S.E.		2.9	2.2	4.2	3.2
% change		0%	+3.1%	+5.2%	-0.5%
1		5.1	6.7	8.5	10.6
2		5.3	7.0	8.2	9.7
3		4.1	5.5	5.9	6.5
4		3.5	4.3	6.0	9.3
5	mM	6.4	6.6	8.8	11.0
6	K^+	5.9	6.6	7.5	8.6
7		5.1	6.4	7.1	8.1
8		4.9	5.7	6.3	7.5
9		5.4	5.6	6.7	8.3
10		6.1	6.6	8.8	10.6
$\bar{x}$		5.18	6.10	7.38	9.02
S.E.		0.28	0.26	0.36	0.47
% change		0%	+17.8%	+42.5%	+74.1%

samples taken and analysed as described above; the results are shown in table 5. The animals were exposed to 32°C for 15 minutes, approximately the LD_{50} time for these animals of 16 minutes (see table 1). The results obtained for the 10°C adapted crayfish were compared with those obtained for 25°C adapted crayfish exposed to the same temperature (32°C) in figures 9 and 10, since exposure to this temperature was just lethal to both groups of animals. The mean changes in haemolymph Na^+ and K^+ were similar in both adaptation groups (see figures 9 and 10). An initial increase in the mean haemolymph Na^+ occurred in 10 and 25°C adapted animals, the increase was maximal after about 10 minutes (+ 5.2%) and 15 minutes (+ 7.5%) respectively (see tables 4 and 5). After attaining these maximal levels the haemolymph Na^+ decreased more rapidly, returning to its control value within the next 5 minutes in the 10°C adapted animals (table 5), and dropping below its control value within the next 15 minutes in the 25°C adapted animals (table 4), see also figure 9).

The haemolymph K^+ of both adaptation groups increased dramatically at 32°C (see figure 10), rising to a mean of 9.02 $\pm$ 0.47 mM in 10°C adapted animals after 15 minutes exposure (see table 5), and 10.16 $\pm$ 0.83 mM in the 25°C adapted animals after 45 minutes exposure (see table 4). In both adaptation groups the initial rise of haemolymph K^+ during the first 5 minutes was slightly slower than that which occurred later. In view of the essentially steady increase in haemolymph K^+ after this brief initial period, this slow initial increase might be due to the initial 'warming up' period of the animal when immersed in the warm water. The haemolymph Na^+ and K^+ changes which occurred in both 10 and 25°C adapted animals were thus of similar natures and magnitudes, differing mainly in their time courses. These results indicate that the events leading to haemolymph monovalent cation changes are similar in 10 and 25°C adapted

Figure 9.

The Effect of Adaptation Temperature on the Haemolymph Sodium Movements during Exposure to $32^{o}C$.

The mean haemolymph Na^{+} concentration of ten 10^{o} and ten $25^{o}C$ adapted crayfish was plotted against the time of exposure to $32^{o}C$. The concentrations were expressed in m moles per 1 and the vertical bars indicate $\pm$ 1 standard error of the mean. The data was obtained from tables 4 (c) and 5.

Mean haemolymph Na^{+} concentration of $10^{o}C$ adapted crayfish....●---●

Mean haemolymph Na^{+} concentration of $25^{o}C$ adapted crayfish....○——○

Figure 9.

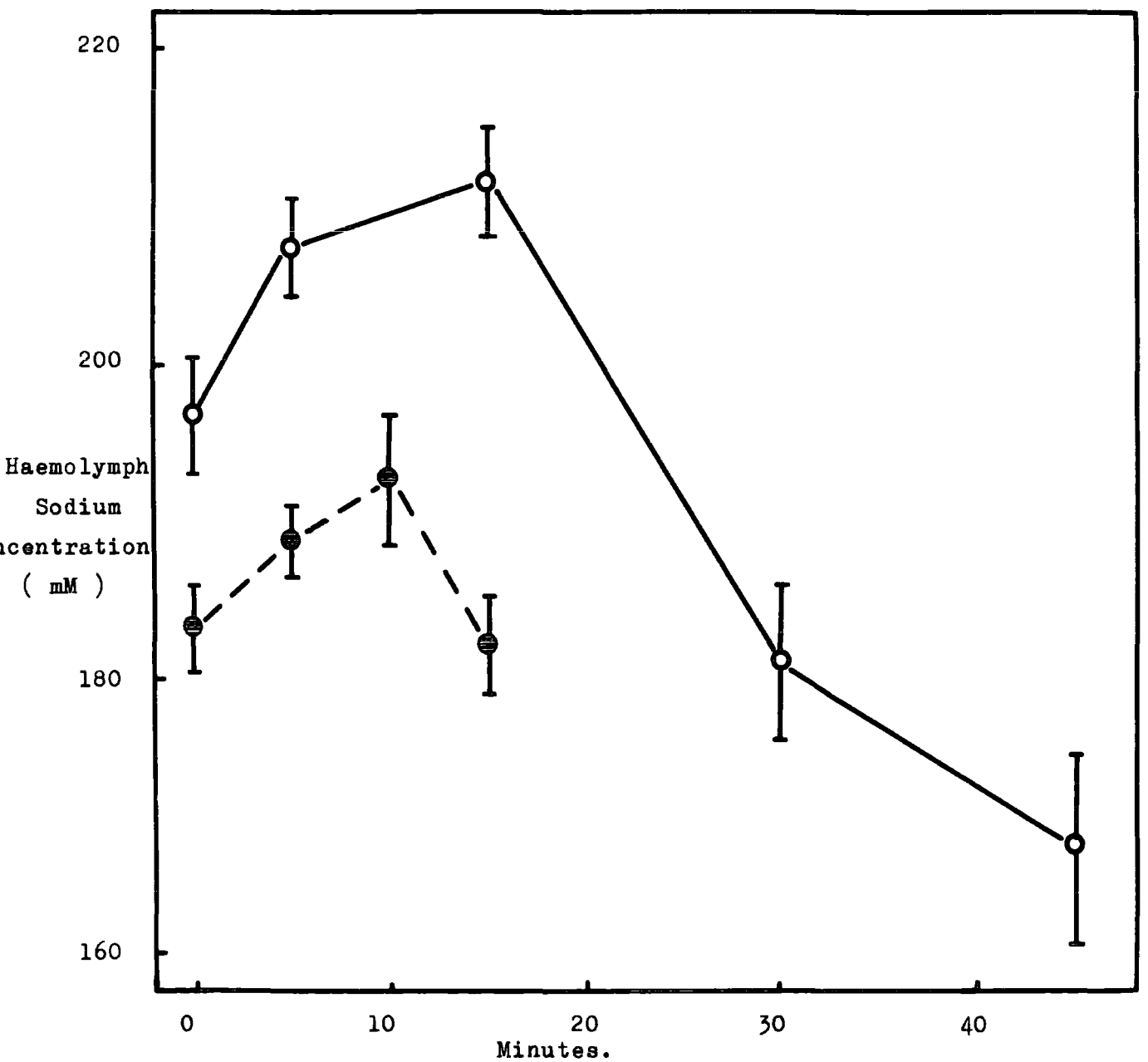

220
200
180
160
Haemolymph
Sodium
ncentration
(mM)
0
10
20
30
40
Minutes.

Figure 10.

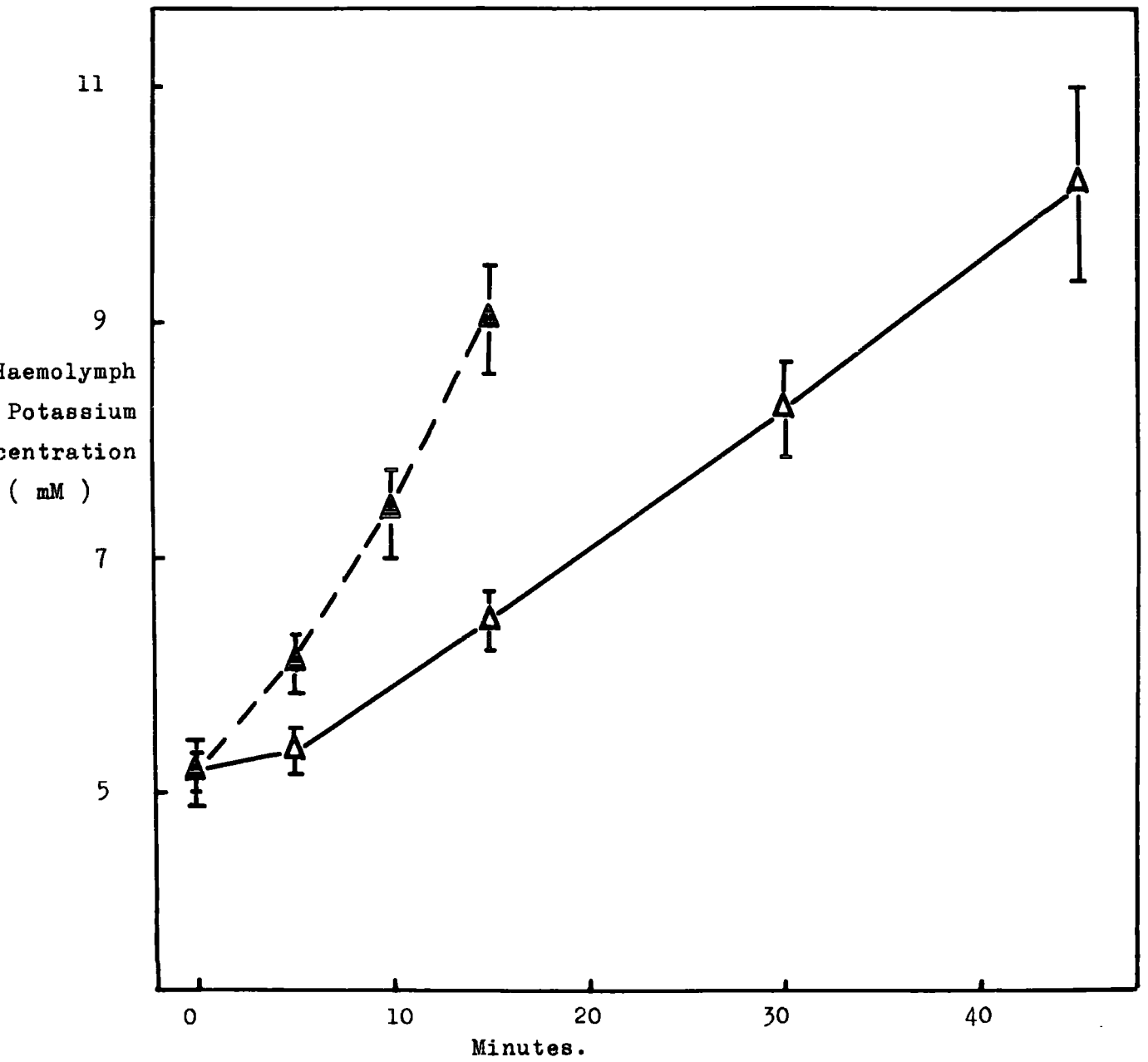
The Effect of Adaptation Temperature on the Haemolymph Potassium Movements during Exposure to $32^{o}C$.

The mean haemolymph K^{+} concentration of ten 10^{o} and ten $25^{o}C$ adapted crayfish was plotted against the time of exposure to $32^{o}C$. The concentration was expressed in m moles per 1 and the vertical bars represent $\pm$ 1 standard error of the mean. The data was obtained from tables 4 (c) and 5.

Mean haemolymph K^{+} concentration of $10^{o}C$ adapted crayfish....▲- - -▲

Mean haemolymph K^{+} concentration of $25^{o}C$ adapted crayfish....Δ———Δ

Figure 10.


11
9
7
5
Haemolymph
Potassium
centration
(mM)
0
10
20
30
40
Minutes.

crayfish, but that the time course of these events is much faster in $10^{o}C$ adapted animals.

3. Muscle Cation Concentrations during Heat Stress

In order to calculate the Na^{+} and K^{+} concentrations of the muscle fibres, the extracellular space of the abdominal muscles was measured as described in the methods. The results are shown in table 6, the mean extracellular space was found to be 18.74 $\pm$ 0.62 ml per 100 g wet weight of muscle. This agrees with the figure of 17.6 $\pm$ 1.6 ml per 100 g wet weight of muscle reported for Orconectes virilis by Van der Kloot (1966). The extracellular space was assumed to be essentially ion-free after rinsing the muscle in deionised water and the following results were calculated allowing for a 18.74% dilution of the muscle fibre ions by extracellular, ion-free water.

The Na^{+} and K^{+} ion concentrations of 10 normal crayfish of each adaptation group were measured, the results are shown in table 7. The mean muscle fibre content of Na^{+} was 39.7 $\pm$ 1.0 and 36.2 $\pm$ 0.8 m moles / Kg cell water for 10 and $25^{o}C$ adapted crayfish respectively. The mean K^{+} content was 122.0 $\pm$ 2.6 and 115.7 $\pm$ 1.7 m moles /Kg cell water for 10 and $25^{o}C$ adapted crayfish. The differences in Na^{+} and K^{+} content between 10 and $25^{o}C$ adapted animals was significant at the 5% level. (Students t test). The Na^{+} and K^{+} content of somatic muscle in the crayfish Orconectes virilis was 79.4 and 117.4 m moles / Kg cell water according to Van der Kloot (1966). The K^{+} figures agree well, but the Na^{+} concentrations differ considerably. The reason for this difference is not clear, though it may be a simple species difference. The consistency of the results and the fact that errors in sampling would tend to increase the Na^{+} content rather than decrease it suggest that the values obtained (see table 7) were valid.

TABLE 6.

Extracellular Tritiated Inulin Space of Crayfish Muscle.

The extracellular space of abdominal flexor muscles taken from 13 crayfish was determined by the method of Van der Kloot (1966) as described in the Methods (Chapter 2). Animals 1 - 7 were 10^{o}C adapted and animals 8 - 13 were 25^{o}C adapted. A Chi squared test showed no significant difference between the extracellular space of the two adaptation groups. ($P < 0.1$).

TABLE 6.

Animal.	ml extracellular space/100g wet muscle.
1	19.3
2	18.2
3	14.6
4	20.4
5	18.9
6	20.8
7	17.9
8	23.2
9	20.0
10	20.4
11	18.4
12	18.8
13	15.0
$\bar{x}$	18.74
S.E.	0.62

Crayfish of known thermal history were exposed to high lethal temperatures between 32 and 35°C and muscle Na^+ and K^+ contents measured in samples taken at the time of cessation of scathognathite beat. The results are shown in table 8. The net muscle fibre Na^+ content increased when the animal was exposed to lethal temperatures. The extent of this increase appeared to depend more upon the time of exposure than the temperature (see table 8). Thus there was a general trend to higher intracellular Na^+ concentrations with longer exposure times to a given lethal temperature (see table 8). It is interesting to compare the mean rates of Na^+ entry into the muscle fibres for the groups of animals treated at different lethal temperatures. The mean rate of net Na^+ gain was an increase of 1.2% over the normal Na^+ level (see table 7) per minute for the animals exposed to 32 and 33°C and 1.0% per minute for the animals exposed to 34 and 35°C. The difference in rates probably reflects the longer exposure times of the animals exposed to 32 and 33°C. The rate of net Na^+ inflow into the muscle fibres of the 10°C adapted crayfish exposed to 34°C was much faster, being about 1.5% per minute, i.e. about 50% faster than the net rate of Na^+ entry into muscle fibres of crayfish adapted to 25°C which had been exposed to the same temperature.

Exposing 25°C adapted crayfish to lethal temperatures between 32 and 35°C resulted in a net loss of K^+ from the muscle fibres (see table 8). The net mean K^+ loss was greater in muscles taken from crayfish exposed to 32 and 33°C for lethal periods than from crayfish exposed to 34 and 35°C for lethal periods. The rate of net K^+ loss was faster in the animals exposed to the higher lethal temperatures, however, being about 0.6% of the initial content per minute compared with about 0.25% per minute loss for the animals exposed to 32 and 33°C. Exposing 10°C adapted animals to 34°C resulted in an apparent gain in muscle K^+, the mean content being 129.7 ± 4.9 as opposed to a control value of 122.0 ± 2.6 m moles / Kg

TABLE 7.

Intracellular Na^+ and K^+ Concentrations of Crayfish Muscle Fibres.

Abdominal flexor muscles were dissected from ten 10^{o} and ten $25^{o}C$ adapted crayfish and analysed as described in the Methods (Chapter 2). A minimum of three independent analyses of each muscle were meaned to give the results. Concentrations of ions were expressed as m moles per Kg cell water.
A 'Student's t' test showed that the mean Na^+ and K^+ concentrations were not significantly different at the 5% level when the two adaptation groups were compared.

TABLE 7.

Animal	10°C Adapted		25°C Adapted.	
	mM Na^+	mM K^+	mM Na^+	mM K^+
1	35.4	127.9	40.7	122.9
2	37.4	131.7	38.1	103.6
3	41.0	123.4	35.8	117.6
4	41.9	122.5	35.1	124.3
5	39.6	103.3	37.0	112.9
6	47.9	125.6	38.5	114.2
7	39.6	117.1	31.9	117.4
8	37.9	122.2	35.2	117.2
9	38.0	126.2	35.0	114.0
10	38.0	119.9	34.4	112.6
$\bar{x}$	39.7	122.0	36.2	115.7
S.E.	1.0	2.6	0.8	1.7

TABLE 8.

Muscle Fibre Na^+ and K^+ Concentrations at the Time of Heat Death.

Groups of five 25°C adapted crayfish were exposed to temperatures between 32 and 35°C, and a further group of five 10°C adapted crayfish were exposed to 34°C. The abdominal flexor muscles were removed and analysed for Na^+ and K^+ immediately after scathognathite beat ceased as described in the Methods (Chapter 2). The values represent the mean of a minimum of three independent determinations on each muscle and were expressed in m moles per Kg cell water.

TABLE 8.

		Animal (25°C Adapted).						
T°C		1	2	3	4	5	$\bar{x}$	S.E.
	SBC time	46	54	107	140	230	115.4	33.5
32	Na^+	40.8	57.8	106.1	117.2	108.7	86.1	15.4
	K^+	105.2	104.3	63.7	75.6	64.6	82.7	9.25
	SBC time	89	106	116	122	132	113	7.3
33	Na^+	80.3	91.2	87.1	77.1	92.4	85.6	3.0
	K^+	94.7	95.4	68.4	93.6	61.7	82.8	7.3
	SBC time	26	30	35	43	59	38.6	5.8
34	Na^+	78.9	56.5	54.9	78.3	72.0	68.1	5.2
	K^+	84.8	92.0	96.7	83.6	88.4	89.1	2.4
	SBC time	10	13	16	22	27	17.6	3.1
35	Na^+	57.8	59.5	43.4	58.3	63.3	56.5	3.4
	K^+	96.2	123.2	111.2	103.2	86.0	104.0	6.3

		Animal (10°C Adapted).						
T°C		1	2	3	4	5	$\bar{x}$	S.E.
	SBC time	3	5	6	7	9	6	1.0
34	Na^+	41.9	39.8	46.1	40.9	47.5	43.2	1.5
	K^+	145.8	131.4	127.4	115.3	128.6	129.7	4.9

cell water (see tables 7 and 8), a 6.3% increase.

In order to investigate the Na^+ and K^+ movements more fully, groups of 10 and 25°C adapted crayfish were exposed to 32°C for predetermined periods and muscle samples taken for analysis. The 10°C adapted animal muscles were taken after 5, 10, 16 or 20 minutes' exposure and the 25°C adapted after 50, 100, 150 or 200 minutes. The results are shown in table 9. There was a net gain of muscle Na^+ in both the 10 and 25°C adapted crayfish as is shown on figure 11 where the mean Na^+ contents of both adaptation groups are plotted against the time of exposure to 32°C. The interesting point concerning these results is that the two curves representing the rate of net sodium gain versus time of figure 11 are dissimilar. The curve representing the 10°C adapted animals is essentially linear, whilst that representing the 25°C adapted animals is sigmoidal. Since the exposure times are essentially equivalent in terms of their effect upon the two adaptation groups (see table 1), this suggested that the gain of Na^+ by the muscle fibres may be a function of time of exposure to lethal temperatures rather than a direct cause of heat death. It is also interesting to note that whilst the overall rate of net Na^+ inflow was faster in the 25°C adapted crayfish, a 1.10% gain over control values per minute compared to 0.83% gain per minute overall for the 10°C adapted animals, the initial net Na^+ gain was slower in the 25°C adapted animals, an increase of 0.65% per minute. The movement of Na^+ down its electrochemical gradient into the muscle fibres is probably a result of an increase in the passive Na^+ permeability of the muscle cell plasma membranes, the results presented in table 9 therefore suggest that the muscle plasma membranes of 25°C adapted crayfish can maintain their normal, low Na^+ permeability better at lethal temperatures than can 10°C adapted crayfish.

TABLE 9.

The Effect of $32^{o}C$ on Na^{+} and K^{+} Concentrations in Crayfish Muscle Fibres.

The intracellular concentrations of Na^{+} and K^{+} in crayfish abdominal flexor muscles taken from 10^{o} and $25^{o}C$ adapted crayfish after timed exposure to $32^{o}C$ were measured as described in the Methods (Chapter 2). Values given represent a minimum of three independent determinations on each muscle. Concentrations were expressed in m moles per Kg cell water.

TABLE 9.

(a) 10°C adapted crayfish exposed to 32°C.

Animal		Exposure time (minutes).			
		5	10	16	20
1		43.0	41.0	46.6	46.3
2		40.2	42.7	44.5	46.8
3	mM	41.5	44.9	46.2	45.8
4				43.5	
5	Na^+			46.9	
$\bar{x}$		41.6	42.9	45.5	46.3
S.E.		0.81	1.13	0.66	0.29
1		123.2	113.6	108.7	110.6
2		121.6	118.1	103.7	113.0
3	mM	122.4	114.4	113.8	110.2
4	K^+			133.9	
5				115.3	
$\bar{x}$		122.4	115.6	115.3	111.2
S.E.		0.46	0.58	5.13	0.87

(b) 25°C adapted crayfish exposed to 32°C

Animal		Exposure time (minutes).			
		50	100	150	200
1		40.8	70.2	108.7	105.6
2	mM	57.8	79.1	111.6	122.1
3	Na^+	45.3	80.2	102.3	120.2
$\bar{x}$		48.0	76.2	107.5	115.9
S.E.		5.09	3.04	2.75	5.21
1		105.2	95.4	75.6	65.3
2	mM	104.3	88.6	78.3	77.2
3	K^+	104.8	93.7	80.7	69.1
$\bar{x}$		104.8	92.6	78.2	70.5
S.E.		0.26	2.04	1.47	3.51

Figure 11.

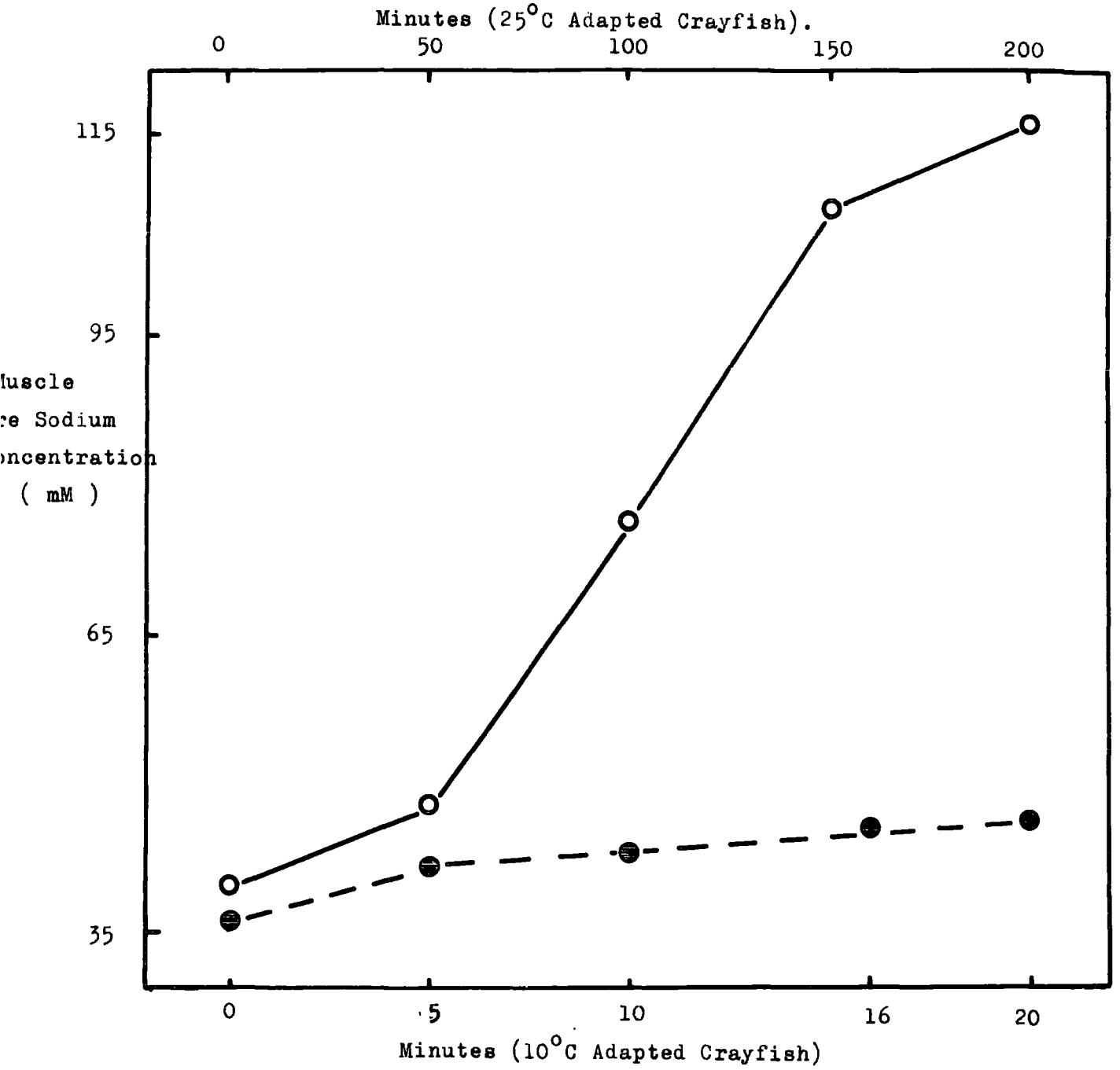
The Effect of Adaptation Temprature on the Muscle Sodium Movements during Exposure to $32^{o}C$.

The mean Na^{+} concentration of the muscle fibres was calculated from the data of table 9 and plotted against the time of exposure of the intact crayfish to $32^{o}C$. Each point represents the mean muscle fibre Na^{+} content expressed in m moles per Kg cell water from a minimum of 3 animals. The vertical bars represent $\pm$ 1 standard error of the mean. The lower abscissa refers to the 10^{o} and the upper abscissa to the $25^{o}C$ adapted crayfish.

$10^{o}C$ adapted crayfish muscle Na^{+} concentration .....●---●

$25^{o}C$ adapted crayfish muscle Na^{+} concentration .....O——O

Figure 11.


Minutes (25°C Adapted Crayfish).
0
50
100
150
200
115
95
65
35
Muscle
re Sodium
oncentration
(mM)
0
5
10
16
20
Minutes (10°C Adapted Crayfish)

The muscle K^+ contents measured from the same samples as those used for muscle sodium measurements are shown in table 9. There was a net loss of muscle potassium in both 10 and 25°C adapted crayfish during exposure to 32°C. The loss of K^+ from the muscle fibres of 25°C adapted animals was almost linear during the first 150 minutes of exposure (see figure 12); the rate of loss being 0.25 m moles intracellular K^+ per Kg cell water per minute. The amount of K^+ lost during the final 50 minutes of exposure was less than the loss during earlier 50 minute exposure periods, 0.154 m moles per Kg cell water per minute. This may have been due to the reduced concentration gradient across the muscle fibre plasma membrane brought about by the reduced intracellular and increased extracellular (see table 4) K^+ concentrations following exposure to 32°C. The muscle fibres of the 10°C adapted crayfish appeared to gain intracellular K^+ during the first five minutes of exposure, K^+ concentration rising from the control value of 122.0 $\pm$ 1.0 (see table 7) to 122.4 $\pm$ 0.46 (see table 9). m moles per Kg cell water. This difference is not significant, however, the results therefore indicating no real K^+ loss during the first five minutes exposure to 32°C. There was an overall rate of loss of intracellular K^+ of almost 0.75 m moles per Kg cell water during the last 15 minutes of exposure, a rate three times as fast as the potassium loss from 25°C adapted crayfish exposed to the same temperature. It is probable that the absence of any loss of intracellular K^+ during the initial exposure period may be explained similarly to the small increase observed in 10°C adapted crayfish exposed to 34°C for short intervals (see table 8), i.e. that the cation pump was stimulated during the 'warming-up' period.

4. Muscle, Haemolymph and Environment Cation Concentrations during Heat Stress.

The net changes of the concentrations of sodium and potassium in the environment, the haemolymph and the muscle fibres of crayfish

Figure 12.

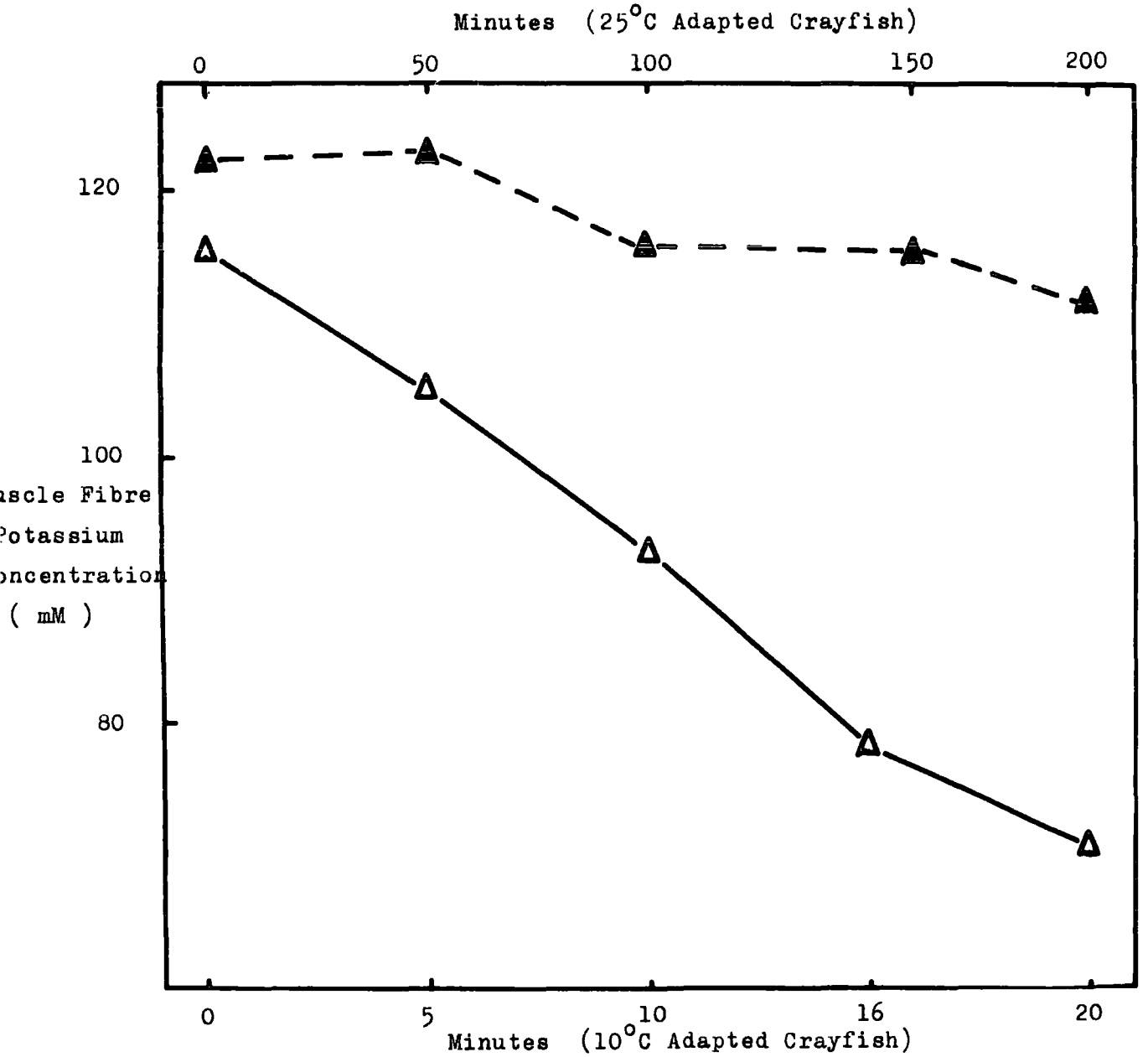
The Effect of Adaptation Temperature on the Muscle Potassium Movements during Exposure to $32^{o}C$.

The mean K^{+} concentration of the muscle fibres was calculated from the data of table 9 and plotted against the time of exposure of the intact crayfish to $32^{o}C$. Each point represents the mean muscle fibre K^{+} concentration expressed in m moles per Kg cell water from a minimum of 3 animals. The vertical bars represent $\pm$ 1 standard error of the mean. The lower abscissa refers to the 10^{o} and the upper abscissa refers to the $25^{o}C$ adapted crayfish.

$10^{o}C$ adpated crayfish muscle K^{+} concentration .....▲- - -▲

$25^{o}C$ adapted crayfish muscle K^{+} concentration .....Δ———Δ

Figure 12.


Minutes (25°C Adapted Crayfish)
0
50
100
150
200
120
100
80
Muscle Fibre
Potassium
Concentration
(mM)
0
5
10
16
20
Minutes (10°C Adapted Crayfish)

of known thermal history have been described above. The following experiments represent an attempt to correlate these movements and to clarify the relationship between them.

Eight crayfish were blotted, weighed and dissected so that the major constituents of the body could be weighed, the results are shown in table 10. The mean weights of the major tissues were expressed as a percentage of the mean whole animal weight so that the approximate percentage weight composition of the crayfish body was obtained. Haemolymph volume was measured as described in the methods and the results are shown in table 11. The results show that the haemolymph represents 24.7 $\pm$ 0.26 and 25.7 $\pm$ 0.23 percent of the body weight of 10 and 25^{o}C adapted crayfish respectively. These figures are in quantitative agreement with the figure of around 25% body weight given by Prosser and Weinstein (1950) and Bryan (1960 a) for Cambarus virilis and Astacus fluviatilis respectively. The haemolymph was therefore taken to represent 25% of the body weight. The other main constituents, muscle, cuticle, ligated intestinal tract (with contents) and hepatopancreas represented 50, 20, 2.5 and 2% of the body weight respectively (see table 10).

The estimation of haemolymph volume was extended to investigate the effect of lethal heat treatment on haemolymph volume. The results are shown in table 11 and indicate that there was a reduction in haemolymph volume in 9 of the 10 experimental animals. The mean figures for the percentage reduction in haemolymph volume of 10^{o}C adapted crayfish exposed to 32^{o}C and 25^{o}C adapted crayfish exposed to 35^{o}C until heat death were 11 and 7.8% respectively. Due to the variability of the data the difference between the adaptation groups cannot be taken as being significant, and therefore an overall mean for both adaptation groups of about 10% haemolymph volume reduction was assumed.

TABLE 10.

Wet weights of the Major Organs and Tissues of *Astacus pallipes*.

Crayfish were blotted dry, weighed, and the major components dissected out. The organs and tissues were blotted dry and weighed. Haemolymph was assumed to make up 25% of the pre-experimental body weight (see table 11 and section 4 of the results, Chapter 2). The weight of the components was summed for comparison with the pre-experimental weight. All weights were expressed in grams, and the percentile composition of crayfish by weight calculated from the mean organ and animal weights.

TABLE 10.

Animal	Sex	Total Muscle Weight	Ligated Gut	Hepato-pancreas	Haemolymph	Cuticle	Deduced weight (components)	Pre-experimental Weight.
1	♀	4.24	0.33	0.17	2.29	1.75	8.78	8.98
2	♂	6.75	0.39	0.30	3.35	2.84	13.63	13.40
3	♀	5.09	0.19	0.25	2.40	2.12	10.04	9.74
4	♀	4.24	0.33	0.21	2.10	1.80	8.68	8.20
5	♀	3.90	0.22	0.14	2.10	1.66	8.02	8.21
6	♀	3.43	0.17	0.10	1.71	1.39	6.80	6.83
7	♀	3.39	0.18	0.05	1.70	1.30	6.62	6.79
8	♂	3.54	0.26	0.12	1.79	1.45	7.15	7.10
Mean Proportion of body weight.		50.1%	2.5%	2.0%	(25%)	20%		
$\bar{x}$							8.71	8.66

TABLE 11.

Haemolymph Volume of *Astacus pallipes* Before and After Heat Death.

The haemolymph volume of 10 crayfish was estimated by the method of Prosser and Weinstein (1950) as described in the Methods (Chapter 2). Animals 1 - 5 were $10^{o}C$ adapted crayfish exposed to $32^{o}C$ and animals 6 - 10 were $25^{o}C$ adapted crayfish exposed to $35^{o}C$. Haemolymph volumes were expressed as ml and also as a percentage of the pre-experimental body weight of the crayfish.

TABLE 11.

Animal	Haemolymph volume before heating (ml) 1	2	$\bar{x}$	% Body Weight	Haemolymph volume after heating. (ml)	% Body Weight	SBC time (minutes)	% change of haemolymph volume.
(a) 10°C adapted crayfish.								
1	9.10	9.65	9.40	25.1	8.45	23.0	15	-10
2	5.56	5.53	5.55	24.9	5.21	24.0	15	-6
3	4.17	4.30	4.38	24.9	3.70	21.9	17	-14
4	4.17	3.83	4.00	23.7	3.36	20.2	17	-16
5	3.13	3.09	3.11	25.1	2.79	22.9	16	-10
$\bar{x}$				24.7		22.4	16	-11
(b) 25°C adapted crayfish.								
6	7.14	7.07	7.11	25.2	5.35	19.1	26	-25
7	5.00	4.35	4.68	25.4	4.81	26.1	15	+3
8	4.00	4.07	4.04	26.9	3.91	26.2	18	-3
9	4.00	3.59	3.77	26.0	3.61	24.9	18	-4
10	3.17	3.20	3.19	25.2	2.86	21.2	23	-10
$\bar{x}$				25.7		23.5	20	-10

TABLE 12.

Water Content of Abdominal Flexor Muscles of *Astacus pallipes*.

Abdominal flexor muscles were weighed before and after drying in a vacuum oven at 60°C for 24 hours and the water content of the muscles calculated. n = the number of animals sampled and the results were expressed as the percentage of the muscle wet weight.

TABLE 12.

Adaptation Temperature (°C)		$\frac{\text{Wet weight - Dry weight}}{\text{Wet weight}} \times 100$	S.E.	n
10	Normal	84.3	1.5	10
	Heat Dead	85.7	1.1	20
25	Normal	85.1	1.9	10
	Heat Dead	84.8	0.9	30
	Mean	84.97	0.7	70

TABLE 13.

Astacus pallipes Muscle Density.

The density of crayfish abdominal flexor muscle was estimated by Archimedes principle. Animals 1 and 2 were 10^{o} and animals 3 - 5 were 25^{o}C adapted crayfish. Density was expressed in grams per ml.

TABLE 13.

Muscle	Wet Weight (g.)	Volume (ml.)	Density (Kg/dm^3)
1	2.47	2.01	1.23
2	1.39	1.17	1.19
3	1.06	0.85	1.25
4	0.82	0.57	1.44
5	1.97	1.65	1.19
$\bar{x}$	1.54	1.25	1.26
S.E.			0.104

The cellular water content was assumed not to alter during heat death since the wet weight/dry weight ratio of the muscles used for analyses in the previous section did not alter when the animals were heated, see table 12. The muscle density was measured by weighing and displacement of water and found to be 1.26 $\pm$ 0.104 Kg/dm^3 (see table 13.)

Five 10^{o}C and five 25^{o}C adapted animals were then exposed to 32 and 35^{o}C respectively as described for environment sampling. The LD_{50} times for the 10 and 25^{o}C adapted animals at these temperatures were 16 and 19.3 minutes respectively (see table 1). Initial samples of the water and haemolymph were obtained for each experimental animal as previously described. After exposing animals to the lethal temperatures for their appropriate LD_{50} time the animals were removed, the water and haemolymph sampled and abdominal muscle dissected out for muscle ion analysis. The results of this experiment are shown in table 14, the methods used for calculation were described in the methods section.

Comparison of the data presented in table 14 with the appropriate equivalent information in tables 3, 4, 8 and 9 indicates a degree of variability in the results. Thus the 10^{o}C adapted crayfish in this experiment lost more K^+ to the environment than the data in table 3 would suggest, 0.0116 mg as opposed to 0.0045 mg. The haemolymph Na^+ concentration differed from those recorded previously in table 4 e (25^{o}C adapted) and table 5 (10^{o}C adapted), though the effect of lethal temperatures was similar to the results previously obtained for both adaptation groups. The concentrations of Na^+ and K^+ in the muscle fibres following lethal heat treatment were similar to those obtained in previous experiments (see tables 8 and 9). These results therefore indicate that there was some variability in the effects of high lethal temperatures on individual animals.

TABLE 14.

The Effect of High Lethal Temperatures on Haemolymph, Muscle and Environment Na^+ and K^+ Concentrations in Warm and Cold Adapted Crayfish

Five 10^oC adapted crayfish were exposed to 32^oC for 16 minutes and five 25^oC adapted crayfish were exposed to 35^oC for 20 minutes, i.e. a lethal heat treatment for both groups (see table 1). The relevant exposure tims is denoted on the table by the bracketed figure.

Haemolymph Na^+ and K^+ were estimated from samples taken immediately before and after the exposure; ion concentrations were expressed in m moles per l.

Muscle intracellular Na^+ and K^+ were measured in abdominal flexor muscles dissected from crayfish immediately after heat exposure. The values represent the mean of a minimum of three independent determinations on each muscle and concentrations were expressed as m moles per Kg cell water.

Crayfish were heated in a known volume of water (200 ml) which was sampled before and after the exposure. Samples were analysed as described in the Methods and the net gain of ions was expressed in m moles per l.

TABLE 14.

Animal	Sex	Weight (g)	Haemolymph Na^+ (m moles/l)		Muscle Na^+ (m moles/l)	Environment Na^+ gain (m moles/l)	Adaptation Temperature (°C).
			(0)	(16)	(16)	(16)	
1	♂	13.15	197	204	46.8	0.030	
2	♂	13.20	190	186	46.5	0.035	
3	♀	9.96	190	195	44.3	0.030	
4	♀	8.44	185	179	44.5	0.060	10°C
5	♂	17.12	207	196	46.0	0.040	
$\bar{x}$		12.37	193.8	192.5	45.6	0.039	
S.E.		1.51	3.81	4.32	1.15	0.006	
			(0)	(20)	(20)	(20)	
6	♂	23.25	190	162	52.5	0.21	
7	♀	10.70	177	163	51.4	0.33	
8	♀	6.35	199	171	54.4	0.23	25°C
9	♂	4.40	194	164	52.6	0.18	
10	♂	13.53	182	163	38.5	0.22	
$\bar{x}$		11.65	188.4	164.6	49.9	0.234	
S.E.		3.314	3.98	1.63	2.89	0.025	

Animal	Sex	Weight (g)	Haemolymph K^+ (m moles/l)		Muscle K^+ (m moles/l)	Environment K^+ gain (m moles/l)	Adaptation Temperature (°C)
			(0)	(16)	(16)	(16)	
1	♂	13.15	5.5	10.0	104.7	0.010	
2	♂	13.20	5.0	10.0	113.9	0.010	
3	♀	9.96	5.7	10.3	107.6	0.010	10°C
4	♀	8.44	5.2	10.3	123.4	0.010	
5	♂	17.12	5.3	9.9	119.7	0.018	
$\bar{x}$		12.37	5.34	10.10	113.9	0.0116	
S.E.		1.51	0.12	0.08	7.88	0.0016	
			(0)	(20)	(20)	(20)	
6	♂	23.25	4.9	10.2	109.1	0.005	
7	♀	10.70	5.5	7.5	115.7	0.005	
8	♀	6.35	4.8	10.0	108.0	0.010	25°C
9	♂	4.40	5.4	12.2	111.7	0.025	
10	♂	13.53	4.7	7.6	104.5	0.012	
$\bar{x}$		11.65	5.06	9.50	109.7	0.0114	
S.E.		3.314	0.16	0.88	1.87	0.0036	

The net Na^+ changes which occurred in the 25°C adapted animals exposed to 35°C for 20 minutes were of greater magnitude than those observed in 10°C adapted animals exposed to 32°C for 16 minutes (see table 15). The calculated net haemolymph Na^+ change was 2.69 mg for the 25°C adapted and 0.49 mg for the 10°C adapted animals. The calculated net sodium loss to the water was 1.08 and 0.18 mg respectively for the 25 and 10°C adapted animals. The calculated net Na^+ changes between the haemolymph and water of 25°C adapted crayfish were therefore over five times greater than those which occurred in 10°C adapted animals. The calculated net K^+ changes which occurred in 10 and 25°C adapted crayfish were of similar magnitudes, 0.45 mg and 0.40 mg respectively. The net K^+ loss to the water was the same, 0.09 mg, in both adaptation groups (see table 15). The calculated net loss of muscle K^+ was slightly greater in the 25°C adapted animals, 0.73 mg, than in the 10°C adapted, 0.63 mg (see table 15).

The discrepancies by which the calculated net Na^+ and K^+ movements in the haemolymph, muscle and environment failed to balance are also given in table 15. They are much larger in 25°C adapted animals. The increased Na^+ discrepancy may have been due to the greater magnitude of sodium movements in the warm adapted animals with a constant percentage error in the calculations. This appears unlikely, however, since the magnitude of the K^+ discrepancy is greater in the 25°C adapted animals while the net K^+ movements are similar in both adaptation groups (table 15). This suggests that other tissues may have been involved in the Na^+ and K^+ movements of 25°C adapted crayfish exposed to 35°C, and possibly in the 10°C adapted animals exposed to 32°C also. The calculated Na^+ discrepancies were less than the smallest net changes which occurred in both 10 and 25°C adapted animals, those in the environment, so that for the net Na^+ changes

TABLE 15.

Net Interchange of Na^+ and K^+ between Muscle, Haemolymph and Environment during Heat Death in Astacus pallipes.

The data presented in table 14 was used to calculate the results of this table as described in the Methods (Chapter 2.) The results represent the net gain or loss of Na^+ or K^+ by the muscle haemolymph or water expressed in mg.

TABLE 15.

Na^+		10^oC Adapted.	25^oC Adapted.
Loss from Haemolymph	(mg)	0.49	2.69
Gain by Muscle	(mg)	0.44	0.97
Gain by Environment	(mg)	0.18	1.08
Discrepancy	(mg)	0.13	0.64

K^+		10^oC Adapted	25^oC Adapted.
Gain by Haemolymph	(mg)	0.45	0.40
Loss from Muscle	(mg)	0.63	0.725
Gain by Environment	(mg)	0.09	0.09
Discrepancy	(mg)	0.09	0.24

the three compartments investigated must constitute the main tissues involved in Na^+ movements during heat stress. The net potassium discrepancy for the 10^oC adapted animals was of the same magnitude as the smallest net change which occurred in the three compartments, that of the environment. The discrepancy observed in the net K^+ changes of the 25^oC adapted animals was larger than the net environmental K^+ gain, suggesting that some of the K^+ was moved into a compartment which was not investigated or that experimental error was larger in the K^+ than the Na^+ results. The net K^+ discrepancy was, however, still considerably less than the calculated net K^+ movements in the haemolymph or muscle of 25^oC adapted animals. The results therefore suggest that the muscle, haemolymph and environment are the three main compartments involved in the Na^+ and K^+ movements reported in this chapter for crayfish exposed to lethal temperatures.

DISCUSSION.

The results presented in this chapter indicate that the monovalent cations, sodium and potassium, exhibit a net movement down their respective electrochemical gradients when crayfish were exposed to high temperatures. Heat stress was observed to cause a net loss of Na^+ and K^+ to the environment (figure 7) and energetically net downhill movement of these ions across the muscle/haemolymph barrier which normally maintains electrochemical gradients of these ions across the muscle fibre plasma membrane. (see figures 9 and 10 and tables 4, 5, 8 and 9).

The results of independent experiments on the movement of Na^+ and K^+ correlated reasonably well. Thus the loss of Na^+ to the environment was more rapid during the initial phases of heat death (see table 3) when the haemolymph concentration was elevated (see table 4). Bryan (1960 a and c) has shown that the loss of Na^+ via the excretory organs and the general body surface was greatly increased when the haemolymph Na^+ concentration was elevated by approximately 50%. Although the haemolymph Na^+ concentration in these experiments was not observed to rise by more than 10% during heat stress (see table 4) it seems plausible to argue that a haemolymph Na^+ regulating mechanism would excrete Na^+ more rapidly when haemolymph Na^+ was high than when it was at normal or lower levels, i.e., in the latter phases of heat death. Since Na^+ is lost from the heat stressed crayfish when haemolymph Na^+ concentrations are below the normal level (see tables 3 and 4) a breakdown of the normal Na^+ regulation mechanism must occur at least during the latter phases of heat

death. It may be that the loss of Na^+ to the environment can be completely explained in terms of passive leakage, the more rapid Na^+ loss to the environment during the early phases of heat stress being explained merely on the basis of a bigger concentration gradient. The rapidity of the initial Na^+ loss (over 50% of the total loss of Na^+ during the initial 30% heat stress time for both adaptation groups (see figure 3)) suggests that either the permeability of the crayfish/environment barrier was very rapidly affected by exposure to high temperatures or that the initial Na^+ loss was at least in part due to an active excretion of Na^+ ions triggered by the elevated haemolymph Na^+ concentration.

The rate of net K^+ loss to the environment (see figure 7) could also be correlated with the haemolymph K^+ concentration in a similar fashion. The increase in haemolymph K^+ during heat death (see figure 7) was more rapid during the latter phases of heat death than in the early phases. It can be seen from figure 7 that the rate of net K^+ loss to the environment reflected this. Once again it is not clear at this time whether the potassium loss is due to a passive permeability breakdown or whether some active extrusion of K^+ ions was involved in an attempt by the crayfish to regulate the haemolymph K^+ concentrations to normal low levels.

The effect of adapting the experimental animals to 10 or 25^oC was clear (see figures 7, 8, 9 and 10). Changes in the haemolymph Na^+ and K^+ concentrations and the net loss of these ions to the environment were of a similar nature in both adaptation groups. The two main differences seen between the two adaptation groups were that the net movements of Na^+ and K^+ ions in 10^oC adapted crayfish were more rapid but of smaller magnitude than those observed in the 25^oC adapted crayfish. Thus the 25^oC adapted crayfish which exhibit a higher high temperature resistance than the 10^oC adapted crayfish were found to lose

ions to the environment and gain ions in the haemolymph more slowly than 10°C adapted animals during the same heat stress conditions.

Examination of tables 4 and 5 suggests that it is the haemolymph K^+ concentration which is of particular significance in heat death. Thus when ten 25°C adapted crayfish were exposed to 32°C (table 4) for 45 minutes only two animals were found to survive the experiment by more than a few minutes. These two animals (numbers 2 and 10) were found to have the lowest haemolymph concentrations of the ten experimental animals after 45 minutes' exposure to 32°C. It was also apparent that the mean haemolymph K^+ concentration of crayfish exposed to high temperatures for lethal times, i.e. 25°C adapted at 32, 34 and 35°C and 10°C adapted at 32°C, were found to be of similar magnitude (see tables 4 and 5) being about 9 - 10 mM. The mean haemolymph Na^+ concentrations of the same animals, on the other hand, was found to vary between 157 and 182 mM.

Examination of the muscle fibre Na^+ and K^+ concentrations during heat death (see tables 8 and 9) indicated that these ions were moving down their electrochemical gradients during heat death. Thus Na^+ moved from the haemolymph to the muscle fibre and K^+ moved from the muscle fibre to the haemolymph. The net Na^+ gain of the muscle fibres was much greater in the 25°C adapted animals (220%), than in the 10°C adapted animals (16.6%). Similarly the loss of K^+ by the muscle fibres was greater in the higher adaptation group, being a 36.8% loss from the 25°C as opposed to an 8.9% loss from the 10°C adapted animals. Perhaps the most interesting aspect of these results is that the rate of net Na^+ movement into the muscle fibres is very similar in both adaptation groups (see tables 8 and 9). In fact the muscle fibre Na^+ gain is slightly more rapid in the 25°C adapted animals, apart from the initial sampling period. This is probably a reflection of the higher haemolymph Na^+ concentration of 25°C adapted animals during heat death

(see tables 4 and 5) and suggests that the rate of Na^+ entry depends upon the concentration gradient across the muscle fibre plasma membrane. This in turn suggests that the net movement of Na^+ observed into the muscle fibres is the result of a breakdown in the passive permeability barrier located in this membrane.

The results presented in tables 8 and 9 show that the rate of net K^+ loss from the muscle fibres was considerably more rapid in the 10^oC adapted animals apart from the initial 5 minute reading. This may be due in part to the higher initial muscle fibre K^+ concentration found in 10^oC adapted animals (see table 7), the greater concentration gradient in this group bringing about a more rapid net movement of K^+.

The data discussed above suggest that the movements of Na^+ and K^+ between animal and environment and between haemolymph and muscle fibre might be linked. Experiments were therefore performed in order to make simultaneous measurements of haemolymph, environment and muscle fibre Na^+ and K^+ concentrations during heat death. Treatment of the results of table 14 as described in the Results (section) of this chapter allowed the construction of a 'Balance Sheet' for net Na^+ and K^+ movements during heat death, this was presented in table 15.

The discrepancies observed in table 15 were much larger for the 25^oC adapted animals than the 10^oC adapted animals, this was probably a function of the greater net ion movements observed in the 25^oC adapted animals. It is not possible to calculate meaningful confidence limits for the data in table 15 since, as described in the Results (section 4) assumptions of the initial muscle fibre ion concentrations and the relative proportions of the tissues in the crayfish had to be made separately and mean values used in the calculations. The limited number of animals used in the experiments also make the application of meaningful confidence limits more difficult. The magnitude of the discrepancies noted in table 15, however, do tend to

to suggest that one or more other ion compartments may have been involved in the overall ion movements observed, but that the ion movements observed could be to a large extent explained by the movements of Na^+ and K^+ between the three ionic compartments discussed.

The data of table 15 therefore illustrate three significant points. Firstly the majority of the dramatic Na^+ and K^+ movements observed during heat death occurred between three compartments, the muscle, haemolymph and environment. Secondly the movements of Na^+ ions was much greater in the 25^oC adapted animals than in the 10^oC adapted animals during heat death. The total net movement of Na^+ was over four times greater in the higher temperature adaptation group. Finally, the net K^+ movements observed in both adaptation groups were extremely similar in magnitude in all three compartments. Since the experimental animals were exposed to high lethal temperatures for times approximating to their LD_{50} times (as given in Chapter 1, table 1) this may be interpreted as meaning that in the two adaptation groups, possessing notably differing thermal tolerances, the overall net K^+ movements during heat death were very similar. The overall net Na^+ movements, on the other hand, were not. This last conclusion, together with the conclusions drawn from the changes in haemolymph K^+ during heat death discussed above indicate that the rate and magnitude of the net K^+ ion movements which occur during heat death are of considerable importance in the process of heat death.

CHAPTER 3.

EFFECT OF HIGH TEMPERATURE ON BIOELECTRIC PHENOMENA OF NERVE AND MUSCLE FIBRES.

INTRODUCTION.

Nerve and muscle cells in the non-excited state characteristically exhibit a large potential difference across their plasma membrane known as the resting potential. Current information suggests that there may be two components to the resting potential, an ionic and a metabolic component. The ionic component depends upon the particular distribution of permeable ions across a selectively permeable barrier around the cell, this is probably the cell membrane as seen in electron micrographs. There is now a great deal of evidence for such a barrier which includes electrical considerations such as the high resistance and capacitance of the cell boundary (e.g. Cole and Hodgkin, 1939; Falk and Fatt, 1964) and limited permeability of many ions (e.g. see Collander, 1937). Hodgkin and Keynes (1953) produced experimental evidence of a barrier to radio-active potassium ions located at the boundary of squid giant axons, showing that the mobility of the potassium isotope once inside the giant axons was ~~almost~~ virtually the same as in sea water. The majority of biologists now accept that cells are bounded by a membrane which acts as a barrier to the movement of many ions and molecules.

The potential difference across a barrier due to a single ion species is known as the equilibrium potential (E_c) for that ion. This depends upon the concentration of that ion on either side of the membrane. The net potential across a membrane having an unequal distribution of several permeant ions lies between the extreme E_c values for those ions. The E_c of an ion may be calculated from the Nernst equation:-

$$E_c = \frac{RT}{nF} \cdot \ln \frac{C_2}{C_1}$$

Where R = universal gas constant,

F = the Faraday (electric charge per gram equivalent of univalent ions)

T = the absolute temperature

n = the valency of the ion

E_c = the equilibrium potential

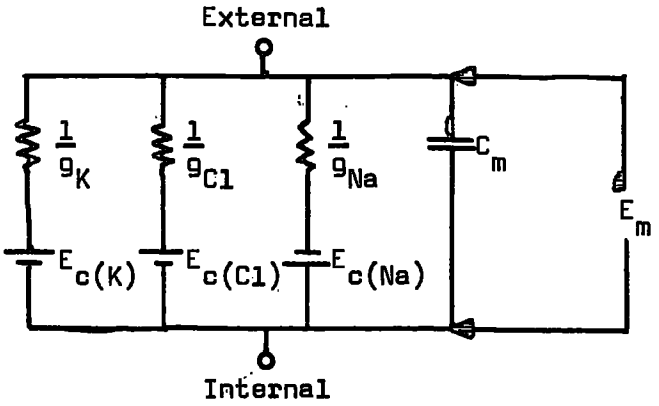
and C_1 and C_2 = the concentration of ion 'C' on either side of the membrane.

Bernstein (1902) applied the Nernst equation to the potassium ion distribution across the plasma membrane of excitable cells and found that he could quantitatively predict the effects of alterations in external potassium ions and temperature on the resting potential of these cells. Further studies showed, however, that both sodium and chloride ions were able to move across the cell membrane and that it was therefore necessary to take into account the relative permeabilities of the permeant ions in order to predict the resting potential. This can be done by using the Goldman Constant Field Equation:-

$$E_m = - \frac{RT}{nF} \ln. \frac{P_{Na}[Na]_o + P_K[K]_o + P_{Cl}[Cl]_i}{P_{Na}[Na]_i + P_K[K]_i + P_{Cl}[Cl]_o}$$

where R, T, F and n have the same meanings as in the Nernst Equation, P_{Na}, P_K, and P_{Cl} = the permeabilities of Na^+, K^+ and Cl^- ions and $[\ ]_i$ and $[\ ]_o$ refer to the intra and extracellular concentrations of ions respectively.

From measurements of various electrical parameters of the plasma membranes of muscle and nerve cells, Hodgkin (1958) constructed an equivalent electrical circuit diagram:-



where $\frac{1}{g_X}$ = the electrical resistance of the membrane to ion 'X'

C_m = the membrane capacitance

E_m = the potential difference across the membrane

and $E_{c(X)}$ = the equilibrium potential across the membrane of ion 'X'.

The conductance (g) of an ion is the reciprocal of the electrical resistance of the membrane to that ion, and is a function of the permeability and concentration of ions on either side of the membrane. This circuit diagram formally indicates that the membrane potential is determined by the ionic "leakage" pathways in the absence of any metabolic component. It further indicates that the leakage resistance for each ion is independently variable, and that there are separate leakage pathways for each permeant ion.

The second component of the resting potential is directly related to cellular metabolism and is known as the 'electrogenic' component. The electrogenic component is thought to be generated by a cation pump located in the cells of many tissues (see Whittam and Wheeler, 1970). This cation pump is often known as the 'sodium' pump, since it pumps sodium from the cells in exchange for extracellular potassium. It is therefore responsible for the maintenance of the characteristic distribution of Na^+ and K^+ ions between the inter and intracellular compartments. This active transport of cations has been shown in many tissues to be the property of a $Na^+ + K^+$ activated

adenosine triphosphatase (E.C. 3.6.1.3) (Skou, 1957, Whittam and Wheeler, 1970). Evidence has been presented in some tissues that the rate at which this pump works is related to intracellular Na^+ concentration. The higher the intracellular Na^+ concentration, the higher the pump activity. Evidence has been presented by Adrian and Slayman (1966) and Bittar (1966) that an unequal number of Na^+ and K^+ ions are exchanged during the operation of the Na^+ pump, more Na^+ ions leaving the cell than K^+ ions entering per mole of adenosine triphosphate (ATP) used. This would result in the net transfer of positive charge to the extracellular space, so increasing the resting potential of the cell. It is only when unequal numbers of charged particles are transferred across the membrane that the cation pump can act as an electrogenic pump.

Evidence for the presence of an electrogenic cation pump in Na^+ rich frog muscle has been presented by Connelly (1959), Kernan (1962), Keynes and Rybova (1963), Mullins and Noda (1963), and Mullins and Awad (1965). Carpenter and Alving (1968), Ayrapetyan (1969) and Marmor and Gormon (1970) have demonstrated an electrogenic component in molluscan neurones and Senft (1967) for lobster axons. The presence of an electrogenic component in mammalian muscles has been indicated by the effects of infused ouabain (Locke and Solomon, 1967) and phospholipase C (Albuquerque and Thesleff, 1967) which reduced the resting potential of these cells by over 20 mV. Both of these agents are known to inhibit the cation pump, (Skou, 1957 and De Mello, 1971).

In the resting state the membrane potential of excitable cells tends towards the potassium equilibrium potential, usually 50 - 100 mV, inside negative. During the passage of an action potential the membrane potential tends towards the Na^+ equilibrium potential. Studies by many

authors (e.g. Cole and Curtis, 1942; Del Castillo and Stark, 1951; Hodgkin and Huxley, 1952; Hodgkin, 1958 and Frankenhauser, 1960) have indicated the sequence of membrane events which occur. When the membrane is depolarised there is a rise in the Na^+ conductance, g_{Na}. This moves the membrane potential closer to the Na^+ equilibrium potential, usually in the range of 0 - 60 mV, inside positive. The increase in g_{Na} is transient and followed by an increase in the K^+ conductance, g_K, of the membrane leading to the repolarisation of the membrane. The action potential may therefore be explained in terms of alterations in the conductances of permeant ions.

The ionic requirements of excitable cells vary from one group of animals to another depending upon their environment and mode of life. The ionic requirements of crustacean nerve and muscle fibres have been investigated by many authors including Fatt and Katz (1953), Fatt and Ginsborg (1958), Reuben, Girardier and Grundfest (1962 and 1964), Girardier, Reuben, Brandt and Grundfest (1963), Ozeki, Freeman and Grundfest (1966), Machne and Orozco (1967), Reuben, Brandt, Girardier and Grundfest (1967), Strickholm and Wallin (1967), Wallin, (1967 a and b, 1969), Obaru (1968) and Strickholm, Wallin and Shrager (1969). Their results indicate that Na^+ plays a minor role in the resting potential of crayfish nerve and muscle, though it is involved in the action potential. Van der Kloot (1966) and Strickholm and Wallin (1967) have demonstrated that K^+ is highly permeable in the excitable cell membrane, changes in extracellular K^+ concentrations having marked effects on the transmembrane resistance and membrane potential. The permeability of the axonal membranes to chloride is small at normal pH, but changes in a variety of abnormal ionic and pH environments (Strickholm and Wallin, 1967). The permeability of the sarcolemma to Cl^- is considerably smaller than its potassium permeability (Hinkle, Heller and Van der Kloot, 1971),

and is confined mainly to invaginations of the sarcolemma (Orentlicher and Reuben, 1971).

The effects of temperature on crustacean muscle fibre electrical properties has been investigated by Fatt and Katz (1953). They found that raising the temperature of an *in vitro* preparation of crab muscle within physiological temperature limits resulted in an increased resting potential, and a reduced membrane and myoplasmic resistance. Raising the temperature to lethal levels resulted in an inability to produce action potentials and an irreversible drop in the membrane resistance. Furthermore, just as the adaptation temperature affects the upper temperature limits the animal can survive, so Fatt and Katz (1953) found that adapting the crabs to higher environmental temperatures increased the heat resistance of the *in vitro* muscle preparation.

The aims of the following experiments were to examine the effects of high lethal temperatures on nerve and muscle tissue, to investigate the effects of adapting crayfish to different temperatures on the excitable tissue heat tolerance and to examine the effects of the changes in haemolymph ion concentration described in the previous chapter upon the excitable tissues.

MATERIALS AND METHODS.

Experimental animals were acclimatised to either 10 or 25°C as previously described. The lateral abdominal extensor muscles were exposed by ventral dissection and mounted in the experimental chamber with their tergal attachments intact. The experimental chamber was a rectangular perspex depression in a larger perspex vessel (see figure 13 a). During an experiment constant temperature was maintained by the rapid flow of water through the outer perspex vessel (about 0.5 l per minute) and a constant flow of saline (between 20 and 25 ml per minute). The temperature of the circulating water and saline was controlled to within $\pm$ 0.1°C as described in chapter 1 and the temperature continuously monitored by means of the thermocouple and 'Scalamp' galvonometer previously described. (calibration curve shown in figure 15).

The level of saline in the experimental chamber was regulated by aspirating saline from the opposite end of the chamber to that at which fresh saline entered. The volume of saline in the chamber depended upon the size of the preparation, but was normally between 6 and 12 ml. The constant flow of saline was used to eliminate the effects of Na^+ and K^+ exchange, between the muscle and saline, on the saline composition at high lethal temperatures. The saline used was a modification of Van Harreveld's saline, it contained 195 mM NaCl, 5.4 mM KCl, 2.6 mM $MgCl_2$, 13.5 mM $CaCl_2$, 1.2 mM $NaHCO_3$, and 10 mM Tris-Maleate buffered to pH 7.5 at the experimental temperature. This saline was found to be suitable, preparations remaining active for over 6 hours if kept cool.

1. Measurement of the Muscle Fibre Resting Potential.

Glass micropipettes were constructed from Jencons H 15/10 Pyrex capillary tubing (O.D. circa 1 mm) by means of a Palmer micro-electrode puller. They were filled with absolute ethanol under reduced

Figure 13.

Experimental Methods for Measuring the Resting Potential and the Effective Membrane Resistance of Crayfish Muscle Fibres.

(a) Diagram to show the arrangement of the apparatus used to measure the resting potential (RP) of crayfish abdominal extensor muscles. The muscles, still attached to the terga, were mounted in a perspex chamber which was surrounded by a water jacket. The RP was measured via a glass micropipette filled with 3 M KCl and displayed on a cathode ray oscilloscope. The tip resistance of the micropipette was measured by the method of Frank and Fuortes (1956).

(b) Diagram to show the arrangement of the apparatus used to measure the effective membrane resistance (EMR) of crayfish abdominal extensor muscles. The EMR was determined by injecting a sine wave of known amplitude into the circuit so that the signal was divided between a known resistance (R_n) and the combined EMR and micropipette tip resistance after Schanne, Kawata, Schafer and Lavallee (1966).

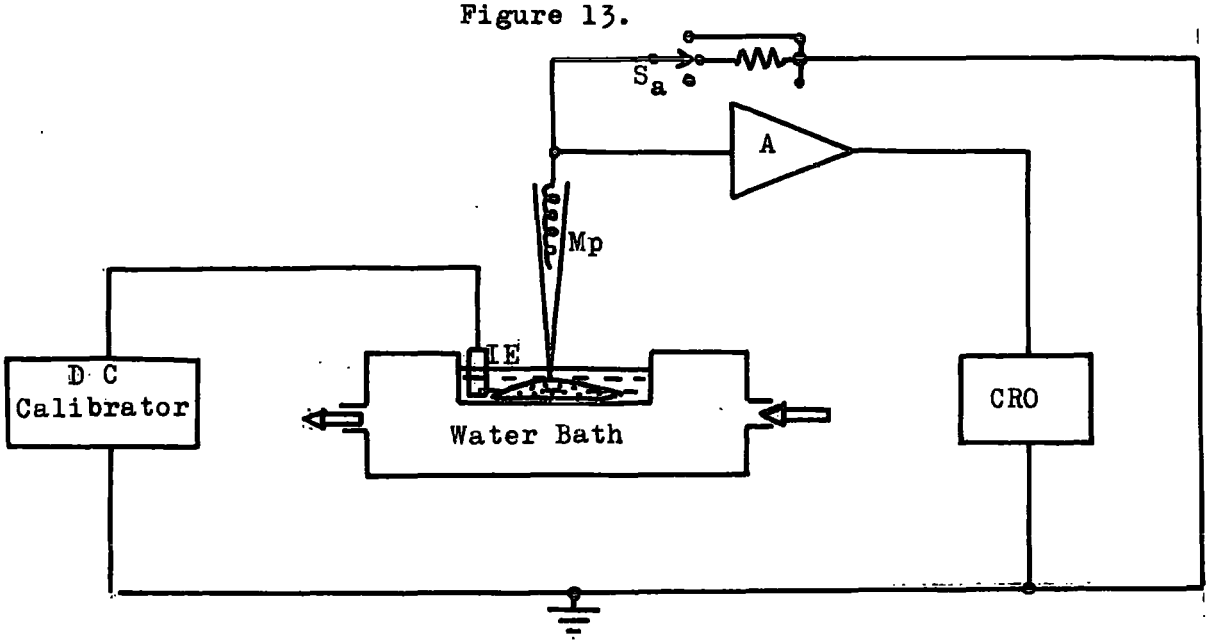
A = High input impedence cathode follower with a gain of X 1.

CRO = Cathode ray oscilloscope.

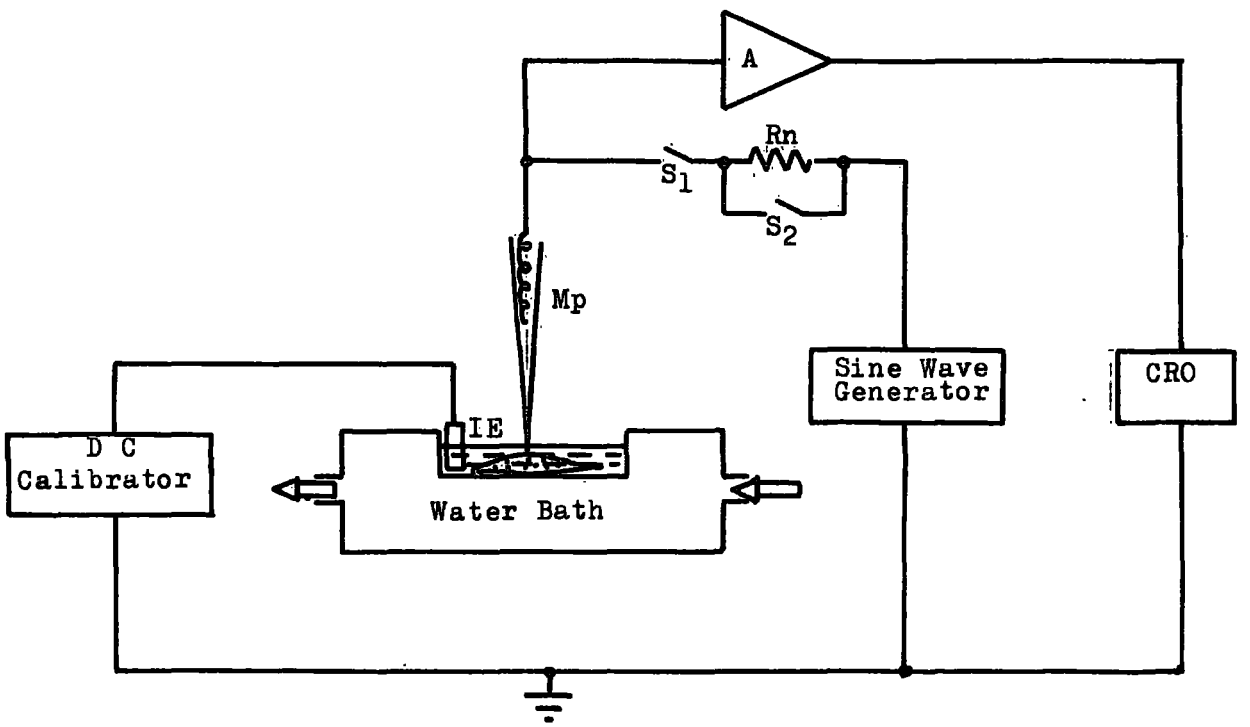
Mp = Micropipette. IE = Indifferent electrode.

S_1 , S_2 , S_3 = switches Rn = 10 M Resistor (1 % tolerance).

Figure 13.


S_a
A
Mp
IE
D C
Calibrator
Water Bath
CRO

(a)


A
Rn
S_1
S_2
Mp
Sine Wave
Generator
CRO
IE
D C
Calibrator
Water Bath

(b)

pressure, the ethanol being replaced by distilled water and then 3 M KCl by diffusion. Care was taken to clean the glass tubing before use and to filter all filling solutions to avoid tip blockage. This method of filling micropipettes is described in detail by Tasaki, Polley and Orrego (1954). Oliveira Castro and Machado (1969) have shown that this method of filling produces fine tipped micropipettes which cause less damage to cells than larger tipped micropipettes. Tip potentials were measured by the method of Adrian (1956) and micropipettes with tip potentials greater than $\frac{5}{8}$ mV were discarded. Micropipette resistance was measured by the method of Frank and Fuortes (1955) (see figure 13 a). Only pipettes with a resistance of between 5 and 15 MΩ were used. This is because micropipettes with low resistances have larger tip diameters which are likely to cause damage to cells (Oliveira Castro and Machado, 1969) and those with higher resistances are prone to pick up interference.

The micropipettes were connected to a high input impedance cathode follower unit (Electrophysiological Instruments Ltd.) via a non-polarisable Ag/AgCl wire electrode. The length of the input wire was kept very short and shielded by a cathode driven shield to reduce input capacitance. The cathode follower was connected to a Tektronix 502 A cathode ray oscilloscope. The saline in the experimental chamber was earthed by means of an Ag/AgCl electrode connected to the oscilloscope via a DC calibrator (Electrophysiological Instruments Ltd.). The cathode follower and micropipette holder were mounted directly on to one side of a Zeiss Sliding Micromanipulator.

The resting potential (RP) was measured by lowering the micropipette vertically to pierce a muscle fibre, the potential appeared as a sudden change on the oscilloscope screen which could be measured by means of the DC calibrator. A Zeiss binocular dissecting

microscope was used to observe micropipette insertion. The resistance and tip potential of the micropipettes were tested frequently during the course of an experiment and discarded if they became blocked or broken or developed an increased tip potential. Readings were restricted to the top three layers of muscle fibres to avoid breaking micropipettes on the terga and to ensure that the fibres were fully equilibrated to the saline temperature.

2. Measurement of the Muscle Fibre Membrane Resistance.

The method used to measure the transverse membrane resistance was that of Schanne, Kawata, Schäfer and Lavallée (1966), (see figure 13 b). The muscle fibre was penetrated with switch 1 open and the resting potential measured, switches 1 and 2 were then closed so that the total output of the sinewave generator was measured. The frequency of the sinewave was 15 Hz, this low frequency being necessary to avoid loss of signal in the micropipette (Schanne, Lavallée, Laprade and Gagné, 1968), and the amplitude (peak to peak) was 300 mV. Switch 2 was then opened and the generator voltage was divided between R_n and the micropipette resistance in series with the effective resistance of the cell membrane. The voltage drop across the latter was measured on the oscilloscope. The resistance of the micropipette is critical in the calculation of the membrane resistance, it was therefore measured after each insertion by simply withdrawing the micropipette and measuring the voltage drop across the electrode. The resistance of the micropipette was calculated by the equation:-

$$R_{el} = \frac{R_n}{V'_A - 1}$$

where R_{el} = the resistance of the micropipette

R_n = the known resistance, 50 MΩ.

V'_A = the ratio of the generator output over the voltage drop observed across the micropipette.

The effective membrane resistance (EMR) was calculated by the equation:-

$$EMR = \frac{R_n}{V'_B - 1} - R_{el}$$

where V'_B = the ratio of the generator voltage over the voltage drop observed across the micropipette and the muscle cell in series

and R_n and R_{el} have the same meanings as before.

This method allowed rapid, sequential measurements of the muscle fibre membrane resistance and resting potential to be made.

3. Measurement of the Spontaneous Activity in the Ventral Nerve Cord.

The ventral nerve cord of an adapted crayfish was exposed by removal of the abdominal pleura, the cephalothorax removed, and the preparation mounted in the muscle bath previously described (see figure 13 a). Arrangements for temperature control and saline flow were also as previously described. A short length of the nerve cord (about 5 mm) was freed anteriorly by careful dissection and aspirated into a suction pipette. The suction pipette was constructed of a 20 gauge stainless steel hypodermic needle mounted on a micromanipulator. The suction pipette was then raised so that a portion of the nerve was exposed to air and allowed to dry partially, so isolating the suction pipette from the grounded saline in the experimental chamber. A small glass collar which had previously been suspended at the top of the pipette was filled with liquid paraffin (B.D.H. Ltd.) and allowed to slide down the hypodermic needle around the exposed portion of the nerve. In this way the recording electrode was isolated electrically from the experimental chamber and the nerve prevented from drying out entirely.

Action potentials were displayed on a Tektronix 502 A cathode ray oscilloscope after preamplification by a Tektronix 122 Low-Level

Preamplifier. Permanent records of activity were made photographically using a Cossor Oscilloscope camera on Ilford 5G91 film. The signals were also recorded on a tape recorder, the recorded signals being replayed into a digital counter to count the number of action potentials in a given period.

TABLE 16.

The Resting Potential and Effective Membrane Resistance of Crayfish Muscle Fibres.

The resting potential (RP) and effective membrane resistance (EMR) of *in vitro* preparations of abdominal extensor muscles from four 10^{o} and four 25^{o}C adapted crayfish were measured as described in the Methods (Chapter 3). The preparations were maintained in a modified Van Harreveld's saline at the adaptation temperature of the animal from which they were obtained. The sampling time is the time which had elapsed after the isolation of the preparations, when either 30 RP or 15 EMR + 15 RP measurements were made.

TABLE 16.

Animal	Adaptation Temperature (°C)	Mean Resting Potential (mV) (n=30) at sampling time (minutes).		
		0	60	120
1	10	-69.7 ± 0.80	-68.1 ± 0.71	-68.8 ± 0.48
2	10	-69.5 ± 0.66	-69.0 ± 0.73	-68.9 ± 1.00
3	25	-70.4 ± 0.47	-69.9 ± 0.61	- 69.1 ± 0.75
4	25	-72.3 ± 0.71	-71.4 ± 0.82	-70.1 ± 1.03

Animal	Adaptation Temperature (°C)	Mean Effective Membrane Resistance (MΩ) (n=15) at sampling time (minutes).	
		0	60
5	10	1.864 ± 0.261	1.787 ± 0.415
6	10	1.737 ± 0.146	1.752 ± 0.327
7	25	1.560 ± 0.274	1.646 ± 0.392
8	25	1.811 ± 0.236	1.772 ± 0.232

RESULTS

1. The Muscle Fibre Resting Potential

The RP of fresh abdominal extensor muscle fibres was measured at the same temperature as that at which the animal had been adapted. Thirty individual readings, which were taken in approximately 10 minutes, were made immediately after dissection, thirty more readings were made 60 minutes after dissection and a further thirty readings were made 120 minutes after dissection. Two preparations from each adaptation group were used. The results are shown in table 16. There was no significant change in the RP, the RP being about 70 mV in all preparations. Dudel and Rudel (1968) found that crayfish muscle could be stored in this saline for several days if kept at 8°C without affecting their activity.

2. The Effect of 32°C on the Muscle Fibre Resting Potential

Abdominal extensor muscles were dissected out and 30 RP measurements made, either at 10 or 25°C, depending on the adaptation temperature of the animal used. The results are given in table 17 as the mean value, and are taken to represent the normal mean RP of the preparation. The preparation temperature was raised to 32°C, this taking about 30 seconds according to the thermocouple readings, and RPs measured and their time of measurement recorded. The readings so obtained were grouped into 5 minute intervals so that the mean RP and the standard error of that mean could be determined for each five minute interval. These results are given in table 17 for a group of four 10° and a group of four 25°C adapted crayfish. Figure 14 shows the RP plotted against exposure time to 32°C.

The results shown in table 17 and figure 14 indicate that the RP of both 10 and 25°C adapted crayfish muscle fibres fell when exposed to 32°C. The rate at which the RP fell depended upon the adaptation

TABLE 17.

The Effect of High Temperature on the Resting Potential of Crayfish Muscle Fibres.

The abdominal extensor muscles from four 10^{o} (animals 1 to 4) and four 25^{o}C adapted crayfish (animals 5 to 8) were dissected out and mounted as described in the Methods (Chapter 3). A series of control resting potential (RP) measurements were made at the adaptation temperature of the animal from which the preparation was obtained and the temperature was then raised to 32^{o}C. Measurements of the RP were made with respect to time of exposure to 32^{o}C and the readings were grouped into 5 minute intervals for presentation. The RP was expressed in mV, the inside of the fibres being negative. The mean LD_{50} for 10^{o}C adapted animals (table 17a) at 32^{o}C is 16 minutes and for 25^{o}C adapted animals (table 17b) is 205 minutes.

TABLE 17 (a).

Time (minutes).		Animal. 1	2	3	4	$\bar{x}$
Control	RP	71.9	69.4	69.9	68.5	70.12
	SE	0.50	0.71	0.50	0.56	0.33
	n	30	31	30	30	121
0 - 4	RP	73.4	76.75	72.8	71.7	74.75
	SE	1.2	0.9	1.6	1.6	0.73
	n	10	13	10	11	44
5 - 9	RP	65.1	70.9	64.8	66.7	67.84
	SE	1.75	0.4	2.0	1.7	0.78
	n	15	12	15	15	57
10 - 14	RP	56.9	69.3	60.9	54.2	61.69
	SE	1.3	1.3	2.0	2.1	1.00
	n	15	15	16	15	61
15 - 19	RP	55.5	63.4	55.4	45.4	55.15
	SE	1.6	1.4	2.2	2.2	1.23
	n	15	16	15	15	61
20 - 24	RP	45.9	56.6	48.0	40.3	49.64
	SE	1.4	1.7	3.2	1.2	1.33
	n	14	15	19	15	63
25 - 29	RP	33.6	47.0	38.2	33.0	43.32
	SE	2.1	3.2	2.4	1.0	1.54
	n	15	15	15	15	60
30 - 34	RP	24.9	36.3	34.7	24.2	31.07
	SE	2.2	2.0	2.6	2.1	2.68
	n	9	15	15	11	50
35 - 40	RP	17.2	25.1	22.75	16.0	20.26
	SE	3.2	2.1	1.7	3.7	2.49
	n	9	13	16	10	48

TABLE 17 (b)

Time (minutes)		Animal 5	6	7	8	$\bar{x}$
Control	RP	70.1	72.0	72.8	67.2	70.53
	SE	0.60	0.73	0.66	0.66	0.40
	n	30	30	32	30	122
0 - 4	RP	77.4	75.7	76.5	73.8	75.85
	SE	0.9	1.0	0.62	1.2	0.79
	n	15	15	15	15	60
5 - 9	RP	77.2	75.5	74.9	73.0	75.15
	SE	1.25	1.4	1.4	1.3	0.33
	n	15	15	15	15	60
10 - 14	RP	80.2	71.9	71.8	72.1	73.81
	SE	1.4	1.3	1.9	1.4	1.00
	n	15	15	15	15	60
15 - 19	RP	80.3	73.3	71.2	-	74,67
	SE	1.1	2.3	1.6	-	0.99
	n	15	9	15	0	39
20 - 24	RP	72.3	-	69.6	66.4	69.43
	SE	1.4	-	1.9	2.6	1.96
	n	15	0	15	15	45
25 - 29	RP	72.0	68.6	66.7	66.2	68.53
	SE	1.6	2.4	1.5	1.7	1.80
	n	15	13	12	15	55
30 - 34	RP	68.3	-	52.5	58.7	61.20
	SE	1.1	-	2.9	1.5	1.24
	n	16	0	15	15	46
35 - 39	RP	58.0	32.8	41.4	52.8	46.24
	SE	2.3	3.6	2.7	1.5	2.13
	n	15	12	14	15	56
40 - 44	RP	38.3	25.7	-	36.7	34.25
	SE	2.4	2.1	-	1.82	1.49
	n	12	10	0	15	37
45 - 47	RP	26.3	-	-	-	26.3
	SE	2.39	-	-	-	2.39
	n	6	0	0	0	6

Figure 14.

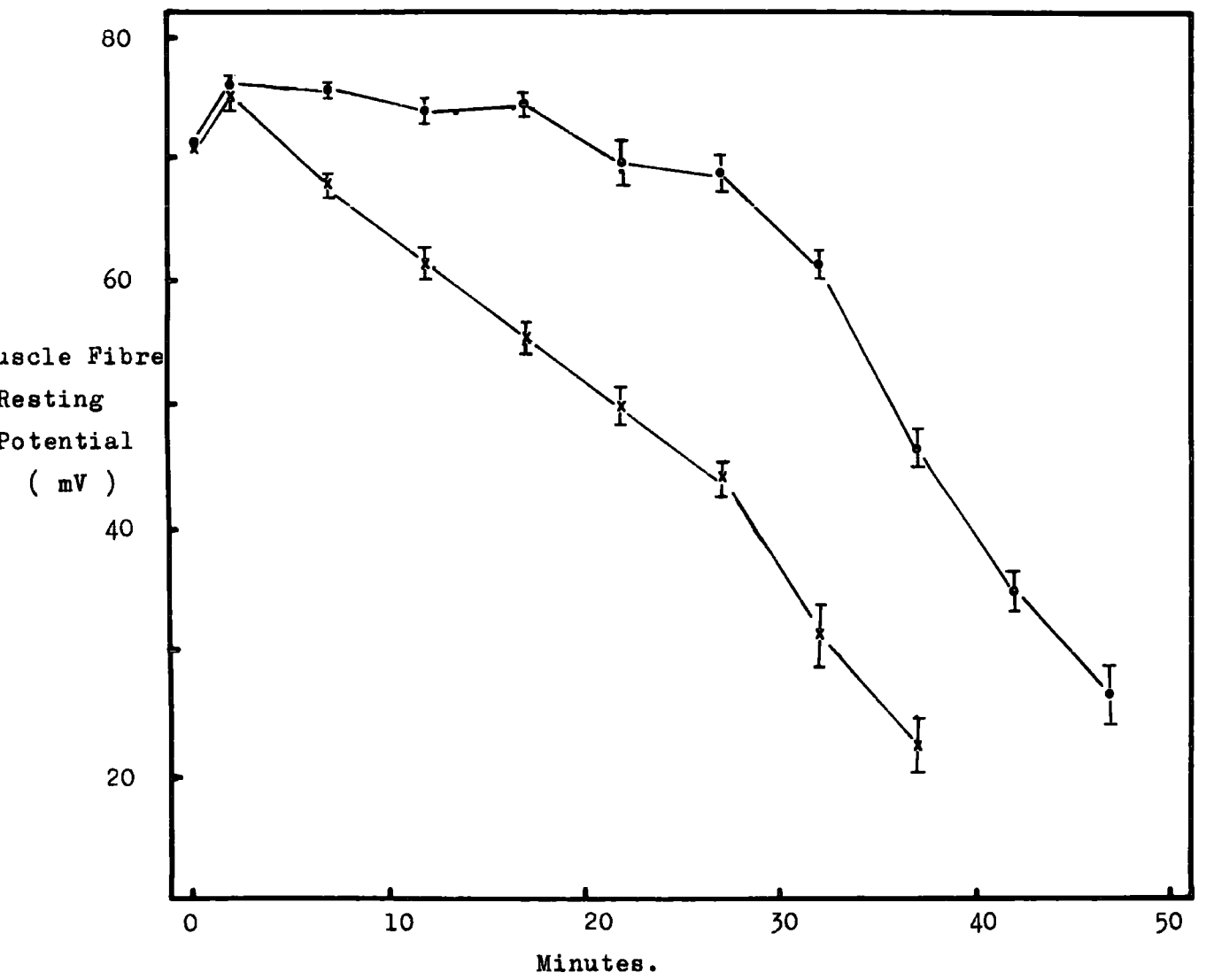
The Effect of Temperature Adaptation on the Sensitivity of the Resting Potential of Crayfish Muscle Fibres to 32°C.

Four 10° and four 25°C adapted crayfish abdominal extensor muscle fibre preparations were exposed to 32°C as described in the Methods. The mean RPs of each adaptation group were calculated from the data of table 17 for 5 minute intervals and plotted against the exposure time. The vertical bars represent ± 1 standard error of the mean.

Mean 10°C adapted crayfish RP ..... ✕——✕

Mean 25°C adapted crayfish RP ..... ⊖——⊖

Figure 14.


80
60
uscle Fibre
Resting
Potential
(mV)
40
20
0
10
20
30
40
50
Minutes.

temperature of the animal from which the preparation was obtained, the rate of depolarisation was similar in preparations taken from animals of the same adaptation temperature. The RP of the muscle fibres taken from 10°C adapted crayfish fell more rapidly than that of the muscle fibres from 25°C adapted animals. After 30-34 minutes exposure to 32°C the RP of muscle fibres taken from 10°C adapted crayfish was between 24 and 36 mV (group mean = 31.07 mV) whilst that of muscle fibres taken from 25°C adapted animals was between 58 and 68 mV (group mean 61.02 mV) (see table 17). The normal RP was similar in both groups, group means were 70.12 and 70.53 mV for 10 and 25°C adapted animals respectively. The RP of the 10°C adapted animals' muscle fibres was therefore much more sensitive to 32°C than that of 25°C adapted animal muscle fibres.

There were, however, strong similarities in the pattern of events which was observed in the two adaptation groups. Both groups exhibited an initial hyperpolarisation of the muscle fibres on exposure to 32°C which was followed by depolarisation. The timing of these changes was different in the two adaptation groups as can be seen in figure 14. The initial hyperpolarisation was much longer in the 25°C adapted animal preparations, lasting at least 10-14 minutes and in one case 25-29 minutes (see table 17). The period of hyperpolarisation in the 10°C adapted crayfish was much shorter, being less than 5-9 minutes in 3 of the 4 preparations (see table 17).

The pattern of depolarisation of the muscle fibres exposed to 32°C differed in the two adaptation groups. Following the initial hyperpolarisation (after 5 minutes exposure) the RP fell by approximately 1.25 mV per minute in the 10°C adapted animal preparations. The rate of depolarisation then increased slightly to 2 mV per minute

for the final 10 minutes of exposure. In the 25°C adapted preparations a rapid rate of depolarisation was not seen until after 35 minutes of exposure when a rate of about 3 mV per minute was found. The RP of the muscle fibres from 10°C adapted animals was therefore always markedly less than that of the 25°C adapted muscle fibres after the first 5 minutes of exposure to 32°C.

3. The Effect of 32°C on the Effective Membrane Resistance of the Muscle Fibre.

In order to determine the 'normal' values for the RP and the EMR of muscle fibres, measurements were made on three 10 and two 25°C adapted animals as described in the Methods, at 10 and 25°C respectively. At time zero, table 18, the saline was replaced with saline at 32°C and further measurements of the RP and the EMR were made. It is notable that the RP of the muscle fibres fell more rapidly in all cases than in the experiments in which the RP alone was measured (compare tables 17 and 18). The reason for this appears to be that the fibres either suffered some damage during the measurement of the EMR, or that passing 300 mV sine wave had a depolarising effect upon the fibres. Since control experiments indicated that the EMR and RP did not change significantly in 60 minutes (see table 16) it appears that the rapid depolarisation observed was probably a combination of the effects of high temperature and some factor in the membrane resistance measuring technique. The general pattern of initial hyperpolarisation followed by depolarisation of the fibres was again observed, however, and the differences between the two adaptation groups were similar to those described previously.

The group mean EMR values obtained from table 18 are shown in figure 15. These results show that the EMR of muscle fibres from 10°C adapted animals decreased more rapidly than that of muscle fibres taken

TABLE 18.

The Effect of High Temperature on the Effective Membrane Resistance of Crayfish Muscle Fibres.

In vitro preparations of abdominal extensor muscles from three 10^{o} and two 25^{o}C adapted crayfish were mounted in the experimental chamber as described in the Methods (Chapter 3). Control measurements of the effective membrane resistance (EMR) were made while the preparation was maintained at the adaptation temperature of the crayfish from which it was obtained, and then raised to 32^{o}C. The EMR of the muscle fibres was then measured against time as described in the Methods and the results grouped into 5 minute intervals for presentation. Measurements of the resting potential (R^{p}) were made during the EMR measurements. The EMR was expressed in M and the RP in mV (inside negative).

TABLE 18 (a). $10^{o}C$ Adapted Crayfish.

Time (minutes)		Animal 1	2	3	$\bar{x}$
	RP	69.9	71.7	70.3	70.79
	SE	1.05	0.72	1.25	0.68
Control	EMR	2.624	1.789	1.644	2.033
	SE	0.301	0.171	0.134	0.121
	n	23	30	16	69
	RP	65.2	69.6	73.75	69.09
	SE	1.1	0.1	1.1	0.92
1 - 5	EMR	1.317	1.289	1.729	1.420
	SE	0.179	0.179	0.418	0.191
	n	11	10	8	29
	RP	-	61.1	58.25	59.83
	SE	-	2.82	4.51	2.51
6 - 10	EMR	-	0.952	1.306	1.109
	SE	-	0.148	0.284	0.251
	n	0	10	8	18
	RP	56.1	40.5	27.56	43.23
	SE	3.7	5.4	5.1	2.86
11 - 15	EMR	1.165	1.069	0.756	1.024
	SE	0.216	0.230	0.505	0.237
	n	9	8	6	23

TABLE 18 (b). 25°C Adapted Crayfish.

Time (minutes).		Animal 4	5	$\bar{x}$
	RP	71.6	73.9	72.73
	SE	1.19	1.3	1.21
Control	EMR	1.489	1.727	1.608
	SE	0.247	0.220	0.241
	n	16	16	32
	RP	72.6	79.3	75.81
	SE	2.5	1.5	2.64
1 - 5	EMR	1.679	1.759	1.717
	SE	0.274	0.210	0.205
	n	11	10	21
	RP	67.8	77.3	72.55
	SE	2.81	2.46	2.13
6 - 10	EMR	1.330	1.760	1.545
	SE	0.266	0.283	0.274
	n	10	10	20
	RP	63.7	72.2	67.95
	SE	3.3	3.4	4.10
11 - 15	EMR	1.022	1.870	1.446
	SE	0.136	0.270	0.261
	n	10	10	20
	RP	63.1	65.1	63.98
	SE	2.58	4.23	1.87
16 - 20	EMR	1.158	1.039	1.105
	SE	0.185	0.207	0.192
	n	10	8	18
	RP	55.9	57.8	56.85
	SE	1.7	3.8	1.75
21 - 25	EMR	0.865	0.779	0.822
	SE	0.162	0.177	0.141
	n	10	10	20
	RP	48.4	45.5	46.81
	SE	4.0	2.1	2.2
26 - 30	EMR	0.616	0.448	0.523
	SE	0.080	0.056	0.110
	n	9	11	20
	RP	37.25	36.9	37.06
	SE	4.0	4.1	3.37
31 - 35	EMR	0.636	0.280	0.438
	SE	0.046	0.043	0.126
	n	8	10	18

Figure 15.

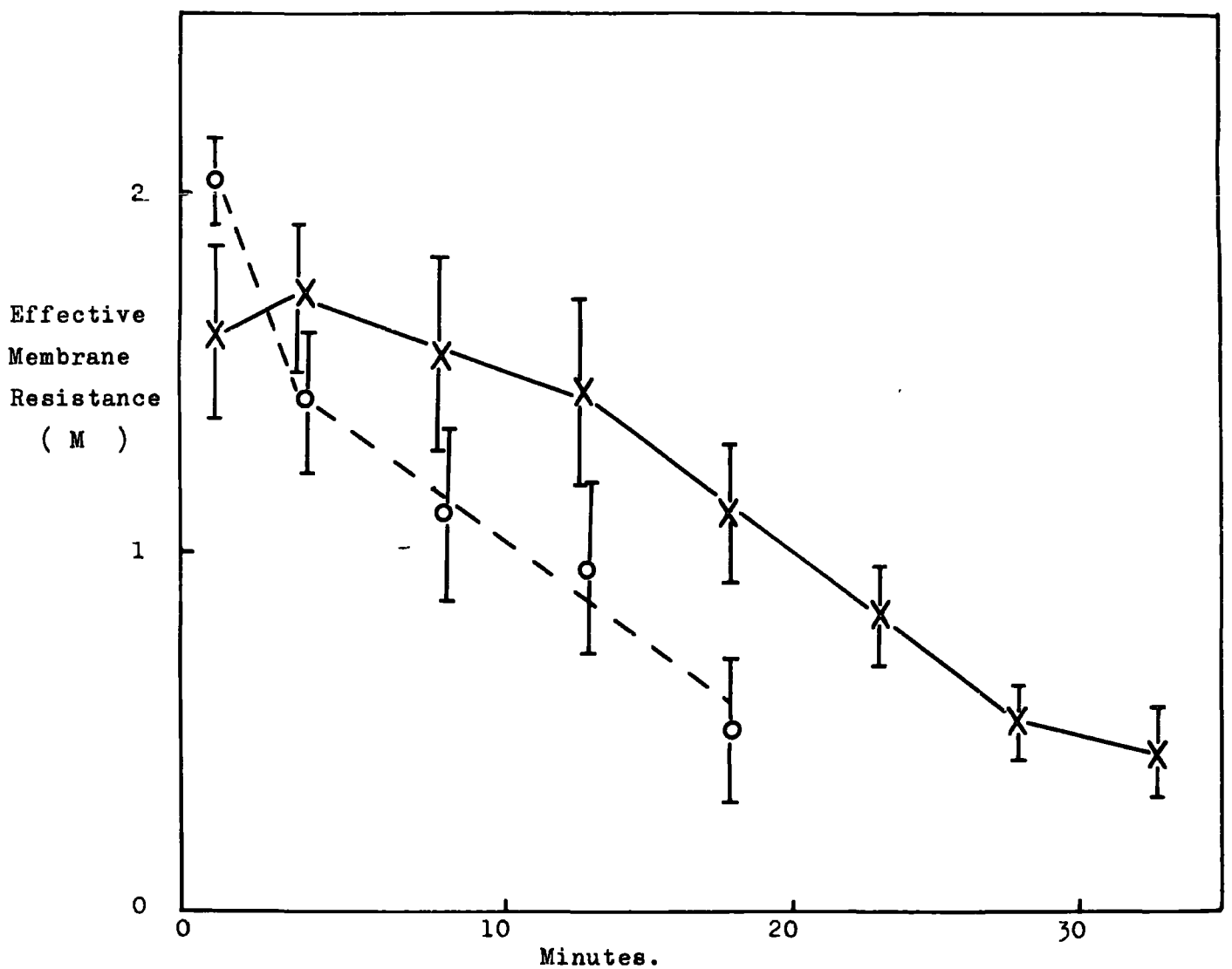
The Effect of Temperature adaptation on the Sensitivity of the Muscle Membrane Resistance to 32°C.

Three 10° and two 25°C adapted crayfish abdominal extensor muscle preparations were exposed to 32°C as described in the Methods. The mean Effective Membrane Resistance (EMR) of each adaptation group was calculated from the data of table 18 for 5 minute intervals and plotted against the exposure time. The vertical bars represent ± 1 standard error of the mean.

Mean 10°C adapted crayfish EMR ..... O— — —O

Mean 25°C adapted crayfish EMR ..... X———X

Figure 15.



from 25°C adapted crayfish. The mean EMR of 10°C adapted animal muscle fibres fell from 2.033 ± 0.121 to 1.024 ± 0.237 MΩ within 15 minutes of exposure to 32°C. The mean EMR of 25°C adapted animal muscle fibres decreased from 1.608 ± 0.241 to 0.438 ± 0.126 MΩ within 35 minutes of exposure to 32°C. The exposure time required for the muscle fibre EMRs to fall to half of their initial value were 12 and 25 minutes for the 10 and 25°C adapted animals respectively. The EMR of the muscle fibres from 10°C adapted crayfish was therefore considerably more sensitive to exposure to 32°C than the EMR of muscle fibres taken from 25°C adapted crayfish.

It is interesting to note that the data of table 18 indicate that the membrane resistance decreased before the muscle fibres depolarised. In preparations taken from 10°C adapted animals the EMR fell rapidly in the initial 5 minutes of exposure to 32°C, (from 2.033 to 1.420 MΩ), a fall of about 45%. The RP of the same muscle fibres did not fall significantly during this time (70.79 to 69.08 mV, see table 18). Comparing the group means for EMR and RP obtained for muscle fibres from 10°C adapted crayfish during the latter phases of heat death, i.e. 6-10 and 11-15 minute means given in table 18, it is evident that the EMR decreased relatively little from 1.109 to 1.024 MΩ (about 7.5%). The RP fell from -59.83 to -42.23 mV (about 57%) during the same period, the largest depolarisation rate observed. In the preparations taken from 25°C adapted animals, the muscle fibre RP only depolarised after about 15 minutes exposure to 32°C. They then depolarised at a steady rate of 1.6 to 2.0 mV per minute additional exposure time. The EMR of the muscle fibres began to fall at about the same time as the RP, falling from 1.446 to 1.105 MΩ in the period between 11-15 and 16-20 minutes exposure to 32°C, a fall of almost 25%. The EMR

continued to fall at a rate of about 0.06 MΩ per minute until it reached a value of about 0.500 MΩ after 26-30 minutes exposure to 32°C. It was therefore apparent in these experiments that the depolarisation of the muscle fibres on exposure to 32°C was preceeded by a fall in the EMR indicating an increased membrane ionic conductance.

4. The Effect of Extracellular Ions on the Muscle Fibre Resting Potential

The effect of extracellular K^+ concentration on the RP of muscle fibres was studied in both adaptation groups. The results are shown in figure 16. The changes in the extracellular K^+ concentration were of the order of the changes observed in haemolymph K^+ concentration during heat death. The concentrations of Cl^- ions in the saline was kept constant by replacing NaCl with KCl since the Na^- concentration is not critical in its effect on the muscle fibre RP (Fatt and Katz, 1953). Raising the extracellular K^+ concentration depolarised the muscle fibres. Increasing the saline K^+ from 5.4 to 10 mM resulted in a fall in the RP of 22 and 23 mV in 25 and 10°C adapted crayfish preparations respectively. The slope of the curve in figure 16 was 56.7 mV per tenfold change in extracellular K^+ which compares closely with the value of 58.5 mV predicted by the Nerst Equation for a K^+ electrode. This indicates that K^+ is the major permeant ion.

A second series of experiments were performed in which four 10°C adapted animals were exposed to 32°C until scathognathite beat ceased. The abdominal extensor muscles were then rapidly exposed and immersed in saline at 10°C. Measurements of the RP were then made in 'normal' saline, containing 5.4 mM KCl and 195 mM NaCl and then in 'heat death' saline containing 182 mM NaCl and 9.0 mM KCl, and other saline constituents remaining unchanged. The composition of the 'heat death' saline was determined by taking the mean haemolymph Na^+ and K^+ values recorded for 10°C adapted animals exposed to 32°C for 16 minutes

TABLE 19.

The Resting Potential of Muscle Fibres from Heat Dead Crayfish.

Four 10°C adapted crayfish were exposed to 32°C until scathognathite beat cessation and their abdominal extensor muscles removed and mounted for resting potential (RP) measurements as described in the Methods (Chapter 3). The RP was measured in modified Van Harreveld's saline at 10°C which contained 5.4 mM KCl + 195 mM NaCl. The saline was then changed to one containing 9.0 mM KCl + 182 mM NaCl which was made up to emulate the haemolymph monovalent cation concentrations observed in 10°C adapted crayfish which had been exposed to 32°C until the scathognathite beat ceased (see table 5). The RPs were expressed in mV, inside negative.

TABLE 19.

Animal	Normal Saline			'Heat Dead' Saline.		
	RP(mV)	SE	n	RP(mV)	SE	n
1	71.3	1.34	30	49.7	3.38	30
2	69.0	0.90	32	46.6	2.77	30
3	73.1	1.37	30	52.1	2.92	30
4	70.0	1.63	30	48.1	4.23	30
$\bar{x}$	70.8	0.47	122	49.1	2.72	120

Figure 16.

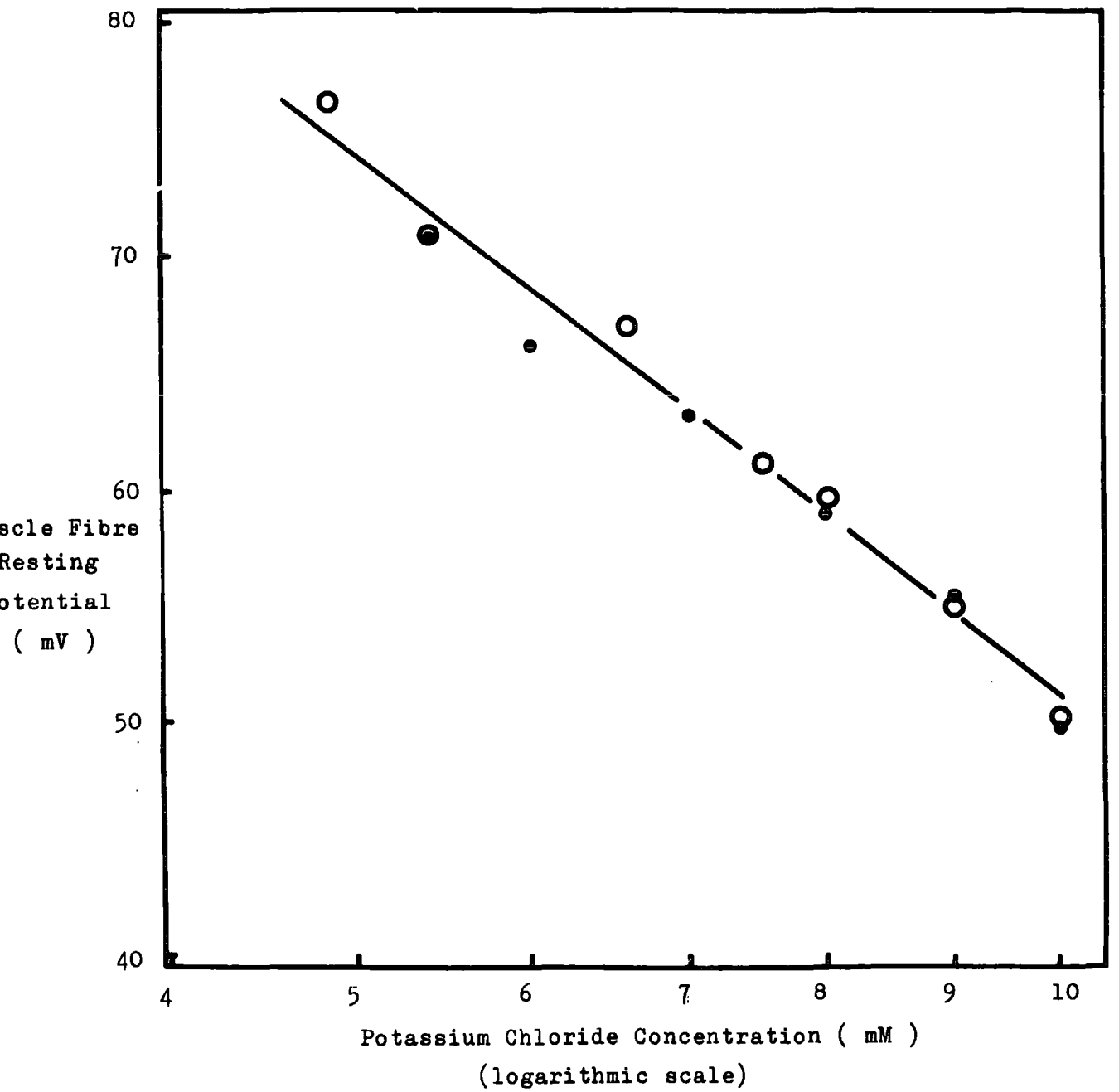
The Effect of Extracellular Potassium on the Resting Potential of Crayfish Muscle.

The extracellular K^+ concentration of abdominal extensor muscle preparations was varied over the range of concentrations observed in the haemolymph during heat death. The chloride concentration was kept constant. The RP was plotted against the logarithm of the K^+ concentration. The measurements were made on two 10^o and two 25^oC adapted crayfish preparations; the temperature being maintained at the adaptation temperature of the source animal. The curve was fitted by the method of least squares, the slope being 56.7 mV per tenfold change in extracellular K^+.

10^oC adapted crayfish preparations ..... O

25^oC adapted crayfish preparations ..... ●

Figure 16.


80
70
60
50
40
scle Fibre
Resting
otential
(mV)
4
5
6
7
8
9
10
Potassium Chloride Concentration (mM)
(logarithmic scale)

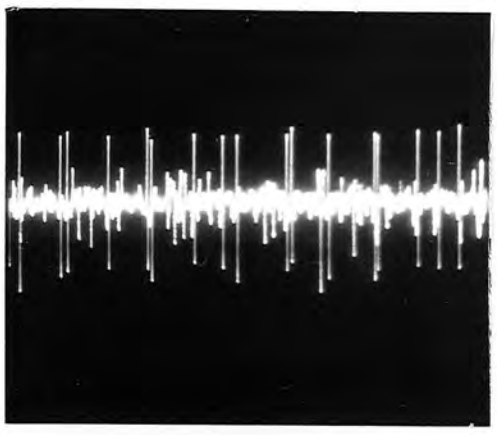
(see table 5). The results are shown in table 19. It can be seen that the preparations all have an RP of about -70 mV in 'normal' saline, i.e. they were very similar to the values obtained from unheated animals (see table 16).

When the preparations were immersed in 'heat death' saline, RPs of 45-50 mV were found. The RP obtained in normal muscle fibres when extracellular K^+ was 9 mM was about 55 mV, see figure 16, as can be seen from table 19, however the RP of muscle fibres from 'heat dead' animals, in the 'heat death' saline was about 49 mV and therefore considerably lower than would be predicted. Since the extracellular Na^+ concentration is not of critical significance to the RP due to its low permeability (Hinkle, Heller and Van der Kloot, 1971) it appears that heating the muscles in vivo potentiated the effect of raising the extracellular K^+.

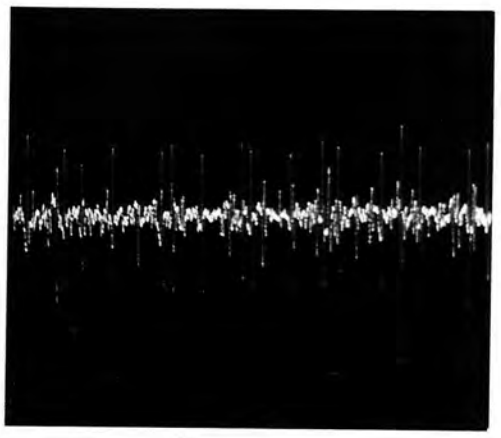
5. The Effect of 32°C Raised Extracellular Potassium on the Spontaneous Activity of the Ventral nerve Cord.

The ventral nerve cord of a 25°C adapted crayfish was exposed and the recording apparatus set up as described in the Methods. The temperature of the preparation was increased stepwise to 32°C and the spontaneous activity monitored, the results are shown in figure 18. The mean spontaneous action potential frequency at 25°C was 29.2 per second, rising to a mean frequency of 34.8 per second at a temperature of 32°C. This represents a Q_{10} value of 1.27, indicating that the increase in temperature had a relatively small effect upon the spontaneous activity of the nerve cord. After 30 minutes in 'normal' saline at 32°C, the normal saline was replaced with a saline in which the KCl concentration had been increased to 10 mM, the temperature was maintained at 32°C. This saline was made up to mimic the haemolymph K^+

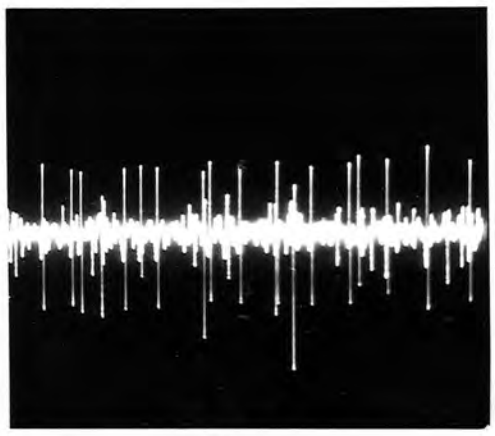
Figure 17



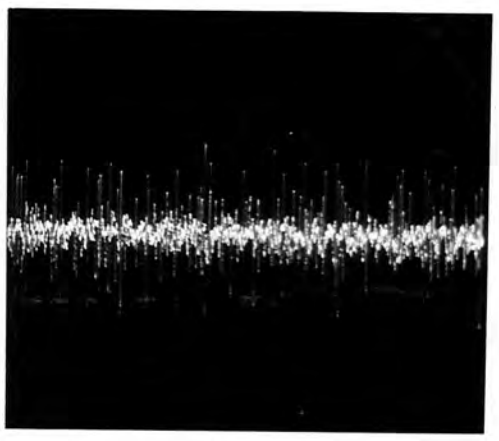
i



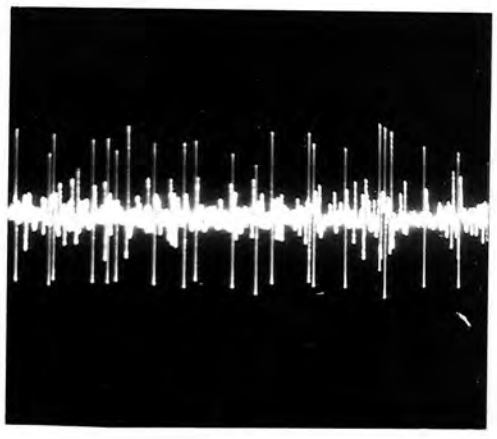
iv



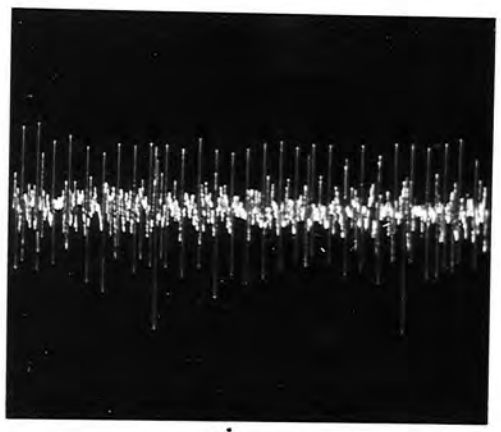
ii



v



iii



vi

Figure 17.

The Effect of High Temperature and Extracellular Potassium on Spontaneous Action Potentials of the Ventral Nerve Cord.

The ventral nerve cord of two 25°C adapted crayfish was exposed and activity displayed on a Tektronix oscilloscope described in the Methods. Photographs of the activity were taken with a Cossor Oscilloscope Camera. Each trace represents an 0.5 second sweep.

Trace i. Activity in normal saline at 25°C.

Trace ii. Activity in normal saline after 10 minutes' exposure to 32°C.

Trace iii. Activity in normal saline after 30 minutes' exposure to 32°C.

Trace iv. Activity in normal saline at 25°C.

Trace v. Activity after 30 seconds exposure to saline containing 10 mM K^+.

Trace vi. Activity after 180 seconds exposure to saline containing 10 mM K^+.

Traces i, ii, and iii were obtained from one animal and traces iv, v and vi were obtained from another.

Figure 18.

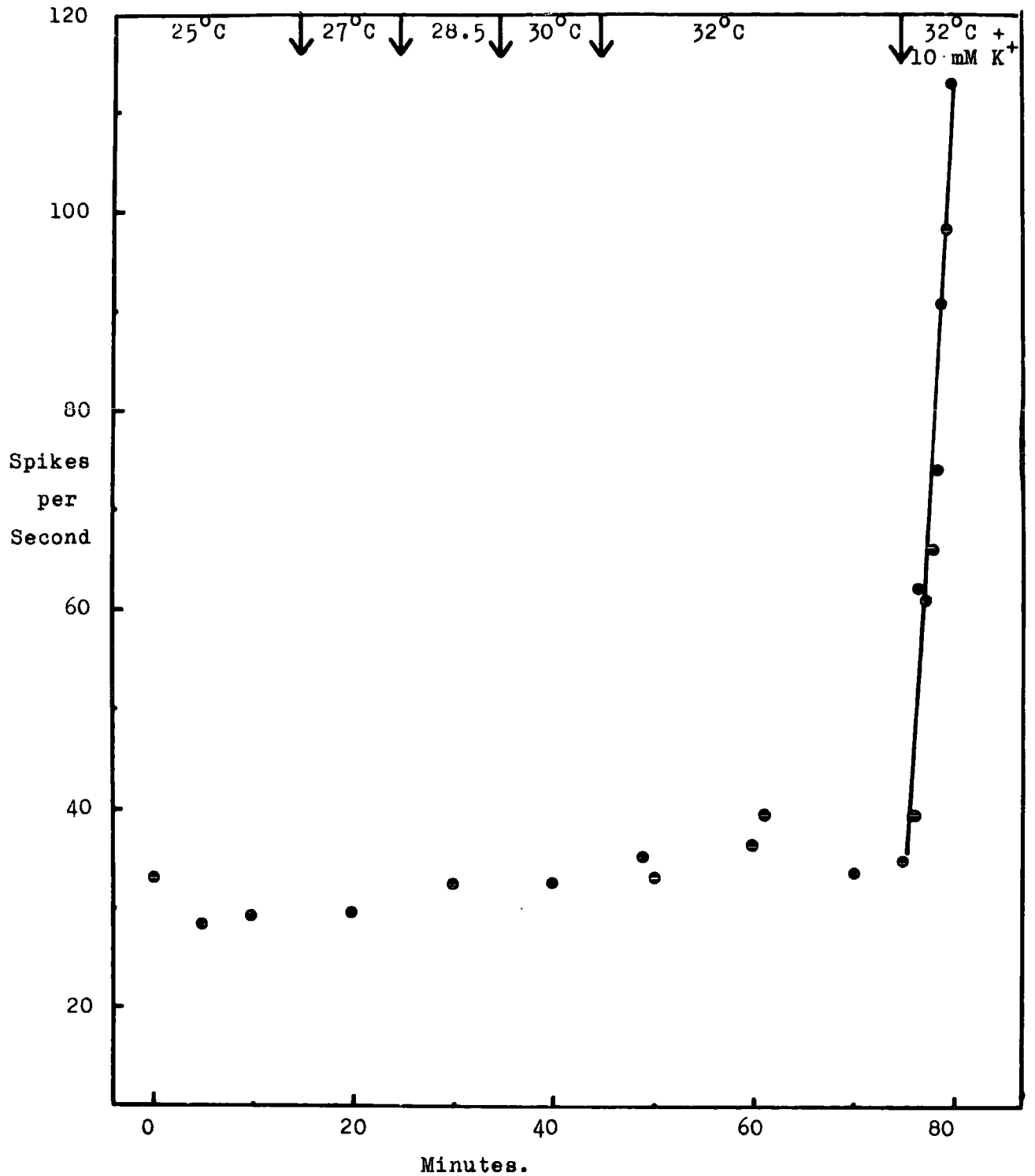
The Effect of Exposing the Ventral Nerve Cord of a Crayfish to High Temperature and Extracellular Potassium.

The frequency of spontaneous action potentials in the ventral nerve cord of a 25°C adapted crayfish was monitored as described in the Methods, and plotted against the duration of the experiment while

1) the temperature was raised 'step-wise' from 25° to 32°C

2) the saline was replaced by a 'high K^+' saline containing 10 mM K^+, the temperature being maintained at 32°C.

Figure 18.


120
100
80
Spikes
per
Second
60
40
20
25°C
27°C
28.5
30°C
32°C
32°C +
10 mM K+
0
20
40
60
80
Minutes.

concentrations observed in 25°C adapted crayfish exposed to 32°C for 45 minutes (see table 4), that is to say the approximate time of heat death. The effect of the high K^+ saline at 32°C was marked, the frequency of action potentials rising from about 34 per second to over 56.5 per second within 5 minutes of exposure, see figure 18.

A series of experiments on 3 crayfish adapted to 10°C and 3 crayfish adapted to 25°C was performed to confirm the previous observations on the effect of temperature. The mean spontaneous action potential frequency of the nerve cords were measured over a period of 30 minutes in saline at the adaptation temperature of the animal from which the preparation was obtained, and then for a further 30 minute period during which the nerve cord was exposed to 32°C. The results are shown in table 20. The Q_{10} values all lie between 1.1 and 1.33, so confirming that increasing the temperature of the ventral nerve cord in normal saline does not markedly affect the spontaneous action potential frequency. (see also figure 17)

To determine whether heating the ventral nerve cord in vivo produced a different effect, one crayfish of each adaptation group was exposed to 32°C until cessation of scathognathite beat. The ventral nerve cord was then exposed and the spontaneous activity monitored at either 10 or 25°C as appropriate. These results are also presented in table 20. After 5 minutes in normal saline, the saline K^+ concentration was raised to 10 mM. The results in table 21 show that the spontaneous activity frequency observed during the initial 5 minutes in normal saline was quite comparable to the frequencies observed in unheated animals. Furthermore, the rapid increase in the spontaneous activity which occurred when extracellular K^+ concentration was increased to 10 mM indicated that the ability of the nerves to respond to depolarisation with action potentials was not critically impaired. (see also figure 17)

TABLE 20.

The Effect of High Temperature on the Spontaneous Activity of the Crayfish Ventral Nerve Cord.

The ventral nerve cords of three 10^{o} and three $25^{o}C$ adapted crayfish were exposed by ventral dissection and the spontaneous activity monitored as described in the Methods (Chapter 3). Recordings were made of the spontaneous activity at the adaptation temperature of the experimental animal and at $32^{o}C$. The activities quoted were the mean values for the activity observed during a 30 minute exposure period at each temperature. The Q_{10} values were calculated from the mean activities.

TABLE 20.

Animal	Adaptation Temperature (^{o}C)	Spontaneous Frequency of Spikes (number per second).		Q_{10}
		25^{o}C	32^{o}C	
1		30.7	33.7	1.14
2	25	32.0	39.3	1.325
3		33.5	39.5	1.26
		10^{o}C	32^{o}C	
4		27.75	38.8	1.18
5	10	28.2	39.2	1.16
6		28.45	39.95	1.20

TABLE 21.

The Effect of High Extracellular K^+ on the Spontaneous Activity of the Crayfish Ventral Nerve Cord.

The ventral nerve cord of one 10^{o} and one $25^{o}C$ adapted crayfish were exposed by ventral dissection and the spontaneous activity monitored as described in the Methods (Chapter 3). Measurements of the spontaneous activity were made over five minute periods while the nerve cord was immersed in:

(1) Modified Van Harreveld's saline (5.4 mM KCl + 195 mM NaCl)

(2) High K^+ Van Harreveld's saline (10 mM KCl + 190 mM NaCl).

The temperature of the preparation was maintained at the adaptation temperature of the animal from which the preparation was obtained.

TABLE 21.

Time (minutes)	Saline	25°C Adapted frequency (spikes/sec).	10°C Adapted frequency (spikes/sec).
0 - 1		36.75	29.3
1 - 2	Normal saline	32.7	25.8
2 - 3	Na^+ = 195 mM	32.8	26.2
3 - 4	K^+ = 5.4 mM	33.5	26.8
4 - 5		36.2	25.7
% change after 5 minutes		-1.5	-12.3
5 - 6		43.3	33.7
6 - 7	High K^+ saline	47.7	34.5
7 - 8	Na^+ = 190 mM	51.9	47.2
8 - 9	K^+ = 10 mM	48.2	56.4
9 - 10		50.4	47.5
% change from control after 5 minutes		+39.2	+84.8

The effect of the concentration of extracellular K^+ on the spontaneous activity of the ventral nerve cord was investigated in 3 animals from each adaptation group. More than one animal was necessary for this since it was observed that nerve cords exposed to high extracellular K^+ did not always recover completely. The time of exposure appeared to be the critical factor, the nerves becoming damaged if exposed for long periods (i.e. longer than about 3 minutes). These experiments were all performed at 25°C and the data are presented in figure 1q. It can be seen from this figure that the initial spontaneous activity of the ventral nerve cord varied in a predictable fashion with the extracellular K^+ concentration. The slope of the curve of figure 1q indicates that the spontaneous activity of the ventral nerve cord increased 3.5 times per ten-fold increase in extracellular K^+.

TABLE 22.

The Effect of High Temperatures on the ATPase activity of Crayfish Nerves.

Nerve cords were dissected from crayfish, homogenised and the microsomal fraction purified by differential centrifugation.

(a) A microsomal preparation was obtained from the nerve cords of 7 crayfish, aliquots were either preincubated at 35°C for 30 minutes, or stood on ice prior to incubation. Duplicate aliquots were incubated with either 3 mM $MgCl_2$ + 2 mM ATP or 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl + 2 mM ATP at 30°C for the times shown. ATPase activity was expressed as n moles Pi liberated per aliquot.

(b) A microsomal preparation was obtained from the nerve cords of four 8°C adapted crayfish. Duplicate aliquots of resuspended microsomes were preincubated for 10 minutes at 25°, 34°, 35° or 40°C, and then incubated for 30 minutes at 25°C with either 3 mM $MgCl_2$ + 2 mM ATP or 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl = 2 mM ATP. ATPase activity was expressed as μ moles Pi liberated per mg protein per hour.

(c) A microsomal preparation was obtained from the nerve cords of four crayfish which had been killed by 30 minutes exposure to 35°C. Duplicate aliquots of resuspended microsomes were incubated at 30°C with either 3 mM $MgCl_2$ + 2 mM ATP or 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl + 2 mM ATP for the times shown. ATPase activity was expressed as n moles Pi liberated per aliquot.

TABLE 22.

(a)

35°C Preinc. Time	Total ATPase 0'	Total ATPase 30'		Mg^{++} ATPase. 0'	Mg^{++} ATPase. 30'
Incubation time			Incubation time		
2	23	17	2	2	5
5	39	30	10	11	15
10	80	61	20	38	26
15	130	108	30	54	43
20	150	133	40	-	60
25	173	202	50	85	51
30	205	223	60	93	65
35	268	265	70	93	79

(b)

10' Preinc. Temp (°C)	Mg^{++} act. ATPase	$Na^{+} + K^{+}$ act. ATPase
25	0.54	3.8
34	0.55	3.4
35	0.47	3.4
40	0.49	3.6

(c)

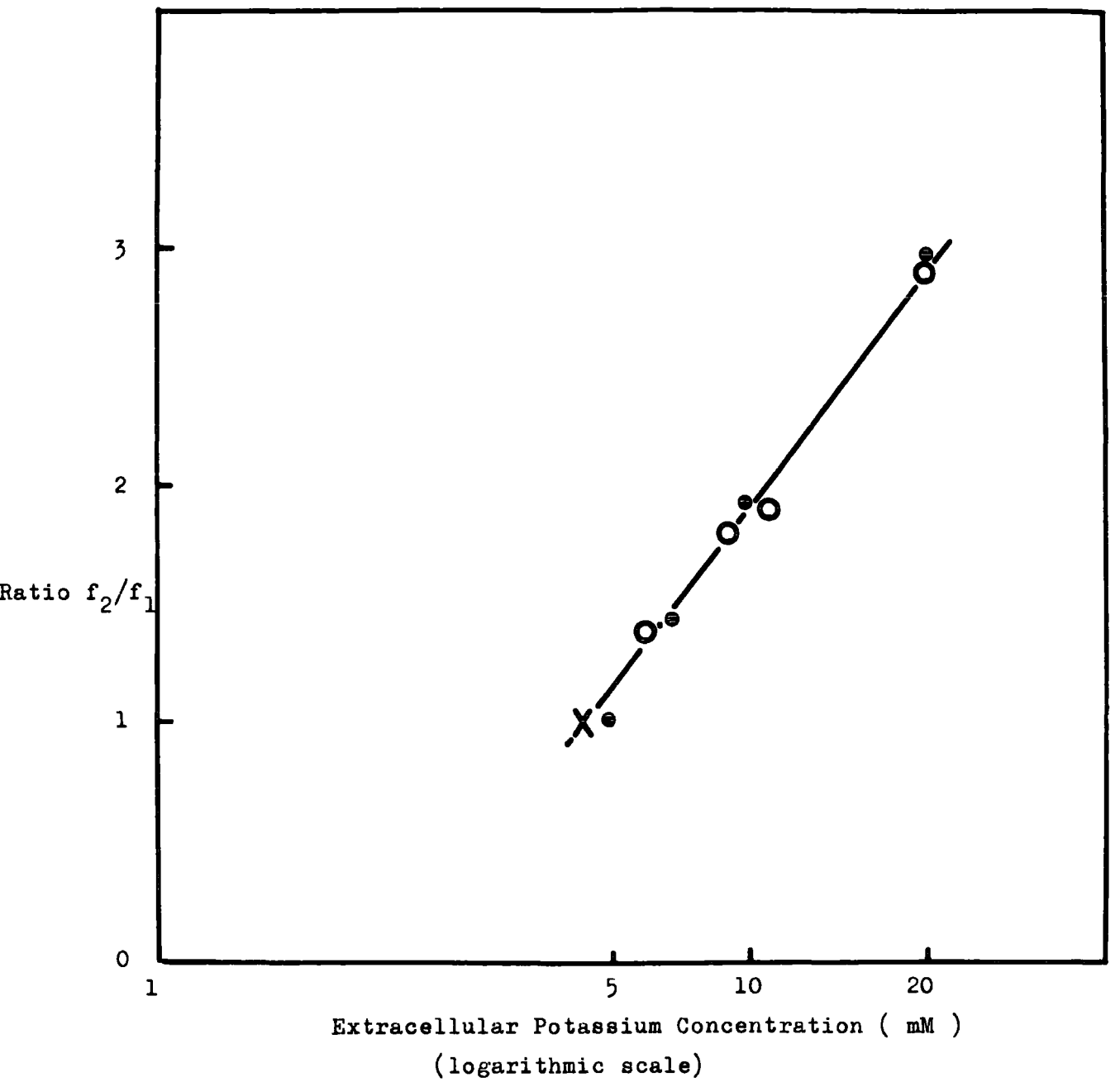
Incubation time (minutes).	Mg^{++} act. ATPase	$Na^{+} + K^{+}$ act. ATPase.
10	10	31
15	15	50
20	21	68
25	26	93
30	36	114
40	33	132

Figure 19.

The Effect of Extracellular Potassium on the Spontaneous Activity of the Crayfish Ventral Nerve Cord.

The ratio of the spontaneous activity observed during the initial 30 seconds of exposure to high K^+ (f_2) over the spontaneous activity recorded in normal saline (f_1) was plotted against the logarithm of the extracellular K^+ concentration. The results were obtained from the experiments with three 10^o (O) and three 25^oC (X) adapted crayfish, the temperature of the preparations was maintained at the adaptation temperature of the source animal. The curve was fitted by the method of least squares, the slope being a 3.5 times increase in spontaneous activity per tenfold change in the extracellular K^+ concentration.

Figure 19.


3
2
1
0
Ratio f_2/f_1
1
5
10
20
Extracellular Potassium Concentration (mM)
(logarithmic scale)

DISCUSSION

The mean RP of crayfish abdominal extensor muscle fibres bathed in modified Van Harreveld saline at the appropriate adaptation temperature was found to be -70.12 ± 0.33 and -70.53 ± 0.40 mV for 10 and 25°C adapted animals respectively (see table 17). These values are similar to those given in the literature for crayfish muscle fibres, e.g.

Species	Muscle	Resting Potential	Reference
Astacus fluviatilis	Carpopodite extensors of walking legs	-74 mV	i
Procambarus spp.	Single fibres from walking legs	-59 mV	ii
Orconectes limosus Rafinesque	Contractor epimeralis	-70 mV	iii
Orconectes virilis	Superior extensor abdominalis medialis	-74.5 mV	iv

References: -

(i) Fatt and Ginsborg (1958). (ii) Reuben, Girardier and Grundfest (1964)

(iii) Dudel, Morad and Rudel (1968). (iv) Hinkle, Heller and Van der Kloot (1971)

The effect of temperature on the resting potential and membrane resistance of muscle fibres from Carcinus maenas and Portunus depurator was investigated by Fatt and Katz (1953). They reported that exposing the muscle fibres to temperatures approaching or exceeding the upper physiological limits of the crabs resulted in hyperpolarisation of the muscle fibres. This was associated with a large decrease in the membrane resistance which became irreversible if the temperature exceeded the physiological range of the crab. Adaptation of the crabs to temperatures

in the upper region of their range resulted in increased resistance of the muscle fibres to high lethal temperatures.

The results presented in this Chapter agree quite well with the results of Fatt and Katz (1953) described above. Exposing crayfish muscle fibres to high lethal temperatures resulted in hyperpolarisation (see table 14) and a large fall in the effective membrane resistance, (see table 15). Fatt and Katz (1953) did not give their exposure times, but it is probable that the exposure times were brief since no depolarisation of the muscle fibres was reported. The results presented in table 18 show that the depolarisation observed in these experiments followed an increase in the membrane conductance. The time course of the sequence of initial hyperpolarisation, fall in membrane resistance and depolarisation of the muscle fibres differed with the adaptation temperature of the crayfish, being more rapid in muscle fibres taken from the animals adapted to the lower temperature.

The initial hyperpolarisation could be caused by a number of events, the two most likely are (1) the activation of an electrogenic pump and (2) a change in the ionic permeability of the plasma membrane. Electrogenic pumps must move unequal numbers of charged particles across a membrane to produce a potential difference. In the muscle fibres this probably means that the cation pump moves more Na^+ out of than K^+ into the cells, so building up a net increased negative potential inside the cell. The presence of a $Na^+ + K^+$ activated ATPase has been demonstrated in crayfish sarcolemma by Bowler and Duncan (1968), and further evidence for its presence is presented in chapters 4 and 5. This enzyme is stimulated by intracellular Na^+ (see Whittham and Wheeler, 1970), and will also be stimulated by a rise in temperature. It is therefore possible to argue that an electrogenic pump will be stimulated by either of these events. Intracellular Na^+ concentra-

tions might rise during heat exposure due to an increased passive Na^+ permeability. There is no direct evidence of an immediate increase in sarcolemmal Na^+ permeability in the membrane resistance data of table 18, for while the 10°C adapted animal muscle fibres show a fall in EMR in the first 5 minutes of exposure, the 25°C adapted muscle fibres do not, even though they are hyperpolarised during this period. It appears likely that the initial hyperpolarisation is caused in the first place by a stimulation of an electrogenic pump by the rise in temperature, this may be correlated with the elevated haemolymph Na^+ concentrations observed during the early phases of heat death (see tables 4 and 5). The fall in sarcolemmal resistance must be due to an increased passive permeability to small ions. Such an increase would allow Na^+ ions into the muscle fibres which would stimulate the Na^++K^+ activated ATPase and hence tend to hyperpolarise the fibres, and also allow K^+ ions to leave the fibre which will ultimately lead to the depolarisation of the muscle fibres.

This sequence of events can be correlated with the haemolymph and muscle ion changes during heat death described in Chapter 2. Crayfish exposed to temperatures at which their LD_{50} was in excess of 15 minutes showed an initial increase in haemolymph Na^+, there being a relatively small increase in haemolymph K^+ at the same time. This was followed by a fall in haemolymph Na^+ and a rapid increase in haemolymph K^+ (see tables 4 and 5). The initial hyperpolarisation of the muscle fibres can be correlated with an activation of an electrogenic pump which pumped Na^+ out of the cells, so increasing haemolymph Na^+, and intracellular K^+. An increase of intracellular K^+ during the initial phases of heat death was observed in animals exposed to lethal temperatures for a short time, see table 8 (10°C adapted crayfish exposed to 34°C) and table 9 (10°C adapted crayfish exposed to 32°C). Further exposure to

lethal temperatures brought about an increase in intracellular muscle Na^+ (see tables 8, 9 and 15) and a decrease in intracellular K^+ which can be correlated with the increased membrane conductance. Increased membrane conductance means that the passive permeability of the membrane to small ions will be increased, thus entry of Na^+ into the cells will allow K^+ to escape and the cells depolarise. The haemolymph Na^+ concentration would then decrease and the haemolymph K^+ concentration increase, which is in fact what was observed (see tables 4 and 5). The evidence presented in the next Chapter and the results of Bowler and Duncan (1968) indicate that the Na^++K^+ activated ATPase is more resistant to high lethal temperatures than the whole animal, and that the cation pump would still be active during the depolarisation of the muscle fibres. It would therefore oppose the ionic movements brought about by increased passive permeability and so tend to maintain the polarisation of the muscle fibres, being stimulated by the increased intracellular Na^+. Depletion of substrate and further increases in the passive permeability of the sarcolemma would be expected to overcome the cation pump however, leading to the depolarisation of the fibre. This cation pump activity probably explains the delay between the increased sarcolemmal conductance and depolarisation of the muscle fibres. The ion movements between muscle and haemolymph reported in Chapter 2 therefore correlate quite well with the hypothesis advanced above to explain the electrophysiological events observed during exposure of muscle fibres to lethal temperatures apart from the observed small initial rise in haemolymph K^+. The data presented in table 15 suggest that there might have been another compartment apart from the muscle and haemolymph which was involved in the K^+ movements during the heat death which could have been responsible for the slow haemolymph K^+ increase during the initial phases of heat death. The observed increase in muscle fibre K^+

after brief periods of exposure to lethal temperatures tends to support this view, indicating that muscle fibre K^+ loss was not responsible for the initial rise of haemolymph K^+.

It is therefore suggested that the depolarisation of the muscle fibres during exposure to high lethal temperatures was caused by an increase in the passive permeability of the sarcolemma, and that the changes in the activity of the cation pump and the passive permeability of the sarcolemma are to a large extent responsible for the haemolymph Na^+ and K^+ changes reported in Chapter 2. This implies that the greater heat resistance of muscle fibres taken from warm adapted animals was due to an ability to maintain their normal low passive permeability for longer than the cold adapted crayfish muscle fibres.

It is interesting to note that the resting potential of muscle fibres taken from heat dead animals was normal when immersed in 'normal' saline at room temperature and was not significantly different from those of muscle fibres taken from unheated crayfish (see table 19). Hinkel, Heller and Van der Kloot (1971) found that when crayfish muscle fibres were depolarised the permeability constants of the sarcolemma altered. This suggests that the increased Na^+ permeability of the sarcolemma during heat death was reversible. Returning the muscle fibres to cool saline with normal Na^+ and K^+ concentrations restored the RP indicating that the muscle fibres were not irreversibly damaged by exposing crayfish to lethal temperatures until cessation of scathognathite beat. This also indicates that the Na^++K^+ activated ATPase of the sarcolemma had not been permanently inactivated during heat death, since the normal RP values indicated that the intracellular K^+ and Na^+ levels had been adjusted to their normal levels.

The effect of increasing the K^+ concentration of the saline (see figure 16) was to depolarise the muscle fibres. This agreed

with the findings of Hinkle, Heller and Van der Kloot (1971) in that the depolarisations produced by increasing the extracellular K^+ concentration were similar to those predicted by the Nernst Equation, indicating that the fibres approximate K^+ electrodes. No spontaneous contractions of the muscle fibres were observed when the extracellular K^+ was increased to the concentrations observed in the haemolymph during heat death. It is therefore unlikely that the rapid limb twitches observed during the latter stages of heat death can be explained by muscular dysfunction.

The effects of increasing the extracellular K^+ concentration of the ventral nerve cord <u>in vitro</u> were marked. There was a large increase in the spontaneous activity (see figure 18 and table 21) which could be related to the extracellular K^+ concentration during the initial period of exposure. The sensitivity of the ventral nerve cord to extracellular K^+ is illustrated by the steep curve of figure 18 which indicated a 3.5 fold increase in spontaneous activity for a ten-fold increase in extracellular K^+. Exposure to high lethal temperature (32^oC) for a prolonged period (30 minutes) had only a small effect on the frequency of the spontaneous activity, indicating a Q_{10} of about 1.2 for nerve cords from animals of both adaptation groups (see table 20). Three experiments performed by Bowler and Duncan (personal communication) on the temperature sensitivity of crayfish nerve cord ATPases support these results. Their results (see table 22) showed that:

1. Preincubation ATPase preparations from crayfish nerve cords at 35^oC for 30 minutes, a treatment sufficient to kill 25^oC adapted crayfish by Bowler's criteria (see Bowler, 1963 a), did not greatly affect the activity of either the Mg^{++} or the Na^++K^+-activated ATPase.
2. Preincubating ATPase preparations obtained from 8^oC adapted crayfish nerve cords for 10 minutes at temperatures up to 40^oC did not markedly inactivate either ATPase.

3. ATPase preparations obtained from the nerve cords of crayfish which had been killed by 30 minutes exposure to 35°C were shown to possess considerable ATPase activity.

In view of the central role which the ATPases of the neurilemma are known to play in nerve functions, and the low Q_{10} of the spontaneous activity, it seems reasonable to suggest that the nerve cord of the crayfish is not greatly affected by temperature during heat death in the crayfish. The elevated haemolymph K^+ concentrations which were observed during the latter phases of heat death however, (see Chapter 2) would cause dramatic changes in the spontaneous activity of the nerve cord. Such an increase in the spontaneous activity can be correlated with the loss of co-ordination and the onset of limb twitching and fibrillation which was observed during the latter phases of heat death (see Chapter 1).

The results presented in this chapter therefore suggest that the following sequence of events occurred during heat death:

1. An increase in the activity of a sarcolemmal cation pump leading to an initial hyperpolarisation of the muscle fibres.
2. An increase in the passive permeability of the sarcolemma leading to depolarisation of the muscle fibre, elevated haemolymph K^+ and lowered haemolymph Na^+.
3. An increase in the spontaneous activity of the nervous system brought about by the elevated haemolymph K^+ and leading to the breakdown of neural co-ordination, and ultimately, death.

The results have one other important implication in that the ability of warm adapted crayfish to resist high temperatures is due to their ability to resist the breakdown of the sarcolemmal passive permeability at these high temperatures. It has been proposed by Duncan

(1965 and 1967) and Bowler and Duncan (1968) that the passive permeability of excitable cell membranes is controlled by a membrane-bound Mg^{++} activated ATPase (E C 3.6.1.3). The results therefore suggest that temperature adaptation may alter the thermal sensitivity of this enzyme. An investigation into the temperature sensitivity of sarcolemmal ATPases was carried out and has been described in Chapter 4.

CHAPTER 4

INACTIVATION OF SARCOLEMMAL ADENOSINE TRIPHOSPHATASES BY HIGH TEMPERATURE

INTRODUCTION

Skou (1957) was the first to describe an adenosine triphosphatase (ATPase) in particles from peripheral nerves of the crab. Similar enzyme systems have since been described in many tissues, e.g. brain (Hess and Pope, 1957); erythrocytes (Post, 1959 and Post, Merritt, Kinsolving and Albright, 1960); muscle (Schwartz, 1962); electroplax (Albers and Koval, 1962); liver (Emmelot and Bos, 1962); intestine (Taylor, 1962) and frog skin (Bonting and Caravaggio, 1963). The membrane bound ATPase activity of these cells may be divided into two components with different properties (Skou, 1957, 1960, 1962; Post, Merritt, Kinsolving and Albright, 1960; Dunham and Glynn, 1961). These differences may be summarised as follows, the first component is inhibited by cardiac glycosides and very low concentrations of calcium ions. The presence of magnesium, Na^+ and K^+ ions is essential for activity. The second component is not inhibited by cardiac glycosides, is stimulated by low calcium concentrations and does not require the presence of Na^+ or K^+ ions. These two components have been termed the Na^+-K^+ activated and the Mg^{++} activated ATPases respectively.

There has been some discussion as to whether these two components are in fact part of the same enzyme system. Thus Ribo and Ponz (1969) have suggested that the Mg^{++} activated ATPase activity in the small intestine of the rat is merely the Na^+-K^+ activated ATPase functioning inefficiently. Skou and Hilberg (1965) suggested that the activity of the two components was due to the same enzyme, and Marchesi and Palade (1967) were unable to show two separate sites for the two

enzyme components of the erythrocyte membranes. The results presented by many other authors who have subjected the partially purified enzyme to a variety of different treatments (e.g. Nakao, Nagano, Adachi and Nakao, 1963; Jorgensen, 1967; Bowler and Duncan, 1968; Rubenstein and Scholefield, 1969; Ellory and Smith, 1970) support the view that the two components are separate enzymes. The kinetic data of Robinson (1967) and Garrahan, Pouchan and Regan (1969) also support this view.

It has been clearly established that the Na^++K^+ activated ATPase is involved in the active translocation of Na^+ and K^+ ions across the plasma membrane (Skou, 1957; Dunham and Glynn, 1961; Levi and Pisareva, 1970; Lowe, 1970). The physiological role of this enzyme has been reviewed by Skou (1965) and by Katz and Epstein (1968). The enzyme has been shown to have a relatively high substrate specificity (Schöner, Beusch and Kramer, 1968) and adenosine triphosphate (ATP) as opposed to other high energy phosphates. Whittam and Wheeler (1970) have reported that a minimum of 20% of the total energy production of human erythrocytes and squid giant axons is utilised for Na^+ extrusion by this enzyme, In view of the significant role that this enzyme plays in cellular metabolism it is not surprising that investigations into the structure and mechanism of this enzyme are numerous in the current literature.

The mechanism of the Na^++K^+ activated ATPase in effecting active ion transport is still unknown. Recent hypotheses of mechanisms include those of Garrahan, Pouchan and Regan, 1969; Weiss, 1969a, b, c and d; Massari and Azzone, 1970 a and b; Rossi and Azzone, 1970; Scarpa and Azzone, 1970; and Whittam and Wheeler, 1970. It appears that the presence of Na^+ ions at the inner surface of the plasma membrane together with the Mg^{++}-ATP substrate complex is required to initiate functional enzyme activity. Bader, Post and Bond, (1968) and Blostein (1968) have presented evidence for the existence of phosphorylated

intermediates and Fahn, Koval and Albers (1968) and Post, Kume, Sen, Tobin and Orcutt (1968) have produced evidence for two forms of phosphorylated intermediate. These authors have suggested that K^+ ions catalyse the breakdown of one of the phosphorylated intermediates into inorganic phosphate and the $Na^+ + K^+$ activated ATPase. This phosphorylated intermediate is suggested to be an acyl phosphate due to its sensitivity to alkaline hydrolysis and to hydrolysis by purified acyl phosphate or hydroxylamine (Whittam and Wheeler, 1970).

Weiss (1969,c) has produced a possible molecular model for an ATPase ion pump, and Eisenman, Ciani and Szabo (1968) have investigated some molecular ion carrier properties using artificial lipid bilayer membranes which show some resemblance to naturally occurring ion translocating mechanisms. There are many problems inherent in attempting to elucidate the structural details of a complex enzyme by kinetic studies, but this has been attempted (e.g. Robinson, 1967a, 1967b, 1968 and 1969; Garrahan, Pouchan and Rega, 1969). These kinetic studies have lead to some confusion as to whether or not the ATPase is allosteric (see Priestland and Whittam, 1968 and Robinson 1968, for example). Somogyi (1968) has concluded from enzymic digestion studies that the enzyme is allosteric. On the basis of his results he concluded that the enzyme shows conformational changes such that the presence of $Na^+ + Mg^{++} + ATP$ stabilises one form, and the presence of $K^+ + Mg^{++} + ATP$ stabilises a second conformation.

The molecular weight of the ATPase is difficult to determine because the enzyme has not been purified to date. Mizuno, Nagano, Nakao, Tashima, Fujita and Nakao (1968) have used differential centrifugation techniques to arrive at a proposed molecular weight of 500,000 for pig brain ATPase. Kepner and Macey (1968), using <u>in vacuo</u> radiation techniques, estimated the molecular weights of human erythrocyte and

guinea pig brain ATPases to be about 250,000. Accurate determination of ATPase molecular weight will probably have to wait until the enzyme can be purified, however. The major problem in the purification of ATPases is that they appear to be closely associated with membrane phospholipids. The basis of this relationship is not clear, but Wheeler and Whittam (1970) have shown that human erythrocyte ATPase specifically requires phosphatidylserine;. Smith and Kemp (1968) have also shown that the addition of phospholipase C affects the ATPases of Goldfish intestinal mucosa. Tanaka and Sakamoto (1969) have shown that the structural organisation of the phospholipid is required to activate ATPases of bovine cerebral cortex. These results suggest that the structural configuration of the ATPase may be dependent upon phospholipid/enzyme interactions. In view of Somogyi's results (1968) and the hypothesis advanced by Weiss (1970a, b, c and d), it is possible that the structural relationship between phospholipid and ATPase is closely related to an allosteric enzyme mechanism.

The function of the Mg^{++} activated ATPase has received less attention, as has its structure and mechanism. Duncan (1965, 1966 and 1967) and Bowler and Duncan (1968) have suggested that this enzyme is involved in the control of passive membrane permeability. Klein and Breland (1966) have reported a Mg^{++} activated ATPase in the microsomal fraction of Acanthamoeba species homogenates which was insensitive to cardiac glycosides. No $Na^{+} + K^{+}$ activated ATPase was found. They suggested that the Mg^{++} activated ATPase was responsible for active, aerobic K^{+} transport which supports the view that this enzyme is involved in the control of ion transport across cell membranes.

The experiments described in this chapter were designed to determine whether or not these membrane localised ATPases were involved in the physiological process of adaptation and/or in heat death in the crayfish.

MATERIALS AND METHODS

Astacus pallipes were adapted to either 10 or 25°C as described in Chapter 1. The abdominal flexor muscles were rapidly dissected free, weighed and minced in ice-cold 0.25 M sucrose + 1 mM ethylene diaminotetracetic acid (EDTA) in 50 mM histidine-HCl buffered to pH 7.25. The minced muscle was then homogenised in a Potter-Elvejehm homogeniser with a teflon pestle (clearance 0.10-0.15 mm) with 5 passes of the pestle at approximately 1,000 r.p.m. The homogenate was kept cold during homogenisation by surrounding the Pyrex homogeniser tube with ice. The resulting homogenate was then transferred to centrifuge tubes and centrifuged at 2,000 g for 10 minutes in a refrigerated M.S.E. Mistral 2L centrifuge. The supernatant was then separated from the cell nuclei and debris and transferred to clean, precooled, centrifuge tubes. This supernatant was then spun at 14,000 g for 20 minutes in a refrigerated M.S.E. 'Superspeed 40' (head number 2409) to remove mitochondria. The supernatant was spun at 14,000 g to ensure that no mitochondria remained. The supernatant from the second mitochondrial spin was spun at 107,000 g for 60 minutes in the 'Superspeed 40' (Head number 2409) to obtain the microsomal pellet. All RCF values were determined from the centre of the centrifuge tubes, and both centrifuges were maintained at 0-4°C during centrifugation. The microsomal pellet was resuspended in a known volume of ice-cold 50 mM Histidine-HCl, pH 7.25, by gentle hand homogenisation. The volume of this suspension depended upon the nature of the experiment in hand and the amount of muscle excised.

In all experiments apart from those using preincubated muscle blocks, 10 ml aliquots of the resuspended microsomal fraction were pre-incubated either with or without ions in 25 mm diameter boiling tubes at the appropriate temperature for a stated time. The whole

muscle blocks were preincubated in similar tubes immediately after dissection and before homogenisation. Preincubated samples were rapidly cooled by transferring the preincubation test tubes to an ice bath. The preparation was allowed a 10 minute temperature equilibration period. The reaction was started by the addition of 1.0 ml aliquots of ions and Tris-ATP in 50 mM histidine-HCl, pH 7.25, which was at the reaction temperature. The final concentrations of reagents in the reaction mixture in all experiments was either:

1. 3 mM $MgCl_2$ + 2 mM Tris-ATP, or
2. 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl + 2 mM Tris-ATP.

These concentrations were determined experimentally by incubation resuspended microsomal pellets in 2 mM Tris-ATP with various Na^+ and K^+ ~~chloride~~ concentrations (see figure 20) and selecting concentrations which gave maximal ATPase activity.

Incubation time was normally 30 minutes, enzyme activity was stopped by the addition of 1.0 ml of ice-cold, 12% (w/v) trichloracetic acid (TCA) and the tubes were immediately transferred to an ice bath. The proteins precipitated were removed by centrifuging at 2,000 g for 10 minutes at 0^oC. The amount of inorganic phosphate liberated was estimated by the method of Fiske and Subbarow (1925) in duplicate samples from each incubation mixture using a Hilger-Watt spectrophotometer. A 'blank' was run in each experiment by adding the TCA before the ions + Tris-ATP mixture and keeping it on ice. The blank value was substracted from the experimental values to give the amount of inorganic phosphate produced non-enzymatically.

Tris-ATP was purchased as a solid from Sigma Chemical Co., St. Louis, Missouri, U.S.A. for the early experiments. The disodium salt of ATP was purchased from the same source and converted into Tris-ATP by treatment with acid, ion-exchange, Dowex resin and stored

Figure 20.

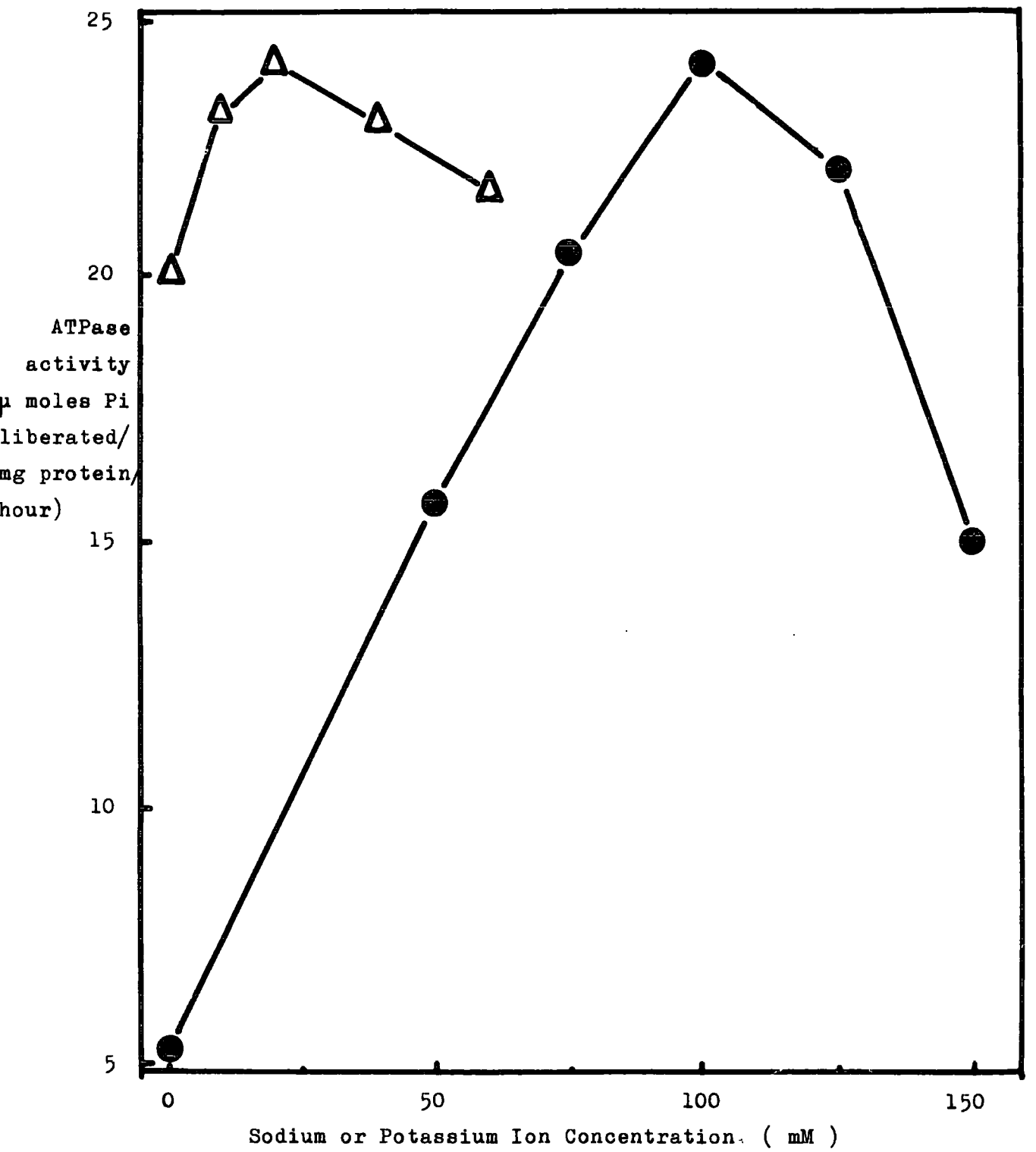
The Effect of Sodium and Potassium Concentrations on the Crayfish Microsomal Adenosinetriphosphatase Activity.

The microsomal fraction of crayfish abdominal flexor muscle was prepared and incubated for 10 minute intervals with ions and adenosine triphosphate (ATP) as described in the Methods. The ATPase activity was expressed as μ moles Pi liberated per mg protein per hour and plotted against the concentration on Na^+ or K^+ in the incubation medium.

△ ..... 3 mM $MgCl_2$ + 100 mM NaCl + 2 mM Tris-ATP + X mM KCl

● ..... 3 mM $MgCl_2$ + 20 mM KCl + 2 mM Tris ATP + X mM NaCl

Figure 20.


25
20
15
10
5
ATPase activity μ moles Pi liberated/ mg protein/ hour)
0
50
100
150
Sodium or Potassium Ion Concentration (mM)

frozen at -20°C for later experiments. 'Sigma' grade histidine and Fiske and Subbarow reagent were obtained from Sigma, all other salts used were obtained from B.D.H. Ltd., Poole, Dorset, and were 'Analar' grade. Solutions were made up in glass distilled water. Protein estimations were made on aliquots of resuspended microsomal homogenate by the microbiuret method of Itzaki and Gill (1964). Bovine serum albumen (fraction V) supplied by Sigma was used as the protein standard.

RESULTS

1. Thermal inactivation of sarcolemmal ATPases during 10 minute pre-incubation periods

These experiments were designed to investigate the effects of high lethal temperatures on the sarcolemmal ATPases of the abdominal flexor of Astacus pallipes. Bowler and Duncan (1968) performed similar experiments, but only on preparations from 25°C adapted animals. The present experiments were performed on both 10 and 25°C adapted animals. Bowler and Duncan found that the Mg^{++} activated ATPase was inactivated at lower temperatures than the $Na^{+}+K^{+}$ activated ATPase. They also found that the thermal inactivation of the Mg^{++} activated ATPase correlated well with the death of the whole animal over the same temperature range (29 - 35°C). Regarding the established ability of crayfish to alter their resistance to high temperature, it seemed possible that the thermal resistance of the sarcolemmal ATPases might also change with temperature adaptation.

Aliquots of resuspended microsomal pellets were preincubated in the absence of ions for 10 minutes at temperatures between 10 and 38°C The subsequent ATPase activities were then determined in 5 preparations from 10°C and 4 preparations from 25°C adapted crayfish. The results have been presented in Arrhenius plots of the mean ATPase activities for the Mg^{++} and the $Na^{+}+K^{+}$ activated ATPases in figures 21 and 22. The Arrhenius μ values obtained from these figures are shown in table 23 for comparison with the figures obtained by Bowler and Duncan (1968) and for the thermal inactivation of the whole animal.

The Mg^{++} activated ATPase from both 10 and 25°C adapted crayfish muscles were found to be inactivated at lower temperatures than the $Na^{+}+K^{+}$ activated ATPases. This was particularly clear in preparations taken from 10°C adapted crayfish. In these preparations the Mg^{++} activated ATPase was partially inactivated at temperatures 2-4°C lower

Figure 21.

The Effect of High Temperature on $10^{o}C$ adapted Crayfish Muscle ATPases.

Microsomal preparations were obtained from five $10^{o}C$ adapted crayfish and preincubated for 10 minute intervals at temperztures between $10^{o}C$ and $38^{o}C$. The ATPase activity of duplicate aliquots during 30 minutes incubation at $10^{o}C$ was either:

1) 2 mM ATP + 3 mM $MgCl_2$

2) 2 mM ATP + 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl

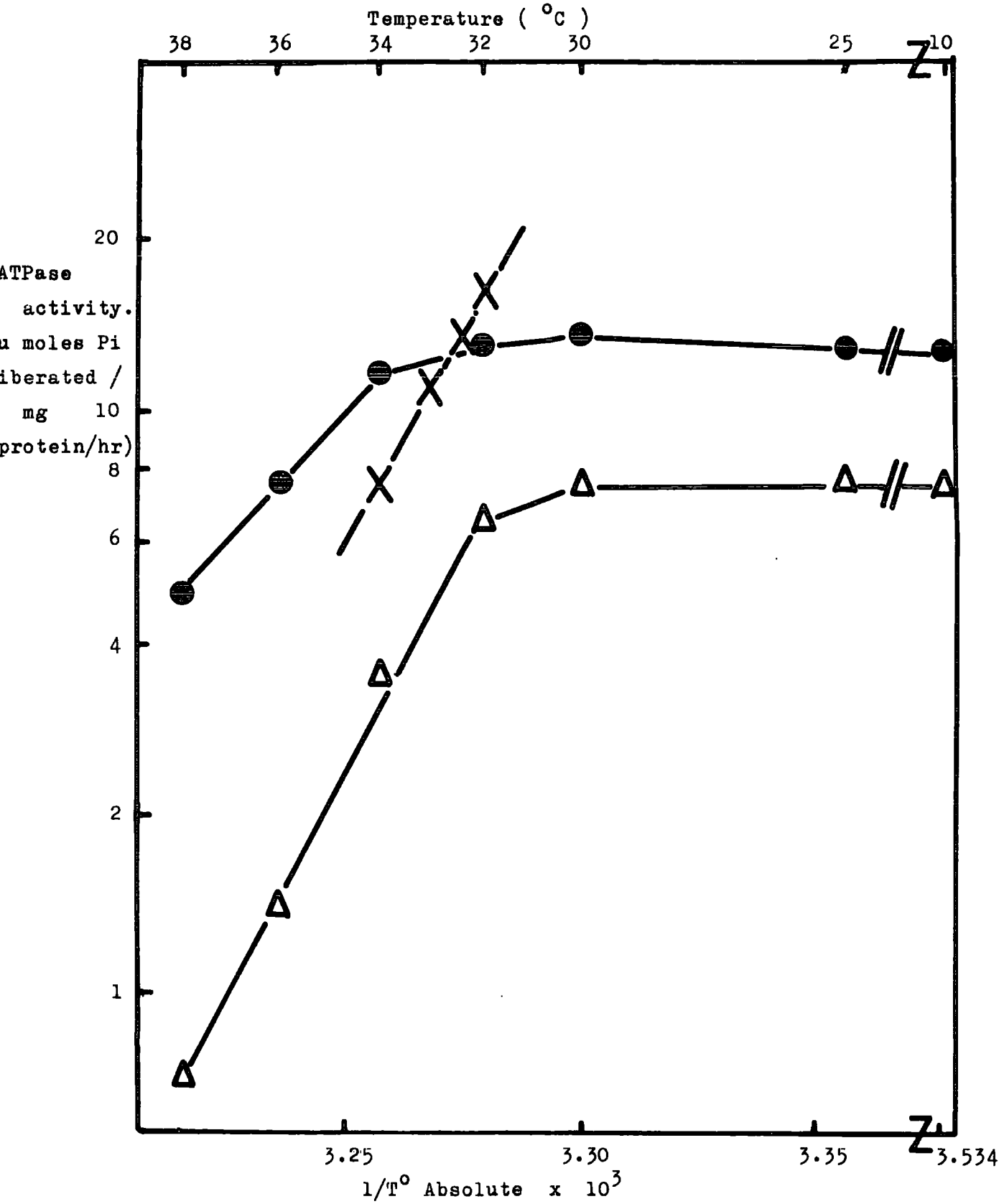
was expressed as μ moles Pi liberated per mg protein per hour, and used to draw an Arrhenius plot. The thermal inactivation of the whole animal (see figure 4) was included in the figure for comparison.

$Na^{+}K^{+}$ activated ATPase ..... ●

Mg^{++} activated ATPase ..... Δ

Whole animal inactivation ... X

Figure 21.


Temperature (°C)
38
36
34
32
30
25
10
20
ATPase
activity.
u moles Pi
iberated /
mg
protein/hr)
10
8
6
4
2
1
3.25
3.30
3.35
3.534
1/T° Absolute x 10³

than the temperature at which the first partial inactivation of the $Na^{+}+K^{+}$ activated ATPase was observed (see figure 21). In preparations from 25°C adapted animals the sensitivity of the Mg^{++} activated ATPase to high temperatures was less than the sensitivity of the same enzyme taken from 10°C animals, but still greater than that of the $Na^{+}+K^{+}$ activated ATPase (see figure 22). The sensitivity of the Mg^{++} activated ATPase to 10 minutes exposure to high temperatures therefore varied with the adaptation temperature of the animal from which the preparation was obtained, the enzyme from the warm adapted animals being more resistant to high temperatures than the enzyme from cold adapted animals. The temperature sensitivity of the $Na^{+}+K^{+}$ activated ATPase from both adaptation groups was very similar, the Arrhenius μ values obtained from figures 21 and 22 for this enzyme were very similar (42 and 48 K calories $mole^{-1}$), indicating that the thermal sensitivity of this enzyme was not affected by temperature adaptation of the whole animal.

Table 21 includes Arrhenius μ values obtained by plotting the LD_{50} values of 10 and 25°C adapted crayfish on an Arrhenius plot. Whilst the significance of the values so obtained is not clear, heat death being a complex process in such highly organised animals as crayfish; it seems reasonable to suggest that the Arrhenius μ value so obtained will reflect the thermal inactivation of a critically heat sensitive process. It is therefore considered valid to compare the Arrhenius μ value obtained from the LD_{50} figures (table 1) with those obtained for heat sensitive enzymes. The values given in table 23 show that the thermal inactivation of the whole animal and the Mg^{++} activated ATPase are similar, and depend upon the adaptation temperature of the animal. The termal inactivation of the $Na^{+}+K^{+}$ activated ATPase was not similar to that of the whole animal, and did not change greatly

Figure 22.

The Effect of High Temperature on 25°C Adapted Crayfish Muscle ATPases.

Microsomal preparations were obtained from five 25°C adapted crayfish and preincubated for 10 minute intervals at temperatures between 25° and 38°C. The ATPase activity of duplicate aliquots during 30 minutes incubation at 25°C wtih either;

1) 2 mM ATP + 3 mM $MgCl_2$

2) 2 mM ATP + 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl

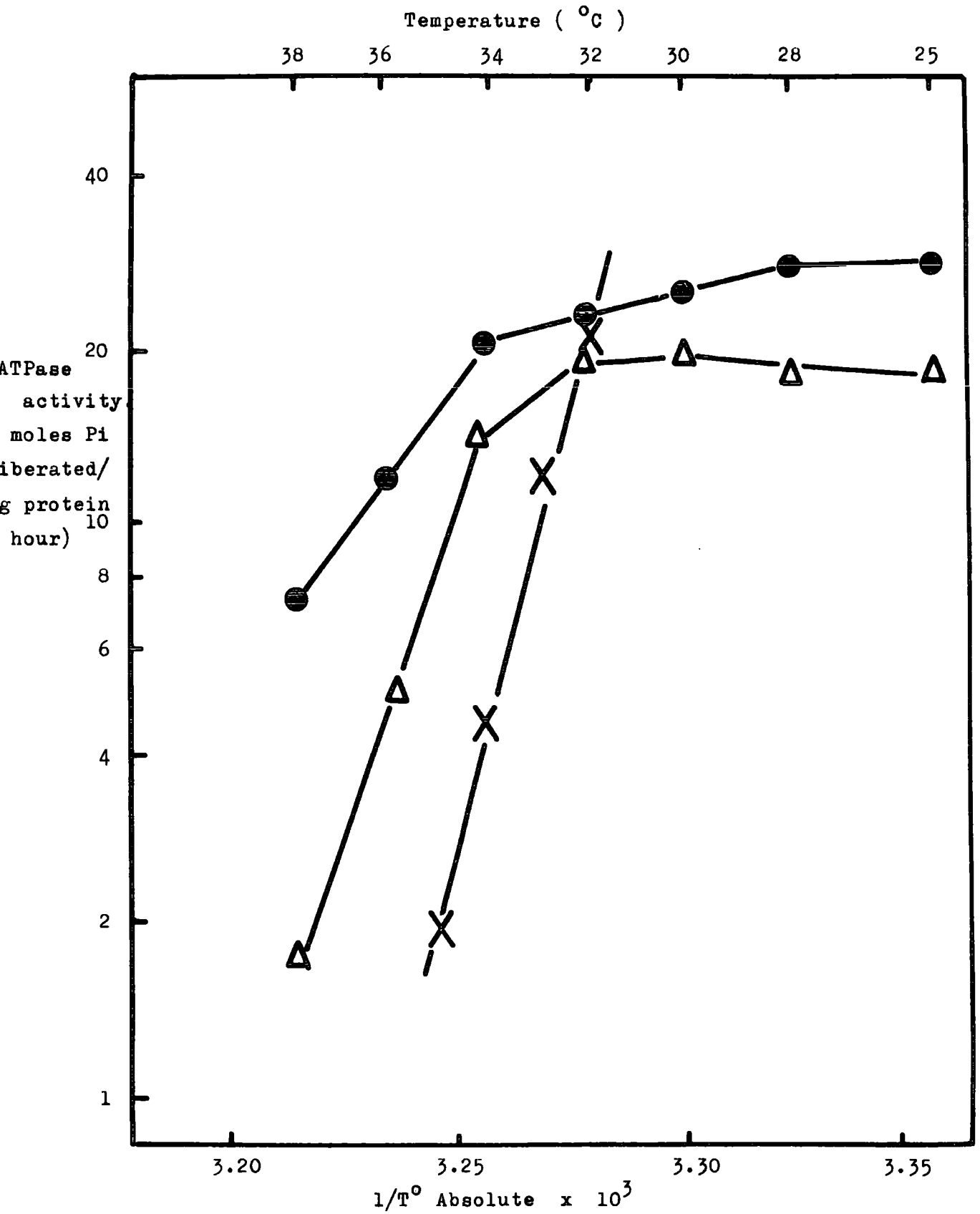
was expressed as μ moles Pi liberated per mg protein per hour, and used to draw an Arrhenius plot. The thermal inactivation of the whole 25°C adapted animal (see figure 4) was included in the figure for comparison.

$Na^+ + K^+$ activated ATPase ..... ●

Mg^{++} activated ATPase ..... Δ

Whole animal inactivation X

Figure 22.


Temperature (°C)
38
36
34
32
30
28
25
40
20
10
8
6
4
2
1
ATPase
activity
moles Pi
liberated/
g protein
hour)
3.20
3.25
3.30
3.35
1/T° Absolute x 10³

with the adaptation of the animal. The results obtained in these experiments also agreed well with those obtained previously by Bowler and Duncan (1968).

A further point of interest was the correlation of the temperature which gave 50% inactivation of the Mg^{++} activated ATPase during 10 minutes preincubation and the temperature which gave an LD_{50} of 10 minutes. The $10^{o}C$ adapted crayfish had a projected LD_{50} of 10 minutes when exposed to $33.1^{o}C$ (see figure 4) and the Mg^{++} activated ATPase was 50% inactivated after 10 minutes preincubation at $33.4^{o}C$ (see figure 21). The $25^{o}C$ adapted crayfish had an LD_{50} of 10 minutes when exposed to $35.8^{o}C$ and the Mg^{++} activated ATPase was 50% inactivated after 10 minutes preincubation at $34.9^{o}C$ (see figure 22).

These results therefore indicate that the sarcolemmal Mg^{++} activated ATPase has a similar temperature sensitivity to that of the whole animal, and that thermal adaptation of the whole animal affects the thermal sensitivity of this enzyme. This suggests that the sarcolemmal Mg^{++} activated ATPase is implicated in temperature adaptation and heat death in the crayfish.

2. Thermal inactivation of Sarcolemmal ATPases at $34^{o}C$

These experiments were designed to further elucidate the effects of high lethal temperature on the sarcolemmal ATPases. The temperature chosen for these experiments, $34^{o}C$, was selected because it was lethal over a relatively brief period for the $25^{o}C$ adapted crayfish, but not too brief for study for the $10^{o}C$ adapted animals. The results show that the thermal inactivation of the $Na^{+}+K^{+}$ activated ATPase at $34^{o}C$ was similar in preparations from 10 and $25^{o}C$ adapted crayfish (see figures 23 and 24). The enzyme was 37% inactivated after 25 minutes preincubation at $34^{o}C$ in both adaptation groups. The inactivation of the Mg^{++} activated ATPase under the same conditions was much greater and depended upon the adaptation temperature of the

Figure 23.

The Effect of the Duration of Exposure to 34°C on 10°C Adapted Crayfish Muscle ATPases.

Microsomal preparations from four 10°C adapted crayfish were prepared and preincubated for 0 - 25 minutes at 24°C as described in the Methods. The ATPase activity of duplicate aliquots of microsomes was measured during a 30 minute incubation at 10°C with either

1) 2 mM ATP + 3 mM $MgCl_2$, or

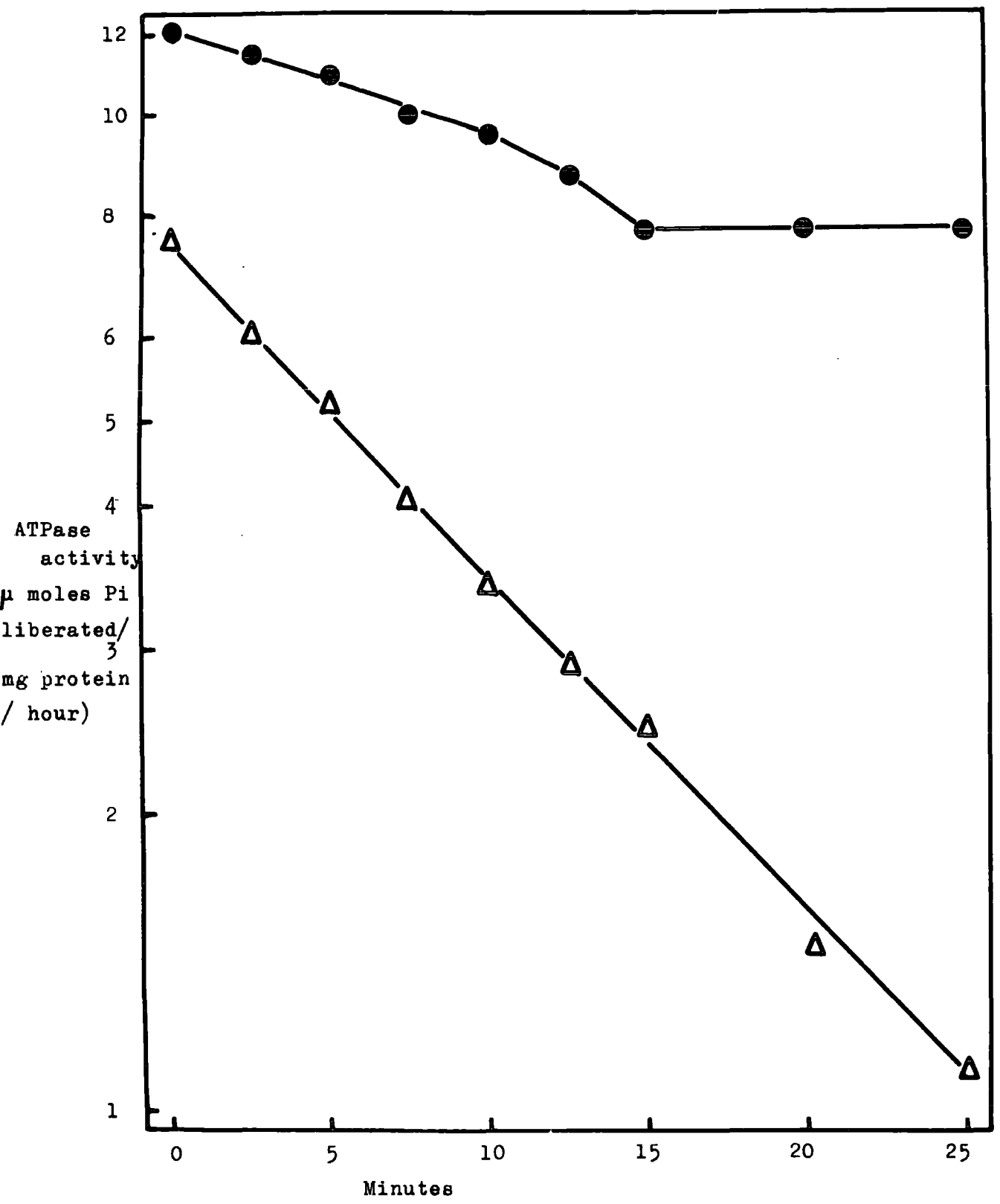
2) 2 mM ATP + 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl

The ATPase activity was expressed as μ moles Pi liberated per mg protein per hour and plotted against the preincubation time on a semi-log plot.

$Na^+ + K^+$ activated ATPase ..... ●

Mg^{++} activated ATPase ..... Δ

Figure 23.


12
10
8
6
5
4
3
2
1
ATPase activity
μ moles Pi liberated/ mg protein / hour)
0
5
10
15
20
25
Minutes

animal from which the preparation was obtained. Preincubation for 25 minutes at 34°C reduced the Mg^{++} activated ATPase activity of preparations from 10°C adapted crayfish by 85%, and that of preparations from 25°C adapted animals by 67%. It can also be seen from figures 23 and 24 that whilst the activity of the Mg^{++} activated ATPase from 10°C adapted animals fell in a steady, logarithmic fashion with preincubation time, the activity of preparations from 25°C adapted animals was relatively unaffected by 5 minutes preincubation at 34°C, but after longer incubation activity fell at a steady, logarithmic rate with preincubation time. These results confirm the findings of the first section in that the Mg^{++} activated ATPase was more sensitive to high temperatures, and that its sensitivity depended upon the previous thermal history of the animal from which the preparation was obtained.

If the Mg^{++} activated ATPase inactivation were to be considered as the primary lesion of heat death in the crayfish, then the LD_{50} time of the whole animal might be expected to correlate with the 50% inactivation time of the enzyme at this temperature. The enzyme 50% inactivation time from figures 22 and 23 are 9.2 and 17.7 minutes for preparations from 10 and 25°C adapted animals respectively. The LD_{50} time are 7.5 and 44 minutes (see table 1) for 10 and 25°C adapted animals respectively. These figures indicate that the times correlate well for the 10°C adapted animals and preparations, but not for the 25°C adapted animals and preparations. The possible reasons for this discrepancy are discussed at the end of this chapter with reference to the results obtained in previous chapters.

3. Preincubation of Whole Muscle Blocks at 34°C

This experiment was d esigned to investigate the validity of comparing the isolated, microsomal enzyme activity with that of the

Figure 24.

The Effect of Duration of Exposure to 34°C on 25°C Adapted Crayfish Muscle ATPases.

Microsomal preparations from three 25°C adapted crayfish were prepared and preincubated for 0 - 25 minutes at 34°C as described in the Methods. The ATPase activity of duplicate aliquots of microsomes was measured during 30 minute incubation with either

1) 2 mM ATP + 3 mM $MgCl_2$, or

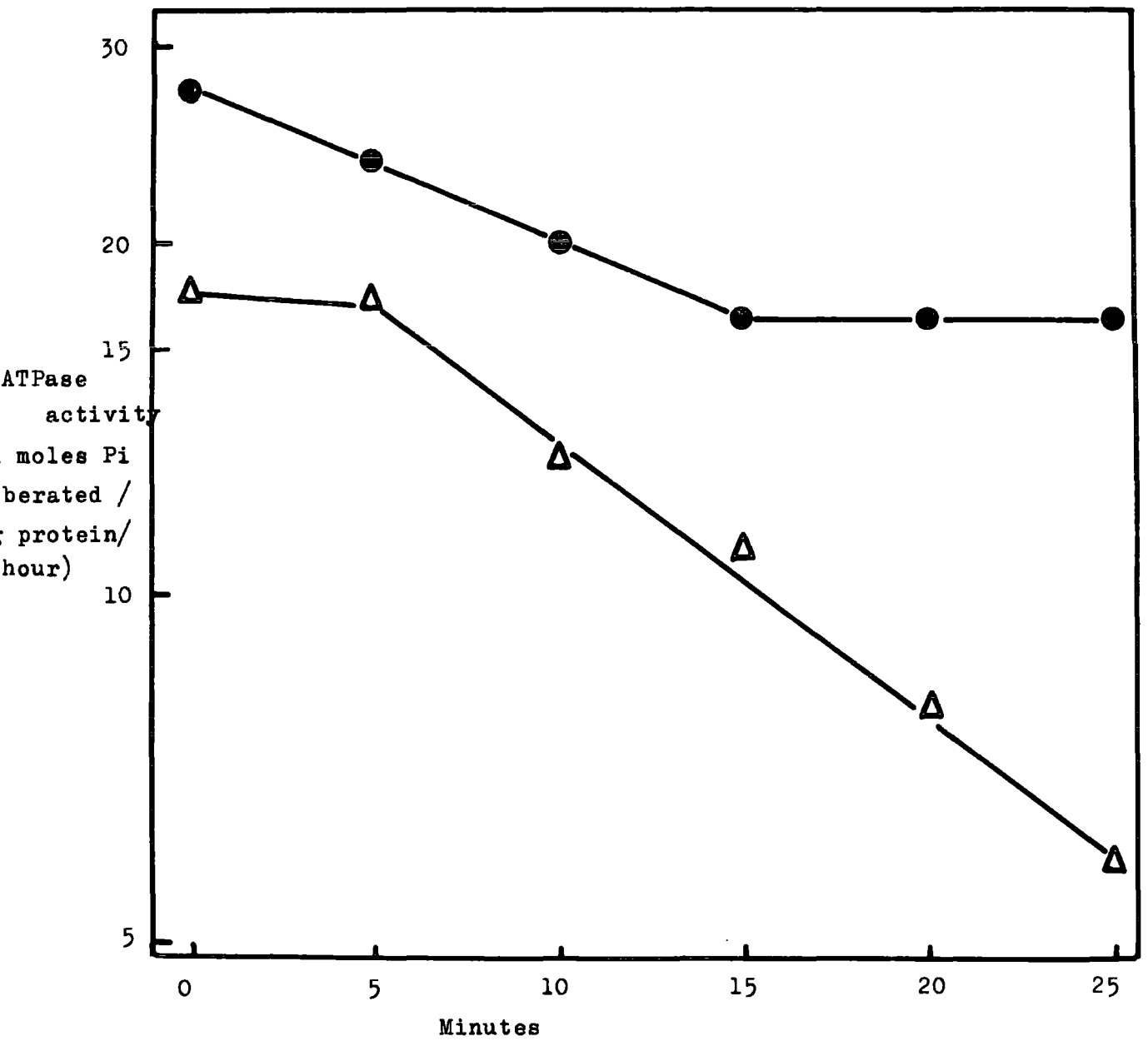
2) 2 mM ATP + 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl..

The ATPase activity was expressed as μ moles Pi liberated per mg protein per hour and plotted against the preincubation time on a semi-log plot.

$Na^+ + K^+$ activated ATPase ..... ●

Mg^{++} activated ATPase ..... ▲

Figure 24.


30
20
15
ATPase
activity
moles Pi
berated /
protein/
hour)
10
5
0
5
10
15
20
25
Minutes

intact cells. It was considered that, if the results from this experiment resembled those of the preceeding section, direct comparison of the effects of high lethal temperatures on the whole animal would probably be valid. If they did not, the limitations of such a comparison might be elucidated. The preincubation of the muscle blocks was performed in saline at 34°C prior to mincing the muscle. The resuspended microsomal pellet was then incubated immediately. The results are shown on figure 25. The results were similar in general outline to those obtained for the microsomal preincubations. The Mg^{++} activated ATPase was more sensitive to 34°C than the $Na^{+}+K^{+}$ activated ATPase, and both enzymes were inactivated progressively with increasing preincubation time. The degree of inactivation brought about by 25 minutes preincubation at 34°C was similar in both the microsomal and the muscle block preincubated preparations, about 60% inactivation of the Mg^{++} activated ATPase and 30% inactivation of the $Na^{+}+K^{+}$ activated ATPase (see figures 22 and 24). Thus the results show that the sarcolemmal ATPases of intact muscle cells were inactivated by preincubation at 34°C in a similar fashion to the inactivation observed in microsomal preparations.

4. Ion Protection of Sarcolemmal ATPases at High Temperatures

It is known that the presence of appropriate ions or substrate can protect ATPases from adverse conditions (Skou and Hilberg, 1965, Cooper and McIlwain, 1967, Schoner and Schmidt, 1969). It therefore seemed interesting to test the possible protective abilities of ions during preincubation of the microsomal homogenate at high lethal temperatures. The reason for the protective action of ions and substrate, according to Schoner and Schmidt (1969), is that they protect the phosphorylated acceptor group of the ATPases against nucleophilic attack. Since the presence of substrate would lead to

Figure 25.

Thermal Inactivation of Muscle ATPase by Preincubating Whole Muscle Blocks.

The abdominal flexor muscle of a 10°C adapted crayfish was removed and cut into four sections. These were preincubated in modified Van Harreveld's saline at 34°C for 0, 10, 25 and 35 minutes respectively. The microsomal fraction of the muscle blocks was then prepared and duplicate aliquots of the microsomes incubated at 10°C for 30 minutes with either;

1) 2 mM ATP + 3 mM $MgCl_2$, or

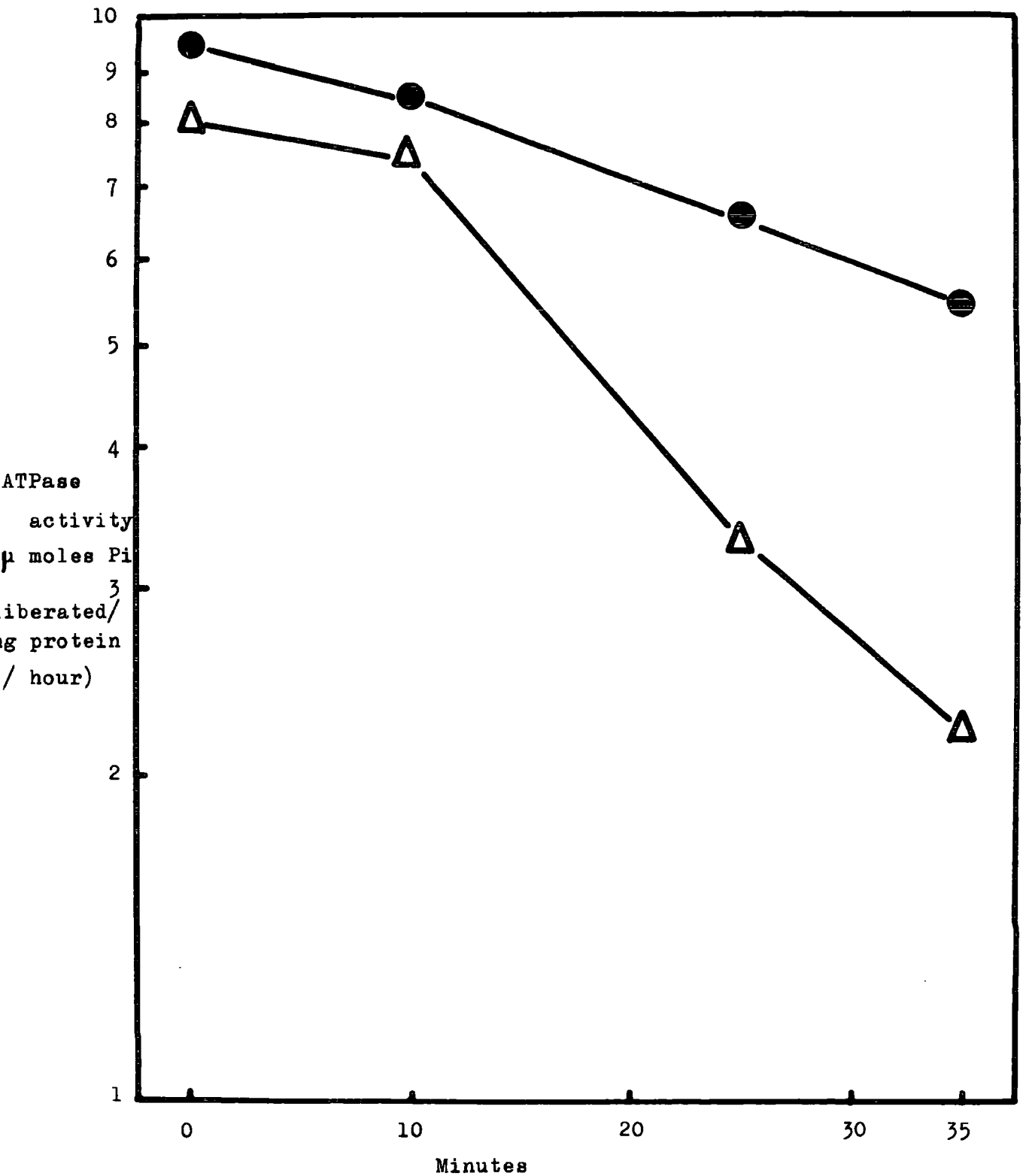
2) 2 mM ATP + 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl

as described in the Methods. ATPase activity was expressed as μ moles Pi liberated per mg protein per hour and plotted against preincubation time on a semi-logarithmic plot.

$Na^+ + K^+$ activated ATPase activity ..... ●

Mg^{++} activated ATPase activity ..... Δ

Figure 25.


10
9
8
7
6
5
4
3
2
1
ATPase
activity
μ moles Pi
liberated/
g protein
/ hour)
0
10
20
30
35
Minutes

enzyme activity during preincubation, the protective effect on ions was investigated. It was hoped that these experiments would give further information concerning the applicability of *in vitro* results to the *in vivo* condition of these enzymes.

The effects of ion protection were investigated by the addition of either 3 mM $MgCl_2$ or 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl to the resuspended microsomal aliquots. Incubation for 30 minutes at 10^oC was started by the addition of substrate (2 mM Tris-ATP). Two experiments were performed in which the preincubation time was 10 minutes and the preincubation temperature varied between 10 and 38^oC, (see figure 25) and two more experiments in which the preincubation temperature was 34^oC and the time of preincubation varied between 0 and 25 minutes (see figure 26). The experimental animals were adapted to 10^oC.

The results which have been presented in figures 25 and 26 are directly comparable with the results shown in figure 20 and 22. The presence of ions during preincubation appeared to protect the ATPases most effectively. The Na^++K^+ activated ATPase was activated rather than inhibited by exposure to high temperatures. Comparing the results of figure 25 with those of figure 20 it can be seen that 10 minutes preincubation with ions at 38^oC resulted in an activation of 108%, preincubation under the same condition s without ions giving a 73% inactivation. Similarly it can be seen from a comparison of figures 23 and 26 that preincubation of the Na^++K^+ activated ATPase with ions at 34^oC resulted in a progressive activation of the enzyme at higher temperatures, whilst when this enzyme was preincubated under the same conditions without the ions present, it was progressively inactivated. The Na^++K^+ activated ATPase was thus protected and even activated by preincubation to high temperatures in the presence of ions.

Figure 26.

Protection of Crayfish Muscle ATPases Exposed to High Temperature by Ions.

Duplicate microsomal aliquots were prepared from the abdominal flexor muscles of two 10°C adapted crayfish as described in the Methods. The microsomes were preincubated for 10 minutes at temperatures between 10° and 38°C with either

1). 3 mM $MgCl_2$, or

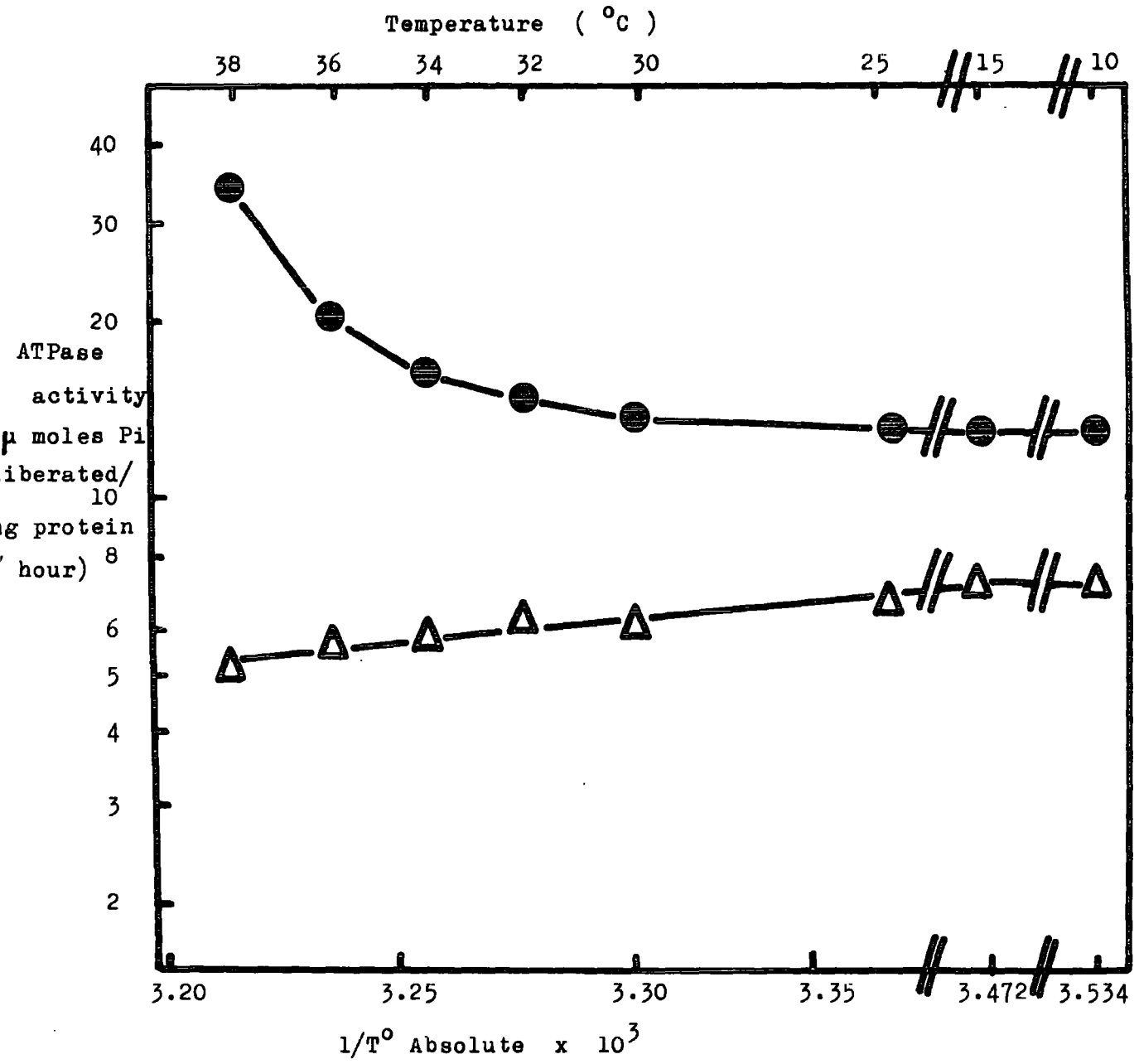
2) 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl.

The duplicate aliquots of microsomes were then incubated with 2 mM ATP for 30 minutes at 10°C and the ATPase activity was expressed as μ moles Pi liberated per mg protein per hour. The logarithm of the ATPase activity was plotted against the reciprocal of the absolute preincubation temperature to give an Arrhenius plot.

$Na^+ + K^+$ activated ATPase activity ..... ●

Mg^{++} activated ATPase activity ..... Δ

Figure 26.


Temperature (°C)
38
36
34
32
30
25
15
10
40
30
20
ATPase
activity
μ moles Pi
liberated/
10
g protein
hour)
8
6
5
4
3
2
3.20
3.25
3.30
3.35
3.472
3.534
1/T° Absolute x 10³

The Mg^{++} activated ATPase was also protected by the presence of ions in the preincubation mixture. Thus 10 minutes preincubation at $38^{o}C$ caused an inactivation of only 30% (see figure 26) compared with an inactivation of over 90% when the microsomal pellet was preincubated without ions (see figure 21). When the Mg^{++} ATPase was incubated at $34^{o}C$ in the presence of ions there was a small initial activation during the first 5 minutes of preincubation, followed by a small inactivation (14% inactivation after 25 minutes preincubation) (see figure 26). Preincubating the Mg^{++} ATPase without ions at $34^{o}C$ (see figure 23) produced no activation of the emzyme and a much greater inactivation (85% inactivation after 25 minutes preincubation).

These results confirm that the presence of ions protects the sarcolemmal ATPases against high temperatures. If the protection was due to the protection of the phosphorylated acceptor group as Schoner and Schmidt (1969) have suggested, the ions must have been binding to the enzyme. The surprising feature of these results was the activation of the $Na^{+}+K^{+}$ activated ATPase. This was possibly due to a conformational change in the enzyme during preincubation in the presence of ions, the new enzyme conformation displaying more activity. There is, however, no evidence that the enzyme was functioning as an effective cation pump following this treatment. Thus it is possible that the observed activation represented an increased substrate untilisation which was not matched by increased ion translocation. In fact the se experiments demonstrate quite clearly that it is not possible to apply the previous results of this chapter uncritically to the events occurring <u>in vivo</u> during heat death.

Figure 27.

Protection of Crayfish Muscle ATPases Exposed to 34°C by Ions.

Duplicate microsomal aliquots were prepared from the abdominal flexor muscles of two 10°C adapted crayfish as described in the Methods. The microsomes were preincubated at 34°C for 0, 5, 10, 15, 20 or 25 minutes with either;

1) 3 mM $MgCl_2$, or

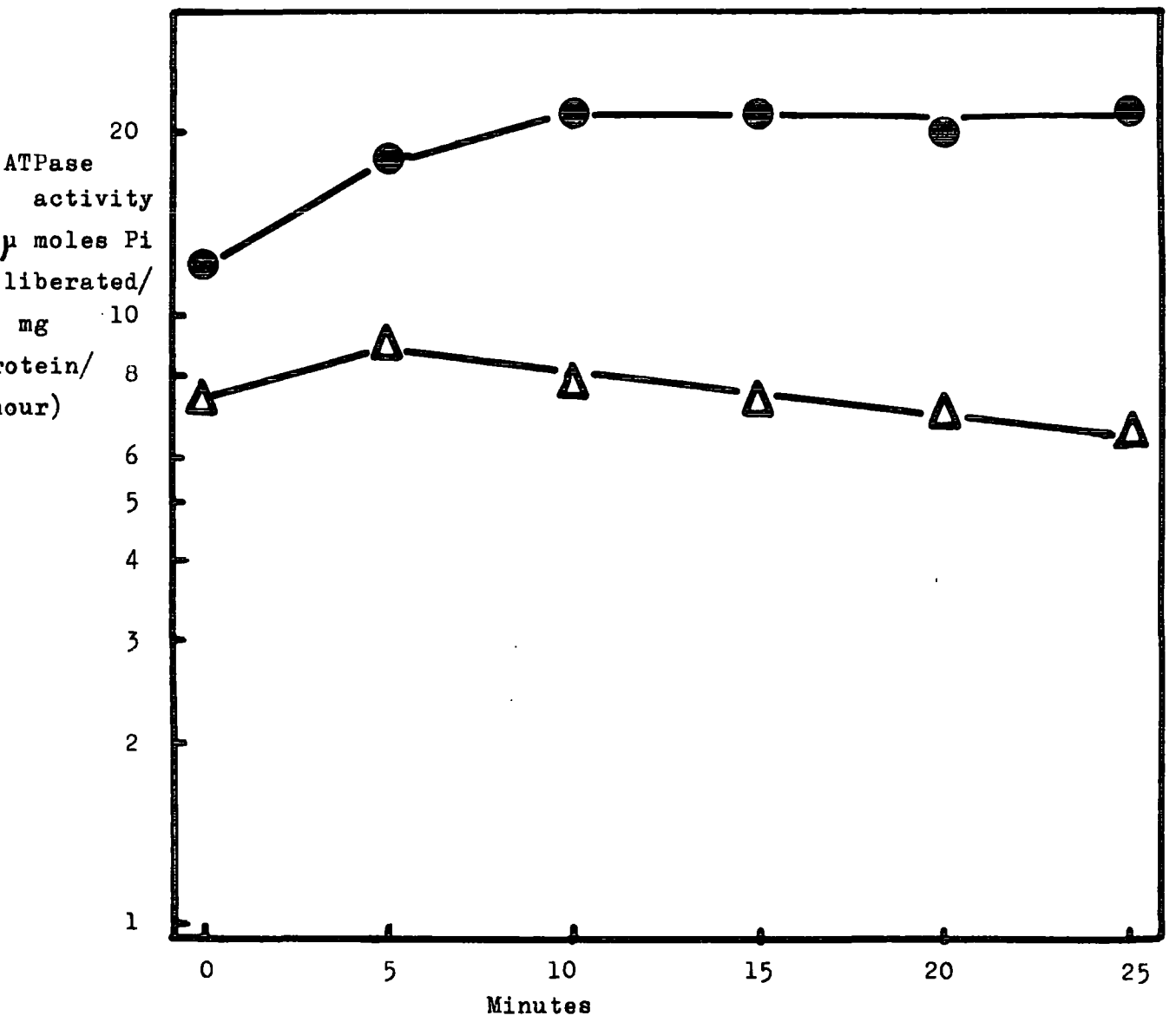
2) 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl.

The duplicate aliquots of microsomes were then incubated with 2 mM ATP for 30 minutes at 10°C and the ATPase activity expressed as μ moles Pi liberated per mg protein per hour. The logarithm of the ATPase activity was plotted against the preincubation time.

$Na^+ + K^+$ activated ATPase activity ..... ●

Mg^{++} activated ATPase activity ..... Δ

Figure 27.


ATPase
activity
μ moles Pi
liberated/
mg
rotein/
our)
20
10
8
6
5
4
3
2
1
0
5
10
15
20
25
Minutes

DISCUSSION

The experiments which investigated the effects of high lethal temperatures on muscle blocks and the protective effect of ions during preincubation at high temperatures demonstrate how difficult it is to relate data obtained from this preparation to the whole animal. Indeed, Whittam and Wheeler (1970) have observed that "it is becoming apparent that conclusions relating to the function of cells and organs cannot be uncritically drawn from work with preparations of membranes from fragmented cells". Nevertheless, it would appear that an examination of these enzymes might help to elucidate some of the events occurring during heat death and temperature acclimatisation in the whole animal.

The results show clearly that the Mg^{++} activated ATPase was inactivated by milder heat treatment than the $Na^{+}+K^{+}$ activated ATPase. This was true regardless of the adaptation temperature of the crayfish from which the sarcolemmal microsomes were prepared. The effect of high temperatures (32 - 38°C) on the $Na^{+}+K^{+}$ activated ATPase was similar regardless of the adaptation temperature of the animal from which the enzyme was obtained. However, the effect of these temperatures on the Mg^{++} activated ATPase was found to be related to the adaptation temperature of the animal from which the enzyme was obtained (see figures 21 and 22). Adaptation of the experimental animals to higher temperatures resulted in an increased ability of the Mg^{++} activated ATPase to resist exposure to high temperatures. Furthermore, the thermal inactivation of the Mg^{++} activated ATPase exhibited strong similarities to the thermal inactivation of the whole animal (see table 23). It is interesting to note that the Arrhenius μ values for the thermal inactivation of the microsomal preparations and heat death of the whole animal compare closely with the figures obtained by Bowler and Duncan (1968).

TABLE 23.

A Comparison of the Thermal Inactivation of Whole Crayfish and Crayfish Muscle Microsomal ATPases.

The Arrhenius $\bar{\mu}$ values for the thermal inactivation of the whole animal (see figure 4) and crayfish muscle microsomal ATPases (see figures 20 and 21) were presented in tabular form for comparison. The values obtained by Bowler (1963 a) and Bowler and Duncan (1968) for these figures have also been included for comparison.

TABLE 23.

Adaptation Temperature (^{o}C)	Preinc. time (minutes)	Whole Animals	Mg^{++} ATPase	$Mg^{++} + Na^{+} + K^{+}$ ATPase.
		K calories $mole^{-1}$		
10	10	70	70	42
25	10	143	103	48
25	8	140	111	48
25	18	140	114	40

It is also of interest to relate the observed changes in the effects of high temperatures on the sarcolemmal ATPases to the adaptation temperatures of the crayfish and the results obtained in previous chapters. The similarity between the Arrhenius μ values for the whole animal and the Mg^{++} activated ATPase over the same temperature range mentioned above suggests that this enzyme is implicated in the events leading to heat death of the crayfish. This thermal inactivation of the Mg^{++} activated ATPase can be correlated with the breakdown in passive sarcolemmal permeability indicated by the large Na^{+} and K^{+} ion movements during heat death (see Chapter 2), and the observed increase in sarcolemmal conductivity during exposure to high temperatures (see Chapter 3). The sarcolemmal $Na^{+}+K^{+}$ activated ATPase was not drastically inactivated even by prolonged exposure to $34^{o}C$ (see figures 22 and 23). Bowler and Duncan (personal communication) found that this was true of crayfish axolemmal $Na^{+}+K^{+}$ activated ATPase also, and in view of the preponderance of this enzyme which they found in the nerve axons, this probably explains the small effect of temperature on the spontaneous activity of the ventral nerve cord reported in Chapter 3 (see figure 17 and table 20), since this enzyme would be able to prevent any ions movements brought about by inactivation of an axolemmal Mg^{++} activated ATPase.

These results therefore suggest that the Mg^{++} activated ATPase controls the passive permeability of the muscle fibre cell membrane. The following hypothesis is advanced. When the Mg^{++} activated ATPase is inactivated by heat, the resulting increase in sarcolemmal passive permeability, which is reflected by the dramatic increase in the sarcolemmal conductivity (see figure 15), allows Na^{+} and K^{+} to move down their respective electrochemical gradients across the sarcolemma (see tables 8 and 9). This causes large changes in the haemolymph Na^{+} and K^{+} concentrations during heat death (see tables

4 and 5). The increased haemolymph K^+ concentration seems to be of the greatest significance during heat death since the observed haemolymph K^+ levels at the time of heat death were similar in both adaptation groups, and these levels have been shown to bring about a large increase in the spontaneous activity of the ventral nerve cord (see figure 19). It is therefore proposed that the thermal inactivation of the Mg^{++} activated ATPase observed in this chapter is implicated in heat death of the crayfish. The results also indicate that the resistance of the Mg^{++} activated ATPase to thermal inactivation depended upon the adaptation temperature of the animal and hence suggest that the sarcolemmal Mg^{++} activated ATpase is implicated in the mechanism of physiological resistance adaptation to temperature. The membrane-bound ATPases are known to be dependent upon membrane phospholipids (Wheeler & Whittam, 1970; Naguchi and Freed, 1971, and Tanaka, Sakamoto and Sakamoto, 1971). De Mello (1971) has shown that membrane phospholipids are involved in the control of passive permeability of mammalian myocardial cells. The thermal inactivation of the Mg^{++} activated ATPase might therefore reflect the breakdown of either protein or phospholipid, or a change in their structural relationship which causes a change in the configuration of the enzyme.

CHAPTER 5

KINETIC STUDIES OF THE SARCOLEMMAL ATPASES

INTRODUCTION

Crayfish, in common with many other aquatic poikilotherms, exhibit a phenomenon known as metabolic temperature compensation. This compensatory effect of adaptation on the overall metabolic rate of animals is well known. It is of great importance to poikilotherms which are exposed to widely fluctuating habitat and therefore body temperatures, and manifests itself as a relatively small difference in the metabolic rates of animals maintained for long periods at different environmental temperatures. In this way poikilotherms achieve some degree of metabolic homeostasis which is of great importance in the exploitation of their habitat. Studies by previous authors have shown that the phenomenon of metabolic temperature compensation is accompanied by changes in the metabolic rate as measured by oxygen consumption and by enzymes. Since certain enzymes have been shown to be capable of acting in a regulatory fashion, it is possible that thermal compensation might well be mediated by changes in a few key enzymes.

The enzymic changes which have been reported to date are of two main types. Firstly the amount of enzyme present may vary with the temperature in a compensatory fashion. This has been ~~shown~~ claimed by Eckberg (1962) in crucian carp gills, Baslow and Nigrelli (1964) in brain cholinesterases of the killifish and Freed (1965) on the phosphofructokinase of cold and warm adapted goldfish, for example. Secondly the kinetics of the enzyme may change in a compensatory fashion. This has been shown by Hochachka and Somero (1968) for

lactate dehydrogenases of lake and brook trout, by Behrisch and Hochachka (1969a) for lungfish fructose diphosphatase and by Behrisch and Hochachka (1969b) for rainbow trout fructose diphosphatase for example. These two compensatory mechanisms are not mutually exclusive, and it might be expected that the two types of compensation could complement each other's negative thermal modulation.

The enzyme/substrate affinity is one of the critical rate-determining factors of enzymic reactions, as is the substrate concentration. The importance of the substrate concentration has been noted by Somero (1969) who stated "At the low substrate levels normally present in the cell, enzyme-substrate affinity .....is of primary importance in governing the rate of catalysis". In the experiments described in this chapter the substrate affinity of enzyme preparations from 10 or 25^{o}C adapted crayfish was measured in order to determine whether temperature adaptation caused compensatory changes in the kinetics of the sarcolemmal ATPases. An attempt was also made to determine the <u>in vivo</u> substrate concentrations in relation to the adaptation temperature of the animal.

THEORY

The relationship between the rate of an enzyme catalysed reaction and the substrate concentration is described by the Michaelis-Menton equation:-

$$v = \frac{V_{max}}{1 + \frac{K_m}{S}} \qquad \ldots\ldots\ldots\ldots (1)$$

where v = the initial velocity of the reaction,

V_{max} = the theoretical maximal velocity of the reaction,

S = the substrate concentration,

and K_m = the Michaelis constant.

When two enzymes utilising the same substrate and producing the same product are considered, then one may stoichometrically express the situation as follows:-

$$E_1 + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C_1 \qquad C_1 \xrightarrow{k_{1a}} E_1 + P$$

$$E_2 + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C_2 \qquad C_2 \xrightarrow{k_{2a}} E_2 + P$$

where E_1 and E_2 = enzyme 1 and 2 respectively,

C_1 and C_2 = appropriate intermediates,

P = the common product,

S = the common substrate,

and k_1, k_2, k_{1a}, k_{-1}, k_{-2}, and k_{2a} = the appropriate rate constants.

Theoretical considerations of this situation refer specifically to the initial velocity of the reaction, and thus no competition for the substrate by the two enzymes is proposed. To quote Reiner (1969) "We assume that no conservation equation is required (for/the substrate). In other words, in the range through which substrate con-

centration may be varied, the amount of substrate bound to the enzymes is still quite small compared with the amount of free substrate. To put it another way, two enzymes, while using the same substrate, are not competing for it". In this case, according to the stoichometric scheme:-

$$v_{tot} = k_{1a}C_1 + k_{2a}C_2 \quad \text{.....(2)}$$

$$= \frac{V_{max\ 1} \cdot S}{K_{m\ 1} + S} + \frac{V_{max\ 2} \cdot S}{K_{m2} + S} \quad \text{.....(3)}$$

where v_{tot} = the combined velocity of both reactions and the subscripts $_1$ and $_2$ refer to the stoichometric scheme above.

Since both terms on the right of equation (3) describe rectangular hyperbolas then plotting the combined activity of the two enzymes (v_{tot}) against S will not give a rectangular hyperbola. Further, the reciprocal of equation (3):-

$$\frac{1}{v_{tot}} = \frac{\left(\frac{1 + K_{m\ 1}}{S}\right)\left(\frac{1 + K_{m\ 2}}{S}\right)}{V_{max\ 1} + V_{max\ 2} + \left(\frac{V_{max\ 1} \cdot K_{m2} + V_{max\ 2} \cdot K_{m\ 1}}{S}\right)} \quad \text{...(4)}$$

~~Which~~ does not necessarily describe a straight line.

According to Dixon and Webb (1957) the shape of the curve obtained from such a situation will depend upon the relative values of $K_{m\ 1}$ and $K_{m\ 2}$. If these two constants are the same, then a straight line is obtained with a double reciprocal plot, with intercepts $1/V_{max\ 1} + 1/V_{max\ 2}$ on the abscissa and $-1/K_{m\ 1}$ ($= -1/K_{m\ 2}$) on the ordinate. If the two K_m values are not the same then the resulting double reciprocal plot will be hyperbolic in form and very difficult to interpret. Also, when the substrate concentration is low enough, the double reciprocal plot would approximate to a straight line, for only

the enzyme with the higher K_m would appreciably contribute to the observed activity. Reiner (1969) has also considered this situation; he suggests that a hyperbolic double reciprocal plot can be considered as two joined slopes; i.e. that part of the graph at high substrate concentrations which he describes as follows:-

$$\text{Slope at high S} = \frac{V_{max\ 1} \cdot K_{m\ 1} + V_{max\ 2} \cdot K_{m\ 2}}{V_{max\ 2}} \quad \ldots\ldots\ldots(5)$$

and the part at low substrate concentrations

$$\text{Slope at low S} = \frac{K_{m\ 1} \cdot K_{m\ 2}}{V_{max\ 1} \cdot K_{m\ 2} + V_{max\ 2} \cdot K_{m\ 1}} \quad \ldots\ldots\ldots(6)$$

From these considerations it is clear that the form of the double reciprocal plot of the combined enzyme activity is complex and not amenable to simple interpretation. The task is simplified if the $V_{max\ 1}$ and $V_{max\ 2}$ values or the quantities of the two enzymes present are widely disparate, for in these cases the observed activity will consist almost entirely of the activity of one enzyme. If the properties of both enzymes are to be studied in detail then some method of separating and purifying the two enzymes is necessary.

MATERIALS AND METHODS

Abdominal flexor muscles were dissected from 10 and 25°C adapted crayfish,minced and homogenised as described in Chapter 4. The microsomal pellet was obtained and resuspended as described previously. All solutions and reagents used in this preparation were made up as before. The resuspended microsomal pellets were incubated with either 1) 3 mM $MgCl_2$

or 2) 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl

ATP was added as the tris salt. Incubation times were 10 minutes for the preparations from 25°C adapted crayfish and 15 minutes for the preparations taken from 10°C adapted crayfish. The incubation temperature was 10 or 25°C. The enzyme activity was estimated by duplicated inorganic phosphate measurements as described in Chapter 4.

Estimation of Nucleoside Triphosphate

Nucleoside triphosphates were extracted from 5-10 mg (wet weight) strips of abdominal flexor muscle in 5 ml of boiling distilled water. The resulting extract was made up to 10 ml with distilled water and aliquots taken for assay. The assay procedure was as follows:

A solution of 3-phosphoglyceric acid, magnesium ions, triethanolamine and 0.2 ml nucleoside triphosphate solution was made up to 3.0 ml with 0.3 mg β-diphosphopyridine mucleotide (reduced form (β-DPNH), pH 7.25 and mixed well. The optical density at a wavelength of 340 nm. was measured in a Hilger-Watt spectrophotometer (fused silica cuvettes with a 1 cm light path). A mixture of glyceraldehyde phosphate dehydrogenase and phosphoglyceric phosphokinase suspension in 0.04 ml ammonium sulphate was added to the cuvette with a Shandon-Terumo microsyringe and the contents mixed by inversion. The optical density was measured at a wavelength of 340 nm against a water blank as before, and the final, constant reading obtained recorded.

The principle of the method is as follows; the enzyme phosphoglyceric phosphokinase (PKG) was used to catalyse the following reactions:-

ATP + 3-phosphoglycerate $\rightleftharpoons$ ADP + 1,3-diphosphoglycerate.

The enzyme glyceraldehyde phosphate dehydrogenase (GAPD) then catalysed the second reaction:-

1,3-diphosphoglycerate + DPNH $\rightleftharpoons$ glyceraldehyde-3-phosphate + DPN + Pi

By measuring the decrease in optical density at 340 nm resulting from the oxidation of DPNH to the unreduced form, DPN, the amount of nucleoside triphosphate in the sample may be estimated:-

μ moles nucleoside triphosphate per ml original aliquot =

$$\frac{\text{change in optical density}}{6.22} \times 3.0$$

The enzymes and solutions used in this procedure were supplied in kit form by Sigma Chemical Co., (U.S.A.) in Kit number 366.

RESULTS

1. The Mg^{++} Activated ATPase

The results are shown in figures 28 to 31. The K_m and V_{max} values obtained from these figures are given in table 24. It is evident from these figures that the Mg^{++} activated ATPase obeys normal Michaelis-Menton kinetics as outlined in the Theory section above, giving good straight line plots. The results of table 24 suggest that at any given incubation temperature the V_{max} of the enzyme was fairly independant of the adaptation temperature of the animal. Thus the V_{max} for the Mg^{++} activated ATPase incubated at $10^{o}C$ were 8.3 and 7.3 μ moles Pi liberated per mg protein per hour for preparations from 10 and $25^{o}C$ adapted animals respectively. When incubated at $25^{o}C$, the V_{max} values were 34.5 and 29.4 μ moles Pi liberated per mg protein per hour for preparations from 10 and $25^{o}C$ adapted animals respectively (see table 24). These figures indicate that the V_{max} of the Mg^{++} activated ATPase obtained from $10^{o}C$ adapted crayfish was 13.7 and 17.3% greater than the V_{max} of the same enzyme obtained from $25^{o}C$ adapted crayfish when incubated at 10 and $25^{o}C$.

The enzyme/substrate affinity of the Mg^{++} activated ATPase increased with decreasing incubating temperature (see table 24). The decrease in K_m value for a $15^{o}C$ incubation temperature reduction was greater in preparations obtained from $25^{o}C$ adapted crayfish, 0.364 to 0.133 mM ATP, see table 24, a 63.5% reduction. The decrease in Km value for preparations obtained from $10^{o}C$ adapted animals for the same drop in incubation temperature was from 0.137 to 0.105 mM ATP, a 20.5% reduction.

The K_m of the Mg^{++} activated ATPase was also modified by the adaptation temperature of the crayfish. It can be seen from table 24 that the K_m of this enzyme at each of the incubation temperatures

Figure 28.

The Effect of Substrate Concentration on 10°C Adapted Crayfish Muscle ATPase Velocity at an Incubation Temperature of 10°C.

Microsomal preparations from four 10°C adapted crayfish were prepared as described in the Methods. Duplicate microsomal aliquots were incubated at 10°C for 15 minutes with either:

1. 3 mM $MgCl_2$ + ATP, or
2. 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl + ATP.

The concentration of ATP in the incubation medium was between 0.1 and 1.5 mM. The ATPase activity was expressed as μ moles Pi liberated per mg protein per hour and the results of four experiments meaned. The reciprocal of the ATPase velocity was plotted against the reciprocal of the ATP (substrate) concentration to give a Lineweaver-Burke plot.

$Na^+ + K^+$ activated ATPase velocity ..... O Δ

Mg^{++} activated ATPase velocity ..... Δ O

Total (Mg^{++} + Na^+ + K^+ activated) ATPase velocity ..... X x

The dashed curve represents the theoretical $Na^+ + K^+$ activated ATPase velocity (see text for explanation).

Figure 28.

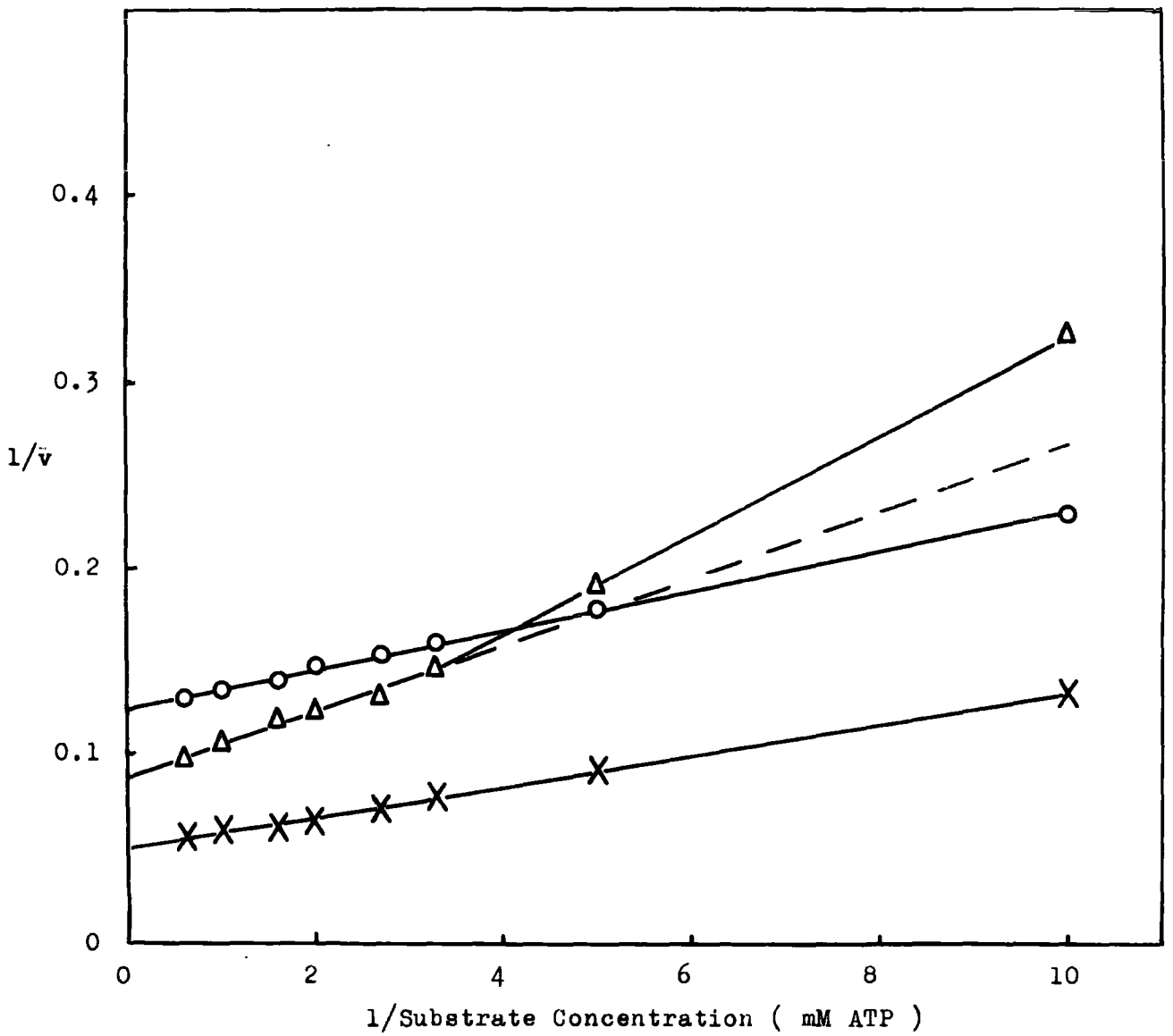


Figure 29.

The Effect of Substrate Concentration on 10°C Adapted Crayfish Muscle ATPase Velocity at an Incubation Temperature of 25°C.

Microsomal preparations from three 10°C adapted crayfish were prepared as described in the Methods. Duplicate microsomal aliquots were incubated at 25°C for 15 minutes with either:

1. 3 mM $MgCl_2$ + ATP or
2. 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl + ATP.

The concentration of ATP in the incubation medium was between 0.1 and 1.5 mM. The ATPase activity was expressed as μ moles Pi liberated per mg protein per hour and the results of three experiments meaned. The reciprocal of the ATPase velocity was plotted against the reciprocal of the ATP (substrate) concentration to give a Lineweaver-Burke plot.

$Na^+ + K^+$ activated ATPase velocity .....Δ

Mg^{++} activated ATPase velocity .....O

Total ($Mg^{++} + Na^+ + K^+$ activated) ATPase velocity ..... X x

The dashed curve represents the theoretical $Na^+ + K^+$ activated ATPase velocity (see text for explanation).

Figure 29.

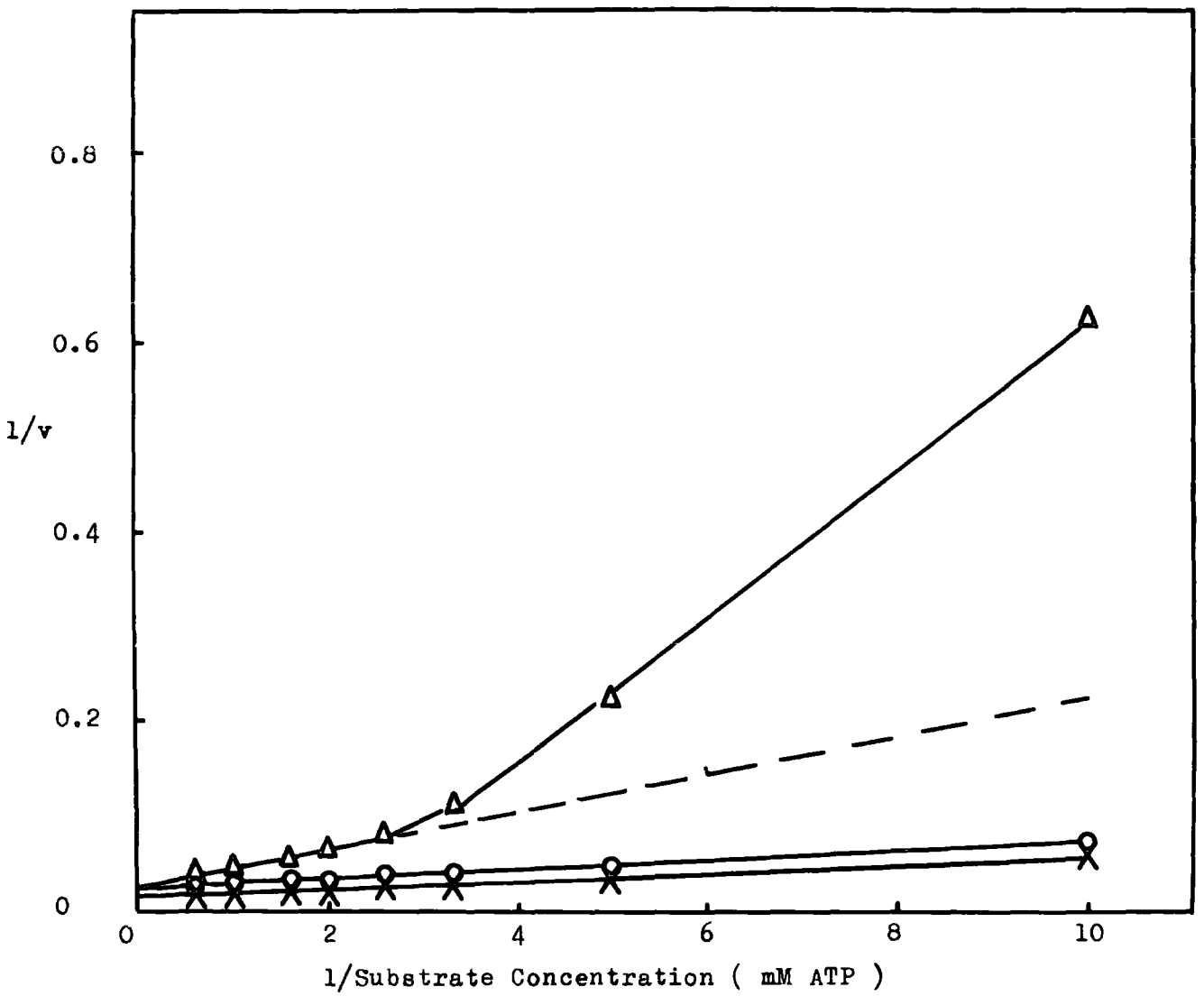


Figure 30.

The Effect of Substrate Concentration on 25°C Adapted Crayfish Muscle ATPase Velocity at an Incubation Temperature of 10°C.

Microsomal preparations from three 25°C adapted crayfish were prepared as described in the Methods. Duplicate microsomal aliquots were incubated at 10°C for 10 minutes with either:

1. 3 mM $MgCl_2$ + ATP, or
2. 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl + ATP.

The concentration of ATP in the incubation medium was between 0.1 and 1.5 mM. The ATPase activity was expressed as μ moles Pi liberated per mg protein per hour and the results of three experiments meaned. The reciprocal of the ATPase velocity was plotted against the reciprocal of the ATP (substrate) concentration to give a Lineweaver-Burke plot.

$Na^+ + K^+$ activated ATPase velocity ..... Δ

Mg^{++} activated ATPase velocity ..... O

Total ($Mg^{++} + Na^+ + K^+$ activated) ATPase velocity ..... X x

The dashed curve represents the theoretical $Na^+ + K^+$ activated ATPase velocity (see text for explanation).

Figure 30.

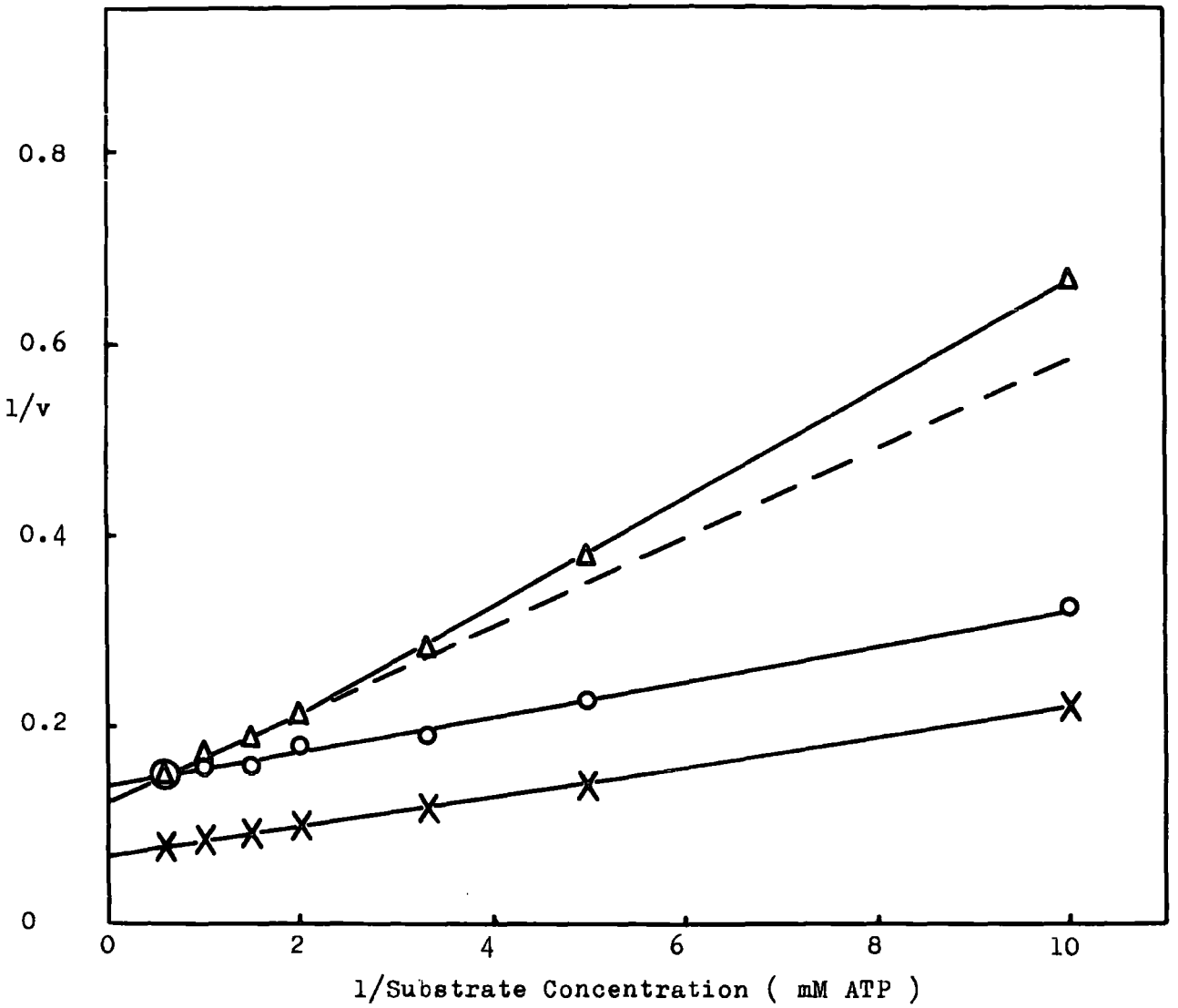


Figure 31.

The Effect of Substrate Concentration on 25°C Adapted Crayfish Muscle ATPase Velocity at an Incubation Temperature of 25°C.

Microsomal preparations from four 25°C adapted crayfish were prepared as described in the Methods. Duplicate microsomal aliquots were incubated at 25°C for 10 minutes with either:

1. 3 mM $MgCl_2$ + ATP, or
2. 3 mM $MgCl_2$ + 20 mM KCl + 100 mM NaCl + ATP.

The concentration of ATP in the incubation medium was between 0.1 and 1.5 mM. The ATPase activity was expressed as μ moles Pi liberated per mg protein per hour and the results of four experiments meaned. The reciprocal of the ATPase velocity was plotted against the reciprocal of the ATP (substrate) concentration to give a Lineweaver-Burke plot.

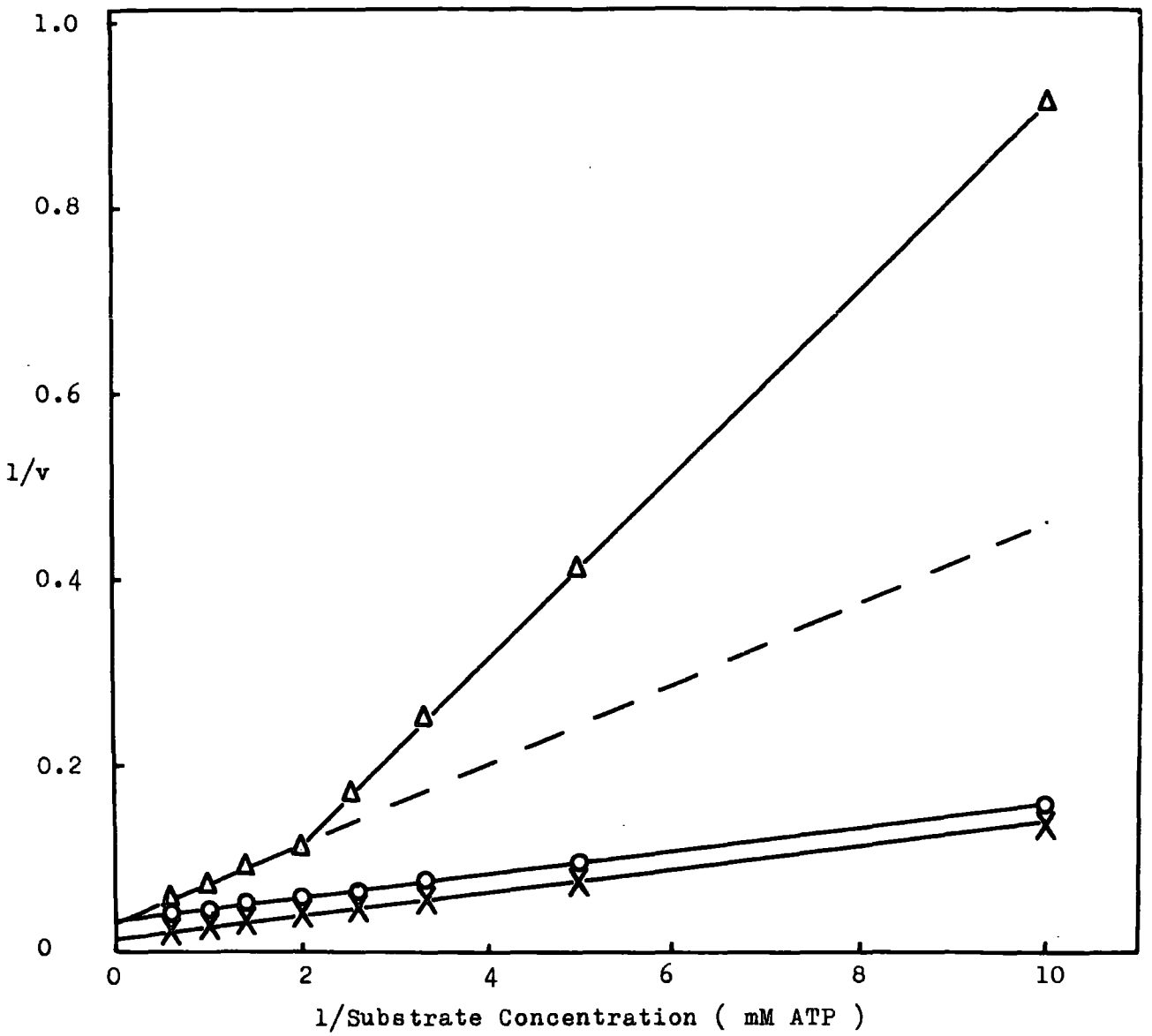
$Na^+ + K^+$ activated ATPase velocity ..... Δ

Mg^{++} activated ATPase velocity ..... O

Total ($Mg^{++} + Na^+ + K^+$ activated) ATPase velocity .... X x

The dashed curve represents the theoretical $Na^+ + K^+$ activated ATPase velocity (see text for explanation).

Figure 31.



was lower in preparations obtained from 10°C adapted crayfish. This means the affinity of the enzyme for the substrate was increased by adapting the animal to lower environmental temperatures.

These results therefore indicate that the K_m of the Mg^{++} activated ATPase was modified by both the adaptation temperature of the animal from which the preparation was obtained and the incubation temperature. The direction in which these changes occurred was such that they tended to reduce the effects of large temperature changes and would therefore aid the survival of the animal in an environment in which the temperature varied.

2. The $Na^+ + K^+$ activated ATPase

The estimation of the activity of the $Na^+ + K^+$ activated ATPase was complicated by the presence of the Mg^{++} activated ATPase. The usual method of estimating the $Na^+ + K^+$ activated ATPase activity, by subtracting the Mg^{++} activated ATPase activity from the total activity was used. The double reciprocal plot of the $Na^+ + K^+$ activated ATPase activity so obtained was not straight, however, curving upwards at low substrate concentrations (see figures 28 to 31). This was very similar to the pattern reported by Robinson (1967) for rat brain ATPases. Robinson calculated the theoretical V_{max} and K_m values for the rat brain $Na^+ + K^+$ activated ATPase from equation (3) in the Theory section as follows:

$$v_{tot\ 1} = \frac{V_{max\ 1} \cdot S'}{K_{m\ 1} + S'} + \frac{V_{max\ 2} \cdot S'}{K_{m\ 2} + S'}$$

$$v_{tot\ 2} = \frac{V_{max\ 1} \cdot S''}{K_{m\ 1} + S''} + \frac{V_{max\ 2} \cdot S''}{K_{m\ 2} + S''}$$

where $v_{tot\ 1}$ and $v_{tot\ 2}$ = the combined reaction velocities at substrate concentrations S' and S" respectively. Other terms as in Theory Section.

TABLE 24.

Kinetics of the Crayfish Muscle Microsomal ATPases.

The maimum velocity (V_{max}) and Michaelis constant (K_m) values for the muscle ATPases were calculated from figures 28 - 31 as described in the Theory section (Chapter 5). The V_{max} values were expressed as μ moles $\bar{P}i$ liberated per mg protein per hour and the K_m values as mM ATP.

TABLE 24.

Adaptation Temperature		10°C		25°C	
Incubation temperature		10°C	25°C	10°C	25°C
Mg^{++} activated ATPase	V_{max}	8.3	34.5	7.3	29.4
	K_m	0.105	0.137	0.133	0.364
$Na^{+}+ K^{+}$ activated ATPase	V_{max}	12.3	35.8	7.9	45.4
	K_m	0.269	0.676	0.367	0.197

Knowing the numerical values of the terms $v_{tot\ 1}$ and $v_{tot\ 2}$, and also $V_{max\ 1} \cdot S/K_{m\ 1} + S$ (referring to the Mg^{++} activated ATPase) it was possible to put:-

$$\frac{V_{max\ 2} \cdot S'}{K_{m\ 2} + S'} = X \qquad \text{and} \qquad \frac{V_{max\ 2} \cdot S''}{K_{m\ 2} + S''} = Y$$

where X and Y are numerical terms.

Knowing S' and S", the values of $V_{max\ 2}$ and $K_{m\ 2}$ (referring to the $Na^+ + K^+$ activated ATPase) can be calculated from these simultaneous equations. Having calculated these values, and knowing that the double reciprocal plot of $1/v$ against $1/S$ to be a straight line, those two values may then be used to draw in the theoretical activity of the enzyme on the graph. This was done for figures 28 to 31, the calculated curve being drawn in a dotted line for comparison with the observed activity which was drawn as a solid line. It can be seen from these figures that the calculated and observed activities coincided at high substrate concentrations, diverging only at the lower substrate concentrations. The values given in table 24 for the K_m and V_{max} of the $Na^+ + K^+$ activated ATPase were measured from figures 28 to 31 using the values obtained at the higher substrate concentrations where the theoretical and the observed activities coincided.

The V_{max} of the $Na^+ + K^+$ activated ATPase varied with the incubation temperature similarly to the Mg^{++} activated ATPase. Thus the V_{max} at a given incubation temperature did not differ greatly between the two adaptation groups. At an incubation temperature of 25^oC the V_{max} values were 35.8 and 45.4 μ moles Pi liberated per mg protein per hour for enzymes from 10 and 25^oC adapted crayfish respectively, (see table 24). At an incubation temperature of 10^oC the V_{max} values were 12.3 and 7.9 μ moles Pi liberated per mg protein

TABLE 25.

Nucleoside Triphosphate Concentrations in Crayfish Abdominal Flexor Muscles.

Samples of crayfish abdominal flexor muscles were obtained from five 10^{o} and five 25^{o}C adapted crayfish and the nucleoside triphosphate concentration estimated by the method described in the Methods (Chapter 5). The concentration of nucleoside triphosphate was expressed in $\bar{\mu}$ moles per g wet weight of muscle.

TABLE 25.

Animal	Nucleoside triphosphate content of muscles (μ moles/g wet weight).	
	10°C Adapted	25°C Adapted.
1	0.960	0.876
2	1.460	0.587
3	0.816	1.360
4	1.060	0.889
5	0.965	0.675
$\bar{x}$	1.052	0.877
S.E.	0.104	0.234

per hour for enzymes from 10 and 25°C adapted crayfish respectively (see table 24). These figures indicate that the V_{max} of the $Na^+ + K^+$ activated ATPase obtained from 10°C adapted crayfish was 15.6% higher and 21.0% lower than the V_{max} of the same enzyme obtained from 25°C adapted crayfish when incubated at 10 and 25°C respectively.

The K_m of the $Na^+ + K^+$ activated ATPase varied with the incubation temperature so that the K_m was lowest at the lower incubation temperature. Reducing the incubation temperature of the enzyme from 25°C adapted animals from 25 to 10°C resulted in a fall of the K_m from 1.970 to 0.367 mM ATP (see table 24). A similar reduction in the incubation temperature of the $Na^+ + K^+$ activated ATPase obtained from 10°C adapted animals resulted in a fall in the K_m value from 0.676 to 0.269 mM ATP (see table 24). The K_m of this enzyme also changed at a given incubation temperature with the adaptation temperature of the animal from which it was obtained. Thus it is evident from table 23 that the K_m of the $Na^+ + K^+$ activated ATPase obtained from 10°C adapted crayfish was lower than the K_m of the enzyme obtained from 25°C adapted crayfish at both incubation temperatures. The $Na^+ + K^+$ activated ATPase therefore exhibited negative thermal modulation in so far as the kinetics of the enzyme changed with both the incubation temperature of the enzyme and the adaptation temperature of the animal in such a way as to compensate for thermal changes.

3. Nucleoside Triphosphate Concentrations in Crayfish Abdominal Flexor Muscles

The results of the nucleoside triphosphate estimation for 5 crayfish from each adaptation group are shown in table 25. The results show that the average amounts of total mucleoside triphosphates present in 10 and 25°C adapted animal abdominal flexor muscles were 1.052 and 0.877 μ moles per g wet weight respectively. The wet weight/dry weight and extracellular space measurements of Chapter 2 (see

TABLE 26.

Estimated *in vivo* Activity of the Crayfish Muscle Microsomal ATPases.

The velocity of the microsomal ATPases was calculated from the data of figures 28 to 31 on the basis of an *in vivo* ATP concentration of 1.34 mM (see table 25). Velocities were expressed in μ moles Pi liberated per hour per mg protein.

The van't Hoff Q_{10} values were computed from

$$Q_{10} = \frac{k_2}{k_1}^{10 (t_2-t_1)}$$

where k_1 = the rate of reaction at temperature t_1,

and k_2 = the rate of reaction at temperature t_2.

TABLE 26.

Adaptation Temperature (^{o}C)	10		25	
Incubation Temperature (^{o}C)	10	25	10	25
Velocity of Mg^{++} activated ATPase (ATP = 1.34 mM) in μ moles Pi liberated mg protein/ hour	7.69	33.3	6.76	23.81
Mg^{++} activated ATPase	2.66		2.32	
Q_{10}	2.17			
Velocity of Na^{+}+ K^{+} activated ATPase (ATP = 1.34 mM) in μ moles Pi liberated/mg protein/hour	10.01	23.26	6.76	16.67
Na^{+}+ K^{+} activated ATPase	1.76		1.83	
Q_{10}	1.41			

tables 6 and 12) were used to calculate the amount of water in the muscle fibres and the nucleoside triphosphate concentrations converted to mM. Ax^2 test showed that the difference in concentration between the 10 and 25^{o}C adapted crayfish were not significant, the overall mean for the 10 crayfish muscles was 0.952 μ moles per g wet weight muscle, which represents 1.34 mM mucleoside triphosphate. This suggests that the sarcolemmal ATPases have a reasonable substrate supply which is adequate for over half maximal velocity.

TABLE 27.

Estimation of Substrate Utilisation of Crayfish Muscle Microsomal ATPases during *in vitio* Incubations.

The percentage substrate utilisation of the combined abdominal flexor muscle ATPase activities was calculated for a substrate concentration of 0.1 mM ATP. The rates of reaction were calculated from the data of Figures 28 - 31 and assumed to be constant. The reaction times were 10 minutes for 25°C adapted crayfish and 15 minutes for 10°C adapted crayfish. The percentage deviation of the observed $Na^+ + K^+$ activated ATPase velocity from the calculated velocity at an ATP concentration of 0.1 mM is included for comparison.

TABLE 27.

Adaptation Temperature of source animal. (^{o}C)	Incubation times (minutes)	Incubation temperature of enzyme preparation (^{o}C)	Percent substrate utilisation (S = 0.1 mM)	Percentage Deviation (S = 0.1 mM)
10	15	10	18.5	2.6
		25	38.5	15.8
25	10	10	11.25	6.25
		25	18.5	11.9

DISCUSSION

The results indicated that the Mg^{++} activated ATPase obeyed normal Michaelis-Menten kinetics but that the $Na^{+}+K^{+}$ activated ATPase did not (see figures 27 to 30). The experimental points obtained for the $Na^{+}+K^{+}$ activated ATPase consistently deviated from the predicted straight line double reciprocal plot at low substrate concentrations. This deviation was similar to that observed by Robinson (1967) for rat brain $Na^{+}+K^{+}$ activated ATPase, the activity being less than expected at low substrate concentrations. The reason for this deviation is not clear, and it raises several questions. The first point is that the combined activity of the two enzymes gave a straight line double reciprocal plot (see figures 27 to 30). This is contrary to equation (4) in the Theory section, since if the two separate enzyme components obeyed Michaelis-Menten kinetics the resulting plot should be curvilinear. (A straight line can only be obtained if the K_m values of the two enzymes are the same, or if one of the enzymes is present in a great excess, neither condition obtaining in this case). This suggests that the two enzymes were not, in fact, acting entirely independently. An alternative possibility is that the method used was at fault. The experimental method did not measure the true initial velocity of the reaction as required by the theory, but was evolved bearing in mind the limited supply of experimental animals. Calculations were therefore made of the amount of substrate utilised by the combined activity of the two enzymes during the incubation period, the percentage substrate utilisation is given in table 27. It can be seen from this table that substrate utilisation was significant at the lowest substrate concentration used (0.1 mM ATP), in one case it was almost 40%. The percentage deviation of the double reciprocal plot from the calculated straight line (see figures 28 to

31) was included in table 27 for comparison with the percentage substrate utilisation. It can be seen that the percentage deviation is highest when the substrate utilisation is greatest, but no definite correlation between the two factors emerged. The possibility therefore exists that the observed deviations from the straight line double reciprocal plot were due to substrate depletion during measurements of combined enzyme activity. The straight line plot of the combined enzyme activity and the lack of a definite correlation between the percentage substrate utilisation and the percentage deviation suggest that something more might have been involved, however. This is supported by the findings of Bourgoigne, Klahr, Yates, Guerra and Bricker (1970). These authors found that the $Na^{+}+K^{+}$ activated ATPase of dogfish erythrocyte membranes, estimated by similar methods to those used in this work, gave a straight line double reciprocal plot. Since these authors incubated their ATPase preparations for 20 minutes at $37^{o}C$ to obtain their results, the substrate utilisation must have been quite high. It is therefore possible that the sarcolemmal ATPases of the crayfish are not entirely independent.

In common with many key enzymes of poikilotherms (e.g. see Somero, 1969, and Hazel and Prosser, 1970) both the sarcolemmal ATPases exhibit negative thermal modulation of their substrate affinity (see table 24). The results indicate that the enzyme/substrate affinity of the Mg^{++} activated ATPase changes to a greater degree than that of the $Na^{+}+K^{+}$ activated ATPase when the animals were adapted to differentt temperatures (see table 24). The consequence of these large changes in the enzyme/substrate affinity of the Mg^{++} activated ATPase is that cold adapted animals possess an enzyme which has a greater reaction velocity at a given substrate concentration than the same enzyme from warm adapted animals. Bearing in mind the effect of temperature

on enzymic reactions, this means that the reduction in the enzyme's K_m value acts as a compensatory mechanism.

The nucleoside triphosphate concentrations in the muscle fibres was estimated to be 1.34 mM (see table 25). The velocity of the ATPases at this substrate concentration were measured from figures 27 to 30 and are shown in table 26. This assumes that the nucleoside triphosphate is all available to the ATPases as a suitable substrate. The Q_{10} values for the enzymes have been calculated and included on table 25. The Q_{10} values of the Mg^{++} activated ATPase are all in excess of 2.0, indicating that this enzyme is more sensitive to the effects of temperature than the $Na^{+}+K^{+}$ activated ATPase whose Q_{10} values are all less than 2.0. The most interesting point about this table is that it illustrates the compensatory nature of the kinetic changes observed in the sarcolemmal ATPases at a substrate concentration approximating that found <u>in vivo</u>. Thus the Q_{10} value obtained when the incubation temperature was changed from 10 to 25°C was smaller for both enzymes when the animals were allowed to adapt to the appropriate incubation temperature. This means that the velocity of the enzymic reactions at an incubation temperature of 10°C was higher when the enzyme was prepared from a 10°C adapted animal rather than a 25°C adapted animal; and similarly, the velocity of the reactions at an incubation temperature of 25°C was lower when the enzyme was prepared from a 25°C adapted rather than a 10°C adapted animal.

The results of this chapter therefore indicate that the kinetics of the sarcolemmal ATPases change with the adaptation temperature of the animal. These changes tend to compensate for the change in environmental temperature. The results also indicate that the Mg^{++} activated ATPase is more sensitive to temperature than the $Na^{+}+K^{+}$ activated ATPase which suggests that modification of the kinetic properties of the Mg^{++} activated ATPase are of more significance than kinetic changes of the $Na^{+}+K^{+}$ activated ATPase during temperature acclimatisation.

GENERAL DISCUSSION AND CONCLUSIONS

Fresh water poikilotherms are often exposed to large seasonal variations in environmental, and hence body temperature. In order to survive they have evolved various behavioural and physiological mechanisms which compensate for the temperature fluctuations. The compensation is not perfect, however, and if the environmental temperature varies too much the animals die. This work was particularly concerned with the reason for the death of an aquatic poikilotherm at temperatures exceeding its physiological range, and the ability of the animals to alter their physiological temperature range after a period of temperature acclimatisation, i.e. 'resistance adaptation'. The animal used for this work was the freshwater crayfish, Astacus pallipes Lereboullet, an animal of convenient size which can be successfully maintained in the laboratory and shows the phenomenon of resistance adaptation (see Bowler, 1963a). The primary aims of this work were to determine the primary lesion of heat death and to investigate the phenomenon of resistance adaptation in Astacus pallipes Lereboullet

The ability of crayfish to adapt was determined by exposing cold (10°C) and warm (25°C) adapted animals to high lethal temperatures which were just in excess of their physiological range, i.e. 31 - 35°C. It was discovered that the time of cessation of scathognathite beat served as a reliable criterion of heat death, the crayfish being unable to recover if returned to their adaptation temperature at that point. A fairly precise criterion of heat death was required because the precise time of heat death is exceedingly difficult to determine. Warm adapted crayfish were shown to have a greater resistance to lethal temperatures than the cold adapted crayfish. For example, the LD_{50} times for 25 and 10°C adapted crayfish exposed to 32°C were 205 and 16 minutes respectively (see table 1). These results therefore agreed

with the findings of Bowler (1963a) in that warm adapted crayfish had a greater heat resistance than cold adapted animals. The results also agreed quantitatively with Bowler's results in that when the logarithm of the LD_{50} times for 25°C adapted crayfish was plotted against the reciprocal of the absolute temperature the Arrhenius μ value of the slope was very similar in both studies (143 and 140 Kcalories $mole^{-1}$) (see table 23).

The ability of the crayfish to adapt to temperature and the LD_{50} times having been established, experiments were performed to investigate ion movements between various compartments of the crayfish during heat death. Bowler (1963b) reported that there was a net gain of Na^+ and K^+ by the environment during heat death of 25°C adapted crayfish at 35°C. In the present study, warm and cold adapted crayfish were exposed to a lethal temperature of 34 and 32°C respectively, and there was a net loss of both Na^+ and K^+ from both types of adapted animals. The net Na^+ loss was much greater than the net K^+ loss, which agreed with Bowler (1963b), this was probably due to the much higher haemolymph Na^+ concentration. The haemolymph Na^+, K^+, Mg^{++} and Ca^{++} concentrations of 25°C adapted crayfish exposed to temperatures between 30 and 35°C were measured. No significant changes were observed in the haemolymph Ca^{++} and Mg^{++} concentrations apart from a slight increase during the final phases of heat death (see table 4). This was probably due to the 10% reduction of haemolymph volume which occurred during heat death (see table 11).

The haemolymph Na^+ and K^+ concentrations changed significantly during heat death, and in a predictable manner (see table 4) which could be correlated with the gain of these ions by the environment. Haemolymph Na^+ increased during the initial phases of heat death, the elevated Na^+ levels probably giving rise to the rapid

initial Na^+ gain by the environment (see figures 7 and 8.) After a period of exposure to lethal temperature which depended upon the severity of the heat stress the haemolymph Na^+ fell to below its normal levels which may be correlated with slower rate of Na^+ gain by the environment during the latter phases of heat death. Haemolymph K^+ concentrations increased during heat death, the initial increase was slower than the rate of increase observed during the latter phases of heat death (see figure 8). This can be correlated with the K^+ loss to the environment, the rate of which increased as death approached. It is suggested that the loss of Na^+ and K^+ to the environment reflects a passive leakage from the haemolymph, probably across the gills, the rate of ion loss therefore depending upon the haemolymph concentration of that ion. The changes in haemolymph Na^+ and K^+ concentrations which were observed were similar to those described by Bowler (1963b) in 25^oC adapted crayfish exposed to 35^oC. A comparison of the haemolymph Na^+ and K^+ concentration changes observed in warm and cold adapted crayfish exposed to 32^oC indicated that the general sequence of changes was similar in both adaptation groups. However, the ion movements were more rapid in the cold adapted crayfish.

Haemolymph analyses suggested that during heat death Na^+ was moving from the haemolymph and K^+ into the haemolymph. Ten crayfish were dissected and the components of the body weighed, the results indicated that muscle constituted about 50% of the body weight (see table 10). Muscle therefore appeared to be a possible major source of haemolymph K^+ and the destination of haemolymph Na^+ during heat death. Analyses of the muscle fibre Na^+ and K^+ concentrations of normal and heat dead animals showed that there was a gain of muscle Na^+ and a loss of muscle K^+ during heat death (see tables 7 and 8). The ion movements were more rapid in the muscles of cold adapted animals when 10 and 25^oC

adapted animals were exposed to the same lethal temperature (see figures 11 and 12). Analyses of muscles taken from crayfish which had been exposed to lethal temperatures for sublethal periods indicated that the exchange of ions between the haemolymph and muscle fibres began during the early phases of heat death.

In order to correlate the observed Na^+ and K^+ movements in the three compartments examined, measurements of the environmental gain and haemolymph and muscle fibre changes of Na^+ and K^+ ions were made simultaneously on a group of warm and a group of cold adapted crayfish. Calculations based on the Na^+ and K^+ concentrations in the three compartments before and after lethal heat treatment (see table 14) and reasonable assumptions of the volume of the haemolymph and muscle compartments were made. These calculations indicated that the observed ion movements between the environment, haemolymph and muscle cells which occurred during heat death constituted the major compartments in which Na^+ and K^+ ion exchange took place (see table 15).

The preceeding observations of Na^+ and K^+ ion movements during heat death led to the following conclusions; first, that Na^+ and K^+ ions moved down their respective electrochemical gradients during heat death due to a breakdown of passive permeability barriers between haemolymph and muscle and between haemolymph and environment. Second, that the breakdown of these passive permeability barriers occurred during the early phases of heat death and might be associated with the primary lesion of heat death in the crayfish. Third, that the increased sarcolemmal passive permeability was responsible for the major part of the rise of haemolymph Na^+ and the fall of haemolymph K^+.

A further point of interest arising from these ion movements was raised by three separate observations. First, the concentration of K^+ in the haemolymph of crayfish at the time of heat death was ob-

served to be similar regardless of the adaptation temperature or lethal temperature used (9 - 10 mM). Second, in an experiment in which ten 25°C adapted crayfish were exposed to 32°C for sequential haemolymph sampling, two animals survived. These two animals showed a very much smaller increase of haemolymph K^+ than did the eight animals which died (see table 4c). Third, the amount of K^+ which moved either into or out of the three compartments, environment, haemolymph and muscle, was very similar in two groups of animals adapted to different temperatures during death at different temperatures (see table 15). No such correlation existed between the Na^+ ion movements during heat death. These observations suggested that the elevated haemolymph K^+ concentrations observed during the latter phases of heat death might be a causative agent of heat death.

In view of the well established importance of the extracellular K^+ concentrations to the functioning of crayfish nerve (see for example Strickholm and Wallin, 1967) and muscle (see for example Fatt and Ginsborg, 1958), the effect of increasing the K^+ content of the extracellular saline was investigated on nerve and muscle cells. Increasing the extracellular K^+ concentration caused the muscle cells to depolarise in accordance with the Nernst equation, a rise from the normal level of 5.4 mM to 10 mM K^+ caused the muscle fibre resting potential to fall from about 72 mV to about 55 mV (inside negative) (See figure 16). This increase of extracellular K^+ was not sufficient in itself to bring about the K^+ contractures observed by Van der Kloot (1966) and seemed unlikely to cause death. The effect of increasing the extracellular K^+ on the spontaneous activity of the nerve cord was a large, rapid increase in the spontaneous activity (see figure 18). This can be correlated with the progressive loss of co-ordination observed in crayfish undergoing heat death, and the rapid limb twitching which occurred at or about the

time of heat death. Increasing the temperature of the nerve cord to lethal temperatures had little effect on the spontaneous activity (see table 20). It is therefore suggested that the immediate cause of heat death in the crayfish is the malfunction of the nervous system caused by the high haemolymph K^+ concentration.

In order to investigate the primary lesion of heat death a series of electrophysiological measurements were made on muscle fibres exposed to a lethal temperature (32^oC). The resting potential of the muscle fibres increased about 5 mV immediately after the temperature was increased, but depolarised when they were exposed for longer periods. In muscle fibre preparations taken from cold adapted crayfish the initial hyperpolarisation lasted for less than 10 minutes, whilst in muscle fibres taken from warm adapted crayfish the initial hyperpolarisation lasted for about 25 minutes before depolarisation began.(see figure 14). The muscle fibres of 25^oC adapted crayfish were thus more resistant to 32^oC than the muscle fibres taken from 10^oC adapted animals. The causes of the changes in the resting potential were not related to the extracellular ionic concentrations since these were maintained constant throughout the experiment, and therefore reflect changes in the muscle fibres themselves. To elucidate these changes, the effective membrane resistance of the muscle fibres was measured during exposure to 32^oC. The sarcolemmal resistance fell during this heat treatment, indicating a rise in the ionic conductance of the sarcolemma (see figure 15). Again, the muscle fibres taken from warm adapted animals were more resistant to exposure to 32^oC than muscle fibres taken from cold adapted crayfish. The fall in sarcolemmal resistance occurred within 5 minutes of exposure to 32^oC in the 10^oC adapted, but not until after about 15 to 20 minutes exposure in the 25^oC adapted fibres. It was interesting to note that the increase in sarcolemmal conductivity during exposure to 32^oC occurred

before the depolarisation of the muscle fibres. The depolarisation of the muscle fibres during heat treatment was therefore interpreted as the result of an increase in the sarcolemmal permeability to Na^+ and K^+ ions.

The initial hyperpolarisation of the muscle fibres was probably caused by the activation of an electrogenic cation pump. The activity of such a pump, pumping K^+ into and Na^+ out of the muscle fibres could also explain the increased haemolymph Na^+ concentration which was observed during heat death. These electrophysiological measurements therefore confirm that the passive permeability of the sarcolemma increased dramatically during the early phases of heat death. It was not possible to be more precise about the timing of the change in sarcolemmal permeability from the electrophysiological measurements since the isolated muscles appeared to be more sensitive to heat than the whole animal. This was probably due to the necessity for dissecting the muscles out of the crayfish to perform the experiments, so that the muscles were exposed to the high temperatures in an artificial environment.

It has been suggested that the passive permeability of the plasmalemma of excitable cells is controlled by a Mg^{++} activated adenosine triphosphatase (ATPase) (EC 3.6.1.3) (see Duncan, 1965 and 1967, and Bowler and Duncan, 1968). Inactivation of this enzyme would therefore cause an increase in the passive permeability of the sarcolemma. The electrogenic cation pump activity is probably due to the presence of another membrane-bound enzyme, a Na^++K^+ activated ATPase (EC 3.6.1.3); this enzyme has been shown to be present in crayfish sarcolemma by Bowler and Duncan (1968). Thermal inactivation of this enzyme would also bring about an effective increase in the sarcolemmal ion permeability since it normally regulates the intracellular Na^+ concentration by pumping Na^+ against its electrochemical gradient,

the inactivation of this enzyme would therefore lead to an effective increase in the sarcolemmal Na^+ permeability which would allow the outward movement of K^+ ions. An investigation into the sensitivity of the sarcolemmal ATPases was therefore undertaken.

Partially purified microsomal preparations of sarcolemmal ATPases were preincubated for 10 minutes at temperatures between 10 and 38°C. Arrhenius plots of the activity of the enzymes following preincubation showed the Mg^{++} ATPase to be more heat sensitive than the $Na^+ + K^+$ activated ATPase. Furthermore, the sensitivity of the Mg^{++} ATPase to lethal temperatures depended upon the adaptation temperature of the animal from which the enzyme was prepared. The Mg^{++} activated ATPase from 10°C adapted crayfish was 50% inactivated by 10 minutes preincubation at 33.4°C, the enzyme from 25°C adapted crayfish was 50% inactivated by 10 minutes preincubation at 34.8°C (see figures 21 and 22). The sensitivity of the $Na^+ + K^+$ activated ATPase was not altered by the adaptation temperature of the source animal. The Arrhenius μ value for the thermal inactivation of the sarcolemmal ATPases was compared with the value obtained for the thermal inactivation of the whole animal over the same temperature range. The μ value for the Mg^{++} activated ATPase and the whole animal varied with the adaptation temperature of the crayfish and were similar in magnitude. Thus the μ value was 70 K calories $mole^{-1}$ for both the whole animal and the Mg^{++} ATPase taken from 10°C adapted crayfish. The μ value was 143 K calories $mole^{-1}$ for whole 25°C adapted animals and 103 K calories $mole^{-1}$ for the Mg^{++} activated ATPase taken from these animals. These figures indicated that the thermal sensitivity of the whole animal and the sarcolemmal Mg^{++} activated ATPase were similar over the range of 30 - 35°C.

The validity of such a comparison rests upon the premise

that the heat death of the whole animal will reflect the inactivation of the most heat sensitive component of the animal. Ushakov (1964) has proposed that the heat resistance of a cell is limited by its least resistant protein system, and microbiologists working on unicellular organisms have often compared the thermal inactivation of cells with the thermal inactivation of proteins (e.g. Byfield, Chang Lee and Bennett, 1969). The problem which arises when comparing the effect of heat on a highly organised metazoan with the effect of heat on a subcellular fraction is whether the response of the subcellular fraction in the experimental in vitro conditions is the same as it is in the intact animal. The results indicated that the temperature which caused 50% inactivation of the Mg^{++} activated ATPase during a 10 minute preincubation period was similar to the temperature which caused cessation of stethognathite beat after 10 minute exposure. This suggested that the Mg^{++} activated ATPase inactivation was indeed correlated with the heat death of the whole animal. It was therefore suggested that the Mg^{++} activated ATPase was responsible for the maintenance of the normal sarcolemmal passive permeability, and that its inactivation by heat caused the increased sarcolemmal permeability previously noted. The change in the temperature sensitivity of the Mg^{++} activated ATPase which occurred when the animal was allowed to adapt to different temperatures suggested that this enzyme was one of the factors which changed during resistance adaptation.

Investigations were made to study the effects of adapting crayfish to either 10 or 25°C on the kinetics of the sarcolemmal ATPases. It was found that the kinetics changed ina predictable fashion, the substrate affinity of the enzymes was greater in the cold adapted crayfish (see table 24). These changes were of a compensatory nature in that they reduced the effects of changes in the environmental temperature.

The results of this work have therefore led to the following conclusions:

1. The primary lesion of heat death in the crayfish, Astacus pallipes Lereboullet, was a breakdown of sarcolemmal passive permeability control caused by the thermal inactivation of the sarcolemmal Mg^{++} activated ATPase. This caused the breakdown of the normal sarcolemmal passive permeability which allowed Na^{+} ions to move into and K^{+} ions to move out of the muscle fibres. The K^{+} from the muscle fibres entered the haemolymph, causing an increase in haemolymph K^{+} and the K^{+} loss to the environment. The rise in haemolymph K^{+} resulted in a dramatic increase in the spontaneous activity of the nerves, leading to an inability of the crayfish to co-ordinate which was presumed to cause death.

2. The sarcolemmal Mg^{++} activated ATPase was implicated in resistance adaptation in the crayfish. The thermal sensitivity and the kinetics of this enzyme changed in a predictable fashion with the adaptation temperature of the crayfish.

SUMMARY

1. The aims of this work were to investigate the events leading to heat death in the crayfish, Astacus pallipes Lereboullet. It was shown that the time of heat death of crayfish at a temperature of between 32 and 35°C depended upon the previous thermal history of the animal. Crayfish adapted to higher temperatures exhibited a greater resistance to high lethal temperatures. The factors involved in heat death and thermal adaptation were therefore shown to be related.

2. Analyses of the Na^+ and K^+ contents of the haemolymph, muscle and environment of the crayfish indicated that significant changes occurred in these compartments during heat death. Furthermore, these changes were observed during the early phases of heat death. Further experiments indicated that the movements of monovalent cations which were observed in these three compartments were linked in that virtually all the ion movements observed could be reasonably explained in terms of these three compartments.

3. Electrophysiological observations on the effect of lethal temperatures on an in vitro abdominal muscle preparation indicated that high temperatures caused a large increase in the conductance of the sarcolemma, followed by depolarisation of the muscle fibres. These observations explained the dramatic increase in haemolymph K^+ and loss of haemolymph Na^+ during the early phases of heat death. Furthermore, the muscles taken from warm adapted crayfish were more resistant to high temperature than muscles taken from cold adapted crayfish.

4. In vitro preparations of abdominal muscles were exposed to a physiological saline in which the concentration of K^+ was adjusted to the haemolymph K^+ concentration at the time of heat death. The muscle fibres were depolarised, but the depolarisation was insufficient to inactivate the muscles. Measurements of the spontaneous

frequency of action potentials in an in vitro preparation of the ventral nerve cord of the crayfish indicated that the nerve cord activity was not greatly altered by exposure to lethal temperatures. Exposing the nerve cord to a physiological saline containing K^+ concentrations similar to those observed in the haemolymph heat dead crayfish resulted in a large increase in the frequency of the spontaneous action potentials. Prolonged exposure of the nerve cord to high K^+ resulted in an irreversible decline in activity.

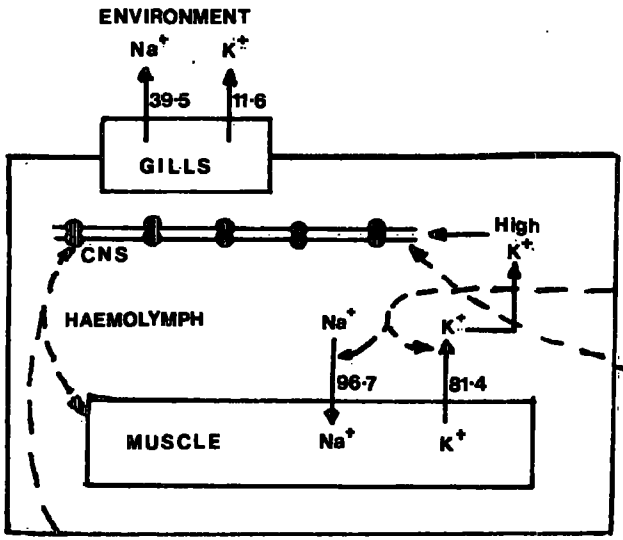
5. The thermal sensitivity of sarcolemmal Mg^{++} and $Na^+ + K^+$ activated ATPases was investigated. The Mg^{++} activated ATPase was found to be more sensitive to high temperatures, and its sensitivity was found to depend upon the previous thermal history of the crayfish from which the preparation was obtained. The thermal sensitivity of the $Na^+ + K^+$ activated ATPase was not altered significantly by the previous thermal history of the crayfish. Thermal inactivation of the Mg^{++} activated ATPase occurred over a similar temperature range to the heat death range of the whole animal, and had a very similar Arrhenius μ value which varied predictably with the adaptation temperature of the crayfish.

6. Kinetic studies on the sarcolemmal ATPases indicated that the kinetics of the Mg^{++} activated ATPases altered with the adaptation temperature of the animal from which the preparation was obtained in a manner which would make the enzyme more efficient at the appropriate adaptation temperature.

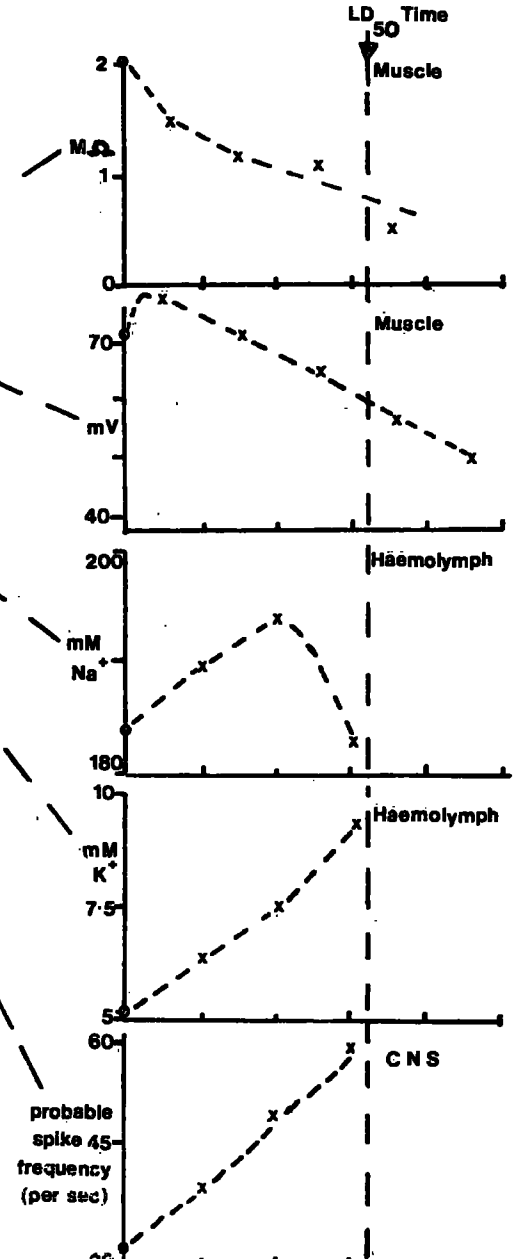
7. The conclusions drawn from these results were that heat death in the crayfish is a sequential event which proceeds as follows. Exposing a crayfish to a high lethal temperature causes the inactivation of a sarcolemmal Mg^{++} activated ATPase. This is associated with an increase in sarcolemmal conductance which allows the movement

of Na^+ and K^+ down their respective electrochemical gradients. The notable increase in haemolymph K^+ causes a large increase in the spontaneous activity of the ventral nerve cord, and ultimately irreversible damage to the nervous system leading to death. The increased heat resistance of warm adapted crayfish as compared with cold adapted animals is at least in part due to the greater heat resistance of the sarcolemmal Mg^{++} activated ATPase. The findings are summarised diagrammatically in figure 32.

Figure 32.



1. Lethal high temperature inactivates sarcolemmal Mg^{++}-ATPase.
2. Sarcolemmal conductance (= permeability) increase.
   - 2a. Muscle fibres depolarise.
3. Haemolymph Na^+ and K^+ change.
4. High haemolymph K^+ causes increased CNS activity.
5. Progressive loss of co-ordination.
6. Death.



A SUMMARY OF THE PROPOSED EVENTS LEADING TO DEATH OF A 10°C-ADAPTED *ASTACUS pallipes Lereboullet* AT 32°C.

The Na^+ and K^+ fluxes : p moles per minute per gram of crayfish (from tables 14 and 15).

REFERENCES.

Adrian, R.H. (1956) The effect of internal and external potassium concentrations on the membrane potential of frog muscle. J. Physiol. (London) 133: 631-658

Adrian, R.H. and C. L. Slayman. (1966). Membrane potential and conductance during transport of sodium, potassium and rubidium in frog muscle. J. Physiol. (London) 184: 970-1023.

Albers, R.W. and G. J. Koral (1962). Properties of the sodium-dependent ATPase of Electrophorus electricus. Life Sciences 1: 219-222.

Albuquerque, E.X. and S. Theslefi, (1967). Influence of phospholipase C on some electrical properties of the skeletal muscle membrane. J. Physiol. (London) 190: 123-137

Anderson, R.L. and J.A. Mutchmor (1968). Temperature acclimatization and its influence on the nervous system in 3 species of cockroach. J. Insect. Physiol. 14: 243-251.

Atwood, H.L. (1957). Influence of NO_3^- and other anions on fast and slow contractions of crab muscle. Can. J. Physiol. and Pharm. 46: 109

Ayrapetyan, S.N. (1969). The effect of temperature on the membrane potential of giant neurons of snails. Биофизика 14: 663-664.

Bader, H., R.L. Post and G.H. Bond. (1968). Comparison of sources of phosphorylated intermediate in transport ATPase of cell membranes. Biochem. Biophys. Acta 118: 116-123.

Banks, E.C., V. Damjanovic and C.A. Vernon, (1972). The so-called thermodynamic compensation law and thermal death. Nature, (London) 240: 147-148

Baslow, M.H., and R.F. Nigrelli, (1964). The effects of thermal acclimation on brain cholinesterase activity of the Killifish, Fundulus heteroclites. Zoologia (New York) 49, 41-57.

Behrisch, H.W. (1969). Temperature and the regulation of enzyme activity in poikilotherms. Fructose diphosphatase from migrating salmon. Biochem. J., 115: 687-696.

Behrisch, H.W. (1971). Temperature and the regulation of enzyme activity in poikilotherms. Regulatory properties of fructose diphosphatase from the muscle of the alaskan king-crab. Biochem. J., 121: 399-409.

Behrisch, H.W., and P.W. Hochachka, (1969). a. Temperature and the regulation of enzyme activity in poikilotherms. Properties of rainbow-trout fructose diphosphatase. Biochem.J., 111: 287-295.

Behrisch, H.W. and P.W. Hochachka, (1969) b. Temperature and the regulation of enzyme activity in poikilotherms. Properties of Lungfish fructose diphosphatase. Biochem. J., 112: 601-607.

Belehrádek, J. (1931). Le mechanisme phyoicochronique de l'adaptation thermique. Protoplasma 12: 406-424.

Belehrádek, J. (1935). Temperature and Living Matter. Protoplasma - Monograph 8, 1-227.

Berger, E. (1931). Über die Anpassung eines Susswasser und eines Brackwasserkrebses an Medieu von verschiedenem Zalzgehalt. Pflüg. Arch. ges Physiol. 228: 790-807.

Bernstein, J. (1902). Untersuchungen zur Thermo-dynamik der Bioelektrischen Strome. Pflug. Arch. ges. Physiol. 92: 521-502

Bittar, E. E. (1966). The effect of inhibitors and uncouplers on the sodium pump of Maia muscle fibre. J. Physiol. (London) 187: 81-103.

Bliss, D.E., M. E. Wang and E. A. Martinez. (1966). Water balance in the land crab. Geocarcinus lateralis, during the intermoult cycle. Amer. Zool. 6; 197-212.

Blostein, R. (1968). Relationships between erythrocyte membrane phosphorylation and adenosine triphosphate hydrolysis. J. Biol. Chem. 243: 1957-1965.

Bogucki, M. (1934). Recherches sur la regulation de la composition menerale du sang chez l'ecrivesse (Astacus fluviatilis L.) Arch int Physiol. 38: 1720179.

Bonting, S.L. and L. L. Caravaggio. (1963). Studies on the sodium-potassium activated adenosinetriphosphatase v. correlation of enzyme activity with cation flux in six tissues. Arch. Biochem. Biophys. 101: 37-46.

Bourgoigne, J., S. Klahr, J. Yates, L. Guerra and N.S. Bricker, (1970). Characteristics of an adenosinetriphosphatase system of dogfish erythrocyte membranes. Amer. J. Physiol. 218: 314-320.

Bowjberg. R.V. (1952). Comparative ecology and physiology of the crayfish. Orconectes propinquus and Cambarus fodiens. Physiol. Zool. 25: 34-55.

Bowler, K. (1963) a. A study of the factors involved in acclimatisation to temperature and death at high temperatures in Astacus pallipes. I Experiments on intact animals. J. cell and Comp. Physiol, 62: 119-132.

Bowler, K. (1963) b. A study of the factors involved in acclimatisation to temperature and death at high temperatures in Astacus pallipes. II Experiments at the tissue level. J. Cell and Comp. Physiol. 62: 133-146.

Bowler, K. and C.J. Duncan. (1967). Evidence implicating a membrane ATPase in the control of passive permeability of excitable cells. J. Cell Physiol. 70: 121-124.

Bryan, G.W. (196). a. Sodium regulation in the crayfish Astacus fluviatilis. I. The normal animal. J. Exper. Biol. 37: 83-99

Bryan, G.W. (1960) b. Sodium regulation in the crayfish Astacus fluviatilis. II Experiments with sodium depleted animals. J. Exper. Biol. 37: 100-112.

Bryan, G.W. (1960), c. Sodium regulation in the crayfish Astacus fluviatilis. III, Experiments with NaCl-loaded animals. J. Exper. Biol. 37: 113-128.

Byfield, J.E., Y. Chang Lee and L.R. Bennett. (1969). Thermal instability of Tetraliymena ribosomes: effects on protein synthesis. Biochem. Biophys. Res. Comm. 37: 806-812.

Carlisle, D.B. (1955). On the hormonal control of water balance in Carcinus. Pubbl. Staz Zool Napoli 27: 227-231.

Carpenter, D.O. and B.O. Alving. (1968). A contribution of an electrogenic Na^+ pump to membrane potential in Aplysia neurons. J. Gen. Physiol. 52: 1-21

Chapman, D. (1965). The Structure of Lipids. Methuen, London.

Cole, K.S. and A.L. Hodgkin. (1939). Membrane and protoplasm resistance in the squid giant axon. J. Gen. Physiol. 22: 671-687

Connelly, C.M. (1959). Recovery processes and metabolism in nerve in 'Biophhsical Sciences - A Study Programme" pp 475-484. John Wiley & Sons Inc., New York.

Conway, E.J. and Hingerty, D. (1948). Relations between sodium and potassium levels in mammalian muscle and blood plasma. Biochem. J., 42: 372-376.

Cooper, J.R. and H. McIlwain (1967). The $Na^+ + K^+$ activated ATPase of cerebral microsomal fractions: Treatments with disrupting agents. Biochem. J. 102: 675-681.

Curtis, H.J. and K. S. Cole (1942). Membrane resting and action potentials from the squid giant axon. J. Cell. Comp. Physiol. 19: 135-144.

Davison, T.F. (1971). The relationship between age, acclimatisation temperature and heat death point in adult Calliphora erythrocephala. J. Insect Physiol. 17: 575-585.

Davison, T.F. and Bowler (1971). Changes in the functional efficiency of flight muscle sarcosomes during heat death of adult Calliphora erythrocephala. J. Cell Physiol. 78: 37-48.

Dean, R.B. (194)). Anaerobic uptake of potassium by frog muscle. Proc. Soc. Exp. Biol and Med. 45, 8170819.

Dehinel, P.A. (1960). Effects of temperature and salinity on the oxygen consumption of two intertidal crabs. Biol. Bull. 118:215-249.

Del Castillo, J and L. Stark. (1952). Local responses in single medullated nerve fibres. J. Physiol. (London) 118: 207-215

De Mello, W.C. (1971). The influence of phospholipase C on membrane potential and conductance of heart fibres. Biophys. Soc. Abstracts p 254a.

De Mello, J and O. Hutter (1965). The anion conductance of crustacean muscle. Physiol. Soc. 5 - 6 November.

Dixon, M and E.C. Webb (1958). "Enzymes", Pub. Longman's, Green & Co. (London), Chapter iv pp 62-182

Dudel, J., M. Morad and R. Rüdel. (1968). Contractions of single crayfish muscle fibres induced by controlled changes of membrane potential. Pflügers Archiv. 299: 38-51.

Dudel J. and R. Rüdel (1968). Temperature dependence of electro-mechanical coupling in crayfish muscle fibres. Pflügers Archiv. 301: 16-30

Duncan, C.J. (1965). Cation permeability control and depolarisation in excitable cells. J. Theoret. Biol. 8: 403-418.

Duncan, C.J. (1966). Properties and stabilisation of the lysosomal membrane. Nature (London) 210: 1229-1230.

Duncan, C.J. (1967). The Molecular Properties of Excitable Cells. Pergamon Press, Oxford.

Dunham, E.T. and I. Glynn, (1961). Adenosinetriphosphatase activity and the active movements of alkali metal ions. J. Physiol. (London) 156: 274-293.

Eckberg, D.R. (1962). Anaerobic and aerobic metabolism in the gills of the crucian carp adapted to high or low temperatures. Comp. Biochem. Physiol. 5: 123-128

Edwards, G.A. and L. Irving, (1943). The influence of temperature and season upon the oxygen consumption of the land crab, Emerita talpoida Say. J. Cell. Comp. Physiol. 21; 169-182.

Eisenman, G., S.M. Ciani and G. Szabo, (1968). Some theoretically expected and experimentally observed properties of lipid bilayer membranes containing neutral molecular carriers of ions. Fed. Proc. 27: 1289-1304.

Elkes, J. and J.B. Finean, (1953). X-ray diffraction studies on the effect of temperature on the structure of myelin in the sciatic nerve of frog. Exp. Cell. Res. 4: 69-75.

Ellory, J.C. and M.W.Smith (1969). Deoxycholate stimulation of goldfish intestinal (Na^++ K^+) ATPase and its relation to digoxin binding. Biochim. Biophys. Acta, 193: 137-145.

Emmelot P and C.J. Bos (1962). Adenosine triphosphatase in the cell membrane fraction from rat liver. Biochim. Biophys. Acta, 58: 374-375.

Evans, P.R. and K. Bowler (1973). Thermal death and the denaturation of proteins. Subcell. Biochem. 3: 91-93.

Fähn, S., G.J. Koral and R.W. Albers. (1968). Sodium-potassium activated adenosinetriphosphatase of Electrophorus electric organs. J. Biol. Chem. 243: 1993-2002.

Falk, G. and P. Fatt, (1964). Linear electrical properties of striated muscle fibres observed with intracellular electrodes. Proc. Roy. Soc, (London) 160: 69-123.

Fatt, P and B.L. Giusborg. (1958). The ionic requirements for the production of action potentials in crustacean muscle fibres. J. Physiol. (London) 142: 516-543.

Fatt P. and B. Katz. (1953). The electrical properties of crustacean muscle fibres. J. Physiol. (London), 129: 171-204

Fiske, C.H. and Y. Subbarowe. (1925). The colorimetric determination of phosphorus. J. Biol. Chem. 66: 375-407.

Fraenkel, G.S. and H.S. Hopf. (1940). The physiological action of abnormally high temperatures on poikilothermic animals. I Temperature adaptation and the degree of saturation of the phosphatides. Biochem. J., 34: 1085-1092.

Frank, K. and M.G.F. Fuortes (1955). Potentials recorded from the spinal cord with microelectrodes. J. Physiol. (London) 130: 625-637.

Frankenhauser, R. (1960). Quantitative description of sodium currents in myelinated nerve fibres of Xenopus larvis. J. Physiol. (London) 151: 491-501.

Freed, J.M. (1965). Changes in the activity of cytochrome oxidase during adaptation of goldfish to different temperatures. Comp. Biochem. Physiol. 14: 651-659.

Gainer, H. (1968). The role of calcium in excitation - contraction coupling of lobster muscle. J. Gen. Physiol. 52: 88-110.

Gainer, H. and H. Grundfest. (1968). Permeability of alkali metal cations in lobster muscle. A comparison of electro-physiological and osmometric analyses. J. Gen. Physiol. 51: 399-425.

Garrahan, P.J., M.I. Pouchan and A.F. Rega, (1969). Potassium activated phosphatase from red blood cells. The mechanism of potassium activation. J. Physiol. (London) 202: 305-327.

Gaughran, E.R.L. (1947). The saturation of bacterial lipids as a function of temperature. J. Bact. 53: 506.

Girardier, L., J.L.Reuben, P.W. Brandt and H. Grundfest, (1963). Evidence for an anion-permselective membrane in crayfish muscle fibres and its possible role in excitation contraction coupling. J. Gen. Physiol. 47: 189-214.

Glynn, I. (1959) Sodium and potassium movements in nerve, muscle and red blood cells. Internat. Rev. Cyvol. 8: 449-480

Grainger, J.N.R. (1958). First stages in the adaptation of poikilotherms to temperature change in "Physiological Adaptation", Editor C.L. Prosser, Amer. Physiol. Soc, Washington, D.C., U.S.A.

Grainger, J.N.R. (1969). Heat death in Arianta arbustorium, Comp. Biochem. Physiol. 29: 665-670.

Gunn, D.L. (L942). Body temperature in poikilothermic animals. Biol. Rev. 17: 293-314.

Gustavson, K.H. (1956). The Chemistry and Reactivity of Collagen. Academic Press, New York, U.S.A.

Hays, E.A., M. A. Lang and H. Gainer, (1968). A re-examination of the donnau distribution as a mechanism for membrane potentials and potassium and chloride ion distributions in crab muscle fibres. Comp. Biochem. Physiol. 26: 761-792.

Hazel, J and C. L. Prosser, (1970). Interpretation of Inverse Acclimation to temperature. Z. Vergl. Physiologie, 67: 217-228

Heilbrunn, L.V., (1924) The colloid chemistry of protoplasm. American Journal of Physiology 190-199.

Heller, H. and B. Smith (1948). The water balance principle of crustacean eye-stalk extracts. J. Exp. Biol. 25: 388-398.

Hess, H. and A. Pope. (1957). Effect of metal cations on the adenosinetriphosphatase activity of rat brain. Fed. Proc. 16: 196-202

Hinkle, H., P. Heller and W. Van der Kloot (1971). The influence of potassium and chloride ions on the membrane potential of single muscle fibres of the crayfish. Comp. Biochem. Physiol. 40: 181-201.

Hochachka, P.W. (1967). Organisation of metabolism during temperature compensation. In "Molecular Aspects of Temperature Adaptation". Edited by C. L. Prosser, Amer. Ass. Adv. Sci. Publ. 84, 297-309

Hochachka, P.W. (1968). Action of temperature at branch points in glucose and acetate metabolism. Comp. Biochem. Physiol. 25: 107-118.

Hochachka, P.W. and G. N. Somero. (1968). The adaptation of enzymes to temperature. Comp. Biochem. Physiol. 27: 659-668.

Hodgkin. A.L. (1958). Ionic movements and electrical activity in giant nerve fibres. Proc. Roy. Soc. (London) 148: 1-37.

Hodgkin, A.L. and A. F. Huxley. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. J. Physiol. (London). 117: 500-544.

Hodgkin, A.L. and R.D. Keynes. (1953). The mobility and diffusion coefficient of potassium in giant axons from Sepia. J. Physiol. (London) 119: 513-528.

Huf, E. (1934). Über den Einfluss der Narkose auf den Wasser und Mineralhaushalt bei Susswassertieren. Pflügers Arch. iv. 235: 129-140.

Huntsman, A.G. (1924) Limiting factors for marine animals. II Resistance of larval lobsters to extremes of temperature. Contrib. Can. Biol 2: 91-93.

Itzhaki, R.F. and D. M. Gill, (1964). A micro-bivret method for estimating proteins. Analyt. Biochem. 9: 401-410.

Johnson, F.H., H. Eyring and M.J. Pollisar, (1954). The kinetic basis of molecular biology. John Wiley & Sons, New York.

Johnstone, P.V. and B.I. Roots. (1964). Brain lipid fatty acids and temperature acclimation. Comp. Biochem. Physiol. 11: 303-309.

Jørgensen, P.L. (1968). Regulation of the (Na^++ K^+)-activated ATP hydrolyzing enzyme system in the rat kidney. I. The effect of adrenolectomy and the supply of sodium on the enzyme syste. Biochim. Biophys. Acta. 151: 212-224.

Kamemoto, F.I., K. M. Kato and L.E. Tucker. (1966). Neurosecretion and salt and water balance in the Annelida and Crustacea. Am. Zoologist 6: 213-219.

Kamemoto, F.I. and J. K. Ono, (1969) Neuroendocrine regulation of salt and water balance in the crayfish Procambarus clarkii Comp. Biochem. Physiol. 29: 393-401.

Katz, A.I. and F. H. Epstein. (1967). Physiologic roles of sodium-potassium activated adenosinetriphosphatase in the transport system of cations across biologic membranes. New England J. Med. 278: 253-261.

Kemp, P. and M.W. Smith (1970). Effect of temperature acclimatisation on the fatty acid composition of goldfish intestinal lipids. Biochem. J. 117: 9-15.

Kepner, G.R. and R. I. Macey. (1968). Molecular weight estimation of membrane bound ATPase by in vacuo radiation inactivation. Biochem. Biophys. Res. Comm. 30: 582-587.

Kernan, R.P. (1962). The role of lactate in the active excretion of sodium by frog muscle. J. Physiol. (London) 162: 129-137.

Keynes, R.D. and R. Rybova. (1963). The coupling between sodium and potassium fluxes in frog sartorius muscle. J. Physiol. (London) 168: 58-63.

Klein, R.L. and A.P. Breland. (1966). Active cation transport and ATP hydrolysis in Acanthamoeba sp. Comp. Biochem. Physiol. 17: 39-47.

Knipprath, W.G. and J. F. Mead (1968). The effect of the environmental temperature on the fatty acid composition and the in vivo incorporation of 1-^{14}C-acetate in the goldfish. (Carassius anratus L). Lipids 3: 121-128.

~~Krebs~~

Kusakina, A.A. (1963). Changes in the thermostability of muscle proteins of the salmon in the course of smoltification. Cytology (U.S.S.R.) 5: 88-91.

Langridge, J. (1963). Biochemical aspects of temperature response. Ann. Rev. Plant Physiol. 14: 441-462.

Lehman, H. and B.T. Scheer. (1956). Hormonal control of metabolism in crustacea VI. The effect of eyestalk removal on phosphate uptake by Hemigrapsus nudus. Physio. Comp. Oecol. 4: 164-172.

Lehninger, A.L. (1964). The Mitochondrion. Benjamin Inc. Press, New York, U.S.A.

Levi, A.A. and L.N. Pisareva, (1970). Direct evidence of sodium release in the course of $Na^+ + K^+$ - dependent ATPase reaction. Biochem. Biphys. Res. Comm 38: 465-471.

Ling, G.N. (1967). Chapter 2, p.19 in "Thermobiology", Edited by A. H. Rose, Published by Academic Press, London.

Locke, S. and H. C. Solomon. (1967). Relation of the resting potential of rat gastrocnemius and soleus muscles to innervation, activity and the Na^++K^+ Pump. J. Exp. Zool. 166: 377-386.

Lockwood, A.P.M. (1960). Some effects of temperature and concentration of the medium on the ionic regulation of the isopod, Asellus aquaticus. J. Exp. Biol. 37: 614-630.

Lockwood, A.P.M. (1968). Aspects of the Physiology of Crustacea. Oliver and Boyd, Edinburgh.

Lowe, A.G. (1968). Linkage of ATPase active transport of Na^+ and K^+ in animal cells. Nature 219: 934.

Machne X. and R. Orozco, (1967). Effects of temperature and calcium variation on membrane potentials of crayfish axons. Amer. J. Physiol. 214: 672-676.

Marchesi, V.T. and G.E. Palade, (1967). The localisation of Mg^{++}-Na^+-K^+-activated ATPase on red cell ghost membranes. J. Cell. Biol. 35: 385-404.

Marmor, G.F. and A.L.F. Gorman (1970). Membrane potential as the sum of ionic and metabolic components. Science 167: 65-67.

Massari, S. and G.F. Azzone (1970) a. The mechanism of ion translocation in mitochondria. I.Coupling of K^+ and M^+ fluxes. Eur. J. Biochem. 12: 301-309.

Massari, S. and G.F. Azzone (1970) b. The mechanism of ion translocation in mitochondria. II.Active transport and proton pump. Eur. J. Biochem. 12: 310-318.

McLeese, D.W. (1956). Effects of temperature, salinity and oxygen on the survival of the American lobster. J. Fish. Res. Board, Can. 13: 247-272.

Mizuno, N., K. Nagano, T. Nakao, Y. Tashima, M. Fujita and M. Nakao (1968). Approximation of the molecular weight of (Na^++K^+) ATPase. Biochem. Biophys. Acta 168: 311-320.

Mullins, L.J. and M.Z. Awad. (1965). The control of the membrane potential of muscle fibres by the sodium pump. J. Gen. Physiol. 48: 761-775.

Mullins, L. J. and K. Noda, (1963). The influence of sodium free solutions on the membrane potential of frog muscle fibres. J. Gen Physiol. 47: 117-122.

Nakao, T., K. Nagano, K.Adachi and M. Nakao, (1963). Separation of two adenosine triphosphatases from erythrocyte membrane. Biochem. Biophys. Res. Comm. 13: 444-448.

Noguchi, T. and S. Freed (1971). ATPase - Lipids required for Mg^{++} dependent activity stimulated by sodium and potassium. Nature (New Biology) 230: 148-150.

Obara, S. (1968). Effects of some organic cations on generator potential of crayfish stretch receptor. J. Gen. Physiol. 52: 363-386.

Oliveiro-Castro, G.M. and R.D. Machado, (1969). Control of glass microelectrodes for intracellular recordings. Experentia 25: 556-557.

Orentlicher, M., and J.P. Reuben, (1971). Localisation of ionic conductances in crayfish muscle fibres. J. Memb. Biol. 4: 209-226.

Ozeki, M., A.R. Freeman and H. Grundfest, (1966). The membrane components of crustacean neuromuscular systems. II. Analysis of interactions among the electrogenic components. J. Gen. Physiol. 49: 1335-1347.

Post, R.L. (1959). Relationship of an ATPase in human erythrocyte membrane to the active transport of sodium and potassium. Fed. Proc. 18: 121-122.

Post, R.L., S. Kirna, A.K. Sen, T. Tobin and B. Orcutt (1968). Symposium on membrane proteins. New York Heart Association and Heart Fund.

Post, R.L., C.R. Merritt, C.R.Kinsolving and C.D. Albright (1960). Membrane adenosine triphosphatase as a participant in the active transport of sodium and potassium in the human erythrocyte. J. Biol. Chem. 235: 1796-1802.

Precht, H. (1957). Concepts of temperature adaptation of unchanging reaction systems of cold-blooded animals. Published by American Physiol. Soc. Washington, D.C. U.S.A.

Prosser, C.L. and S.J.F. Weinstein, (1950). Comparison of blood volume in animals with open and with closed circulatory systems. Physiol. Zool. 123: 113-124.

Ramamurthi, R. and B.T. Scheer, (1967). A factor influencing sodium regulation in crustaceans. Life Sciences 6: 2171-2175.

Reiner, J.M. 1969. 'Behaviour of Enzyme Systems', (2nd edition), Van Nostrand Reinhold Co. New York.

Reuben, J.P., P.W. Brandt, H. Garcia and H. Grundfest, (1967). Excitation-contraction coupling in crayfish. Am. Zoologist, 7: 623-645.

Reuben, J.P. L. Girardier and H. Grundfest, (1962). The chloride permeability of crayfish muscle fibres. Biol. Bull. 123: 509-521.

Reuben, J.P., L. Girardier and H. Grundfest (1964). Water transfer and cell structure in isolated crayfish muscle fibres. J. Physiol. (London), 47: 1141-1174.

Ribo, J.M. and F. Ponz, (1969). Role of the Mg^{++} activated ATPase in the enzymatic ATPase system depending on Na^{+} and K^{+} from the small intestine of rat. Revista Espanole de Fisiologa 25: 257-266.

Riegel, J.A. (1968). Analysis of the distribution of sodium, potassium and osmotic pressure in the urine of crayfish. J. Exp. Biol. 48: 587-596.

Rigby, B.J. (1968). Temperature relationships of poikilotherms and the melting temperature of molecular collagen. Biol. Bull. 135: 223-229.

Roberts, J.L. (1957). Thermal acclimation of metabolism in the crab, Portunus crassipes Randall. II. Mechanisms and the influence of season and latitude. Physiol. Zoology, 30, 242-255.

Robinson, J.D. (1967) a. Adenosinediphosphate metabolism in brain microsomes: structural organisation and enzymic activity. J. Neurochem. 14: 1143-1154.

Robinson, J.D. (1967) b. Kinetic studies on a brain microsomal ATPase. Evidence suggesting conformational changes. Biochemistry 6: 3250-3258.

Robinson, J.D. (1968). Regulating ion pumps to control cell volume. J. Theoret. Biol. 19: 90-96.

Robinson, J.D. (1969) a. Effects of Phlorizin on membrane cation-dependent ATPase and p-nitrophenyl phosphatase activity. Mol. Pharmacol. 5: 584-592.

Robinson, J.D. (1969) b. Kinetic studies on a brain microsomal ATPase. II Potassium-dependent phosphatase activity. Biochem. 8: 3348-3354.

Roots, B.I. and Prosser C.L. (1962). Temperature acclimatisation and the nervous system in fish. J. Exp. Biol. 39: 617-629.

Rosenberg, B., G. Kemeny, R.C. Switzer and T.C.Hamilton, (1971). Quantitative evidence for protein denaturation as the cause of thermal death. Nature (London) 232: 471-473.

Rossi, E. and G.E. Azzone. (1970). The mechanism of ion translocation in mitochondria. III Coupling of K^+ efflux with ATP synthesis. Evr. J. Biochem. 12: 319-327.

Rubenstein, B. and P.G. Scholefield, (1969). The relationship between Mg^{++}-stimulated ATPase activity and the glycogen content of rat liver. Can. J. Biochem. 47: 339-345.

Scarpa, A. and G.E. Azzone (1970). The mechanism of ion translocation in mitochondria. IV Coupling of K^+ efflux with Ca^{++} uptake. Eur. J. Biochem. 12: 328-335.

Schanne, O., H. Kawata, B. Schäfer and M. Lavallée, (1966). A study on the electrical resistance of the frog sarborius muscle. J. Gen. Physiol. 49: 897-912.

Schaune, O., M. Lavallée, R. Laprade and S. Gagné, (1968). Electrical properties of glass microelectrodes. Proc. Inst. Electr. Electron. Eng., 56: 1072-1082.

Scholles, W. (1933). Über die Mineralregulation wasserblender Evertebraten. Z. verg. Physiol. 19: 522-554.

Schöner, W., R. Beusch and R. Kramer, (1968). On the mechanism of Na^+ and K^+-stimulated hydrolysis of ATP. 2. Comparison of nucleotide specifities of Na^+- and K^+- activated ATPase and Na^+- dependent phosphorylation of cell membranes. Eur. J. Biochem. 7: 102-110.

Schöner, W. and H. Schmidt, (1969). Inhibition of (Na^+ + K^+)-activated ATPase by N,N'-dicyclohexyl carbamide. Febs. Letters, 5: 285-287.

Schwartz, A. (1962). A sodium and potassium stimulated adenosinetriphosphatase from cardiac tissues. 1. Preparation and properties. Biochem. Biophys. Res. Comm. 9: 301-306.

Senft, J.P. (1967). Effects of some inhibitors on the temperature dependent component of resting potential in lobster axon. J. Gen. Physiol. 50: 1835-1848.

Sharma, M.L. (1969). Trigger mechanism of increased urea production by the crayfish, Orconectes rusticus, under osmotic stress. Comp. Biochem. Physiol., 30: 309-321.

Shaw, J. (1959). The absorption of sodium ions by the crayfish, Astacus pallipes Lereboullet. I. The effect of external and internal sodium concentrations. J. Exp. Biol. 36: 126-144.

Shaw, J. (1960). a. The absorption of sodium ions by the crayfish, Astacus pallipes Lereboullet. II. The effect of the external anion. J. Exp. Biol. 37: 534-547.

Shaw, J. (1960). b. The absorption of sodium ions by the crayfish, Astacus pallipes Lereboullet. III The effect of other cations in the external solution. J. Exp. Biol. 37: 548-556.

Shaw, J. (1960) c. The absorption of chloride ions by the crayfish, Astacus pallipes Lereboullet. J. Exp. Biol. 37: 557-572.

Skou, J.C. (1957). Influence of some cations on adenosine-triphosphatase from peripheral nerves. Biochim. Biophys. Acta, 23: 394-401.

Skou, J.C. (1960). Further investigations on a Mg^{++} + Na^{+}- activated adenosinetriphosphatase, possibly related to the active, linked transport of Na^{+} and K^{+} across the nerve membrane. Biochim. Biophys. Acta, 42: 6-23.

Skou, J.C. (1962). Preparation from a mammalian brain and kidney of the enzyme system involved in active transport of Na^{+} and K^{+}. Biochim. Biophys. Acta, 58: 314-325.

Skou, J.C. and C. Hilberg. (1965). The effect of sulphydryl-blocking reagents and of urea on the (Na^{+}+ K^{+})-activated system. Biochim. Biophys. Acta, 110: 359-369.

Smith, M.W. and P. Kemp. (1969). Phospholiphase C. induced changes in intestinal adenosinetriphosphatase prepared from goldfish acclimated to different temperatures. Biochem. J. 114: 659-662.

Somero, G.N. (1969) a. Enzyme mechanisms of temperature compensation: Immediate and evolutionary effects of temperature on enzymes of aquatic poikilotherms. The American Naturalist 103: 517-530.

Somero, G.N. (1969) b. Pyruvate kinase variants of the alaskan king crab. Evidence for a temperature-dependent inter-conversion between two forms having distinct and adaptive kinetic properties. Biochem. J. 114: 237.

Somogyi, J. (1968). The effect of proteases on the (Na^{+}+ K^{+})-activated adenosine triphosphatase system of rat brain. Biochim. Biophys. Acta, 151: 421-428.

Spoor, W.A. (1955). Loss and gain of heat tolerance by the crayfish. Biol. Bull. 108: 77-87.

Steinbach, H.B. (1940). Sodium and potassium in frog muscle. J. Biol. Chem. 133: 695-701.

Strickholm, A. and B.G. Wallin (1967). Relative ion permeabilities in the crayfish giant axon determined from rapid external ion changes. J. Gen. Physiol. 50: 1929-1953.

Strickholm, A., B.G. Wallin and P. Shrager, (1969). The pH dependency of relative ion permeabilities in the crayfish giant axon. Biophys. J. 9: 873-883.

Takenaka, T. and K. Yumoto (1968). Excitability of crayfish giant axons in media with trivalent cations in place of divalent cations. Proc. Jap. Acad. 44: 564-568.

Tanaka, R. and T. Sakamoto (1969). Molecular structure in phospholipid essential to actuate (Mg^{++}+ Ka^{+}+ K^{+}) ATPase and (K^{+}+ Mg^{++}) dependent phosphatase of bovine cerebral cortex. Biochim. Biophys. Acta. 193: 384-395.

Tanaka, R., T. Sakamoto and Y. Sakamoto, (1971) Mechanism of lipid activation of Na^{+}, K^{+}, Mg^{++} activated ATPase and K^{+}, Mg^{++} activated phosphatase of bovine cerebral cortex. J. Memb. Biol. 4: 42-51.

Tasaki, I., E.H. Polley and F. Orrego, (1954). Action potentials from individual elements in cat geniculate and striate cortex. J. Neuro physiol. 17: 454-474.

Taylor, E.W. (1962). Spontaneous activity in the cardioaccelerator nerves of the crayfish, Astacus pallipes Lereboullet. Comp. Biochem. Physiol. 33: 859-869.

Todd, M.E. and P.A. Dehnel. (1960). Effect of temperature and salinity on the heat tolerance of two grapsoid crabs, Hemigrapous nudus and Hemigrapsus oregonensis. Biol. Bull. 118: 150-172.

Ushakor, B.P. (1964). Thermostability of cells and proteins of poikilotherms and its significance in speciation. Physiol. Rev. 44: 518-560.

Van der Kloot, W. (1966). The exchange of radioactive cations by somatic and cardiac muscles of the crayfish. Comp. Biochem. Physiol. 17: 1019-1043.

Vernberg, F.J. (1959). Studies on the physiological variation between tropical and temperate zone fiddler crabs of the genus Uca. III The influence of temperature acclimatisation on oxygen consumption of whole animals. Biol. Bull. 117: 582-593.

Wallin, B.G. (1967) a. Intracellular ion concentrations in crayfish axons. Acta. Physiol. Scand. 70: 419-430.

Wallin, B.G. (1967) b. The relation between external potassium concentration, membrane potential and internal ion concentrations in crayfish axons. Acta Physiol. Scand. 70: 431-448.

Wallin, B.G. (1969). Water permeability in resting and stimulated crayfish nerve. J. Gen. Physiol. 54: 462-468.

Weiss, D.E. (1969) a. Energy transducing reactions in biological membranes. I. Energy transduction in ion- and electron-exchange polymers. Aust. J. biol. Sci. 22: 1337-1354.

Weiss, D.E. (1969) b. Energy transducing reactions in biological membranes. II A molecular mechanism for the permeability changes in nerve during the passage of an action potential. Aust. J. biol. Sci. 22: 1355-1371.

Weiss, D.E. (1969) c. Energy transducing reactions in biological membranes. III A model of the sodium pump and its possible relevance to some other active transport processes. Aust. J. biol. Sci. 22: 1373-1387.

Weiss, D.E. (1969) d. Energy transducing reactions in biological membranes. IV A model of oxidative phosphorylation and ion accumulation in mitochondria based on a thioacylation mechanism. Aust. J. biol. Sci. 22: 1389-1412.

Wheeler, K.P. and R. Whittam, (1970). ATPase activity of the sodium pump needs phosphatidylserine. Nature (London) 225:449.

Whittam, R. and K.P. Wheeler (1970). Transport across all membranes. Annual Rev. Physiol. 32: 21-60.

Woodhead, P.H.J. and A.D. Woodhead, (1959). The effects of low temperature on the physiology and distribution of the cod, Gadus morhua (L) in the Barents Sea. Proc. Zool. Soc. Lond. 133: 181-199.